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Abstract

Resistance against first and second generation (irreversible) ErbB inhibitors is an unsolved problem in clinical oncology. The purpose of this study was to examine the effects of the irreversible ErbB inhibitors pelitinib and canertinib on growth of breast and ovarian cancer cells. Although *in vitro* growth-inhibitory effects of both drugs exceeded by far the effects of all reversible ErbB blockers tested (lapatinib, erlotinib, gefitinib), complete growth-inhibition was usually not reached. To define the mechanism of resistance, we examined downstream signaling pathways in drug-exposed cells by Western blot analysis. Although ErbB phosphorylation was reduced by pelitinib and canertinib, activation of the AKT/mTOR pathway remained essentially unaltered in drug-resistant cells. Correspondingly, transfection of tumor cells with constitutively activated AKT was found to promote resistance against all ErbB inhibitors tested, whereas dominant negative AKT reinstalled sensitivity in drug-resistant cells. In a next step, we applied PI3K/AKT/mTOR blockers including the dual PI3K/mTOR kinase inhibitor NVP-BEZ235. These agents were found to cooperate with pelitinib and canertinib in producing *in vitro* growth inhibition in cancer cells resistant against ErbB-targeting drugs. In conclusion, our data show that ErbB drug-refractory activation of the PI3K/AKT/mTOR pathway plays a crucial role in resistance against classical and second-generation irreversible ErbB inhibitors, and NVP-BEZ235 can override this form of resistance against pelitinib and canertinib.

Key words: Breast cancer, drug resistance, irreversible EGFR/ErbB/HER inhibitor, ovarian cancer, NVP-BEZ235, PI3K/AKT/mTOR

Introduction

ErbB receptors have been implicated as key triggers of oncogenesis in various solid tumors [1]. Therefore, these receptors represent potential molecular targets for therapeutic intervention [2]. Indeed, several ErbB-targeting antibodies and small molecule-type inhibitors of ErbB receptors are currently tested in clinical trials and some of these agents are already used in clinical practice [1, 2]. One highlighting example is the ErbB2-targeting antibody trastuzumab that has improved treatment outcome in patients with breast cancer expressing ErbB2 [3]. Unfortunately, however, about 70% of these patients progress despite treatment, suggesting that resistance is an important issue. Some of the trastuzumab-resistant patients (20-30%) may still respond to the ErbB1/2 inhibitor lapatinib [3]. However, responses are usually short-lived and most patients relapse after a variable latency period. Therefore, research is focusing on the development of more potent ErbB receptor blockers. One novel type of targeted drugs are the irreversible ErbB receptor inhibitors. These agents may exert higher anticancer activity compared to first-generation (reversible) ErbB-targeting drugs [4, 5], and there was some hope that resistance against these inhibitors would occur less frequently [6]. Unfortunately, however, ErbB-expressing tumor cells may become resistant even against irreversible ErbB-targeting drugs. Although the exact mechanisms of resistance against these novel (irreversible) ErbB blockers remain unknown, it has been hypothesized that additional pro-oncogenic signaling pathways may play an important role [6]. Therefore, a better understanding of mechanisms underlying resistance against irreversible ErbB inhibitors is crucial for improvement in therapy of ErbB expressing neoplasms.

All ErbB receptors except ErbB3 activate various downstream signaling molecules including the phosphatidylinositol 3-kinase (PI3K) and its downstream mediators AKT

and mTOR, and the mitogen-activated protein kinase (MAPK) cascade proteins C-RAF, MEK, and ERK [7, 8]. Both, the MAPK- and the PI3K signaling pathway, have been involved in resistance of tumor cells against ErbB-targeting drugs [9-11]. However, the relative contribution of these two pathways to the development of resistance is still a matter of debate.

We examined the effects of two potent irreversible ErbB inhibitors, pelitinib (EKB-569) and canertinib (CI-1033), on MAPK and PI3K/AKT downstream cascades in ErbB drug-sensitive and drug-resistant breast and ovarian cancer cells. Pelitinib blocks ErbB1 by covalent interaction with the ATP binding pocket (IC_{50} 8 – 83nM) and inhibits ErbB2 to lesser extent (IC_{50} 378 – 1255 nM) [4, 12-14]. Canertinib inactivates ErbB1, -2 and -4 at IC_{50}-concentrations of 0.8 – 30nM (ErbB1), 19 – 127nM (ErbB2), and 7 – 388nM (ErbB4), respectively [4, 15, 16-18].

We here show that wiping out AKT phosphorylation is essential and sufficient, whereas abrogation of ERK phosphorylation is not required for the anticancer effects of these ErbB-targeting drugs. Thus, pAKT may be a biomarker for resistance against irreversible ErbB inhibitors in breast and ovarian cancer cells. Most importantly, resistance against ErbB blockers can be overcome by co-application of PI3K/AKT/mTOR inhibitors such as NVP-BEZ235. These data suggest that further studies exploring the effects of targeting of both ErbB receptors and PI3K/AKT/mTOR in breast and ovarian cancer are warranted.

Materials and methods

Reagents

Recombinant epidermal growth factor (EGF) and heregulin-β1 (HRG-β1) were purchased from Sigma (St. Louis, MO) or Thermo Fisher Scientific (Fremont, CA). The irreversible ErbB inhibitor pelitinib (EKB-569) was a kind gift from Wyeth (Cambridge, MA) and the irreversible ErbB inhibitor canertinib (CI-1033, PD-183805) was kindly provided by Pfizer (Groton, CT). The reversible ErbB inhibitors gefitinib, erlotinib, and lapatinib, the PI3K/mTOR inhibitor NVP-BEZ235, and MEK1/2 inhibitor AZD6244 were purchased from ChemieTek (Indianapolis, IN), the IGF-IR inhibitor picropodophyllin, AKT1/2 inhibitor Akti-1/2, and mTOR inhibitor rapamycin from Calbiochem (San Diego, CA), and the MEK1/2 inhibitor U0126 from Cell Signaling Technology (Boston, MA). All kinase blockers were dissolved in DMSO and diluted 1:1,000 or 1:2,000 in medium before use. Fetal calf serum (FCS), DMEM, α-MEM, and RPMI 1640 were purchased from Gibco (Karlsruhe, Germany).

Cell lines

Ten mammary cancer cell lines (BT-20, BT-474, MCF-7, MDA-MB-231, MDA-MB-361, MDA-MB-453, SK-BR-3, SW527, T47D, ZR-75-30) and 11 ovarian cancer cell lines (A2774, A2780, A2780ADR, CAOV3, OVCAR-3, PA-1, SKOV3, H134, HEY, HOC-7, TR-170), as well as CEM leukemia cells were used in this study. A specification of cell lines is shown in Supplementary Table S1. Cells were maintained in DMEM, α-MEM, or RPMI 1640 medium (Supplementary Table S1) with 10% FCS, antibiotics, and 2 mM glutamine (Gibco). Cells were cultured for up to 20 passages. They were tested for absence of *Mycoplasma* (Venor GeM, Minerva Biolabs, Berlin, Germany). Cell morphology and expression of marker antigens (ErbBs, estrogen receptor, retinoic

acid receptors, fatty acid synthase) were checked to exclude cross-contamination of cell lines (Supplementary Table S1).

Cell growth assays

For growth stimulation, cells were plated at 1.5x10³/well (SK-BR-3) or 3x10³/well (T47D) in 96-well plates, were allowed to attach, and were then exposed to 100 ng/ml EGF for up to 6 days in culture medium containing 5% FCS. Cell numbers were examined using a formazan dye assay (Biomedica, Vienna, Austria). For assessing growth inhibition, cells were plated at 1.5x10³/well in 96-well culture plates. After adhesion, kinase inhibitors were added at various concentrations in 5% FCS in the absence of EGF. After 72 hours, cell numbers were determined. In a separate set of experiments, cell growth and inhibitory drug effects were confirmed by determining cellular DNA content by CyQUANT assay (Invitrogen, Paisley, UK). The cell numbers, given in percent of untreated control, were plotted against the logarithm of the concentration of each drug. Sigmoidal curves were calculated with the Boltzmann equation using GraphPad Prism 4.03 software and the drug concentrations causing 50% reduction of the cell numbers (IC_{50}) were obtained from these curves. The type of interaction (synergistic, additive or antagonistic) between ErbB drugs combined with PI3K/AKT/mTOR antagonists in inhibition of cell growth was determined at the IC_{50}-levels using the geometric isobologram method. To this end, the IC_{50}-values of the single drugs were plotted on the x or y axis, respectively, and a line connecting these two points was drawn. Synergism between both drugs is encountered if the isoeffective point of the drug combination falls below that line, whereas antagonism occurs if the point lies above it [19].

Western blotting

Cells plated in media containing 5% FCS at $5x10^5$ in 60 mm dishes were allowed to adhere and were either immediately exposed to various concentrations of kinase inhibitors for various time periods, or were depleted from FCS for 24 hours before adding drugs. In select experiments, drug-treated cells were challenged with 100 ng/ml EGF alone or together with 1 nM HRG-β1 for up to 20 minutes before lysis. Ligand-dependent phosphorylation of the ErbB system and of downstream effectors was examined in untreated, serum-depleted cells that had been challenged with EGF and/or HRG-β1 as described above. Cells were then lysed, and protein fractions (30 µg/lane) subjected to SDS-PAGE, blotted onto PVDF membranes, and immunostained as described [20] using antibodies against total or phosphorylated (p) ErbB1, ErbB2, ErbB3, ErbB4, MEK1/2, ERK1/2, PTEN, AKT, GSK3ß, mTOR, S6, α/β tubulin, and actin. Membranes were then incubated with peroxidase-tagged secondary antibodies. Antibody-binding was made visible by enhanced chemiluminescence. Specifications of antibodies are shown in Supplementary Table S2.

DNA constructs and transfections

Hyperactive (HA) MEK1 lacking nuclear export signal domain (amino acids 32–51) and harboring activating amino acid substitutions at two phosphorylation sites (S218E, S222D) was kindly provided by H. Kiyama (Osaka City Univ., Japan) [21] and murine HA AKT1 carrying the myristoylated signal of Lck, which targets it to the membrane was kindly provided by D. Efremov (International Center for Genetic Engineering and Biotechnology, Rome, Italy) [22]. Dominant negative, kinase-inactive AKT that harbors a K179M mutation was obtained from M. Sibilia (Medical Univ. Vienna, Austria) [23]. Vectors with or without green fluorescent protein (GFP) were used as negative control plasmids. For transient transfection, $8x10^5$ SK-BR-3, BT-474 or T47D cells were plated

in 6-well plates and allowed to attach. Then, plasmid DNA diluted in Opti-MEM I (Invitrogen) containing Lipofectamine LTX (Invitrogen) was incubated for 30 minutes at room temperature to allow formation of Opti-MEM-DNA-LTX transfection complexes, added to cells and left in cultures for 24 hours. Cells were then allowed to grow for another 24 hours in the absence of exogenous plasmid DNA. These transfected, polyclonal cell populations were trypsinized, and subjected to cell growth assays or to Western blot analysis as described above without further clonal selection or enrichment.

Statistical analyses

The levels of significance in differences between IC_{50}-values produced by pelitinib in individual SK-BR-3 transfectants were determined by one-factorial analysis of variance (ANOVA) followed by Scheffe test. In all other experiments, the Student's t-test for unpaired samples was applied to examine the significance in differences of growth in drug-treated cells. p-values ≤ 0.05 were considered significant.

Results

Irreversible ErbB blockers are stronger inhibitors of growth of breast and ovarian cancer cells compared to reversible ErbB-targeting drugs

The growth-inhibitory effects of irreversible and reversible ErbB-targeting drugs were examined and compared in various breast and ovarian cancer cell lines. Results obtained by formazan dye assay were found to match well with the CyQUANT assay in all cases tested. As shown in Table 1, the first generation ErbB1 inhibitors gefitinib and erlotinib, and the ErbB1/2 inhibitor lapatinib were marginally or not at all effective in inducing growth arrest (IC_{50}-values: >5 µM). However, the irreversible ErbB inhibitors pelitinib and canertinib were found to block growth of most breast and ovarian cancer cells. The IC_{50}-values produced by canertinib, a drug targeting all three ErbB kinases (ErbB1, -2, and -4) [4, 15, 16-18], were comparable to those produced by pelitinib, which targets ErbB1 and –2 [4, 12-14]. Despite their more potent effects, IC_{50}-values produced with pelitinib and canertinib varied from cell line to cell line (IC_{50}: 1.20–15.40 µM). Whereas the breast cancer cell lines MDA-MB-453, SK-BR-3, ZR-75-30 and MDA-MB-361, and the ovarian carcinoma lines H134, A2780 and HOC-7 were inhibited at concentrations (IC_{50}: 1.2–4.4 µM) that are within a pharmacological range [24, 25], the breast carcinoma cell lines BT20, MCF-7, and T47D, and the ovarian carcinoma line CAOV3 showed no response at reasonable drug concentrations (IC_{50}: >5µM) (Table 1, Supplementary Fig. S1). None of the examined cell lines were found to harbor mutations in the *EGFR/ErbB1* gene (W. Shabbir and T.W. Grunt unpublished observation). In control experiments, proliferation of ErbB-negative CEM leukemia cells [26] was not affected by pelitinib or canertinib (Supplementary Fig. S1) suggesting that drug-dependent growth inhibition was indeed mediated by blockade of the ErbB system and not by interference of the drugs with other kinases. The

sensitivity of breast cancer cells against the growth inhibitory action of irreversible ErbB drugs correlated with ErbB2 (pelitinib $p<0.05$, canertinib $p<0.01$), but not with ErbB1, -3, or -4 protein levels (Table 2). Unfortunately, in the clinical setting, only about 30% of ErbB2 overexpressing breast cancers actually do respond to ErbB-targeted therapy [27, 28]. This indicates that ErbB2 overexpression is not the only molecular determinant for sensitivity against ErbB-specific drugs.

Irreversible ErbB blockers inhibit ErbB receptor phosphorylation in drug-sensitive SK-BR-3 and in drug-resistant T47D cells

The efficacy of pelitinib and canertinib in abolishing ErbB receptor phosphorylation was examined in SK-BR-3 and T47D cells. Serum-depleted, quiescent cell cultures were exposed to the drugs and then challenged with EGF, which binds ErbB1 and primarily activates ErbB1 and -2, and with heregulin-β1 (HRG-β1) that binds ErbB3 and -4 and activates ErbB2, -3, and -4, and to lesser extent ErbB1 [29, 30]. As shown in Fig. 1a, the drugs completely abrogated both ligand-activated and constitutive phosphorylation of ErbB1 and ErbB3 as well as ErbB4 in the two cell lines, and they blocked ligand-activated phosphorylation of ErbB2 in T47Dcells. In contrast, neither pelitinib nor canertinib could abolish constitutive phosphorylation of ErbB2 in SK-BR-3 and T47D cells. Instead, the drugs were found to downregulate the steady-state level of ErbB2 in a dose-dependent manner in both cell lines. Next, we examined ErbB family receptors and their downstream mediators in ErbB drug-sensitive SK-BR-3 cells and in drug-resistant T47D cells. Notably, despite marked differences in ErbB receptor expression, SK-BR-3 (much ErbB1 and -2, less ErbB3, no ErbB4) and T47D (little ErbB1 and -2, much ErbB3 and -4) exhibited similar levels of spontaneous AKT and ERK1/2 expression/phosphorylation, and expressed equal amounts of PI3K antagonist PTEN in the presence of serum (Fig. 1b). In serum-free conditions, however, cells

were unable to maintain high levels of pAKT and pERK1/2 despite appreciable baseline phosphorylation of ErbB2 and –3, and required EGF to fully activate ErbB1, -2, and -3 (but not ErbB4) phosphorylation and to induce AKT and ERK1/2 phosphorylation (Fig. 1c). This suggests that a certain threshold level of composite phosphorylation of ErbB1, -2, and -3 is required for full phosphorylation of AKT and ERK1/2. Surprisingly, EGF-induced stimulation of serum-supported cell growth, which is characterized by appreciable levels of AKT and ERK1/2 phosphorylation (Fig. 1b), was stronger in T47D than in SK-BR-3 (Fig. 1d). Thus, although T47D cells are resistant to the growth-inhibitory action of ErbB drugs, they are responsive to EGF stimulation. In both cell lines, the ErbB system is functional and responsive to ligand- and inhibitor-mediated modulation, irrespective of the relative sensitivity/resistance of the cells against the growth inhibitory action of the irreversible ErbB-targeting drugs.

Resistance of breast and ovarian cancer cells against irreversible ErbB-targeting drugs correlates with drug-refractory PI3K/AKT signaling

Pelitinib and canertinib rapidly and completely erased phosphorylation of AKT in sensitive (MDA-MB-453, SK-BR-3 and BT-474), but not in resistant breast cancer cells (MDA-MB-231, MCF-7 and T47D). In fact, drug-refractory cells up- rather than down-regulated pAKT levels upon drug exposure and maintained these levels during the whole treatment period (Fig. 2a-c). We asked whether the residual AKT activity can be caused by autocrine insulin-like growth factor-I (IGF-I) activity, which might cause stimulation of PI3K/AKT and thus may cause resistance against ErbB-targeting drugs [31, 32]. However, as visible in Supplementary Fig. S2, the IGF-IR inhibitor picropodophyllin (PPP) was unable to impair pelitinib-resistant phosphorylation of AKT in T47D cells, although it moderately reduced the growth of these cells. Thus, IGF-IR is not contributing to constitutive pAKT levels in these cells. PI3K/AKT signaling also

appears to determine ErbB drug resistance in ovarian carcinoma cells. In fact, Figure 2d demonstrates that the ability of pelitinib to block pAKT was more pronounced in sensitive HOC-7 than in resistant CAOV-3 cells (see also Table 1 and Supplementary Fig. S1E,F). By contrast, drug-sensitivity of breast and ovarian cancer cells did not correlate with drug-mediated inhibition of p-ERK1,2 levels (Fig. 2, Table 2). These data suggest that silencing phosphorylation of AKT, but not of ERK, may be crucial for the growth inhibitory effects of ErbB-blocking drugs in breast and ovarian cancer cells, and that AKT-related signaling cascades may contribute to resistance against these drugs.

Constitutively active AKT1 induces resistance, whereas dominant negative AKT promotes sensitivity of breast cancer cells against pelitinib

To assess the potential role of AKT as a resistance-related signaling molecule in cancer cells, we employed a constitutively active variant of *AKT1*. Drug-sensitive SK-BR-3 were transfected with this constitutively active *AKT1* gene [22] or with *MEK1* [21]. Overexpression/hyperphosphorylation of transgenes and downstream effector molecules (GSK3β, mTOR, S6, ERK1,2) was confirmed by Western blot analysis of the unselected polyclonal transfectant cell populations. Unlike wildtype, control-vector-, and MEK1-transfected cells, AKT1-transfectants exhibited an excess of pAKT that was refractory to the inhibitory activity of pelitinib. Accordingly, phosphorylation of AKT-downstream targets (GSK3β, mTOR, S6) was not or only slightly affected by pelitinib in these cells. In turn, forced expression of hyperactive MEK1 was found to phosphorylate ERK1/2 and to make pERK1/2 expression unresponsive to pelitinib (Fig. 3a). These transfectants were used to directly examine the relative contribution of MAPK and PI3K signaling to drug resistance against ErbB-targeting drugs. As shown in Fig. 3b and 3c, pelitinib was found to inhibit growth of wildtype, sham-, vector-,

GFP-, and MEK1-transfected SK-BR-3 cells with similar potency, but did not inhibit the growth of AKT1-transfected cells. Induction of pelitinib-resistance by transient transfection of constitutively active *AKT1* was also observed in unselected, polyclonal BT-474 cells (Fig. 3d,e). In contrast, introduction of the dominant negative, kinase-inactive K179M mutant of *AKT* into T47D cells reduces phosphorylation of downstream S6 ribosomal protein and induces sensitivity against pelitinib in these cells (Fig. 3f-h). Collectively, our data demonstrate 1) that forced expression of dominant active MEK1 and AKT1 effectively stimulates MAPK and PI3K pathways in SK-BR-3 cells, respectively, and renders these pathways resistant to pelitinib, 2) that PI3K/AKT-, but not MAPK-hyperactivity, confers growth-resistance of the cells against pelitinib, and 3) that blockade of AKT downstream signaling can reinstall pelitinib-sensitivity in drug-resistant cells.

NVP-BEZ235 and other PI3K/AKT/mTOR antagonists abrogate PI3K signaling in ErbB drug-resistant breast cancer cells

This prompted us to examine drugs that silence the PI3K signaling cascade at distinct levels in SK-BR-3 and T47D cells. NVP-BEZ235 inhibits all four PI3K isoforms and both mTOR complexes (mTORC1, mTORC2) [33], rapamycin blocks mTORC1 and mTORC2 [34], whereas Akti-1/2 inhibits AKT1 and AKT2. These agents decreased phosphorylation of AKT and of S6 in a dose-dependent manner, but left pERK1/2 levels unaffected (Supplementary Fig. S3). Interestingly, low concentrations of NVP-BEZ235 inhibited pAKT(Ser473), but not pAKT(Thr308), which was even up-regulated in T47D cells. This may be explained by higher affinity of NVP-BEZ235 to mTOR than to PI3K [33]. NVP-BEZ235-mediated blockade of mTORC1 abolishes mTOR-dependent negative feedback-regulation of PI3K and PDK1 and thus stimulates phosphorylation of AKT at Thr308. In contrast, concurrent NVP-BEZ235-mediated

blockade of mTORC2 leads to reduced pAKT(Ser473) levels [35]. Accordingly, dephosphorylation of Thr308 was observed only at higher doses of NVP-BEZ235, which inhibit both mTOR and PI3K. In addition, we examined the MEK1/2 antagonists AZD6244 and U0126 and found that both inhibitors efficiently silenced the MAPK cascade in SK-BR-3 and T47D cells (Supplementary Fig. S4).

NVP-BEZ235 and other PI3K/AKT/mTOR antagonists selectively block growth in ErbB drug-refractory breast cancer cells and override resistance against ErbB blockers

ErbB drug-resistant T47D were more responsive to growth arrest by the PI3K pathway blockers NVP-BEZ235, rapamycin and Akti-1/2 than ErbB drug-sensitive SK-BR-3 cells (Fig. 4a-c). Moreover, cotreatment of T47D cells with PI3K- and ErbB drugs significantly reduced the IC_{50}-values for the ErbB drugs from 13–15µM in the absence, to a pharmacologically useful dose range of <4.6 µM in the presence of low amounts (0.01 – 0.5µM) of PI3K antagonists. The responsiveness of ErbB drug-sensitive SK-BR-3 cells was also significantly enhanced by some of the PI3K-silencing drugs (Fig. 4a,b,d). Notably, isobologram analysis revealed strong synergisms for the combinations of NVP-BEZ235 or rapamycin with ErbB drugs, and weak synergism for Akti-1/2 plus ErbB blockers (Fig. 4e). Moreover, growth of MDA-MB-231 breast cancer cells, which are resistant to ErbB drugs (Table 1, Supplementary Fig. S1) and express wildtype PIK3CA (Table 2), was also synergistically blocked by cotreatment with pelitinib or canertinib and NVP-BEZ235 (Fig. 4f). On the other hand, the MEK1/2 inhibitors AZD6244 and U0126 did not inhibit the growth of T47D or SK-BR-3 cells (IC_{50}-values far beyond 60 µM), nor did they improve the responsiveness of these cells against pelitinib and canertinib (Fig. 4a-d); they rather induced slight antagonism (Fig. 4e). Thus, unlike MEK1/2 inhibitors, PI3K/AKT/mTOR antagonists cooperate with

ErbB drugs and render breast cancer cells sensitive to pelitinib and canertinib. This demonstrates that PI3K/AKT/mTOR antagonists, but not MEK1/2 inhibitors, can overcome resistance against novel irreversible ErbB blockers.

Discussion

Novel small molecular drugs that irreversibly inhibit ErbB family receptors reveal higher efficacy than first-generation reversible ErbB inhibitors (Table 1) [4, 5], and there was some hope that ErbB-positive tumor cells would less frequently exhibit resistance against these drugs [6]. Unfortunately, however, we demonstrate that several breast and ovarian cancer cells are insensitive to the growth inhibitory effects of irreversible ErbB blockers pelitinib and canertinib, which target ErbB1/2 or ErbB1/2/4, respectively [4, 12-16, 36]. Very high concentrations of pelitinib may block several other non-ErbB kinases as well, whereas canertinib appears to retain specificity for the ErbB kinases, even at high concentrations [37, 38]. Interestingly, the growth of ErbB-negative CEM leukemia cells was completely unresponsive to ErbB inhibitors, even at very high doses (Supplementary Fig. S1), suggesting that the biological drug effects seen in the tumor cells were not caused by non-ErbB related inhibitory activities of the drugs. We thought that elucidating the molecular mechanisms of unresponsiveness of ErbB-positive tumors against such irreversible ErbB inhibitors may be helpful for the development of strategies to overcome resistance. In line with previous studies [9, 10], the sensitivity of breast cancer cells to ErbB-targeting drugs significantly correlated with ErbB2 expression levels (pelitinib $p<0.05$, canertinib $p<0.01$), but did not correlate with expression of ErbB1, -3, or -4 (Table 2). Moreover, the lack of activity of the ErbB1 blockers erlotinib and gefitinib argues against ErbB1 inhibition as a critical determinant of ErbB drug sensitivity in breast and ovarian cancer cells. Pelitinib and canertinib inhibit both ligand-stimulated and constitutive phosphorylation of ErbB1, -3, and –4. However, the drugs cannot abrogate constitutive phosphorylation of ErbB2. This suggests that pelitinib and canertinib bind to the ErbB2 kinase only, if the domain is in the inactive, but not if it is

in the active conformation. Interestingly, there is a precedent to this. Kumar et al. [39] recently reported that lapatinib binds to the inactive, but not to the active, conformation of ErbB1. Nevertheless, pelitinib and canertinib attenuated the steady-state level of the ErbB2 protein in both cell lines. Interestingly, a similar pelitinib- and canertinib-dependent downregulation of ErbB2 has recently been observed in ovarian cancer [40]. This lets us speculate that complexing between inhibitor and target may result in a conformational change that would trigger target degradation. This entails reduced overall levels of pErbB2 leading to deficient transphosphorylation/activation of ErbB3 (Fig. 1a). ErbB3, when dimerized with other ErbB members (mainly ErbB2), is known to predominantly stimulate the PI3K/Akt growth and survival pathway [45]. Overall, our data are consistent with the notion that interference with the ErbB3-PI3K axis is essential for ErbB drugs to affect cell growth. The ErbB system was functional and responsive to EGF in both ErbB blocker-sensitive SK-BR-3 cells and -resistant T47D cells. Pelitinib and canertinib blocked ErbB phosphorylation in responsive and in drug-resistant cancer cell lines (Fig. 1a). Thus, differential ErbB inhibitory efficacy or differential cellular drug uptake did not account for the variable sensitivity of various cancer cells to ErbB-targeting drugs.

Recent data suggest that mutations in *PTEN* may contribute to resistance of tumor cells against various anti-cancer agents [41-43]. In the current study, mutation in *PTEN*, rarely seen in breast cancer patients [41], was found in one of nine cell lines studied. This line (MDA-MB-453) revealed an E307K exchange in PTEN and was most sensitive to the ErbB-targeting drugs. This suggests that *PTEN* mutations not always confer resistance against ErbB inhibitors. Moreover, we found that drug-sensitive SK-BR-3 cells and drug-resistant T47D cells express similar levels of wildtype PTEN protein (Fig. 1b). Therefore, loss of PTEN e.g. due to promoter hypermethylation [42] or proteasomal degradation [43] apparently did not account for

differential drug sensitivity. Another resistance-related gene is PIK3CA. However, in this study, *PIK3CA* mutations [41] were found in both drug-sensitive and drug-resistant breast cancer cells (Table 2) suggesting that *PIK3CA* mutations are not the sole determinant for development of resistance against ErbB-targeting drugs.

The relative contribution of the MAPK and the PI3K pathways to resistance against ErbB-targeting drugs in breast and ovarian cancer cells is still a matter of debate. Several reports suggested that constitutive PI3K signaling confers ErbB drug resistance [9, 32, 44, 45]. Other studies have identified MAPK signaling as a potential driving force that contributes to resistance [46-49]. Depending on the tumor cell type other mechanisms may also play a role in drug resistance [50, 51]. The issue gets further complicated by the occurrence of multiple crosstalk regulations between MAPK and PI3K pathways [52, 53]. We observed that resistance of breast and ovarian cancer cells against ErbB blocking agents correlates with drug-refractory phosphorylation of AKT, but not with drug effects on ERK1,2. In some resistant cells, pelitinib and canertinib even caused a dose-dependent increase in expression of pAKT (Fig. 2). This may be explained by drug-mediated silencing of MAPK, which abolishes negative MAPK-PI3K feedback loops [52, 53]. Therefore, our data suggest that silencing of AKT signaling is crucial for ErbB drugs to exert anticancer activity in malignant breast and ovarian disease.

Persistent AKT signaling can generally be caused by several mechanisms including activation by other ligand/receptor systems. However, the IGF-I/IGF-IR cascade, which has been linked to resistance against ErbB blockers [31, 32], did not account for unresponsiveness of T47D against irreversible ErbB drugs, since the IGF-IR inhibitor PPP, though moderately impairing cell proliferation, did not diminish pAKT levels in these cells. Notably, T47D cells harbor the H1047R activating mutation in the PIK3CA catalytic domain of PI3K, which could contribute to constitutive AKT activity. However,

our data show that cell lines with mutant (e.g. MDA-MB-453) and wildtype PIK3CA (e.g. SK-BR-3) not only can reveal similar constitutive and drug-modulated pAKT levels, but may also be equally sensitive to pelitinib and canertinib (compare Fig. 1b,c and 2a with Table 2). On the other hand, MDA-MB-231 contain wildtype, whereas T47D express mutant PIK3CA; yet both cell lines are resistant to pelitinib and canertinib. Moreover, the PI3K/mTOR inhibitor NVP-BEZ235 efficiently blocks growth of both cell lines on its own (T47D: IC_{50} 0.058µM, MDA-MB-231: IC_{50} 0.067µM) and synergizes with the ErbB targeting drugs pelitinib and canertinib (Fig. 4f), irrespective of the mutation status of PIK3CA. Thus, the activation status of AKT appears to be independent of the presence of *PIK3CA* mutations in the cell lines studied**.** In fact, it is quite questionable whether activating *PIK3CA* mutations stimulate AKT downstream signaling and exert oncogenic activity. It has also been shown that *PIK3CA* mutations exert relatively mild effects on tumor cell growth [54]. Moreover, recent reports [28, 55, 56] failed to demonstrate a correlation between the presence of *PIK3CA* mutations and expression of pAKT, nor did they provide evidence that such mutations shorten survival or elevate chemoresistance in breast tumor patients [55]. Consistently, recent evidence indicates that when PDK1, which is overexpressed in a large proportion of breast cancers [8], is activated by mutant PIK3CA, it phosphorylates SGK3 rather than AKT [56]. Therefore, PIK3CA mutations may at best contribute to drug-refractory AKT signaling and growth resistance, but are definitely not the sole causes for it. This is further supported by the fact that the PI3K/mTOR inhibitor NVP-BEZ235 sensitizes resistant breast cancer cells against ErbB drugs irrespective of their PIK3CA mutation status.

In order to directly examine the relative contribution of MAPK and PI3K pathways in ErbB drug resistance we overexpressed constitutively active MEK1 or constitutively active AKT1 in ErbB inhibitor-sensitive SK-BR-3 and BT-474 cells. AKT1 rendered the

cells fully resistant to pelitinib, whereas MEK1 had no effect (Fig. 3b-e). Conversely, dominant negative AKT is able to reinstall drug-sensitivity in pelitinib-resistant T47D cells (Fig. 3f-h). Importantly, these experiments directly demonstrate 1) that pAKT down-regulation is required and is sufficient for growth arrest induced by ErbB inhibitors, 2) that ErbB drug sensitivity is independent from MAPK blockade, and 3) that resistance occurs independent of the molecular mechanisms of AKT hyperactivity. Thus, drug-dependent downregulation of pAKT may serve as a biomarker for breast and ovarian cancer cell sensitivity against irreversible ErbB inhibitors. Notably, we have found that ErbB drug-resistant cells are particularly sensitive to the PI3K/AKT/mTOR inhibitors NVP-BEZ235, rapamycin and Akti-1/2. In contrast, the MEK1/2 antagonists AZD6244 or U0126 did not affect growth of these cells (Fig. 4a,c). Most importantly, NVP-BEZ235, rapamycin and Akti-1/2, when used in combination with ErbB blockers, significantly increased the antiproliferative efficacy and reduced the IC_{50}-values of pelitinib and canertinib in T47D cells into a pharmacologically reasonable range (Fig. 4a,d). This effect was obviously dependent on mTOR inhibition, since rapamycin, which targets mTOR, was as efficient as the other compounds, which either block upstream PI3K along with mTOR (NVP-BEZ235) or inhibit AKT, which is also upstream of mTOR (Akti-1,2).

In summary, our data show that 1) abrogation of PI3K/AKT/mTOR reinstalls ErbB drug sensitivity and 2) that ErbB/PI3K/mTOR co-targeting using irreversible ErbB inhibitors along with NVP-BEZ235 or mTOR blockers represents a promising strategy to overcome resistance against irreversible ErbB inhibitors. Thus, from our data it may be tempting to speculate that NVP-BEZ235, which is currently in phase I/II clinical trials for advanced breast cancer, may have clinical utility when combined with novel ErbB-targeting drugs.

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Figure Legends

Fig. 1 Ligand- and drug-dependent modulation of ErbB- and downstream effector phosphorylation, and of growth of ErbB drug-sensitive SK-BR-3 and -resistant T47D cells. **a** Pelitinib (left) and canertinib (right) inhibit ErbB phosphorylation in drug-sensitive and -resistant cells. Serum-depleted cells were exposed to drugs and then stimulated for 3min with 100ng/ml EGF and 1nM HRG-β1. Serum- (**b**) and EGF-dependent (**c**) ErbB, AKT and ERK expression/phosphorylation. Note: both lines express PTEN. **d** EGF stimulates growth of T47D, but not of SK-BR-3. Cell numbers given as optical density-values. Means±SD, n≥3

Fig. 2 Drug-mediated down-regulation of pAKT, but not of pERK1,2, as biomarker for drug efficacy in breast (**a-c**) and ovarian (**d**) cancer cells. Sensitive and resistant cells grown in the presence or absence of 5% serum were exposed for 6h to various concentrations of pelitinib (**a**) or canertinib (**b**). Cells were then either directly lysed in RIPA buffer or stimulated with EGF (100ng/ml, 20min) before lysis. **c** Rapid and persistent down-regulation of pAKT and pGSK3β in pelitinib-sensitive, but not in -resistant cells

Fig. 3 Exogenous expression of hyperactive (HA) AKT1, but not of HA MEK1 induces ErbB drug resistance, whereas dominant negative (DN) AKT promotes sensitivity. **a** Wildtype SK-BR-3 (SKBR3) transiently transfected with vector (SKBR3pcDNA3), HA MEK1 (SKBR3MEK1) or HA AKT1 (SKBR3AKT1) were treated with or without pelitinib and analyzed for MEK1,2, ERK1,2, AKT, GSK3β, mTOR, and S6 expression/phosphorylation. Dose-dependent growth inhibition (**b,d**) and resulting IC_{50}-values (**c,e**) after 72h pelitinib-exposure of wildtype (SKBR3, BT474), mock-

transfected (SKBR3LTX), empty vector-transfected (SKBR3pcDNA3, BT474pcDNA3), GFP-transfected (SKBR3GFP), HA MEK1-transfected (SKBR3MEK1) or HA AKT1-transfected (SKBR3AKT1, BT474AKT1) SK-BR-3 and BT-474 cells. **f** Wildtype T47D (T47D) transiently transfected with GFP (T47DGFP) or DN AKT (T47DDNAKT) were treated with or without pelitinib and analyzed for AKT and S6 expression/phosphorylation. Dose-dependent growth inhibition (**g**) and resulting IC_{50}-values (**h**) after 72h pelitinib-exposure of wildtype (T47D), GFP-transfected (T47DGFP) or DN AKT-transfected (T47DDNAKT) T47D cells. Means±SD, n≥3. One-factorial ANOVA and Scheffe-test. ***, p<0.001 for SKBR3AKT1 *vs.* SKBR3GFP (**c**), for BT474AKT1 *vs.* BT474pcDNA3 (**e**), and for T47DDNAKT *vs.* T47DGFP (**h**)

Fig. 4 Differential effects of drugs blocking PI3K/AKT/mTOR or MAPK signaling on growth and ErbB drug sensitivity of breast cancer cells. ErbB drug-resistant T47D (**a**) or -sensitive SK-BR-3 cells (**b**) were exposed for 72h to various concentrations of PI3K/mTOR inhibitor NVP-BEZ235 (BEZ), mTOR inhibitor rapamycin (Rapa), AKT1/2 inhibitor Akti-1/2 (Akti), or MEK1/2 inhibitors AZD6244 (AZD) or U0126 (U0) alone (**a**,**b**, left panels) or together with pelitinib (Pel) (**a**,**b**, central panels) or canertinib (Can) (**a**,**b**, right panels) and the IC_{50}-values (**c**,**d**) were determined from the dose-response relationships given in (**a**) and (**b**) (details see Material and methods). Means±SD, n≥3. Student's t-test, one-tailed, unpaired, equal variances. (**c**) ***, p<0.001 for T47D *vs.* SKBR3. (**d**) **, p<0.01 and ***, p<0.001 for ErbB drug (pelitinib, canertinib) alone *vs.* ErbB drug plus PI3K pathway inhibitor (BEZ, Rapa, Akti). **e** Isobologram analysis. The IC_{50}-isoeffective points are shown. Combinations of pelitinib or canertinib with NVP-BEZ235, rapamycin and Akti1/2 yield synergistic effects, respectively, whereas ErbB drugs combined with U0126 reveal slight antagonism. **f** ErbB drug-resistant MDA-MB-231 cells, which express wildtype PIK3CA, were exposed for 72h to various

concentrations of the PI3K/mTOR inhibitor NVP-BEZ235 (BEZ) alone (upper left) or together with pelitinib (Pel) (upper central) or canertinib (Can) (upper right) and the IC_{50}-values were determined (lower left) (details see Material and methods). Means±SD, n≥3. Student's t-test, one-tailed, unpaired, equal variances. **, p<0.01 for ErbB drug (pelitinib, canertinib) alone *vs.* ErbB drug plus BEZ. Isobologram analysis at the IC_{50}-isoeffective points reveal that combinations of pelitinib (lower central) or canertinib with BEZ (lower right) yield synergistic growth inhibition, respectively.

Table 1 The antiproliferative efficacy (IC_{50}) of novel, irreversible ErbB inhibitors compared to first-generation, reversible ErbB blockers

	IC_{50} (µM)				
Type of inhibition	**Irreversible**		**Reversible**		
Targets	**ErbB1/2**	**ErbB1/2/4**	**ErbB1/2**	**ErbB1**	**ErbB1**
Drug name	**Pelitinib**	**Canertinib**	**Lapatinib**	**Erlotinib**	**Gefitinib**
Breast cancer					
MDA453	2,95	2,38	6,53	13,87	6,20
SKBR3	3,65	2,45	5,81	8,28	5,96
ZR7530	4,26	3,54	7,31	>20,00	7,61
MDA361	4,38	4,34	>20,00	17,40	16,53
BT474	4,45	5,70	8,58	>20,00	12,62
SW527	5,04	5,77	12,57	>20,00	18,36
MDA231	5,43	6,85	15,38	>20,00	>20,00
BT20	6,43	8,88	19,18	>20,00	>20,00
MCF7	8,16	7,86	10,03	>20,00	>20,00
T47D	15,40	12,64	>20,00	>20,00	>20,00
Ovarian cancer					
H134	1,20	2,37	10,04	>20,00	>20,00
A2780	1,51	2,70	19,72	>20,00	11,31
HOC7	2,92	2,21	18,34	>20,00	>20,00
A2780ADR	4,28	5,10	>20,00	>20,00	17,32
TR170	5,37	6,54	>20,00	>20,00	>20,00
PA1	5,47	4,76	18,49	>20,00	>20,00
A2774	5,60	6,86	>20,00	>20,00	>20,00
SKOV3	5,86	7,43	15,85	>20,00	>20,00
OVCAR3	6,84	5,16	>20,00	>20,00	>20,00
HEY	8,00	6,27	>20,00	>20,00	>20,00
CAOV3	12,92	13,16	19,96	>20,00	>20,00

Table 2 Overview on ErbB expression, downstream pathways and responsiveness of breast cancer cells against the irreversible ErbB1/2 inhibitor pelitinib and the irreversible pan-ErbB inhibitor canertinib

										Correlation ErbB - IC_{50}			
										Pelitinib		Canertinib	
	MDA453	**SKBR3**	**ZR7530**	**MDA361**	**BT474**	**MDA231**	**BT20**	**MCF7**	**T47D**	**r[a]**	**p-value**	**r**	**p-value**
ErbB Expression[b]													
ErbB1	0,03	2,98	?[c]	0,42	1,00	2,42	3,97	0,13	0,41	-0,20	-[d]	-0,01	-
ErbB2	1,49	1,89	1,80	1,41	1,00	0,75	0,29	0,03	0,12	-0,76	<0.05	-0,90	<0.01
ErbB3	0,52	0,54	?	0,10	1,00	0,24	0,70	0,43	0,83	0,33	-	0,42	-
ErbB4	1,56	0,07	?	0,78	1,00	0,48	?	0,87	2,45	0,73	-	0,71	-
Downstream Mutation[e]													
PTEN	E307K	wt[f]	wt	wt	wt	wt	wt	wt	wt				
PIK3CA	H1047R	wt	wt	E545K,K567R	K111N	wt	P539R	E545K	H1047R				
KRAS	wt	wt	wt	wt	wt	G13D	wt	wt	wt				
BRAF	wt	wt	I326T	wt	wt	G464V	wt	wt	wt				
Growth Inhibition by irreversible ErbB Inhibitors (IC_{50}, µM)[g]													
Pelitinib	2,95	3,65	4,26	4,38	4,45	5,43	6,43	8,16	15,40				
Canertinib	2,38	2,45	3,54	4,34	5,70	6,85	8,88	7,86	12,64				
Downstream Effect of irreversible ErbB Inhibitors[g]													
pAKT	decreased	decreased	?	?	decreased	increased	?	increased	increased				
pERK	not affected	decreased	?	?	decreased	not affected	?	decreased	decreased				

[a]correlation coefficient; [b]own data, obtained from densitometry of Western blot autoradiographs and expressed as optical density relative to BT474; [c]not determined; [d]not significant; [e]Ref. 41; [f]wildtype; [g]this report