Expression of Epidermal Growth Factor Receptor or ErbB3 Facilitates Geldanamycin-Induced Down-Regulation of ErbB2

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Abstract

Overexpression of the epidermal growth factor receptor (EGFR), ErbB2, and ErbB3 promotes growth and anti-apoptotic signaling. Overexpression of ErbB2 in breast cancer is associated with poor clinical outcome, and ways of down-regulating ErbB2 are important as therapeutic approaches. In contrast to EGFR, ErbB2 has been shown to be endocytosis deficient. However, down-regulation of ErbB2 can be induced by incubation of cells with geldanamycin and geldanamycin derivatives, counteracting the stabilizing function of heat shock protein 90 on ErbB2. In the present study, we have made use of stably transacted isogenic cell lines expressing ErbB2 only or ErbB2 together with EGFR and/or ErbB3. We now show that whereas ErbB2 can be down-regulated by incubation with geldanamycin in cells expressing ErbB2 only, the rate of geldanamycin-induced down-regulation increases significantly when the cells additionally express EGFR and/or ErbB3. This increase does, however, not correlate with activation/phosphorylation of ErbB2. The potential of heterodimer formation in ErbB2-positive breast cancer cells could thus turn out to be prognostically predictive with respect to outcome of treatment with geldanamycin derivatives. (Mol Cancer Res 2009;7(2):275–84)

Introduction

The epidermal growth factor (EGF) receptor (EGFR) family includes four members: EGFR/ErbB1, ErbB2, ErbB3, and ErbB4. The ErbB proteins share a common structural organization with an extracellular ligand-binding domain, a single-membrane spanning region and a cytoplasmic tyrosine kinase domain (reviewed in refs. 1, 2). The different ErbB proteins do, however, have different properties. EGFR, ErbB3, and ErbB4 bind multiple ligands (reviewed in refs. 3-5), whereas no known ligand interacts with ErbB2 (6, 7). Ligand binding induces conformational changes causing exposure of the extracellular dimerization arm allowing formation of homodimers and/or heterodimers (reviewed in refs. 2, 4). ErbB2 has a constitutively exposed dimerization arm (2) and is therefore the preferred dimerization partner (8, 9). Heterodimers containing ErbB2 have enhanced and prolonged signaling (10), which is induced by phosphorylation of COOH-terminal tyrosine residues serving as docking sites for adaptor and effector proteins. Each ErbB protein exhibits a unique phosphotyrosine profile that allows binding of different enzymes and adaptors (reviewed in refs. 5, 11, 12). The tyrosine kinase activity of ErbB3 is deficient (13), making ErbB3 incapable of forming active signaling homodimers. Phosphorylation of ErbB3 thus depends on dimerization with other ErbB proteins (14, 15), and the ErbB2-ErbB3 complex is reportedly the most potent in oncogenic signaling (7, 10, 16). It is well established that the EGFR is rapidly endocytosed on ligand binding followed by recycling or degradation (reviewed in refs. 17). All other ErbB proteins, however, show impaired ligand-induced endocytosis (18-20), and ErbB2 has clearly been shown to be internalization resistant (21-23). In contrast to EGFR and ErbB2, ErbB3 was shown to have a rapid turnover at the plasma membrane, and this turnover was reported to depend on the ubiquitin ligase Nedd1 and on proteasomal activity (24, 25).

ErbB2 has its dimerization loop constitutively exposed, and overexpression of ErbB2 efficiently drives heterodimerization (reviewed in ref. 26). Due to its internalization resistance, overexpressed ErbB2 also inhibits endocytosis of EGFR-ErbB2 heterodimers (27). ErbB2 is thereby a causal factor in carcinogenic processes. For this reason, mechanistic insight into how ErbB2 can be down-regulated is called for. In the current work, we have investigated whether other ErbB proteins (EGFR and ErbB3) affect the efficiency of geldanamycin-induced down-regulation of ErbB2.
Geldanamycin, which is a microbial fermentation product isolated from *Streptomyces hygroscopicus*, has been explored as a potential drug in cancer treatment (28), and the geldanamycin derivate 17-allylamino-17-demethoxygeldanamycin appears to be promising in treatment of cancers with overexpression of ErbB2 (reviewed in ref. 29). Geldanamycin binds to the conserved ATP/ADP-binding pocket of the heat shock protein 90 (Hsp90) chaperone (30, 31). The Hsp90 chaperone complex, which stabilizes several client proteins (ErbB2, Akt, p53, etc.), cycles between at least two conformations depending on whether ADP or ATP is bound to Hsp90 (reviewed in ref. 32). In the ADP-bound state, Hsp90 is associated with Hsp70 as well as other proteins, whereas in the ATP-bound state Hsp70 is released. When geldanamycin is bound to Hsp90, the chaperone complex resembles the ADP-bound state with Hsp70 associated with Hsp90, and geldanamycin-induced degradation of ErbB2 has been suggested to be accompanied by recruitment of the ubiquitin ligase CHIP to Hsp70 (33, 34). This reportedly leads to polyubiquitination and proteasomal degradation (35). However, recent observations show that ErbB2 is internalized intact in a clathrin-dependent manner and eventually degraded in lysosomes on exposure to geldanamycin (36, 37).

In contrast to ErbB2, mature wild-type EGFR and ErbB3 do not associate with Hsp90 (38, 39). Because it is of interest to understand whether/how EGFR and ErbB3 affect geldanamycin-induced endocytosis of ErbB2, we have in the present study compared geldanamycin-induced endocytosis of ErbB2 in cells expressing ErbB2 only with geldanamycin-induced endocytosis of ErbB2 in cells expressing ErbB2 and other members of the ErbB family. Such studies have been possible because we have taken advantage of porcine aortic endothelial (PAE) cells not harboring endogenous ErbB proteins. PAE cells have been stably transfected with a plasmid encoding wild-type EGFR (40, 49, 54).

### Table 1. PAE Cells Used in the Current Study

<table>
<thead>
<tr>
<th>Cell line</th>
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<tr>
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**NOTE:** The PAE cell lines used in the current study were constructed by stable transfection of PAE cells lacking endogenous ErbB proteins. The PAE.B2 cell line is a previously described PAE cell line stably transfected with a plasmid encoding phosphorylated.

**Results**

ErbB2 Is Endocytosed on Incubation with Geldanamycin in Cells Not Expressing Other ErbB Proteins

To investigate to what extent ErbB2 undergoes geldanamycin-induced down-regulation in cells not expressing other ErbB proteins, we used PAE cells stably transfected with a plasmid encoding ErbB2 (PAE.ErbB2 cells; see Table 1 for overview of PAE cells used in the current study and Supplementary Fig. S1 for Western blots showing the expression of ErbB proteins in each cell line). As different cells show different sensitivity to geldanamycin, we initially confirmed that the concentration used (3 μmol/L) did not induce apoptosis (Supplementary Fig. S2). Flow cytometry analysis was then used to visualize down-regulation of ErbB2 from the plasma membrane. On incubation with geldanamycin for 24 h, a shift in the peak...

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**FIGURE 1.** Geldanamycin-induced endocytosis and down-regulation of ErbB2 in cells expressing ErbB2 only. A. PAE.ErbB2 cells were incubated with or without geldanamycin (3 μmol/L) for 24 h at 37°C, and the cells were fixed and immunostained using mouse anti-ErbB2 antibody. Down-regulation of ErbB2 from the plasma membrane was investigated by flow cytometry. B. PAE.ErbB2 cells were incubated with or without geldanamycin (3 μmol/L) for 4 h, fixed, and immunocytochemically labeled with mouse anti-ErbB2 antibody (TAB250) followed by phycoerythrin-conjugated goat anti-mouse antibody. Down-regulation of ErbB2 from the plasma membrane was investigated by flow cytometry. C. PAE.ErbB2 cells were incubated with or without geldanamycin (3 μmol/L) for 4 h, fixed, and immunocytochemically labeled with mouse anti-ErbB2 antibody (TAB250) followed by Cy5-conjugated donkey anti-mouse antibody and analyzed by confocal microscopy. Arrows, labeling for ErbB2 at the plasma membrane in cells not treated with geldanamycin; arrowheads, vesicular labeling for ErbB2 in cells treated with geldanamycin. D. Rabbit anti-ERK antibody was used as loading control.
representing ErbB2 showed geldanamycin-induced down-regulation (Fig. 1A). Moreover, confocal microscopy analysis showed vesicular localization of ErbB2 on incubation with geldanamycin (Fig. 1B). Previous studies have shown that incubation with geldanamycin induces clathrin-dependent endocytosis of ErbB2 (37) and that ErbB2 in cells incubated with geldanamycin localizes to early as well as to late endosomes (36, 37). Geldanamycin-induced endocytosis of ErbB2 was confirmed by immunoelectron microscopy experiments. Whereas labeling for ErbB2 mainly localized to the plasma membrane in PAE.ErbB2 cells not incubated with geldanamycin (Supplementary Fig. S3), ErbB2 localized to vesicular compartments with morphology resembling early endosomes, as well as late multivesicular endosomes, in cells incubated with geldanamycin for 1 h at 37°C (Fig. 2). It should be noted that the vesicular localization of ErbB2 on incubation of cells with geldanamycin could be observed both when using antibodies to the extracellular part of ErbB2 (Fig. 1B and 2) and when using antibodies to the intracellular part of ErbB2 (Supplementary Fig. S4). This is consistent with the notion that full-length ErbB2 was endocytosed (36, 37). ErbB2 was eventually degraded on incubation of the cells with geldanamycin as shown by Western blots using antibody to an intracellular epitope (Fig. 1C) or antibody to an extracellular epitope (Fig. 1D) in ErbB2. Altogether, these results showed that ErbB2 could be internalized and degraded when cells expressing ErbB2 only were incubated with geldanamycin.

EGFR and ErbB3 Increased the Efficiency of Geldanamycin-Induced Down-Regulation of ErbB2 from the Plasma Membrane

To investigate whether coexpression of the EGFR or ErbB3 could affect the rate of geldanamycin-induced down-regulation of ErbB2 from the plasma membrane, we took advantage of PAE.ErbB2 cells stably transfected with plasmids encoding ErbB2 in addition to EGFR and/or ErbB3 (for an overview of the cells used, see Table 1). These cells (PAE.ErbB2, PAE.ErbB2.EGFR, PAE.ErbB2.ErbB3, and PAE.EGFR.ErbB2) were used in
addition to SKBr3 cells endogenously expressing EGFR, ErbB2, and ErbB3. Down-regulation of ErbB2 from the plasma membrane on incubation with geldanamycin for 6 and 12 h was investigated by flow cytometry (Fig. 3). In PAE.ErbB2 cells, ErbB2 was down-regulated by ~50% after 12 h. However, in cells where either EGFR or ErbB3 was coexpressed with ErbB2 (PAE.ErbB2.EGFR and PAE.ErbB2.ErbB3 cells), down-regulation of ErbB2 further increased significantly on incubation with geldanamycin. Likewise, the geldanamycin-induced down-regulation of ErbB2 in SKBr3 cells was strongly increased compared with in PAE.ErbB2 cells, which express comparable amounts of ErbB2. The same result was observed when measuring geldanamycin-induced down-regulation of ErbB2 in PAE.EGFR.ErbB2 cells, a clone that was selected based on initial transfection with a plasmid encoding EGFR and then further stably transfected with a plasmid encoding ErbB2 (Supplementary Fig. S5). This argues that the enhanced down-regulation reported was not a property of one selected cell clone only. Together, these results show that the presence of EGFR or ErbB3 facilitates geldanamycin-induced down-regulation of ErbB2.

Incubation of PAE.EGFR.ErbB2 Cells with Geldanamycin Did Not in Itself Down-Regulate or Phosphorylate the EGFR

To investigate whether incubation of cells expressing ErbB2 and EGFR with geldanamycin also increased down-regulation of wild-type EGFR, we incubated PAE.EGFR.ErbB2 (Fig. 4) and SKBr3 (Supplementary Fig. S6) cells with geldanamycin. Flow cytometry analysis showed that the amount of EGFR at the plasma membrane was not significantly reduced by incubation with geldanamycin for 12 h. However, as expected, the amount of EGFR at the plasma membrane was reduced when PAE.EGFR.ErbB2 (Fig. 4) or SKBr3 (Supplementary Fig. S6) cells were incubated with EGF. We therefore further examined whether geldanamycin affected EGF-induced down-regulation of EGFR.
of EGFR. As shown in Fig. 4, the EGFR was more efficiently down-regulated when PAE.ERG.ErbB2 cells were incubated with both geldanamycin and EGFR than with EGFR only. This is consistent with the notion that geldanamycin-induced down-regulation of ErbB2 reduces the number of EGFR-ErbB2 heterodimers and thereby facilitates EGFR-induced down-regulation of endocytosis-competent EGFR homodimers. When PAE.ERG.ErbB2 cells were incubated with geldanamycin or EGFR alone or with both geldanamycin and EGFR before the level of ErbB2 at the plasma membrane was measured by flow cytometry, we found no increased down-regulation of ErbB2 on incubation with both EGFR and geldanamycin compared with incubation with geldanamycin only (Fig. 4). We thus conclude that EGFR activation as such is not essential in geldanamycin-induced down-regulation of ErbB2.

To examine whether incubation of cells with geldanamycin affected phosphorylation of the EGFR or ErbB2 when EGFR and ErbB2 were coexpressed, Western blotting was done using antibodies recognizing phosphotyrosines in the EGFR and in ErbB2. By using an antibody recognizing pY1173, the EGFR was found not to be phosphorylated when PAE.ERG.ErbB2 cells were incubated with geldanamycin (Fig. 5A). Similar results were obtained when using antibodies recognizing other phosphotyrosines (pY1045, pY1068, and pY1086) in the EGFR (Supplementary Fig. S7). Neither did we detect changes in phosphorylation of ErbB2 in PAE.ERG.ErbB2 cells incubated with geldanamycin when using antibody to pY1248 (Fig. 5B). To make sure this lack of induced phosphorylation was not restricted to the specific tyrosines examined and not limited to a certain cell line, we immunoprecipitated EGFR and ErbB2 from PAE.ERG.ErbB2 cells incubated with or without geldanamycin or EGFR and performed Western blotting with an anti-phosphotyrosine antibody (Fig. 5C). Both EGFR and ErbB2 were weakly phosphorylated in nonstimulated cells. The apparently reduced phosphorylation seen in Fig. 5C compared with in Fig. 5A and B is most likely due to different experimental conditions, such as Western blotting using whole-cell lysates (Fig. 5A and B), versus immunoprecipitation of receptors (Fig. 5C) and to the efficiency of the different antibodies used to detect phosphorylation. As found with the EGFR pY1173 and the ErbB2 pY1248 antibodies, general tyrosine phosphorylation of EGFR and ErbB2 was not induced when cells were incubated with geldanamycin for short periods of time. Phosphorylation was, however, induced in cells incubated with EGFR only and in cells incubated with geldanamycin and EGFR (Fig. 5A-C).

**Incubation with Geldanamycin Induced Down-Regulation, but not Activation, of ErbB3**

As expression of ErbB3 increased geldanamycin-induced down-regulation of ErbB2 (Fig. 3), we further studied whether geldanamycin also affected the level ErbB3. To study a possible geldanamycin-induced down-regulation of ErbB3, we used PAE.ERG.ErbB3, PAE.ErbB2.ErbB3, and SKBr3 cells. Flow cytometry analysis showed that incubation with geldanamycin for 5 h caused down-regulation of ErbB3 from the plasma membrane in all cell lines, although this effect was most pronounced in PAE.ErbB2.ErbB3 cells (Fig. 6). Down-regulation of ErbB3 is down-regulated from the plasma membrane on incubation with geldanamycin. PAE.ErbB2.ErbB3, PAE.ERG.ErbB3, and SKBr3 cells were incubated with geldanamycin (GA; 3 μmol/L), cycloheximide (CHX; 25 μg/mL), for 5 h at 37°C. On incubation, the cells were fixed and immunostained using mouse anti-ErbB3 (Ab-5) antibody followed by phycoerythrin-conjugated goat anti-mouse antibody and analyzed by flow cytometry. Average median fluorescence intensities of three parallels were plotted as ratio of control. Bars, SD. Representative of three experiments.
of ErbB3 could potentially be caused by geldanamycin-induced endocytosis of ErbB3. However, ErbB3 has a short half-life at the plasma membrane due to ligand-independent turnover caused by the ubiquitin ligase Nrdp1 and proteasomal activity (24, 25). A potential geldanamycin-induced block in synthesis and/or maturation of ErbB3 could therefore readily result in reduced levels of ErbB3 at the plasma membrane. We therefore compared the effect of geldanamycin with the effect of cycloheximide. Our results show that ErbB3 was down-regulated from the plasma membrane with approximately the same efficiency on incubation with geldanamycin and cycloheximide (Fig. 6). However, this does not necessarily mean that the mechanism of down-regulation is the same, and we can thus not conclude whether the effects of geldanamycin are solely through endocytic down-regulation or through decreased synthesis. Both geldanamycin- and cycloheximide-induced down-regulation of ErbB3 was most pronounced in PAE.ErbB2.ErbB3 cells, suggesting that the half-life of ErbB3 varies in the different cell lines.

Western blotting, using antibody to phospho-ErbB3 (pY1289), was used to examine whether activation of ErbB3 was involved in geldanamycin-induced down-regulation of ErbB2. As shown (Fig. 7A), we did not find geldanamycin-induced phosphorylation of ErbB3 (pY1289), whereas ErbB3 was phosphorylated when PAE.ErbB2.ErbB3 cells were incubated with the ErbB3 ligand Heregulin. Immunoprecipitation of ErbB2 or ErbB3 from PAE.ErbB2.ErbB3 cells followed by Western blotting with anti-phosphotyrosine (pY) antibody. The membranes were stripped and incubated again with mouse anti-ErbB2 (BD Transduction) and rabbit anti-ErbB3 antibodies.

Discussion

ErbB2 has been shown to be endocytosis resistant (21-23), and overexpression of ErbB2 has further been reported to inhibit endocytosis of the EGFR (23, 27). ErbB2 is stabilized...
by interactions with Hsp90 during synthesis and on maturation, and incubation of cells with the Hsp90-binding drug geldanamycin has been found to destabilize ErbB2 (43). This destabilization was proposed to occur as a result of ubiquitination, internalization, and degradation of ErbB2 (35, 38, 44). Lerdrup et al. suggested that the geldanamycin-destabilized ErbB2 could be endocytosed clathrin-dependently (36), but to what extent other ErbB proteins modulate or interfere with geldanamycin-induced endocytosis of ErbB2 has been unclear. In the current study, we used PAE cells lacking endogenous ErbB proteins to create stably transfected cells expressing ErbB2 only or ErbB2 together with EGFR and/or ErbB3. The use of such cell lines has the advantage that one avoids comparing nonisogenic cells.

Our current results show that endocytosis of ErbB2 can happen in PAE.ErbB2 cells expressing ErbB2 only. Surprisingly however, we found that when EGFR and/or ErbB3 were coexpressed with ErbB2, the rate of geldanamycin-induced down-regulation of ErbB2 increased significantly. A mutant ErbB2 not binding Hsp90 was reported to be constitutively active and tyrosine phosphorylated and to promote trans-activation within ErbB2-ErbB3 heterodimers (45). In vitro experiments have further shown that treatment with geldanamycin can induce phosphorylation of ErbB2 (39). One could therefore envision that geldanamycin-induced phosphorylation of EGFR, ErbB2, and ErbB3 could initiate a signaling cascade leading to endocytosis of ErbB2. However, we could not find increased phosphorylation of EGFR, ErbB2, or ErbB3 on incubation of cells expressing ErbB2 with geldanamycin. This is consistent with previous data showing that incubation with geldanamycin caused decreased EGFR phosphorylation due to disruption of constitutively active EGFR-ErbB2 heterodimers (46). Furthermore, it has been shown previously that although sensitivity to geldanamycin depended on Hsp90-binding sequences within the kinase domain of ErbB2 (38, 47, 48), geldanamycin sensitivity was independent of ErbB2 kinase activity (38). Our current results thus support the conclusion that down-regulation of ErbB2 does not depend on geldanamycin-induced phosphorylation of ErbB proteins.

EGF-induced endocytosis of EGFR is highly inefficient in PAE.EGFR.ErbB2 cells compared with in PAE cells expressing EGFR only (27), and our finding that geldanamycin speeds up EGF-induced endocytosis of the EGFR in PAE.EGFR.ErbB2 cells could therefore be consistent with the notion that geldanamycin efficiently disrupts ErbB2 heterodimers (46). Our finding that EGF-induced phosphorylation of EGFR and ErbB2 lasted for shorter periods in cell incubated with geldanamycin supports such geldanamycin-induced disruption of heterodimers. However, based on the cross-linking experiments, we could not establish whether ErbB2 homodimers and EGFR-ErbB2 heterodimers were dissolved with different kinetics.

FIGURE 8. Incubation with geldanamycin causes activation of p38 MAPK, but p38 MAPK is not instrumental in geldanamycin-induced down-regulation of ErbB2 from the plasma membrane. A. PAE.EGFR.ErbB2 cells were treated with geldanamycin (GA; 3 μmol/L) for the indicated times. Cells incubated with 100 μmol/L cisplatin (Cis) for 4 h were used as positive control. Cells were lysed and subjected to Western blotting using mouse anti-phospho-p38 MAPK, anti-β-tubulin (loading control), and horseradish peroxidase-conjugated secondary antibodies. Representative of three experiments. B. PAE.EGFR.ErbB2 cells were preincubated with or without the p38 MAPK inhibitor SB203580 (10 μmol/L) for 30 min at 37°C before incubation with geldanamycin (3 μmol/L) for 12 h with or without SB203580. The cells were then fixed and immunostained with antibody to ErbB2 (TAB250), and the amount of ErbB2 at the plasma membrane was investigated by flow cytometry. Each sample was run in three parallels. Bars, SD. Representative of three experiments. C. Cell lysates of PAE.EGFR.ErbB2 cells treated with siRNA to p38 MAPK or scrambled control siRNA (Scr) were subjected to Western blotting using antibodies to p38 MAPK and to β-tubulin (loading control). D. PAE.EGFR.ErbB2 cells incubated with siRNA to p38 MAPK or scrambled control siRNA were incubated without (Ctrl) or with geldanamycin for 6 h. The cells were then fixed and immunostained with antibody to ErbB2 (TAB250), and the amount of ErbB2 at the plasma membrane was investigated by flow cytometry. Each sample was run in three parallels. Bars, SD. Representative of two experiments.
Contrasting results with respect to phosphorylation of ErbB proteins on incubation of cells expressing ErbB2 with geldanamycin have been reported. It was shown that a mutant ErbB2 unable to bind Hsp90 was constitutively tyrosine phosphorylated (45), and it was further reported from in vitro experiments that geldanamycin induced phosphorylation of ErbB2 (39). Dote et al. did, however, show that incubation with the geldanamycin derivative 17-dimethylaminoethylamino-17-demethoxygeldanamycin induced a decreased phosphorylation of EGFR in cells expressing EGFR and ErbB2 only, whereas 17-dimethylaminooethylamino-17-demethoxygeldanamycin had no effect on phosphorylation of EGFR in cells expressing EGFR, ErbB2, and ErbB3 (46). Consistently, we found no increased phosphorylation of EGFR, ErbB2, or ErbB3 in geldanamycin-incubated cells. Because mechanisms controlling subcellular trafficking of ErbB proteins are still poorly understood, more focus on signals controlling trafficking of ErbB proteins will be important in future studies. Importantly, heterodimer formation in ErbB2-positive cancer cells could turn out to be prognostically predictive with respect to outcome of treatment with geldanamycin derivatives.

### Materials and Methods

#### Materials

Human recombinant EGF was from Bachem Feinchemikalien. Bis(sulfosuccinimidyl)suberate was from Pierce. Lactacystin was from Sigma-Aldrich or Tocris Bioscience. FuGENE 6 was from Roche Diagnostics, whereas Alexa Fluor 488-conjugated Annexin V, G418, ToPro3, and Zeocin were from Invitrogen. Other chemicals were from Sigma-Aldrich unless otherwise noted.

#### Antibodies

Mouse anti-ErbB2 antibody (TAB250 to extracellular domain) and rabbit anti-ErbB2 antibody (clone PADZ4881 to the intracellular domain) were from Invitrogen. Mouse anti-ErbB2 (Ab-8 to intracellular domain) and mouse anti-EGFR (Ab-3 to extracellular domain) antibodies were from Lab Vision. Mouse anti-ErbB3 (Ab-5 to extracellular domain) and mouse anti-ErbB2 (Ab-3 to intracellular domain) antibodies were from Calbiochem Merck. Rabbit anti-phospho-ErbB2 (pY1248) and mouse anti-phospho-EGFR (pY1173) antibodies were from Millipore. Rabbit anti-phospho-ErbB3 (pY1289), mouse anti-phospho-EGFR (pY1068), rabbit anti-phospho-EGFR (pY1045), rabbit anti-p38 MAPK, and mouse anti-phospho-p38 MAPK antibodies were from Cell Signaling Technologies. The rabbit anti-EGFR, rabbit anti-phospho-EGFR (pY1086), and rabbit anti-tubulin antibodies were from Abcam, whereas rabbit anti-ErbB3, rabbit anti-extracellular signal-regulated kinase (ERK), and mouse anti-phosphotyrosine antibodies were from Santa Cruz Biotechnology. Sheep anti-EGFR antibody was from Fitzgerald Industries International. Mouse anti-ErbB2 (clone 42 to the extracellular domain) and mouse anti-clathrin heavy chain antibodies were from BD Biosciences. Peroxidase-conjugated donkey anti-mouse, peroxidase-conjugated donkey anti-rabbit, peroxidase-conjugated donkey anti-sheep, Cy5-conjugated goat anti-mouse, and phycoerythrin-conjugated goat anti-mouse antibodies were from Jackson ImmunoResearch Laboratories.

#### Plasmids

The pcDNA3.1 Hygro(+) ErbB3 was generated by PCR amplification of the pLXSN ErbB3 plasmid (a gift from David Riese, Purdue School of Pharmacy and Purdue Cancer Research Center) using the following gene-specific primers 5'-AAGCTAGGAGTAGCTGTGGGCGAAC-3' and 5'-TTTCTAGATTACGTTCTCTGGGCATTAGCC-3' (Invitrogen), including restrictions sites for NheI and XbaI. The PCR product was directly cloned into the pCR-Blunt II-TOPO (Invitrogen), and positive clones were digested with NheI and

![FIGURE 9. Oligomeric/dimeric ErbB2 complexes disappear with the same rate in PAE.ErbB2 and PAE.ErbB2.EGFR cells. A. PAE.ErbB2 and PAE.ErbB2.EGFR cells were treated with geldanamycin (GA; 3 μmol/L) for 0, 15, 60, or 120 min at 37°C before plasma membrane proteins were cross-linked using 3 mmol/L bis(sulfosuccinimidyl)suberate in PBS. Cells were lysed and subjected to Western blotting using mouse anti-ErbB2 (BD Transduction) antibody. The membrane was stripped and restained with mouse anti-clathrin heavy chain (CHC) antibody as loading control. Representative of three experiments. B and C. The intensity of each band in A was estimated using Kodak Molecular Imaging software. Each band was then normalized to the loading control. Each of the 0 time point values were set to 1. Average ± SD at each time point from three separate experiments are plotted as ratio of the 0 time point sample in PAE.ErbB2 (B) and PAE.ErbB2.EGFR (C) cells.](image-url)
XhoI. The fragment was then ligated into pcDNA3.1 Hygro (+) (Invitrogen); pcDNA3.1-ErbB2 has been described previously (27). pCMV.HERC encoding EGFR cDNA was provided by Dr. Reiner Lammers (Medical Clinic IV).

Stable Transfection of Cells
PAE cells were from Carl-Henrik Heldin, Ludwig Institute for Cancer Research and stably transfected PAE cells expressing wild-type EGFR (PAE.B2; refs. 40, 49) were from Alexander Sorkin, University of Colorado Health Sciences Center. PAE and PAE.B2 cells were stably transfected with the plasmid pcDNA3.1-ErbB2. Stably transfected cells were established by standard single-cell cloning procedures (50) and Zeocin selection (30 μg/mL). The cell lines named PAE.B2 (expressing EGFR only) and PAE.ErbB2 (expressing ErbB2 only) were further stably transfected with the plasmid pcDNA3.1-ErbB3 using standard single-cell cloning procedures (50) and hygromycin (60 μg/mL) selection resulting in the additional generation of PAE.EGFR.ErbB2 and PAE.ErbB2.ErbB3 cells. A different clone expressing EGFR and ErbB2 (PAE.ErbB2.EGFR) was made by transfection of PAE.ErbB2 cells with pcMV.HERC essentially as described above and with G418 (400 μg/mL) selection. Clones were characterized by flow cytometry and Western blot analysis.

Cell Culture and Treatment
The mammary carcinoma cell line SKBr3 (American Type Culture Collection), which endogenously expresses EGFR, ErbB2, and ErbB3, was grown in DMEM (Lonza) containing penicillin-streptomycin mixture (0.5 × ; Lonza), L-glutamine (2 mmol/L; Lonza) and fetal bovine serum (15% v/v). The PAE cells were grown in Ham’s F-12 (Cambrex Bio Science) (2 mmol/L; Lonza) and fetal bovine serum (15% v/v). The PAE is established by standard single-cell cloning procedures (50) and hygromycin (60 μg/mL) for EGFR, Zeocin (30 μg/mL) for ErbB2, and hygromycin (60 μg/mL) for ErbB3.

Determination of Apoptosis
Cells treated as described in the figure legends were trypsinized and labeled with Alexa Fluor 488-conjugated Annexin V according to the description by the manufacturers. Before analysis by flow cytometry, ToPro3 was added to detect dead cells.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Expression of Epidermal Growth Factor Receptor or ErbB3 Facilitates Geldanamycin-Induced Down-Regulation of ErbB2

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