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BIBX1382BS, but Not AG1478 or PD153035, Inhibits the ErbB Kinases at Different Concentrations in Intact Cells

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The activation of ErbB tyrosine kinase receptors (ErbB1, -2, -3, and -4) by ligand-induced homo- or heterodimerization regulates cell growth, death, and differentiation. AG1478 and PD153035 (also know as AG1517) have been adopted as specific ErbB1 inhibitors based on their high specificity for ErbB1 as compared to ErbB2 in *in vitro* kinase assays. We compared their ability to inhibit ErbB receptor signaling in intact cells to that of a novel ErbB receptor kinase inhibitor, BIBX1382BS. Neither AG1478 nor PD153035 displayed any specificity for ErbB1-mediated signaling induced by transforming growth factor α (TGF-α) as compared to signaling initiated through the other ErbB kinases. In contrast, BIBX1382BS was more potent at inhibiting signaling induced by TGF-α than that induced by neuregulin1-β1 or anti-ErbB2 agonist antibodies. Interestingly, this compound blocked antibody-induced ErbB4 homodimer activation at even lower concentrations than ErbB1-triggered signaling. Thus, BIBX1382BS, but not AG1478 and PD153035, can be employed to differentiate between the ErbB kinases in intact cells when used at appropriate concentrations.

Key Words: **ErbB receptor kinase; EGFR; inhibitor; neuregulin; TGF-α; activating antibodies.**

ErbB receptor signaling controls cellular differentiation, growth, and apoptosis (1–4) e.g., during embryonic development and tumor progression (5). The ErbB receptor family comprise four receptor tyrosine kinases (RTKs): ErbB1 (the epidermal growth factor (EGF) receptor), ErbB2 (Neu or HER2), ErbB3 and ErbB4. Each of these receptors has an extracellular ligand binding domain, a transmembrane domain and an intracellular tyrosine kinase domain (5). ErbB3 has, however, no intrinsic kinase activity (6–8). The ErbB receptors are activated by ligand-induced receptor dimerization leading to the activation of the intrinsic tyrosine kinases and downstream signaling molecules, e.g., phosphoinositol 3′-kinase, Src, phospholipase C γ and the extracellular regulated kinases (ERK) 1/2. The ligands for the ErbB receptors can be divided into three groups (9). The first group of ligands, EGF, transforming growth factor alpha (TGF-α) and amphiregulin, utilize ErbB1 as the primary receptor. The second group binds either ErbB1 or ErbB4 and includes betacellulin, heparin-binding EGF-like growth factor and epiregulin. The third group, the neuregulins (NRGs), binds ErbB3 or ErbB4 (10–15). There is no known ligand for ErbB2, but this receptor is the preferred dimerization partner for other ligand-bound ErbB receptors (16, 17).

Inhibitors of the ErbB1 kinase, AG1478 and PD153035 (also known as AG1517), have over 100-fold higher specificity for ErbB1 as compared to ErbB2 or unrelated tyrosine kinases in *in vitro* kinase assays (18, 19). It has also been suggested that they are specific for ErbB1 compared to ErbB2 in intact cells because they inhibit the ligand-induced activation of ErbB1 at lower concentrations than those required to block ligand-induced ErbB2 activation in cancer cell lines overexpressing ErbB1 or ErbB2, respectively (20, 21). No attempt has, however, been made to compare the specificity of these compounds for ErbB1 versus the other ErbB receptor kinases in the same cell lines. In addition to the ErbB signaling, these inhibitors have been used as tools to determine the involvement of the ErbB1 kinase in signal transduction pathways initiated by unrelated receptors such as the insulin growth factor receptor (22) and G-protein-coupled-receptors (23). In this study, the specificity of AG1478, PD153035 and the novel inhibitor BIBX1382BS for the ErbB receptor kinases is addressed in intact cells. A panel of breast cancer cells expressing endogenous or exogenous ErbB receptors at variable levels was treated with TGF-α or NRG1-β1 as well as activating antibodies against ErbB2 and ErbB4. The concentrations of the ErbB1 kinase inhibitors needed to block the activation of the ErbB receptors and the downstream sig-

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naling molecules ERK1 and ERK2 were then determined.

MATERIALS AND METHODS

Reagents. Lyophilized recombinant human ligands (TGF-α, Sigma, St. Louis, MO; NRG1-β1 EGF domain; R & D Systems, Abingdon, UK) were reconstituted in phosphate buffered saline (PBS) containing 1% BSA (TGF-α) or 0.1% BSA (NRG1-β1) at 100 μg/ml and stored at −20°C. The ErbB kinase inhibitors, AG1478 (16 mM, Calbiochem, La Jolla, CA) and PD153035 (10 mM, Calbiochem), were dissolved in dimethyl sulfoxide (DMSO) and stored at −20°C. BIBX1382BS (24) was kindly provided by Dr. van Meel (Boehringer Ingelheim, Vienna, Austria) and dissolved at 10 mM in 0.1 N HCl.

Cell lines, culture conditions, and treatments. A previously described subclone of the MCF-7 breast carcinoma cell line (25), and the CAMA-1, SKBR-3, T47D and MDA-MB-157 breast cancer cell lines (kindly provided by Per Briand and Jiri Bartek, Copenhagen, Denmark) were used. MCF-7 cells stably overexpressing ErbB1 (M1-ErbB1), ErbB2 (M1-ErbB2), ErbB4 (M1-ErbB4), or transfected with empty vector (M1-pEBS7) have been characterized previously (26). Cells were cultured at 37°C in humidified air atmosphere with 5% CO_2. RPMI 1640 medium with L-alanyl-L-glutamin (GibcoBRL, Paisley, United Kingdom) supplemented with 10% heat inactivated fetal calf serum (FCS) (BioWhittaker Europe, Verviers, Belgium) streptomycin (100 μg/ml) and penicillin (100 U/ml) was used as growth medium. The medium used for passage of T47D was supplemented with 250 ng/ml insulin (Roche Molecular Biochemicals, Basel, Switzerland). The medium used for propagation of the M1-ErbB1, M1-ErbB2, M1-ErbB4, and M1-pEBS7 cell lines was supplemented with 150 μg/ml hygromycin B (Calbiochem, La Jolla, CA).

To determine the effects of ErbB1 kinase inhibitors on ErbB receptor activation, 750,000 cells/well were plated in 6-well plates 18 h before the start of the experiment. Cells were pretreated with indicated concentration of kinase inhibitors or DMSO (0.01%) 1 h prior to the addition of ErbB ligands or activating antibodies against ErbB2 (N28, NeoMarkers, Fremont, CA) or ErbB4 (H4.77.16, NeoMarkers). After 5 min (ligands) or 15 min (antibodies) at 37°C, the cells were harvested and analyzed by immunoblotting. All experiments were conducted in medium containing 5% FCS.

Immunoblot analysis. Cells were harvested in lysis buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 1 mM EGTA, 1% NP-40, 0.25% sodium deoxycholate, 100 mM pefabloc, 1 mM Na_3VO_4, 1 mM NaF, 1 μg/ml each aprotinin, leupeptin, pepstatin), equal amounts of protein were separated by sodium dodecyl sulfate (SDS)–7% polyacrylamide gel electrophoresis (PAGE) and transferred to hybond ECL membrane (Amersham Pharmacia Biotech, Little Chalfont, UK). ErbB1 and ErbB2 were detected as previously described (26) employing monoclonal antibodies sc-03 and sc-284 (Santa Cruz, CA). Antibodies against phospho-ErbB1 (05-483, Upstate Biotechnology, Lake Placid, NY), phospho-ErbB2 (06-229, Upstate Biotechnology), phospho-tyrosine (4G10, Upstate Biotechnology) ERK1/2 (New England Biolabs, Beverly, MA) and phospho-ERK1/2 (New England Biolabs) were all used according to the manufacturer's instructions. As previously described, the sc-03 antibody directed against ErbB1 cross-reacts with ErbB2 and the 05-483 antibody directed against phosphorylated ErbB1 also recognizes phosphorylated ErbB2 and ErbB4 (26). Heat shock cognate protein 70 (Hsc70) was detected employing the antibody N69A (kindly provided by Dr. B. A. Margulis, St. Petersburg, Russia) diluted 1:4000 in PBS containing 0.5% low-fat milk powder and 0.1% Tween 20. Peroxidase-conjugated secondary antibodies were from DAKO (Glostrup, Denmark). The chemiluminescence reaction was performed and the membranes exposed to ECL hyperfilm according to the manufacturer's instructions (Amersham Pharmacia Biotech, Little Chalfont, UK).

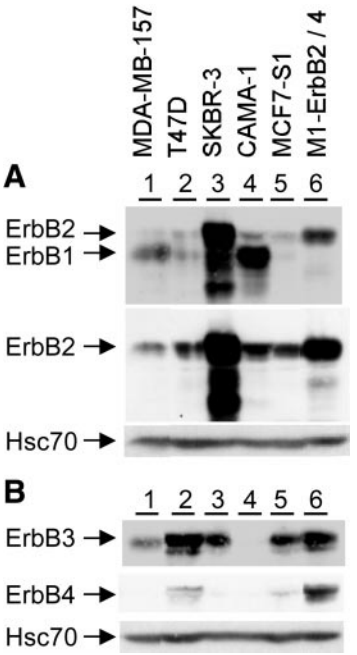

FIG. 1. Expression of ErbB receptors in a panel of breast cancer cell lines. Protein samples (75 μg) prepared from subconfluent human breast cancer cells (MDA-MB-157, T47D, SKBR-3, CAMA-1, and MCF-7) were analyzed by immunoblotting using antibodies directed against (A) ErbB1 or ErbB2 and (B) ErbB3 or ErbB4. The antibody directed against ErbB1 (sc-03) cross-reacts with ErbB2 (26). Therefore, the expression of ErbB1 in SKBR-3 was partly masked by the high expression level of ErbB2, but ErbB1 was readily detectable at lower exposure times and by flow cytometry (not shown). Protein samples from MCF-7 cells transfected with (A) ErbB2 (M1-ErbB2) or (B) ErbB4 (M1-ErbB4) were employed as controls for the specificity of the antibodies (lanes 6). The membranes were probed for heat shock cognate protein 70 (Hsc70) to control protein loading. Similar results were obtained by flow cytometry analysis of non-permeabilized cells indicating that the expressed receptors were localized to the plasma membrane (not shown).

RESULTS AND DISCUSSION

Equal Concentrations of AG1478 and PD153035 Are Required to Block the Phosphorylation of ERK 1/2 Triggered by TGF-α or NRG1-β1

To determine the specificity of the ErbB1 kinase inhibitors AG1478 and PD153035 for ErbB1 in intact cells, five breast cancer cell lines with variable expression levels of the four ErbB receptors were used (Fig. 1). The cells were treated with the ErbB ligands TGF-α, which almost exclusively activates ErbB1 homodimers, or with NRG1-β1, which preferentially activates ErbB2/ErbB3 or ErbB2/ErbB4 heterodimers (27). ErbB receptor activation was analyzed by immunoblotting with a monoclonal antibody recognizing phosphorylated ErbB1, ErbB2 and ErbB4 or a monoclonal antibody specific for phosphorylated ErbB2 (26). Activation of the downstream signaling was detected by employing antibodies recognizing phosphorylated

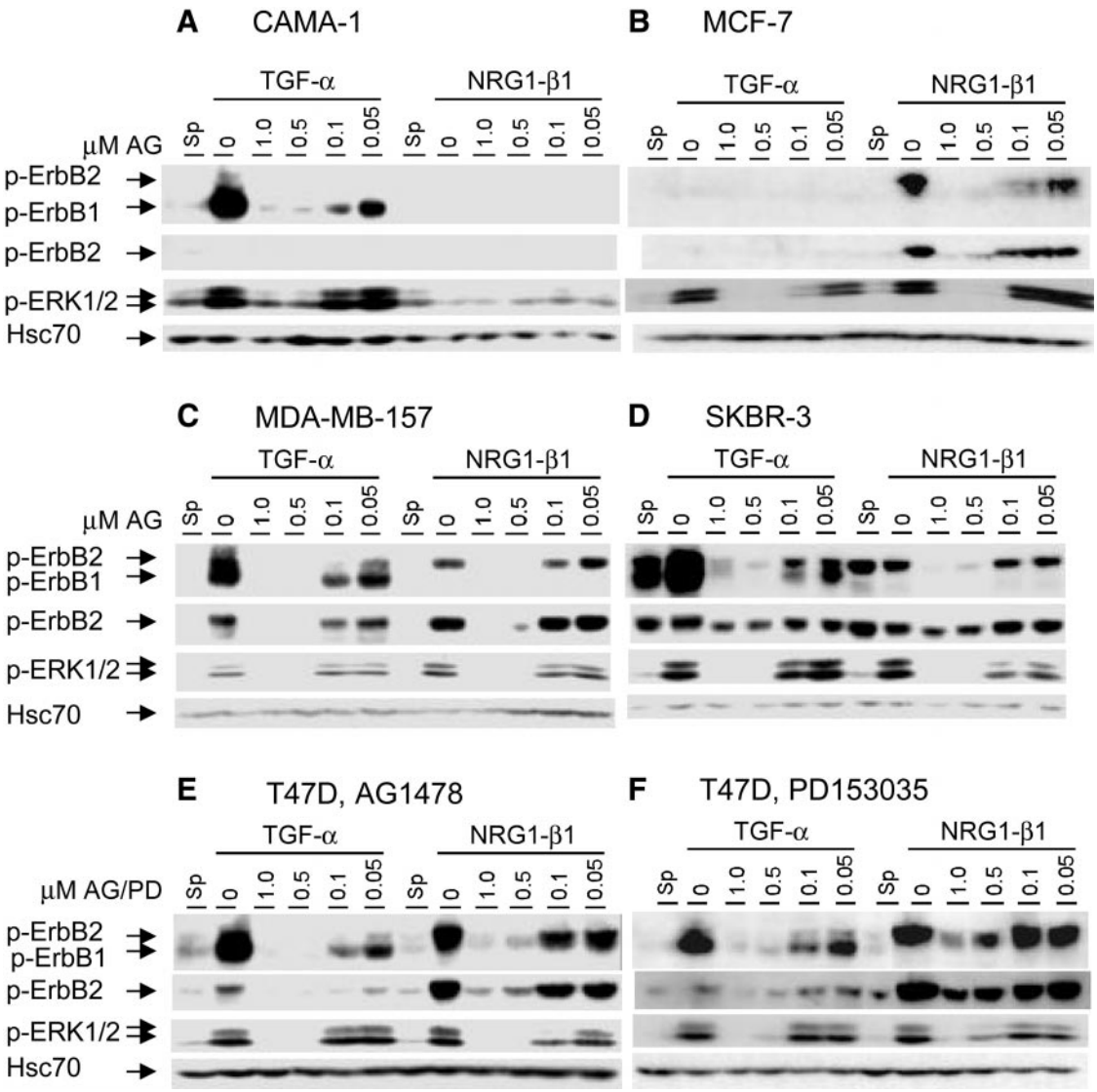

FIG. 2. Equal concentrations of AG1478 and PD153035 are required to block the phosphorylation of ERK 1/2 triggered by TGF-α or NRG1-β1. Subconfluent CAMA-1 (A), MCF-7 (B), MDA-MB-157 (C), SKBR-3 (D) and T47D (E, F) cells were treated with indicated concentrations of AG1478 (A–E) or PD153035 (F) for 1 h prior to the addition of TGF-α or NRG1-β1 at a final concentration of 100 ng/ml or were left untreated (Sp). After 5 min at 37°C, the cells were harvested and analyzed by immunoblotting for phospho-ErbB1, phospho-ErbB2 and phospho-ERK1/2. The antibody directed against phospho-ErbB1 (05-483) cross-reacts with phospho-ErbB2 but the proteins can be identified based on different molecular weights (26). The membranes were probed for Hsc70 to control protein loading. An equal amount of DMSO (0.01%) was added to all wells. The experiments were repeated with similar results, and comparable results were obtained with PD153035 for all cell lines.

ERK1 and ERK2 that are activated by most ErbB receptor dimers.

In the CAMA-1 cell line, which expresses ErbB1 and ErbB2 but not ErbB3 or ErbB4 (Fig. 1), TGF-α treatment resulted in the phosphorylation of ErbB1 and the activation of ERK1/2 (Fig. 2A). Consistent with the lack of ErbB3 and ErbB4 expression, this cell line did not respond to NRG1-β1 treatment. At concentrations of 0.5 μM or higher, AG1478 completely inhibited TGF-α-induced ERK1/2 phosphorylation in CAMA-1 cells. The MCF-7 cell line expresses moderate levels of ErbB2, ErbB3 and ErbB4, while the expression level of ErbB1 is barely detectable (Fig. 1). In this cell line, TGF-α resulted in a scarcely measurable phosphorylation of ErbB1, but a clear activation of ERK1/2 was, however, observed (Fig. 2B). NRG1-β1 increased the phosphorylation of ErbB2 and ERK1/2. In spite of the very low expression of ErbB1, the ErbB1 inhibitors AG1478 and PD153035 inhibited NRG1-β1-induced ErbB2 and ERK1/2 activation at 0.5 μM or higher concentrations corresponding to the concentration needed to block TGF-α signaling in the CAMA-1 cells. The MDA-157 cell line expresses moderate levels of

ErbB1, ErbB2 and ErbB3 (Fig. 1). These cells responded to TGF-α with increased phosphorylation of ErbB1, ErbB2 and ERK1/2 and to NRG1-β1 with increased phosphorylation of ErbB2 and ERK1/2 (Fig. 2C). Similar inhibitor concentrations were required for the complete inhibition of the phosphorylation of ErbB1, ErbB2 and ERK1/2 triggered either by TGF-α or NRG1-β1 in the MDA-MB-157 cells. The SKBR-3 cell line expresses ErbB1 and ErbB3 at moderate levels (Fig. 1) and overexpresses ErbB2 resulting in constitutive phosphorylation of this receptor (Fig. 2D). TGF-α treatment resulted in the phosphorylation of ErbB1 and ERK1/2 in the SKBR-3 cells. In contrast, NRG1-β1 treatment did not result in the further activation of ErbB2 whereas ERK1/2 was activated by the ligand, possibly through the activation of ErbB2/ErbB3 heterodimers (Fig. 2D). Also in this cell line 0.5 μM of the inhibitors completely inhibited the phosphorylation of ERK1/2 regardless of the ligand used to stimulate the cells. The two antibodies detecting phosphorylated ErbB2 are likely to recognize different tyrosine phosphorylated residues on ErbB2: the phosphorylation of ErbB2 was almost completely abolished by AG1478 at 0.5 μM or higher concentration when detected with the antibody that recognizes both phosphorylated ErbB1 and ErbB2. In contrast, the reduction in the level of phosphorylated ErbB2 appeared much less dramatic when tested with the antibody specific for phosphotyrosine-ErbB2. Finally, the T47D cell line, which expresses all four ErbB receptors at moderate levels, were employed (Fig. 1). As for all the other cell lines 0.5 μM of AG1478 was sufficient to block ERK1/2 activation regardless of whether the cells had been stimulated with TGF-α or NRG1-β1 (Fig. 2E). As was observed in the SKBR-3 cell line, the compound inhibited TGF-α-induced phosphorylation of ErbB1 at slightly lower concentrations than those blocking NRG1-β1-induced ErbB2 phosphorylation, especially when visualized with the antibody specific for phosphorylated ErbB2. For all cell lines, similar results were obtained when PD153035 was employed instead of AG1478 (Fig. 2F and data not shown) and when an antibody against phosphorylated tyrosine residues was used to detect phosphorylated ErbB receptors (data not shown).

Thus, there were very minor differences in the concentrations of AG1478 or PD153035 needed to block ErbB1 and ErbB2 phosphorylation upon treatment with ErbB ligands with preference for activation of either the ErbB1 or the ErbB2 kinases. Furthermore, when ERK1/2 was used as an indicator of ErbB receptor activation, there was no difference in the concentrations of the inhibitors necessary for inhibition of TGF-α- or NRG1-β1-induced signaling.

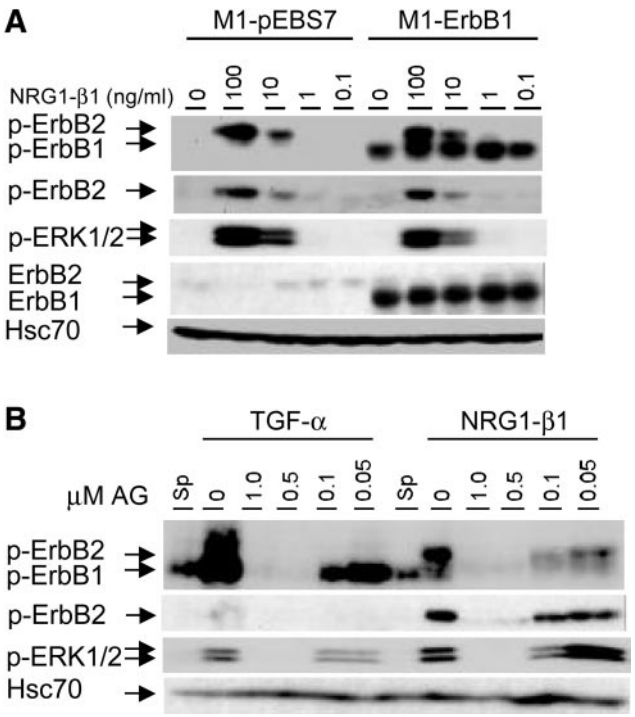

FIG. 3. Neuregulin signaling is not affected by ErbB1 expression levels in MCF-7 cells. (A) MCF-7 cells transfected with ErbB1 (M1-ErbB1) or vector transfected control cells (M1-pEBS7) were treated with NRG1-β1 at indicated concentration. After 5 min treatment at 37°C, the cells were harvested and analyzed by immunoblotting as described in the legend to Fig. 2. (B) M1-ErbB1 cells were pretreated with AG1478 at the indicated concentrations. After 1 h, TGF-α or NRG1-β1 was added to a final concentration of 100 ng/ml and after 5 min at 37°C, the cells were harvested and analyzed by immunoblotting as described in the legend to Fig. 2.

NRG1-β1 Signaling Is Not Affected by ErbB1 Expression Levels in MCF-7 Cells

The NRG-β1-induced ERK1/2 activation was completely inhibited by AG1478 and PD153035 in spite of some residual ErbB2 phosphorylation in the MCF-7, SKBR-3 and T47D cells. This suggests that a threshold level of activated ErbB2 must be reached in order to transduce a downstream signal. Alternatively, the ErbB1 kinase could be necessary for NRG1-β1 signaling, although NRG1-β1 did not result in detectable levels of phosphorylated ErbB1 in any of the cell lines (Fig. 2). To test if the expression level of ErbB1 influenced NRG1-β1 signaling, the MCF-7 cells, which had the lowest endogenous ErbB1 expression level among 14 breast cancer cell lines tested (data not shown) were transfected with ErbB1 cDNA. Overexpression of ErbB1 in the MCF-7 cells resulted in the constitutive phosphorylation of the receptor (Fig. 3A). However, after NRG1-β1 treatment, ErbB2 and ERK1/2 were phosphorylated to the same extent in the ErbB1 overexpressing cell line as in the vector transfected control cell line (Fig. 3A). Thus, NRG1-β1 signaling was not

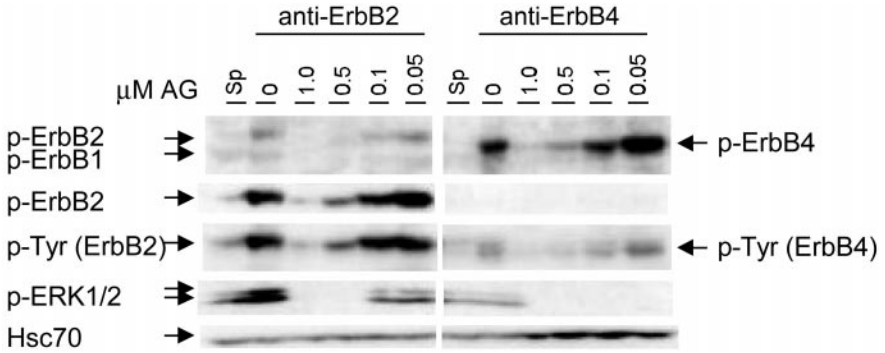

FIG. 4. ErbB1 kinase inhibitors block the activation of ErbB2 or ErbB4 homodimers. T47D cells were grown overnight, treated with AG1478 at indicated concentrations for 1 h and activating antibodies against ErbB2 or ErbB4 were added to the final concentration of 2 μg/ml. After 15 min at 37°C, the cells were harvested and analyzed by immunoblotting for phospho-ErbB1, phospho-ErbB2, phospho-tyrosine and phospho-ERK1/2. The antibody directed against phospho-ErbB1 (05-483) cross-reacts with phospho-ErbB2 and phospho-ErbB4, but the three proteins are separable by the difference in their molecular weights (26).

affected by ErbB1 expression levels. When the ErbB1 overexpressing cell line was treated with AG1478 before ligand stimulation, identical concentrations were needed to block TGF-α- and NRG1-β1-induced ErbB receptor and ERK1/2 phosphorylation (Fig. 3B). Thus, it is unlikely that the effective inhibition of NRG1-β1-induced signaling by AG1478 and PD153035 was due to the involvement of ErbB1 in this signaling pathway.

The ErbB1 Kinase Inhibitors Block the Activation of ErbB2 and ErbB4 Homodimers

Although we did not observe any phosphorylation of ErbB1 upon NRG1-β1 simulation, it has been reported that this ligand is capable of activating ErbB1 containing receptor dimers (28). As we did not find an ErbB1 devoid cell line that expressed the NRG1-β1 binding receptors ErbB3 or ErbB4, it could not be excluded that the low endogenous levels of ErbB1 in the MCF-7 cells were necessary for NRG1-β1 signaling. To directly test whether the inhibitors could block ErbB1 independent signaling we then used agonist antibodies against ErbB2 or ErbB4 that specifically activate homodimers of ErbB2 or ErbB4, respectively (29, 30). In the T47D cells the anti-ErbB2 antibody (29) induced ErbB2 and ERK1/2 phosphorylation, which was abrogated by AG1478 at 0.5–1.0 μM (Fig. 4). Similarly, anti-ErbB4 antibody activated ErbB4, but failed to induce ERK1/2 phosphorylation (30) (Fig. 4). Neither antibody increased the phosphorylation of ErbB1 (Fig. 4). Similar results were obtained with PD153035 (data not shown), and in the MCF-7 cell line with barely detectable levels of ErbB1. Thus, AG1478 and PD153035 do not act as specific ErbB1 inhibitors in intact cells, but appear rather as *pan*-ErbB receptor tyrosine kinase inhibitors capable of inhibiting ErbB1, ErbB2 and ErbB4 signaling at sub-micromolar concentrations.

A Novel ErbB1 Kinase Inhibitor Shows Differential Specificity for ErbB1, ErbB2, and ErbB4 in Vivo

AG1478 and PD153035 are quinazolines with a highly related chemical structure (18, 19). A novel pyrimido-pyrimidine derivative, BIBX1382BS, has recently been developed by Boehringer Ingelheim (24). The specificity of this compound was tested by treating the T47D cell line with either the natural ligands TGF-α, NRG1-β1, or the homodimer activating antibodies against ErbB2 or ErbB4. As for AG1478 and PD153035, BIBX1382BS blocked signaling induced by TGF-α, NRG1-β1 or activating antibodies (Fig. 5). However, 3 μM of BIBX1382BS that was sufficient to block signaling by TGF-α had no effect on the phosphorylation of ErbB2 or ERK1/2 upon stimulation with NRG1-β1 or anti-ErbB2 antibodies (Fig. 5 and data not shown). Ten μM BIBX1382BS blocked, however, also ErbB2 activity efficiently. Also in the MCF-7 cell line, approximately threefold higher concentrations of BIBX1382BS were required to hinder ErbB receptor and ERK1/2 phosphorylation upon NRG1-β1 treatment than after stimulation with TGF-α (data not shown). Interestingly, ErbB4 homodimer activation induced by the agonist antibody was inhibited by BIBX1382BS already at 1 μM. Thus contrary to AG1478 and PD153035, this compound inhibits the ErbB4, ErbB1 and ErbB2 kinases at different concentrations *in vivo*. The data further suggest that the kinase activity of ErbB4 is not necessary for NRG1-β1 signaling in the T47D cell line.

In this study, we provide evidence that the commonly used ErbB1 kinase inhibitors, AG1478 and PD153035, are not specific for the ErbB1 kinase when compared with the ErbB2 or ErbB4 kinase in intact cells. This conclusion is based on the ability of the inhibitors to equally well block ErbB receptor phosphorylation and ERK1/2 activation induced by TGF-α,

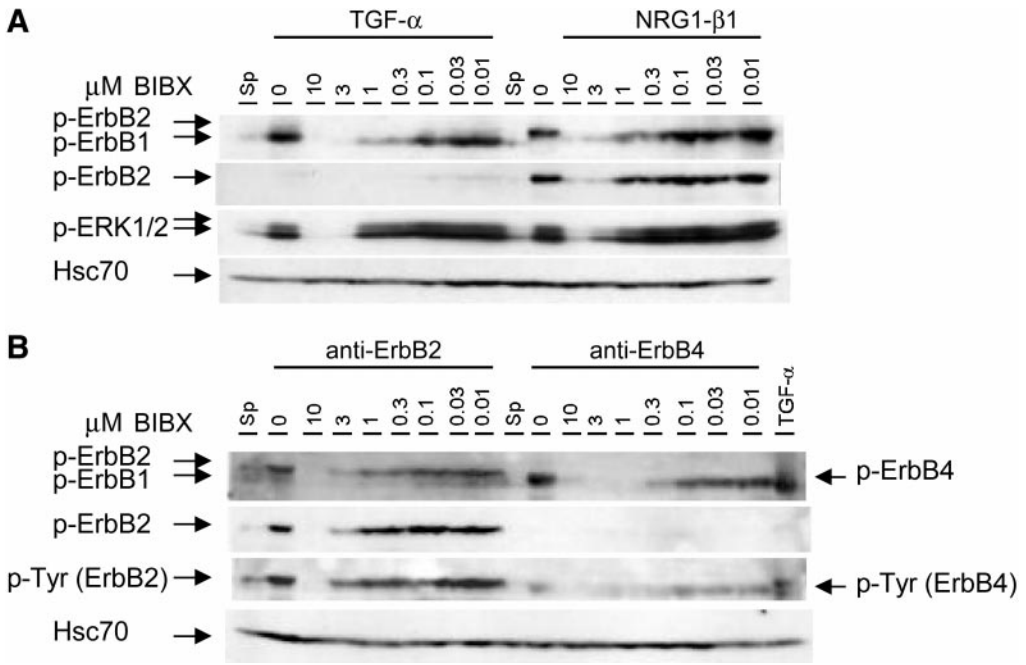

FIG. 5. BIBX1382BS shows differential specificity for ErbB1, ErbB2 and ErbB4 *in vivo.* Indicated concentrations of BIBX1382BS were added to subconfluent T47D cells. After 1 h, ligands (TGF-α or NRG1-β1) or agonist antibodies (anti-ErbB2 or anti-ErbB4) were added at the final concentrations of 100 ng/ml or 2 μg/ml, respectively. After 5 min (ligands) or 15 min (antibodies) at 37°C, the cells were harvested and analyzed by immunoblotting as described in the legend to Fig. 4.

NRG1-β1, or homodimer activating antibodies against ErbB2 or ErbB4. Furthermore, this is supported by the notion that the same concentration of the inhibitors was needed to block NRG1-β1 induced signaling in cell lines with a wide variation of ErbB1 expression levels and that neither NRG1-β1 nor the agonist antibodies resulted in detectable ErbB1 phosphorylation in the cell lines tested. In contrast, the novel compound, BIBX1382BS, can be used to differentiate between ErbB4 and other ErbB receptor kinases when used at very low concentrations. Furthermore, in cell lines devoid of ErbB4, BIBX1382BS appears more suitable than AG1478 and PD153035 for studies on the differential role of the ErbB1 and the ErbB2 kinases.

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