

Epidermal Growth Factor Receptor (HER1) Tyrosine Kinase Inhibitor ZD1839 (Iressa) Inhibits HER2/*neu* (*erbB2*)-overexpressing Breast Cancer Cells *in Vitro* and *in Vivo*¹

Stacy L. Moulder,² F. Michael Yakes,² Senthil K. Muthuswamy,³ Roberto Bianco, Jean F. Simpson, and Carlos L. Arteaga⁴

Departments of Medicine [S. L. M., F. M. Y., R. B., C. L. A.], Cancer Biology [C. L. A.], Pathology [J. F. S.], and Vanderbilt-Ingram Cancer Center [C. L. A.], Vanderbilt University School of Medicine, Nashville, Tennessee 37232-6307, and Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115 [S. K. M.]

ABSTRACT

Aberrant signaling by the epidermal growth factor receptor [EGFR (HER1, *erbB1*)] and/or HER2/*neu* tyrosine kinases is present in a cohort of breast carcinomas. Because HER2 is constitutively phosphorylated in some breast tumors, we speculated that, in these cancers, transmodulation of HER2 may occur via EGFR signaling. To test this possibility, we examined the effect of EGFR-specific kinase inhibitors against the HER2-overexpressing human breast tumor lines BT-474, SKBR-3, MDA-361, and MDA-453. ZD1839 (Iressa) is an ATP-mimetic that inhibits the purified EGFR and HER2 kinases *in vitro* with an IC₅₀ of 0.033 and >3.7 μM, respectively. The specificity of ZD1839 against EGFR was confirmed in Rat1 fibroblasts transfected with EGFR or HER2 chimeric receptors activated by synthetic ligands without the interference of endogenous receptors. Treatment of all breast cancer cell lines (except MDA-453) with 1 μM ZD1839 almost completely eliminated HER2 phosphorylation. In contrast, the incorporation of [γ -³²P]ATP *in vitro* onto HER2 receptors isolated from BT-474 cells was unaffected by 1 μM ZD1839. EGFR is expressed by BT-474, SKBR-3, and MDA-361 but not by MDA-453 cells, suggesting that ZD1839-mediated inhibition of the EGFR kinase explained the inhibition of HER2 phosphorylation *in vivo*. In SKBR-3 cells, ZD1839 exhibited a greater growth-inhibitory effect than Herceptin, a monoclonal antibody against the HER2 ectodomain. In both SKBR-3 and BT-474 cells, treatment with ZD1839 plus Herceptin induced a greater apoptotic effect than either inhibitor alone. Finally, ZD1839 completely prevented growth of BT-474 xenografts established in nude mice and enhanced the antitumor effect of Herceptin. These data imply that EGFR tyrosine kinase inhibitors will be effective against HER2-overexpressing breast tumor cells that also express EGFR and support their use in combination with HER2 antibodies, such as Herceptin, against mammary carcinomas with high levels of the *HER2* proto-oncogene.

INTRODUCTION

The EGFR⁵ (HER1, *erbB1*), HER2/*neu* (*erbB2*), HER3 (*erbB3*), and HER4 (*erbB4*) are members of the *erbB* family of transmembrane tyrosine kinases. Except for HER2, binding of receptor-specific ligands to the ectodomain of EGFR, HER3, and HER4 results in the formation of homodimeric and heterodimeric kinase-active complexes into which HER2 is recruited as a preferred partner (1–3). Although

HER2 is unable to directly interact with HER-activating ligands, it is well established that its kinase can potentiate signaling of HER2-containing heterodimers and/or increase the binding affinity of ligands to EGFR and HER3 (4–9). Aberrant EGFR and HER2 signaling has been causally associated with enhanced breast cancer cell proliferation and shorter survival in patients with mammary carcinomas (10–13). For HER2, the most common change in human breast tumors involves overexpression of HER2 mRNA or protein with or without amplification of the *HER2/neu* locus (14). Recently, trastuzumab (Herceptin), a humanized monoclonal antibody that binds the ectodomain of HER2, was shown to induce regression of HER2-overexpressing breast cancers (15, 16), thus validating HER2 as a therapeutic target within the HER (*erbB*) network.

Studies with breast cancer cell lines and human tumors have demonstrated constitutive phosphorylation of HER2 (17, 18). The biochemical basis for this constitutive activation is not clear, but it is consistent with the reported ability of wild-type *neu*, the rat homologue of human HER2, to multimerize and become activated when present at high concentrations in cells (19). It is unclear, however, whether this spontaneous dimerization and activation of HER2 occurs in human tumors. Another possible mechanism for activation of the HER2 tyrosine kinase in human breast cancers is the coexpression of ligand-activated EGFR, resulting in transactivation of the HER2 tyrosine kinase. This is supported by studies in which EGF was able to activate HER2 only when EGFR was also present in the same cell, suggesting transmodulation of HER2 as a result of its heterooligomerization with EGFR in response to EGF (20–22). Indeed, cooperation between EGFR and HER2 has been shown to induce accelerated transformation in studies with fibroblasts and transgenic mice (23, 24). In cells that coexpress HER2, ligand-activated EGFR preferentially recruits HER2 into a heterodimeric complex that exhibits an increased rate of recycling, stability, and signaling potency compared with EGFR homodimers (2, 5). In a recent report, the anti-EGFR quinazoline AG1478 suppressed mammary tumors and *neu* phosphorylation in MMTV/TGF α \times MMTV/*neu* (the mouse homologue of human HER2) bigenic mice and mammary tumors, respectively (25), suggesting that blockade of the EGFR kinase can prevent EGFR-HER2/*neu* cooperation. On the other hand, inactivation of HER2/*neu* with single-chain HER2 antibodies or vectors encoding kinase-dead *neu* has been shown to impair EGFR-mediated transformation (26, 27). Moreover, breast tumors that co-overexpress EGFR and HER2 exhibited a worse outcome than tumors that overexpressed either receptor alone (12). If receptor synergy is operational in breast cancers, interruption of EGFR function with EGFR-specific small molecular weight tyrosine kinase inhibitors may disrupt EGFR-HER2 cross-talk and result in HER2 inactivation as well. This has clinical implications because the inactivation of HER2 through the inhibition of EGFR may also increase the antitumor effect of Herceptin.

In this study, we present evidence that blockade of EGFR function with the EGFR-specific tyrosine kinase inhibitor ZD1839 (Iressa; Ref. 28) inhibits phosphorylation of the HER2 receptor and growth and

Received 8/30/01; accepted 11/1/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by NIH Grant R01 CA80195 (to C. L. A.), a Department of Veteran Affairs Clinical Investigator Award (to C. L. A.), a Massachusetts State Breast Cancer Grant (to S. K. M.), and Vanderbilt-Ingram Cancer Center Support Grant CA68485. S. L. M. is the recipient of a Translational Research Fellowship Award from the American Association for Cancer Research.

² Co-first authors.

³ Present address: Cold Spring Harbor Laboratory, 1 Bungtown Road, Cold Spring Harbor, NY 11724.

⁴ To whom requests for reprints should be addressed, at Division of Oncology, Vanderbilt University, 777 Preston Research Building, Nashville, TN 37232-6307. Phone: (615) 936-3524; Fax: (615) 936-1790; E-mail: carlos.arteaga@mcmail.vanderbilt.edu.

⁵ The abbreviations used are: EGFR, epidermal growth factor receptor; TGF, transforming growth factor; GSK, glycogen synthase kinase; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; PMSF, phenylmethylsulfonyl fluoride; PI3K, phosphatidylinositol-3 kinase; BrdUrd, bromodeoxyuridine; HA, hemagglutinin antigen; FKBP, FK506-binding protein; Cdk, cyclin-dependent kinase; mAb, monoclonal antibody.

survival of HER2-overexpressing breast carcinoma cells. We also demonstrate that in these cells, targeting the HER family signaling network at two separate molecular sites, the catalytic domain of the EGFR tyrosine kinase with ZD1839 and the ectodomain of HER2 with Herceptin, results in a greater antitumor effect both *in vitro* and *in vivo* than either agent alone. This is the first demonstration of a combined molecular approach to inhibit the HER network in HER2-overexpressing breast tumor cells, thus providing a basis for further combinations of rational anti-signaling strategies in human breast cancer.

MATERIALS AND METHODS

Cell Lines, Kinase Inhibitors, and Antibodies. The human breast cancer cell lines MDA-453, MDA-361, BT-474, and SKBR-3 were obtained from the American Type Tissue Culture Collection (Manassas, VA). All cells were maintained in Improved Minimal Essential Medium (IMEM; Life Technologies, Inc., Rockville, MD) containing 10% FCS (Hyclone, Logan, UT) at 37°C in a humidified, 5% CO₂ atmosphere. WT3 cells are 32D myeloid cells stably transfected with an EGFR cDNA and were kindly provided by James Staros (Vanderbilt University). These cells were grown in RPMI 1640 (Life Technologies, Inc.) containing 15% FCS. Rat1 fibroblasts transfected with ErbB-1 (EGFR) or ErbB-2 (HER2) chimeric receptors have been described previously (29). AP1510 was from ARIAD Pharmaceuticals (Cambridge, MA). TGF- α was from R&D Systems, Minneapolis, MN). The quinazoline ZD1839 (Iressa) was provided by Steven Averbuch (AstraZeneca Pharmaceuticals, Wilmington, DE), and Herceptin was purchased from the Vanderbilt University Hospital Pharmacy.

For immunoblot analysis and/or immunoprecipitations, the following antibodies were used: GSK-3 β and p27 (Transduction Laboratories, Lexington, KY); HER2/neu and HER3 (Neomarkers, Fremont, CA); P-Tyr (Upstate Biotechnology, Lake Placid, NY); Rb and cyclin D1 (PharMingen, San Diego, CA); MAPK, P-GSK3 β , Akt, and P-Akt (New England Biolabs, Beverly, MA); P-MAPK (Promega Corp., Madison, WI); and p85 α , Cdk2, and Cdk4 (Santa Cruz Biotechnology, Santa Cruz, CA). Protein in cell lysates was measured by bicinchoninic acid (Pierce, Rockford, IL) or Bio-Rad (Bio-Rad Laboratories, Hercules, CA) assays. The HA antibody HA.11 was from BabCo (Richmond, VA).

Monolayer Growth and Anchorage-independent Growth Assays. For monolayer growth, cells were seeded at a density of 3–4 $\times 10^4$ cells in 12-well plates. Twenty-four h later, ZD1839 was added to the cells. Fresh medium \pm ZD1839 was replaced on day 3. On day 5, cells were harvested by trypsinization and counted with a Zeiss Coulter Counter (Beckman Coulter, Miami, FL). Colony-forming assays in soft agarose were performed as described previously (30). Tumor cell colonies measuring ≥ 50 μ m were counted after 7 days using an Omnicon 3800 colony counter and Tumor Colony Analysis V2.IIA software (Imaging Products International, Inc.).

Flow Cytometric Analysis of EGFR Cell Surface Expression. Breast cancer cells were plated in 100-mm dishes. WT3 cells, containing approximately 2 $\times 10^5$ EGF binding sites/cell, were used as positive controls. Cells were lifted with 0.02% EDTA in Ca²⁺- and Mg²⁺-free PBS, aliquoted into two sets of Eppendorf tubes (5 $\times 10^5$ cells/tube), and washed three times with staining buffer (5% heat-inactivated FCS in PBS). One set of cells was treated with 200 μ g/ml of the 528 EGFR monoclonal antibody (Santa Cruz Biotechnology) dissolved in 200 μ l of staining buffer. This antibody recognizes an epitope in the extracellular portion of the EGFR. A second set of cells was treated with 1 mg/ml of a nonspecific IgG2A (Sigma Chemical Co.), incubated for 20 min on ice, and washed three times with staining buffer. This was followed by a 20-min incubation with 0.5 mg/ml of a FITC-labeled goat antimouse IgG_{2A} (Southern Biotechnology) and 5 μ l of 7-amino-actinomycin D (2 mg/ml) dissolved in 200 μ l of staining buffer. All incubations were done in the dark on ice to avoid antibody and EGFR internalization. Flow cytometry of FITC-labeled cells was performed using a FACS/Calibur Flow Cytometer (Becton Dickinson, Mansfield, MA).

TUNEL Assays. BT-474 and SKBR-3 cells in IMEM/10% FCS were treated with ZD1839, Herceptin or a combination of the two drugs. Adherent cells were harvested by scraping and pooled with floating cells. TUNEL assay was performed using the APO-BrdU kit (Phoenix Flow Systems, San Diego,

CA). Flow cytometric detection of FITC-positive cells was performed using a FACS/Calibur flow cytometer.

Immunoblot Analysis and Immunoprecipitation. Cells were washed twice with ice-cold PBS, scraped in EBC lysis buffer [50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 0.5% NP40, 100 mM NaF, 200 μ M Na₃VO₄, and 10 μ g/ml each aprotinin, leupeptin, PMSF, and pepstatin], and incubated for 20 min at 4°C while rocking. Lysates were cleared by centrifugation (10 min at 12,000 rpm, 4°C). Fifty μ g protein were resolved by SDS-PAGE, transferred to nitrocellulose, and subjected to immunoblot analysis as described previously (30). Immunoreactive bands were detected using chemiluminescence (Roche Molecular Biochemicals, Indianapolis, IN). For immunoprecipitations, 1 mg of total protein from cell lysates was incubated overnight with primary antibody at 4°C; protein A-Sepharose (Sigma Chemical Co.) or protein G-Sepharose (Pharmacia) was then added for 2 h at 4°C while rocking. The precipitates were washed four times with ice-cold PBS, resuspended in 6 \times Laemmli sample buffer, and resolved using SDS-PAGE followed by immunoblot analysis.

In Vitro Kinase Assays. The HER2 *in vitro* kinase reaction was performed as described previously (31) with modifications. BT-474 cells were lysed in EBC buffer. One mg of total protein was precipitated with the HER2 antibody Ab-7 (Neomarkers) and protein A-Sepharose beads. Immunoprecipitates were washed three times with 20 mM HEPES (pH 7.5), 0.5 M LiCl and resuspended in kinase assay buffer [20 mM HEPES (pH 7.5), 10 mM MgCl₂, and 2 mM MnCl₂]. Kinase activity in the immune complexes was tested by adding 5 μ Ci [γ -³²P]ATP (specific activity, 3000 Ci/mmol; Amersham Pharmacia) and 10 μ M cold ATP (Sigma Chemical Co.). After a 10-min incubation at room temperature, the reaction was stopped by heating and adding Laemmli buffer; kinase products were resolved by SDS-PAGE, transferred to nitrocellulose, and exposed to X-ray film. The same nitrocellulose membrane was rinsed and then subjected to a HER2 immunoblot procedure (above).

For determination of PI3K activity, BT-474 cells were seeded at a density of 5 $\times 10^5$ cells/100-mm dish 24 h before treatment with ZD1839. After treatment, the cells were washed twice with 137 mM NaCl, 20 mM Tris (pH 7.5), 1 mM CaCl₂, 1 mM MgCl₂, and lysed in the wash buffer supplemented with 10% glycerol, 1% NP40, 1 mM PMSF, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 10 mM NaF, and 200 μ M Na₃VO₄. Cell extracts precleared by centrifugation were precipitated overnight with a P-Tyr monoclonal antibody (Upstate Biotechnology) and protein A-Sepharose. Immune complexes were washed three times with 1% NP40 in PBS; two times with 100 mM Tris (pH 7.5), 0.5 M LiCl; two times with 10 mM Tris (pH 7.5), 100 mM NaCl, 1 mM EDTA; and two times with kinase assay buffer [10 mM Tris (pH 7.5), 100 mM NaCl, 4 mM MgCl₂, 1 mM EDTA, and 0.5 mM EGTA]. All wash buffers contained 200 μ M Na₃VO₄. The beads were suspended in 40 μ l of kinase buffer, followed by addition of 10 μ Ci of [γ -³²P]ATP and sonication in 0.2 mg/ml phosphatidylinositol 4,5-bisphosphate (Avanti). The kinase reaction proceeded for 10 min at room temperature and was terminated with stop buffer (1:1 methanol:HCl), followed by extraction with chloroform. The reaction products were separated by TLC on 1% oxalate pretreated TLC with chloroform:methanol:acetone:glacial acetic acid:water (60:20:23:18:1) and detected by autoradiography.

Immunofluorescent Localization of p27^{KIP1}. BT-474 cells were seeded on coverslips in six-well plates at a density of 4 $\times 10^4$ cells/well. After a 12-h incubation with ZD1839, cells were washed with PBS, fixed with 4% paraformaldehyde in PBS for 10 min, washed, and stored overnight at 4°C. The cells were permeabilized with 0.1% Triton X-100/PBS for 15 min, washed, and then incubated for 1 h with a p27 monoclonal antibody (Transduction Laboratories) diluted 1:250 in 0.05% Triton X-100/PBS. After extensive washes, the cells were incubated for 45 min with antimouse Cy3 IgG (Jackson ImmunoResearch Labs., West Grove, PA) diluted 1:500 in 0.05% Triton X-100/PBS. The cells were then washed six times with 0.05% Triton X-100/PBS, stained with 1 mg/ml Hoechst, and mounted in AquaPoly Mount (PolySciences, Inc.). Cy3 immunofluorescence was recorded with a Princeton Instruments cooled digital CCD camera on a Zeiss Axiophot upright microscope.

Xenograft Studies in Athymic Mice. Five-week-old female Balb/C athymic nude mice (Harlan Sprague Dawley, Madison, WI) were implanted with 0.72-mg, 60-day release, 17 β -estradiol pellets (Innovative Research, Sarasota, FL). The next day, 2.5 $\times 10^7$ BT-474 cells suspended in (300 μ l) Growth Factor Reduced Matrigel (BD Biosciences, Bedford, MA) were injected s.c. in the right flank. Once tumors reached a volume ≥ 200 mm³, seven to eight mice/group were randomly allocated to treatment with: (a) vehicles: 0.05%

Tween 80 by oral gavage daily and sterile PBS by i.p. injection twice a week; (b) 10 mg/kg Herceptin in sterile PBS, administered by i.p. injection twice a week, and Tween 80 vehicle daily; (c) ZD1839 in 0.05% Tween 80, administered at 200 mg/kg/day by oral gavage, and sterile PBS i.p. twice a week; and (d) Herceptin and ZD1839. Tumor diameters were serially measured with calipers, and tumor volumes were calculated by the formula: volume = width² × length/2. After 4 weeks of treatment, 2 mice/group were injected with a single dose of BrdUrd (100 mg/kg), and their tumors were harvested 2 h later. Other tumors were homogenized using a Polytron homogenizer (Brinkmann, Westbury, NY) in TNE lysis buffer [50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 2 mM EDTA, 1 mM Na₃VO₄, 1 mM PMSF, 1 μg/ml pepstatin A, 2 μg/ml aprotinin, and 0.5 μg/ml leupeptin]. After homogenization, 1% (v/v) NP40 was added. Equivalent amounts of protein from the tumor lysates were next subjected to immunoblot analysis. For histological analysis, excised tumors were embedded in paraffin, sectioned, and stained with H&E. BrdUrd labeling of tumor cell nuclei was visualized by staining the sections with BrdUrd antibody (Biogenics, San Ramon, CA) using a streptavidin-biotin stain (Histo-mouse kit; Zymed, South San Francisco, CA). Additional sections were used to stain for apoptotic cells using the DNA *in situ* nick end-labeling (TUNEL) immunohistochemical method (Intergen, Purchase, NY) according to the manufacturer's instructions.

RESULTS

ZD1839 Is Specific for EGFR *in Vivo*. ZD1839 inhibits the EGFR (isolated from A431 cell membranes) and HER2 (baculovirus-expressed HER2 kinase domain) tyrosine kinases *in vitro* with an IC₅₀ of 0.033 μM and >3.7 μM, respectively (27).⁶ To confirm this specificity in intact cells, we used Rat1 cells stably transfected with HA-tagged EGFR (ErbB1) or HER2 (ErbB2) chimeric receptors. These chimeras contain the FKBP in their COOH terminus and the low affinity nerve growth factor receptor in the ectodomain to prevent activation by autocrine ligands. As described previously (29), addition of the bivalent FKBP ligand AP1510 resulted in phosphorylation of the chimeric EGFR or HER2 receptors without a change in receptor levels (Fig. 1, Lanes 1 and 2). Consistent with the *in vitro* IC₅₀ for EGFR, addition of ≤0.1 μM ZD1839 markedly inhibited ligand-induced EGFR phosphorylation, whereas >1 μM was required to block HER2 phosphorylation, thus confirming that ZD1839 is specific for EGFR *in vivo*.

ZD1839 Inhibits HER2 Phosphorylation and Growth in HER2-overexpressing Breast Tumor Cells That Coexpress EGFR. We next studied the effects of ZD1839 on SKBR-3, BT-474, MDA-361, and MDA-453 human breast cancer cells, which are known to overexpress HER2 (17, 32). SKBR-3, BT-474, and MDA-361 cells also express EGFR, whereas MDA-453 cells do not (32–35). Treatment of SKBR-3, MDA-361, and BT-474 cells with 1 μM ZD1839 resulted in substantial loss of HER2 tyrosine phosphorylation without a change in HER2 levels (Fig. 2A). In contrast, treatment with 10 μM ZD1839 was required to moderately reduce HER2 phosphorylation in the EGFR-negative MDA-453 cells. The ability of low concentrations of ZD1839 to inhibit HER2 phosphorylation in MDA-361, SKBR-3, and BT-474 cells implied a dissociation between the effects of the inhibitor on the HER2 kinase *in vitro* versus *in vivo*. Therefore, we next performed a more detailed dose response of ZD1839 on HER2 receptors in intact BT-474 cells versus HER2 receptors isolated from the same cell line. Treatment of BT-474 cells with 1 μM ZD1839 resulted in near complete inhibition of HER2 phosphorylation *in vivo* (Fig. 2B). In contrast, increasing the concentrations of ZD1839 to 2–5 μM only modestly reduced the phosphorylation of isolated HER2 receptors in an *in vitro* kinase reaction (Fig. 2C), suggesting that ZD1839 was a far better inhibitor of the HER2 kinase in intact cells. This result suggests that ZD1839 uses an indirect mechanism for HER2 inhibi-

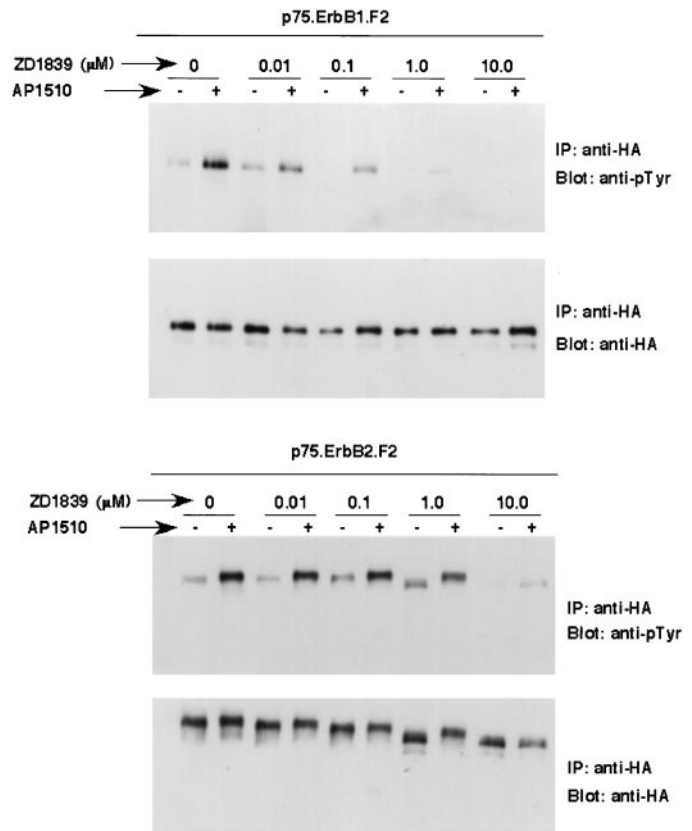


Fig. 1. ZD1839 specifically blocks EGFR (ErbB1) phosphorylation. Rat1 fibroblasts expressing EGFR (ErbB1) or HER2 (ErbB2) fused to one copy of FKBP were stimulated with 500 nM AP1510 for 15 min. Where indicated, 0.01–10 μM ZD1839 was added immediately before AP1510. Cell lysates were collected, and 500 μg were used for precipitation with an anti-HA antibody, followed by P-Tyr or HA immunoblot procedures as indicated in "Materials and Methods." IP, immunoprecipitation.

tion in intact cells but does not directly inhibit the catalytic activity of HER2.

All four cell lines were incubated with various concentrations of ZD1839 in both cell monolayer and anchorage-independent growth assays. Treatment of adherent SKBR-3 and BT-474 cells with ≤1 μM concentrations of ZD1839 for 5 days substantially impaired cell proliferation compared with controls. Higher concentrations of ZD1839 (10 μM) were required to inhibit MDA-361 cells but had no effect on MDA-453 cells (Fig. 3A). Similarly, 1 μM ZD1839 markedly inhibited SKBR-3 and BT-474 colony formation, whereas 10-fold higher concentrations were required to reduce MDA-361 and MDA-453 colony growth. However, in this assay the IC₅₀ of ZD1839 was 2 μM and >5 μM for MDA-361 and MDA-453 cells, respectively (data not shown).

ZD1839 Modulates Cell Cycle and Survival Regulatory Molecules. The HER (erbB) signaling network activates several signaling pathways that subvert the G₁-to-S transition as well as disable proapoptotic molecules (1, 3), thus leading to dysregulated proliferation and enhanced tumor cell survival. In line with its ability to inhibit HER2 kinase function, a 24-h treatment of BT-474 cells with 1 μM ZD1839 increased the G₁ fraction from 74 to 88% and reduced the proportion of cells in S from 15 to 4%. Simultaneous with the accumulation of cells in G₁ was complete elimination of both active Akt and MAPK, as measured with phosphospecific antibodies, without changes in the content of total Akt and MAPK. Consistent with the inhibition of Akt activity, phosphorylation of GSK-3β, a target of the Akt kinase, was reduced. Cyclin D1 and Cdk4 were also reduced, whereas protein levels of the Cdk inhibitor p27 were up-regulated

⁶ Alan Wakeling, AstraZeneca Pharmaceuticals, personal communication.

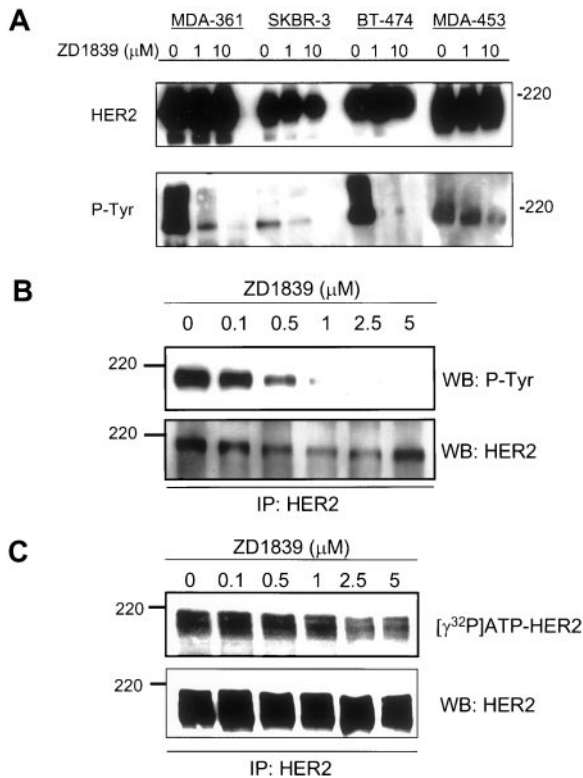


Fig. 2. ZD1839 preferentially inhibits HER2 phosphorylation *in vivo* but not *in vitro*. **A**, HER2-overexpressing human breast cancer cell lines in 10% FCS were treated for 20 h with 1–10 μM ZD1839. After washes, cell lysates were lysed in EBC buffer containing phosphatase and protease inhibitors. Equivalent amounts of protein (50 $\mu\text{g}/\text{lane}$) were subjected to SDS-PAGE, followed by a HER2 immunoblot procedure (*top*), or 500 μg were precipitated first with HER2 antibodies followed by SDS-PAGE and P-Tyr immunoblot analysis (*bottom*). **B**, rapidly proliferating BT-474 cells were treated with the indicated concentrations of ZD1839 for 20 h. Cells were lysed, and lysate was precipitated with the HER2 antibody AB-7 and protein A-Sepharose. Immune complexes were divided in half, and each aliquot was subjected to P-Tyr or HER2 immunoblot procedures. **C**, HER2 was isolated from a BT-474 cell lysate by precipitation with the HER2 antibody Ab-7. The immune complexes were divided in identical amounts in kinase assay buffer and incubated with [γ - ^{32}P]ATP for 10 min in the presence or absence of 0.1–5 μM ZD1839. Radiolabeled HER2 (*top*) and HER2 protein levels (*bottom*) were detected by autoradiography and HER2 immunoblot, respectively, as indicated in “Materials and Methods.” For all panels, molecular weights in thousands are indicated next to the immunoreactive and ^{32}P -labeled bands. WB, Western blot.

(Fig. 4A). In addition to altering p27 levels, treatment with ZD1839 induced redistribution of p27 from the cytosol to the nucleus as detected by immunofluorescence (Fig. 4B). These effects on Akt, MAPK, and p27 were not seen in the EGFR-negative MDA-453 cells (Fig. 4C).

Because of (a) the effect of ZD1839 on Akt, serine/threonine kinase activated by PI3K (36); (b) the unique ability of HER3, present in the BT-474 cells, to couple to the PI3K pathway directly relative to EGFR and HER2 (37); and (c) the constitutive association of HER2/HER3 reported in tumor cells with HER2 overexpression (17), we examined the effect of ZD1839 on the coupling of HER2 with HER3 and on PI3K activity. Under basal growth conditions, HER3 coprecipitated with HER2 antibodies (Fig. 5A, *left*), indicating the presence of constitutive HER2/HER3 complexes. HER3 exhibited a low level of basal tyrosine phosphorylation and was constitutively associated with p85 α , the regulatory subunit of PI3K (Fig. 5A, *right*). Treatment with 1 μM ZD1839 for 20 h eliminated the association of HER3 with HER2, the association of HER3 with p85 α , and the low level of HER3 basal phosphorylation. Consistent with the loss of association of HER3 with PI3K, ZD1839 eliminated PI3K activity in the HER2-overexpressing cells as measured by the ability of P-Tyr precipitates from BT-474 cell lysates to induce the formation of phosphatidyl-

inositol 3,4,5 trisphosphate from phosphatidylinositol 4,5 bisphosphate *in vitro* (Fig. 5B).

Combination of ZD1839 with HER2 Antibody (Herceptin) Induces Apoptosis of Breast Tumor Cells. We next examined the antitumor effect of combined blockade of the HER network with ZD1839 and Herceptin, a humanized IgG1 that binds to the extracellular juxtamembrane domain of HER2 (38). ZD1839 at concentrations that did not affect the HER2 kinase directly ($\leq 1 \mu\text{M}$) inhibited SKBR-3 colony formation in soft agarose. Herceptin also inhibited colony formation but less than ZD1839, and the combination was not more potent than ZD1839 alone (Fig. 6A). In this assay, BT-474 cells were exquisitely sensitive to either ZD1839 or Herceptin with an IC_{50} of 0.2 μM and 0.1 $\mu\text{g}/\text{ml}$, respectively (data not shown). Because of the exquisite sensitivity of BT-474 colonies to either inhibitor, we were unable to demonstrate a synergistic or supra-additive effect of the combination against these cells in this assay. Because the colony-forming assay measures both anchorage-independent proliferation and survival, we next focused on an effect of the combination on tumor cell apoptosis as measured by DNA double-strand breaks in the presence of terminal deoxynucleotidyl transferase. In SKBR-3, treat-

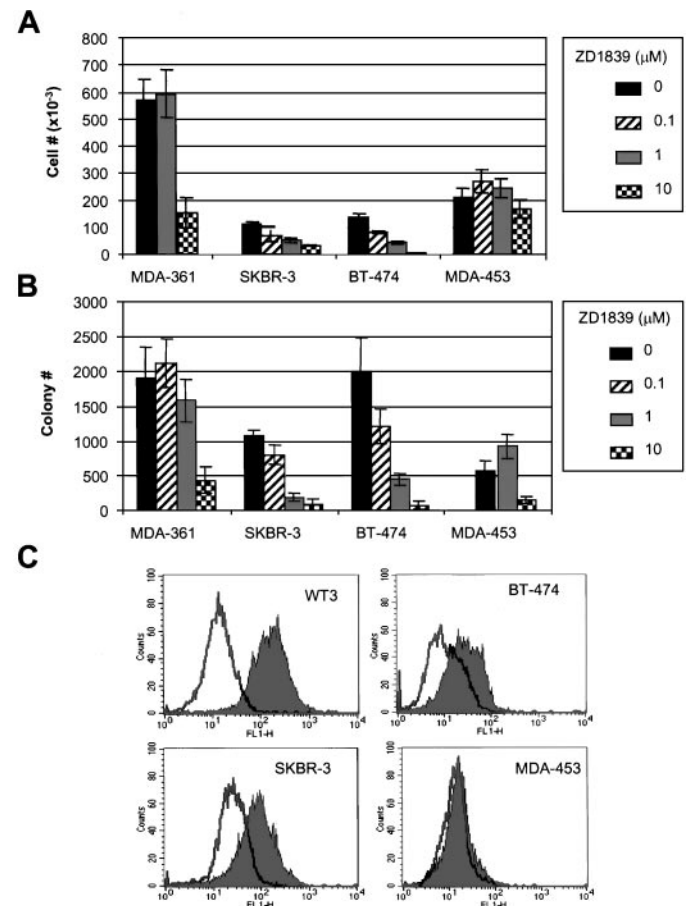


Fig. 3. ZD1839 preferentially inhibits growth of EGFR-positive, HER2-overexpressing breast tumor cells. Breast cancer cell lines were plated in monolayer (**A**) or in soft agarose (**B**) in the presence of absence of 0.1–10 μM ZD1839. Proliferation and colony formation ($\geq 50 \mu\text{m}$) were assessed 5 or 7 days later, respectively, as described in “Materials and Methods.” Each column represents the mean cell number (**A**) or colony number of triplicate dishes (**B**); bars, SD. **C**, detection of EGFR by flow cytometry. Cells were prepared as described in “Materials and Methods” and incubated with the 528 EGFR monoclonal antibody or a nonspecific IgG $_{2A}$, each followed by FITC-labeled goat antimouse IgG $_{2A}$. FITC-labeled cells were detected using flow cytometry. The *clear peaks* mark the IgG $_{2A}$ -stained populations, and the *dark area* marks the EGFR-positive cells labeled with 528 mAb. Except for MDA-453 cells, all cells exhibited an FITC-positive population that stained with 528 mAb but not with the nonspecific control IgG. In MDA-453 cells, both populations overlapped, indicating lack of cell surface EGFR.

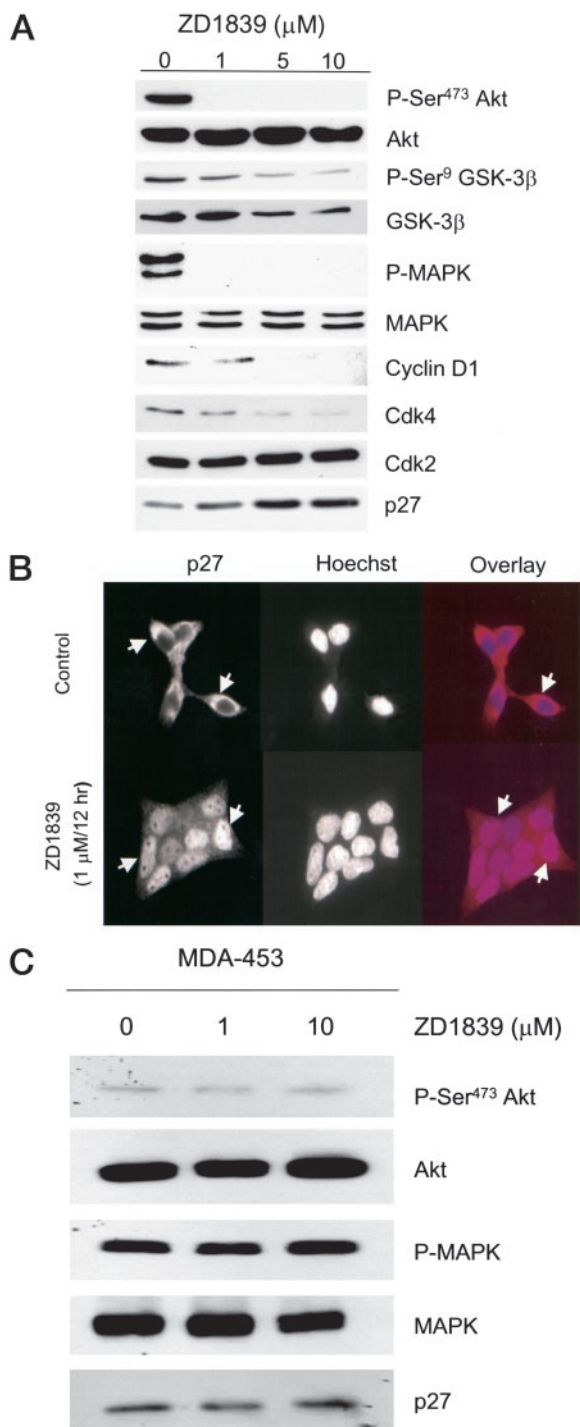


Fig. 4. ZD1839 modulates cell cycle and survival regulatory molecules. *A*, BT-474 cells were incubated for 20 h with 1–10 μM ZD1839. Cells were lysed in EBC buffer; 75 μg of total protein/lane were separated by SDS-PAGE and subjected to immunoblot analysis with the indicated antibodies. *B*, localization of p27 by immunofluorescence. BT-474 cells were treated with 1 μM ZD1839 for 12 h, fixed onto coverslips in 4% paraformaldehyde, and stained with a p27 mAb, followed by antimouse Cy3 as indicated in "Materials and Methods." Nuclei were stained with Hoechst. Treatment with ZD1839 induces translocation of Cy3 fluorescence from the cytosol to the nucleus (p27 column). Overlay of the red Cy3 over the Hoechst image results in loss of the blue nuclear Hoechst stain, indicating translocation of p27 to the nucleus. *C*, MDA-453 cells were treated with 1–10 μM ZD1839 for 24 h and then subjected to the same immunoblot procedures as in *A*.

ment with either Herceptin or ZD1839 for 72 h induced a 3-fold increase in the basal level of apoptosis with the combination inducing a 6–7-fold supra-additive effect. On the other hand, neither inhibitor induced apoptosis *per se* of BT-474 cells. However, BT-474 cells

treated with both Herceptin and ZD1839 exhibited a 5-fold increase in apoptosis compared with untreated controls (Fig. 6B).

ZD1839 Abrogates TGF- α -induced BT-474 Cell Proliferation Better Than Herceptin. With some exceptions, it is generally accepted that the EGFR kinase is required and sufficient for transmodulation of HER2. A truncated EGFR mutant lacking the EC domain of the receptor can associate with full-length HER2 (39). In addition, heterodimerization of the cytoplasmic domains of EGFR and HER2 is sufficient to activate both kinases (29), but the soluble EGFR extracellular domain is unable to hetero-oligomerize with the soluble ectodomain of HER2 (40). These results suggest that conserved regions in the EGFR kinase domain are involved in EGFR/HER2 heterodimerization. Therefore, we next studied the ability of either ZD1839 or Herceptin to block TGF- α -induced proliferation of BT-474 cells. Addition of 20 ng/ml (3 nM) TGF- α resulted in a 2.5-fold increase in cell number over 5 days. This increase was completely abrogated by 1 μM ZD1839 but not by a saturating concentration (20 $\mu\text{g}/\text{ml}$) of Herceptin (Fig. 7).

ZD1839 Prevents Growth of Established BT-474 Xenografts and Enhances the Antitumor Effect of Herceptin. We finally examined the effect of ZD1839 alone or in combination with Herceptin against BT-474 tumors *in vivo*. These tumors have been shown to be arrested or eliminated by treatment with Herceptin (41). Once x-

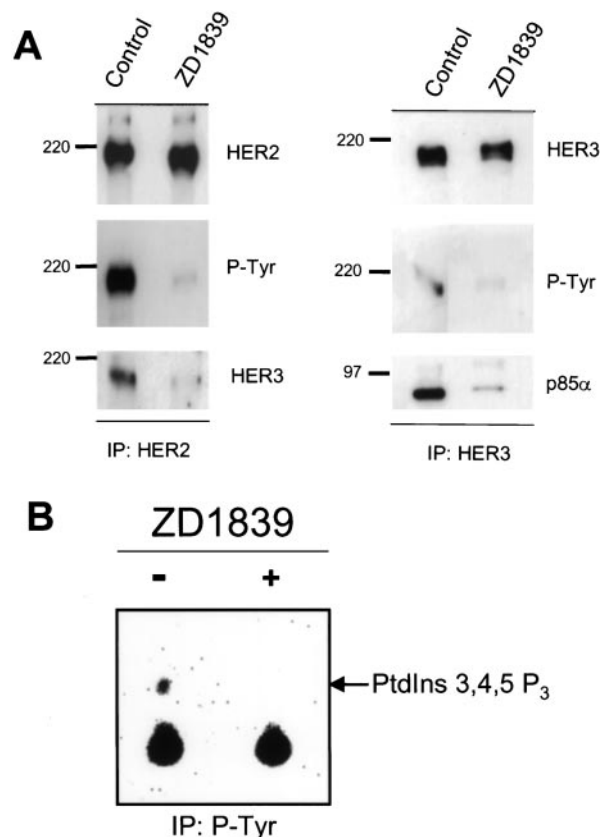


Fig. 5. ZD1839 disrupts HER2-HER3 association and PI3K activity. *A*, subconfluent, exponentially growing BT-474 cells in IMEM/10% FCS were treated or not with 1 μM ZD1839 for 20 h. After cell lysis, HER2 or HER3 were precipitated (IP) with specific antibodies. Precipitates were divided equally and subjected to immunoblot analysis using the indicated antibodies. Molecular weights in thousands are shown on the left of each panel. ZD1839 inhibited the basal association of HER3 with HER2 and with p85 α without affecting HER3 or HER2 levels. *B*, after similar treatment, BT-474 cells were lysed, and cell lysates were precipitated with a P-Tyr monoclonal antibody and protein A-Sepharose as indicated in "Materials and Methods." Immune complexes were next tested for PI3K activity in an *in vitro* reaction containing phosphatidylinositol 4,5-trisphosphate and [γ - ^{32}P]ATP. Reaction products were separated by TLC and detected by autoradiography. The site of migration of PtdIns3,4,5P $_3$ is indicated.

nografts had reached a volume $\geq 200 \text{ mm}^3$, tumor-bearing nude mice were randomly allocated to either no treatment, ZD1839, Herceptin, or the combination. ZD1839 completely prevented tumor growth but did not induce complete remissions ($n = 8$). Herceptin alone induced complete remission in two of seven, whereas the combination resulted in three of eight complete responses ($P = 0.06$, Student's unpaired t test; Fig. 8). No mice exhibited treatment-related toxicity. Three mice treated with ZD1839 plus Herceptin, in which tumors regressed completely, remained tumor free for >6 months after discontinuation of therapy and had no detectable tumor at necropsy.

To determine whether the different treatments were antiproliferative versus cytotoxic, two mice/group were pulsed with BrdUrd prior to harvesting the tumors on day 28 of therapy. Examination of 10 random fields showed a high proportion of BrdUrd-positive nuclei (28.7%) in control xenografts. All treatments resulted in a reduction of the proportion of cells in S, as measured by BrdUrd staining (Fig. 8B), but this decrease was more marked in the tumors treated with ZD1839. The antiproliferative effect of the combination was statistically greater than that induced by Herceptin alone (10.4 versus 22% BrdUrd-positive tumor cells, respectively; $P = 0.001$, Student's unpaired t test). The total number of apoptotic bodies in 10 high-power fields as detected by TUNEL analysis was not different among tumors from any of the treatment groups (data not shown). Finally, we examined for evidence of inhibition of HER2 signaling as a result of

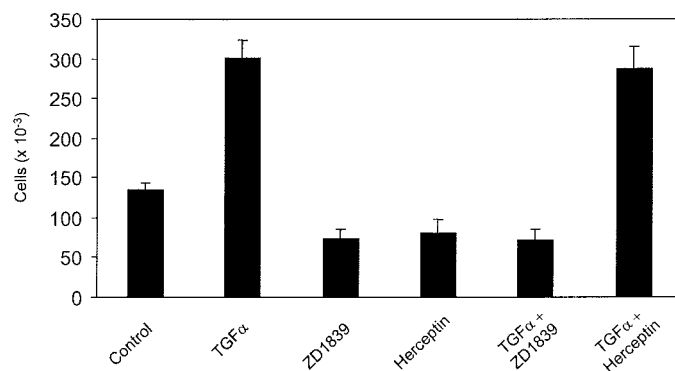


Fig. 7. ZD1839 blocks TGF- α -induced proliferation of HER2-overexpressing breast tumor cells. BT-474 cells were plated in full growth medium in the presence or absence of 20 ng/ml TGF- α , 1 μM ZD1839, or 20 $\mu\text{g/ml}$ Herceptin, either alone or in the indicated combinations. Medium and reagents were replenished on day 3. Cell number was assessed on day 5 as indicated in "Materials and Methods." Each column represents the mean cell number of three wells; bars, SD.

treatment. HER2 content as measured by immunoblot analysis of tumor lysates was roughly equal in all groups. In all treatment groups, Rb was hypophosphorylated, and both active MAPK and Akt were inhibited with no change in total MAPK and Akt tumor levels (Fig. 8C). These data suggest that, in addition to arresting or eliminating xenografts, all three treatments interfered with HER2 signaling *in situ*.

DISCUSSION

We have examined the antitumor effect of ZD1839 (Iressa), an EGFR-specific tyrosine kinase inhibitor, against human breast cancer cells with *HER2/neu* gene amplification (17, 32). Two of these cell lines, SKBR-3 and BT-474, have been shown to be HER2-dependent in that inhibition of the proto-oncogene product with HER2-specific antibodies or HER kinase inhibitors leads to growth arrest and/or tumor cell death both *in vitro* and *in vivo* (30, 41–43). The concentration of ZD1839 that was required to inhibit HER2 phosphorylation in intact cells and to inhibit at least 50% (IC_{50}) of the growth of EGFR-positive SKBR-3, BT-474, and MDA-361 cells was lower than that required to inhibit the HER2 kinase *in vitro*. These results strongly imply that the inhibition of the HER2 kinase *in vivo* by ZD1839 is not attributable to a direct competition for ATP binding to HER2. This argument is further supported by the following results: (a) the inability of $\leq 1 \mu\text{M}$ ZD1839 to inhibit the catalytic activity of HER2 in an *in vitro* kinase reaction using HER2 receptors isolated from BT-474 cells (Fig. 2C); (b) the requirement of 10 μM ZD1839 to inhibit the phosphorylation of HER2-FKBP chimeric receptors in response to AP1510 (Fig. 1); and (c) the inability of 1 μM ZD1839 to inhibit HER2 phosphorylation and downstream signaling pathways in the EGFR-negative MDA-453 cells (Figs. 2A and 4C). Although growth inhibition was seen in all cell lines with 10 μM ZD1839, it is possible that at these concentrations, ZD1839 cross-reacts nonspecifically with HER2 and other EGFR-homologous kinases. Of note, maximal steady-state (trough) plasma levels of ZD1839 of $\sim 1 \mu\text{M}$ have been achieved in patients at the recommended clinical dose of (0.5 mg/day) ZD1839.⁷ This suggests that concentrations $> 1 \mu\text{M}$, at which ZD1839 could interact with other targets nonspecifically, may not be practically achievable. Therefore, at concentrations that would not have been predicted from an *in vitro* EGFR kinase reaction, EGFR-specific inhibitors also block, albeit indirectly, the HER2 kinase in cells that also coexpress EGFR. These results also suggest that, in addition to EGFR levels, the coexpression of HER receptors

⁷ Steven Averbuch, personal communication.

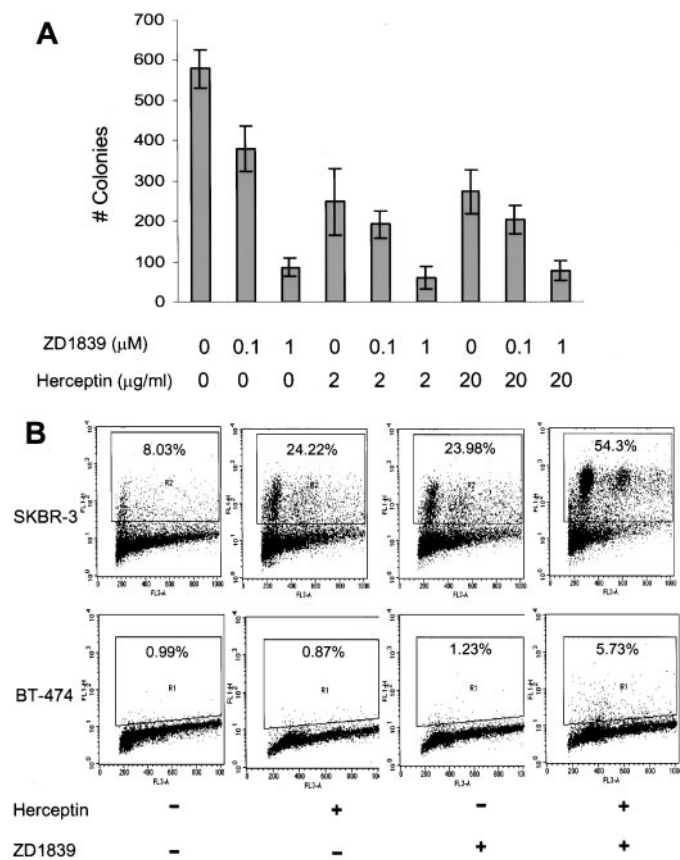


Fig. 6. Combined effects of ZD1839 and Herceptin. A, SKBR-3 cells were plated in a soft agarose colony-forming assay in the presence of the indicated concentrations of ZD1839 and/or Herceptin as described in "Materials and Methods." Each column represents the mean number of $\geq 50 \mu\text{m}$ -diameter colonies of three dishes; bars, SD. Results were reproduced in two independent experiments. B, SKBR-3 and BT-474 cells were treated with 10 $\mu\text{g/ml}$ Herceptin, 1 μM ZD1839, or the combination for 72 h. Adherent and floating cells were harvested and assayed for evidence of apoptosis by Apo-BrdUrd analysis in the presence of terminal deoxynucleotidyl transferase. The percentage of FITC-positive apoptotic cells in the R2 (SKBR-3) and R1 (BT-474) gated areas were quantitated by flow cytometry and are shown in parentheses.

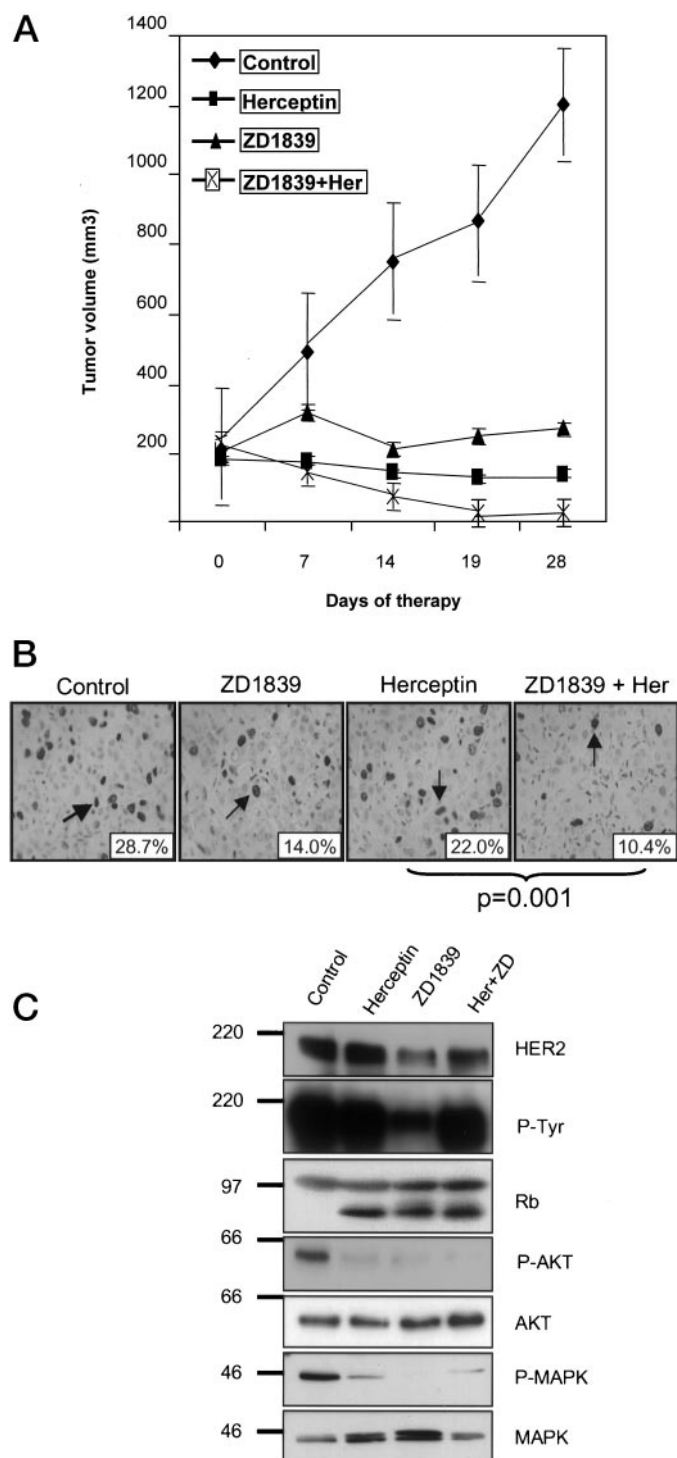


Fig. 8. A, ZD1839 enhances the antitumor effect of Herceptin *in vivo*. Estrogen-supplemented nude mice bearing $\geq 200 \text{ mm}^3$ s.c. BT-474 xenografts were randomly allocated to treatment with vehicles (◆), ZD1839 (△), Herceptin (■), or the combination (×) for the next 28 days. Tumor volumes were measured serially as indicated in "Materials and Methods." Each data point represents the mean tumor volume of seven to eight mice; bars, SD. B, inhibition of tumor cell proliferation. On day 28 of treatment, two mice/group were randomly selected and pulsed with BrdUrd before tumor harvesting. Tumor sections were stained with a BrdUrd antibody, and BrdUrd-positive nuclei were counted in 10 high-power microscopic fields. Numbers in insets denote the percentage of BrdUrd-positive nuclei relative to the total number of tumor cell nuclei in the same fields. The total number of nuclei counted in each group were 1214 (controls), 1014 (ZD1839), 1810 (Herceptin), and 975 (ZD1839 plus Herceptin). $P = 0.001$, Student's unpaired *t* test. C, inhibition of HER2 signaling *in situ*. On day 28, some tumors were harvested, snap-frozen in liquid nitrogen, and later homogenized in a buffer containing protease and phosphatase inhibitors as described in "Materials and Methods." An equivalent amount of protein from the tumor lysates were subjected to HER2, P-Tyr, Rb, P-Akt, total Akt, P-MAPK, and total MAPK immunoblot analyses. Left, molecular weights in thousands.

and their functional interaction with EGFR in different tumor cell backgrounds might dictate the true antitumor effect of some ATP-competitive inhibitors of the EGFR tyrosine kinase.

Interestingly, ZD1839 also disrupted the previously reported constitutive association of HER2 with HER3 in cells that overexpress HER2 (17, 43). Treatment with ZD1839 resulted in the uncoupling of HER3 from p85 and loss of the basal PI3K activity in HER2-overexpressing cells (Fig. 5). This result is somewhat surprising in that there is no strong rationale to suspect an interaction of the quinazoline with the less EGFR-homologous, kinase-impaired HER3 receptor. It has been reported, however, that the anti-EGFR quinazolines AG1478 and AG1517, highly similar in structure to ZD1839, can induce the formation of inactive EGFR homodimers as well as inactive EGFR/HER2 heterodimers (44). Lichtner *et al.* (45) reported recently that ZD1839 was also capable of inducing the formation of signaling-inactive EGFR complexes. In SKBR-3 cells, the inactive heterodimerization of EGFR/HER2 mediated by AG1517 was temporally associated with loss of the growth response to the HER3 ligand heregulin (44). In another study, treatment of PC12 rat cells with EGF induced primary EGFR/Neu heterodimers and secondary Neu/ErbB3 (HER2/HER3) heterodimers (46). The formation of EGF-induced secondary receptor heterodimers was eliminated by the EGFR-specific kinase inhibitor AG1478 (47) but modestly inhibited by the Neu kinase inhibitor AG879 (46), suggesting that EGFR-mediated transmodulation of Neu (the rat homologue of human HER2) is essential for the formation of Neu/ErbB3 secondary dimers. This result raises the speculation that in cells with high levels of constitutively active HER2 that also express EGFR and HER3, the inhibition of EGFR/HER2 cross-talk with ZD1839 may lead secondarily to the uncoupling of HER2/HER3, as implied by the result shown in Fig. 5. Taken together, these data suggest that some EGFR kinase inhibitors, such as ZD1839, may have the ability to prevent HER2 from heterodimerizing with other HER receptors in a dominant-negative manner and thus widely affect signaling by the HER (erbB) network.

Blockade of the EGFR kinase with ZD1839 was markedly more effective than Herceptin in inhibiting TGF- α -induced cell proliferation in BT-474 cells (Fig. 7). This result is also consistent with the report by Gamett *et al.* (46); the HER1-specific kinase inhibitor AG1478 almost completely eliminated EGF-induced (*trans*) phosphorylation of Neu, whereas AG879, a direct inhibitor of Neu autophosphorylation with no activity against EGFR (47, 48), had a modest effect. In another study, treatment of OVCA420 ovarian cancer cells with EGF induced phosphorylation of HER2 that was not blocked by mAb 4D5, the mouse homologue of Herceptin (49). In this same study, mAb C225, an anti-EGFR humanized IgG1 that binds to the ectodomain of the receptor, induces receptor homodimerization, blocks ligand binding to EGFR, and was unable to block basal or EGF-induced tyrosine phosphorylation of HER2 (49). In addition, a constitutively active mutant form of EGFR, lacking the extracellular domain and the hydrophobic leader sequence, associated strongly with HER2, despite its inability to insert into the plasma membrane (39). Finally, EGF induces efficient homodimerization of purified EGFR extracellular domains but is unable to stabilize EGFR/HER2 heterooligomers of receptor extracellular domains (40). Taken together, these data and the results shown in Fig. 7 point to the role of the EGFR kinase on HER2 transmodulation. If so, targeting the kinase domain with therapeutic inhibitors might be a more effective strategy to rapidly disable lateral signaling within the HER network than the use of bivalent antibodies against the ectodomain of the receptor.

Combined treatment with ZD1839 and Herceptin resulted in enhanced tumor cell apoptosis (Fig. 6) and larger tumor reduction than either inhibitor alone (Fig. 8). It is conceivable that the combination exerts a more effective or sustained inhibition of survival signals

up-regulated by the HER signaling network. Elucidation of these pathways to explain the supra-additive antitumor effect will require further investigation. Nonetheless, these results suggest that simultaneous blockade of different molecular sites within the HER network may diminish potential compensatory mechanisms by tumor cells compared with more limited targeted approaches against this signaling network. Of note, ZD1839 arrested tumor growth (Fig. 8) and markedly inhibited BrdUrd incorporation in tumor cells but failed to completely eradicate eight of eight BT-474 xenografts (Fig. 9). On the other hand and as published previously (41), Herceptin induced two of seven complete tumor regressions but induced less detectable inhibition of tumor cell proliferation as measured by BrdUrd incorporation. This result suggests the possibility that Herceptin may induce cancer regressions by tumor cell nonautonomous mechanisms as suggested by Clynes *et al.* (50). In this stimulating report, BT-474 xenografts were established in mice deficient in the Fc receptor Fc γ RIII, which is involved in the recruitment of immune cells that mediate antibody-dependent cell-mediated cytotoxicity. In these mice, the antitumor effect of Herceptin against BT-474 xenografts was drastically reduced. Moreover, HER2 antibodies engineered to disrupt Fc binding did not arrest tumor growth *in vivo* (50), suggesting that Fc receptor-dependent mechanisms contribute substantially to the cytotoxic effect of Herceptin. Nonetheless, the data presented in Fig. 9 clearly demonstrate that Herceptin and ZD1839 inhibited signaling molecules downstream HER2 in the BT-474 xenografts. This is the first demonstration of an anti-signaling effect of Herceptin *in vivo*. This tumor cell-autonomous effect may complement the potential Fc receptor-mediated mechanism of tumor elimination proposed by Clynes *et al.* (50).

In summary, we have presented data to support the use of EGFR-specific tyrosine kinase inhibitors against EGFR-positive, HER2-overexpressing human breast tumor cells. ZD1839, the ATP-competitive inhibitor of the EGFR tyrosine kinase used in these studies, effectively disrupted HER2/HER3 interactions, completely prevented TGF- α -induced mitogenesis of tumor cells with high HER2 levels, and synergized with the HER2 antibody Herceptin against SKBR-3 and BT-474 cells. Taken together, these data support the use of EGFR kinase inhibitors in combination with Herceptin in patients bearing breast cancers with HER2 overexpression. Whether the combination will be more effective against tumors that also express a threshold level of EGFR, as implied by the studies herein, will require prospective investigation.

Acknowledgments

We thank ARIAD Pharmaceuticals for providing AP1510 as well as Drs. Alan Wakeling and Steven Averbuch for critical review of the manuscript. Iressa is a trademark of the AstraZeneca group of companies.

References

- Olayioye, M. A., Neve, R. M., Lane, H. A., and Hynes, N. E. The ErbB signaling network: receptor heterodimerization in development and cancer. *EMBO J.*, *19*: 3159–3167, 2000.
- Graus-Porta, D., Beerli, R. R., Daly, J. M., and Hynes, N. E. ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. *EMBO J.*, *16*: 1647–1655, 1997.
- Yarden, Y., and Sliwkowski, M. X. Untangling the ErbB signalling network. *Nat. Rev. Mol. Cell. Biol.*, *2*: 127–137, 2001.
- Karunakaran, D., Tzahar, E., Beerli, R. R., Chen, X., Graus-Porta, D., Ratzkin, B. J., Seger, R., Hynes, N. E., and Yarden, Y. ErbB-2 is a common auxiliary subunit of NDF and EGF receptors: implications for breast cancer. *EMBO J.*, *15*: 254–264, 1996.
- Lenferink, A. E., Pinkas-Kramarski, R., van de Poll, M. L., van Vugt, M. J., Klapper, L. N., Tzahar, E., Waterman, H., Sela, M., van Zoelen, E. J., and Yarden, Y. Differential endocytic routing of homo- and hetero-dimeric ErbB tyrosine kinases confers signaling superiority to receptor heterodimers. *EMBO J.*, *17*: 3385–3397, 1998.
- Tzahar, E., Waterman, H., Chen, X., Levkowitz, G., Karunakaran, D., Lavi, S., Ratzkin, B. J., and Yarden, Y. A hierarchical network of interreceptor interactions determines signal transduction by Neu differentiation factor/neuregulin and epidermal growth factor. *Mol. Cell. Biol.*, *16*: 5276–5287, 1996.
- Pinkas-Kramarski, R., Soussan, L., Waterman, H., Levkowitz, G., Alroy, I., Klapper, L., Lavi, S., Seger, R., Ratzkin, B. J., Sela, M., and Yarden, Y. Diversification of Neu differentiation factor and epidermal growth factor signaling by combinatorial receptor interactions. *EMBO J.*, *15*: 2452–2467, 1996.
- Sliwkowski, M. X., Schaefer, G., Akita, R. W., Lofgren, J. A., Fitzpatrick, V. D., Nuijens, A., Fendly, B. M., Cerione, R. A., Vandlen, R. L., and Carroway, K. L., III. Coexpression of erbB2 and erbB3 proteins reconstitutes a high affinity receptor for heregulin. *J. Biol. Chem.*, *269*: 14661–14665, 1994.
- Wang, L. M., Kuo, A., Alimandi, M., Veri, M. C., Lee, C. C., Kapoor, V., Ellmore, N., Chen, X. H., and Pierce, J. H. ErbB2 expression increases the spectrum and potency of ligand-mediated signal transduction through ErbB4. *Proc. Natl. Acad. Sci. USA*, *95*: 6809–6814, 1998.
- Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A., and McGuire, W. L. Human breast cancer: correlation of relapse and survival with amplification of the *HER-2/neu* oncogene. *Science (Wash. DC)*, *235*: 177–182, 1987.
- Salomon, D. S., Brandt, R., Ciardiello, F., and Normanno, N. Epidermal growth factor-related peptides and their receptors in human malignancies. *Crit. Rev. Oncol. Hematol.*, *19*: 183–232, 1995.
- Harris, A. L., Nicholson, S., Sainsbury, J. R., Farndon, J., and Wright, C. Epidermal growth factor receptors in breast cancer: association with early relapse and death, poor response to hormones and interactions with neu. *J. Steroid Biochem.*, *34*: 123–131, 1989.
- Pierce, J. H., Arnstein, P., DiMarco, E., Artrip, J., Kraus, M. H., Lonardo, F., Di Fiore, P. P., and Aaronson, S. A. Oncogenic potential of erbB-2 in human mammary epithelial cells. *Oncogene*, *6*: 1189–1194, 1991.
- Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ullrich, A., *et al.* Studies of the *HER-2/neu* proto-oncogene in human breast and ovarian cancer. *Science (Wash. DC)*, *244*: 707–712, 1989.
- Baselga, J., Tripathy, D., Mendelsohn, J., Baughman, S., Benz, C. C., Dantis, L., Sklarin, N. T., Seidman, A. D., Hudis, C. A., Moore, J. J., Rosen, P. P., Twaddell, T., Henderson, I. C., and Norton, L. Phase II study of weekly intravenous recombinant humanized anti-p185HER2 monoclonal antibody in patients with HER2/neu-overexpressing metastatic breast cancer. *J. Clin. Oncol.*, *14*: 737–744, 1996.
- Cobleigh, M. A., Vogel, C. L., Tripathy, D., Robert, N. J., Scholl, S., Fehrenbacher, L., Wolter, J. M., Paton, V., Shak, S., Lieberman, G., and Slamon, D. J. Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. *J. Clin. Oncol.*, *17*: 2639–2648, 1999.
- Alimandi, M., Romano, A., Curia, M. C., Muraro, R., Fedi, P., Aaronson, S. A., Di Fiore, P. P., and Kraus, M. H. Cooperative signaling of ErbB3 and ErbB2 in neoplastic transformation and human mammary carcinomas. *Oncogene*, *10*: 1813–1821, 1995.
- Thor, A. D., Liu, S., Edgerton, S., Moore, D., II, Kasowitz, K. M., Benz, C. C., Stern, D. F., and DiGiovanna, M. P. Activation (tyrosine phosphorylation) of ErbB-2 (HER-2/neu): a study of incidence and correlation with outcome in breast cancer. *J. Clin. Oncol.*, *18*: 3230–3239, 2000.
- Samanta, A., LeVea, C. M., Dougall, W. C., Qian, X., and Greene, M. I. Ligand and p185-neu density govern receptor interactions and tyrosine kinase activation. *Proc. Natl. Acad. Sci. USA*, *91*: 1711–1715, 1994.
- Stern, D. F., and Kamps, M. P. EGF-stimulated tyrosine phosphorylation of p185neu: a potential model for receptor interactions. *EMBO J.*, *7*: 995–1001, 1988.
- Wada, T., Qian, X. L., and Greene, M. I. Intermolecular association of the p185neu protein and EGF receptor modulates EGF receptor function. *Cell*, *61*: 1339–1347, 1990.
- Goldman, R., Levy, R. B., Peles, E., and Yarden, Y. Heterodimerization of the erbB-1 and erbB-2 receptors in human breast carcinoma cells: a mechanism for receptor transregulation. *Biochemistry*, *29*: 11024–11028, 1990.
- Kokai, Y., Myers, J. N., Wada, T., Brown, V. I., LeVea, C. M., Davis, J. G., Dobashi, K., and Greene, M. I. Synergistic interaction of p185c-neu and the EGF receptor leads to transformation of rodent fibroblasts. *Cell*, *58*: 287–292, 1989.
- Muller, W. J., Arteaga, C. L., Muthuswamy, S. K., Siegel, P. M., Webster, M. A., Cardiff, R. D., Meise, K. S., Li, F., Halter, S. A., and Coffey, R. J. Synergistic interaction of the *Neu* proto-oncogene product and transforming growth factor α in the mammary epithelium of transgenic mice. *Mol. Cell. Biol.*, *16*: 5726–5736, 1996.
- Lenferink, A. E., Simpson, J. F., Shawver, L. K., Coffey, R. J., Forbes, J. T., and Arteaga, C. L. Blockade of the epidermal growth factor receptor tyrosine kinase suppresses tumorigenesis in MMTV/Neu + MMTV/TGF- α bigenic mice. *Proc. Natl. Acad. Sci. USA*, *97*: 9609–9614, 2000.
- Graus-Porta, D., Beerli, R. R., and Hynes, N. E. Single-chain antibody-mediated intracellular retention of ErbB-2 impairs Neu differentiation factor and epidermal growth factor signaling. *Mol. Cell. Biol.*, *15*: 1182–1191, 1995.
- Qian, X., Dougall, W. C., Hellman, M. E., and Greene, M. I. Kinase-deficient neu proteins suppress epidermal growth factor receptor function and abolish cell transformation. *Oncogene*, *9*: 1507–1514, 1994.
- Baselga, J., and Averbuch, S. D. ZD1839 (“Iressa”) as an anticancer agent. *Drugs*, *60*: 33–40; discussion 41–42, 2000.
- Muthuswamy, S. K., Gilman, M., and Brugge, J. S. Controlled dimerization of ErbB receptors provides evidence for differential signaling by homo- and heterodimers. *Mol. Cell. Biol.*, *19*: 6845–6857, 1999.
- Lenferink, A. E. G., Busse, D., Flanagan, W. M., Yakes, F. M., and Arteaga, C. L. ErbB2/neu kinase modulates p27Kip1 and cyclin D1 through multiple signaling pathways. *Cancer Res.*, *61*: 6583–6591, 2001.

31. Hong, R. L., Spohn, W. H., and Hung, M. C. Curcumin inhibits tyrosine kinase activity of p185neu and also depletes p185neu. *Clin. Cancer Res.*, *5*: 1884–1891, 1999.
32. Kraus, M. H., Popescu, N. C., Amsbaugh, S. C., and King, C. R. Overexpression of the EGF receptor-related proto-oncogene erbB-2 in human mammary tumor cell lines by different molecular mechanisms. *EMBO J.*, *6*: 605–610, 1987.
33. Lewis, G. D., Figari, I., Fendly, B., Wong, W. L., Carter, P., Gorman, C., and Shepard, H. M. Differential responses of human tumor cell lines to anti-p185HER2 monoclonal antibodies. *Cancer Immunol. Immunother.*, *37*: 255–263, 1993.
34. Arteaga, C. L., Hurd, S. D., Dugger, T. C., Winnier, A. R., and Robertson, J. B. Epidermal growth factor receptors in human breast carcinoma cells: a potential selective target for transforming growth factor α -*Pseudomonas* exotoxin 40 fusion protein. *Cancer Res.*, *54*: 4703–4709, 1994.
35. Aguilar, Z., Akita, R. W., Finn, R. S., Ramos, B. L., Pegram, M. D., Kabbinar, F. F., Pietras, R. J., Pisacane, P., Sliwkowski, M. X., and Slamon, D. J. Biologic effects of heregulin/neu differentiation factor on normal and malignant human breast and ovarian epithelial cells. *Oncogene*, *18*: 6050–6062, 1999.
36. Datta, S. R., Brunet, A., and Greenberg, M. E. Cellular survival: a play in three Acts. *Genes Dev.*, *13*: 2905–2927, 1999.
37. Fedi, P., Pierce, J. H., di Fiore, P. P., and Kraus, M. H. Efficient coupling with phosphatidylinositol 3-kinase, but not phospholipase C γ or GTPase-activating protein, distinguishes ErbB-3 signaling from that of other ErbB/EGFR family members. *Mol. Cell. Biol.*, *14*: 492–500, 1994.
38. Carter, P., Presta, L., Gorman, C. M., Ridgway, J. B., Henner, D., Wong, W. L., Rowland, A. M., Kotts, C., Carver, M. E., and Shepard, H. M. Humanization of an anti-p185HER2 antibody for human cancer therapy. *Proc. Natl. Acad. Sci. USA*, *89*: 4285–4289, 1992.
39. Chantry, A. The kinase domain and membrane localization determine intracellular interactions between epidermal growth factor receptors. *J. Biol. Chem.*, *270*: 3068–3073, 1995.
40. Ferguson, K. M., Darling, P. J., Mohan, M. J., Macatee, T. L., and Lemmon, M. A. Extracellular domains drive homo- but not hetero-dimerization of erbB receptors. *EMBO J.*, *19*: 4632–4643, 2000.
41. Baselga, J., Norton, L., Albanell, J., Kim, Y. M., and Mendelsohn, J. Recombinant humanized anti-HER2 antibody (Herceptin) enhances the antitumor activity of paclitaxel and doxorubicin against HER2/neu overexpressing human breast cancer xenografts. *Cancer Res.*, *58*: 2825–2831, 1998.
42. Sliwkowski, M. X., Lofgren, J. A., Lewis, G. D., Hotaling, T. E., Fendly, B. M., and Fox, J. A. Nonclinical studies addressing the mechanism of action of trastuzumab (Herceptin). *Semin. Oncol.*, *26*: 60–70, 1999.
43. Lane, H. A., Beuvink, I., Motoyama, A. B., Daly, J. M., Neve, R. M., and Hynes, N. E. ErbB2 potentiates breast tumor proliferation through modulation of p27(Kip1)-Cdk2 complex formation: receptor overexpression does not determine growth dependency. *Mol. Cell. Biol.*, *20*: 3210–3223, 2000.
44. Arteaga, C. L., Ramsey, T. T., Shawver, L. K., and Guyer, C. A. Unliganded epidermal growth factor receptor dimerization induced by direct interaction of quinazolines with the ATP binding site. *J. Biol. Chem.*, *272*: 23247–23254, 1997.
45. Lichtner, R. B., Menrad, A., Sommer, A., Klar, U., and Schneider, M. R. Signaling-inactive epidermal growth factor receptor/ligand complexes in intact carcinoma cells by quinazoline tyrosine kinase inhibitors. *Cancer Res.*, *61*: 5790–5795, 2001.
46. Gamett, D. C., Pearson, G., Cerione, R. A., and Friedberg, I. Secondary dimerization between members of the epidermal growth factor receptor family. *J. Biol. Chem.*, *272*: 12052–12056, 1997.
47. Levitzki, A., and Gazit, A. Tyrosine kinase inhibition: an approach to drug development. *Science (Wash. DC)*, *267*: 1782–1788, 1995.
48. Osherov, N., and Levitzki, A. Epidermal-growth-factor-dependent activation of the src-family kinases. *Eur. J. Biochem.*, *225*: 1047–1053, 1994.
49. Ye, D., Mendelsohn, J., and Fan, Z. Augmentation of a humanized anti-HER2 mAb 4D5 induced growth inhibition by a human-mouse chimeric anti-EGF receptor mAb C225. *Oncogene*, *18*: 731–738, 1999.
50. Clynes, R. A., Towers, T. L., Presta, L. G., and Ravetch, J. V. Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. *Nat. Med.*, *6*: 443–446, 2000.

Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

Epidermal Growth Factor Receptor (HER1) Tyrosine Kinase Inhibitor ZD1839 (Iressa) Inhibits HER2/ *neu* (*erb* B2)-overexpressing Breast Cancer Cells *in Vitro* and *in Vivo*

Stacy L. Moulder, F. Michael Yakes, Senthil K. Muthuswamy, et al.

Cancer Res 2001;61:8887-8895.

Updated version Access the most recent version of this article at:
<http://cancerres.aacrjournals.org/content/61/24/8887>

Cited articles This article cites 48 articles, 30 of which you can access for free at:
<http://cancerres.aacrjournals.org/content/61/24/8887.full#ref-list-1>

Citing articles This article has been cited by 100 HighWire-hosted articles. Access the articles at:
<http://cancerres.aacrjournals.org/content/61/24/8887.full#related-urls>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link
<http://cancerres.aacrjournals.org/content/61/24/8887>.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.