

Advances in Brief

ZD1839, a Specific Epidermal Growth Factor Receptor (EGFR) Tyrosine Kinase Inhibitor, Induces the Formation of Inactive EGFR/HER2 and EGFR/HER3 Heterodimers and Prevents Heregulin Signaling in HER2-overexpressing Breast Cancer Cells¹

Judit Anido, Pablo Matar, Joan Albanell, Marta Guzmán, Federico Rojo, Joaquin Arribas, Steve Averbuch, and Jose Baselga²

Laboratory of Oncology Research, Medical Oncology Service, Vall d'Hebron University Hospital, Barcelona 08035, Spain [J. An., P. M., J. Al., M. G., F. R., J. Ar., J. B.]; Universidad Autonoma de Barcelona, Barcelona, Spain [J. B.]; and AstraZeneca Pharmaceuticals, Wilmington, Delaware [S. A.]

Abstract

Purpose: ZD1839 is a tyrosine kinase inhibitor of the epidermal growth factor receptor (EGFR) that has shown clinical activity against EGFR-expressing tumors. Our aim was to explore the effects of ZD1839 in breast cancer cell lines expressing different levels of EGFR and the closely related HER2 receptor.

Experimental Design: We studied the growth-inhibitory effects of ZD1839 in a series of breast carcinoma cell lines. In HER2-overexpressing BT-474 breast cancer cells, we studied the effects of ZD1839 on cell growth and heterodimerization of receptors under basal and ligand-stimulated conditions.

Results: ZD1839 was an equally potent inhibitor of growth in breast cancer cells expressing high levels of EGFR and HER2. In BT-474 breast cancer cells, ZD1839 abolished EGF- and heregulin-induced activation of ErbB receptors and downstream signaling molecules. Because ZD1839 does not inhibit the HER2 tyrosine kinase *in vitro*, and because heregulin is a ligand that activates HER2 by binding to HER3 and HER4 but does not bind to the EGFR, our findings suggested that ZD1839 interfered with HER2 function in intact cells. Searching for mechanisms, we report that

ZD1839 induces the formation of inactive unphosphorylated EGFR/HER2 and EGFR/HER3 heterodimers. Furthermore, ZD1839 completely abolishes basal and heregulin-induced formation of active phosphorylated HER2/HER3 heterodimers.

Conclusions: ZD1839 inhibits the growth of HER2-overexpressing breast cancer cells, possibly by sequestration of HER2 and HER3 receptors in an inactive heterodimer configuration with the EGFR. Our findings suggest that there is a strong rationale to conduct clinical trials of ZD1839 in patients with HER2-overexpressing breast tumors.

Introduction

The ErbB family of receptors (also known as type I receptor TKs³) plays a major role in promoting breast cancer cell proliferation and malignant growth (1). This receptor family is comprised of four homologous receptors: (a) the EGFR (ErbB1/EGFR/HER1); (b) ErbB2 (HER2/*neu*); (c) ErbB3 (HER3); and (d) ErbB4 (HER4). These receptors are composed of an extracellular binding domain, a transmembrane lipophilic segment, and an intracellular protein TK domain with a regulatory COOH-terminal segment. HER3, however, is different from the other members in that it has a deficient TK domain.

There is a rich cross-talk among the ErbB family that regulates the cellular effects mediated by these receptors. At least six different ligands, known as EGF-like ligands, bind to the EGFR. These ligands include EGF, transforming growth factor α , amphiregulin, heparin-binding EGF, betacellulin and epiregulin (2–4). A second class of ligands, collectively termed heregulins, binds directly to HER3 and/or HER4 (5–7). After ligand binding, the ErbB receptors become activated by dimerization between two identical receptors (homodimerization) or between different receptors of the same family [heterodimerization (8, 9)]. In contrast with the other ErbB receptor members, a soluble ligand of HER2 has not yet been identified. However, HER2 is known to be the preferred coreceptor for the EGFR, HER3, and HER4 (2, 10). This preference for heterodimerization within the ErbB receptor family explains how HER2 signals in absence of cognate ligand. After receptor dimerization, activation of the intrinsic protein kinase activity and tyrosine auto-

Received 5/15/02; revised 11/8/02; accepted 12/17/02.

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 Supported in part by Spanish Health Ministry Grant “Fondo de Investigación Sanitaria” 99/0020-01 (to J. Al. and J. B.). J. An. is a recipient of a predoctoral grant from the “Fundación Científica de la Asociación Española Contra el Cáncer.” P. M. is a recipient of “Fundación Carolina Post-doctoral Fellowship Program.”

² To whom requests for reprints should be addressed, at Medical Oncology Service, Vall d'Hebron University Hospital, Paseo Vall d'Hebron 119-129, Barcelona 08035, Spain. Phone: 34-93-274-6077; Fax: 34-93-274-6059; E-mail: baselga@hg.vhebron.es.

³ The abbreviations used are: TK, tyrosine kinase; MAPK, mitogen-activated kinase; EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; MAb, monoclonal antibody; PI3k, phosphatidylinositol 3'-kinase; Stat, signal transducers and activators of transcription.

phosphorylation occur, recruiting and phosphorylating several intracellular substrates involving the Ras-Raf-MAPK, the PI3k-Akt, and other signaling pathways that regulate multiple biological processes including apoptosis and cellular proliferation (3, 11–16).

The EGFR and HER2 are frequently overexpressed in breast cancer, and their overexpression is associated with a more aggressive clinical behavior (17–19). As a consequence, inhibiting receptor TK function may be a fruitful approach to the therapy of breast cancer. ZD1839 is an oral nonpeptide anilinoquinazoline compound that selectively inhibits the TK activity of the EGFR with an IC₅₀ of 0.02 μM and requires a dose almost 200-fold higher to inhibit HER2 (3.7 μM; Ref. 20). In tumor cell lines in culture, ZD1839 completely abolishes autophosphorylation of the EGFR, resulting in the inhibition of the activation of downstream signaling molecules. ZD1839 inhibits the growth of cell lines that express high levels of the EGFR and induces complete regression of well-established xenografts (20–22). ZD1839 has recently entered clinical trials in cancer patients, and antitumor activity has been demonstrated against several human cancers, such as non-small cell lung cancer and prostate and ovarian carcinomas (23–25).

To characterize the potential role of ZD1839 in the treatment of breast cancer, we have studied its effects in a variety of breast carcinoma cell lines expressing different levels of EGFR and HER2 receptors. Our studies demonstrate that ZD1839, despite being a specific inhibitor of the EGFR TK in *in vitro* kinase assays, is a potent inhibitor of HER2-overexpressing breast cancer cells. Searching for potential mechanisms that would explain the high sensitivity of HER2-overexpressing cells to ZD1839, we have observed that ZD1839 promotes the formation of inactive (unphosphorylated) EGFR/HER2 and EGFR/HER3 heterodimers. ZD1839-treated breast cancer cells are no longer stimulated by the non-EGFR ligand heregulin, suggesting that ZD1839 may exert its antitumor effects by sequestration of HER2 and HER3, a favorite heterodimerization partner of HER2, into inactive EGFR-based heterodimers.

Materials and Methods

Compounds. EGFR TK inhibitor ZD1839 (26) was kindly provided by AstraZeneca (Wilmington, DE). Recombinant human EGF and heregulin were obtained from Oncogene Research Products (Cambridge, MA).

Cell Cultures. The human cell lines BT-474 (27, 28), SK-BR-3 (27, 29), T47-D (27, 30), MDA-MB-468 (27, 31), A431 (29), MDA-MB-435S (30), MDA-MB-231 (29, 30), and MDA-MB-453 were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained as monolayers at 37°C in 5% CO₂/air in DMEM:Ham's F-12 containing 10% fetal bovine serum and glutamine (2 mM), and, for BT-474, MDA-MB-435S, and MDA-MB-361, insulin (0.01 μg/ml) was added as reported previously (32).

Growth Assays. Cells were plated in 6-well dishes and treated the next day with increasing doses of ZD1839 (0.1, 1, and 10 μM). The media with compounds were replaced on the third day of treatment. After 5 days of treatment, cells were counted with a Coulter counter.

Table 1 Levels of EGFR and HER2 in the cell lines tested

	EGFR		HER2	
	OD (×10 ³) ^a	% ^b	OD (×10 ³) ^a	% ^c
MDA-MB-435S	0	0	0	0
T47-D	123	1	1272	24
MDA-MB-231	1851	17	167	3
BT-474	164	2	5344	100
MDA-MB-453	0	0	2237	42
SK-BR-3	119	1	2720	51
MDA-MB-468	3100	29	0	0
A431	10600	100	233	4

^a Optical densitometry values.

^b Relative level of expression calculated as percentage of A431.

^c Relative level of expression calculated as percentage of BT-474.

Western Blot Analyses. Western blot assays were performed as reported previously (32), with minor modifications. In brief, tumor cell lines were grown in 6-well dishes until subconfluence. The monolayers were then exposed to ZD1839 for 2, 12, and 24 h; the media were removed; and the cultures were washed twice with ice-cold PBS and lysed in 600 μl of radioimmunoprecipitation assay lysis buffer [20 mM sodium phosphate (pH 7.4), 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 5 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and leupeptin, 250 μg/ml sodium orthovanadate, 1 mM MgCl₂, and 25 mM NaF]. To evaluate the effects of ZD1839 under ligand-mediated stimulation, monolayers were incubated in serum-free medium for 24 h, exposed to ZD1839 for 2 h, and then treated with either EGF or heregulin (100 ng/ml) for 15 min. Media were removed, and cells in culture were washed and lysed as described above. After removal of cell debris by centrifugation, protein concentration was determined by Lowry assay (DC Protein Assay; Bio-Rad, Hercules, CA). Lysate samples containing equal amounts of protein were then added to SDS-PAGE loading buffer with 5% β-mercaptoethanol and heated for 5 min at 100°C.

Electrophoretic transfer to nitrocellulose membranes was followed by immunoblotting with antibodies against phospho-p44/42 MAPK (Thr202/Tyr204), p44/42 MAPK, phospho-Akt (Ser 473), Stat3, phospho-Stat3 (Tyr 705) [all from New England BioLabs Inc., Beverly, MA], anti-Akt1/PKBα, anti-Akt2/PKBβ, anti-phospho-ErbB2/HER-2 (Y1248), anti-EGFR (non-phospho-Y1173), anti-phospho-EGFR (Y1173), anti-erbB-3/HER-3 (Upstate Biotechnology), mouse MAb CB-11 to HER2 (BioGenex, San Ramon, CA), p38 MAPK (Cell Signaling Technology), and anti-phospho-tyrosine (clone 4G10; Upstate Biotechnology).

Finally, membranes were hybridized with the appropriate horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom) and detected via chemiluminescence using the SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL).

In Table 1, X-ray films were quantified using MacBAS software (Fujifilm).

Coimmunoprecipitation Assays. BT-474 and MDA-MB-453 cells were plated until subconfluence and then starved in serum-free medium for 24 h. The monolayers were then exposed to ZD1839 for 2 h and treated with either EGF or

heregulin (100 ng/ml) for 15 min. Media were removed, and cultures were washed twice with ice-cold PBS and lysed in 600 μ l of coimmunoprecipitation lysis buffer [20 mM sodium phosphate (pH 7.4), 150 mM NaCl, 1% NP40, 10% glycerol, 1 mM EDTA, 5 mM phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin and leupeptin, 250 μ g/ml sodium orthovanadate, and 40 mM NaF]. After removal of cell debris by centrifugation, protein concentration was determined by Lowry assay (DC Protein Assay; Bio-Rad). Lysate samples containing equal amounts of protein were incubated with the anti-EGFR MAb C225 (Ref. 5; kindly provided by Imclone Systems), anti-HER2 c-neu(Ab-2) (Oncogene Research Products), or anti-erb-3/HER3 (Upstate Biotechnology) overnight at 4°C with slow agitation. The next day, 50 μ l of protein A-Sepharose and 50 μ l of protein G-Sepharose were added for 2 h at 4°C, and then the immunocomplexes were pelleted by centrifugation, washed twice with coimmunoprecipitation lysis buffer, and resuspended in 150–200 μ l of Laemmli sample buffer. The samples were subjected to SDS-PAGE (7% acrylamide) and electrophoretically transferred to a nitrocellulose membrane. Western blotting was performed as described above.

Apoptosis. Apoptosis was measured by fluorescence-activated cell-sorting analysis following a standardized protocol reported previously (33). Briefly, 4.1×10^6 BT-474 cells were plated in 10-cm culture plates. Cells were starved in serum-free medium for 24 h, and then the treatment with ZD1839 was started. After incubation at 37°C in 5% CO₂ for 72 h, cells were washed twice with ice-cold PBS and then collected with 10 mM PBS-EDTA and centrifuged for 5 min at 1000 rpm. Media were removed, and cells were fixed overnight at 4°C by adding ice-cold 70% ethanol while gently vortexing. Cells were centrifuged at 1000 rpm for 5 min at 10°C, and ethanol was aspirated. Actin was stained by resuspending the pellet in 40 μ l of FITC-phalloidin (10 μ g/ml) in PBS (Sigma) for 1 h in the dark at room temperature and washed once with PBS. DNA was stained with cell cycle buffer [0.1% sodium citrate, 0.3% NP40, 50 μ g/ml propidium iodide (Sigma), and 20 μ g/ml RNaseA (Sigma)] for 30 min at room temperature in the dark.

Results

Effects of ZD1839 on Proliferation and Apoptosis in Breast Cancer Cells. We studied the effects of various concentrations of the EGFR TK inhibitor ZD1839 on the proliferation of a panel of breast cancer cell lines with different levels of expression of EGFR and HER2 (MDA-MB-435S, T47-D, MDA-MB-231, BT-474, MDA-MB-453, SK-BR-3, MDA-MB-468; Table 1). The vulvar carcinoma cell line A431, which expresses about 2×10^6 receptors/cell, was used in these experiments as a positive control for EGFR expression.

ZD1839 inhibited the growth of tested cell lines at different dose levels (Fig. 1A). As expected, ZD1839 markedly inhibited the proliferation of cell lines with high levels of EGFR expression (A431 and MDA-MB-468). Interestingly, ZD1839 was equally potent at inhibiting the growth of BT-474 and SK-BR-3 cells, which had high levels of HER2 and low EGFR levels. In the case of BT-474 cells, the growth-inhibitory effects of ZD1839 were similar to the observed with A431 cells. On the other hand, minimal growth-inhibitory effects were observed in

the cell line MDA-MB 453, which expresses high levels of HER2 but has very minimal or absent EGFR levels. Taken together, these results suggest that the presence of EGFR is required for ZD1839 to inhibit the growth of HER2-overexpressing cells.

A correlation between concentrations of ZD1839 that inhibited cell proliferation (Fig. 1A) and receptor activation (phosphorylation; Fig. 1B) was observed in three representative tumor cell lines with different expression levels of EGFR and HER2 (BT-474, A431, and MDA-MB-453). The expression levels of EGFR and HER2 were not affected by ZD1839 at different time points (2, 12, and 24 h; Fig. 1, B and C).

In BT-474 cells, the marked inhibition of growth induced by ZD1839 was associated with an induction of apoptosis. Treatment with ZD1839 for 72 h resulted in a 16.7% apoptotic fraction (Fig. 2). Because inhibition of Akt (see below) may not be sufficient for the induction of apoptosis by ErbB inhibitors, we decided to study the effects of ZD1839 on p38 MAPK levels. p38 MAPK is a stress-activated member of the MAPK family that has been linked to the induction of apoptosis in cells treated with ErbB TK inhibitors (33, 34). In BT-474 cells, EGF administration (100 ng/ml for 15 min) resulted in a decrease in the total levels of p38 MAPK. Pretreatment with ZD1839 (1 μ M for 2 h) resulted in higher levels of p38 MAPK than those seen under basal and EGF-stimulated conditions (Fig. 3).

Effects of ZD1839 on Receptor Phosphorylation and Receptor-dependent Signaling Pathways in Intact Cells.

Growth inhibition by ZD1839 in BT-474 and SK-BR-3 HER2-overexpressing cells occurred at ZD1839 concentrations that do not suppress HER2 TK activity *in vitro* (35, 36). This finding suggested that ZD1839 interferes with HER2 function in intact cells by an additional mechanism. To explore the *in vivo* effects of ZD1839, we first analyzed the effects of ZD1839 on HER2 and EGFR phosphorylation in BT-474 cells in the presence or absence of exogenously added ligand (EGF or heregulin). EGF binds to the EGFR and promotes the formation of EGFR/EGFR homodimers and EGFR/HER2 heterodimers and, less frequently, EGFR/HER3 and EGFR/HER4 heterodimers (2–4). Heregulin binds to HER3 and HER4, but not to the EGFR, and induces the formation of HER3/HER3 or HER4/HER4 homodimers and HER2/HER3 or HER2/HER4 heterodimers (2, 6, 37).

ZD1839 inhibited the phosphorylation of the EGFR and HER2 as measured in Western blot assays (Fig. 1B). The addition of EGF induced EGFR phosphorylation and, to a minor degree, phosphorylation of HER2, indicating transactivation of HER2 by the EGFR (Fig. 4A). As expected, ZD1839 markedly inhibited EGF-induced EGFR phosphorylation. ZD1839 also inhibited EGF-induced HER2 phosphorylation by 36%, a further suggestion that HER2 is transactivated by the EGFR (Fig. 4A). There was no visible change in the phosphorylation levels of EGFR and HER2 under heregulin-stimulated conditions with or without ZD1839, possibly as a consequence of the basally high level of constitutive HER2 phosphorylation on these cells (Fig. 4B).

To study the effects downstream of the receptor of ZD1839 in BT-474 cells, we analyzed the signaling pathway molecules p42/44 MAPK (3), Akt (13–15), and Stat3 (38, 39), which are activated by ErbB receptors and play an important role in

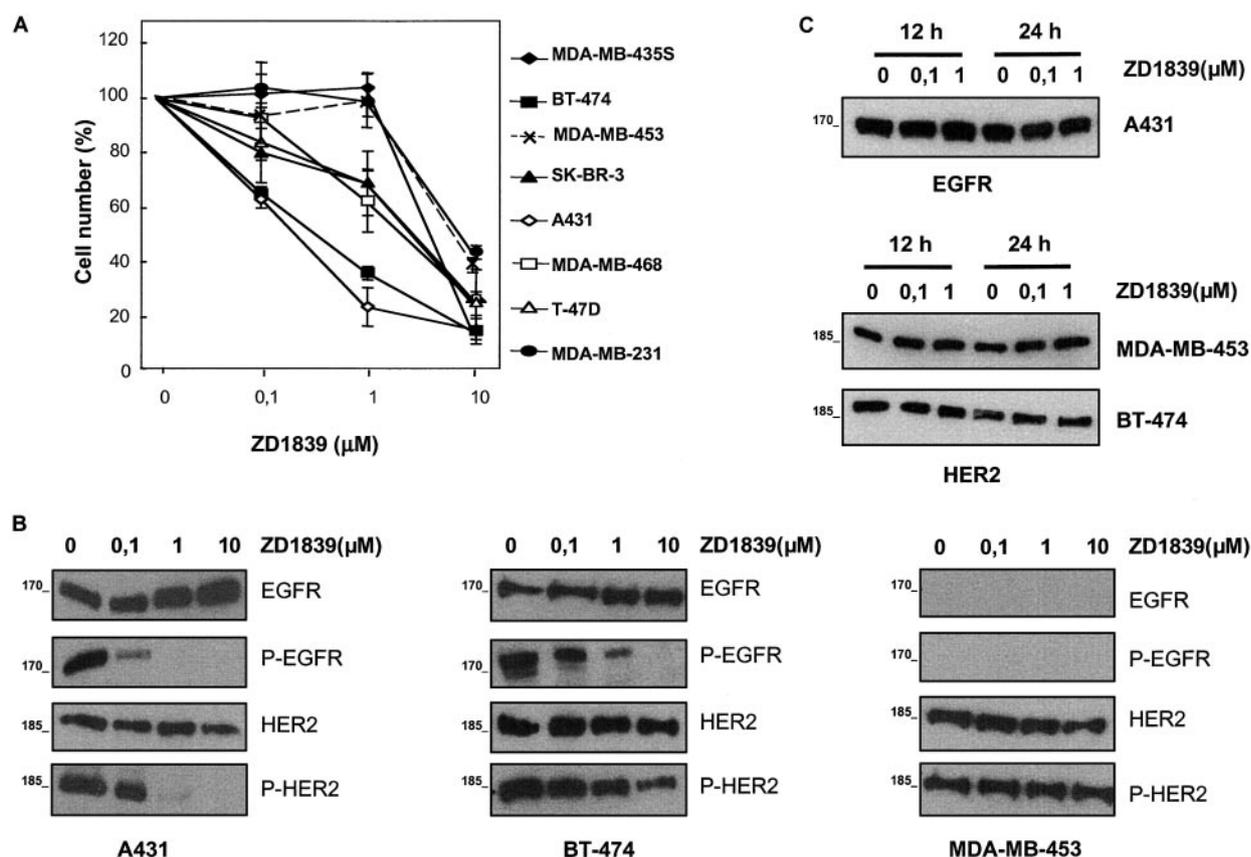


Fig. 1 Effects of ZD1839 in tumor cells with varying degrees of EGFR and HER2 expression. **A**, cell proliferation. Cells were plated in 6-well dishes and treated the next day with increasing doses of ZD1839 (0.1, 1, and 10 μM). After 5 days of treatment, cells were counted with a Coulter counter. Each *data point* represents the mean \pm SE of triplicate experiments expressed as a percentage of cell number compared with the same cell line grown in absence of the compound. **B**, EGFR and HER2 expression levels and activation. Cells were grown in 6-well dishes until subconfluence and then incubated in serum-free medium for 24 h. The monolayers were then exposed to ZD1839 for 2 h. Protein lysates were quantified, and equal amounts of total protein were subjected to SDS-PAGE, transfer to nitrocellulose, and immunoblot analysis using specific antibodies against the phosphorylated and total forms of EGFR and HER2, as described in "Materials and Methods." **C**, EGFR and HER2 expression levels in cells exposed to ZD1839 for 12 and 24 h.

receptor-mediated proliferation and apoptosis. EGF induced phosphorylation of MAPK, Akt, and Stat3, and this phosphorylation was completely prevented in the presence of ZD1839 (Fig. 4A). This inhibition of downstream signaling occurred at the same ZD1839 concentration that inhibited EGF-induced phosphorylation of EGFR and HER2. Heregulin treatment resulted in a marked phosphorylation of MAPK and a slight increase in Akt phosphorylation, but it did not result in Stat3 phosphorylation (Fig. 4B). The lack of Stat3 phosphorylation by heregulin is probably an indication that these two ligands may have differential activation of signaling pathways (40). ZD1839 prevented heregulin-induced activation of MAPK, but it had minimal effect on activation of Akt (Fig. 4B). No change was detected in the total levels of MAPK, Akt, or Stat3 under the same experimental conditions with ZD1839 (data not shown).

To provide additional evidence for the mechanism of inhibition by ZD1839 on EGFR-driven HER2 transactivation, we analyzed the effects of ZD1839 on the MDA-MB-453 cell line, which expresses HER2, HER3, and HER4 but not EGFR. As expected, ZD1839 did not affect cell proliferation unless given

at very high dose levels (Fig. 1A) and it did not affect HER2 phosphorylation (Fig. 4, C and D). Also, in this tumor cell line, ZD1839 did not affect Akt and MAPK activation in response to both ligands. These findings strongly suggest that EGFR expression is required for inhibition by ZD1839 in receptor downstream signaling and antiproliferative effects.

ZD1839 Induces the Formation of EGFR/HER2 and EGFR/HER3 Unphosphorylated Heterodimers. Because ZD1839 prevented signaling by both EGF and heregulin, we decided to analyze whether ZD1839 was interfering with the heterodimerization process. BT-474 cell lysates treated with different concentrations of ZD1839 (1 and 10 μM) under basal and EGF- and heregulin-stimulated conditions were immunoprecipitated with anti-EGFR, anti-HER2, anti-HER3, and anti-HER4 antibodies and analyzed by Western blot. HER4 receptors were not detected in BT-474 cells by immunoprecipitation.

Under basal conditions, and in the presence of EGF and heregulin, ZD1839 induced, in a dose-dependent fashion, the formation of EGFR/HER2 and EGFR/HER3 heterodimers, as demonstrated by the enhanced coimmunoprecipitation of HER2

A. CONTROL

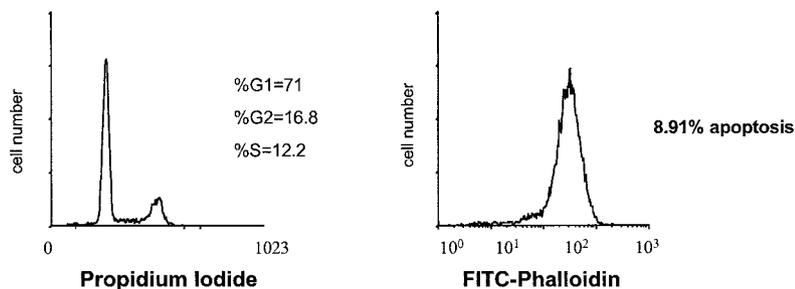
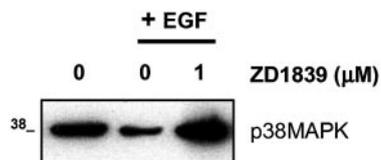
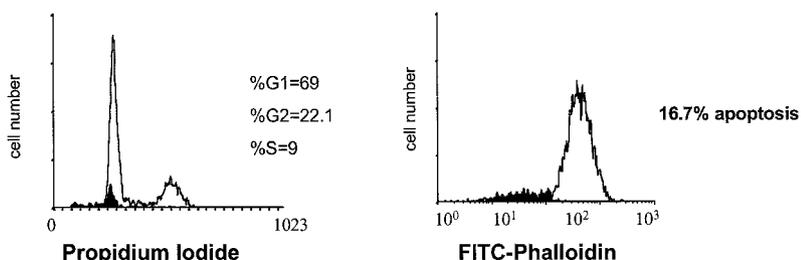
B. ZD1839 (1 μ M)

Fig. 3 Effects of ZD1839 on p38 MAPK in BT-474 breast cancer cells. Cells were grown in 6-well dishes until subconfluence and then incubated in serum-free medium for 24 h. The monolayers were then exposed to ZD1839 (1 μ M) for 2 h and treated with EGF (100 ng/ml) for 15 min. Protein lysates were quantified, and equal amounts of total protein were then subjected to SDS-PAGE, transfer to nitrocellulose, and immunoblot analyses using anti-p38 MAPK antibodies.

and HER3 with the EGFR (Fig. 5A, IV and V, and Fig. 5C, IV). The EGFR/HER2 and EGFR/HER3 heterodimer complexes induced by ZD1839, unlike ligand-induced heterodimer formation, had low or absent phosphorylation, as seen in Fig. 5A, III (blotting with anti-phospho-HER2) and VI, where the *bottom band* corresponds to the EGFR, and the *top band* corresponds to HER2 and HER3. This finding indicates that ZD1839 promotes the formation of unphosphorylated inactive EGFR/HER2 and EGFR/HER3 dimers. Concomitantly, ZD1839 treatment resulted in a decrease in HER2/HER3 heterodimer formation (Fig. 5B, II, and Fig. 5C, II).

As expected, EGF treatment resulted in the phosphorylation of EGFR-based dimers, as demonstrated by the disappearance of unphosphorylated EGFR (Fig. 5A, I) and the marked increase in phosphorylated EGFR (Fig. 5A, II) and other receptors that coimmunoprecipitate with EGFR (Fig. 5A, VI). All of these effects were prevented in a dose-dependent fashion by pretreatment with ZD1839.

In BT-474 cells, which express both EGFR and HER2 (Table 1), heregulin induced the formation of HER2/HER3

Fig. 2 Analysis of the apoptotic fraction in BT-474 cells treated with ZD1839. BT-474 cells were plated in DMEM:Ham's F-12 supplemented with 10% fetal bovine serum, glutamine, and insulin. The next day, cells were incubated in serum-free media for 24 h, and then the monolayers were left untreated (A) or they were exposed to ZD1839 (1 μ M) for 72 h (B). As described in "Materials and Methods," cells were collected and stained with FITC-phalloidin for actin and propidium iodide for DNA and analyzed by flow cytometry. *Black-shaded curve*, the cleaved actin population with low fluorescence levels with FITC-phalloidin, which allows us to identify the same population of cells stained with propidium iodide, representing the apoptotic sub-G₀ population. *White-shaded curve*, the uncleaved actin population stained with FITC-phalloidin, with increased fluorescence. This correlates with the nonapoptotic cells stained with propidium iodide.

heterodimers, which were highly phosphorylated (Fig. 5B, I and II, and Fig. 5C, I). ZD1839 treatment completely abolished the formation of heregulin-induced active phosphorylated heterodimers, as demonstrated by the decrease in the phosphotyrosine levels in HER2 (Fig. 5B, I) and HER3 (Fig. 5C, I) coimmunoprecipitates. On the contrary, in MDA-MB-453 cells, which express high levels of HER2 and undetectable levels of EGFR (Table 1), ZD1839 did not prevent the formation of HER2/HER3 heterodimers (Fig. 5D).

Taken together, the induction of inactive EGFR/EGFR (41), EGFR/HER2, and EGFR/HER3 dimers by ZD1839 provides a unifying explanation for the inhibitory effects of ZD1839 on both EGF- and heregulin-induced receptor activation and receptor downstream signaling. Under EGF stimulation, ZD1839 directly prevents receptor activation and promotes inactive EGFR-based homodimers or heterodimers (Fig. 6A). Under heregulin stimulation, ZD1839 prevents HER2 activation by sequestering HER2 and HER3 in inactive EGFR-based heterodimers, and as a consequence, preventing the formation of active HER2/HER3 heterodimers (Fig. 6B).

Discussion

We have demonstrated that ZD1839, a specific EGFR TK inhibitor, has a marked antiproliferative effect on breast cancer cells that express high levels of HER2. In addition, ZD1839 inhibits HER2 phosphorylation, EGF-mediated receptor activation, and the downstream signaling pathways MAPK and PI3k-Akt. Our results are in concordance with recent findings by other groups (42–44). However, the unexpected finding that ZD1839 prevented HER2 activation and signaling by heregulin, a ligand that binds to HER3 and HER4 but not to the EGFR, led us to further explore the effects of ZD1839 on receptor heterodimerization.

There are several potential explanations for the prevention

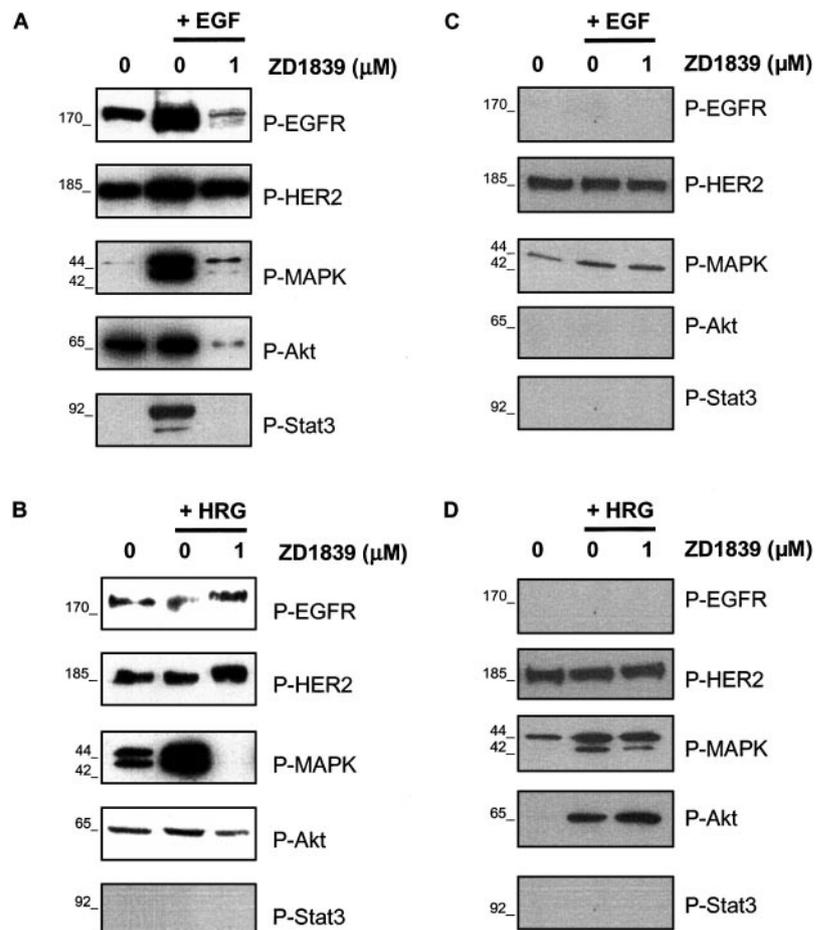


Fig. 4 Effects of ZD1839 on EGF- and heregulin-stimulated signaling pathways. BT-474 (A and B) and MDA-MB-453 (C and D) cells were grown in 6-well dishes until subconfluence and then incubated in serum-free medium for 24 h. The monolayers were then exposed to ZD1839 (1 μM) for 2 h and treated with either EGF (A and C) or heregulin (B and D; 100 ng/ml) for 15 min. Protein lysates were quantified, and equal amounts of total protein were then subjected to SDS-PAGE, transfer to nitrocellulose, and immunoblot analyses using specific antibodies against the phosphorylated forms of EGFR, HER2, MAPK, Akt, and Stat3 as described in "Materials and Methods."

of HER2 activation by a specific EGFR inhibitor. In PC12 rat cells that express *neu*, the rat equivalent of HER2, it has been shown that EGF can induce the formation of primary EGFR/*Neu* heterodimers and, as a consequence, activate the formation of secondary *Neu*/HER3 heterodimers (45). The prevention of active EGFR/HER2 heterodimers by EGFR TK inhibitors could therefore result in lack of formation of secondary HER2/HER3 signaling heterodimers. In support of this hypothesis, it has been reported previously that the less specific anti-EGFR TK inhibitors AG1478 and AG1517 can induce the formation of inactive EGFR homodimers as well as inactive EGFR/HER2 heterodimers (46). ZD1839 also induces the formation of inactive EGFR homodimers (41), and it has been demonstrated that ZD1839 disrupts HER2/HER3 signaling (43). Although the inhibition of HER2/HER3 signaling by ZD1839 could be explained by prevention of the formation of active EGFR/HER2 heterodimers, in our studies we have observed that ZD1839 treatment results in the formation not only of inactive EGFR/HER2 heterodimers but also of inactive EGFR/HER3 heterodimers. Our findings suggest that both HER2 and HER3 are directly sequestered by EGFR into EGFR-based inactive heterodimers. This double sequestration may explain the very efficient prevention by ZD1839 of basal and heregulin-induced HER2/HER3 heterodimerization in HER2-overexpressing BT-

474 cells. This proposed mechanism would imply that only those HER2-expressing cells that have significant levels of EGFR would be inhibited by ZD1839. In support of this hypothesis, in MDA-MB-453 cells, which express HER2 and HER3 but not EGFR, ZD1839 did not inhibit growth and did not interfere with HER2/HER3 heterodimerization. Hence, ZD1839 has a double and complementary mechanism of action against ErbB receptors: a direct inhibition of EGFR TK on one hand; and sequestration of HER2 and HER3 into inactive EGFR/HER2 and EGFR/HER3 heterodimers on the other hand.

The finding that ZD1839, a specific *in vitro* EGFR TK inhibitor, inhibits other ErbB receptors in intact cells may also have clinical implications. It establishes a rationale to study ZD1839 in the clinic against several tumor types with a receptor expression profile not limited to presence of the EGFR. Our studies also suggest that the presence of inactive (unphosphorylated) EGFR heterodimers may be a pharmacodynamic marker of ZD1839 activity that could potentially be used to predict sensitivity to ZD1839 or other TK inhibitors.

Our findings also provide a rationale to explore the use of ZD1839 in combination with other anti-HER2 agents that may exert their activity by different and nonoverlapping mechanisms. In support of this approach, several groups, including ours (43, 44, 47), have shown a marked enhancement of the

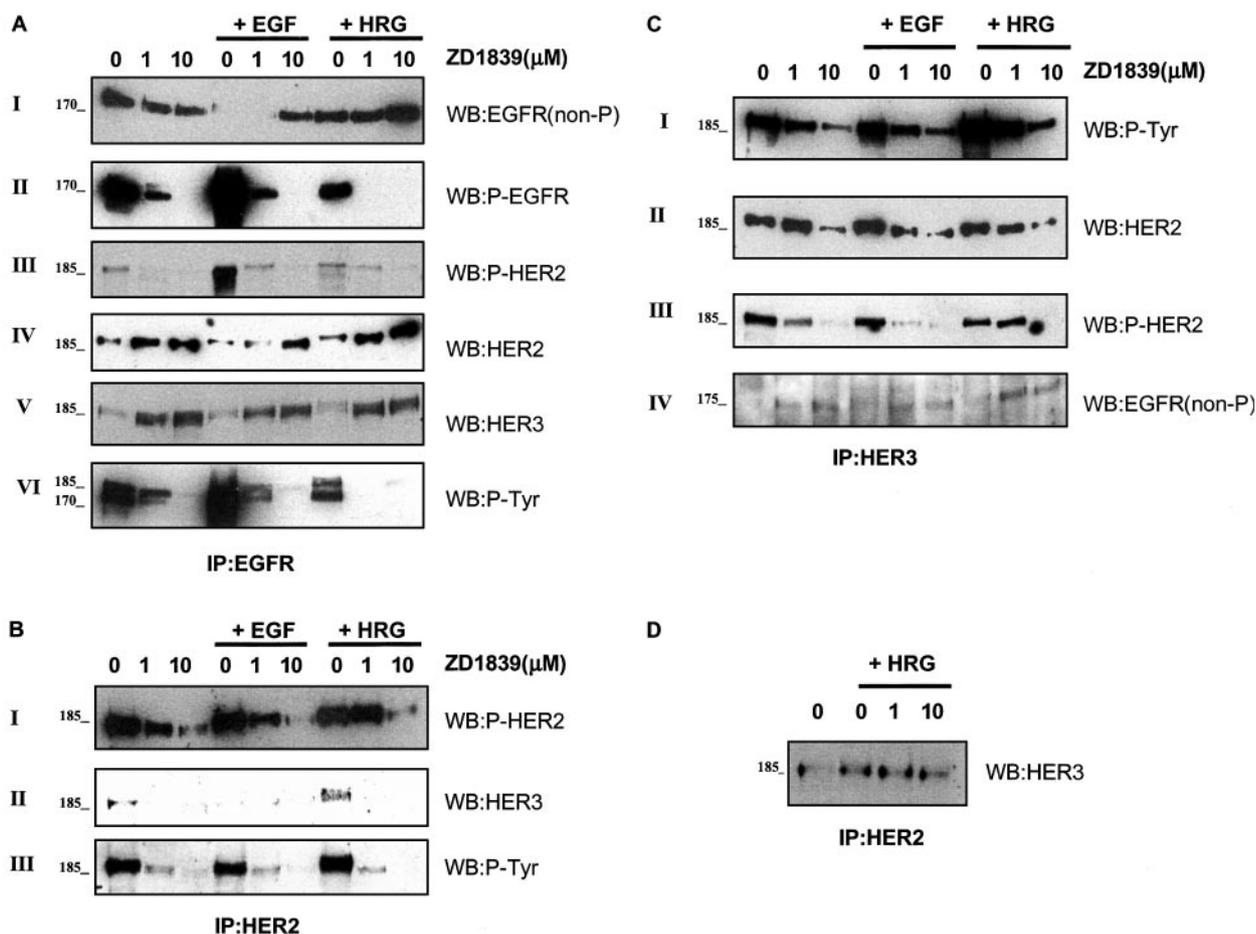


Fig. 5 Effects of ZD1839 on heterodimer formation under EGF- and heregulin-stimulated conditions. BT-474 cells were grown in 6-well dishes until subconfluence and then starved in serum-free medium for 24 h. The monolayers were then exposed to ZD1839 (1 and 10 μM) for 2 h and treated with either EGF or heregulin (100 ng/ml) for 15 min. Protein lysates were quantified, and equal amounts of total protein were then subjected to immunoprecipitation using anti-EGFR (A), anti-HER2 (B), or anti-HER3 (C) antibodies. The immunoprecipitates were subjected to gel electrophoresis and Western blotting with anti-EGFR(non-phospho), antiphospho-EGFR, antiphospho-Tyr, anti-HER2, antiphospho-HER2, and anti-HER3 as described in "Material and Methods." MDA-MB-453 cells were grown in 6-well dishes until subconfluence and then starved in serum-free medium for 24 h. The monolayers were then exposed to ZD1839 (1 and 10 μM) for 2 h and treated with heregulin (100 ng/ml) for 15 min. Protein lysates were quantified and then subjected to immunoprecipitation using anti-HER2 antibody (D). The immunoprecipitates were subjected to gel electrophoresis and Western blotting with anti-HER3 antibody. Each lane contains equal amounts of immunoprecipitate.

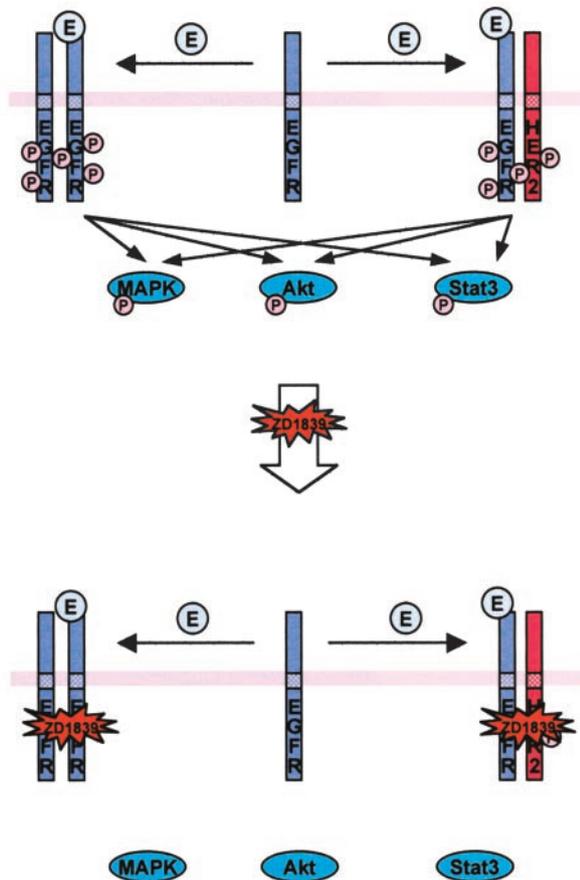
antitumor effects of the anti-HER2 MAb trastuzumab (Herceptin) when given in combination with ZD1839, and clinical trials with this combination are currently under way in patients with advanced HER2-overexpressing breast cancer. In addition to ZD1839, there are a number of EGFR TK inhibitors that are currently under clinical development. Some of these agents, including GW2016 (48, 49) and CI-1033 (50), are potent *in vitro* inhibitors not only of the EGFR but also of HER2 and HER4 (CI-1033). A relevant question will be whether these *in vitro* pan-ErbB inhibitors will be of greater efficacy than receptor-specific EGFR TK inhibitors, or whether they will instead have similar *in vivo* efficacy.

Signal transduction by the ErbB family of receptors is mediated by two major pathways: the Ras-Raf-MAPK and PI3k-Akt pathways that regulate cell proliferation and survival, among other important cellular functions. In the present study, we have observed that ZD1839 inhibits EGF-mediated activa-

tion of MAPK and Akt and heregulin-mediated MAPK activation. However, ZD1839 does not prevent heregulin-mediated Akt activation. Akt activation in response to heregulin is thought to be mostly mediated by HER3 because this receptor has multiple binding sites for the p85 regulatory subunit of PI3k (40, 51). Because ZD1839, even at high doses, failed to completely abolish phospho-tyrosine in HER3 immunoprecipitates (Fig. 5C), it is possible that ZD1839 does not prevent heregulin-dependent phosphorylation of HER3 at the p85 binding sites, which, in turn, would result in Akt activation.

In addition to Ras-Raf-MAPK and PI3k-Akt, other pathways may be important in regulating apoptosis, such as the one constituted by p38 MAPK, a stress-activated member of the MAPK family (52, 53). In studies performed with the pan-ErbB receptor inhibitor CI-1033, apoptosis was seen only in cells with basally activated p38 MAPK (33). In cells with low levels of p38 MAPK expression, treatment with ErbB inhibitors did not

A. SIGNALING VIA EGF



B. SIGNALING VIA HEREGULIN

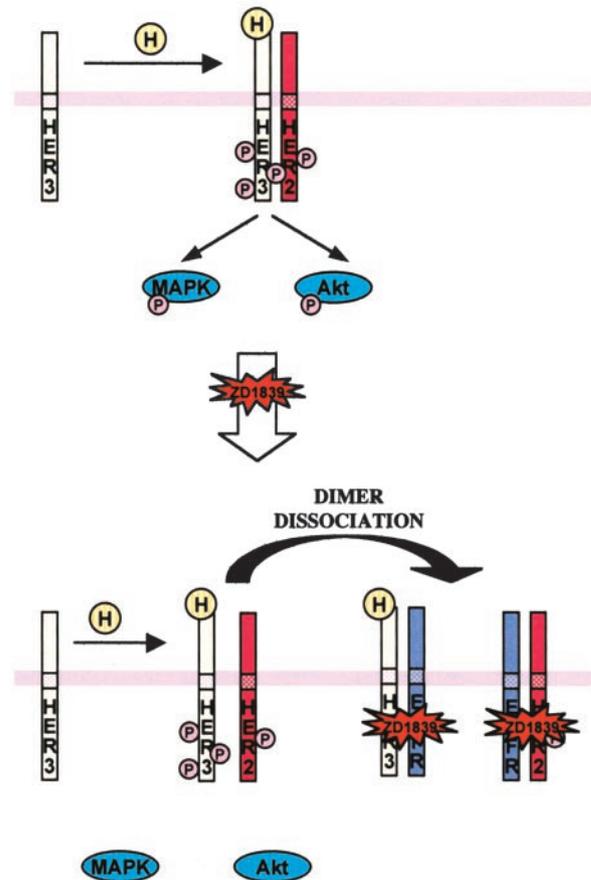


Fig. 6 Hypothetical model showing the effect of ZD1839 in EGF and heregulin receptor signaling. A, EGF treatment results in phosphorylated EGFR-based dimers, which is prevented by pretreatment with ZD1839. B, ZD1839 treatment completely abolishes the formation of heregulin-induced phosphorylated HER2/HER3 heterodimers by promoting the sequestration of HER2 and HER3 into inactive (unphosphorylated) EGFR/HER2 and EGFR/HER3 heterodimers. E, EGF; H, heregulin.

result in apoptosis. However, prior or concomitant administration of gemcitabine, a nucleoside analogue that induces p38 MAPK levels, resulted in a marked apoptotic effect by the receptor TK inhibitor (33). In our studies with BT-474 cells that basally express p38 MAPK, ZD1839 resulted in an increase of p38 MAPK and in an apoptotic effect. One possible implication of this finding is that it may be of interest to study in patients whether there is a correlation between levels of p38 MAPK expression in the tumor and response to ZD1839 or other anti-ErbB receptor kinase inhibitors.

We also demonstrate for the first time that an EGFR TK inhibitor prevents activation of the Stat3 transcription factor. Activated Stat3 is a required participant in v-src oncogenesis in cells, and in a wide array of human cancers, Stat3 is persistently activated (54). Based on our findings, we are also studying basal and on-therapy Stat3 activation levels in tumor samples of patients treated with ZD1839.

We have shown in early clinical studies that ZD1839 can be given safely to patients at doses that fully prevent receptor

activation (32, 55). In these initial studies, ZD1839 has been shown to have clinical activity in non-small cell lung cancer, a tumor that frequently expresses high levels of the EGFR (23, 25, 56). Our findings suggest that in addition to targeting EGFR-dependent tumors, there is a strong rationale to study the clinical efficacy of ZD1839 in HER2-positive breast tumors, and clinical trials of ZD1839 in patients with breast cancer are currently under way.

References

1. Yarden, Y., and Sliwkowski, M. Untangling the ErbB signalling network. *Nat. Rev. Mol. Cell. Biol.*, 2: 127–137, 2001.
2. Klapper, L. N., Glathe, S., Vaisman, N., Hynes, N. E., Andrews, G. C., Sela, M., and Yarden Y. The ErbB-2/HER2 oncoprotein of human carcinomas may function solely as a shared coreceptor for multiple stroma-derived growth factors. *Proc. Natl. Acad. Sci. USA*, 96: 4995–5000, 1999.
3. Alroy, I., and Yarden, Y. The ErbB signaling network in embryogenesis and oncogenesis: signal diversification through combinatorial ligand-receptor interactions. *FEBS Lett.*, 410: 83–86, 1997.

4. Burden, S., and Yarden, Y. Neuregulins and their receptors: a versatile signaling module in organogenesis and oncogenesis. *Neuron*, *18*: 847–855, 1997.
5. Klapper, L. N., Vaisman, N., Hurwitz, E., Pinkas-Kramarski, R., Yarden, Y., and Sela, M. A subclass of tumor-inhibitory monoclonal antibodies to ErbB-2/HER2 blocks crosstalk with growth factor receptors. *Oncogene*, *14*: 2099–2109, 1997.
6. Holmes, W. E., Sliwkowski, M. X., Akita, R. W., Henzel, W. J., Lee, J., Park, J. W., Yansura, D., Abadi, N., Raab, H., Lewis, G. D., *et al.* Identification of heregulin, a specific activator of p185^{erbB2}. *Science (Wash. DC)*, *256*: 1205–1210, 1992.
7. Peles, E., Bacus, S. S., Koski, R. A., Lu, H. S., Wen, D., Ogdan, S. G., Levy, R. B., and Yarden, Y. Isolation of the Neu/HER-2 stimulatory ligand: a 44 kd glycoprotein that induces differentiation of mammary tumor cells. *Cell*, *69*: 205–216, 1992.
8. Lemmon, M. A., and Schlessinger, J. Regulation of signal transduction and signal diversity by receptor oligomerization. *Trends Biochem. Sci.*, *19*: 459–463, 1994.
9. Carraway, K. L., and Cantley, L. C. A new acquaintance for ErbB3 and ErbB4: a role for receptor heterodimerization in growth signaling. *Cell*, *78*: 5–8, 1994.
10. Klapper, L. N., Kirschbaum, M. H., Sela, M., and Yarden, Y. Biochemical and clinical implications of ErbB/HR signaling network of growth factor receptors. *Adv. Cancer Res.*, *77*: 25–79, 2000.
11. Riese, D. J., and Stern, D. F. Specificity within the EGF family/erbB receptor family signaling network. *Bioessays*, *20*: 41–48, 1998.
12. Lewis, T. S., Shapiro, P. S., and Ahn, N. G. Signal transduction through MAP kinase cascades. *Adv. Cancer Res.*, *74*: 49–139, 1998.
13. Liu, W., Li, J., and Roth, R. A. Heregulin regulation of Akt/protein kinase B in breast cancer cells. *Biochem. Biophys. Res. Commun.*, *261*: 897–903, 1999.
14. Muthuswamy, S. K., Gilman, M., and Brugge, J. S. Controlled dimerization of erbB receptors provides evidence for differential signalling by homo- and heterodimers. *Mol. Cell. Biol.*, *19*: 6845–6857, 1999.
15. Burgering, B. M., and Coffey, P. J. Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature (Lond.)*, *376*: 599–602, 1995.
16. Chan, T. O., Rittenhouse, S. E., and Tsichlis, P. N. AKT/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation. *Annu. Rev. Biochem.*, *68*: 965–1014, 1999.
17. 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.
18. Salomon, D., 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.
19. Harris, A. L., Nicholson, S., Sainsbury, J. R. C., 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.
20. Woodburn, J. R., Barker, A. J., Wakeling, A., Valcaccia, B. E., Cartledge, S. A., and Davies, D. H. 6-Amino-4-(3-methyl-phenylamino)-quinazoline: an EGF receptor tyrosine kinase inhibitor with activity in a range of human tumour xenografts. *Proc. Am. Assoc. Cancer Res.*, *37*: 390–391, 1996.
21. Woodburn, J. R., Kendrew, J., Fennell, M., and Wakeling, A. E. ZD1839 (“Iressa”) a selective epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI): inhibition of *c-fos* mRNA, an intermediate marker of EGFR activation, correlates with tumor growth inhibition. *Proc. Am. Assoc. Cancer Res.*, *41*: 402, 2000.
22. Ciardiello, F., Caputo, R., Bianco, R., Damiano, V., Pomato, G., De Placido, S., Bianco, A. R., and Tortora, G. Antitumor effect and potentiation of cytotoxic drugs activity in human cancer cells by ZD-1839 (Iressa), an epidermal growth factor receptor-selective tyrosine kinase inhibitor. *Clin. Cancer Res.*, *6*: 2053–2063, 2000.
23. Ferry, D., Hammond, L., Ranson, M., Kris, M., Miller, V., Murray, P., Tullo, A., Feyereislova, A., Averbuch, S., and Rowinsky, E. Intermittent oral ZD1839 (Iressa), a novel epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI), shows evidence of good tolerability and activity: final results from a Phase I study. *Proc. Am. Soc. Clin. Oncol.*, *19*: 3a, 2000.
24. Baselga, J., Herbst, R., LoRusso, P., Rischin, D., Ranson, M., Plummer, R., Rayond, E., Maddox, A., Kaye, S. B., Kieback, D. G., *et al.* Continuous administration of ZD1839 (Iressa), a novel oral epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI), in patients with five selected tumor types: evidence of activity and good tolerability. *Proc. Am. Soc. Clin. Oncol.*, *19*: A686, 2000.
25. Fukuoka, M., Yano, S., Giaccone, G., Nakagawa, K., Tamura, T., Douillard, J.-Y., Nishiwaki, Y., Vansteenkiste, J., Kudo, S., Rischin, D., *et al.* A multi-institutional randomized phase II trial of ZD1839 (“Iressa”) for previously treated patients with advanced non-small cell lung cancer (IDEAL 1). *J. Clin. Oncol.* In press.
26. Baselga, J., and Averbuch, S. D. ZD1839 (“Iressa”) as an anticancer agent. *Drugs*, *60* (Suppl. 1): 33–40, 2000.
27. 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.
28. Bos, M., Mendelsohn, J., Kim, Y. M., Albanell, J., Fry, D. W., and Baselga, J. PD153035, a tyrosine kinase inhibitor, prevents epidermal growth factor receptor activation and inhibits growth of cancer cells in a receptor number-dependent manner. *Clin. Cancer Res.*, *3*: 2099–2106, 1997.
29. Arteaga, C. L., Winnier, A. R., Poirier, M. C., Lopez-Larraza, D. M., Shawver, L. K., Hurd, S. D., and Stewart, S. J. p185c-erbB-2 signal enhances cisplatin-induced cytotoxicity in human breast carcinoma cells: association between an oncogenic receptor tyrosine kinase and drug-induced DNA repair. *Cancer Res.*, *54*: 3758–3765, 1994.
30. Wosikowski, K., Schuurhuis, D., Johnson, Paull, K. D., Myers, T. G., Weinstein, J. N., and Bates, S. E. Identification of epidermal growth factor receptor and c-erbB2 pathway inhibitors by correlation with gene expression patterns. *J. Natl. Cancer Inst. (Bethesda)*, *89*: 1505–1515, 1997.
31. Filmus, J., Pollak, M. N., Cailleau, R., and Buick, R. N. MDA-468, a human breast cancer cell line with a high number of epidermal growth factor (EGF) receptors, has an amplified EGF receptor gene and is growth inhibited by EGF. *Biochem. Biophys. Res. Commun.*, *128*: 898–905, 1985.
32. Albanell, J., Codony-Servat, J., Rojo, F., Del Campo, J. M., Sauleda, S., Anido, J., Raspall, G., Giral, J., Rosello, J., Nicholson, R. I., Mendelsohn, J., and Baselga, J. Activated extracellular signal-regulated kinases: association with epidermal growth factor receptor/transforming growth factor α expression in head and neck squamous carcinoma and inhibition by anti-EGF receptor treatments. *Cancer Res.*, *61*: 6500–6510, 2001.
33. Nelson, J. M., and Fry, D. Akt, MAPK (Erk1/2), and p38 act in concert to promote apoptosis in response to ErbB receptor family inhibition. *J. Biol. Chem.*, *276*: 14842–14847, 2001.
34. Murillo, H., Schmidt, L. J., and Tindall, D. J. Tyrphostin AG825 triggers p38 mitogen-activated protein kinase dependent apoptosis in androgen-independent prostate cancer cells C4 and C4-2. *Cancer Res.*, *61*: 7408–7412, 2001.
35. Woodburn, J. The epidermal growth factor receptor and its inhibition in cancer therapy. *Pharmacol. Ther.*, *82*: 241–250, 1999.
36. Woodburn, J. R., Barker, A. J., Gibson, K. H., Ashton, S. E., Wakeling, A. E., Curry, B. J., Scarlett, L., and Henthorn, L. R. ZD1839, an epidermal growth factor tyrosine kinase inhibitor selected for clinical development. *Proc. Am. Assoc. Cancer Res.*, *38*: 633, 1997.
37. Wen, D., Peles, E., Cupples, R., Suggs, S. V., Bacus, S. S., Luo, Y., Trail, G., Hu, S., Silbiger, S. M., Levy, R. B., *et al.* Neu differentiation

- factor: a transmembrane glycoprotein containing an EGF domain and an immunoglobulin homology unit. *Cell*, 69: 559–572, 1992.
38. Grandis, J., Drenning, S. D., Zeng, Q., Watkins, S. C., Melhem, M. F., Endo, S., Johnson, D. E., Huang, L., He, Y., and Kim, J. D. Constitutive activation of Stat3 signaling abrogates apoptosis in squamous cell carcinogenesis *in vivo*. *Proc. Natl. Acad. Sci. USA*, 97: 4227–4232, 2000.
 39. Olayioye, M., Beuvink, I., Horsch, K., Daly, J., and Hynes, N. ErbB receptor-induced activation of stat transcription factors is mediated by Src tyrosine kinases. *J. Biol. Chem.*, 274: 17209–17211, 1999.
 40. Olayioye, M., Neve, R., Lane, H., and Hynes, N. The ErbB signaling network: receptor heterodimerization in development and cancer. *EMBO J.*, 19: 3159–3167, 2000.
 41. 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.
 42. Moasser, M. M., Basso, A., Averbuch, S. D., and Rosen, N. The tyrosine kinase inhibitor ZD1839 (“Iressa”) inhibits HER2-driven signaling and suppresses the growth of HER2-overexpressing tumor cells. *Cancer Res.*, 61: 7184–7188, 2001.
 43. Moulder, S. L., Yakes, M., Muthuswamy, S. K., Bianco, R., Simpson, J. F., and Arteaga, C. Epidermal growth factor receptor (HER1) tyrosine kinase inhibitor ZD1839 (Iressa) inhibits HER2/Neu (erb-2)-overexpressing breast cancer cells *in vitro* and *in vivo*. *Cancer Res.*, 61: 8887–8895, 2001.
 44. Normanno, N., Campiglio, M., De Luca, A., Somenzi, G., Maiello, M., Ciardiello, F., Gianni, L., Salomon, D. S., and Menard, S. Cooperative inhibitory effect of ZD1839 (“Iressa”) in combination with trastuzumab (“Herceptin”) on human breast cancer cell growth. *Ann. Oncol.*, 13: 65–72, 2002.
 45. Gamett, D., Pearson, G., Cerione, R., and Friedberg, I. Secondary dimerization between members of the epidermal growth factor receptor family. *J. Biol. Chem.*, 272: 12052–12056, 1997.
 46. 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.
 47. Anido, J., Albanell, J., Rojo, F., Codony-Servat, J., Arribas, J., and Baselga, J. Inhibition by ZD1839 (Iressa) of epidermal growth factor (EGF) and heregulin induced signaling pathways in human breast cancer cells. *Proc. Am. Soc. Clin. Oncol.*, 20: 1712A, 2001.
 48. Keith, B. R., Allen, P. P., Alligood, K. J., Crosby, R. M., Lackey, K., Gilmer, T. M., and Mullin, R. J. Anti-tumor activity of GW2016 in the ErbB-2 positive human breast cancer xenograft, BT-474. *Proc. Am. Assoc. Cancer Res.*, 42: 803, 2001.
 49. Rusnak, D. W., Afleck, K., Gilmer, T. M., Jowett, A., Lackey, K., Spector, N., and Xia, W. The effects of the novel EGFR/ErbB-2 tyrosine kinase inhibitor, GW2016, on the growth of human normal and transformed cell lined. *Proc. Am. Assoc. Cancer Res.*, 42: 803, 2001.
 50. Fry, D. W., Bridges, A. J., Denny, W. A., Doherty, A., Greis, K. D., Hicks, J. L., Hook, K. E., Keller, P. R., Leopold, W. R., Loo, J. A., McNamara, D. J., Nelson, J. M., Sherwood, V., Smaill, J. B., Trumpp-Kallmeyer, S., and Dobrusin, E. M. Specific, irreversible inactivation of the epidermal growth factor receptor and erbB2, by a new class of tyrosine kinase inhibitor. *Proc. Natl. Acad. Sci. USA*, 95: 12022–12027, 1998.
 51. Prigent, S. A., and Gullick, W. Identification of c-erbB-3 binding sites for phosphatidylinositol 3'-kinase and SHC using an EGF receptor/c-erbB-3 chimera. *EMBO J.*, 13: 2831–2841, 1994.
 52. Kummer, J. L., Rao, P. K., and Heidenreich, K. A. Apoptosis induced by withdrawal of trophic factors is mediated by p38 mitogen-activated protein kinase. *J. Biol. Chem.*, 272: 20490–20494, 1997.
 53. Zhuang, S., Demirs, J. T., and Kochevar, I. E. p-38 mitogen-activated protein kinase mediates bid cleavage, mitochondrial dysfunction, and caspase-3 activation during apoptosis induced by singlet oxygen but not by hydrogen peroxide. *J. Biol. Chem.*, 275: 25939–25948, 2000.
 54. Bromberg, J., Wrzeszczynska, M., Devgan, G., Zhao, Y., Pestell, R. G., Albanese, C., and Darnell, J. E., Jr. Stat3 as an oncogene. *Cell*, 98: 295–303, 1999.
 55. Albanell, J., Rojo, F., Averbuch, S., Feyereislova, A., Mascaro, J. M., Herbst, R., LoRusso, P., Rischin, D., Sauleda, S., Gee, J., Nicholson, R. I., and Baselga, J. Pharmacodynamic studies of the EGF receptor inhibitor ZD1839 (“Iressa”) in skin from cancer patients: histopathological and molecular consequences of receptor inhibition. *J. Clin. Oncol.*, 20: 110–124, 2002.
 56. Nakagawa, K., Yamamoto, N., Kudoh, S., Negoro, S., Takeda, K., Tamura, T., and Fukuoka, M. A Phase I intermittent dose-escalation trial of ZD1839 (Iressa) in Japanese patients with solid malignant tumours. *Proc. Am. Soc. Clin. Oncol.*, 19: 183a, 2000.

Clinical Cancer Research

ZD1839, a Specific Epidermal Growth Factor Receptor (EGFR) Tyrosine Kinase Inhibitor, Induces the Formation of Inactive EGFR/HER2 and EGFR/HER3 Heterodimers and Prevents Heregulin Signaling in HER2-overexpressing Breast Cancer Cells

Judit Anido, Pablo Matar, Joan Albanell, et al.

Clin Cancer Res 2003;9:1274-1283.

Updated version Access the most recent version of this article at:
<http://clincancerres.aacrjournals.org/content/9/4/1274>

Cited articles This article cites 55 articles, 22 of which you can access for free at:
<http://clincancerres.aacrjournals.org/content/9/4/1274.full#ref-list-1>

Citing articles This article has been cited by 50 HighWire-hosted articles. Access the articles at:
<http://clincancerres.aacrjournals.org/content/9/4/1274.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://clincancerres.aacrjournals.org/content/9/4/1274>.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.