The Tyrosine Kinase Inhibitor ZD1839 ("Iressa") Inhibits HER2-driven Signaling and Suppresses the Growth of HER2-overexpressing Tumor Cells

Mark M. Moasser, Andrea Basso, Steven D. Averbuch, and Neal Rosen

ABSTRACT

The epidermal growth factor receptor (EGFR) is commonly overexpressed in many human tumors and provides a new target for anticancer drug development. ZD1839 ("Iressa"), a quinazoline tyrosine kinase inhibitor selective for the EGFR, has shown good activity in preclinical studies and in the early phase of clinical trials. However, because it remains unclear which tumor types are the best targets for treatment with this agent, the molecular characteristics that correlate with tumor sensitivity to ZD1839 have been studied. In a panel of human breast cancer and other epithelial tumor cell lines, HER2-overexpressing tumors were particularly sensitive to ZD1839. Growth inhibition of these tumor cell lines was associated with the dephosphorylation of EGFR, HER2, and HER3, accompanied by the loss of association of HER3 with phosphatidylinositol 3-kinase, and down-regulation of Akt activity. These studies suggest that HER2-overexpressing tumors are particularly susceptible to the inhibition of HER family tyrosine kinase signaling and suggest novel strategies to treat these particularly aggressive tumors.

INTRODUCTION

Deregulated expression of tyrosine kinases through mutation, amplification, or deregulation is a hallmark of malignancy. Overactivity of these signaling proteins can lead to abnormal growth control and cellular transformation. Thus, selective inhibitors of specific tyrosine kinases might prove to be effective anticancer agents. Studies of the HER family of tyrosine kinases have been at the forefront of these efforts.

Four members of the HER family of transmembrane RTKs are known: EGFR (HER1, ErbB1), HER2 (Neu, ErbB2), HER3 (ErbB3), and HER4 (ErbB4). These proteins share a common structural organization and an overall amino acid homology of 40–50%. Growth factor signaling by the HER family of RTKs is marked by complexity at every step because of the multiplicity of ligands, receptors, effectors, and downstream pathways. More than 25 ligands are known, which bind these RTKs with various affinities, although a high-affinity ligand specific for HER2 has not yet been reported, and this receptor may not have a physiological ligand. Although the four members of the HER family share many structural and functional characteristics, differences in tissue expression and different phenotypes in gene-disrupted mice reveal that each member performs nonredundant functions, and it remains unclear what unique functions distinguish the individual family members (reviewed in Ref. 1). The HER family of RTKs controls a complex process of lateral signaling through ligand-induced homo- and heterodimerization.

The Tyrosine Kinase Inhibitor ZD1839 ("Iressa") is a synthetic anilinoquinazoline tyrosine kinase inhibitor selective for EGFR. It inhibits EGFR in vitro with an IC50 of 27–33 nM and is competitive with ATP and noncompetitive with peptide substrates. It has much lower activity against related RTKs, with an IC50 of 2 μM and 20 μM for HER2 and KDR, respectively. Because ZD1839 has good oral bioavailability and demonstrated antitumor activity in a broad range of mouse xenograft models, it was selected for clinical development (9). Phase I trials of ZD1839 in patients with solid tumors refractory to standard chemotherapeutic agents have shown good tolerability and evidence of antitumor activity against such tumors (10, 11). These successes have led to additional clinical development, and additional clinical trials are currently underway to better determine its potential in the treatment of cancer patients.

We have studied the molecular basis for the sensitivity of some tumors to ZD1839. We find that, although this agent selectively inhibits EGFR activity, tumors with HER2 overexpression are particularly sensitive to it. Treatment of HER2-overexpressing tumors with ZD1839 results in dephosphorylation of HER2 and profound down-regulation of the PI3k/Akt signaling pathway attributable to dephosphorylation of HER3. These results indicate that the clinical potential of this agent is not limited to tumors with EGFR overexpression and, in fact, suggest that it may represent a novel modality in the treatment of patients with HER2-overexpressing tumors.
MATERIALS AND METHODS

Tumor cell lines were obtained from the American Type Culture Collection maintained in 1:1 mixture of DME:F12 medium supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, 4 mM glutamine, and 10% heat-inactivated fetal bovine serum, and incubated at 37°C in 5% CO2. DU4475 cells were grown in RPMI 1640 with similar supplements. MCF10A cells were grown in DME:F12 supplemented with 5% donor horse serum, 0.5 µg/ml hydrocortisone, 10 µg/ml insulin, and 3 nM EGF. For growth assays, cells were seeded in six-well clusters at 20–50,000 cells/well. Later (24 h), the cells were placed in fresh medium containing ZD1839 and allowed to grow for 4–10 days, depending on the cell line. When control wells were near semiconfluent, cells were harvested by trypsinization and counted using a Coulter counter.

Western blots were performed by harvesting total cellular lysates in RIPA buffer [10 mM sodium phosphate (pH 7.2), 150 mM NaCl, 0.1% SDS, 1% NP-40, 1% sodium deoxycholate, and protease inhibitors], separating 50 µg of each lysate by SDS-PAGE, and transferring to membrane. Immunoblot analyses were performed using antibodies specific for EGFR (Trans. Labs; E12020), HER2 (Neomarkers; Ab-15), HER3 (Neomarkers; Ab-2), HER4 (Neomarkers; Ab-2), and phospho-Akt (Cell Signaling; 9271), and visualized by enhanced chemiluminescence and autoradiography. Quantitative analysis of Akt activity was performed by imaging the autoradiograms in a Bio-Rad GelDoc 2000 in white light and quantitating relative band densities using Quantity One software (Bio-Rad).

Transfections were performed as follows. SkBr3 cells were seeded at 2 million cells/10-cm dish and were transfected with 2 µg of pSG5-P110CAAX (kindly provided by Julian Downward, ICRF, London, United Kingdom) the following day or pSG5 control using Lipofectam reagent (Life Technologies, Inc.). After 24 h, cells were placed in medium containing indicated concentrations of ZD1839 for 30 min, and total lysates were harvested and analyzed by Western blotting as described above.

Receptor phosphorylation assays were performed as follows. RIPA lysates were immunoprecipitated with the relevant antibodies, separated on SDS-PAGE, transferred to membrane, and immunoblotted using antiphosphotyrosine antibodies (Santa Cruz; PY99). Immunoprecipitations were performed using monoclonal anti-EGFR (Calbiochem; GR13), monoclonal anti-HER2 (Neomarkers; Ab-4), and monoclonal anti-HER3 (Neomarkers; Ab-4) antibodies. All of these antibodies were against epitopes within the extracellular domains of the receptors, and previous titering experiments had established the amount of lysate and antibody that would maximally immunoprecipitate the target protein.

RESULTS

Sensitivity of EGF-dependent Growth to ZD1839. Because ZD1839 is a potent inhibitor of EGFR in vitro, we initially studied its efficacy in inhibiting EGFR–dependent cell growth. MCF10A cells are nontransformed breast epithelial cells that require EGF to proliferate. The monolayer growth of these EGF-driven untransformed cells is inhibited by ZD1839 with an IC50 of 0.8 µM making it difficult to establish a definite correlation between EGFR overexpression and drug sensitivity. However, it is evident from MDA-MB-468 cells that EGFR overexpression is not sufficient to determine sensitivity to ZD1839. There is no apparent correlation between the expression of HER3 or HER4 with tumor sensitivity in these cells.

Receptor Dephosphorylation. The four members of the HER kinase family are closely related. Although ZD1839 is a relatively selective inhibitor of EGFR in vitro, it may have broader effects in cells. Therefore, we studied the effects of ZD1839 on the tyrosine phosphorylation of each receptor family member in the drug-sensitive SkBr3 cells, which are growth-inhibited with an IC50 of 0.8 µM. At concentrations >1 µM, and in <30 min, ZD1839 reduces the basal phosphorylation of EGFR but also of HER2 and HER3 (Fig. 2A).

Down-Regulation of the PI3K/Akt Pathway. EGFR and HER2 activate a number of cytoplasmic signal transduction pathways of which the Ras/MAP kinase pathway has been the best defined. We have studied whether inhibition of this pathway may be mediating the antitumor effects of ZD1839. However, in MCF-7 and MDA-MB-468 cells, 1 µM of ZD1839 inhibits EGFR-induced MAP kinase signaling (data not shown) yet has no effects on cell growth at this concentration. Thus far we have not found a correlation between MAP kinase inhibition and ZD1839 sensitivity.

Another downstream signaling event initiated by HER kinase stimulation is recruitment and activation of PI3K at the cell membrane leading to activation of the cytosolic serine/threonine kinase Akt. ZD1839 down-regulates Akt activity within 30 min in some tumor cell lines. This rapid down-regulation of Akt activity is predominantly seen in tumors that are sensitive to ZD1839 such that down-regulation of Akt activity is a marker of tumor response to ZD1839 (Figs. 1 and 3). In fact, Akt down-regulation is a better correlate of tumor sensitivity than HER2 overexpression, because A431 cell growth and Akt activity are very sensitive to ZD1839, yet these cells do not overexpress HER2. DU145 cells are also partially sensitive to ZD1839 and show a decline in Akt activity but no overexpression of HER2. There is no decline in total Akt expression in these experiments (data not shown).

To determine whether ZD1839 treatment down-regulates Akt activity through down-regulation of PI3K activity, we transfected the ZD1839-sensitive SkBr3 cells with an activated PI3K construct. P110-CAAX is a modified p110 that has a COOH-terminal CAAX motif. This motif signals post-translation prenylation leading to membrane localization and activation. Expression of constitutively active PI3K prevents the down-regulation of Akt activity by ZD1839 supporting the hypothesis that ZD1839 down-regulates Akt activity through inhibition of PI3K activity (Fig. 4). Although the resistance to ZD1839 in p110-CAAX-transfected cells appears incomplete here, this represents only the subset of cells that were successfully transfected, because these are transient transfections.

The activation of PI3K by HER family proteins is thought to occur predominantly through association of the SH2 domain of the p85 regulatory subunit of PI3K with several tyrosine-phosphorylated binding sites on the cytoplasmic domain of HER3. Because ZD1839 treatment of HER2-overexpressing tumors results in HER3 dephos-
phorylation, we hypothesized that this would lead to loss of HER3 association with p85, and our experiments corroborate this hypothesis. Immunoprecipitation of EGFR, HER2, and HER3 from the HER2-overexpressing SkBr3 cells results in coimmunoprecipitation of p85 with HER3 but not with EGFR or HER2, and this association is lost at concentrations of ZD1839 >1 μM in parallel with the reduction in the phosphorylation of HER3 (Fig. 2B).

Combination Treatment of HER2-overexpressing Tumor Cells. Because ZD1839 may have clinical application in the treatment of patients with HER2-overexpressing breast cancers, and such patients may benefit from combination therapies, we tested the efficacy of monoclonal anti-HER2 antibodies (trastuzumab [Herceptin]) in combination with ZD1839 in cell culture. Treatment of SkBr3 cells with trastuzumab and ZD1839 shows that the growth inhibitory activities of these two agents are additive (Fig. 5).

DISCUSSION

Selective inhibition of tyrosine kinase activity is a promising strategy for cancer treatment. ZD1839 is one of the first tyrosine kinase inhibitors to be tested in patients with advanced solid tumors. Initial studies have shown promising results, and additional clinical trials are in progress to additionally define the activity and toxicities of this new treatment modality. We have been interested in how this agent inhibits the growth of tumor cells and which subset of tumors may be most effectively treated by this agent. In a survey of human tumor cell lines, we find that tumors with HER2 overexpression are particularly sensitive to ZD1839. There is no correlation with HER3 or HER4 expression. Although in vitro this agent shows the highest activity against EGFR, it is apparent from our analysis of two EGFR-overexpressing tumor types, MDA-MB-468 and A431, that overexpression of EGFR is not sufficient to determine sensitivity to ZD1839. It remains possible that the consequences of EGFR overexpression are modified by other molecular lesions in the tumor. For example, MDA-MB-468 cells may be driven by overexpression and overactivity of EGFR, but their relative resistance to ZD1839 may be attributable to the failure to down-regulate Akt activity, because these cells have a deletion of the PTEN tumor suppressor protein (12). Thus, the...
assumption that EGFR overexpression per se will determine sensitivity to EGFR-targeted therapies such as ZD1839 may turn out to be oversimplistic.

Although ZD1839 shows relative selectivity for EGFR in vitro, our data show that in tumors it leads to reduced basal phosphorylation of EGFR, HER2, and HER3. The reduction in phosphorylation of these receptors occurs at low μM concentrations and correlates with the antitumor activity of this agent. At these concentrations, ZD1839 is not selective for EGFR in vitro and, in fact, inhibits the HER2 kinase domain in vitro with an IC50 of 1.2–3.7 μM. Therefore, it is difficult to know, based on these studies alone, whether ZD1839 inhibits tumors through the inhibition of EGFR or HER2, or perhaps both. Because HER2-overexpressing tumors are thought to be driven by overactivity of HER2, the sensitivity of these tumors to ZD1839 would suggest that the inhibition of HER2 is most important in these tumors, and this remains a distinct possibility based on these studies. However, because HER2 functions preferentially as a heterodimer with other HER family members including EGFR resulting in receptor transphosphorylation EGFR may play an important role in the growth of HER2-overexpressing tumors. HER2 readily dimerizes with EGFR in vivo (2), and cells transfected with EGFR and HER2 have increased basal phosphorylation of these two receptors (13). In transgenic models of breast cancer driven by HER2 and transforming growth factor α overexpression, inhibition of EGFR by a selective tyrosine kinase inhibitor suppresses tumor formation (14). It is possible that the oncogenic activity of HER2 requires phosphorylation by EGFR. Understanding the precise role and importance of the EGFR in human HER2-overexpressing breast cancers requires additional studies.

EGFR and HER2 affect the activity of a number of cytoplasmic signal transduction pathways, and it remains unclear which pathways are most important for maintaining the transformed phenotype in HER2-overexpressing tumors. Numerous effector molecules associate with phosphotyrosine residues within the cytoplasmic domains of HER kinases. These include Grb2, Shc, Shp1, PLCγ, Cbl, p85 subunit of PI3k, and others that associate selectively with specific members of the HER kinases on specific phosphotyrosine residues (reviewed in Ref. 1). We have shown that the down-regulation of the PI3k/Akt pathway correlates with the antitumor effects of ZD1839. This suggests that the PI3k/Akt pathway may be important in driving tumor growth, especially in HER2-overexpressing tumors. Additional studies are required to determine the effects of ZD1839 on other effector molecules and signaling pathways. Although ZD1839 is effective in inhibiting the EGFR-Ras-MAP kinase pathway, this is not sufficient for inhibition of growth in some tumor cells. Future studies will attempt to define the role of this pathway in maintaining tumor growth.
in vitro expression. However, these cells express HER4, which contains a p85 sensitive to ZD1839 and may be driving the PI3k activity in these tumors. HER3 is expressed in most of the tumor cell lines that are tested for HER2 overexpression, and these tumors are associated with a particularly poor prognosis. Moreover, our data suggest that the clinical efficacy of HER2-overexpressing tumors and additive activity with therapeutic anti-HER2 antibodies. The clinical relevance of these findings awaits clinical trials of ZD1839 in patients with HER2-overexpressing tumors.

REFERENCES

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