Featured Article

Resistance to Gefitinib in PTEN-Null HER-Overexpressing Tumor Cells Can Be Overcome through Restoration of PTEN Function or Pharmacologic Modulation of Constitutive Phosphatidylinositol 3′-Kinase/Akt Pathway Signaling

Qing-Bai She, David Solit, Andrea Basso, and Mark M. Moasser[1]

Department of Medicine and Sloan-Kettering Institute, Memorial Sloan-Kettering Cancer Center, New York, New York 10021

Abstract

Purpose: **Tyrosine kinase (TK) inhibitors are emerging as a promising new approach to the treatment of HER overexpressing tumors, however optimal use of these agents awaits further definition of the downstream signaling pathways that mediate their effects. We reported previously that both EGFR- and Her2-overexpressing tumors are sensitive to the new EGFR-selective TK inhibitor gefitinib (ZD1839, "Iressa"), and sensitivity to this agent correlated with its ability to down-regulate Akt. However, EGFR-overexpressing MDA-468 cells, which lack PTEN function, are resistant to ZD1839, and ZD1839 is unable to down-regulate Akt activity in these cells.**

Experimental Design: **To study the role of PTEN function, we generated MDA468 cells with tet-inducible PTEN expression.**

Results: **We show here that the resistance of MDA-468 cells to ZD1839 is attributable to EGFR-independent constitutive Akt activation caused by loss of PTEN function in these cells. Reconstitution of PTEN function through tet-inducible expression restores ZD1839 sensitivity to these cells and reestablishes EGFR-stimulated Akt signaling. Although restoration of PTEN function to tumors is difficult to implement clinically, much of the effects of PTEN loss are attributable to overactive PI3K/Akt pathway signaling, and this overactivity can be modulated by pharmacologic approaches. We show here that pharmacologic down-regulation of constitutive PI3K/Akt pathway signaling using the PI3K inhibitor LY294002 similarly restores EGFR-stimulated Akt signaling and sensitizes MDA-468 cells to ZD1839.**

Conclusions: **Sensitivity to ZD1839 requires intact growth factor receptor-stimulated Akt signaling activity. PTEN loss leads to uncoupling of this signaling pathway and results in ZD1839 resistance, which can be reversed with reintroduction of PTEN or pharmacologic down-regulation of constitutive PI3K/Akt pathway activity. These data have important predictive and therapeutic clinical implications.**

Introduction

A subset of human tumors is driven by overexpression and overactivity of HER family proteins. The EGFR[2] is one of four structurally related members (EGFR/HER1/erbB1, HER2/Neu/erbB2, HER3/erbB3, and HER4/erbB4) that comprise this family of transmembrane tyrosine kinase growth factor receptors that signal diverse biological processes, such as growth, proliferation, differentiation, and apoptosis. HER family RTKs signal through homo and heterodimeric complexes with considerable differences in signaling activities between the various dimerization partners (1). These activated RTK complexes in turn activate a number of cytoplasmic signaling pathways, including the Ras/Raf/MAPK pathway, the PI3K (phosphoinositol 3′-kinase)—Akt pathway, the Stat pathway, and the PKC pathway (2).

Overactivity of HER family proteins is oncogenic in experimental models (3, 4), and overexpression of HER1 (EGFR) or HER2 is seen in many human tumors, including tumors of the breast, lung, ovaries, kidney, and glioblastomas (5). Breast and ovarian cancers with Her2 overexpression are particularly aggressive and have a poorer prognosis (6). The pathways by which overactive HER proteins mediate their tumorigenic effects are not fully identified, and because mutations or other functional abnormalities of each of these downstream pathways are also commonly seen in human tumors, HER proteins may in fact be capable of mediating transformation through multiple cytoplasmic signaling pathways.

Because HER family overexpression is commonly seen in human tumors, much attention has focused on developing targeted therapies for this subset of human cancers. We have been studying the activities and mechanisms underlying the antitumor activities of the novel EGFR-selective tyrosine kinase inhibitor gefitinib (ZD1839, 'Iressa'). Although this agent is highly spe-

Received 3/5/03; revised 6/17/03; accepted 6/25/03.
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] To whom requests for reprints should be addressed, at Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, Box 478, New York, NY 10021. Phone: (212) 639-5025; Fax: (212) 717-3627; E-mail: moasserm@mskcc.org.

[2] The abbreviations used are: EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3′-kinase; MEK, mitogen-activated protein/extracellular signal-regulated kinase kinase; RT-PCR, reverse transcription-PCR; CMV, cytomegalovirus.

cific for EGFR, we reported previously that both EGFR- and HER2-overexpressing tumor cells are sensitive to this agent *in vitro* (7). The biochemical activities of this agent extend beyond the realm of EGFR because in Her2-overexpressing cells, ZD1839 reduces phosphorylation of EGFR, as well as HER2 and HER3 (7). In tumors cells that overexpress either EGFR or HER2, dephosphorylation of these receptors by ZD1839 is accompanied by down-regulation of PI3K and inactivation of Akt. In this analysis of tumor cell lines, we found that down-regulation of the PI3K/Akt pathway was predominantly seen in HER-overexpressing tumor cells and closely correlates with the growth sensitivies of these tumor cell lines to ZD1839. This suggests that inhibition of the PI3K/Akt pathway is an important effector of the antitumor activities of ZD1839. In the current study, we have sought to determine whether inhibition of Akt pathway signaling is required for the sensitivity of HER-overexpressing tumor cells to ZD1839. Using the EGFR-overexpressing MDA-468 breast cancer cells, we show that the resistance of these tumor cells to ZD1839 is caused by PTEN loss with consequent hyperactivation of Akt and uncoupling of the Akt pathway from EGFR. Reconstitution of PTEN in these cells reestablishes EGFR-driven Akt signaling and restores ZD1839 sensitivity, confirming the essential role of Akt signaling in mediating response and resistance to ZD1839.

Materials and Methods

Cell Culture and Reagents. MDA-468 cells were obtained from the American Type Culture Collection; maintained in 1:1 mixture of DME:F12 media 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% CO_2. For growth assays, cells were seeded in six-well clusters at 50,000 cells/well. A full day later, cells were placed in fresh media containing indicated concentrations of drugs and allowed to grow for 5–7 days and subsequently harvested by trypsinization and counted using a Coulter counter. Reagents used were ZD1839 (AstraZeneca Pharmaceuticals, Cheshire, United Kingdom), LY294002 (Calbiochem), and doxycycline (Sigma).

Protein Assays. Western blots were performed by harvesting total cellular lysates in radioimmunoprecipitation assay buffer [10 mM Na phosphate (pH 7.2), 150 mM NaCl, 0.1% SDS, 1% NP40, 1% Na deoxycholate, and protease inhibitors], separating 50 μg of each lysate by SDS-PAGE, transferring to membrane, and immunoblotting using specific primary and secondary antibodies and enhanced chemoluminescence visualization. Antibodies for Akt, phospho-Akt, GSK3, phospho-GSK3, 4EBP1, and phospho-4EBP1 were from Cell Signaling. Antibodies for phosphotyrosine and PTEN were from Santa Cruz Biotechnology. Other antibodies include anti-phospho MAPK (Promega) and Her2 (NeoMarkers).

PCR. RT-PCR was performed using 1 μg of total cellular RNA, oligodT primed reverse transcriptase synthesized cDNA, followed by RNase H treatment. The transcribed PTEN message was amplified using the primers ACACGACGGGAAGACAAGTT and TGGTGATGATGACCGGTATG, which amplify a 500-bp fragment of the PTEN cDNA ending with the Myc and His tags of the pcDNA4-To-MycHic backbone.

Transfections. Stable transfections were performed using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. MDA-468 cells were stably transfected with pcDNA6/TR (Invitrogen), selected in Blasticidin, and expression of the tet repressor confirmed in transient transfection assays. These cells were named MDA468TR. The 1.2-kb human PTEN cDNA was cloned by RT-PCR, sequence confirmed, and ligated into the pcDNA4/TO/MycHis vector (Invitrogen) under control of the CMV promoter containing tet operator sequences. The pcDNA4-PTEN vector was stably transfected into MDA468TR cells, transfectants selected in Zeocin, and inducible expression of the PTEN protein product confirmed by Western blot analysis of doxycycline-treated cells. In this vector system, doxycycline binds with and interferes with the tet repressor leading to unhindered activity of the CMV promoter and expression of the PTEN cDNA insert.

Results

Despite the potent activity of ZD1839 against the EGFR tyrosine kinase, MDA-468 cells are relatively resistant to this agent when compared with other EGFR- or Her2-overexpressing tumor cells, and ZD1839 fails to inhibit Akt activity in these cells (7). However, the lack of functional PTEN in these cells could account for ZD1839 resistance, because in the absence of PTEN function, the Akt pathway would be driven by overactive phosphoinositide signaling and insensitive to upstream EGFR signaling. To study our hypothesis regarding the critical role of the PI3K/Akt pathway further, we restored PTEN function in MDA-468 cells and studied whether this brings the Akt pathway under the control of EGFR and resensitizes these cells to ZD1839.

Reintroduction of PTEN Expression in MDA-468 Cells. MDA-468 cells lack PTEN expression and function because of a frameshift mutation in one allele and loss of the other allele of the PTEN gene (8). Re-expression of PTEN in these cells through transfection would provide the necessary model to study the hypotheses that sensitivity and resistance to ZD1839 is mediated through the PI3K/Akt pathway. However, PTEN-null tumor cells do not tolerate reintroduction of PTEN and specifically in MDA-468 cells; introduction of wild-type PTEN inhibits cell growth and causes apoptosis (9). Therefore, to optimally study the role of PTEN in MDA-468 cells, we established MDA-468 cells with tet-inducible expression of PTEN. Initially, MDA-468 cells were engineered to express the Tet Repressor and named MDA-468TR. MDA-468TR cells were then transfected with the pcDNA4-TO-MycHis vector containing the 1.2-kb wild-type human PTEN cDNA under control of the CMV promoter and tet-operator sequences. Transfectants were selected and expanded in the absence of doxycycline, and inducible PTEN expression was confirmed by the addition of doxycycline. PTEN expression in MDA-468TR-PTEN cells is maximally induced by 3 h using 100 ng/ml doxycycline (Fig. 1*A*). Induction of PTEN expression in MDA-468TR-PTEN cells by the addition of doxycycline leads to inhibition of cell growth and apoptosis. This is consistent with previous reports in these and other PTEN-null tumor cell lines (see "Discussion"). The PTEN-induced inhibition of growth becomes significant after 5–6 days of PTEN expression (Fig. 1*C*). Therefore, there is a

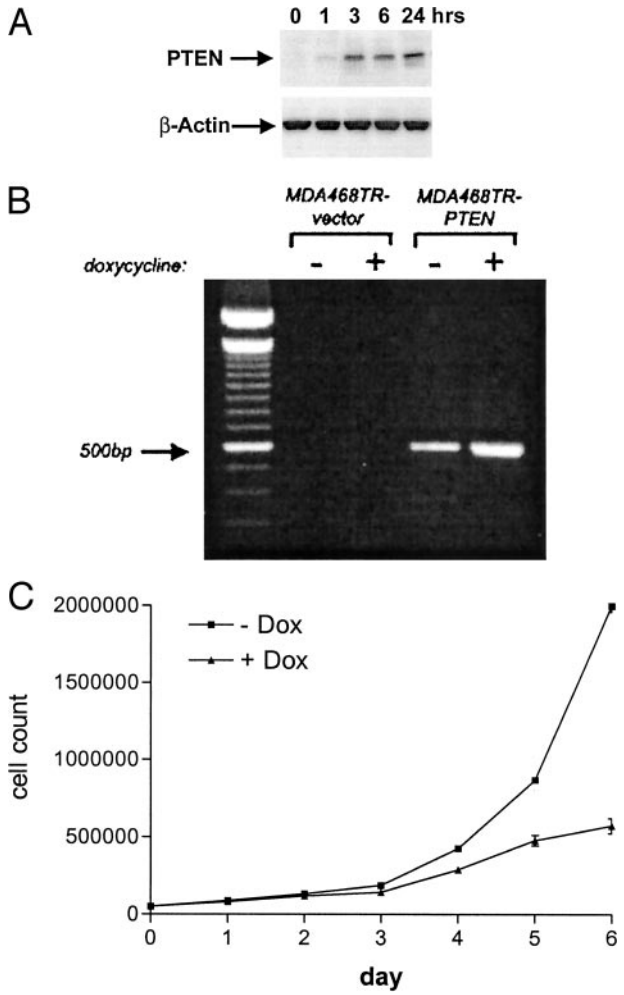

Fig. 1 In *A*, MDA-468TR-PTEN cells were treated with 100 ng/ml doxycyline and harvested at the indicated times. Lysates were separated by SDS-PAGE and immunoblotted using anti-PTEN and anti-β-actin antibodies. In *B*, 1 μg of total cellular RNA was used in RT-PCR reaction as described in "Materials and Methods," using primers specific for the vector-induced PTEN message. PCR products were separated by 1.5% agarose gel electrophoresis and imaged under a UV light source. In *C*, MDA-468TR-PTEN and vector controls were seeded in six-well clusters in various concentrations of ZD1839 and in the presence or absence of 100 ng/ml doxycycline and grown for 5 days and counted. Results are average of duplicates.

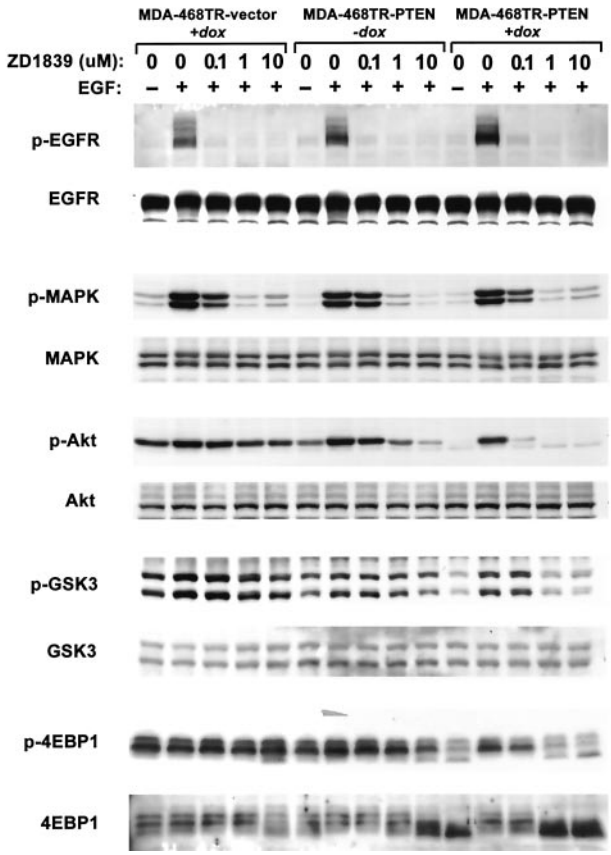

Fig. 2 MDA-468TR-PTEN (with and without 100 ng/ml doxycycline) and MDA-468TR-vector (with dox) were serum starved overnight and the following day treated for 1 h with the indicated concentrations of ZD1839, and cells were harvested rapidly on ice after a 10 nM EGF stimulus. Lysates were separated by SDS-PAGE and immunoblotted using the indicated antibodies. EGFR, MAPK, and Akt activity were assayed at 5 min after the EGF stimulus, whereas activity of the downstream targets GSK-3 and 4EBP1 were assayed at 30 min after the EGF stimulus.

window of 5–6 days during which we were able to study the role of PTEN in mediating sensitivity and resistance to ZD1839. MDA-468TR cells were also transfected with pcDNA4-TO-MycHis parent vector, and stable clones were expanded and used as vector-only controls. Although Western blot analysis shows that MDA-468TR-PTEN cells have no detectable expression of PTEN in the absence of doxycyline, more sensitive expression analysis using RT-PCR shows that there is some expression of the PTEN RNA transcript in these cells (Fig. 1*B*). This indicates that the pcDNA4-TO-MycHis CMV promoter is not completely silenced by the tet-repressor. The reverse PCR primer used in this analysis primes the tagged end of the PTEN cDNA insert donated by the pcDNA4-TO-MycHis backbone. Therefore, this RT-PCR analysis is specific for the transfected PTEN RNA and does not amplify the endogenous and mutated PTEN RNA in these cells.

PTEN Expression Restores EGFR-stimulated Akt Signaling and ZD1839 Sensitivity. MDA-468TR-PTEN cells (with and without doxycycline) and vector controls were treated with increasing concentrations of ZD1839, and the ability of ZD1839 to inhibit EGFR-stimulated Akt signaling was assayed. ZD1839 inhibits EGFR activation effectively in all three experimental arms and inhibits MAPK signaling equally in all three arms (Fig. 2). Although ZD1839 does not inhibit Akt signaling in vector controls, it partially inhibits it in uninduced MDA-468TR-PTEN cells and fully inhibits it in doxycycline-induced MDA-468TR-PTEN cells (Fig. 2). The partial inhibition in uninduced cells is consistent with minimal basal expression of PTEN in these cells, as already discussed. Similar results are seen with GSK-3 and 4-EBP1 activity, which function downstream of Akt. Although ZD1839 does not inhibit activity of GSK-3 or 4EBP1 in vector controls or uninduced MDA-468TR-

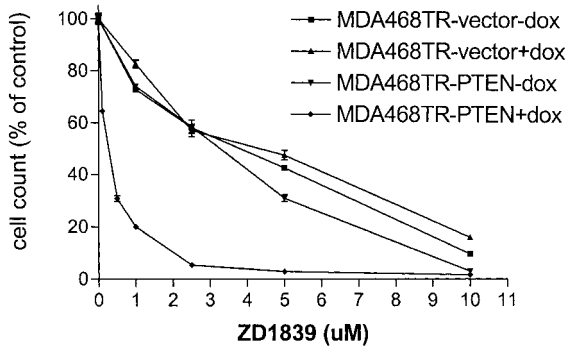

Fig. 3 MDA-468TR-PTEN and vector controls were seeded in six-well dishes and the following day placed in various concentrations of ZD1839 in the presence or absence of 100 ng/ml doxycycline and incubated for 6 days. Cells were trypsinized and counted. Results are the average of duplicates and are shown as a percentage of untreated control cells. The cell count indicated on the *Y* axis has been normalized for each arm to the 0 concentration count to enable a direct comparison of the ZD1839 IC_{50}s in the presence or absence of PTEN expression. The raw total counts in the PTEN-induced arm are lower because induction of PTEN by itself is antiproliferative.

PTEN cells, it does inhibit them in doxycycline-induced MDA-468TR-PTEN cells (Fig. 2). These experiments show that in the absence of PTEN, Akt signaling is uncoupled from EGFR signaling in these cells, and reintroduction of PTEN expression reestablishes EGFR-stimulated Akt pathway signaling and restores the ability of ZD1839 to inhibit Akt signaling.

The *in vitro* ZD1839 growth sensitivity of MDA468TR-PTEN cells was assayed in the presence of doxycycline and compared with controls lacking doxycycline and with vector controls (Fig. 3). With PTEN induction, MDA-468TR-PTEN cells are 10–20-fold more sensitive to ZD1839 (IC_{50} of 0.2 μM compared with 3.5 μM in uninduced cells and compared with 5 μM in vector control cells). The slightly lower IC_{50} in uninduced MDA468TR-PTEN cells compared with vector controls is consistent with a low level of PTEN protein expression in these uninduced cells. These data confirm that loss of PTEN in MDA-468 cells accounts for their resistance to ZD1839 and that restoration of PTEN function can reestablish sensitivity to ZD1839.

Pharmacologic Inhibition of PI3K Also Sensitizes MDA468 Cells to ZD1839. Although these studies suggest that expression of PTEN can sensitize certain PTEN-null tumor cells to ZD1839, this strategy has limited clinical implications because restoration of PTEN function in the tumors of patients is not realistic at this time. However, since one of the main functions of PTEN is to counteract PI3K in the regulation of phosphoinositide signaling, a principle consequence of PTEN loss is unopposed PI3K signaling and overactivity of the PI3K/Akt pathway. Therefore, in PTEN-deleted tumor cells, more balanced phosphoinostide signaling may also be achieved using pharmacologic inhibitors of PI3K kinase, and this intervention may produce some of the effects we see with PTEN transfection. To test this hypothesis, we performed ZD1839 growth sensitivity assays and EGFR signaling assays in MDA-468 cells in the presence or absence of the PI3K inhibitor LY294002. In this analysis, we find that LY294002 sensitizes these cells to

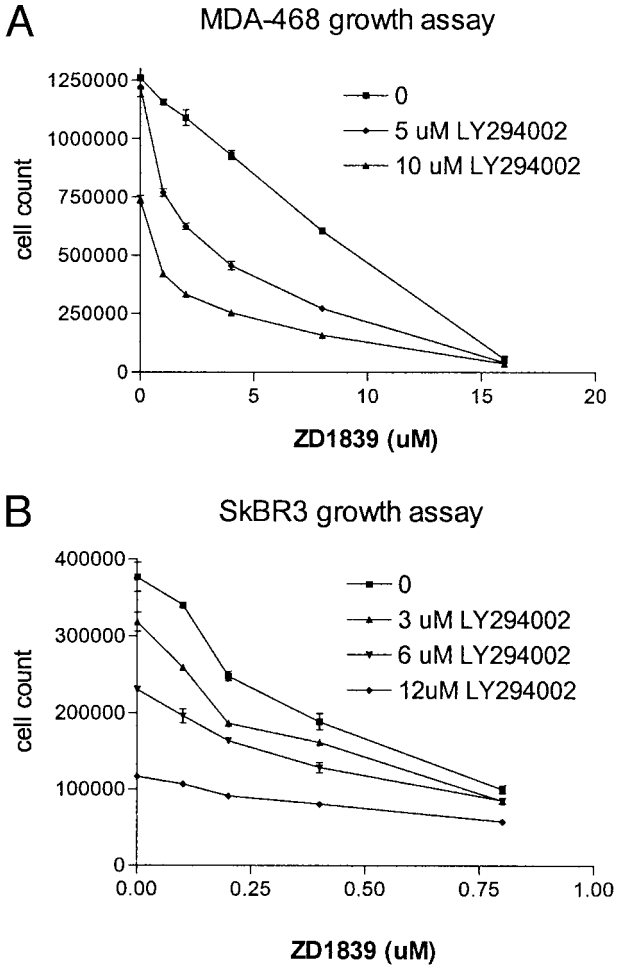

Fig. 4 In *A*, MDA-468 cells were seeded in six-well clusters and the following day placed in various concentrations of ZD1839 and 0, 5, or 10 μM LY294002. Cells were grown for 6 days and counted. Results are the average of duplicates. In *B*, the same experiment was repeated using SkBr3 cells.

ZD1839 by ~4-fold (Fig. 4*A*). This is not simply attributable to additive growth inhibitory effects of the two drugs, because the addition of 5 μM LY294002 alone has no significant growth inhibitory effects, yet it sensitizes these cells to ZD1839 (IC_{50}s of 2 μM compared with 8 μM).

Treatment of MDA-468 cells with LY294002 also reduces basal Akt activity and reestablishes EGFR-stimulated Akt signaling (Fig. 5). In untreated MDA-468 cells, EGFR autophosphorylation and the consequent stimulation of MAPK signaling are inhibited by ZD1839. However, Akt signaling is constitutively elevated and uncoupled from EGFR stimulation with very little effects from ZD1839 (Fig. 5). LY294002 treatment of MDA-468 cells reduces the constitutive basal activation of Akt, but the EGFR-stimulated activation of Akt is not suppressed; therefore, EGFR-stimulated Akt signaling is reestablished, and this signaling is inhibited by ZD1839. The 50% reduction in Akt activity with 1 μM ZD1839 correlates with doses of ZD1839 that inhibit the growth of LY294022-treated MDA-468 cells. These data demonstrate that treatment of MDA-468 cells with

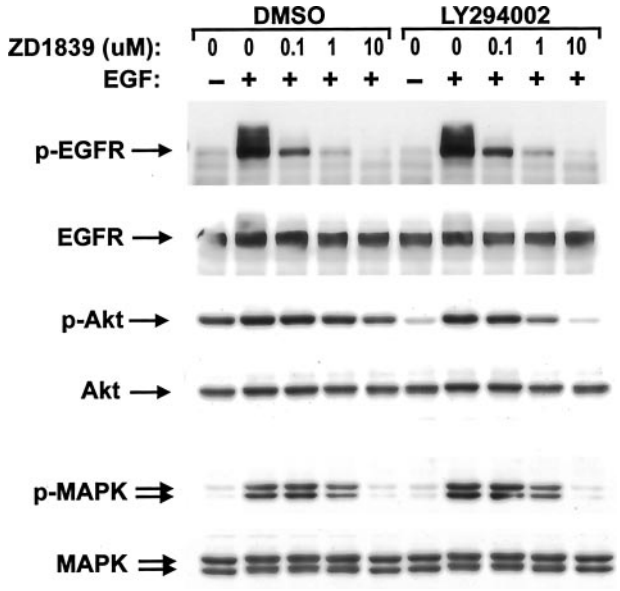

Fig. 5 MDA-468 cells were serum starved overnight and the following day treated with 5 μM LY294002 or DMSO and the indicated concentrations of ZD1839 for 1 h. Cells were harvested rapidly on ice after a 5-min 10 nM EGF stimulus, and cell lysates were separated by SDS-PAGE and immunoblotted using the indicated antibodies.

LY294002 can also reestablish EGFR-stimulated Akt signaling and sensitize these cells to ZD1839.

To determine whether LY294002 has similar effects on growth factor overexpressing tumor cells, which have intact PTEN function and are already sensitive to ZD1839, we performed ZD1839 growth sensitivity assays on the Her2-overexpressing SkBr3 cells in the presence or absence of LY294002. This analysis shows simply additive growth inhibitory effects, and although SkBr3 cells are relatively sensitive to ZD1839, LY294002 does not further enhance their sensitivity (Fig. 4*B*). The IC_{50} for ZD1839 is ~0.6 μM in the presence or absence of LY294002 in these cells.

Discussion

Overexpression of EGFR and other HER family tyrosine kinase receptors is a hallmark of many types of human tumors, and much attention has focused on developing novel antitumor agents that target this family of oncogenic proteins. We have been studying the mechanisms of activity of the synthetic anilinoquinazilone tyrosine kinase inhibitor gefitinib (ZD1839, 'Iressa'). This agent is a highly specific ATP-competitive inhibitor of EGFR tyrosine kinase *in vitro,* has antitumor activity in preclinical animal models, and has shown evidence of clinical antitumor activity in early phases of clinical trials (10, 11). However, the optimal clinical use of this and similar agents remains to be determined by much additional preclinical studies that seek to determine the cellular basis underlying its antitumor activity. We reported previously that although ZD1839 is highly specific for EGFR (HER1) *in vitro,* it leads to dephosphorylation of EGFR, HER2, and HER3 *in vivo* (7). In an analysis of a panel of tumor cell lines, we found that HER-overexpressing tumor cells were particularly sensitive to this agent, including both EGFR- and HER2-overexpressing tumors (7). In these receptors overexpressing tumor cells, the dephosphorylation of the TK receptors is accompanied by loss of association of HER3 with PI3K and down-regulation of Akt. The activation of Akt by EGFR is known to be mediated through recruitment of the p85 subunit of PI3K, predominantly to phosphorylated tyrosine residues on HER3, leading to phosphorylation of membrane inositides and recruitment and phosphorylation of Akt. Our observations have been confirmed by additional studies (12–14). In our analysis, we found down-regulation of Akt in ZD1839 sensitive but not resistant cells. This suggests the hypothesis that inhibition of Akt signaling mediates the antitumor effects of this agent. In the current study, we sought to prove this hypothesis.

To study the role of the PI3K/Akt pathway further, we used MDA468 breast cancer cells as a model. MDA-468 breast cancer cells have overexpression of EGFR, and although ZD1839 effectively inhibits EGFR phosphorylation in these cells, they are relatively resistant to ZD1839. However, these cells also lack PTEN function, have elevated Akt activity, and ZD1839 does not significantly affect Akt signaling in these cells, leading us to believe that their resistance to ZD1839 is attributable to loss of PTEN. In the current study, we reintroduce PTEN function to MDA468 cells through tet-inducible transfection. An inducible model is necessary for this analysis because reintroduction of PTEN into PTEN-null tumor cells, including MDA468 cells, leads to inhibition of growth and apoptotic cell death (9, 15, 16). Therefore, we established a tet-inducible model to optimally focus on the role of PTEN. Our tet-inducible PTEN transfectants have some "leakage" of the tet-repressor and minimal basal expression of PTEN, and although this is below the sensitivity of our Western blotting, it is apparent by RT-PCR and by evidence of Akt down-regulation in all clones assayed. This is not caused by clonal variation because three vector-transfected clones show no such variation in Akt activity (data not shown). The induction of PTEN expression partially inhibits cell growth with a lag time of 5–6 days, which identifies a window of time during which we were able to study ZD1839 sensitivity. Here, we find that introduction of PTEN restores sensitivity to ZD1839. The ZD1839 IC_{50} of 0.2 μM in doxycycline-induced MDA468TR-PTEN cells is similar to the ZD1839 IC_{50}s of EGFR-overexpressing A431 cells or HER2-overexpressing SkBr3 cells (7) and is 10–20-fold lower than uninduced MDA468TR-PTEN cells or vector controls. This confirms that the resistance of MDA468 cell growth to ZD1839 is caused by loss of PTEN.

Reintroduction of PTEN in MDA-468 cells restores ZD1839 growth sensitivity, and this is accompanied by reestablishment of EGFR-stimulated Akt signaling. Although EGFR stimulation is known to activate PI3K, the pathway downstream of PI3K is constitutively activated in MDA-468 cells because of loss of PTEN function and overactive phosphoinositide signaling, and EGF stimulation induces very little increase in Akt activity in these cells, leaving the Akt pathway insensitive to extracellular stimuli (see Fig. 2). However, reconstitution of PTEN function decreases basal Akt activity without a concomitant reduction in EGFR-stimulated Akt activity, thus resensitizing the Akt pathway to extracellular stimuli, which in the case of HER family signaling, is sensitive to ZD1839. The doses of

ZD1839 that inhibit EGFR-stimulated Akt activity in PTEN transfectants correlate well with the doses that cause growth inhibition in these same cells, further supporting that this signaling activity is physiologically relevant. The role of basal Akt activity is less clear from this experimental model. Even minimal induction of PTEN expression in MDA-468 cells leads to near complete inactivation of basal Akt activity with only minimally appreciable further reductions affected by ZD1839 treatment, and these reductions do not correlate with inhibition of cell growth (data not shown). Although the study of basal Akt activity is revealing in some cell lines, its inactivation in PTEN-induced MDA-468 cells makes it difficult to study in the basal state, and considering the apoptotic consequences of sustained PTEN expression in these cells, this low basal Akt activity may not reflect a state of cellular homeostasis. Therefore, a direct comparison of basal Akt activity between this cell model and homeostatic cell lines with intact PTEN function cannot be made. However, we find that inhibition of EGFR-stimulated Akt signaling correlates well with inhibition of growth in this PTEN-inducible cell model.

The transfection experiments shown here confirm the essential role of PTEN function in mediating the antitumor effects of ZD1839. Although this may help identify patients with ZD1839-resistant tumors, it has limited therapeutic implications, because restoring PTEN function in patients' tumors is currently impractical. However, because overactivity of the Akt pathway in PTEN-null tumors is principally caused by unopposed PI3K activity, pharmacologic inhibition of the PI3K/Akt pathway may mimic many of the effects of PTEN restoration. Indeed, we show here that treatment of MDA-468 PTEN-null tumor cells with an inhibitor of PI3K similarly restores EGFR-stimulated Akt signaling and resensitizes these cells to ZD1839. This suggests that the disease in patients with PTEN-null, HER-overexpressing tumors may also be effectively sensitized to ZD1839 by concomitant treatment with inhibitors of PI3K. This strategy is probably not applicable to all HER-overexpressing tumors because we found no synergistic enhancement with the LY294002 and ZD1839 combination in SkBr3 cells, which have intact PTEN function. This is likely because EGFR-stimulated Akt signaling is intact in these cells and effectively inhibited by ZD1839.

These data demonstrate the critical role of the PI3K/Akt pathway in the antitumor effects of ZD1839. However, they do not show that inhibition of this pathway is sufficient for ZD1839 sensitivity. Numerous pathways are known to be engaged by activated HER proteins, including the Ras/Raf/MEK/MAPK pathway, Jak/Stat signaling pathway, the PKC pathway, the PLCγ pathway, and other cytosolic pathways, and the additional role of these pathways in mediating the effects of ZD1839 remain to be worked out. ZD1839 inhibits MAPK signaling in most tumor cell lines that we have studied, including both sensitive and resistant cell types, and clearly, MAPK down-regulation is not sufficient to mediate response to ZD1839. It remains possible that inhibition of MAPK signaling is necessary, in addition to inhibition of Akt signaling, for sensitivity to ZD1839. We think this is unlikely because we see no difference in sensitivity to MEK inhibitors in MDA-468TR-PTEN cells in the presence and absence of PTEN induction, and we see no effect of MEK inhibitors on PI3K/Akt signaling and no effects of PTEN expression on MAPK signaling in MDA-468 cells (data not shown). A more complicated interdependency among these two pathways remains possible. In addition, the role of other signaling pathways cannot be dismissed. It is possible that signaling through other known or unknown EGFR-dependent pathways is also disrupted by constitutive PI3K activity, and such pathways may also be important in mediating the growth inhibitory effects of ZD1839. Additional studies are needed to determine the individual roles of the different signaling pathways.

This work has relevant clinical implications. It identifies PTEN loss as a marker of ZD1839 resistance and suggests that patients with PTEN-null tumors are unlikely to respond to this treatment. In addition, it also suggests that inhibitors of PI3K or Akt signaling may be effective agents in this setting and if given in combination with ZD1839 may reverse ZD1839 resistance in PTEN-null HER protein driven tumors. Validation of these hypotheses requires additional clinical correlative studies and awaits the development of clinically effective inhibitors of PI3K or Akt signaling.

References

1. Tzahar, E., Waterman, H., Chen, X., Levkowitz, G., Karunagaran, 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.

2. Bogdan, S., and Klambt, C. Epidermal growth factor receptor signaling. Curr. Biol., *11:* R292–R295, 2001.

3. Muller, W. J., Sinn, E., Pattengale, P. K., Wallace, R., and Leder, P. Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene. Cell, *54:* 105–115, 1988.

4. Matsui, Y., Halter, S. A., Holt, J. T., Hogan, B. L., and Coffey, R. J. Development of mammary hyperplasia and neoplasia in MMTV-TGF alpha transgenic mice. Cell, *61:* 1147–1155, 1990.

5. Wilks, A. F. Protein tyrosine kinase growth factor receptors and their ligands in development, differentiation, and cancer. Adv. Cancer Res., *60:* 43–73, 1993.

6. 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., and Press, M. F. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science (Wash. DC), *244:* 707–712, 1989.

7. 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.

8. Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S. I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., Bigner, S. H., Giovanella, B. C., Ittmann, M., Tycko, B., Hibshoosh, H., Wigler, M. H., and Parsons, R. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science (Wash. DC), *275:* 1943–1947, 1997.

9. Lu, Y., Lin, Y. Z., LaPushin, R., Cuevas, B., Fang, X., Yu, S. X., Davies, M. A., Khan, H., Furui, T., Mao, M., Zinner, R., Hung, M. C., Steck, P., Siminovitch, K., and Mills, G. B. The PTEN/MMAC1/TEP tumor suppressor gene decreases cell growth and induces apoptosis and anoikis in breast cancer cells. Oncogene, *18:* 7034–7045, 1999.

10. Kris, M. G., Ranson, M., Ferry, D., Hammond, L., Averbuch, S., Ochs, J., and Rowinsky, E. Phase I study of oral ZD1839, a novel inhibitor of epidermal growth factor receptor tyrosine kinase (EGFR-TK): evidence of good tolerability and activity. Clin. Cancer Res., *5:* 3749s, 1999.

11. Ranson, M., Hammond, L. A., Ferry, D., Kris, M., Tullo, A., Murray, P. I., Miller, V., Averbuch, S., Ochs, J., Morris, C., Feyereislova, A., Swaisland, H., and Rowinsky, E. K. ZD1839, a selective oral epidermal growth factor receptor-tyrosine kinase inhibitor, is well tolerated and active in patients with solid, malignant tumors: results of a phase I trial. J. Clin. Oncol., *20:* 2240–2250, 2002.

12. Moulder, S. L., Yakes, F. M., Muthuswamy, S. K., Bianco, R., Simpson, J. F., and Arteaga, C. L. Epidermal growth factor receptor (HER1) tyrosine kinase inhibitor ZD1839 (Iressa) inhibits HER2/neu (erbB2)-overexpressing breast cancer cells *in vitro* and *in vivo*. Cancer Res., *61:* 8887–8895, 2001.

13. Normanno, N., Campiglio, M., De, L. 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.

14. Anderson, N. G., Ahmad, T., Chan, K., Dobson, R., and Bundred, N. J. ZD1839 (Iressa), a novel epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, potently inhibits the growth of EGFR-positive cancer cell lines with or without erbB2 overexpression. Int. J. Cancer, *94:* 774–782, 2001.

15. Weng, L. P., Smith, W. M., Dahia, P. L., Ziebold, U., Gil, E., Lees, J. A., and Eng, C. PTEN suppresses breast cancer cell growth by phosphatase activity-dependent G1 arrest followed by cell death. Cancer Res., *59:* 5808–5814, 1999.

16. Li, D. M., and Sun, H. PTEN/MMAC1/TEP1 suppresses the tumorigenicity and induces G1 cell cycle arrest in human glioblastoma cells. Proc. Natl. Acad. Sci. USA, *95:* 15406–15411, 1998.

Clinical Cancer Research

Resistance to Gefitinib in PTEN-Null HER-Overexpressing Tumor Cells Can Be Overcome through Restoration of PTEN Function or Pharmacologic Modulation of Constitutive Phosphatidylinositol 3′-Kinase/Akt Pathway Signaling

Qing-Bai She, David Solit, Andrea Basso, et al.

Clin Cancer Res 2003;9:4340-4346.