Resistance to Small Molecule Inhibitors of Epidermal Growth Factor Receptor in Malignant Gliomas[1]

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ABSTRACT

Epidermal growth factor (EGF) receptor (EGFR) is commonly amplified and/or mutated in high-grade gliomas. Abnormal signaling from this receptor tyrosine kinase is believed to contribute to the malignant phenotypes seen in these tumors. Highly specific small molecule inhibitors of this receptor tyrosine kinase have been developed and may potentially improve the treatment of these highly aggressive brain tumors. A glioma cell line overexpressing EGFR was developed to mimic the situation of a malignant glioma with amplified EGFR, and this line was used to characterize the response to specific EGFR inhibitors. Treatment of our *in vitro* glioma model with the EGFR kinase inhibitors ZD1839 (Iressa) or PD153035, synthetic anilinoquinazolines with high specificity for EGFR, resulted in significant suppression of EGFR autophosphorylation even with very low levels of drug. However, significantly higher levels of drug were required to fully inhibit signaling through the phosphatidylinositol 3′-kinase/AKT and mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (ERK) pathways. Interestingly, not all downstream signaling pathways displayed this resistance to inhibition. EGF-dependent activation of signal transducers and activators of transcription-3 occurred at low doses of EGFR inhibitors. The uncoupling of EGFR autophosphorylation and signaling through AKT and ERK was not dependent on EGFR overexpression. In addition, although this response was seen in other glioma and the SK-BR3 breast cancer cell lines, it was not universally present. The SQ20B head and neck squamous carcinoma cell line demonstrated loss of EGF-dependent AKT and ERK activation even at low doses of inhibitor. Despite significant loss of EGF-dependent autophosphorylation, the inability of low levels of EGFR inhibitor to suppress some downstream signaling pathways in our model glioma cell line permitted continued EGF-responsive decreases in the expression of the cyclin-dependent kinase inhibitor p27KIP and EGF-dependent proliferation/cell cycle progression. Although the mechanism responsible for the differential sensitivity of the various signal transduction pathways to EGFR inhibitors remains unclear, signaling through erbB2 does not appear to be involved. The ability of certain tumor cells to maintain signaling through AKT and ERK under EGFR inhibition may represent a potential mechanism of resistance by which a tumor cell may escape the antiproliferative activity of this new class of drugs.

INTRODUCTION

Astrocytic tumors are the most common primary brain tumor in adults. Despite the wide range of treatments, including surgery, radiotherapy, and chemotherapy, for high-grade astrocytomas or gliomas, the majority of patients will eventually fail therapy (1, 2). The outcome is even worse for patients with GBMs,[3] the highest grade gliomas, with which long-term survivors are exceedingly rare (3). Because of disappointing results with conventional therapies, newer approaches are needed to significantly improve patient outcome in this aggressive disease.

One of the most prominent abnormalities seen in malignant gliomas involves amplification/mutation of the EGFR (or erbB1). EGFR is a prototypical RTK. It is a M_r 170,000 protein that consists of an extracellular ligand-binding domain, a transmembrane domain, an intracellular tyrosine kinase domain, and a COOH-terminal regulatory domain containing sites of autophosphorylation. Upon binding of its ligand, EGFR will undergo homo- and/or heterodimerization with other members of this receptor family including erbB2, erbB3, and erbB4, resulting in addition of phosphate moieties to specific tyrosines, which can serve as docking sites for downstream effectors (reviewed in Ref. 4). Approximately 50% of GBMs display EGFR gene amplification, and a significant proportion of nonamplified GBMs overexpress this RTK (5). About 40% of tumors with amplified EGFR express a variant receptor termed EGFRvIII with loss of exon 2–7 leading to a large deletion in the extracellular ligand-binding domain and ligand-independent kinase activity (6, 7). Various mouse models have demonstrated that abnormal EGFR signaling can result in transformation of astrocytes and formation of gliomas (8–10). In addition, abnormal EGFR signaling can alter the malignant phenotype of cell lines derived from human malignant gliomas. Overexpression of EGFRvIII in the U87 glioma cell line results in increased tumorigenic potential and increased resistance to chemotherapeutic agents (11, 12). In addition, inhibition of EGFR signaling through expression of the erbB2 ectodomain conferred greater radiosensitivity to the U87 glioma cell line, further supporting a role for EGFR signaling on a potentially important glioma phenotype (13).

Given the frequency at which abnormalities in EGFR signaling are present in malignant gliomas and the demonstrated roles of these changes in the transformation of astrocytes and the production of phenotypes associated with increased malignancy of gliomas, this receptor is an attractive target for therapeutic manipulation. Specific inhibitors of this receptor system have recently been developed. One of the first agents reported to have a highly specific and potent activity for inhibiting the tyrosine kinase activity of EGFR was PD153035 (14). This agent, a synthetic anilinoquinazoline, was found to be extremely effective at reversibly binding the ATP-binding pocket in the kinase domain of EGFR. However, poor solubility of this drug has limited its clinical development. Other derivatives of this compound have subsequently been generated that maintain high specificity and potency for EGFR but with a better solubility profile and have been developed for potential clinical use. ZD1839 (Iressa) is an EGFR inhibitor that recently gained Food and Drug Administration approved for non-small cell lung cancer and is also currently being tested in clinical trials for a number of other tumors including malignant gliomas (15). Preclinical studies with ZD1839 as a single agent demonstrate antiproliferative activity *in vitro* and antitumor activity *in vivo* (16). In addition, this agent can potentiate the effects of chemotherapy and radiation therapy (17, 18). Phase I studies indicate that

[3] The abbreviations used are: GBM, glioblastoma multiforme; EGFR, epidermal growth factor receptor; RTK, receptor tyrosine kinase; PI-3K, phosphatidylinositol 3′-kinase; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; IGF-IR, insulin-like growth factor-I receptor; STAT, signal transducers and activators of transcription; MuLV, murine leukemia virus; P-EGFR, phosphorylated EGFR; P-AKT, phosphorylated AKT; P-ERK, phosphorylated ERK; P-STAT3, phosphorylated STAT3; MEK, mitogen-activated protein kinase kinase.

it is well tolerated (19, 20). However, to date, there have been relatively little published preclinical data on ZD1839 for brain tumors (21), in general, and gliomas, specifically.

Because of the promise of these new EGFR-specific therapies and the high incidence of EGFR signaling abnormalities in malignant gliomas, we sought to determine the relative efficacy of these EGFR inhibitors in an *in vitro* glioma model system with overexpression of EGFR. We hypothesized that these specific inhibitors would be very efficient at inhibiting EGFR tyrosine kinase activity and ligand-dependent downstream signaling in our glioma system. However, surprisingly, whereas even low levels of EGFR inhibitors could very efficiently block receptor autophosphorylation, EGF-dependent activation of AKT and ERK was more resistant to these inhibitors, requiring significantly higher levels of drug. This response may represent a novel mechanism by which resistance to these EGFR kinase inhibitors can develop. This paper presents the characterization and potential implications of this phenomenon where receptor autophosphorylation and activation of downstream signaling pathways are uncoupled in response to the quinazoline class of EGFR inhibitors.

MATERIALS AND METHODS

Cell Lines Used. All cell lines [LN229, SF767, and U343 (glioma); SK-BR3 (breast carcinoma); and SQ20B (head and neck squamous carcinoma)] were cultured in high-glucose DMEM (Mediatech, Herndon, VA) containing 10% FCS (Gibco, Invitrogen Corp., Carlsbad, CA) and penicillin (100 units/ml)/streptomycin (100 μg/ml) unless otherwise indicated. The MuLV-based expression vector pBABE/puro containing wild-type EGFR was used to overexpress this RTK in the LN229 glioma cell line. Briefly, pantropic, replication-defective viral particles for the vector alone and vector containing EGFR were generated after transient transfection into 293T cells along with expression vectors for MuLV *gag* and *pol* genes as well as the vesicular stomatitis virus G glycoprotein gene (22). These defective retroviral vectors were used to infect LN229. After selection in puromycin (1 μg/ml), drug-resistant cell pools were generated that either contain the vector alone (LN229/puro) or overexpress EGFR (LN229/EGFR; Fig. 1).

Growth Factor and Inhibitor Treatments. For the experiment with growth factor treatment, cells were starved (no or 0.5% FCS, as indicated) before exposure to EGF (100 ng/ml; Discovery Labware; BD Biosciences,

Bedford, MA). Cells were then harvested at the indicated times after treatment with EGF. Stock solutions of ZD1839 (10 mM; Astra Zeneca) and PD153035 (5 mM; Tocris Cookson, Ellisville, MO) in DMSO were used at the indicated final concentrations in media. DMSO was added at the same concentration in the corresponding no inhibitor controls. For experiments using these EGFR inhibitors, drug was added 2 h before addition of EGF. Herceptin (Genentech, South San Francisco, CA) was obtained from the pharmacy at Hospital of the University of Pennsylvania and reconstituted as recommend by the manufacturer. LN229/EGFR was treated with Herceptin at a concentration 20 μg/ml for 36 h with medium containing no serum (0.5% BSA was used as a carrier) before harvest or additional treatment with EGFR inhibitors and EGF.

Immunoblot Analysis. All immunoblots were done according to standard procedures. Appropriate cells were lysed in radioimmunoprecipitation assay buffer [50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton, 0.1% SDS, and 0.5% deoxycholate] containing a mixture of protease and phosphatase inhibitors. Total protein concentration was determined by using the Coomassie Plus Protein Assay reagent (Pierce, Rockford, IL), and equal amounts of protein were resolved by SDS-PAGE and electrotransferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). Blots were probed with antibodies against EGFR (sc-03; Santa Cruz Biotechnology, Santa Cruz, CA), P-EGFR (P-Tyr1173; Upstate Biotechnology, Waltham, MA), AKT/P-AKT (P-Ser473; Cell Signaling Technology, Beverly, MA), ERK/P-ERK (Sigma, St. Louis, MO), STAT3/P-STAT3 (Cell Signaling Technology), erbB2/phosphorylated erbB2 (P-Tyr1048; Cell Signaling Technology), and p27KIP (Santa Cruz Biotechnology) according to the manufacturer's recommendations. Where appropriate, blots were also probed with antibody against actin (Santa Cruz Biotechnology) as a loading control. Horseradish peroxidase-conjugated secondary antibodies and the SuperSignal West Dura Extended Duration Substrate (Pierce) were used for chemiluminescent detection. After application of chemiluminescent substrate, blots were exposed to standard X-ray film for appropriate lengths of time.

Cell Cycle Analysis. To assess proliferative response to EGF, cell cycle profiles were determined with or without the addition of EGF at varying concentrations of PD153035 and ZD1839 in the presence or absence of Herceptin. The LN229/EGFR cell line was seeded at a density of 5×10^5 cells in a 10-cm Petri dish and allowed to attach overnight (approximately 12–16 h). Cells were then starved in 0.5% serum for 24 h before treatment with PD153035 or ZD1839 (levels as indicated). For the Herceptin experiment, cells were treated with Herceptin for 36 h before addition of inhibitor. Two h after addition of inhibitor, cells were treated ±EGF (100 ng/ml) in medium containing 0.5% FCS. After confirmation that the cultures were subconfluent, cells were harvested 24 h after addition of EGF. Treated cells were harvested, fixed, and stained with propidium iodide in preparation for analysis on a flow cytometer (FACScan; Immunocytometry Systems, BD Biosciences, San Jose, CA). Briefly, after trypsinization, cells were washed in PBS, fixed with ice-cold 70% ethanol for >1 h, and washed again in PBS, and then DNA was quantitatively stained with PBS containing 5 μg/ml propidium iodide and 50 μg/ml RNase A for 1 h. Data analysis was performed using the ModFitLT 3.0 software (Immunocytometry Systems, BD Biosciences) for modeling cell cycle distribution.
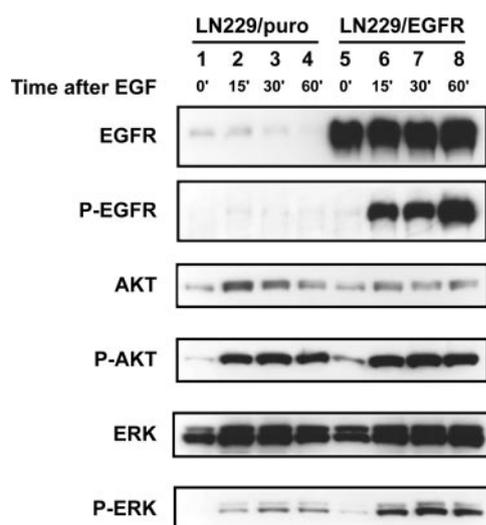
RESULTS

Development of a Model Glioma Cell Line with Overexpression of EGFR. We replicated the amplification of EGFR commonly seen in malignant gliomas in an *in vitro* model system by overexpressing EGFR in an established glioma cell line to better study the effects of overexpression and inhibition of this RTK. The LN229/EGFR glioma cell line was generated by introducing the cDNA for full-length EGFR into the parental LN229 line via a MuLV-based retroviral expression vector. Immunoblot analysis shows that LN229/EGFR cells overexpress EGFR in comparison with the vector-infected controls (LN229/puro; Fig. 1, *Lanes 1* and *5*). This receptor was functional and responded as expected to the addition of exogenous EGF with receptor autophosphorylation and activation of downstream signals including the PI-3K/AKT and MEK/ERK pathways (Fig. 1).

Fig. 1. Establishment of a glioma cell line with overexpression of EGFR. Immunoblots of lysates from LN229/puro (*Lanes 1–4*) and LN229/EGFR (*Lanes 5–8*) cells resolved by SDS-PAGE are shown. Cells were treated as follows before harvesting: no EGF (*Lanes 1* and *5*); EGF for 15 min (*Lanes 2* and *6*); EGF for 30 min (*Lanes 3* and *7*); and EGF for 60 min (*Lanes 4* and *8*). Equal amounts of protein were loaded in each lane. Blots were from duplicate gels probed with antibodies against EGFR, P-EGFR, AKT, P-AKT, ERK, and P-ERK.
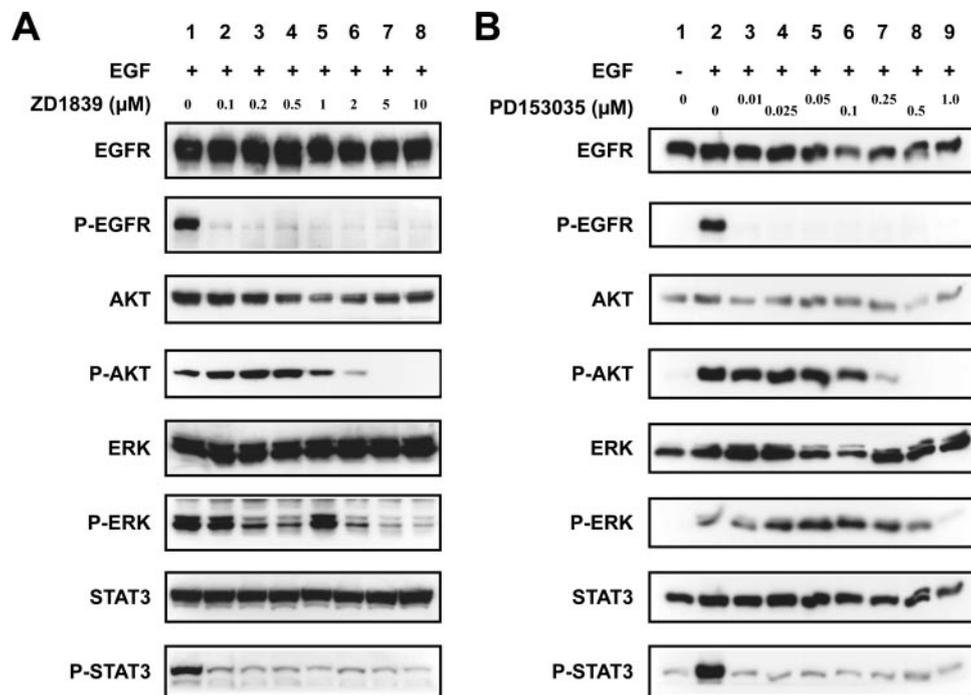
Fig. 2. Response of EGF-stimulated LN229/EGFR cells to EGFR inhibitors. Immunoblots of lysates from LN229/EGFR cells are shown. Cells were harvested 15 min after addition of EGF (100 ng/ml). Cells were treated with increasing levels of ZD1839 (A) or PD153035 (B), as indicated, 2 h before EGF addition. Equal amounts of protein were loaded in each lane. Blots were from duplicate gels probed with antibodies against EGFR, P-EGFR, AKT, P-AKT, ERK, P-ERK, STAT3, and P-STAT3.

Effect of EGFR Inhibitors on Autophosphorylation and Signaling through AKT and ERK in LN229/EGFR Cells. To determine the effectiveness of small molecule inhibitors of EGFR in our glioma model system, ZD1839 was used to treat the LN299/EGFR cell line. Initially, LN299/EGFR was serum-starved for 24 h to reduce the baseline levels of signaling through downstream signal transduction pathways. The cells were then treated for 2 h with increasing concentrations of ZD1839 before addition of EGF (100 ng/ml). Immunoblot analyses of cells harvested 15 min after addition of ligand revealed that ZD1839 was very effective at inhibiting autophosphorylation of EGFR even with relatively low concentrations of drug (as low as 0.1 μM; Fig. 2A). However, higher levels of ZD1839 (on the order of 2–5 μM) were required before significant inhibition of AKT and ERK phosphorylation was achieved (Fig. 2A). Although an isolated increase in phosphorylation of ERK was noted in these cells treated with 1 μM ZD1839 (Fig. 2A, Lane 5), this anomalous result was not consistently seen on repeat experiments. The precise reason for the increased ERK phosphorylation remains elusive, but clearly, cells treated with intermediate levels of ZD1839 in the 0.2–0.5 μM range still permitted signaling through ERK compared with cells treated with extremely high levels of drug (Fig. 2A, Lanes 3, 4, and 8). PD153035, another EGFR inhibitor of the anilinoquinazoline class with a structure similar to ZD1839, was also assessed to determine whether this response is compound specific or common to this class of inhibitors. Like the results seen with ZD1839, low concentrations of PD153035 (as low as 0.01 μM) were capable of inhibiting EGFR autophosphorylation, whereas significantly higher levels of inhibitor (up to 0.25–0.50 μM) were required before AKT and ERK phosphorylation was affected (Fig. 2B). Of note, the dose-response curves for ZD1839 and PD153035 are different and qualitatively consistent with the reported *in vitro* IC$_{50}$ values for these compounds (14, 15).

EGF-Dependent STAT3 Signaling Is Suppressed by Low Concentrations of EGFR Inhibitors. Although EGF-dependent autophosphorylation of EGFR is clearly more sensitive than AKT and ERK activation to EGFR kinase inhibitors in the LN229/EGFR cells, we were interested in determining whether other EGF-dependent downstream signals are similarly resistant to this class of drug. The STAT protein family comprises transcriptional activators that can be activated by phosphorylation, which promotes dimerization and nuclear localization in response to cytokines (reviewed in Ref. 23). Growth factors such as EGF have also been shown to activate the STAT proteins (24, 25). In the LN229/EGFR glioma cells, EGF stimulation results not only in activation of PI-3K/AKT and MEK/ERK pathways but also in activation of STAT3 (Fig. 2B, Lanes 1 and 2). To determine the kinetics for inhibition of STAT3 activation by EGFR inhibitors, STAT3 immunoblot analyses of the LN229/EGFR lysates assessed in the previous section were performed. Whereas ligand stimulation results in EGFR autophosphorylation and activation of downstream signaling pathways including PI-3K/AKT, MEK/ERK, and STAT3 (Fig. 2B, Lanes 1 and 2), phosphorylation/activation of STAT3 was markedly more sensitive to both ZD1839 and PD153035 than phosphorylation/activation of either AKT or ERK (Fig. 2, A and B). In fact, loss of EGF-dependent phosphorylation/activation of STAT3 in response to the EGFR inhibitors appears to be tightly coupled with the suppression of EGFR autophosphorylation. Therefore, our results show that signaling pathways activated by EGFR are not all similarly affected by the EGFR kinase inhibitors.

Uncoupling of Autophosphorylation and Activation of Downstream Signals in Response to EGFR Inhibitors Does Not Require EGFR Overexpression. Because it remains possible that the observed differential effect of the EGFR inhibitors on autophosphorylation and signaling requires high levels of receptor, we sought to determine whether this response was still present in a glioma cell line lacking overexpression of EGFR. Therefore, the LN229/puro glioma cell line, which does not overexpress EGFR, was used to assess response to these kinase inhibitors. The LN229/puro line expresses sufficient levels of EGFR to permit activation of downstream signaling pathways in response to EGF similar to that seen with LN229/EGFR (Fig. 1, Lanes 1–4). When LN229/puro was treated with increasing concentrations of PD153035 before stimulation with EGF ligand, AKT and ERK phosphorylation were not suppressed at a lower concentration of drug that proved sufficient to inhibit EGF-dependent autophosphorylation on the LN229/EGFR cell line (Fig. 3). A similar result was also obtained with ZD1839 (data not shown).
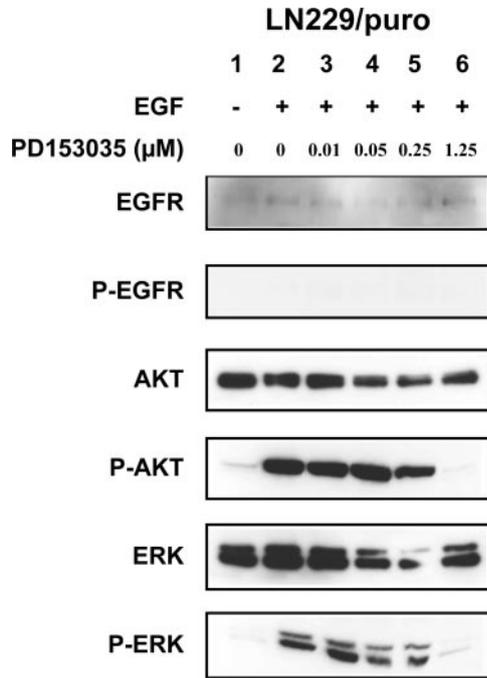
Fig. 3. Response of EGF-stimulated LN229/puro cells to an EGFR inhibitor. Immunoblots of lysates from LN229/puro cells are shown. Cells were harvested 15 min after addition of EGF (100 ng/ml). Cells were treated with increasing levels of PD153035, as indicated, 2 h before EGF addition. Equal amounts of protein were loaded in each lane. Blots were from duplicate gels probed with antibodies against EGFR, P-EGFR, AKT, P-AKT, ERK, and P-ERK.

Effect of EGFR Kinase Inhibitors on Multiple Glioma and Other Tumor Cell Lines. To determine whether this differential effect of the EGFR inhibitors on autophosphorylation and signaling is only seen in the LN229 glioma cell line or whether it is seen more generally, other glioma cell lines including SF767, SF763, U343, and U251 were similarly assayed. These glioma cell lines were treated with the inhibitor PD153035 before stimulation with EGF. In each case, a general pattern was seen where loss of receptor autophosphorylation occurred at a lower concentration of PD153035 than loss of downstream signaling. Two representative glioma cell lines (SF763

and U343) are displayed (Fig. 4, *A* and *B*). The U343 glioma cell line is mutant for PTEN, accounting for the high basal amounts of activated AKT and very little additional increase in the levels of P-AKT after EGF addition (Fig. 4*B*). However, the uncoupling of the loss of receptor autophosphorylation and ERK signaling in response to PD153035 is still clearly present (Fig. 4*B*). To determine whether this phenomenon is seen in other tumor types, the SK-BR3 breast and SQ20B head and neck carcinoma cell lines were also assessed. In these cell lines, expression of EGFR and the ability of EGF to stimulate receptor autophosphorylation and downstream signaling were confirmed (data not shown). When these tumor cell lines were treated with PD153035 and stimulated with ligand, differential inhibition of autophosphorylation and signaling was found in the SK-BR3 breast cancer cells (Fig. 5*A*), whereas downstream signaling was less resistant to inhibitor in the SQ20B head and neck cancer cells, as evidenced by a tighter coupling of inhibition of EGFR autophosphorylation and signal transduction pathways (Fig. 5*B*).

EGF-Induced Proliferation and Inhibition of p27KIP Are Maintained Despite Treatment with Low Levels of EGFR Kinase Inhibitor in Our Glioma Model System. As shown previously, a low level of EGFR inhibitors in LN229/EGFR was sufficient to inhibit both autophosphorylation of EGFR and activation of STAT3 but had no effect on activation of PI-3K/AKT and MEK/ERK (Fig. 2). Because some but not all signaling pathways activated by EGFR are resistant to these low levels of EGFR inhibitor, we tested whether this level of PD153035 and ZD1839 could still inhibit EGF-dependent cell cycle progression. We first determined the impact of this level of PD153035 on EGF-induced proliferation in the LN229/EGFR glioma cell line. The importance of the PI-3K/AKT and MEK/ERK pathways on EGF-induced proliferation is well established (reviewed in Ref. 26). Therefore, we hypothesized that despite significant inhibition of EGFR autophosphorylation by 0.05 μM PD153035, EGF should still continue to induce proliferation and cell cycle progression in the LN229/EGFR cells in the presence of these inhibitor levels. LN229/EGFR cells were serum-starved for 24 h, inducing a quiescent state. The quiescent LN229/EGFR cells were then treated with DMSO alone or with 0.05 or 2 μM PD153035 for 2 h before incubation with or without EGF (100 ng/ml). Cells were harvested 24 h later, and cell cycle profile was determined by measuring DNA content on a flow
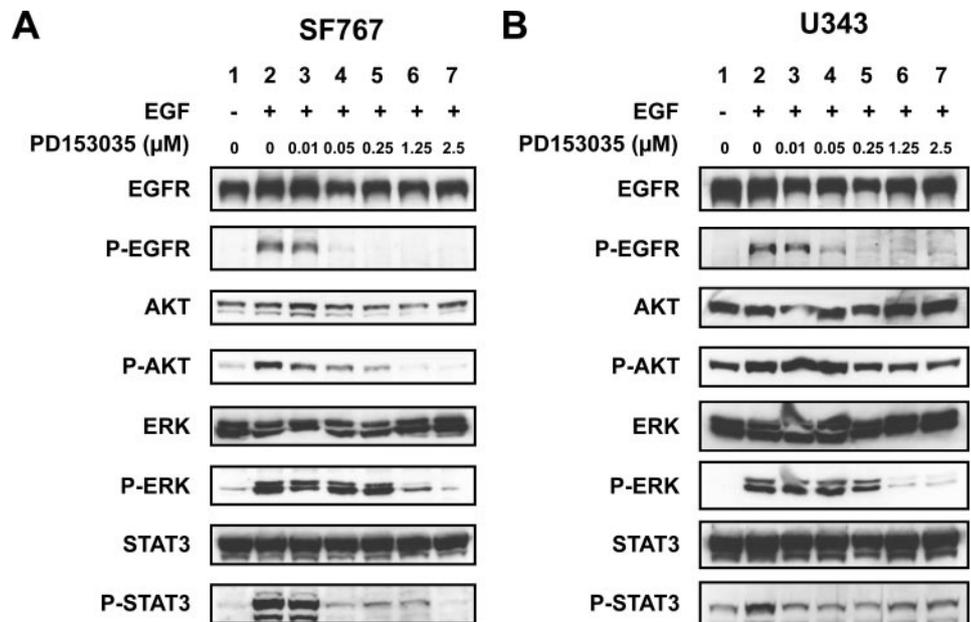

Fig. 4. Response of other glioma cell lines to an EGFR inhibitor. Immunoblots of lysates from SF767 (*A*) or U343 (*B*) glioma cell lines are shown. Cells were harvested 15 min after addition of EGF (100 ng/ml). Cells were treated with increasing levels of PD153035, as indicated, 2 h before EGF addition. Equal amounts of protein were loaded in each lane. Blots were first probed with antibodies against P-EGFR, P-AKT, P-ERK, and P-STAT3. The blot was then stripped by standard procedures and reprobed with antibodies against EGFR, AKT, ERK, and STAT3.
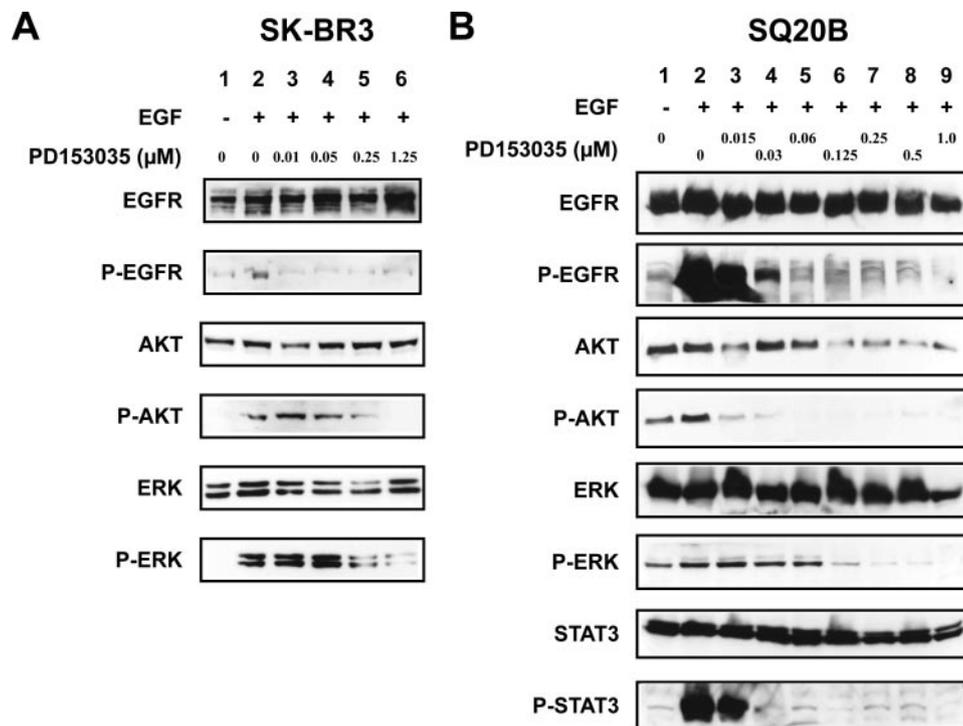
Fig. 5. Response of other tumor cell lines to an EGFR inhibitor. Immunoblots of lysates from SK-BR3 breast carcinoma (*A*) or SQ20B head and neck squamous carcinoma (*B*) cell lines are shown. Cells were harvested 15 min after addition of EGF (100 ng/ml). Cells were treated with increasing levels of PD153035, as indicated, 2 h before EGF addition. Equal amounts of protein were loaded in each lane. Blots were from duplicate gels probed with antibodies against EGFR, P-EGFR, AKT, P-AKT, ERK, P-ERK, STAT3, and P-STAT3, as indicated.

cytometer. Untreated cells entered the cell cycle and underwent S-phase progression in response to EGF, whereas cells treated with 2 μM PD153035 maintained their quiescent state with no significant S-phase entry after EGF treatment (Fig. 6, *A* and *B*). However, when low levels of EGFR inhibitor (0.05 μM PD153035) were assessed, EGF continued to stimulate cell cycle progression and, in fact, induced a higher percentage of cells to enter S phase in comparison with the untreated controls (Fig. 6, *A* and *B*). Similarly, EGF-dependent proliferation was maintained with a low level (0.2 μM) of ZD1839, whereas significant inhibition was accomplished with a high level (20 μM) of ZD1839 (Fig. 6*C*). The low levels of ZD1839 (0.2 μM) and PD153035 (0.05 μM) used in the proliferation experiments are clearly affecting EGFR kinase function because EGF-induced receptor autophosphorylation is markedly suppressed (Fig. 2*A, Lane 3*; Fig. 2*B, Lane 5*). Correlating with the inability of low levels of PD153035 to inhibit EGF-dependent proliferation, the cyclin-dependent kinase inhibitor p27KIP was unaffected by 0.06 μM PD153035 (Fig. 7, *Lanes 4–6*). However, high levels of PD153035 (2 μM) did prevent an EGF-dependent decrease in the levels of p27KIP (Fig. 7, *Lanes 7–9*).

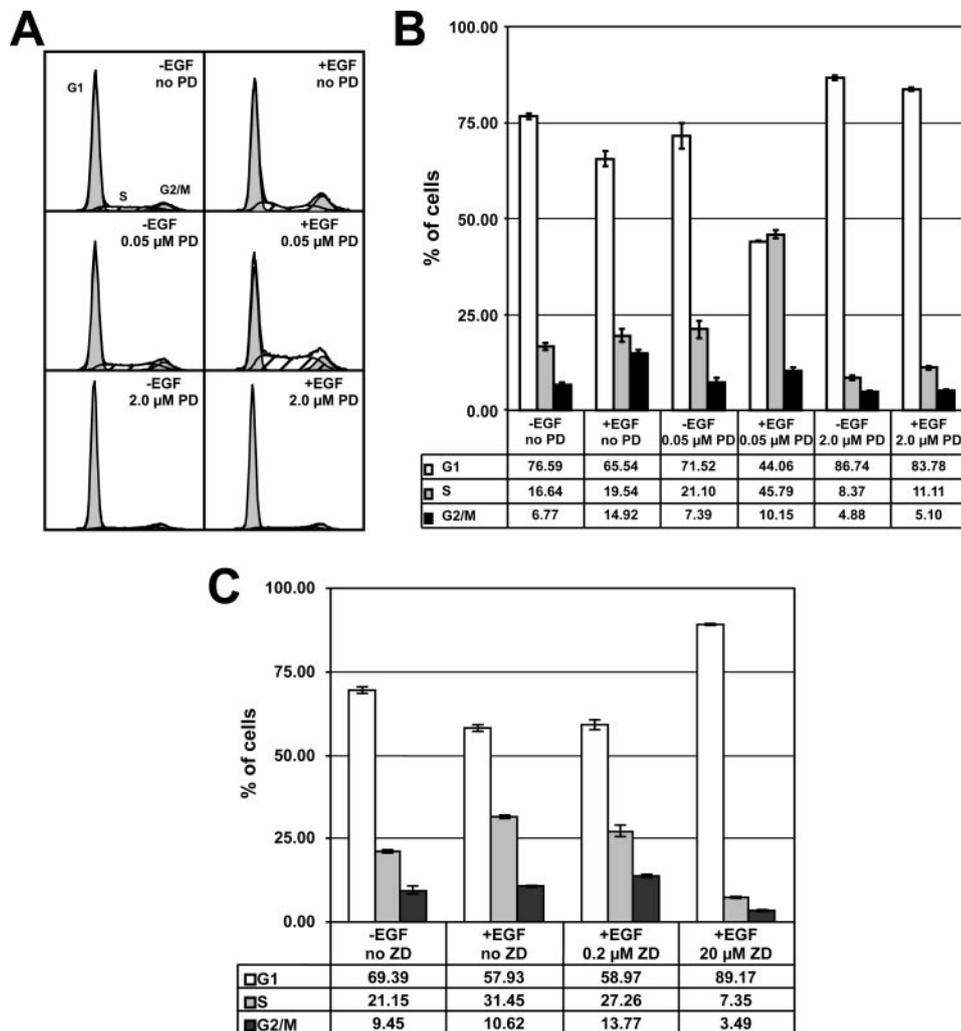**Resistance of EGF-Dependent Signaling and Proliferation to EGFR Kinase Inhibitors Does Not Depend on erbB2 Function.** Although homodimerization of EGFR can result from ligand stimulation, the preferred dimerization partner of EGFR is believed to be erbB2 (27, 28). Because kinase inhibitors such as ZD1839 and PD153035 are highly specific for EGFR and require very high levels before they will also inhibit other EGFR family members such as erbB2, it was conceivable that levels of EGFR inhibitors sufficient to inactivate the EGFR kinase may still allow EGF-dependent signaling through activation of the erbB2 portion of an EGFR-erbB2 heterodimer. Examples of situations similar to this scenario exist both naturally with signaling through erbB2-erbB3 heterodimers and artificially, where a kinase-inactive EGFR mutant is still able to transduce an EGF-dependent signal through erbB2 (29, 30). To assess whether erbB2 is contributing to the resistance of EGF-dependent signaling to the EGFR inhibitors, specific inhibition of erbB2 is required. First, the LN229/EGFR glioma cell line was found to express erbB2 by both

reverse transcription-PCR and immunoblot analyses and could therefore participate in heterodimer formation with EGFR (data not shown). To inhibit erbB2 function, Herceptin, a humanized antibody against erbB2, was used. Treatment with Herceptin (20 μg/ml) for 36 h was sufficient to decrease both the expression of erbB2 (Fig. 8*A, Lanes 1–3*) and EGF-dependent erbB2 autophosphorylation (Fig. 8*A, Lanes 4–6*). Therefore, to determine the role erbB2 plays in this response to EGFR inhibitors, we assessed whether specific inhibition of erbB2 would reduce the resistance of the AKT and ERK pathways to EGFR inhibitors. The LN229/EGFR cells were treated with Herceptin (20 μg/ml) for 36 h under serum-starved conditions before addition of increasing amounts of PD153035 (0.1–0.8 μM) and, finally, EGF. Specific inhibition of erbB2 did not alter the resistance of the AKT and ERK pathways to PD153035 (Fig. 8*B*). Although increasing levels of PD153035 led to a dose-dependent decline in P-AKT and ERK in the absence of Herceptin (Fig. 8*B, Lanes 1–6*), the addition of Herceptin had no additional impact on these signaling pathways (Fig. 8*B, Lanes 7–12*). Similarly, EGF-dependent proliferation is maintained despite erbB2 inhibition with Herceptin when the LN229/EGFR cells are treated with 0.05 μM PD153035 (Fig. 8*C*). Thus, erbB2 does not appear to contribute to the resistance of EGF-dependent signaling pathways and proliferation to the quinazoline class of EGFR kinase inhibitors.

DISCUSSION

In this report, we demonstrate that levels of EGFR inhibitor sufficient to suppress EGFR kinase activity may not be sufficient to inhibit all EGF-dependent signals or EGF-stimulated tumor cell growth. In particular, EGF-dependent activation of AKT and ERK appears to be relatively resistant to EGFR inhibitors, especially in cell lines derived from malignant gliomas. Others have reported that quinazoline-based EGFR inhibitors result in a dose-dependent increase in p27KIP expression in a process that is dependent on decreased PI-3K/AKT activation (31). Consistent with these findings, we show that EGF-stimulated AKT signaling that persists despite treatment with EGFR

Fig. 6. EGF-induced proliferation of LN229/EGFR cells treated with varying levels of an EGFR inhibitor. Cell cycle profile of LN229/EGFR glioma cells 24 h after treatment with differing levels of PD153035 or ZD1839 ± EGF (100 ng/ml). Cells were assayed as described in "Materials and Methods." *A*, representative cell cycle profiles are presented for each level of PD153035 ± EGF addition, as indicated on the *top right* of each panel. G$_1$ (*solid gray*), S (*hatched*), and G$_2$-M (*solid gray*) regions are labeled in the *top left panel*. Cell cycle distribution was determined with curve fitting using the ModFitLT 3.0 software. *B*, graph shows the result of three independent plates of cells treated with varying levels of PD153035 ± EGF addition. The mean values for each phase of the cell cycle are shown on the graph, with the number presented on the table below the graph. *Error bars* represent ±1 SD. *C*, graph shows the result of two to three independent plates of cells treated with varying levels of ZD1839 + EGF addition. A −EGF control was also included. The mean values for each phase of the cell cycle are shown on the graph, with the number presented on the table below the graph. *Error bars* represent ±1 SD.

	-EGF no PD	+EGF no PD	-EGF 0.05 µM PD	+EGF 0.05 µM PD	-EGF 2.0 µM PD	+EGF 2.0 µM PD
G1	76.59	65.54	71.52	44.06	86.74	83.78
S	16.64	19.54	21.10	45.79	8.37	11.11
G2/M	6.77	14.92	7.39	10.15	4.88	5.10

	-EGF no ZD	+EGF no ZD	+EGF 0.2 µM ZD	+EGF 20 µM ZD
G1	69.39	57.93	58.97	89.17
S	21.15	31.45	27.26	7.35
G2/M	9.45	10.62	13.77	3.49

inhibitors was correlated with decreased levels of p27KIP and cell cycle progression. Thus, continued signaling through these pathways despite low levels of EGFR inhibitors is likely to be of importance to the biological behavior of these tumors because EGF-dependent proliferation under these conditions is not only maintained but may in fact be somewhat enhanced.

Similar to our result, Bianco *et al.* (32) recently found that ZD1839 will inhibit autophosphorylation of EGFR in the MDA-468 breast
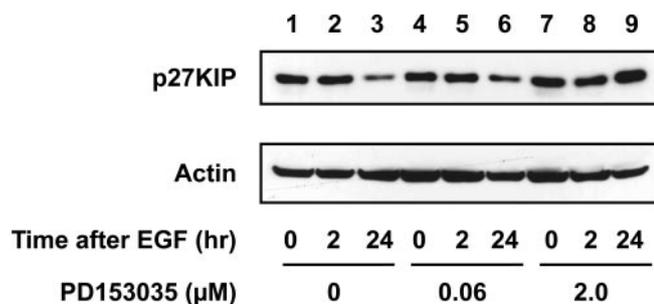

Fig. 7. Suppression of p27KIP expression by EGF despite treatment with an EGFR inhibitor. Immunoblots of lysates from LN229/EGFR glioma cells are shown. Cells were harvested at 0 (*Lanes 1, 4,* and *7*), 2 (*Lanes 2, 5,* and *8*), and 24 h (*Lanes 3, 6,* and *9*) after the addition of EGF (100 ng/ml). Cells were treated with 0 (*Lanes 1–3*), 0.06 (*Lanes 4–6*), and 2.0 µM (*Lanes 7–9*) PD153035, as indicated, 2 h before EGF addition, and drug levels were maintained for the duration of the exposure to EGF. Equal amounts of protein were loaded in each lane. Blot was probed with an antibody against p27KIP. The blot was also probed with actin as a loading control.

cancer cell line at concentrations as low as 0.1 µM, but concentrations greater than 1 µM were required before inhibition of PI-3K/AKT signaling was seen. They show that a mutant PTEN status contributed to the resistance of AKT signaling to ZD1839. However, unlike their results, we find that the MEK/ERK pathway was also resistant to the effects of ZD1839 and PD153035 in our systems. In addition, the LN229 glioma cell line used in our experiments is known to express wild-type PTEN (33) and has a low basal level of P-AKT consistent with the presence of functional PTEN. Thus, mutant PTEN status does not explain the resistance we are seeing in the LN229 glioma cell line.

The precise mechanism by which EGF-dependent signaling is resistant to quinazoline-based EGFR kinase inhibitors in malignant gliomas remains elusive. This resistance is seemingly unaffected by H-*ras* inhibition through the use of a farnesyl transferase inhibitor (data not shown). Thus, it appears unlikely that H-*ras* is contributing significantly to the drug-resistant signaling seen in the glioma cell lines after EGF stimulation. At the level of the receptor, we hypothesize that loss of EGFR kinase function does not preclude signaling through a heterodimer receptor partner in response to ligand stimulation. Precedence for this type of receptor activation and signaling exists in the erbB family, where erbB3 contains an inactive kinase (reviewed in Ref. 34). However, stimulation of erbB3 with its ligand heregulin will result in activation of erbB2 in the context of an erbB3/2 heterodimer (29). Similarly, Deb *et al.* (30) have found that a kinase-inactive EGFR mutant could still transduce a downstream signal after ligand stimulation in the context of an erbB1/2 het-
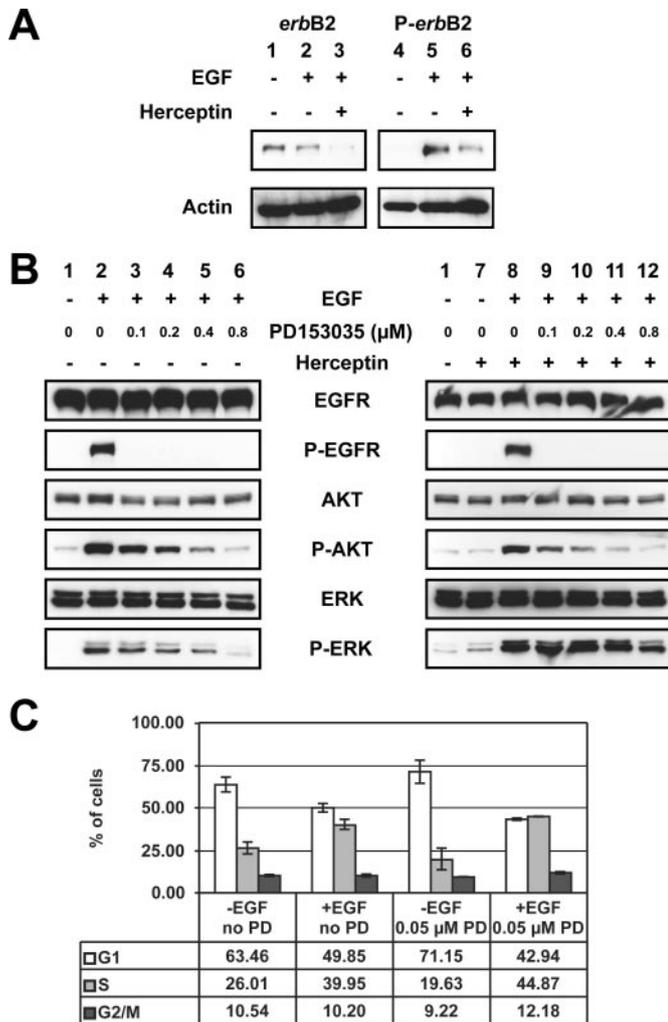
Fig. 8. Lack of erbB2 involvement in the resistance of EGF-dependent AKT and ERK activation and proliferation to an EGFR inhibitor. Immunoblots of lysates from LN229/EGFR glioma cells are shown in *A* and *B*. *A*, cells were serum-starved in media containing 0.5% BSA with (*Lanes 3* and *6*) or without (*Lanes 1, 2, 4,* and *5*) Herceptin (20 μg/ml) for 36 h before treatment with no EGF (*Lanes 1* and *4*) or 100 ng/ml EGF (*Lanes 2, 3, 5,* and *6*) 15 min before harvest. Blots were probed with antibodies against erbB2 (*Lanes 1–3*) and phosphorylated erbB2 (*P-erbB2, Lanes 4–6*). An actin loading control is included for each blot. *B*, cells were serum starved for 36 h without (*Lanes 1–6*) or with (*Lanes 7–12*) Herceptin (20 μg/ml). Control lysate from *Lane 1* is loaded on both gels. Cells were treated with increasing levels of PD153035, as indicated, 2 h before EGF addition. Equal amounts of protein were loaded in each lane. Blots were from duplicate gels probed with antibodies against EGFR, P-EGFR, AKT, P-AKT, ERK, and P-ERK. *C*, graph shows the result of three independent plates of cells treated with low levels of PD153035 (0.05 μM) ± EGF (100 ng/ml) addition in the presence of Herceptin (20 μg/ml). Cells were assayed as described in "Materials and Methods." The mean values for each phase of the cell cycle are shown on the graph, with the number presented on the table below the graph. *Error bars* represent ±1 SD.

erodimer. Based on these observations and the fact that erbB2 has been shown to be the preferred partner for EGFR (27, 28), we initially hypothesized that the mechanism by which EGF-dependent signals are resistant to EGFR inhibitors is attributable to signaling through erbB2. However, despite down-regulation of erbB2 levels and repression of erbB2 phosphorylation by Herceptin, EGF-dependent signaling and proliferation did not become more sensitive to the EGFR inhibitor PD153035 (Fig. 8).

Although erbB2 does not appear to be involved in the drug-resistant signaling to AKT and ERK, the involvement of other potential dimerization partners cannot be excluded. The erbB3 receptor is unlikely to be the critical partner because it has an inactive kinase (35). However, erbB4, the last member of this receptor family, may still be involved. Furthermore, other as-yet-unidentified partners may be responsible for

this effect. Qiu *et al.* (36) have shown previously that the interleukin 6 receptor can associate with and lead to phosphorylation of both erbB-2 and -3. This example illustrates the possibility that members of the erbB family may interact with other receptor types. One potential candidate partner is the IGF-IR. EGFR is capable of transactivating IGF-IR in an EGF-dependent process (37). In addition, Chakravarti *et al.* (38) showed that increased signaling through IGF-IR may be responsible for resistance of a glioma cell line to the EGFR inhibitor AG1478. Experiments aimed at evaluating the role of erbB4, IGF-IR, and other potential receptor partners are ongoing.

The question remains as to whether our observed resistance to the EGFR inhibitors is clinically relevant because higher levels of drug will still inhibit these downstream signals. A recent pharmacokinetic study of ZD1839 in adults has found that daily oral dosing in the 400–600-mg range results in mean plasma concentrations ranging from 478 to 620 ng/ml (1.07–1.39 μM; Ref. 19). In addition, many current protocols have recommended dosing at 250 mg/day, which was found in that same study to yield a mean plasma concentration of approximately 200 ng/ml, or 0.45 μM (19). We find that EGF-dependent AKT and ERK signaling are not significantly inhibited in the LN229/EGFR cells until a ZD1839 concentration of 2–5 μM is reached (Fig. 2A). Therefore, based on our data presented here, drug levels at which resistance to ZD1839 is likely to be seen are clearly possible with either the 250 or 500 mg/day dosing schedule, both of which have been used clinically to date.

Although the PI-3K/AKT and MEK/ERK pathways display significant resistance to the quinazoline-based EGFR inhibitors, EGF-dependent STAT3 activation did not show a similar resistance to these inhibitors. Activation of the JAK/STAT system has been traditionally more correlated with response to cytokines (reviewed in Ref. 23). However, the association of this pathway with RTKs such as EGFR is increasingly appreciated (24, 25). In the cell lines tested in this report, loss of STAT3 phosphorylation always correlated with loss of EGFR autophosphorylation (Figs. 2, 4, and 5). Deb *et al.* (30) found that EGFR homodimers but not EGFR-erbB2 heterodimers can activate STAT3 in an EGF-dependent process. This observation likely explains why STAT3 is so sensitive to the EGFR inhibitors. Because STAT3 activation requires EGFR but not a heterodimer receptor partner, loss of activation would be predicted even with low levels of drug as long as the tyrosine kinase of EGFR is inhibited. Based on this hypothesis, we would predict that in the absence of EGFR amplification/overexpression with normal to elevated levels of erbB2, EGF stimulation would result predominantly in the formation of EGFR-erbB2 heterodimers, and thus STAT3 would not be activated. Consistent with this hypothesis, glioma cell lines (including LN229/puro) that have low expression of EGFR display little or no EGF-dependent STAT3 phosphorylation (data not shown).

Based on the resistance of EGF-dependent signaling to the quinazoline class of EGFR inhibitors, it was expected that EGF-stimulated proliferation would also be similarly resistant. Surprisingly, however, our results suggest not only that ligand-induced proliferation of LN229/EGFR is resistant to 0.05 μM PD153035 (Fig. 6) but also that proliferation actually appeared to be enhanced after treatment with this level of inhibitor. Although we have not yet seen this phenomenon using ZD1839, this is still a concerning result that will require additional study to determine how widely applicable it is. The mechanism by which proliferation is enhanced remains unclear. However, not all downstream signals from EGFR are resistant to this level of EGFR inhibitor. In particular, we have found that STAT3 activation is completely inhibited with 0.05 μM PD153035. Other signals that have not been identified may be similarly inhibited with low levels of drug. It is possible that STAT3 and/or these other as-yet-unidentified signals may be acting to inhibit cell cycle progression in concert with

positive growth signals from PI-3K/AKT and MEK/ERK. Therefore, low levels of PD153035 may be acting to suppress the downstream signals that are growth inhibitory without affecting the signals that promote growth.

In summary, we have identified a potential mechanism of resistance to the quinazoline class of EGFR inhibitors that is prominent in a number of glioma cell lines that were tested. Our data suggest that it is not sufficient to assess the efficacy of these inhibitors by monitoring for suppression of EGFR autophosphorylation but that suppression of downstream signals will also need to be determined to assure full inhibition of this receptor system. Determining the precise molecular mechanism for this resistance will allow additional specific targeting of these resistance machineries that could potentially improve the efficacy of and expand the range of tumors susceptible to the quinazoline class of EGFR inhibitors.

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