Differential Sensitivity of Glioma-versus Lung Cancer-Specific EGFR Mutations to EGFR Kinase Inhibitors

ABSTRACT

Activation of the epidermal growth factor receptor (EGFR) in glioblastoma (GBM) occurs through mutations or deletions in the extracellular (EC) domain. Unlike lung cancers with EGFR kinase domain (KD) mutations, GBMs respond poorly to the EGFR inhibitor erlotinib. Using RNAi, we show that GBM cells carrying EGFR EC mutations display EGFR addiction. In contrast to KD mutants found in lung cancer, glioma-specific EGFR EC mutants are poorly inhibited by EGFR inhibitors that target the active kinase conformation (e.g., erlotinib). Inhibitors that bind to the inactive EGFR conformation, however, potently inhibit EGFR EC mutants and induce cell death in EGFR-mutant GBM cells. Our results provide first evidence for single kinase addiction in GBM and suggest that the disappointing clinical activity of first-generation EGFR inhibitors in GBM versus lung cancer may be attributed to the different conformational requirements of mutant EGFR in these 2 cancer types.

SIGNIFICANCE: Approximately 40% of human glioblastomas harbor oncogenic EGFR alterations, but attempts to therapeutically target EGFR with first-generation EGFR kinase inhibitors have failed. Here, we demonstrate selective sensitivity of glioma-specific EGFR mutants to ATP-site competitive EGFR kinase inhibitors that target the inactive conformation of the catalytic domain. Cancer Discov; 2(5): 458–71. © 2012 AACR.

INTRODUCTION

Glioblastoma (GBM) is the most common malignant brain tumor in adults. Most patients with GBM succumb to their disease within 2 years, and there is a dire need for the development of novel therapeutics (1). Inhibitors of deregulated signaling pathways are active agents in a variety of human cancers (2, 3) and represent a compelling area of drug development for GBM because many of these tumors harbor genetic alterations in growth factor-signaling pathways (4, 5).

The epidermal growth factor receptor (EGFR) is a member of the EGFR family of receptor tyrosine kinases, which also includes HER2 (ErbB2), HER3 (ErbB3), and HER4 [ErbB4 (6)]. EGFR has generated particular interest as a drug target in GBM because of the high frequency of EGFR alterations in this disease (7) and because ATP-site competitive EGFR kinase inhibitors are active agents in patients with EGFR-mutant lung cancer (8). EGFR kinase inhibitors that received regulatory approval for the treatment of lung cancer (erlotinib, gefitinib), however, have shown disappointing results in patients with GBM (9). Reasons for this lack of response in GBM remain poorly understood and include redundancy in signaling pathways (10) and intratumoral heterogeneity (11).

One key difference between EGFR in GBM and lung cancer is the distribution of mutations within the EGFR coding sequence. EGFR mutations in lung cancer reside in the intracellular kinase domain [KD (12)]. EGFR mutations in GBM cluster in the extracellular (EC) domain and include in-frame deletions [such as the common “variant III” (7)] and missense mutations [Fig. 1A (13)]. Both EGFR ecotdomain and KD mutations encode oncoproteins with the ability to transform NIH-3T3 cells in the absence of ligand (13–15). In this study, we examined the role of EGFR for the survival of GBM cells harboring EGFR ecotdomain mutations. We demonstrate that EGFR signals are essential for the survival of these cells and that EGFR EC mutants differ markedly from EGFR KD mutants in their sensitivity to ATP-site competitive EGFR kinase inhibitors.

RESULTS

EGFR-Mutant GBM Cells Are Addicted to EGFR

Missense mutations in the EGFR EC domain are found in 10% to 15% of GBMs (4, 5, 13). To determine whether EGFR signals are essential for the survival of GBM cells endogenously expressing such mutations, we first sequenced the coding region of EGFR in a panel of GBM cell lines. We found 2 lines with EGFR EC mutations. Both mutations
**Figure 1.** EGFR knockdown induces cell death in GBM cells with EGFR EC mutations. A, EGFR domain structure. Mutations indicated in red have been documented in GBM but not in lung cancer, whereas those indicated in blue are seen in non-small cell lung cancer and not in GBM. Roman numerals indicate subdomains within the EC domain. **B**, EGFR-mutant GBM lines are sensitive to EGFR knockdown. The indicated cell lines were acutely transduced with control or 2 different EGFR-targeted shRNAs. The extent of EGFR knockdown was assessed by immunoblot (left). The effects of the hairpins on cell death were assessed by trypan blue exclusion 5 days postinfection (right). pEGFR, tyrosine-phosphorylated EGFR. **C**, HER2 knockdown only induces minimal cell death in EGFR-mutant SF268 GBM cells. Cells were acutely transduced with control, EGFR-targeted, or HER2-targeted shRNAs. The extent of EGFR and HER2 knockdown was evaluated by immunoblot (inset). Cell death was assessed as in panel B. See Supplementary Fig. S2 for the results of HER2 knockdown in EGFR-mutant SKMG3 GBM cells.
resulted in amino acid substitutions at alanine 289, the most common site of EC EGFR missense mutations in human GBMs (Fig. 1A). Alanine was substituted by valine (A289V) in SF268 cells and by aspartic acid (A289D) in SKMG3 cells (Supplementary Fig. S1). We tested whether depletion of the EGFR protein was sufficient to induce cell death in these lines. Acute infection of SKMG3 and SF268 cells with retroviral short hairpin RNA (shRNA) constructs targeting 2 distinct areas of the EGFR mRNA resulted in the loss of EGFR protein expression within 72 hours of infection and robust cell death induction after 5 days. EGFR knockdown in human astrocytes [NHA (16)] and 2 GBM cell lines without the EGFR mutation (SF295, 8-MG-BA) did not induce cell death (Fig. 1B). Of note, SKMG3 cells do not express the tumor suppressor protein phosphatase and tensin homolog (PTEN), confirming our earlier findings that PTEN inactivation is not sufficient to relieve EGFR-mutant cancer cells of their dependence on EGFR for survival (17).

**Figure 2.** Differential sensitivity of EGFR-mutant glioma and lung cancer cell lines to the irreversible EGFR inhibitors HKI-272 and CI-1033. A, HKI-272 induces cell death in GBM cells with EGFR EC mutation (SKMG3, SF268) but not EGFR wild-type (WT EGFR) cancer cell lines or astrocytes (NHA). Cell death was assessed by trypan blue exclusion after 5 days of inhibitor treatment. Cells lines in black express wild-type EGFR, whereas those in red contain EGFR EC mutations. B, CI-1033, unlike HKI-272, does not induce cell death in GBM cells with EGFR EC mutation. SF268 (solid) or SKMG3 (dashed) cells were treated with the indicated doses of HKI-272 (red) or CI-1033 (blue) for 5 days. Cell death was evaluated at day 5 by trypan blue exclusion. C, HKI-272 is more potent than CI-1033 in blocking EGFR phosphorylation in SKMG3 cells with EGFR EC mutation. SKMG3 cells were treated with the indicated doses of CI-1033 or HKI-272, and whole lysates were analyzed by immunoblot with the indicated antibodies. D, CI-1033 is more potent than HKI-272 in blocking EGFR phosphorylation in HCC827 lung cancer cells harboring the (Δ746–750) EGFR KD mutant. HCC827 were treated with the indicated doses of HKI-272 or CI-1033. Lysates of these cells were made and analyzed by immunoblot as indicated. E, CI-1033 is more potent than HKI-272 in inducing cell death in HCC827 lung cancer cells. Cell death was also assessed 5 days after treatment as before.
We conducted similar experiments with shRNA constructs by targeting the EGFR family member HER2 because HER2 can heterodimerize with EGFR and transmit oncogenic signals in certain cellular contexts (18). HER2 knockdown did not induce a significant amount of cell death as measured by the trypan-blue dye exclusion assay and immunoblotting for the cleaved Caspase-3 substrate Poly (ADP-ribose) polymerase (PARP; Fig. 1C and Supplementary Fig. S2). HER2 depletion also did not affect EGFR phosphorylation at tyrosine 1068, suggesting that basal EGFR phosphorylation in SF268 and SKMG3 cells is not the result of trans-phosphorylation by the HER2 kinase.

Several prosurvival functions of EGFR have been attributed to kinase-independent properties of the receptor protein (19). To assess whether EGFR kinase activity is required for the survival of SKMG3 (EGFR-A289D) and SF268 (EGFR-A289V) cells, we treated them with the “second-generation” EGFR kinase inhibitor HKI-272 (20). This drug irreversibly inhibits EGFR (and other ErbB family members) because it forms covalent interactions with cysteines in the ATP cleft of the KD (21). HKI-272 induced cell death in SF268 and SKMG3 cells but not in EGFR wild-type GBM (SF295, 8-MG-BA), lung cancer cells (H460), or human astrocytes (NHA; Fig. 2A).

To extend our observations with HKI-272 to a second EGFR kinase inhibitor, we repeated our experiments with CI-1033. Like HKI-272, CI-1033 is an irreversible, ATP-site competitive inhibitor of ErbB receptors and inhibits phosphorylation of wild-type EGFR in intact cells with similar potency \[IC_{50} 7.4 \text{nM} (22)\] as HKI-272 \[IC_{50} 3 \text{nM} (20)\]. To our surprise, CI-1033 failed to induce cell death in either SF268 or SKMG3 cells (Fig. 2B). Immunoblot of whole-cell lysates from SKMG3 cells treated with either inhibitor showed that CI-1033 inhibited EGFR phosphorylation less effectively than HKI-272 (Fig. 2C).

We wondered whether the differential effect of HKI-272 and CI-1033 on EGFR was unique to GBM cells with EGFR EC mutations. We therefore also compared the activity of both compounds in HCC827 lung cancer cells that harbor a deletion in the EGFR KD (EGFRΔ746-750). In contrast to our findings in GBM cells, CI-1033 more potently inhibited EGFR phosphorylation (Fig. 2D) and more potently induced cell death (Fig. 2E) than HKI-272. Both inhibitors induced cell death at submicromolar concentrations in HCC827 cells, which is consistent with the reported hypersensitivity of the EGFRΔ746-750 mutant to ATP-site competitive EGFR kinase inhibitors in vitro and in patients with lung cancer (23–26). In summary, these results indicate that EGFR-mutant GBM cell lines require EGFR kinase activity for survival and point toward differences in EGFR kinase inhibitor responsiveness between EGFR ectodomain mutants and EGFR KD mutants.

**Enhanced Sensitivity of EGFR Ectodomain Mutants to Lapatinib**

Crystal structures of the EGFR catalytic domain in complex with ATP-site competitive EGFR kinase inhibitors have identified different receptor conformations (27, 28). In complex with the U.S. Food and Drug Administration-approved drug lapatinib/GW572016 [Tykerb (GlaxoSmithKline)], the EGFR KD is in an inactive
conformation [also called “type II” conformation (29)]. In complex with erlotinib/OSI-774 [Tarceva (Genentech)], the EGFR KD adopts an active (or “type I”) conformation [Fig. 3A (30)]. Because HKI-272 binds the inactive conformation of the EGFR KD [“type II” (31)] and CI-1033 likely binds the active conformation (on the basis of analogy to compounds with similar chemical structure), we hypothesized that conformation-specific binding to EGFR might explain the differential response of GBM cell lines with EGFR EC mutants to these 2 compounds. If correct, lapatinib (“type

Figure 3. (continued) D, differential sensitivity of endogenously expressed glioma- versus lung cancer–specific EGFR mutants to lapatinib versus erlotinib. The indicated GBM (top) and lung cancer (bottom) cell lines were treated with various doses of lapatinib or erlotinib. Cells were harvested and analyzed by Western blot with antibodies for pEGFR (top) or tEGFR (bottom). E and F, differential cell death response of GBM (E) and lung cancer (F) cell lines to lapatinib versus erlotinib. Cell death was assessed by trypan blue exclusion assay after 5 days of incubation with the indicated drug, pEGFR, tyrosine-phosphorylated EGFR; tEGFR, total EGFR; WT, wild type.
II inhibitor”) should also show superior activity against EGFR EC mutants than erlotinib (“type I inhibitor”).

To examine this question, we first expressed several EGFR ectodomain mutants in NR6 fibroblasts that do not detectably express EGFR or other ErBB family members and are widely used for the biochemical characterization of EGFR family members (24, 32, 33). After deriving stable sublines for each EGFR allele (Fig. 3B), we examined changes in EGFR phosphorylation in response to equimolar concentrations of erlotinib or lapatinib. Although both inhibitors lowered EGFR phosphorylation in a dose-dependent fashion, lapatinib showed significantly greater potency against all examined EGFR ectodomain mutants (A289D, A289V, G598V, T263P, vIII) and, less dramatically, also against wild-type EGFR (Fig. 3C). We obtained similar results in human astrocytes that express endogenous wild-type EGFR and that we further engineered to overexpress either wild-type EGFR or the 2 most common EGFR ectodomain mutants in GBM (A289V and EGFRvIII; Supplementary Fig. S3).

We next extended our comparison between lapatinib and erlotinib to GBM cell lines endogenously expressing EGFR ectodomain mutants. These included SKMG3 (EGFR-A289D) and SF268 (EGFR-A289V) cells as well as a third line (KNS-81-FD), recently reported to harbor the G598V EGFR ectodomain mutant (COSMIC database). To benchmark our results against previous work on EGFR KD mutants, our experiments also included the lung cancer cell lines HCC827 (EGFRΔ746-750), HCC4006 (EGFRΔ747-749), and H3255 (EGFR-L858R). Similar to our results in NR6 cells and astrocytes, lapatinib was more potent than erlotinib at inhibiting basal phosphorylation of all examined EGFR ectodomain mutants. Erlotinib, on the other hand, was more potent than lapatinib at inhibiting EGFR in lung cancer cell lines with the EGFR KD mutants EGFRΔ746-750 and EGFR-L858R (Fig. 3D), a finding consistent with previous studies (34). Akt and Erk, 2 well-documented effector kinases of the examined EGFR KD mutants, were also more potently inhibited by lapatinib compared with lapatinib in these lines (Supplementary Fig. S4A and S4B). Interestingly, inhibition of EGFR in SKMG3 GBM cells did not result in Akt or Erk inhibition, suggesting that the A289D mutant utilizes other downstream effector pathways (Supplementary Fig. S4C).

We also examined the effects of lapatinib and erlotinib on cell death. Lapatinib, but not erlotinib, induced cell death in all examined GBM cell lines with EGFR ectodomain mutants (Fig. 3E). In EGFR-mutant lung cancer cell lines, erlotinib induced cell death at lower concentrations than lapatinib (Fig. 3F).

**Type II EGFR Inhibitors Effectively Displace ATP from EGFR Extracellular Mutants**

Our results with 4 different EGFR kinase inhibitors suggested that the catalytic domain of EGFR ectodomain mutants might favor an inactive-like conformation that is more accessible to lapatinib or HKI-272 than to erlotinib or CI-1033. To further test this model, we developed an assay that measures the ability of EGFR kinase inhibitors to compete in whole-cell lysates with ATP for binding to the ATP-cleft of the EGFR KD (Fig. 4A).

Coincubation of whole-cell lysates from A289D-EGFR mutant SKMG3 cells with biotinylated ATP and erlotinib demonstrated decreased ATP-binding with increasing erlotinib concentrations. Coincubation of a replicate sample of the same whole-cell lysate with increasing concentrations of lapatinib blocked ATP binding at lower concentrations of lapatinib than erlotinib. As a specificity control, we determined ATP binding to the KD of SRC and found no displacement of ATP-binding by either lapatinib or erlotinib (Fig. 4B). We also repeated these experiments with whole-cell lysates from H3255 lung cancer cells (EGFR-L858R KD mutant) and found that erlotinib blocked ATP binding to the EGFR KD more effectively than lapatinib (Fig. 4C).

Because differences in off-rates between the reversible EGFR kinase inhibitors lapatinib and erlotinib might affect results of the ATP-competition assay, we performed additional experiments with the irreversible EGFR kinase inhibitors CI-1033 and HKI-272. In whole-cell lysates from EGFR-A289D SKMG3 cells, HKI-272 more effectively blocked ATP binding to the EGFR KD than CI-1033 (Supplementary Fig. S6), which was consistent with our model.

Finally, we explored whether a forced change in receptor conformation, induced by ligand binding, might alter the ability of EGFR inhibitors to gain access to the KD (Fig. 4D) and block EGFR phosphorylation. We were able to examine this question in SKMG3 cells harboring the EGFR-A289D mutant because we had previously shown that this mutant, unlike EGFRvIII, does not abrogate the ability of EGFR to respond to EGF (13). When we treated EGFR A289D-mutant SKMG3 cells with lapatinib or erlotinib in the presence of EGF, we indeed found that EGF “desensitized” EGFR to lapatinib and sensitized EGFR to erlotinib: greater lapatinib (right-shift) and lower erlotinib (left-shift) concentrations were required to achieve a similar degree of EGFR inhibition than in the absence of EGF (Fig. 4E). We obtained similar results in receptor-negative NR6 cells reconstituted with EGFR-A289D (Supplementary Fig. S5).

**Lapatinib Fails to Achieve Sufficient Intratumoral Concentrations in Patients with GBM**

Clinical trials with type I EGFR kinase inhibitors (erlotinib, gefitinib) in GBM demonstrated poor inhibition of the EGFR signaling axis in tumor tissue (35, 36). To determine the ability of lapatinib to penetrate into GBM tumor tissue and inhibit EGFR phosphorylation, we conducted a multicenter clinical trial in which patients received 750 mg of lapatinib orally for 7 days before a surgical procedure that was required for tumor recurrence (Fig. 5A; Supplementary Data). A total of 44 patients with recurrent GBM enrolled into the study and underwent surgery (Supplementary Tables S1 and S2). Lapatinib was generally well tolerated (Supplementary Table S3 and Supplementary Data).

Lapatinib concentrations in the plasma sample collected during surgery varied considerably between patients (0.188 to 2.319 μg/mL; Supplementary Table S4), with mean plasma concentrations (mean ± SD: 1.203 ± 0.518 μg/mL) similar to plasma levels reported in the literature for this dosing schedule (37). Tumor concentrations of lapatinib varied considerably between patients (70–3826 nM). The median concentrations for the entire cohort (497 nM) were greater than the IC50, or inhibition of EGFR phosphorylation (~200 nM) but below drug concentrations reported to induce cell death.
Figure 4. Type II EGFR kinase inhibitors more effectively displace ATP from the EGFR KD of EGFR ectodomain mutants than type I inhibitors. A, schematic of ATP competition-binding assessment. Lysates of cells expressing the ATP-binding protein under study are simultaneously treated with a targeted agent (e.g., EGFR TKI) or vehicle, and a biotinylating ATP probe. All ATP-binding proteins will be biotinylated unless the ATP-binding pocket is occupied (e.g., with EGFR TKI). Lysates are subjected to avidin pulldown. The ability of the test compound to compete with ATP for binding to the target protein is assessed by immunoblot of the pulldown using antibodies against the target protein (e.g., EGFR). B and C, results of ATP competition assay in lysates from B) EGFR EC-mutant GBM cells and C) EGFR KD-mutant lung cancer cells. B) Lapatinib more effectively competes with ATP for binding to the EGFR-TK in SKMG3 (EGFR A289D) cell lysates than erlotinib. C) Erlotinib more effectively competes with ATP for binding to the EGFR-TK in H3255 cell lysates (EGFR L858R KD mutation) than lapatinib. Cell lysates were carried through the assay described in panel A. The ATP probe was competed with the indicated doses of erlotinib or lapatinib. After the avidin pulldown, samples were analyzed by immunoblot with antibodies for EGFR. Immunoblots were also probed with antibodies for Src as a control. D, a model of ligand-induced changes in EGFR conformation. In the absence of ligand (serum-free), the conformational equilibrium of EGFR-EC mutants is shifted towards the "inactive" conformation which is preferentially bound by type I inhibitors. In ligand-occupied receptor (EGF), the conformational equilibrium shifts towards the "active" conformation, which is the preferred substrate of type II inhibitors. E, EGF "desensitizes" the EGFR-A289D mutant from lapatinib and "sensitizes" it to erlotinib. SKMG3 GBM cells were serum-starved, stimulated with EGF or vehicle, and subsequently treated with the indicated doses of erlotinib and lapatinib (while still under EGF treatment). Cells were lysed after 30 minutes of drug treatment and analyzed by immunoblot with the indicated antibodies.
**Figure 5.** Lapatinib fails to achieve sufficient intratumoral concentrations in patients with GBM. **A,** design of the multicenter NABTC 04–01 biomarker trial for patients with recurrent GBM. Patients with GBM requiring tumor resection for recurrent disease received preoperative lapatinib (750 mg by mouth twice a day). Lapatinib concentrations in tumor tissue and EGFR phosphorylation were assessed in surgical specimens. See Supplementary Data for details. **B,** intratumoral lapatinib concentrations in GBM are below lapatinib concentrations required to induce cell death in EGFR mutant GBM cells (red line, 1.5 μM). **C,** incomplete inhibition of EGFR phosphorylation in GBM tumor tissue by lapatinib. Shown are levels of total EGFR (x-axis) and phosphorylated EGFR (y-axis) in tumors from patients with GBM who received preoperative lapatinib (red diamonds) versus tumors from GBM patients who did not receive any EGFR kinase inhibitor prior to surgery (open circles). Levels of EGFR phosphorylation and total levels were measured concurrently with a multi-array immunoassay using electrochemiluminescence detection (see top panel). **D,** EGFR expression in GBMs from patients enrolled in the NABTC 04–01 study. Shown are immunoblots of tumor lysates probed with EGFR or a loading control. Full-length (FL) and truncated EGFRvIII (vIII) mutant forms of EGFR are indicated by arrows. **E,** incomplete EGFR inhibition in GBM tumor tissue by lapatinib. Shown are immunoblots of EGFR overexpressing GBMs on the NABTC 04–01 study (labeled “Lapatinib”) versus tumors from GBM patients who did not receive an EGFR kinase inhibitor prior to surgery (“Control”). Total levels of EGFR and EGFR tyrosine phosphorylation were analyzed by immunoblot. Total Erk levels were also examined as a loading control.

Published OnlineFirst March 31, 2012; DOI: 10.1158/2159-8290.CD-11-0284
in cancer cell lines [Fig. 5B; Fig. 6A; Supplementary Fig. 5; Supplementary Tables S4 and S5 (38)].

We assessed EGFR phosphorylation on tyrosine 1173 in all patient samples for which residual frozen tumor was available and compared it with EGFR phosphorylation in 49 tumor samples from patients with GBM who had not received any EGFR kinase inhibitor before surgery (referred to as “no lapatinib” controls; Supplementary Table S6). Because EGFR levels in GBM range over 2 to 3 orders of magnitude (39), we chose an electrochemiluminescent detection method with a broad linear range of detection (MSD mesoscale). This platform offered the additional advantage that it allowed us to determine total and phospho-EGFR signal for each sample in a single well and run all clinical trial and control samples together in a 96-well format. Compared with control samples (Fig. 5C, empty circles), the group of lapatinib-treated tumors (Fig. 5C, red diamonds) showed less EGFR phosphorylation per total EGFR signal (P = 0.006). However, all lapatinib-treated tumors showed residual EGFR phosphorylation greater than levels observed in lapatinib-naïve tumors not overexpressing EGFR.

For all tumors with sufficient residual sample, we also performed immunoblot analysis (n = 27). EGFR immunoblot analysis showed EGFR overexpression in 12 of 27 (44.4%) tumors; a 140-KDa band, consistent with the EGFRIII deletion, was detected in 7 of 27 (25.9%) of tumors, all within the group of tumors overexpressing EGFR (examples are shown in Fig. 5D). Only 1 of these 27 tumors (E0038) harbored a missense mutation in the EGFR ectodomain (T263P; Supplementary Table S7). A comparison of EGFR phosphorylation between lapatinib-treated tumors with EGFR overexpression and control tumors showed that lapatinib-treated GBMs showed lower levels of EGFR phosphorylation than controls with similar levels of EGFR overexpression (Fig. 5E).

All lapatinib-treated tumors demonstrated residual EGFR phosphorylation greater than levels seen in GBM controls lacking EGFR overexpression, which is consistent with our ELISA results. Because all patients underwent operative tumor resection, we could not evaluate the radiographic tumor responses to lapatinib.

**Level of EGFR Inhibition Determines Cell Death Response in EGFR-Mutant GBM Cells**

Studies in cancer cell lines have shown that cell death induction by lapatinib requires drug concentrations of 2 to 3 μM, that is, drug concentrations greater than the IC50 for inhibition of EGFR phosphorylation and inhibition of cell proliferation (38). Detailed dose-response experiments in EGFR-mutant SF268 (Fig. 6A), SKMG3 (Supplementary Fig. S7A), and KNS-81-FD (Supplementary Fig. S7B) GBM cells similarly showed dose-dependent cell death induction only above lapatinib concentrations of 1,500 to 1,750 nM (Supplementary Fig. S7).

Although lapatinib ranks among the most selective ATP-site competitive kinase inhibitors (40), we sought to confirm that this cell death “threshold” reflected a requirement for near-complete EGFR inhibition rather than potential off-target effects of lapatinib. We performed titration experiments with a retroviral EGFR shRNA construct in GBM cells with EGFR EC mutations. At a virus dilution of 1:27, SF268 (EGFR-A289V) GBM cells showed clear reductions in EGFR protein levels and EGFR phosphorylation and > 50% growth inhibition but no evidence for cell death (PARP cleavage, trypan blue exclusion). However, when EGFR protein levels were almost undetectable by immunoblotting (1:3 dilution of the EGFRshRNA virus), we observed robust cell death induction and PARP cleavage (Fig. 6B). We observed similar results in EGFR-A289D–mutant SKMG3 cells (Supplementary Fig. S8). These results demonstrate that even low levels of EGFR activity, which cannot accurately be quantified by immunoblotting with the use of phosphospecific EGFR antibodies, are sufficient to sustain the survival of EGFR-mutant glioma cells.

To further explore the biologic significance of potent EGFR blockade in vivo, we extended our experiments to GBM tumor sphere cultures freshly derived from patients with GBM. Unlike SF268 and SKMG3 cells, these cells form aggressive tumors in immunodeficient mice.

In preliminary experiments, we compared the effects of erlotinib and lapatinib on in vitro cell viability in 2 EGFR-amplified GBM tumor sphere lines (GS676 and GS600), and again found that only lapatinib was able to effectively induce cell death (Supplementary Fig. S9A and S9B). We also assessed the effects of lapatinib on anchorage-independent growth in a slightly larger panel of glioma sphere lines. In all 3 lines with EGFR gene amplification (GS676, GS596, GS600), lapatinib reduced colony formation in a dose-dependent fashion with complete abrogation of colony growth above 2 μM lapatinib (Fig. 6C; Supplementary Fig. S9C). Lapatinib had no effect on colony formation of a PDGFRα-amplified (GS543) glioma sphere line (Fig. 6C).

We then compared the effectiveness of different lapatinib dosing schedules on the growth of subcutaneous GS676 GBM xenografts. After tumors were established, mice were assigned to either treatment with vehicle or 4 different oral lapatinib dosing schedules: 200 mg/kg daily, 600 mg every third day, 800 mg every fourth day, or 1000 mg every fifth day. We designed this dosing schedule on the basis of previous reports that transient potent blockade of oncogenic kinases is able to irreversibly commit cancer cells to cell death (41, 42). We observed maximal growth inhibition (Fig. 6D) and caspase activation (Fig. 6E; Supplementary Fig. S10) in the cohort receiving 1000 mg/kg every fifth day.

**DISCUSSION**

The EGFR kinase inhibitor erlotinib has received regulatory approval for the treatment of EGFR-mutant lung cancer (8, 43), but results with this agent in patients with GBM have been disappointing. Our study provides a potential explanation for the differential activity of erlotinib against these 2 cancer types. In contrast to the most common EGFR kinase mutants in lung cancer, the most common oncogenic EGFR alterations in GBM are relatively insensitive to erlotinib. Instead, these mutants are preferentially inhibited by EGFR inhibitors that can only be accommodated by the inactive conformation of the EGFR catalytic pocket as the result of their bulky aniline substituents [lapatinib, HKI-272 (29, 44)]. Although many novel EGFR kinase inhibitors distinguish themselves from first-generation EGFR kinase inhibitors by their irreversible mode of EGFR binding or
Figure 6. Level of EGFR inhibition determines cell death response in EGFR mutant GBM cells. A, lapatinib induces cell death in SF268 GBM cells (A289V EGFR) at concentrations > 1.5 μM. Cell death was assessed 5 days after treatment by trypan blue exclusion. Similar results are shown in Supplementary Fig. S7A for SKMG3 cells (A289D-EGFR) and in KNS-81-FD cells (G598V-EGFR; Supplementary Fig. S7B). B, cell death induction in SF268 GBM cells (A289V EGFR) requires near-complete EGFR inactivation. SF268 cells were acutely transduced with a dilution series of a lentiviral EGFR-targeted shRNA (dilution factor is indicated in parenthesis) or with a control shRNA (empty vector). A fraction of the cells from each infection was used for immunoblot analysis with the indicated antibodies (left). The remaining cells were re-seeded and allowed to grow for 5 days after infection. The fold number of viable cells (relative to day 1) and fraction of dead cells are shown in the middle and right panel, respectively. Similar results were obtained in SKMG3 GBM cells (Supplementary Fig. S8). C, lapatinib inhibits anchorage-independent growth of EGFR-amplified (GS676, GS596, GS600) but not PDGFRα-amplified, GBM tumor sphere cultures. Anchorage-independent growth of 4 freshly derived GBM tumor sphere cultures was assessed in soft agar in the presence of the indicated concentrations of lapatinib. (See also Supplementary Fig. S9). D, pulsatile lapatinib dosing [1,000 mg/kg every 5 days (Q5d)] is superior to daily lapatinib dosing [200 mg/kg once daily (Qd)] in inducing growth inhibition and cell death induction in EGFR-amplified GS676 GBM tumor sphere lines. GS676 cells were injected subcutaneously into SCID mice and treatment initiated at the indicated lapatinib dosing schedules after tumors were established. Tumor volumes (left) were evaluated at the end of treatment by caliper measurements. The right panel shows quantification of cleaved Caspase-3 staining in tumor sections from the indicated cohorts, that is, vehicle, 200 mg/kg lapatinib, and 1,000 mg/kg lapatinib. Immunohistochemistry images are shown in Supplementary Fig. S10. Q3d, every 3 days; Q4d, every 4 days.
activity against selected kinases in addition to EGFR (8, 45), our results argue for focused clinical development of type II EGFR kinase inhibitors for EGFR-mutant GBM.

The molecular mechanisms for the inhibitor selectivity of EGFR EC versus EGFR KD mutants require further study. Studies of (near) full-length EGFR receptors are beginning to uncover details of the relationship between the EC and KDs of receptor tyrosine kinases (46). It seems unlikely that the conformation of EC EGFR mutants is identical to the inactive-like conformation (i.e., catalytically incompetent) described in structural studies of the isolated KD (29), especially when considering that these mutants possess ligand-independent constitutive activity and transforming ability (13). Instead, we propose that the unliganded EC-domain mutant receptors exist in a dimeric state that retains enough flexibility within the KD to accommodate lapatinib and other type II EGFR kinase inhibitors. This flexibility appears to be compromised in EGFR KD mutants (34).

Although we uncovered a relative vulnerability of “glioma-relevant” EGFR genotypes (wild-type, EGFRwild, A289V/D, G598V, T263P) to lapatinib, oral lapatinib therapy at a dose of 750 mg twice daily failed to prolong progression-free survival in patients with recurrent GBM in our study, and another recent phase I/II trial (47). Neither of the 2 patients with GBM whose tumors showed intratumoral drug concentrations > 1500 nM (E50088 and E50034) and also overexpressed EGFR could be evaluated for therapeutic response (Supplementary Table S8). These results highlight the need to enrich clinical trials with targeted agents in GBM for patients whose tumors harbor the drug-relevant oncogenic lesion, a strategy that is already pursued in the development of kinase inhibitors for several other human cancer types (48).

The experience with BRAF-mutant melanoma illustrates the importance of effective kinase inhibition for therapeutic response (49). Such potent EGFR inhibition is readily achievable in lung cancer because of the direct effects of KD mutations on inhibitor and ATP affinity (44). Further clinical trials are required to explore whether a similar degree of EGFR kinase inhibition can be achieved in EGFR-mutant GBM through alternative lapatinib dosing schedules [e.g., pulsatile dosing (50)], type II EGFR inhibitors with improved central nervous system penetration, or perhaps combination therapies converging on the mutant EGFR protein and its effectors.

METHODS

Cell Lines and Reagents

SF295 and SF268 cells were obtained from the National Cancer Institute. H460, HCC827, and HCC4006 cells were purchased from American Type Culture Collection. KNS-81-FD cells were purchased from Japanese Collection of Research Bioresources. 8-MG-BA and H3255 cells were kindly provided by Dr. Rameen Beroukhim (Broad Institute). SKMG3 cells were provided by Conforma Therapeutics. NHAs were kindly provided by Dr. Russell Pieper (UCSF). NR6 cells were kindly provided by Dr. Harvey Hershman (UCLA). DNA fingerprinting was used for authentication of all glioma cell lines; no further validation was performed. All antibodies with the exception of anti-Actin and Ki-67 were purchased from Cell Signaling Technologies. Anti-Actin antibody was purchased from Sigma-Aldrich. Ki-67 antibody was purchased from Dako. Erlotinib and lapatinib were purchased from LC Laboratories. CI-1033 and HKI-272 were purchased from Selleck Chemicals.

Electrochemiluminescent Detection of EGFR and Tyrosine-Phosphorylated EGFR in Tumor Samples

Phospho (Tyrl1173)/Total EGFR Assay was purchased from Meso Scale Discovery and assay was performed as described in the product insert (catalog number K15104D) with SECTOR Imager 2400 instrument.

Plasmids

Wild-type EGFR was shuttled from pLXSN-EGFR (kindly provided by Dr. David Riese; Purdue University) into pLNCX2 as a XhoI restriction fragment. pLHCX-EGFRIII was kindly provided by Dr. Paul Mishel (UCLA). pLNCX2-EGFR was used as a template to generate A289D, A289V, G598V, and T263P point mutants with Quickchange (Agilent). Lentiviral shRNA constructs targeting EGFR and ErbB2 were purchased from Sigma-Aldrich [EGFRshRNA (1), TRCN0000010329; EGFRshRNA (2), TRCN0000121068; ErbB2, TRCN0000195369].

Retroviral Infections

For transduction of wild-type and mutant EGFR into NR6 fibroblasts, pan-tropic retrovirus was generated by use of the Pantropic Retroviral Expression System from Clontech. To summarize, EGFR cDNAs were cotransfected with pVSVG into the GP2-293 packaging cell line. Viral particles were collected 36 and 60 hours after transfection and target cells were infected for 18 hours with each virus collection. Stable expressors were derived through antibiotic selection. Knockdown of EGFR and ErbB2 was performed with the use of lentiviral shRNAs. Viral particles were produced by cotransfection of shRNA constructs (empty pKLO vector was used as control) with 2 packaging plasmids (pMD2G and pPAX2) into 293T cells. Viral particles were collected at 36 and 60 hours after transfection. Each virus was diluted 1:3 with collection media (Iscove’s media + 10% fetal bovine serum) and infections were carried out with diluted virus for 3 hours. Where noted, virus stock was further diluted as indicated.

Assessment of Cell Death Induction

Cells were seeded on 6-cm dishes and allowed to attach overnight. Cells were then treated with the indicated drugs at the indicated doses for 5 days. Each treatment group was seeded in triplicate. After treatment, both attached and unattached cells were harvested and counted on a ViCell Cell Viability analyzer. The instrument uses trypan blue to assess cell death. Cell death was expressed as the fraction of trypan-blue-positive cells over the total number of cells.

Soft Agar Colony-Formation Assay

Cells were seeded at 5,000 (GS543), 25,000 (GS596), or 50,000 (GS600 and GS676) cells/plate based on predetermined colony formation efficiencies of untreated cells such that each cell line would give rise to similar numbers of colonies under vehicle control conditions. Cells were plated in Neurocult media (Stem Cell Technologies) containing 0.65% Noble agar and growth factor supplements, and each treatment group was conducted in duplicate. Colonies were stained with crystal violet (0.005%) 3 weeks after plating, imaged in a Gel Count (Oxford Optronix), and the images were processed using the Charm algorithm (Oxford Optronix) to obtain colony number and colony size distributions.

ATP Competition Assay

The ability of EGFR TKIs to compete with ATP for binding to EGFR was measured with the Pierce Kinase Enrichment Kit with
ATP Probe and was conducted according to the manufacturer’s protocol with the following modifications. To summarize, cells were harvested and lysed. Lysates then were passed through a desalting column to remove ATP. After this buffer exchange, lysates were incubated with a premade mixture of the appropriate inhibitor at the desired concentration and desthiobiotin-ATP probe to a final concentration of 5 μM. This mixture was then incubated for 5 minutes at room temperature and the reaction terminated by addition of 4 M urea. Avidin agarose beads were then added to the reaction mixtures and allowed to pull down biotinylated proteins for 1 hour at room temperature. Beads were washed 3 times and eluted with 3X Laemmli sample buffer. Pulldowns were then analyzed by immunoblot.

**Immunohistochemistry and Computer-Assisted Image Analysis**

Paraform-embedded sections of tumor xenografts were obtained at 5 μm/slide. Antigen retrieval, immunohistochemical detection, and counter staining were performed by use of the Ventana Discovery Ultra autostainer (Ventana) with primary antibodies against cleaved caspase-3 at a 1:1,000 dilution. To determine apoptotic index, we used total number of nuclei with positive cleaved-Caspase-3 labeling X 100/total number of nuclei on hematoxylin and eosin staining. Histologic fields were captured with a camera (SPOT Imaging Solutions). Digitized images were segmented with segmentation techniques such as density and size thresholding to distinguish negative from positive objects using image analysis software (ImageJ, NIH). The segmentation process resulted in the generation of binary images from which the number of stained objects and total numbers of nuclei were determined. Three separate regions were analyzed in each tumor sample.

**Tumor Xenografts**

Mice were restrained with Institutional Animal Care and Use Committee–approved restraint techniques to expose the flank. The hair was removed with an electric razor, and the injection site was disinfected with 70% ethanol. Then 105 cells, in 100 μL of a 50:50 mixture of growth media and in Matrigel (BD, catalog number 356237), was injected under the skin. Mice were monitored to ensure that tumor growth did not exceed 1.5 cm in diameter.

**Disclosure of Potential Conflicts of Interest**

W.K.A. Yung received grant support from Novartis and Daiichi. M.P. Mehta is an employee of and has ownership interest in Pharmacaycs and has consulted for Tomotherapy. No potential conflicts of interest were disclosed by the other authors.

**Acknowledgments**

The authors thank members of the Mellinghoff laboratory and Dr. Charles Sawyers for helpful discussions in the course of this work and Dr. William Weiss (UCSF) and Dr. Kevan Shokat (UCSF) for sharing unpublished results.

**Grant Support**

This work was supported through U54CA143798 (I.K. Mellinghoff) and U01 CA141502 from the National Cancer Institute. Further funding support was provided by the Leon Levy foundation, the Sonstag Foundation, the Doris Duke Charitable Foundation, and an Advanced Clinical Research Award from the American Society of Clinical Oncology (I.K. Mellinghoff). C. Grommes was supported through an American Brain Tumor Association Basic Research Fellowship Award, M.G. Chheda was supported through NIH-SK08NS062907, B. Oldrini was supported through grants from the American Italian Cancer Foundation and a Memorial Sloan-Kettering Cancer Center (MSKCC) Brain Tumor Center grant, and N. Yannuzzi was the recipient of an American Brain Tumor Association Medical Student Summer Fellowship. Investigators of the NABTC-04-01 Clinical Trial were supported through the following funding sources: 5-U01CA62399-09 (A.B. Lassman and L.M. DeAngelis); NABTC number CA62399 and member number CA62422, GCRC grant number M01-RR00079 (S.M. Chang, K.R. Lamborn, and M.D. Prados); CA62426 (J.G. Kuhn); CA62412, GCRC grant number CA16672 (W.K.A. Yung and M.R. Gilbert); U01CA62407-08 (P.Y. Wi); U01CA62421-08, GCRC grant number M01-RR03186 (M.P. Mehta and H.I. Robbins); U01CA62405, GCRC grant number M01-RR00056 (F. Lieberman); and U01CA62399, GCRC grant number M01-RR0865 (T.F. Cloughesy). This work was supported in part by the MSKCC Experimental Therapeutics Center.

Received November 2, 2011; revised February 9, 2012; accepted February 24, 2012; published OnlineFirst March 31, 2012.

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Glioni EGFR Mutants Selectively Respond to Type II EGFR TKIs


