Preclinical Test of Dacomitinib, an Irreversible EGFR Inhibitor, Confirms Its Effectiveness for Glioblastoma

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Abstract

Glioblastomas (GBM) are devastating tumors in which there has been little clinical improvement in the last decades. New molecularly directed therapies are under development. EGFR is one of the most promising targets, as this receptor is mutated and/or overexpressed in nearly half of the GBMs. However, the results obtained with first-generation tyrosine-kinase inhibitors have been disappointing with no clear predictive markers of tumor response. Here, we have tested the antitumor efficacy of a second-generation inhibitor, dacomitinib (PF299804, Pfizer), that binds in an irreversible way to the receptor. Our results confirm that dacomitinib has an effect on cell viability, self-renewal, and proliferation in EGFR-amplified GBM cells. Moreover, systemic administration of dacomitinib strongly impairs the in vivo tumor growth rate of these EGFR-amplified cell lines, with a decrease in the expression of stem cell–related markers. However, continuous administration of the compound was required to maintain the antitumor effect. The data presented here confirm that dacomitinib clearly affects receptor signaling in vivo and that its strong antitumoral effect is independent of the presence of mutant receptor isoforms although it could be affected by the PTEN status (as it is less effective in a PTEN-deleted GBM line). Dacomitinib is being tested in second line for EGFR-amplified GBMs. We hope that our results could help to select retrospectively molecular determinants of this response and to implement future trials with dacomitinib (alone or in combination with other inhibitors) in newly diagnosed GBMs. Mol Cancer Ther; 14(7): 1548–58. ©2015 AACR.

Introduction

Glioblastoma (GBM), or grade IV astrocytoma, is the most frequent malignant primary brain tumor and one of the most aggressive forms of cancer. Current therapy includes surgical intervention, radiotherapy, and chemotherapy with temozolomide. However, median survival after diagnosis is usually just 12 to 15 months with this standard treatment (1). Therefore, there is a strong medical need to develop new therapeutic strategies for the management of GBMs. Epidermal growth factor receptor (EGFR), also called ErbB1/HER1, plays an important role in tumor development by stimulating cell proliferation and cell resistance to apoptosis and autophagy (2). It also participates in drug and radiotherapy resistance via stimulation of different pathways such as Ras–Raf–ERK and PI3K–AKT–mTOR (3). However, although first-generation EGFR tyrosine-kinase inhibitors (TKIs) such as erlotinib and gefitinib have been proven to be active in the treatment of lung cancer, they have produced poor results in glioma patients (4) and were not able to inhibit EGFR signaling in vivo (5). Nevertheless it has been estimated that the EGFR gene is amplified in 30% to 40% of GBMs and nearly 50% of them overexpress the receptor, whereas this alteration is infrequent in anaplastic astrocytoma or in secondary GBMs (6–8). Different mechanisms could justify the activation of the EGFR signaling pathway in GBM: overexpression that could provoke a local accumulation and activation of the kinase domain or high expression of EGFR ligands such as TGFα (9). However, it is also known that many GBMs with EGFR amplification also carry mutations in EGFR (10, 11). The most common EGFR mutations found in GBMs are in-frame deletion of regions in the extracellular domain like EGFRvIII that is present in 30% to 40% of GBMs with EGFR amplification (11). This mutated gene, EGFRvIII, encodes for a receptor with a constitutively active kinase activity that could produce a distinct set of downstream signals to those associated with wild-type EGFR (12) and that is more tumorigenic in mouse glioma models (9). However, there is controversy whether the expression of the vIII isoform confers resistance or sensitivity to TKIs (9). In contrast, there is a bigger consensus regarding the relevance of PTEN (phosphatase and tensin homolog) status as a biomarker for EGFR inhibitors. PTEN activity attenuates AKT stimulation in response to receptor activation and its gene is lost or mutated in 40% to 50% of gliomas (6–10). AKT phosphorylation is...
correlated with EGFR amplification (13) and it has been proposed that patients carrying wild-type PTEN tumors or low levels of phosphorylated AKT would have a better outcome in response to TKIs (14, 15).

PEZ98904 (dacomitinib) is a second-generation, oral, irreversible, pan-HER TKI, active in erlotinib and gefitinib-resistant nonclinical models of non–small cell lung cancer (NSCLC; ref. 16). This drug has several advantages over gefitinib and erlotinib such as its irreversible action or its ability to target not only EGFR but also HER2 and HER4. Furthermore, this compound displays improved pharmacokinetic properties, including increased bioavailability, half-life, and lower clearance as compared with the first-generation inhibitors (17). A phase II study comparing dacomitinib with erlotinib as a second-line treatment in unscreened NSCLC patients gave positive results although a phase III in the same patient settings did not show any improvement (18). However, encouraging clinical activity has been demonstrated for dacomitinib as initial treatment in patients with advanced NSCLC carrying EGFR-activating mutations (19). These results have reinforced the applicability of dacomitinib for molecularly selected patients and now a phase III trial is ongoing for NSCLC tumors. In GBMs, two phase II studies are being carried out in recurrent tumors with EGFR amplification (NCT01520870 and NCT01112527) and dacomitinib has proven to be efficient in some GBM cell lines and U87 xenografts (20, 21). However, more preclinical data were needed for the development of current and future clinical trials in glioma patients.

In this study, we test the antitumoral efficacy of dacomitinib in a panel of patient-derived GBM cell lines and xenografts (hetero and orthotopic injections) that present the most significant molecular profiles in this tumor. We demonstrate that systemic administration of dacomitinib efficiently impairs EGFR signaling in vivo and has an effect on tumor growth and survival in EGFR-amplified GBM cells, independently of the presence of different mutant receptor isoforms. Moreover, dacomitinib decreases the level of stem cell markers in the treated tumors. However, the presence of wild-type PTEN function and continuous administration of the compound seems to be required for a strong antitumoral effect. Our results provide a major boost to the clinical trials with dacomitinib in GBM patients and suggest possible synergistic approaches as well as predictive markers.

Materials and Methods

Primary cultures
Tissues provided by the Hospital Universitario 12 de Octubre (Madrid, Spain) were obtained after patient’s written consent and with the approval of the ethic committee of the participating Hospital. Primary cultures GBM3 to 7 were established from those samples and processed as cultured previously described (22). Primary lines GBM1 and GBM2 were kindly provided by Dr. Rosella Galli (Neural Stem Cell Biology Unit, Division of Regenerative Medicine, Stem Cells and Gene Therapy, San Raffaele Scientific Institute, Milan, Italy). Their molecular profiles (tested by the authors) are resumed in Supplementary Table S1. They all have been confirmed as gliomas for their capacity to grow as such in xenografts.

Mouse xenograft assays
All mouse experiments were approved by and performed according to the guidelines of the institutional animal care committee of the ISCIII, in agreement with the European Union and national directives. Heterotopic and orthotopic xenografts were performed as previously described (22).

Dacomitinib usage
Dacomitinib (PF-299804), an irreversible anilinoquinazoline derivative, was a kind gift from Pfizer. Its structure has been published previously (23). For the in vitro experiments, it was dissolved in DMSO (20 mmol/L) and used as indicated. For mouse treatments, dacomitinib was dissolved in 20 mmol/L sodium lactate (pH 4; 1.5 mg/mL). Mice were administered dacomitinib (15 mg/kg/day, 5 days/week) or sodium lactate.

Heterotopic xenografts. For the tumor growth curves assays, treatment began when the subcutaneous tumors were noticeable and it was maintained for 3 to 4 weeks. Tumors were measured with a caliper twice a week. For short-term treatment, tumors were removed 5 days after the beginning of drug administration.

Orthotopic xenografts. Lactate or dacomitinib were administered to mice 2 to 3 weeks after the intracranial injections and were maintained until they were sacrificed.

In vitro assays
Cell viability was assessed by a colorimetric assay (WST-1 reagent; Roche) according to the manufacturer’s instruction. Cell proliferation assays were determined by 2 μg/mL 5-Bromo-2’-deoxyuridine (BrdUrd; Sigma-Aldrich) incorporation. In both cases, cells were treated for 3 days before processing. For self-renewal assays, cells were treated with dacomitinib or DMSO for 3 days and then single cells were plated in fresh medium in the absence of drugs at a density of 2.5 cells/μL. The number of neurosphere formed was counted 6 days later.

Quantitative real-time PCR
RNA was extracted from both frozen pellets of cells or frozen tissue sections with the RNeasy Kit (Qiagen). Total RNA (1 μg) was reverse transcribed with the SuperScript II Reverse Transcriptase (Invitrogen) and quantitative real-time PCR.

(qRT-PCR) was performed using the Light Cycler 480 (Roche) with SYBR Premix Ex Taq (TaKaRa). HPRT was used as an internal expression control. Primers used are indicated in the Supplementary Table S2.

Western blot analysis
For immunoblot analysis, cells or small tissue fragments were collected and processed as previously described (22). Primary and secondary antibodies used are shown in Supplementary Tables S3a and S3b.

Mice magnetic resonance imaging
Magnetic resonance imaging (MRI) analysis was performed in mice injected IP with Gd-DOTA (Dotarem; Guerbet). T1W images were acquired in a 4.7 T Biospec BMT 47/40 spectrometer (Bruker) with a spin-echo sequence.

Analysis of mouse tissue sections
Subcutaneous tumors were either embedded in paraffin or cryopreserved in 30% sucrose and cut in a cryostat at 20 μm. Sections for the intracranial xenografts, animals were perfused with 4% paraformaldehyde (PFA; Merck) and brains were cryo-protected and cut in a cryostat at 20 μm coronal sections. Immunostaining was performed as previously described (22).
Primary and secondary antibodies are shown in Supplementary Tables S3a and S3b.

Flow cytometry analysis
Disaggregated tumor cells were stained with anti-CD44-FITC (Immunotools) or AC133-PE (Miltenyi) and analyzed by flow cytometry (BD Biosciences).

Statistical analysis
The survival of nude mice was analyzed by the Kaplan–Meier method and was evaluated with a two-sided log-rank test. The Student t test or ANOVA test were performed for statistical analysis of in vitro studies. Data in graphs are presented as means ± SEM. * represents a P value ≤ 0.05; ** represents a P value ≤ 0.01; *** represents a P value ≤ 0.001. Statistical values of P > 0.05 were not considered significant.

Further information can be found in the Supplementary Methods.

Results
Dacomitinib exerts potent inhibitory effect on EGFR-amplified GBM tumor cells
We first evaluated whether dacomitinib modulated the behavior of a panel of GBM cells, grown in neurosphere culture conditions. The primary GBM cell lines were treated with increasing concentrations of dacomitinib over 72 hours and cell viability was measured. The cytostatic effect observed, reached a plateau at very low concentrations of the compound, and it was especially evident in those cell lines exhibiting EGFR amplification, regardless of the presence of the vIII isoform (Fig. 1A). In accordance with this, dacomitinib significantly inhibited proliferation of all EGFR-amplified (EGFRamp) GBM cells but not GBM5 and GBM6 that have no amplification of the gene (Fig. 1B). To determine whether the EGFR inhibitor affects the self-renewal capacity of GBM cells, the neurosphere cultures were maintained in the presence of dacomitinib for 72 hours, before isolating single cells from dissociated spheres and replating them at semi-clonal (2.5 cells/mL) densities in the absence of the drug. Dacomitinib significantly inhibited the growth of all the EGFRamp primary lines but not the EGFR-wild type (EGFRwt) ones, GBM5 and GBM6 (Fig. 1C).

Dacomitinib inhibits the growth of EGFRamp GBM xenografts
To address whether the in vitro effect of EGFR inhibition translated into a decreased growth of GBM cells in vivo, we injected 1 to 3.0 × 10⁶ cells into the flanks of immunodeficient mice, and when the tumors reached a minimal volume, the animals were separated into two groups: lactate- and dacomitinib-treated mice. Tumor size was measured during 3 to 6 weeks, until animals were sacrificed. Figure 2A shows how dacomitinib severely impairs the growth of EGFRamp GBM lines, but it does not affect the growth of EGFRwt-GBM5. At the endpoint, there was a clear difference in tumor size in the dacomitinib-treated animals, for all the EGFRamp lines (Supplementary Fig. S1). Subsequent analysis of the tumor tissue indicated that in the sensitive GBM cell lines dacomitinib induced a significant reduction in the number of mitotic figures (Fig. 2B) and a clear increase in the number of apoptotic cells (Fig. 2C). These results indicate that dacomitinib prevents GBM proliferation and survival, thereby decreasing the tumor burden of EGFR-dependent GBMs.

Effective blockade of EGFR signaling in dacomitinib-treated xenografts
So far, our results indicate that the effect of dacomitinib is EGFR-dependent as it does not produce a significant inhibition of GBM5, a primary cell line without EGFR amplification and showing low levels of receptor expression (22). To confirm
that dacomitinib is effectively targeting EGFR signaling, we investigated the phosphorylation status of the receptor and its downstream targets in the treated tumors. We first confirmed that several EGFR tyrosine residues were dephosphorylated in the dacomitinib-treated xenografts, both in GBM1 (Fig. 3A and B) and GBM4 (EGFRAMP-vIII positive; Fig. 3B). However, when we performed a phosphorylation screen for a selected panel of published EGFR pathway signal transducers, we only detected a small decrease in AKT and S6 phosphorylation inhibition after dacomitinib treatment (Supplementary Fig. S2). It is important to point out that there was a strong degree of variability between tumors and that the control tissues were rich in necrotic and inflammatory areas (due to the big size of the tumors) that could be affecting the activation of different signaling molecules. For that reason, we decided to treat two new cohorts of mice for 5 days. Three hours after the last injection, lactate or dacomitinib-treated tumors were excised and the levels of activation of the main EGFR downstream cascades were analyzed by Western blot (WB) analysis. Figure 3C shows that there is a clear dephosphorylation of AKT, ERK, and S6 after dacomitinib treatment. Interestingly there is no significant effect on the GBM5 xenografts. A similar result was observed by immunofluorescence (IF) analysis (Fig. 3D) confirming that systemic dacomitinib treatment was able to inhibit EGFR phosphorylation and downstream signaling in receptor-dependent GBMs.

**Dacomitinib inhibits intracranial tumor growth**

To assay if dacomitinib treatment could prevent intracranial growth, we injected 50,000 cells of GBM1 and GBM4 in the striatum of immunodeficient mice. Two (GBM4) to 3 (GBM1) weeks later, the mice were divided into two groups and lactate or dacomitinib was administered systemically. Kaplan–Meier analysis demonstrated that EGFR inhibition prolonged the survival of the GBM-bearing animals (Fig. 4A). Moreover, tumors formed were much smaller in the dacomitinib-treated group, as detected by immunofluorescence staining of the brain tissue with a human-specific anti-vimentin antibody (Fig. 4B) or by contrast-enhanced MRI images (GBM4; Fig. 4C). Subsequent analysis of the tumor tissue confirmed that there was a significant reduction of proliferation (5.8 \pm 0.6 mitosis/field in lactate-treated animals vs 2.5 \pm 0.4 in the dacomitinib-treated ones) and a clear induction of apoptosis in dacomitinib-treated animals (Fig. 4D). Moreover,
Figure 3. Dacomitinib blocks EGFR phosphorylation and signaling in vivo in EGFR amp GBMs. The phosphorylation status of EGFR after long-term treatment with lactate or dacomitinib was analyzed by WB (A) and/or IF (B) in GBM1 and GBM4 tumors. A, relative protein levels (a.u.) are represented in the right panel. Actin was used as a loading control. B, quantification of the fluorescence intensity (a.u.) is shown in the panels. WB (C) or IF (D) analysis of EGFR downstream signaling after short-term treatment of flank tumors. C, relative phosphorylation levels (a.u.) are represented in the right panels. GAPDH was used for normalization. D, quantification of the fluorescence intensity (a.u.) is shown in the right graphs. Scale bar, 50 μm in B and D (bigger panels), 10 μm in D (magnified pictures). *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001.
we confirmed the results obtained in the flank as we observed a strong downregulation of the phosphorylation of EGFR and its downstream targets (Fig. 4D). All these results indicate that systemic dacomitinib treatment is able to cross the blood–brain barrier (BBB) and effectively inhibits EGFR signaling in GBM brain xenografts, clearly impairing tumor burden.

**Effect of other mutations in the response of GBM primary cells to dacomitinib**

Based on the literature, it is not clear how EGFR mutations affect glioma sensitivity to dacomitinib (20, 21). However, the results presented here indicate that vIII expressing tumors are as sensitive as the wt EGFRamp ones. Interestingly, we were able to confirm that dacomitinib is equally efficient in the presence of another EGFR truncation (the vII isoform), that is present in 15% of EGFRamp GBMs (11). We injected $2.0 \times 10^6$ cells of GBM7 into the flanks of immunodeficient mice. When tumors reached a minimal volume, the animals were treated systemically with lactate or dacomitinib, and tumor size was measured during 4 weeks, until animals were sacrificed. Figure 5A shows a clear inhibition of tumor growth in the presence of the drug.

To test if chronic activation of AKT in the absence of PTEN could mediate resistance to EGFR inhibition, we inoculated $2.0 \times 10^6$ cells of GBM2 (a PTEN-deficient primary cell line; ref. 23) into the

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**Figure 4.** Intracranial GBM growth is impaired by systemic dacomitinib treatment. 50,000 GBM1 (A) and GBM4 (B) cells were implanted intracranially into nude mice. Two to 3 weeks later (arrow), the animals started to receive intragastric injections of vehicle (lactate) or dacomitinib (15 mg/kg/day, 5 days/week). Animal survival was evaluated using a Kaplan–Meier survival curve and the differences in survival times were analyzed with a log-rank test ($n = 5$). The images on the right show representative vimentin staining of tumors formed. C, representative contrast-enhanced MRI images at different time points after GBM4 brain injection. D, representative images of activated caspase 3 (Act Casp3), and phosphorylated EGFR, ERK, AKT, and S6 staining in vehicle- and dacomitinib-treated tumor tissues. Quantification of the fluorescence intensity (a.u.) is shown. Scale bar, 200 μm in B; 50 μm in D.
flanks of immunodeicient mice, which were treated as the previous ones. Figure 5B shows how dacomitinib produced only a limited effect on the growth of this EGFR amp GBM line. Alternatively, the animals were treated for only 5 days and tumors were excised 3 hours after the last treatment. Subsequent analysis of the tumor tissues indicated that whereas dacomitinib-treated tumors had statistically significant fewer phospho-EGFR positive cells (Fig. 5C), there was no clear impairment of AKT, ERK, or S6 phosphorylation (Fig. 5C and D). These results suggest that in the absence of PTEN function, dacomitinib can inhibit EGFR tyrosine-kinase activity but this is not sufficient to block downstream signaling and therefore tumor growth. Interestingly the lack of PTEN did not prevent the effect of dacomitinib in vitro (Fig. 1), suggesting that in the tumors there are other signals that compensate the inhibition of EGFR in the absence of PTEN function.

Dacotiminib reduces the aggressiveness and the stemness of EGFRamp GBMs

Histochemical analysis of the flank xenografts indicated an almost complete disappearance of mitosis and a change in the cellular morphology that suggested a less aggressive and more differentiated state on the dacomitinib-treated tumors (Fig. 6A). We therefore performed an RT-PCR analysis of several stem cell–related molecules. Although there was a high degree of variability between tumors, we detected a clear inhibition of some of them in the EGFRamp xenografts that had been treated with dacomitinib in the 5-day schedule (Supplementary Fig. S3) and even more after the long-term treatment (Fig. 6B and Supplementary Fig. S4). However, we were not able to detect any clear increase in differentiation markers in the treated tumors (Supplementary Fig. S4). Interestingly, we observed a significant decrease in the number of cells expressing stem cell–related markers (CD44 and CD133) at
cell surface in EGFR amp tumors that had been treated with dacomitinib, but not in the EGFRwt ones (Fig. 6C). These results suggest that EGFR inhibition is inducing the loss of stem-cell features and that this could be the reason for the less aggressive behavior of dacomitinib-treated tumors. However, EGFR inhibition does not seem to provoke the terminal differentiation of the cells (Supplementary Fig. S5). In fact, the effect of dacomitinib is reversible as tumor growth relapsed after drug removal (Fig. 6D).
suggesting that continuous regimes should be necessary to control GBM growth.

**Discussion**

Treatment options for GBM with standard cytotoxic agents are unsatisfactory, thus the development of effective therapeutic strategies is urgently needed. **EGFR** is amplified or mutated in a large number of glioma patients, but first generation of EGFR inhibitors have failed to show a clinical benefit. In this study we assessed the activity of dacomitinib, an irreversible PAN-HER inhibitor that is being tested in recurrent GBM, in a panel of primary GBM cells grown in vitro (as neurospheres) and in vivo using a nude mice xenograft model.

Our in vitro results indicate that dacomitinib acts in a specific way as it inhibits the proliferation and viability of the **EGFR**-Ramp but not the **EGFR**-wt GBM primary cell lines. Two other studies have recently demonstrated that the drug is able to inhibit EGFR phosphorylation and to reduce viability of different GBM cells (20, 21). Zhu and Shah have indicated that GBM cells, forced to overexpress the vIII mutant isoform of EGFR, do not respond so well to dacomitinib (20). However, our data show that all **EGFR**amp GBMs are sensitive to the drug, regardless of the presence of **EGFR** deletions. This would be in agreement with the other authors that have postulated that tumors harboring certain point mutations, as well as the vIII deletion, would be more responsive to dacomitinib (21). In fact, the presence of EGFRvIII, in a wild-type PTEN context, had been associated with GBM response to erlotinib (15), although this could not be confirmed in subsequent trials (24). Retrospective analysis of the two current phase II clinical trials would help to solve these discrepancies.

GBM cells grown in the absence of serum are enriched in the so-called cancer stem cells (CSC; ref. 25). These cells have a stronger self-renewal capacity than the rest of the tumor, which can be tested in a clonogenic assay. Our results indicate that dacomitinib clearly impairs the self-renewal of GBM-CSCs, but only if they show **EGFR** amplification. Moreover, we have confirmed the effect of EGFR inhibition in vivo as systemic dacomitinib treatment dramatically impairs the growth of **EGFR**-Ramp tumors. The posterior analysis indicated that dacomitinib induced an increase in the number of apoptotic cells. However, cell death does not seem to be the main response of GBM cells as the effect of the drug is reversible. Thus, when dacomitinib was removed, tumors restarted to grow rapidly demonstrating the specific effect of the compound and suggesting that continuous treatment should be needed to prevent tumor growth in patients. Interestingly, we have observed that EGFR dephosphorylation provokes the accumulation of the receptor (see Fig. 3A as an example). EGFR could be exerting some survival function in a kinase independent manner (9), which may represent an undesirable secondary effect of EGFR kinase inhibitors, and this could contribute to the reversibility of its effect.

Histologic analysis suggested that tumors exposed to dacomitinib have a more differentiated phenotype with less aggressive behavior. This is in agreement with the downregulation of several stem cell–related markers in dacomitinib-treated tumors. **EGFR** expression is a marker of proliferating neural stem cells and progenitors (26). Indeed, the presence of the receptor in the membrane marks a highly aggressive subpopulation of GBM-CSCs (27) and **EGFR** signaling has been linked to the expression of stem cell features in GBMs (28). More recently, it has been demonstrated that EGFR is downregulated upon GBM differentiation and that EGFR signaling blockade leads to decreased tumorigenic and stem cell–like potential of GBM neurospheres (29). Therefore, dacomitinib could be targeting specifically the GBM-CSC population. However, we do not observe a clear upregulation of terminally differentiated neural cells upon EGFR inhibition, and this could also explain why the effects of the drug are reversible and tumors restart when the treatment stops.

Two of the most consistently stem cell–related downregulated molecules after EGFR inhibition were LIF and II11. These two cytokines have been associated with the maintenance of self-renewal in normal and tumorigenic stem cells (30–32). These results suggest that EGFR could be modulating the maintenance of GBM stemness in a paracrine way and could correlate with the mosaic distribution of **EGFR** amplification and vIII expression in the tumors (33). Furthermore, part of the tumorigenic capacity of EGFR in GBM could be mediated by the secretion of cytokines (including LIF; ref. 34). Interestingly LIF secretion mediates also the tumorigenic potential of TGFβ in GBMs (32), suggesting a possible cross-talk between the two signaling pathways in such tumors, as it has been proposed by others (35). Therefore, a possible synergism between dacomitinib and TGFβ inhibitors, currently under development for the treatment of GBM, could be envisioned.

There have been doubts about the capability of the EGFR-TKI to reach the brain parenchyma and to cross the BBB (36, 37). Here, we demonstrate that dacomitinib can reach the brain and prevent intracranial growth, confirming the results obtained in the flank. In the case of gefitinib, very high concentrations of the drug were found in the resected tumors, together with an efficient EGFR dephosphorylation. However, the clinical results were discouraging, which seemed to correlate with the inefficient dephosphorylation of the EGFR downstream targets in vivo (both in patients and in the xenografts models; ref. 5). Our data with dacomitinib indicate in turn that this compound can efficiently inhibit tumor growth because it can target the EGFR signaling pathway, provoking the dephosphorylation of the main EGFR downstream cascades. This effect was not observed in the **EGFR**-wt tumors. These findings are in accordance with those found by Zhu and Shah (20) that demonstrated an inhibition of downstream molecules such as PLC. However, they only tested that in vitro (20). Although these authors suggest that multiple genetic lesions determine GBM response to dacomitinib, in fact, they show that the only sensitive GBM cell line to low doses of the compound is that with **EGFR** amplification. Although dacomitinib can inhibit HER2 and HER4 it does not seem to be relevant for GBM blockade as we could not detect expression of those receptors in any of the responsive lines (data not shown), in agreement with other observations (38, 39). Moreover, HER2 is not amplified in GBMs (40). Nevertheless, we also find that in the absence of PTEN function dacomitinib loses part of its antitumor capacity in vivo. However, we cannot discard that additional genetic alteration in GBM2 cells could be affecting the response to dacomitinib. Moreover, certain extracelular mutations in **EGFR** could make the tumors responsive to dacomitinib, even if PTEN is mutated (like in the case of U87 cells; ref. 21). Altogether, these results reinforce the notion that the characterization of **EGFR** status and PTEN function is fundamental to predict GBM response to dacomitinib (14). Another corollary
to all these data would be that PI3K-mTOR inhibitors could synergize with dacomitinib, as it has already been tested in glioma cell lines (20).

References

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Dacomitinib Inhibits Glioblastoma Growth

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dacomitinib could be an active drug in GBM as it is able to inhibit tumor growth in vitro and in vivo of EGFRamp tumors and it is able to reach the brain parenchyma. The growth inhibition is based on the dephosphorylation of the downstream effectors and a possible paracrine effect mediated by stem cell–related cytokines. Moreover, based on our data dacomitinib treatment should be given in a continuous regime and would be effective even in the presence of the EGFR-mutant isoforms. Furthermore, the presence of an active PTEN activity should be checked as a predictive marker. Current clinical trials testing dacomitinib in GBM will shed light on these affirmations but dacomitinib seems to be a promising treatment for newly diagnosed glioblastoma, alone or in combination with cytotoxic agents, molecules that could favor EGFR degradation and TGFβ or PI3K–mTOR inhibitors. It will be particularly relevant to determine if those synergistic therapies would induce then, an irreversible tumor growth inhibition.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

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