

**Cancer Therapy: Preclinical**

See commentary by Yao and Phan, p. 5217

**Brivanib, a Dual FGF/VEGF Inhibitor, Is Active Both First and Second Line against Mouse Pancreatic Neuroendocrine Tumors Developing Adaptive/Evasive Resistance to VEGF Inhibition**Elizabeth Allen<sup>1,2</sup>, Ian B. Walters<sup>3</sup>, and Douglas Hanahan<sup>1,2</sup>**Abstract**

**Purpose:** Preclinical trials of a mouse model of pancreatic neuroendocrine tumors (PNET) were conducted to determine whether dual FGF/VEGF pathway inhibition with brivanib can improve first-line efficacy in comparison with VEGF inhibitors lacking fibroblast growth factor (FGF)-inhibitory activity and to characterize second-line brivanib activity before and after the onset of evasive resistance to VEGF-selective therapy.

**Experimental Design:** An anti-VEGFR2 monoclonal antibody (DC101), an inhibitor of FGF signaling (FGF ligand trap), sorafenib, and brivanib were comparatively evaluated in first-line monotherapy in short and longer term fixed endpoint intervention trials in the RIP-Tag2 mouse model of PNET. Brivanib was also tested second line aiming to block adaptive resistance to selective VEGF therapies, assessing tumor growth, vascularity, hypoxia, invasion, and metastasis. The effects of initiating second-line brivanib therapy prior to or following overt relapse on sorafenib therapy were compared in overall survival trials to first-line therapies.

**Results:** Brivanib produced enduring tumor stasis and angiogenic blockade, both first and second line following the failure of DC101 or sorafenib. Overall survival was significantly extended by brivanib versus sorafenib, both first-line and when second-line therapy was initiated prior to sorafenib failure; second-line brivanib was less beneficial when initiated later, after the initiation of revascularization and incipient tumor progression.

**Conclusions:** Brivanib holds promise and deserves consideration for clinical evaluation as an anti-angiogenic therapy, both in the context of impending failures of VEGF-selective therapy and in a first-line setting aiming to limit the adaptive response to VEGF inhibitors that results in evasive resistance. *Clin Cancer Res*; 17(16); 5299–310. ©2011 AACR.

**Introduction**

Tumor angiogenesis depends on the balance of pro- and antiangiogenic signaling circuits. The most prominent regulatory factor is VEGF-A, which signals via 3 receptor tyrosine kinases (RTK) expressed in endothelial cells. The importance of VEGF signaling for tumor growth has been documented in multiple preclinical studies, as well as in

clinical trials that have led to the approval of 4 VEGF inhibitors for use in specific human cancers (refs. 1–8 and references therein). A second proangiogenic regulatory circuit involves fibroblast growth factors (FGF), and their cognate receptors, in particular FGFR1 (refs. 9–12 and references therein). Although the activity of FGF ligands as angiogenesis inducers has been long recognized, the general importance of FGF signaling for tumor angiogenesis has remained unclear, reflecting the focus on the central role played by VEGF signaling. However, studies in mouse cancer models show a functional role for FGF signaling in tumor angiogenesis (10, 13, 14). Pertinent to the present study are analyses of the roles of FGF/VEGF signaling in a mouse model of pancreatic neuroendocrine tumor (PNET), the RIP1-Tag2 (RT2) line of transgenic mice, which develop multiple tumors under tight developmental regulation (ref. 15; see also Materials and Methods). Previous studies indicate that targeting both the VEGF and FGF signaling pathways inhibits tumor growth in RT2 mice, with VEGF signaling predominating in initiation of tumor angiogenesis, whereas FGF signaling contributes in a

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**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org>).

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### Translational Relevance

Antiangiogenic therapies are producing initial responses followed by progression and modest survival benefits in multiple clinical indications. Preclinical trials in a mouse pancreatic neuroendocrine tumors (PNET) model presaged this outcome, revealing a response period followed by adaptive/evasive resistance to anti-VEGF therapy driven by alternative proangiogenic signals, predominantly fibroblast growth factor (FGF) signaling, which have also been implicated in resistance to human glioblastoma. These results prompted investigation of brivanib, which cotargets FGF/VEGF signaling and is currently in phase 2/3 trials. Brivanib showed second-line efficacy following failure of 2 VEGF receptor (VEGFR) inhibitors in mouse PNET and proved even more efficacious when used first line, suggesting that it delays induction of evasive resistance. Histopathologic evidence suggests that second-line dosing may be more effective when the switch from selective VEGFR inhibitors to brivanib is made concomitant with early signs of revascularization, prior to detectable tumor regrowth. The results warrant consideration in clinical trial designs, aiming to limit evasive resistance to VEGF inhibitors.

collaborative fashion to its maintenance (16). A subsequent study investigating the basis for the observed relapse to progressive disease following a period of response to a VEGF receptor (VEGFR) inhibition revealed upregulation of FGF ligands concomitant with VEGF-independent revascularization of the tumors; layering an anti-FGF therapy (FGF-trap, which captures multiple FGF ligands to limit FGFR signaling) on top of an antibody inhibiting VEGFR2 (DC101, which blocks binding of VEGF to VEGFR2) at the time of relapse attenuated both the revascularization and tumor growth (17). More recently, anti-VEGF therapy has also been shown in multiple tumor models to elicit other forms of adaptive resistance involving recruitment of proangiogenic inflammatory cells (18), heightened invasiveness (19, 20), and/or increased rates of metastasis (20–22). The realization that tumors can develop forms of adaptive resistance that evade continuing blockade of VEGF signaling naturally suggests that agents targeting such evasive resistance mechanisms might render VEGF therapy more enduring (ref. 23 and references therein). Toward that end, we have evaluated an investigational drug, brivanib, a selective RTK inhibitor that targets signaling via VEGFR2 and 3, and FGFR1, 2 and 3 (24–29). Currently, brivanib therapy is being evaluated in phase III clinical trials in colorectal carcinoma and hepatocellular carcinoma (HCC; ref. 5) and in phase II trials for numerous indications including brivanib second-line therapy following sorafenib failure (see ClinicalTrials.gov).

To assess the efficacy of dual targeting of VEGF and FGF signaling by brivanib, we conducted comparative fixed

endpoint, first- and second-line trials utilizing target-selective inhibitors of VEGFR2 (DC101) and FGFRs (FGF-trap) in RT2 mice. Furthermore, first- and second-line brivanib dosing was tested in short and long fixed endpoint trial, and in survival trials, versus sorafenib, a multikinase inhibitor of VEGFR2, platelet-derived growth factor receptor (PDGFR)  $\beta$ , and RAF (30) that is clinically approved for renal cell carcinoma and HCC. Specifically, we assessed whether brivanib therapy could limit the adaptive resistance that characterizes VEGF-targeted therapies and whether there was a differential effect of initiating second-line brivanib prior to or following anti-VEGF therapeutic failure.

### Materials and Methods

#### Mice and trial design

The generation and characterization of the single transgenic RT2 mice, and the immunocompromised RIP1-Tag2; *Rag1*-null (RT2;*Rag1*-null) mice has been previously described (15, 17). Briefly, RT2 mice undergo multifocal stepwise tumorigenesis, producing hyper- and dysplastic islets, a subset of which subsequently undergoes an angiogenic switch, leading in turn to formation of highly angiogenic PNET starting around 10 weeks; mice die at 15 to 16 weeks with a burden of 5 to 15 independent large, red, hemorrhagic PNETs. Trial arms that involved DC101 and their controls utilized RT2;*Rag1*-null mice to obviate potential production of neutralizing antibodies to DC101 that could interfere with its therapeutic activity. Trial designs utilized in this study (intervention, regression, and survival) are depicted in Supplementary Figure S1.

#### Therapeutic agents

DC101 is a rat monoclonal antibody that specifically targets the VEGF signaling pathway by blocking the binding of VEGF to VEGFR2 (31); mice were dosed twice weekly with 1 mg per mouse, as previously described (17). FGF-trap is a fusion of mouse immunoglobulin Fc with a soluble FGFR construct (sFGFR) that captures FGF1, 2, 3, 7, and 10, thus inhibiting ligand-dependent FGFR signaling (16); mice were dosed with an adenovirus vector expressing FGF-trap ( $8 \times 10^8$  plaque-forming units) every 10 days, as previously described (17). Dose escalation studies using sorafenib (30) were previously conducted, indicating a maximal response between 30 and 60 mg/kg, whereas brivanib produced a maximal response between 60 and 90 mg/kg (29); consequently, mice were dosed at approximately the midline level (40 and 75 mg/kg, respectively). Please see additional Materials and Methods in the Supplementary section.

### Results

#### Brivanib monotherapy: survival and fixed endpoint tumor regression trials

Survival trials were conducted to determine whether brivanib monotherapy has potential to extend the life span

of treated animals, and the results of a Kaplan–Meier analysis were plotted. Cohorts of 14 controls and 13 brivanib-treated RT2 mice were treated from 13 weeks until endpoint, revealing a statistically significant survival benefit in brivanib-treated animals ( $19.6 \pm 2.4$  weeks) versus untreated control animals ( $16 \pm 0.85$  weeks; Supplementary Fig. S2A), an appreciable extension of life span in this rapidly progressive and multifocal mouse model of cancer. Because of the efficacy of brivanib in survival trials, fixed endpoint regression trials were next undertaken to determine whether this effect can be attributed to reduced tumor growth in treated animals and also to characterize the time course of therapeutic response. Regression trials were conducted in RT2 mice commencing at 13 weeks of age and ending at 15, 17, or 18.5 weeks. The results indicate that brivanib produces tumor stasis during the entire 5.5 weeks of therapy (Supplementary Fig. S2B).

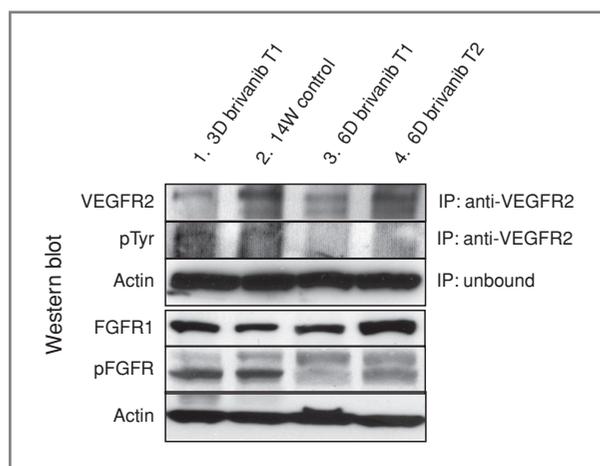
### Target inhibition of VEGFR2 and FGFR

To assess the specific inhibition of pVEGFR2 and pFGFR signaling by brivanib, short-term molecular efficacy trials were conducted, and brivanib-treated and control untreated tumors were assessed for pVEGFR2 and pFGFR levels. Three days of brivanib treatment starting at week 14 did not appreciably reduce vascularization, and tumors expressed high levels of both proteins (Fig. 1, lane 1, pTyr/ip-VEGFR and pFGFR). In contrast, tumors analyzed after 6 days of brivanib treatment starting at 14 weeks showed evident inhibition of both phosphoproteins (Fig. 1, lanes 3–4, pTyr/ip-VEGFR and pFGFR) in contrast

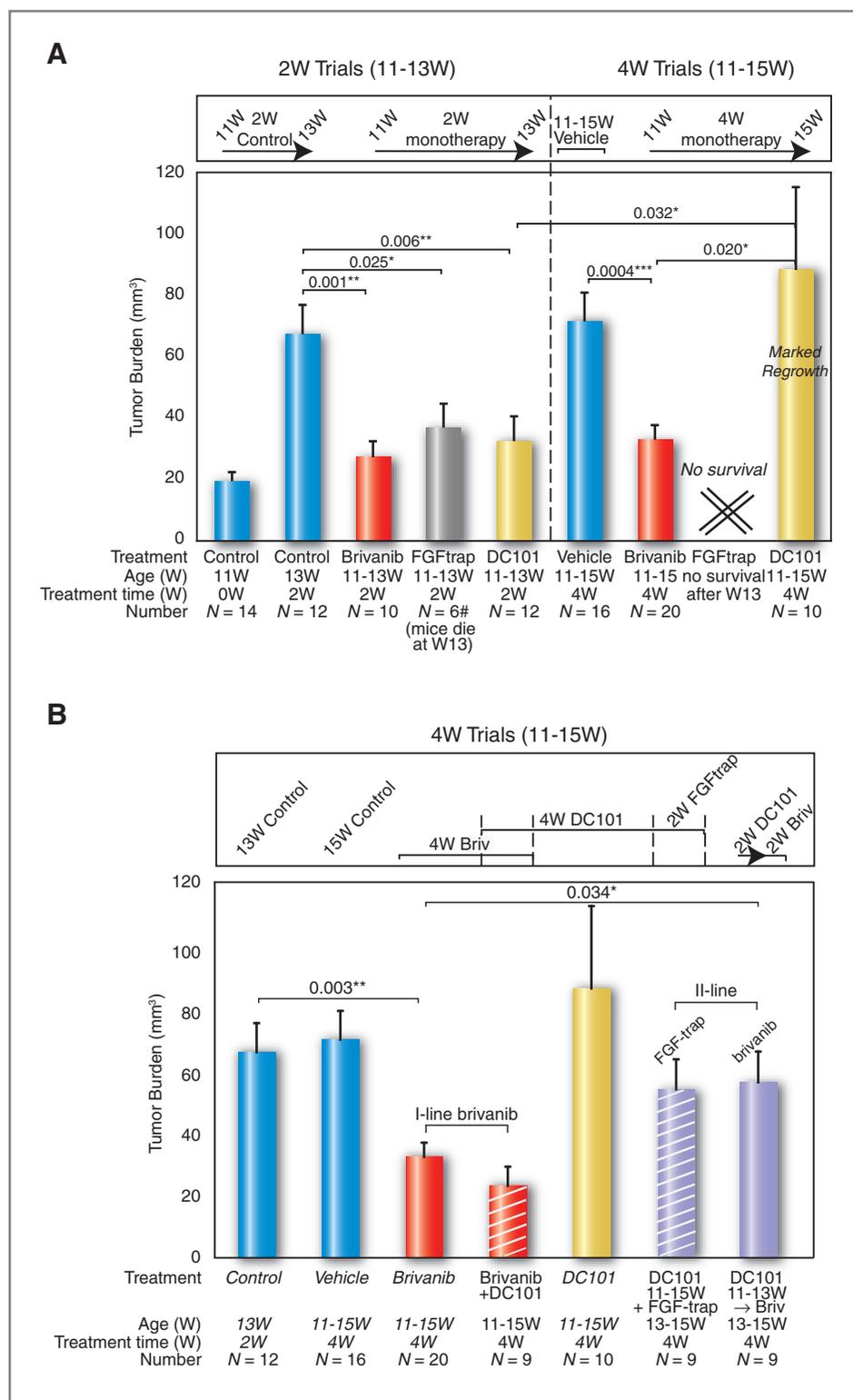
to 14-week control untreated tumors (lane 2, pTyr/ip-VEGFR and pFGFR) and 3-day-treated tumors, indicative of target modulation. Furthermore, longer term brivanib treatment may reduce abundance of VEGFR2-expressing endothelial cells (EC): Expression profiling of RNAs isolated from tumors of 2-week brivanib-treated mice indicated that treatment markedly reduced levels of EC-restricted VEGFR2 mRNA, whereas 6-week-treated tumors also showed considerably reduced levels of VEGFR2 protein in Western blot analysis (data not shown). This notion is also supported by immunostaining of tumor tissue sections using antibodies to an EC-specific marker, Meca32, which also showed reduced abundance of ECs [Fig. 4A (ii and ii')]. Collectively, the data may reflect both inhibition of EC proliferation and vascular dropout consequent to EC death. Although brivanib has been shown to directly inhibit proliferation of cultured human cancer cells that overexpress FGF receptors (32), we have been unable to show antiproliferative effects of brivanib on cultured  $\beta$  tumor cells derived from these PNET tumors (data not shown).

### Comparative efficacy of brivanib monotherapy versus specific VEGFR2 or FGF inhibitors: first-line intervention trials in RT2/RagKO mice

To determine the dual targeting efficacy of brivanib versus single VEGFR2 (DC101) or FGF (FGF-trap) inhibition, intervention trials were conducted in RT2;*Rag1*-null mice (Fig. 2A). DC101 was previously used to show that VEGF/VEGFR2 signaling is primarily responsible for angiogenic switching and neovascularization to enable PNET tumor growth in RT2 (17), and FGF-trap was previously used to block VEGF-independent revascularization in the same model (16). Although DC101 treatment was efficacious in reducing tumor burden during a 2-week treatment period (Fig. 2A, yellow bars), tumors had begun to regrow after another 2 weeks of treatment, reflected in increased tumor burden (Fig. 2A, yellow bars, 4-week trials), indicative of therapeutic failure over this time course, consistent with previous results (17). Regrowth after 4 weeks of therapy with DC101 was concomitant with indications of incipient failure, evidenced by hypoxia and revascularization of some 2-week DC101-treated tumors [Fig. 3B (i)], again consistent with our previous report (17). In comparison, there was a significantly reduced tumor burden in brivanib-only-treated mice after 2 and 4 weeks of treatment (Fig. 2A, red bars) versus age-matched 13- and 15-week old controls. Notably, there was no significant difference in tumor burden between 11-week-old RT2 mice and brivanib-treated mice at 13 and 15 weeks, after 2 or 4 weeks on trial (*i.e.* the tumors did not increase in size vs. the starting time point, a condition of "stable disease"; Fig. 2A, blue bars). Moreover, the tumor burden in brivanib-only-treated mice was significantly lower than that of DC101-treated mice over the 4-week time course. Although surviving mice treated for 2 weeks with FGF-trap monotherapy showed a significant reduction in tumor volume versus similarly treated controls (gray



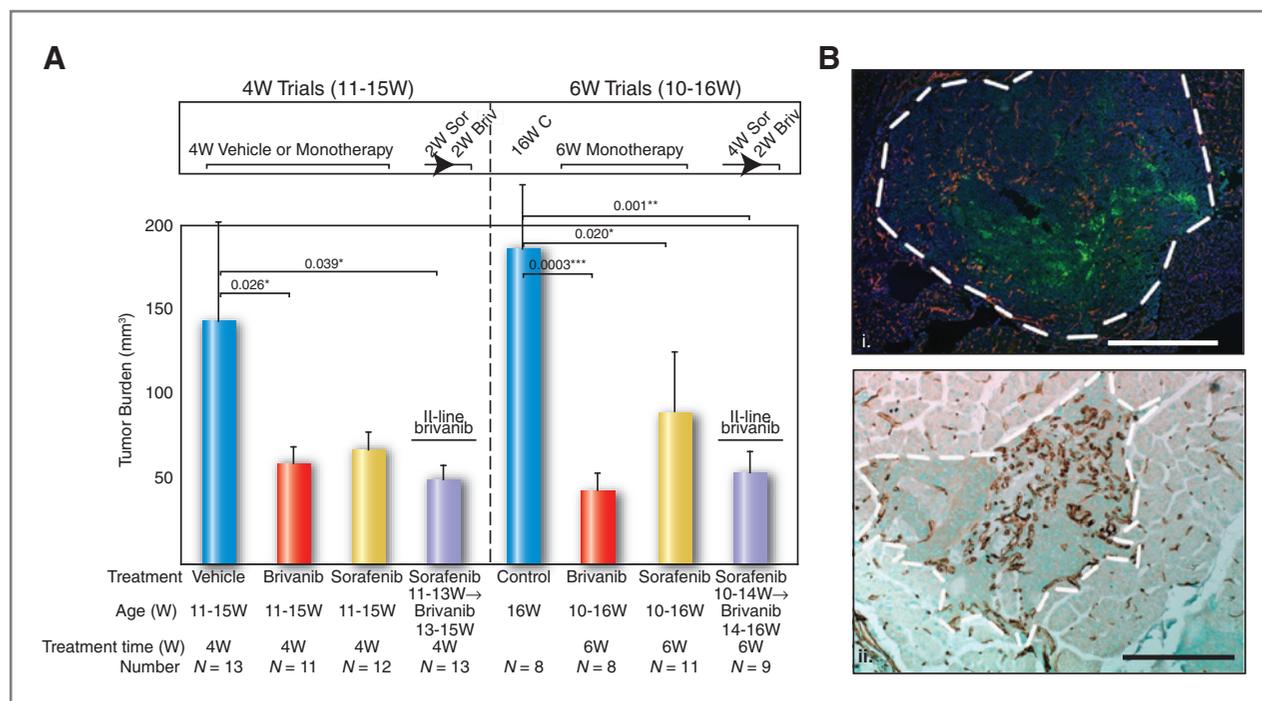
**Figure 1.** Target inhibition of VEGFR2 and FGFR in brivanib-treated tumors. Protein lysates were prepared from short-term brivanib-treated total tumors after 3 days (3D) or 6 days (6D; starting at 14 weeks of age), and equal levels of each are shown in lanes 1, 3, and 4, along with control lysates from 14 week (14W) untreated tumors in lane 2. Lysates were immunoprecipitated (IP) with anti-VEGFR2 and analyzed by Western blotting with anti-VEGFR2 or pTyr; equal fractions of each lysate were blotted with  $\beta$ -actin (top actin) as a measure of relative levels of total protein used for IP. The bottom 3 depict Western blot analysis of equal levels of lysate probed with pFGFR antibody (middle) and sequentially stripped and reprobed with anti-FGFR1 (top), followed by anti- $\beta$ -actin (bottom actin).



**Figure 2.** Comparative efficacy of brivanib versus pure VEGFR2 and FGFR inhibitors. **A**, first-line (I-line) brivanib versus DC101 and FGF-trap. To assess efficacy of brivanib, intervention trials were conducted in compound RIP1-Tag2 (RT2), *Rag1*-null mice for 2 weeks (left) or 4 weeks (right). There was no significant difference in tumor burden at 2 weeks between any of the treatments (brivanib, DC101, and FGF-trap). After 4 weeks, the DC101 arm had significantly larger tumors than at 2 weeks and compared with 2 and 4 weeks of brivanib treatment. FGF-trap had equivocal efficacy, as mice did not survive longer than the first 2 weeks of monotherapy, as indicated by the X in the figure for the 4W FGF-trap trial arm. Mean values  $\pm$  SEM are indicated. Two-tailed Mann-Whitney *U* test for statistical significance: \*,  $P = 0.05-0.01$ ; \*\*,  $P = 0.009-0.001$ ; \*\*\*,  $P < 0.001$ . **B**, second-line brivanib versus combined DC101 and FGF-trap. To assess efficacy of brivanib as a second-line (II-line) therapeutic, an additional intervention trial was conducted in RT2;*Rag1*-null mice. Following 2 weeks of DC101 monotherapy, at which point FGF-dependent revascularization is being induced either FGF-Trap was layered on top of DC101 for 2 weeks of combined therapy or brivanib was substituted for DC101 as a sole second-line therapeutic. An additional arm combined DC101 and brivanib throughout the 4-week long trial, which produced a slightly better but statistically insignificant effect on tumor burden. To facilitate comparison, the monotherapy trial arms (from A) redisplayed here are shown in bold italics and green font. Mean values  $\pm$  SEM are indicated. Two-tailed Mann-Whitney *U* test: \*,  $P = 0.01-0.05$ ; \*\*,  $P < 0.009$ .

bars), this therapy produced red, highly vascular tumors (data not shown) and no demonstrable survival benefit from therapy—mice were found dead or became moribund and were sacrificed prior to the second 2-week

phase of the trial, in contrast to mice from all the other treatment arms. This equivocal efficacy is consistent with a previous study suggesting that FGF signaling is normally secondary to VEGF signaling (16), such that FGF signaling



**Figure 3.** Comparative efficacy of brivanib versus sorafenib, a clinically approved VEGFR/PDGFR inhibitor, in intervention trials in RT2 mice. **A**, intervention trials: brivanib versus sorafenib. Four-week intervention trials conducted in RT2 mice showed similar efficacy for brivanib and sorafenib, and a sequential 2-week regimen of sorafenib followed by brivanib (left). Because a subset of sorafenib-treated tumors evidenced signs of therapeutic failure at 4 weeks [exemplified in **B** (ii)], we assessed brivanib second line following the impending failure after 4 weeks of sorafenib (right). Six weeks of sorafenib resulted in a modest but statistically insignificant increase in tumor burden versus the first- and second-line brivanib arms, which produced stable disease over this same time course. Mean values  $\pm$  SEM are indicated. Two-tailed Mann-Whitney  $U$  test: \*,  $P = 0.05$ – $0.01$ ; \*\*,  $P = 0.009$ – $0.001$ ; \*\*\*,  $P < 0.0009$ . **B**, adaptation in DC101- and sorafenib-treated tumors. **i**, a tumor treated for 2 weeks with DC101 that shows distinct regions of hypoxia and revascularization, visualized by immunofluorescence for pimonidazole (hypoxia; green) and anti-Meca32 (blood vessels; red). The result is representative of several similar tumors in the animal, although those in another DC101-treated member of the cohort showed no signs of revascularization, indicative of a heterogeneity in the adaptive response. **ii**, a tumor after 4 weeks of sorafenib treatment that has begun to undergo evasion from antiangiogenic therapy, as evidenced by revascularization and invasive margins. The blood vessels were visualized by staining with anti-Meca32 and visualized with horseradish peroxidase. Such marked revascularization was infrequent after 4 weeks of sorafenib monotherapy (and was not observed after 4 weeks of brivanib treatment) but was widespread in 2 surviving animals after 7.1 to 7.6 weeks of sorafenib treatment [see Fig. 5B iv]. The white dashed line delineates the neoplastic lesion in both panels. Scale bar represents 400  $\mu$ m (i) and 100  $\mu$ m (ii).

acts (in untreated mice) as a supplementary angiogenesis enhancer.

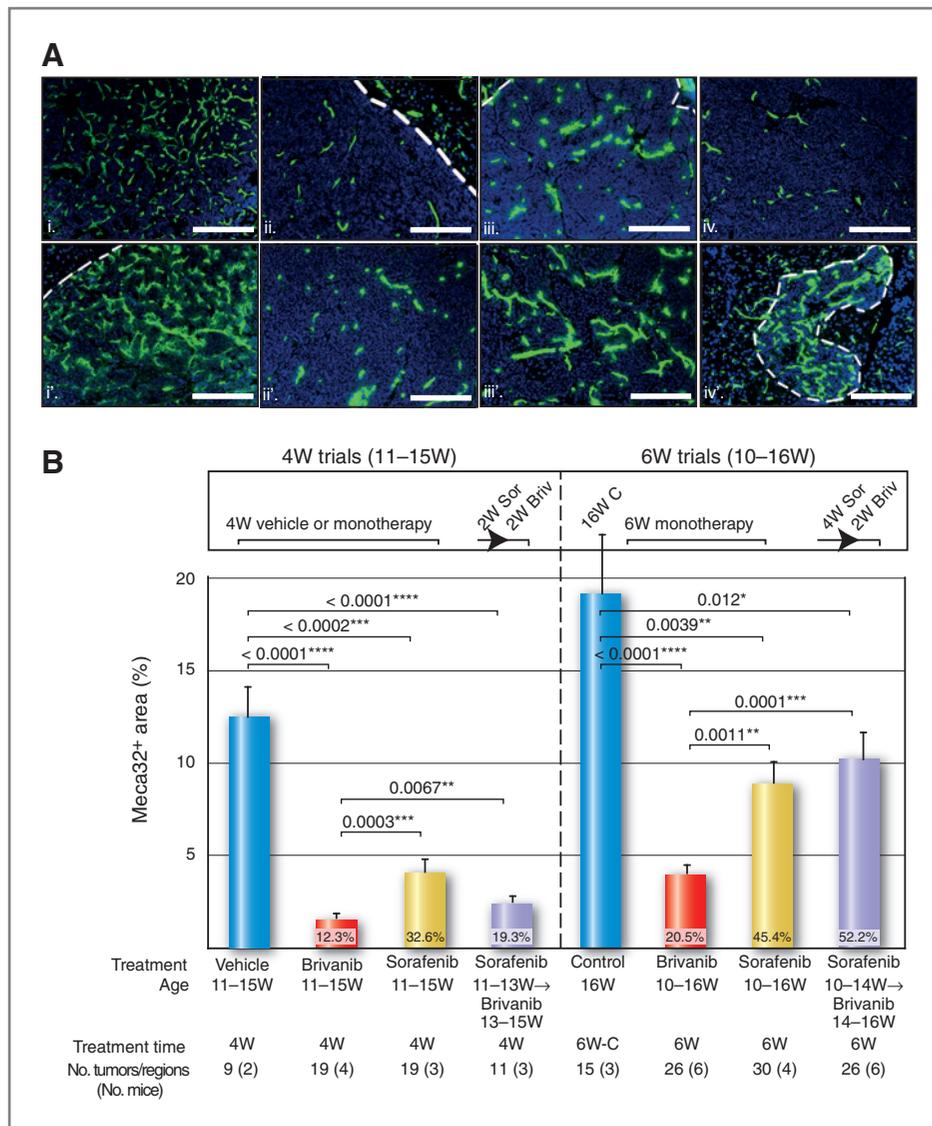
### Brivanib second line versus combined DC101 and FGF-trap therapy

To determine the efficacy of brivanib as a second-line therapeutic following DC101 failure, additional intervention trials were conducted in RT2;*Rag1*-null mice (Fig. 2B). In the interest of clarity and to judge the efficacy of monotherapy versus second-line dosing, trial arms from Figure 2A are redisplayed here and labeled below in green italics. There is a significant difference in efficacy of brivanib as a first- (red bar) versus second-line therapeutic (purple bar). In addition, brivanib produced comparable efficacy as a second-line therapeutic (weeks 13–15) following 2 weeks of DC101 (weeks 11–13) to a second-line combination therapy of DC101 plus FGF-trap (weeks 13–15), again following 2 weeks of DC101 monotherapy. Moreover, brivanib and FGF-trap second-line dosing was found to trend toward superiority to 4 weeks

of continuous DC101 monotherapy, although this result did not reach statistical significance. A trial arm using 4 weeks of combined DC101 and brivanib first-line treatment was conducted to rule out the unlikely possibility that DC101 treatment promotes tumor growth. The result was statistically indistinguishable from brivanib monotherapy, evidencing potent VEGFR2 inhibition by brivanib and a lack of antagonism between the 2 drugs.

### Comparative efficacy of brivanib versus sorafenib, a clinically approved VEGFR/PDGFR inhibitor, in intervention trials

We next assessed efficacy of brivanib as a therapeutic versus a clinically relevant multi-RTK inhibitor, sorafenib. First- and second-line intervention trials were conducted using brivanib and sorafenib in 4- and 6-week fixed endpoint trials in RT2 mice. Fixed endpoint 4-week trials resulted in similar efficacy (assessed as tumor burden and vascularity) between first-line brivanib (Fig. 3A, red bars,



**Figure 4.** Vascularity of brivanib/sorafenib-treated tumors in fixed endpoint trials. **A**, immunostaining analysis of vascularity in 4- and 6-week fixed endpoint brivanib/sorafenib trials. Mice were treated for 4 weeks [from 11–15 weeks of age; top (i–iv)] or for 6 weeks [from 10–16 weeks; bottom, (i'–iv')] and analyzed for tumor vascularity with Meca32 (green). Control untreated tumors showed characteristically high vascularity (i and i'). Brivanib monotherapy (ii and ii'), sorafenib monotherapy (iii and iii'), or 2 weeks of sorafenib followed by 2 weeks of brivanib (iv) or 4 weeks of sorafenib followed by 2 weeks of brivanib (iv') are shown. All 4 week treatments produced markedly less vascularized tumors than controls. After 6 weeks of treatment, the majority of Brivanib-treated tumors (ii') showed no sign of revascularization, in contrast to 6 weeks of sorafenib-treated tumors (iii'); surprisingly, although the 4-week sorafenib → 2-week brivanib arm shows no increase in tumor size (Fig. 3A), some tumors are extensively revascularized (iv'), suggestive of impending relapse to progressive growth. Scale bars represent 100  $\mu$ m. **B**, quantification of vascularity. Multiple tumors and regions within each tumor (see Supplementary Materials and Methods) were immunostained with Meca32, and metamorph analysis was used to quantitatively assess total tumor vascularity. Results from each treatment group were combined. Both 4- and 6-week brivanib monotherapy produced significantly lower levels of vascularity than other treatment groups. Tumor vascularity was markedly higher following 6 weeks of sorafenib monotherapy, as well as from second-line brivanib following sorafenib (4 weeks of sorafenib followed by 2 weeks of brivanib) as compared with first-line brivanib. All treatment regimens produced significantly lower vascularity than untreated tumors. Mean values  $\pm$  SEM are indicated. Two-tailed Mann–Whitney *U* test: \*,  $P = 0.010$ – $0.050$ ; \*\*,  $P = 0.001$ – $0.009$ ; \*\*\*,  $P = 0.0001$ – $0.0009$ ; \*\*\*\*,  $P < 0.0001$ .

4-week trials) and second-line brivanib (2-week first-line sorafenib → 2-week second brivanib, purple bars) treatment, and versus sorafenib monotherapy (yellow bars). However, the 4-week sorafenib monotherapy occasionally produced small, highly vascularized tumors, a sign of incipient therapeutic failure [Fig. 3B (ii)]; thus sorafenib is eliciting adaptive resistance, albeit more slowly than

DC101, after 4 versus 2 weeks of treatment, respectively. To assess efficacy of brivanib as a second-line inhibitor following sorafenib failure (after 4 weeks of therapy), 6-week trials were conducted. While 6 weeks of sorafenib monotherapy produced somewhat larger tumors than brivanib monotherapy, there was no significant difference in tumor burden between treatment arms.

### Adaptation in DC101- and sorafenib-treated tumors

Figure 3B (i) depicts a treated tumor from a time point consistent with the initial onset of evasive resistance to anti-VEGFR2 therapy (after 2 weeks of DC101), still prior to measurable tumor regrowth. Avascular regions of intense hypoxia [pimonidazole (pimo), green] are surrounded by islands of revascularized tumor (visualized with anti-Meca32, an endothelial marker, red). Figure 3B (ii) depicts a rare, revascularized tumor following 4 weeks of sorafenib monotherapy. Recent studies indicate that antiangiogenic therapy can elicit increased invasion (19, 20), and therefore, control and inhibitor-treated tumors were analyzed for invasiveness. Supplementary Figure S3A (i) depicts highly invasive tumor masses resulting from 4 weeks of DC101 monotherapy that has spread throughout multiple pancreatic lobes through all sections analyzed. A similar mass was found in 1 of 5 mice treated with 2 weeks first-line DC101 followed by 2 weeks second-line brivanib; this tumor also extends through the entire depth of analyzed tissue [Supplementary Fig. S3A (ii)]. In contrast, the most invasive tumor found in 1 of 5 Brivanib-treated mice is depicted in Supplementary Figure S3A (iii), appears more focal, and it does not extend as deeply into adjacent tissue. Statistical analysis of invasiveness revealed an increased incidence of highly invasive lesions compared with control untreated tumors for DC101 and the combined DC101 followed by second-line brivanib-treated tumors (Supplementary Fig. S3B). Although first-line brivanib produced more invasive tumors than control, untreated tumors, the brivanib therapy produced fewer invasive tumors than DC101. Although there is no statistical difference in the incidence of micrometastasis between control or treatment arms (Supplementary Fig. S3C), 1 DC101-treated mouse (of 5 total) had high number (20) of liver micrometastases, in contrast to all other cohorts.

### Vascularity of brivanib/sorafenib-treated tumors in fixed endpoint trials

In contrast to the lack of significant differences in tumor burden between the various 4- and 6-week treatment arms, striking differences in vascularity were evident (Fig. 4A and B), most notably following 6 weeks of treatment. Four weeks of therapy produces significantly reduced vascularity for first-line brivanib versus other treatment arms, with the highest levels of vascularity resulting from 4 weeks of sorafenib monotherapy. This difference becomes more pronounced following 6 weeks of treatment (Fig. 4B, metamorph analysis, right vs. left). Surprisingly, although the regimen of 4 weeks of first-line sorafenib followed by 2 weeks of second-line brivanib arm showed no sign of tumor regrowth, tumors became extensively revascularized [Fig. 4A (vi), Meca32, and 4B, right], an indicator of incipient therapeutic failure that motivated further investigation.

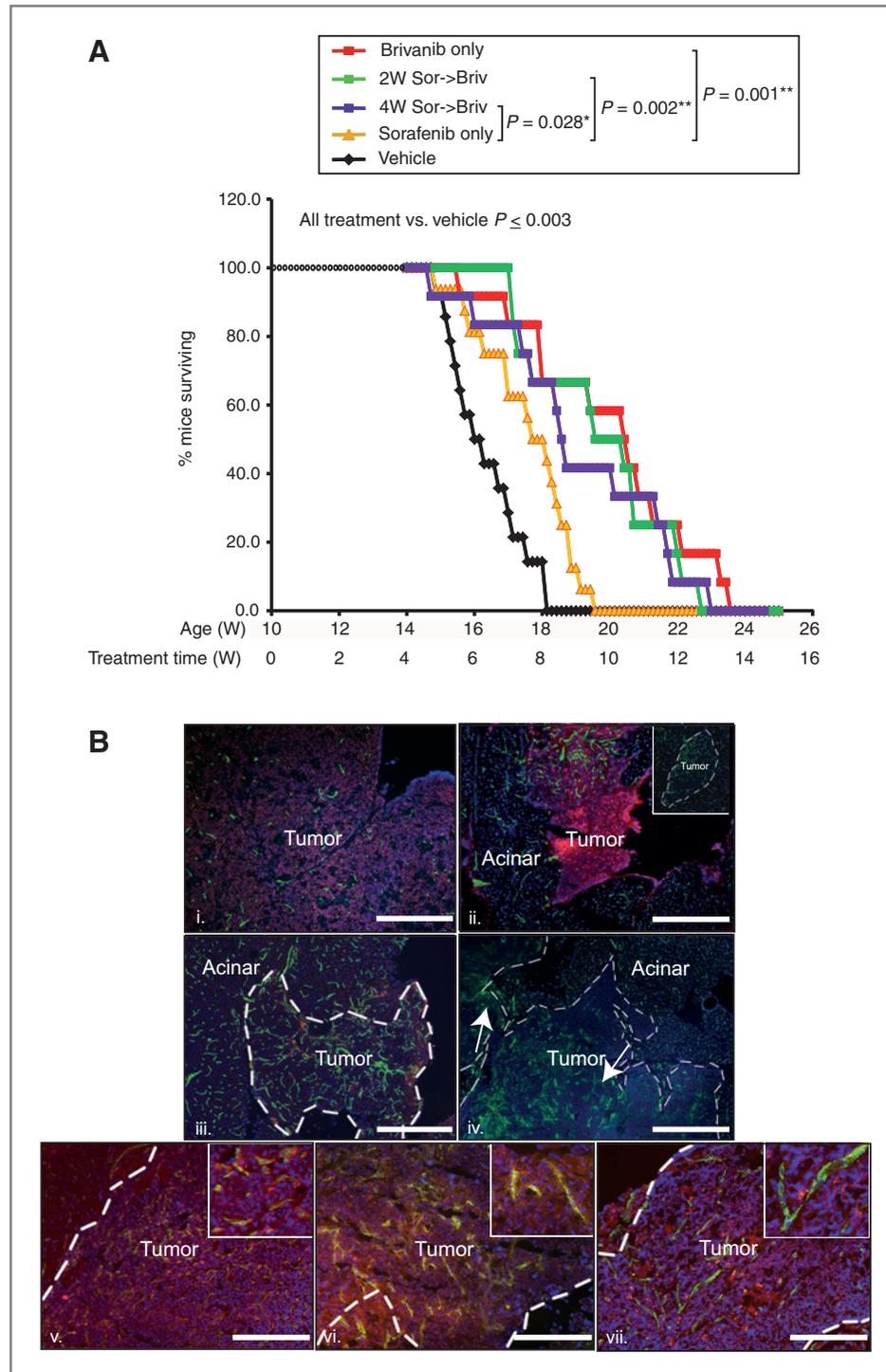
### Survival trials: first- and second-line brivanib versus sorafenib

Results implicating incipient adaptive resistance in the fixed endpoint trials prompted survival trials in which

second-line brivanib dosing commenced prior to sorafenib failure (2 weeks of first-line sorafenib followed by second-line brivanib until end stage) or concomitant with sorafenib failure (4 weeks of first-line sorafenib followed by second-line brivanib until end stage), along with appropriate monotherapy controls. Cohorts of mice were treated starting at 10 weeks of age until death (Fig. 5A) and evaluated by Kaplan–Meier analysis. Although mice were dosed from the 10-week time point, survival statistics are depicted starting at week 14, as dosing for the 4 weeks of first-line sorafenib followed by second-line brivanib cohort begins at that time; prior to that time, the cohort is receiving sorafenib monotherapy. Overall survival calculated starting at 10 weeks is not significantly different than that depicted here (Supplementary Table S2). All treatment arms, including sorafenib monotherapy, led to a significant survival advantage versus control mice. There was, however, a markedly increased survival benefit with the brivanib monotherapy arm and the arm involving earlier brivanib second-line dosing (2 weeks first-line sorafenib followed by second-line brivanib) versus sorafenib monotherapy. There was also a statistically significant benefit derived from delayed initiation of second-line brivanib at the point of histologic sorafenib failure (4 weeks first-line sorafenib followed by second-line brivanib) versus continued sorafenib monotherapy. This delayed second-line therapy produced a shoulder in the survival curve at about 19 weeks, indicating a differential advantage to initiating second-line brivanib even after development of adaptive resistance in a subset of the treated mice. Relative to the untreated cohort, the mean survival benefit for the 3 brivanib regimens was 3.7 weeks for first line, 3.3 weeks for early second line, and 2.8 weeks for delayed second line; in contrast, sorafenib monotherapy increased survival by 1.2 weeks.

### Survival trials: immunohistochemical analysis of end stage-treated tumors

Seeking to evaluate the possible activation of adaptive resistance mechanisms (invasion and revascularization), tissue was collected in the rare instances when animals were found immediately prior to (as evidenced by extreme lethargy and low body temperature) or after death. Tumors from at least 2 mice per treatment arm were processed for analysis. Panel (i) depicts a representative tumor from a mouse treated for 10.6 weeks with brivanib monotherapy (at 20.6 weeks of age) that reveals low vascularity [(i), Meca32, green, Tag oncprotein, red]. However, a second brivanib-treated mouse evidenced signs of therapeutic failure (invasive and revascularized tumors) after 8.6-week brivanib monotherapy [at almost 19 weeks of age, (ii) and inset, Meca32, green, and Tag, red]. An animal treated with 4 weeks of first-line sorafenib followed by 4.4 weeks of second-line brivanib also developed invasive and revascularized tumors [8.4 weeks total, 18.4 weeks of age; (iii), Meca32, green]. Panel (iv) depicts revascularized tumors from mice treated with 7.6 week sorafenib monotherapy—all tumors from 2 end stage animals uniformly appear red,



**Figure 5.** Survival trials: first- and second-line brivanib versus sorafenib. **A**, survival trials/Kaplan–Meier analysis. Survival trials were conducted from week 10 until endpoint in RT2 mice. Because the 4 weeks of first-line sorafenib followed by second-line brivanib until endpoint begins at week 14 (prior to that the mice are in the sorafenib monotherapy arm), all arms are assessed here starting at 14 weeks. All data starting from 10 weeks onward are shown in Supplementary Figure S5 and Supplementary Table S2 and are not substantially different from results depicted here. Cohorts were composed of 12 to 16 mice per arm, and  $P$  values were derived using the log-rank test. Survival was assessed between control vehicle treated mice ( $n = 14$ ;  $16.4 \pm 1.4$  weeks of survival); brivanib monotherapy ( $n = 12$ ;  $20.1 \pm 2.5$  weeks); sorafenib monotherapy ( $n = 16$ ;  $17.6 \pm 1.4$  weeks); 2 week first-line sorafenib followed by second-line brivanib until endpoint ( $n = 12$ ;  $19.7 \pm 2.6$  weeks), and 4-week first-line sorafenib followed by second-line brivanib until endpoint ( $n = 12$ ;  $19.2 \pm 2.6$  weeks). All treated arms show significantly higher survival than untreated mice (see Supplementary Table S2). When compared with sorafenib monotherapy, the arms involving either brivanib monotherapy ( $P = 0.001$ ) and 2 weeks ( $P = 0.002$ ) or 4 weeks ( $P = 0.028$ ) of first-line sorafenib followed by

highly revascularized, and tumor vasculature appears diffuse versus that of the surrounding acinar tissue [(iv) arrows, Meca32, green], which is consistent with the reactivation of VEGF signaling (17). In contrast, revascularized tumors in other treatment arms are white and the blood vessels have a distinct appearance, as do tumors after 6 weeks of sorafenib monotherapy. To confirm that the tumors escaping sorafenib monotherapy had overcome the VEGF/VEGFR2 blockade, immunostaining was conducted using anti-pVEGFR (red) and Meca32 (green). The merged yellow image is more intense for the 7.6-week sorafenib-treated tumors (vi, inset) and for the 7.6-week vehicle-treated control tumors (v, inset), indicating higher expression of pVEGFR in the vessels of these tumors than in brivanib-treated tumors (vii, inset). Thus, it appears that VEGFR signaling is reactivated during evasive resistance to sorafenib monotherapy that arises during these longer survival trials, in contrast to the 6-week sorafenib monotherapy arm and all other tested treatment arms. This may indicate a more heterogeneous response to brivanib than for sorafenib, reflected in the more gradual slope of the survival curve data from first- and second-line brivanib dosing, in contrast to the rapid and uniform failure reflected in the steeply sloped sorafenib monotherapy survival curve (Fig. 5A), similar to untreated animals. It is also notable that brivanib monotherapy only begins to produce signs of revascularization after about 8 weeks (corroborated by the small size of revascularized tumors at this time point, ii), whereas sorafenib and DC101 begin to fail at 4 and 2 weeks, respectively.

Tumor burden was analyzed from rare end stage RT2 mice found immediately prior to or after death (Supplementary Fig. S4). Results indicate that all treatment regimens produced tumors that eventually progressed and grew, producing tumor burden comparable with control endpoint untreated animals, although at a markedly delayed time. Note that sorafenib monotherapy at endpoint produced red tumors, in contrast to the other treatment arms, an evidence for reactivation of VEGF/VEGFR2 signaling in response to sustained sorafenib therapy.

## Discussion

The initial promise of and expectations for antiangiogenic cancer therapy have not been fully realized: Clinical investigations of drugs targeting the proangiogenic VEGF signaling pathway, including 4 clinically approved angiogenesis inhibitors, bevacizumab, sunitinib, sorafenib, and pazopanib, along with others in mid-to late-stage clinical trials, typically show a transient period of tumor stasis or partial regression, followed by a relapse phase characterized by tumor regrowth and progression and variable survival benefit (refs. 33–37) and references therein). Previous studies in the RT2 mouse model of PNET recapitulate this clinical course, revealing that VEGFR2 inhibition leads to a transitory phase of tumor stasis, followed by regrowth associated with upregulation of proangiogenic ligands, including the FGF family (17). These results motivated our evaluation of brivanib, a combined VEGFR2 and FGFR inhibitor, in RT2. Results from trials comparing first-line brivanib monotherapy versus anti-VEGFR2 monotherapy (DC101) or anti-FGF ligand capture (FGF-trap) showed that first-line brivanib monotherapy produced more enduring tumor stasis and vascular inhibition, in contrast to either single pathway inhibitor. As a second-line inhibitor following DC101 therapy, brivanib conducted comparably to a combination therapy consisting of continuous DC101 with FGF-trap layered on at the same time as the switch from DC101 to brivanib in the parallel arm. Moreover, in the majority of samples analyzed [the exception is depicted in Fig. 5C (ii)], brivanib first-line therapy produced no signs of revascularization-mediated evasive resistance (up to 11-week treatment and 21 weeks of age), in contrast to the demonstrable and earlier onset of adaptive resistance via revascularization with DC101. Our previous investigation of another potent angiogenesis inhibitor, sunitinib, revealed that it, too, produced an extended angiogenesis blockade with no apparent tumor revascularization over comparable time courses analyzed here. Notably, both sunitinib and DC101 treatment produced tumors that were more highly invasive and metastatic than untreated tumors (20). We observed a similar trend, again

second-line brivanib until endpoint showed significantly higher survival. Interestingly, the cohort in which initiation of brivanib was delayed (4 week first-line sorafenib followed second-line brivanib until endpoint) produced a pronounced shoulder in the survival curve, indicative of the survival benefit resulting from second-line brivanib therapy, even when initiated "late." Mean values  $\pm$  SD are indicated, log-rank test for statistical significance. \*,  $P = 0.05-0.01$ ; \*\*,  $P < 0.01$ . B, survival trials: immunofluorescent analysis of end stage-treated tumors. Histologic analysis by fluorescent immunostaining (immunofluorescence) was conducted on tumors from mice in the survival trial that were found at end stage to be amenable for excising tumors. All tumors with good morphology from 2 mice per treatment arm were analyzed, with representative images depicted. i, a large, noninvasive tumor from a mouse treated with brivanib monotherapy for 10.6 weeks (until 20.6 weeks of age; anti-Meca32, green; T-antigen, red), which evidenced continued angiogenic blockade by its low vascularity. ii, a tumor from a second end stage brivanib monotherapy mouse (8.6-week treatment, at 18.6 weeks of age; anti-Meca32, green; T-antigen, red, and inset) that evidenced signs of therapeutic evasion including tumor revascularization (Meca32, green) and invasion (T-antigen, red). iii, a revascularized and invasive tumor that resulted from 4 weeks of sorafenib followed by 4.4 weeks of second-line brivanib (iii, 8.4-week treatment total, 18.4 weeks of age; anti-Meca32, green). iv, a revascularized and invasive tumor from an end stage mouse treated with sorafenib monotherapy (7.6-week sorafenib, 17.6 weeks of age; anti-Meca32, green). Note the diffuse vasculature, invasive border, and irregular revascularization. To assess maintenance of the VEGFR signaling blockade, tumors from a 7.6-week vehicle-treated tumor (v), a 7.6-week sorafenib-treated tumor (vi), and a 10.6-week brivanib-treated tumor (vii; both monotherapy) were stained with Meca32 (green) and pVEGFR (red) antibodies; the merged (yellow) images are indicative of VEGFR signaling (yellow) for sorafenib monotherapy (vi and inset) and control vehicle (v and inset) treated tumors, in contrast to predominantly pVEGFR2-negative staining of endothelium in Brivanib-treated tumors (vii, green). Scale bar represents 400  $\mu$ m (i, ii, iv), 200  $\mu$ m (iii), and 100  $\mu$ m (v–vii). Panel iv is false colored to maintain consistency between panels.

using DC101 monotherapy, and with sequential first-line DC101 followed by second-line brivanib therapy in the trials described herein. Interestingly while first-line brivanib therapy also induced more invasive tumors than are typical in untreated mice, the incidence of invasive carcinomas is apparently lower than that which characterizes the adaptive response to the abovementioned drugs and regimens, a result that warrants further investigation.

Brivanib was further evaluated in first- and second-line fixed endpoint studies involving a clinically approved angiogenesis inhibitor, sorafenib, which targets VEGFR1, VEGFR2, and VEGFR3, PDGFR $\beta$ , and (wild-type) RAF; DC101 was again used as a benchmark. Sorafenib elicited adaptive resistance in the form of revascularization, in contrast to brivanib, which did not in these defined endpoint trials; as such, brivanib showed better efficacy first line. Nevertheless, sorafenib monotherapy produced a more enduring response than DC101, as evidenced by blocking tumor revascularization and inducing tumor stasis for longer times: Sorafenib monotherapy began to produce signs of tumor revascularization after 4 weeks, whereas revascularization was already evident at 2 weeks for DC101. Second-line dosing with brivanib following this acquired resistance proved more beneficial than continued first-line monotherapy with either sorafenib or DC101. When second-line brivanib was initiated after 4 weeks of sorafenib treatment in 6-week long fixed endpoint trials, the tumors exhibited revascularization, although they had not yet begun to regrow significantly. In contrast, first-line brivanib monotherapy after 4 and 6 weeks produced no signs of therapeutic failure/evasion in the form of revascularized tumors, concomitant with apparent tumor stasis.

Interestingly, results from fixed endpoint trials imply that some proangiogenic pathways upregulated following the failure of sorafenib are not downregulated by subsequent treatment with brivanib, given that brivanib did not fully block revascularization second line. In contrast, these putative proangiogenic signaling pathways are evidently not induced by first-line brivanib over the same treatment time course, as revascularization was not observed in this case. Given the target profile of brivanib, it seems likely that first-line inhibition of FGFR signaling limits the induction of the revascularization response. However, once the adaptive proangiogenic signaling pathways are induced in the context of VEGFR inhibition, brivanib seemingly cannot suppress them all, implicating other proangiogenic signals not directly targeted by brivanib. Thus, a cascade mechanism may be responsible, wherein upregulation of FGF signaling induces other circuits that then become FGF independent. Follow-up studies will be required to identify the postulated proangiogenic pathway(s) induced in the course of adaptive resistance to VEGF inhibitors. These considerations seem relevant to recent clinical trials, where inhibitors that primarily target the VEGF pathway have been observed to result in upregulation of basic FGF during and/or prior to progression in glioblastoma (38) and in metastatic colorectal carcinomas (39).

Seeking to further probe this interesting difference in response to brivanib first- versus second-line dosing, we asked whether there was a difference in initiating second-line brivanib prior to, or coincident with, pathologically evident sorafenib failure and sought to further distinguish the efficacy of first- versus second-line dosing in survival trials. Survival benefit from earlier second-line brivanib following sorafenib treatment appeared indistinguishable from first-line brivanib monotherapy and was significantly better than sorafenib monotherapy. Survival for delayed second-line brivanib following 4 weeks of sorafenib was also significantly better than sorafenib monotherapy, indicating a survival benefit from second-line brivanib therapy even when it is initiated "late," at a time possibly analogous to the end of "progression-free survival" used to define drug failure and transition to second-line therapy in clinical settings. Moreover, the results suggest that another mechanism for evasion is involved in the failure of sorafenib—circumventing the VEGFR2 blockade. While the other VEGF inhibitors and 6-week sorafenib monotherapy produce white tumors in both first- and second-line dosing, mice treated to end stage (7–8 week) with sorafenib monotherapy had red, hemorrhagic tumors, an indication that the VEGF/VEGFR2 axis was reactivated, as confirmed using a pVEGFR antibody. It is possible that brivanib can extend life span of sorafenib-treated mice, even when initiated "late," because of its more potent VEGF inhibition ( $IC_{50}$  brivanib = 34 nmol/L vs.  $IC_{50}$  sorafenib = 80–160 nmol/L). However, although brivanib produces tumor stasis for an extended time, tumors eventually progress.

Multiple adaptive mechanisms contribute to the development of evasive resistance to antiangiogenic therapy targeting VEGF signaling (18, 21, 37, 40,41–43). One class involves revascularization and another heightened invasion and metastasis. It appears that brivanib primarily impacts revascularization to a lesser extent heightened invasion and metastasis, although further studies will be required to delineate the effects of brivanib on invasion, as well as the impact of such invasiveness on survival. Notably, despite the observed differences in histologic and pathologic responses in this model, sunitinib (20), sorafenib, and brivanib each significantly extended life span and time to progression versus untreated mice, presumably through their common disruption of the tumor vasculature, evoking tumor stasis until one or another form of adaptive resistance kicks in or until the cumulative burden of tumor stasis become overwhelming.

These studies may help inform future therapeutic regimens in patients. Brivanib therapy produced a marked blockade of tumor angiogenesis and significant efficacy in a mouse model of PNET, in both first- and second-line settings. Brivanib was clearly efficacious in the first-line setting of VEGF inhibitor naive mice, encouraging its clinical evaluation as first-line antiangiogenic therapy. Moreover, brivanib had demonstrable benefit in second-line settings in the context of the failure of 2 VEGF pathway inhibitors—an anti-VEGFR2 monoclonal antibody (DC101) and sorafenib. Initial insight into the predictive

value of these preclinical results and implications may come from current clinical trials comparing brivanib and sorafenib in HCC: a first-line head-to-head trial is ongoing and 2 trials in which second-line brivanib therapy is initiated upon progression of sorafenib-treated patients (<http://www.cancer.gov/clinicaltrials/search/results?protocolsearchid=8952330>).

In addition, in regard to second-line strategies, the results of this study suggest that there may be added benefit from an early switch to second-line brivanib before radiographic progression is evident. Although not reflected in differential survival in this highly aggressive multifocal model of PNET, the histopathologic analysis suggests that an early switch to second-line brivanib (or other dual VEGFR + FGFR inhibitors) concomitant with early signs of revascularization could limit the full induction and manifestation of evasive resistance mechanisms involving VEGF-dependent and -independent proangiogenic mechanisms, thereby improving efficacy in some patients and tumor types. For example, perhaps dynamic contrast enhanced-MRI (DCE-MRI) could serve as an indicator of incipient adaptive resistance by revascularization, as suggested by a clinical trial of glioblastoma patients being treated with an investigational VEGFR inhibitor, AZ-2171 (44). Radiologic evidence of revascularization was evident before clinical failure, concomitant with increased levels of FGF2 in the blood (44), suggestive of elevated FGF ligands and FGFR signaling in the tumors. Thus, brivanib might be considered for second-line therapy of glioblastoma following the impending failure of VEGF inhibitors detected by DCE-MRI, well before evident pathologic failure. More generally, if it is clear that a particular drug is destined to fail because of evasion through an identified pathway, it may be pre-

ferential to initiate a relevant second-line therapy prior to the complete failure of the first-line agent, if means to detect such impending failure are available. Preclinical trials in mouse models of other human cancers, as well as clinical trials, should provide insight into the potential benefit of early versus traditional second-line therapy with such dual VEGFR and FGFR inhibitors.

### Disclosure of Potential Conflicts of Interest

I.B. Walters is an employee of Bristol-Myers Squibb. D. Hanahan is a consultant to Onyx Pharmaceuticals, which comarkets sorafenib and is an American Cancer Society research professor. E. Allen has no potential conflict of interest.

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# Clinical Cancer Research

## Brivanib, a Dual FGF/VEGF Inhibitor, Is Active Both First and Second Line against Mouse Pancreatic Neuroendocrine Tumors Developing Adaptive/Evasive Resistance to VEGF Inhibition

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