Pazopanib Reveals a Role for Tumor Cell B-Raf in the Prevention of HER2+ Breast Cancer Brain Metastasis

Brunilde Gril1, Diane Palmieri1, Yong Qian1, DeeDee Smart2, Lilia Ileva3, David J. Liewehr4, Seth M. Steinberg4, and Patricia S. Steeg1

Abstract

Purpose: Brain metastases of breast cancer contribute significantly to patient morbidity and mortality. We have tested pazopanib, a recently approved antiangiogenic drug that targets VEGFR1, VEGFR2, VEGFR3, PDGFRβ, PDGFRα, and c-kit, for prevention of experimental brain metastases and mechanism of action.

Experimental Design: In vitro assays included B-Raf enzymatic assays, Western blots, and angiogenesis assays. For in vivo assays, HER2 transfectants of the brain seeking sublines of MDA-MB-231 cells (231-BR-HER2) and MCF7 cells (MCF7-HER2-BR3, derived herein) were injected into the left cardiac ventricle of mice and treated with vehicle or pazopanib beginning on day 3 postinjection. Brain metastases were counted histologically, imaged, and immunostained.

Results: Treatment with 100 mg/kg of pazopanib resulted in a 73% decline in large 231-BR-HER2 metastases (P < 0.0001) and a 39% decline in micrometastases (P = 0.004). In vitro, pazopanib was directly antiproliferative to 231-BR-HER2 breast cancer cells and inhibited MEK and ERK activation in vitro despite B-Raf and Ras mutations. Enzymatic assays demonstrated that pazopanib directly inhibited the wild type and exon 11 oncogenic mutant, but not the V600E mutant forms of B-Raf. Activation of the B-Raf targets pERK1/2 and pMEK1/2 was decreased in pazopanib-treated brain metastases whereas blood vessel density was unaltered. In the MCF7-HER2-BR3 experimental brain metastasis model, pazopanib reduced overall brain metastasis volume upon magnetic resonance imaging (MRI) by 55% (P = 0.067), without affecting brain metastasis vascular density.

Conclusions: The data identify a new activity for pazopanib directly on tumor cells as a pan-Raf inhibitor and suggest its potential for prevention of brain metastatic colonization of HER2+ breast cancer.

Clin Cancer Res; 17(1); 142–53. ©2010 AACR.

Introduction

The majority of cancer patients succumb to metastatic disease or the consequences of its treatment. Although metastasis to any site in the body is a devastating event, the brain may represent a "final frontier". Brain metastases are 10-fold more prevalent than primary tumors of the brain (1), concentrated in lung and breast carcinomas and melanoma. In breast cancer, brain metastases occur predominately in the HER2+ and triple-negative subtypes (2). The incidence of brain metastatic disease has increased to approximately 35% in patients with HER2+ metastatic breast cancer (3–6). The majority of HER2+ metastatic patients experienced a brain relapse when either responding to treatment systemically or experiencing stable systemic disease, and up to 50% of deaths were due to brain disease (7–9). Current treatments are palliative including steroids, cranial radiotherapy, and surgical resection. Brain metastases are designated an unmet medical need by the U.S. Food and Drug Administration.

The mechanistic basis of brain metastasis has been investigated using brain tropic breast cancer cell lines. Several molecular pathways have been reported to contribute to brain metastatic potential including HER2 (10), VEGF-A (11), integrin αvβ3 (12), and Stat3 (13). We developed a quantifiable brain metastasis mouse model using a brain-seeking variant of the MDA-MB-231 breast carcinoma cell line (231-BR). When injected into the left cardiac ventricle, 231-BR cells produce numerous
Translational Relevance

Brain metastases occur in approximately 35% of HER2+ metastatic breast cancer patients. Many patients now develop brain metastases as a first site of progression with stable systemic disease and, increasingly, brain metastases contribute to patient death. We report that pazopanib, an approved inhibitor of VEGFRs, PDGFR, and c-kit, reduced brain metastatic colonization in two experimental breast cancer models over-expressing the HER2 oncogene. In vitro studies using 231-BR cells revealed an unexpected diminution of tumor ERK activation despite the presence of both Ras and B-Raf mutations; enzymatic assays confirmed a new function for pazopanib as a B-Raf inhibitor. In the 231-BR-HER2 model, reduced brain metastasis formation was accompanied by reduced pERK but no change in vessel density. The data suggest pazopanib as a candidate for brain metastasis prevention. The data also identify a new activity for this drug which may enable new rational combinations.

Materials and Methods

Drugs

Pazopanib and lapatinib were provided by GlaxoSmithKline through a Material Collaborative Research and Development Agreement with NIH. For in vitro experiments, pazopanib and lapatinib were reconstituted in dimethylsulfoxide (DMSO) and stored at −80°C. For in vivo experiments, pazopanib was suspended in 0.5% hydroxypropylmethylcellulose with 0.1% Tween 80 (vehicle). Raf inhibitor was purchased from Calbiochem, and MEK inhibitor (U0126) was purchased from Cell Signaling Technology.

Cell lines and in vitro experiments

Primary human brain microvascular endothelial cells (HBMEC) were purchased and cultured as per the manufacturer's instructions (Cell Systems). The human MDA-MB-231-BR (231-BR) cell line and the HER2 transfectant were previously described (10, 19). The MCF7-HER2 cell line was kindly provided by Dr. Dennis Slamon (University of California Los Angeles) and maintained in RPMI-1640 (Invitrogen) supplemented with 10% fetal bovine serum (FBS). Standard procedures were used for Western blot analysis, cell-cycle, and viability assays. Specific details for these assays and siRNA experiments can be found in Supplementary Materials and Methods.

B-Raf kinase assay

To analyze the effect of pazopanib on B-Raf activity, components of a Raf kinase assay kit (Upstate Biotechnology) were incubated with MEK1, and pMEK levels were determined on Western blots. Details of the techniques are in Supplementary Materials and Methods.

Animal experiments

All experiments were conducted under an approved animal use agreement with the NCI. For 231-BR-HER2 cells: 5- to 7-week-old female Balb/c nude mice (Charles River Laboratories) were inoculated with 1.75 × 10⁶ cells in the left ventricle of the heart. Mice were randomly assigned to receive vehicle or pazopanib (30 or 100 mg/kg) twice daily by oral gavage, starting 3 days postinjection for 21 days. Histological analysis is described in Supplementary Materials and Methods.

For MCF7-HER2-BR3 cells: For establishment of the BR3 cell line, see Supplementary Material and Methods. Athymic nude mice were injected with 1 × 10⁶ cells in the left cardiac ventricle. In addition, mice received a subcutaneous injection of 1.5 mg/kg of depo-estradiol 1 day before tumor cell injection and once a week throughout the duration of the study. On day 3 postinjection, mice were randomized to either vehicle or pazopanib (100 mg/kg) twice daily by oral gavage. After 6 weeks, 10 mice per group were randomly chosen for magnetic resonance imaging (MRI). After MRI, all mice were euthanized and the brain frozen as described in Supplementary Materials and Methods. MRI imaging of brain metastases is described in Supplementary Materials and Methods.
We therefore hypothesized that pazopanib could also target breast cancer cells (231-BR) expressing VEGFR1 (Fig. 1). Pazopanib inhibits 231-BR breast cancer cell growth and tube formation.

Findings confirmed that pazopanib targeted endothelial ERK1/2 and affected the phosphorylation of 70S6K. Our way through decreased phosphorylated MEK1/2 and with disruption of downstream PLCγ1. Pazopanib abrogated the phosphorylation of VEGFR2 the network at 1 and 72 hours, respectively (Supplementary Fig. S1A). In an anchorage-dependent growth was 2 and 1 for both cell lines after 96 hours of culture (Fig. 1A). This dose was considered physiologically relevant because it was in the same range as the in vitro growth inhibition of lapatinib on 231-BR cells (21). We, therefore, deciphered the signaling pathways disrupted by pazopanib on both 231-BR-vector and 231-BR-HER2 cell lines. VEGFR1 phosphorylation was inhibited by pazopanib at 0.5 μmol/L (Fig. 1B). Analysis of downstream pathways focused our attention on the Ras-Raf-ERK1/2 pathway. Despite the fact that 231-BR cells harbor two oncogenic mutations in the Ras-Raf-ERK1/2 pathway (K-Ras G13D and B-Raf G464V in exon 11; ref. 22), a clear decrease in MEK1/2 and ERK1/2 phosphorylation was observed with pazopanib treatment. Downstream targets of MEK1/2 and ERK1/2 were also affected including a decrease in phospho- and total c-Jun phosphorylation, P70S6K, and cyclin D1, and an increase in the cyclin-dependent kinase inhibitor p27.

Cell-cycle analysis was performed using vehicle or 5 μmol/L of pazopanib. At 72 hours of culture, vehicle-treated 231-BR-vector and 231-BR-HER2 cells were spread throughout the cycle, with 58% and 61% of cells in G0/G1 phase, 20% and 18% in S phase, and 22% and 21% in G2/M phase, respectively. In contrast, pazopanib induced a complete cell-cycle arrest in G0/G1 in both cell lines (Fig. 1C). No cleaved caspase 3 staining was observed after culture with pazopanib (data not shown). These results suggest that pazopanib induced a cell-cycle arrest, but not apoptosis. In summary, the principal effect of pazopanib on tumor cells was the inhibition of the constitutively activated Ras-Raf-ERK1/2 pathway that questioned the mechanism of action of this compound.

**Statistical analysis**

Statistical analyses are described in Supplementary methods.

**Results**

**Pazopanib inhibits human brain microvascular endothelial cell growth**

To confirm the antiangiogenic activity of pazopanib, its effect on HBMEC was analyzed. The IC50 for pazopanib for anchorage-dependent growth was 2 and 1 μmol/L after 48 and 72 hours, respectively (Supplementary Fig. S1A). In an in vitro tube formation assay, pazopanib disrupted 50% of the network at 1 μmol/L (Supplementary Fig. S1B and C).

Pazopanib abrogated the phosphorylation of VEGFR2 with disruption of downstream PLCγ1 (Supplementary Fig. S1D). Pazopanib also disrupted the Ras-Raf-ERK pathway through decreased phosphorylated MEK1/2 and ERK1/2 and affected the phosphorylation of 70S6K. Our findings confirmed that pazopanib targeted endothelial cells, affecting cell growth, VEGFR-induced signaling, and tube formation.

**Pazopanib inhibits 231-BR breast cancer cell growth and the ERK1/2 signaling pathway**

The brain-seeking derivative of human MDA-MB-231 breast cancer cells (231-BR) expresses VEGFR1 (Fig. 1). We therefore hypothesized that pazopanib could also directly target tumor cells. In vitro assays were performed using vector (231-BR-vector) and HER2 transfectants (231-BR-HER2) of the 231-BR cell line. In an anchorage-dependent growth assay, the IC50 of pazopanib was 5 μmol/L for both cell lines after 96 hours of culture (Fig. 1A). This dose was considered physiologically relevant because it was in the same range as the in vitro growth inhibition of lapatinib on 231-BR cells (21). We, therefore, deciphered the signaling pathways disrupted by pazopanib on both 231-BR-vector and 231-BR-HER2 cell lines. VEGFR1 phosphorylation was inhibited by pazopanib at 0.5 μmol/L (Fig. 1B). Analysis of downstream pathways focused our attention on the Ras-Raf-ERK1/2 pathway. Despite the fact that 231-BR cells harbor two oncogenic mutations in the Ras-Raf-ERK1/2 pathway (K-Ras G13D and B-Raf G464V in exon 11; ref. 22), a clear decrease in MEK1/2 and ERK1/2 phosphorylation was observed with pazopanib treatment. Downstream targets of MEK1/2 and ERK1/2 were also affected including a decrease in phospho- and total c-Jun phosphorylation, P70S6K, and cyclin D1, and an increase in the cyclin-dependent kinase inhibitor p27.

Cell-cycle analysis was performed using vehicle or 5 μmol/L of pazopanib. At 72 hours of culture, vehicle-treated 231-BR-vector and 231-BR-HER2 cells were spread throughout the cycle, with 58% and 61% of cells in G0/G1 phase, 20% and 18% in S phase, and 22% and 21% in G2/M phase, respectively. In contrast, pazopanib induced a complete cell-cycle arrest in G0/G1 in both cell lines (Fig. 1C). No cleaved caspase 3 staining was observed after culture with pazopanib (data not shown). These results suggest that pazopanib induced a cell-cycle arrest, but not apoptosis. In summary, the principal effect of pazopanib on tumor cells was the inhibition of the constitutively activated Ras-Raf-ERK1/2 pathway that questioned the mechanism of action of this compound.

**Pazopanib directly targets B-Raf**

It was unlikely that pazopanib could decrease pERK1/2 solely through an interaction with established targets (VEGFR1, VEGFR2, VEGFR3, PDGFRα, PDGFRβ, and c-kit), because all of these targets are upstream of Ras and B-Raf that are constitutively activated in 231-BR cells. Consequently, we hypothesized that pazopanib directly targeted B-Raf. In an enzymatic assay, pazopanib was incubated with B-Raf protein and inactive MEK1, and B-Raf activity was measured by the phosphorylation level of its substrate, MEK1 (Fig. 2A). Increasing concentrations of pazopanib inhibited the phosphorylation of MEK1. As a positive control, a nonclinical Raf inhibitor inhibited MEK1 phosphorylation; as a negative control, lapatinib, a kinase pocket inhibitor of HER2 and EGFR, had no effect on phospho-MEK1. Thus, pazopanib directly targeted wild-type B-Raf.

Pazopanib was then tested in an enzymatic assay using total lysate from three tumor cell lines to investigate the spectrum of its B-Raf inhibitory activity (Fig. 2B). The MCF7 breast carcinoma cell line transfected with HER2 exhibits activated wild-type B-Raf downstream of HER2 overexpression (23). The 231-BR cell line expresses the mutated G464V form of B-Raf in exon 11 and the melanoma cell line SKMEL28 harbors the common mutation V600E in exon 15 of B-Raf (24). MEK phosphorylation by B-Raf was inhibited by pazopanib in cell lysates from the HER2-activated, wild-type B-Raf cell line as well as the exon 11 B-Raf mutant cell line, but not from the line harboring a V600E mutation. These data confirmed the inhibitory effect of pazopanib on B-Raf activity and showed that pazopanib had enhanced inhibitory activity for the G464V mutant form of B-Raf. In independent experiments using recombinant proteins, the dissociation constant (Kd) of pazopanib for wild-type B-Raf was 59.2 nmol/L and for B-Raf V600E was 148 nmol/L, a 2.5-fold difference, confirming the lesser activity toward V600E B-Raf. Consistent with its effects as a B-Raf inhibitor, the antiproliferative activity of pazopanib on 231-BR-HER2 cells was similar to that of nonclinical Raf and MEK (U0126) inhibitors (Fig. 2C).

Pazopanib had been previously identified as targeting C-Raf (25). A complex signaling pathway has been described recently in which B-Raf heterodimerizes with C-Raf and each can modulate the other protein’s level of activity (26–28). Experiments were, therefore, conducted to ascertain the importance of B-Raf relative to C-Raf. Specific knockdown of B-Raf using two siRNA constructs was performed in 231-BR-vector cells (Fig. 2D). Neither siRNA construct affected C-Raf expression. Knockdown of B-Raf expression was sufficient to reduce ERK activation, suggesting that C-Raf inhibition is not necessary for the inactivation of this
Similar results were observed using MCF7 cells with wild-type B-Raf activated by HER2 transfection (Fig. 2E). In independent experiments, the $K_i$ of pazopanib for wild-type C-Raf was 91 versus 59.2 nmol/L for B-Raf, identifying approximately 1.5-fold better affinity for B-Raf than for C-Raf\cite{1}. Taken together, the data indicate that pazopanib is a pan-Raf pathway in 231-BR-vector cells.
Figure 2. Pazopanib inhibits B-Raf. A, effect of pazopanib on B-Raf activity. Increasing concentrations (0.022, 0.11, 0.22, 0.44, and 2.2 μmol/L) of pazopanib, lapatinib, or a nonclinical Raf inhibitor were incubated with B-Raf for 20 minutes at 30°C. Inactive MEK1 was added for 30 minutes and the level of MEK1 phosphorylation was analyzed with pMEK1 and total MEK1 antibodies. A Raf inhibitor was used as a positive control and lapatinib was used as a negative control. The (-) column represents the level of MEK1 phosphorylation without the addition of enzyme. In the (+) column, no inhibitors were added, representing the maximum level of pMEK1. B, effect of pazopanib on Raf activity in cell lysates. The same protocol was followed substituting MCF7-HER2, 231-BR, or SKMEL28 cell lysates for B-Raf protein and using the following concentrations of inhibitors: 0.022, 0.22, and 2.2 μmol/L. C, effect on cell viability. 231-BR-vector cells were incubated with increasing concentrations of pazopanib, Raf inhibitor, or MEK inhibitor (U0126) for 96 hours. Cells were assayed for viability using MTT. Data are represented as mean ± SEM. D and E, B-Raf siRNA transfection. 231-BR-vector (D) and MCF7-HER2 (E) cell lines were transfected with 2 different B-Raf siRNA constructs (S1 and S2), or treated with the transfectant agent alone (T). Cell lysates were collected at 48 and 96 hours after transfection.
inhibitor, but that targeting of the B-Raf pathway is likely a significant determinant of its ERK inhibitory activity.

**Pazopanib prevents the brain metastatic outgrowth of the 231-BR-HER2 human breast cancer cell line**

We asked if pazopanib was effective in preventing brain metastasis and investigated its mechanism of action in vivo. The 231-BR-HER2 cell line was injected in the left cardiac ventricle of nude mice and treatment with vehicle, 30 or 100 mg/kg of pazopanib started 3 days after cell injection. Mice were treated twice daily by oral gavage for 21 days. To quantify the effect of pazopanib on metastatic tumor cell colonization of the brain, the number of large metastatic lesions and micrometastases were quantified in hematoxylin and eosin (H&E)-stained brain sections (Table 1 and Supplementary Fig. S2). A cutoff of 300 microns in a single dimension was chosen to separate large metastases from micrometastases because it is approximately proportional to 5-mm MRI detectable lesion in a human brain. The 231-BR-HER2 cells produced a mean of 101.9 micrometastases per brain section in the vehicle-treated mice. Treatment with 30 mg/kg of pazopanib resulted in a 25% decrease to 76.4 micrometastases (NS, nonsignificant); treatment with 100 mg/kg resulted in a 39% decline in micrometastases to 61.7 ($P = 0.004$).

The efficacy of pazopanib in preventing large metastases was more pronounced. The 231-BR-HER2 cells produced a mean of 3.92 large metastases per section in the vehicle-treated mice. Treatment with 30 mg/kg of pazopanib resulted in a 51% decline to 1.93 large metastases ($P = 0.0002$); treatment with 100 mg/kg resulted in a 73% decline to 1.05 large metastases ($P < 0.0001$). Supplementary Figure S2 presents representative pictures from H&E-stained brain sections for each treatment. Both the number and size of the brain metastases were inhibited. Because radiation therapy is the mainstay of treatment for brain metastasis, in vitro experiments were conducted to identify any radiosensitization effect with pazopanib. Pazopanib did not enhance the radiation sensitivity of the 231-BR-vector cell line in vitro (Supplementary Fig. S3).

**Table 1. Pazopanib prevention of brain metastatic colonization**

<table>
<thead>
<tr>
<th>Pazopanib dose, mg/kg</th>
<th>No. of mice</th>
<th>Mean large metastases (95% CI)</th>
<th>$P$</th>
<th>Mean micrometastases (95% CI)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10</td>
<td>3.92 (3.05–4.79)</td>
<td></td>
<td>101.9 (84.2–119.6)</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>11</td>
<td>1.93 (1.59–2.26)</td>
<td>0.0002</td>
<td>76.4 (59.5–93.2)</td>
<td>NS</td>
</tr>
<tr>
<td>100</td>
<td>11</td>
<td>1.05 (0.72–1.39)</td>
<td>&lt;0.0001</td>
<td>61.7 (44.8–78.6)</td>
<td>0.004</td>
</tr>
</tbody>
</table>

*Mice were inoculated with $1.75 \times 10^5$ 231-BR-HER2 cells. Pazopanib or vehicle treatment began 3 days after tumor cell injection for 22 days. Brains were excised and bisected along the sagittal plane. A 10-µm step section every 300 microns through one hemisphere of the brain was analyzed to count every micro- or large (>300 microns in any single dimension) metastasis in each section. The mean number of metastases is reported.

**Pazopanib inhibits 231-BR-HER2 brain metastases through tumor cell Raf, but not antiangiogenic pathways**

It is unknown if pazopanib targeted antiproliferative (Raf-MEK-ERK) and/or antiangiogenic pathways in brain metastasis. The phosphorylation level of the Raf targets MEK1/2 and ERK1/2 in brain metastatic cells was analyzed. Twenty-five micrometastases and all large metastases per brain section for 5 mice per treatment group were scored for antibody staining on a 0 to 3+ intensity scale (Fig. 3). In the mice treated with vehicle, the staining for pERK1/2 was approximately equally divided between the low-intensity (score 0/1+) and high-intensity (2+/3+) groups. In contrast, 30 mg/kg and 100 mg/kg of pazopanib induced a significant increase of approximately 2-fold in the number of brain metastases that were 0/1+ staining, with only 3% to 8% of lesions exhibiting 2+/3+ staining (0/1+ staining: 30 mg/kg vs. vehicle: 92% vs. 56%, $P = 0.0005$; 100 mg/kg vs. vehicle: 97% vs. 56%, $P = 0.0009$). The same trend was observed with pMEK1/2 staining. Pazopanib treatment induced a shift in pMEK1/2 staining from 48% of metastases in the 2+/3+ group in the vehicle group to 15% or less for the treatment groups (0 and 1+ staining: 30 mg/kg vs. vehicle: 85% vs. 52%, $P = 0.005$; 100 mg/kg vs. vehicle: 91% vs. 52%, $P = 0.002$). pAKT staining was not affected by pazopanib treatment, providing a control for specificity (Fig. 3). Therefore, pazopanib effectively decreased the phosphorylation of B-Raf targets without altering markers in a different pathway.

Brain tissues were stained for CD31 to analyze the blood vessel density of metastases among treatment groups, as well as that of the normal unaffected brain (Fig. 4). The blood vasculature was distinct between the normal brain and metastases. Indeed, blood vessel density was slightly but statistically significantly higher in the normal brain compared with the metastases (normal brains of vehicle-treated mice: 61.4 ± 6.53 vessels per high-power field vs. 44.2 ± 2.30 in brain metastases, $P = 0.006$). The percentage of brain area covered by blood vessels was actually larger in the metastases than in normal brain (vehicle-treated mice: 2.90% ± 0.398% of area in normal brain vs. 4.15% ± 0.335% of brain metastases area; Fig. 4). This difference reflected fewer, but dilated blood vessels in the metastases,
Figure 3. Pazopanib inhibits brain metastatic colonization of 231-BR-HER2 cells targeting Raf-MEK-ERK pathway. Brain sections from 5 mice per group were stained for pERK1/2, pMEK1/2, and pAKT. The intensity of staining was quantified using the intensity scores 0, 1+, 2+, or 3+. Representative photographs of the staining are shown for each treatment group. P values are reported where significant (P < 0.01).

Gril et al.  
Clin Cancer Res; 17(1) January 1, 2011  
Clinical Cancer Research
demonstrating that metastatic colonization induced remodeling of blood vasculature.

Pazopanib treatment had no effect on either the normal brain or the metastatic vasculature. In the normal brain, the number of blood vessels per high-power field was 61.4 ± 6.53 in the vehicle group versus 63.5 ± 5.32 and 55.2 ± 5.06 in the 30 mg/kg and 100 mg/kg of pazopanib-treated groups, respectively. The small decrease in blood vessel density with pazopanib treatment did not achieve statistical significance. In the brain metastases, the number of blood vessels was 44.2 ± 2.30 per high-power field in the vehicle-treated group versus 42.2 ± 4.36 and 46.8 ± 4.35 in the 30 mg/kg and 100 mg/kg of pazopanib-treated groups, respectively. The same trend was observed for the percent of area covered by blood vessel (Fig. 4A and B).

Pazopanib prevents the brain metastatic outgrowth of the MCF7-HER2-BR3 human breast cancer cell line

To evaluate the efficacy of pazopanib in an independent brain metastatic model, we developed a brain-seeking cell line using the MCF7 breast cancer cells transfected with HER2. After 3 rounds of intracardiac injection, brain dissection, cell culture, and reinjection into mice, the MCF7-HER2-BR3 cell line produced a single brain metastasis compressing and invading the hypothalamus and thalamus region and sometimes extending further to the pons of the brain in approximately 70% of mice injected.

Similar in vitro experiments, as those performed on the 231-BR model, were conducted using the MCF7-HER2-BR3 cell line. Pazopanib inhibited 50% of cell viability at about 7 μmol/L and a Raf inhibitor and MEK inhibitor produced similar effects (Supplementary Fig. S4A). Pazopanib at low dose induced a slight increase in pERK1/2 but, at higher doses, a decrease was observed (Supplementary Fig. S4B). This trend was previously described in recent articles (26, 28). Cell-cycle analysis showed that after 72 hours of vehicle treatment, 56% of cells were in G0/G1 phase, 31% were in S phase, and 13% are in G2/M phase. Pazopanib (5 μmol/L) induced a complete cell-cycle arrest in G0/G1 phase (Supplementary Fig. S4C).

Using this model, mice were treated with vehicle or 100 mg/kg of pazopanib, starting 3 days postinjection and extending for 6 weeks thereafter. MRI analysis was performed on the brains of mice on day 42 postinjection and tumor volume calculated. Ten mice per treatment arm were randomly chosen for MRI analysis. Four mice from the control arm had to be euthanized 5 days before the end of the experiment, compared with one mouse from the pazopanib-treated arm. For the MRI analysis, 7 of 10 mice per experimental arm developed brain metastases. Metastasis volume for all lesions calculated and representative MRI images are shown (Fig. 5A and B). The average metastatic tumor volume in the vehicle-treated group was 45.4 mm$^3$ (95% CI: 18.1–72.7), whereas the average metastasis size in the pazopanib-treated group was 20.5 mm$^3$ (95% CI: 15.2–25.8). This corresponds to a 55% reduction in metastasis volume, representing a statistical trend ($P = 0.067$).

One hemisphere of the brain was selected to be immediately frozen in optimal cutting temperature (OCT)
compound, randomly, for analysis of blood vessel density and percentage of blood vessel coverage (Fig. 5C and D). Remodeling of blood vasculature was also observed in this model. The blood vessel density was slightly decreased in the metastases compared with normal brain but did not reach statistical significance (P = 0.15). However, blood vessels were much more dilated in metastases; the blood vessels covered 2.00% to 2.16% of normal brain area and 6.02% to 6.46% in the metastases (both P < 0.0001; Fig. 5D). Nevertheless, no effect of pazopanib was observed among metastases or normal brain in terms of blood vessel density (Fig. 5C) or vessel area (Fig. 5D). Figures 5E and F show representative photographs of blood vessels in normal brain and metastatic tissues, respectively. The data confirm vessel remodeling in brain metastases but fail to demonstrate an antiangiogenic effect of pazopanib.

Discussion

We report a new activity for the multikinase inhibitor pazopanib, the inhibition of tumor cell B-Raf activity. B-Raf has been identified as an oncogene and B-Raf mutations are found in approximately 7% of human cancers with the highest frequency in malignant melanoma (29–32). Drugs targeting B-Raf have been reported with distinct profiles of activity against the wild-type and mutated forms of B-Raf and its family members (24, 33, 34). Our findings emanated from the observation that pazopanib inhibited the proliferation of 231-BR tumor cells directly. Pazopanib has been reported to inhibit the proliferation of lung cancer cells and multiple myeloma cells as well (35, 36). Interestingly, inhibition of tumor cell proliferation by pazopanib was accompanied by inhibition of the MEK1/2 and ERK1/2 pathways, despite the fact that 231-BR cells harbor Ras and B-Raf mutations. None of...
pazopanib’s reported targets could account for this effect in 231-BR cells, because all of the receptors lie upstream of Ras and B-Raf mutations. Enzymatic assays confirmed a direct inhibition of wild-type B-Raf activity by pazopanib in the high nanomolar range. Using tumor cell lysates, pazopanib inhibition of B-Raf extends to the G464V mutation found in 231-BR cells as well as wild type B-Raf activated by HER2 overexpression. The B-Raf G464V mutation is relatively rare in cancer, but has been reported in an ovarian cancer cell line (37), and a nearby mutation, G469V, was identified in melanoma2. However, almost no inhibitory activity of pazopanib toward the V600E B-Raf form was observed. Independent enzymatic K_i studies confirmed a 2.5-fold lower sensitivity for the V600E mutation than the wild-type enzyme. These data appear to separate pazopanib from previously described B-Raf inhibitors (24, 33, 34) and suggest that it may have distinct preclinical and clinical activity. Further biological and biochemical analyses of the B-Raf mutational spectrum sensitive to pazopanib inhibition is ongoing using primary tumor xenografts.

Combined with previous data indicating an inhibitory effect of pazopanib on C-Raf (25), our data indicate that this drug is a pan-Raf inhibitor. The interrelationship of B-Raf and C-Raf is complex. Dumaz et al. reported that when Ras is mutated in melanoma cell lines, the cells stop signaling through B-Raf and switch their signaling to C-Raf to activate the MEK/ERK pathway (38). The potential importance of B-Raf to the ERK pathway in 231-BR cells was demonstrated by siRNA knockdown of B-Raf. A potent inhibition of ERK was observed, with no effect on C-Raf expression. These data may appear in contradiction with Dumaz et al. (38), but can be potentially explained by the fact that the 231-BR cell line harbors both a mutant Ras and a mutant B-Raf. B-Raf may become the main mediator downstream of mutant Ras, rather than C-Raf. Similarly, siRNA knockdown of B-Raf in MCF7 cells, where a wild-type B-Raf was activated by HER2 transfection, also showed a significant role for B-Raf in ERK regulation. These data confirm the significance of B-Raf in a more prevalent scenario. It will be of interest to determine whether B-Raf activation by other upstream oncogenic events is also sensitive to pazopanib inhibition.

Brain metastases of breast cancer appear to be increasing in incidence and threaten to limit the gains made by systemic chemotherapy. Using the 231-BR-HER2 model, pazopanib prevented the formation of micrometastases by 55% (P = 0.067). More control than pazopanib-treated mice were eliminated from MRI analysis as they required sacrifice before the end of the experiments; histological analyses of the brains at necropsy revealed large metastases in all mice (data not shown). Inclusion of these mice in the MRI analysis may have improved the efficacy of pazopanib over that reported. The data suggest a role for pazopanib in the prevention of HER2+ brain metastatic colonization which could be tested in the metastatic setting or in the time to the development of a new metastasis in brain metastatic patients treated with drug. Future studies will investigate rational combinations of HER2 therapeutics, such as lapatinib and pazopanib, for the prevention and treatment of brain metastasis. We are attempting to derive brain metastatic sublines of naturally occurring HER2+ breast cancer cell lines for these studies.

Given the multiplicity of activities of pazopanib, we attempted to determine the relative contribution of anti-angiogenesis versus Raf inhibitory activity in the experimental brain metastasis assays. The role of angiogenesis in metastasis has been controversial generally and in the brain (11, 14, 15, 39–42). In the 231-BR-HER2 model, brain metastasis development per se resulted in vascular remodeling. Lower vessel densities and higher percentages of brain area covered by vessels corresponded to fewer but dilated vessels, in agreement with previous reports (14). In the MCF7-HER2-2R3 model, higher percentages of brain area covered by vessels were also observed. However, no effect of pazopanib at either dose tested was found on these parameters in both models. It remains possible that other aspects of vascular permeability and remodeling may be affected in the brain, and that pazopanib may exert anti-angiogenic responses in other organ sites of metastasis.

In contrast, a significant decrease in pMEK1/2 and pERK1/2 staining was observed in pazopanib-treated 231-BR-HER2 brain metastases, confirming the in vivo modulation of Raf activity. For technical reasons, pMEK1/2 and pERK1/2 staining could not be performed on the MCF7-HER2-2R3 model. Indeed, each brain was bisected along the sagittal plane and each hemisphere fixed differently for the various analyses presented herein. Unfortunately, it turned out that the metastases favored one hemisphere, leaving inadequate amounts of metastatic tissue for the pMEK1/2 and pERK1/2 staining. Characterization of the signaling pathways altered by pazopanib in MCF7-HER2 mammary fat pad xenografts in vivo is ongoing; results to date showed a significant decrease in pERK1/2 after pazopanib treatment. The data indicate that the recently discovered B-Raf inhibitory activity of pazopanib is active in vivo, and may be of preventive significance. These data may permit new clinical trials to be developed and rational combinations tested.

Disclosure of Potential Conflicts of Interest

B. Gril: collaborative research agreement with GlaxoSmithKline. The other authors disclosed no potential conflicts of interest.

2M. Herlyn, The Wistar Institute, Personal Communication.
3B. Gril, D. Palmieri, Y. Qian, L. Ileva, P. Choyke, M. Bernardo, D.J. Liewehr, S.M. Steinberg, and PS Steeg: The B-Raf status of tumor cell lines is a determinant of both anti-tumor and anti-angiogenic effects of pazopanib. In preparation.
Acknowledgments

The authors thank Drs. Tona M. Gilmer, Stephen D. Rubin, and Rakesh Kumar, GlaxoSmithKline, for their enzymatic data and helpful discussions. We thank Dr Dennis J. Slamon, UCLA, for the MCF7-HER2 cell line. We thank Emily Hua for her assistance with the flow cytometry experiment.

Grant Support

P.S.S. receives research support from the Intramural program of the National Cancer Institute (NCI) and by grant W81XWH-06-0033 from the Department of Defense Breast Cancer Research Program. This work was supported by the Intramural program of the National Cancer Institute (NCI), by Grant W81XWH-06-0033 from the Department of Defense Breast Cancer Research Program, and by funding from GlaxoSmithKline.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received June 16, 2010; revised September 17, 2010; accepted October 17, 2010. Published OnlineFirst November 16, 2010.

References

9. Carbone W, Ansonge O, Sibson N, Mushel R. The vascular base-
11. Carbonell WS, Ansonge O, Sibson N, Mushel R. The vascular base-
15. Carbonell WS, Ansonge O, Sibson N, Mushel R. The vascular base-
23. Pietras R, Fendly B, Chazin V, Pegram M, Howell S, Samdon L. Anti-
26. Poulikakos PI, Zhang C, Bollag G, Shokat KM. Raf inhibitors transactivate RAF dimers and ERK signalling in cells with wild-
31. Allen LF, Sebott-Leopold J, Meyer MB. CI-1040 (PD184352), a tar-
32. Bollo rb JA, Wilh esm H, Carter C, Kelley SL. Role of Raf kinase in cancer: therapeutic potential of targeting the Raf/MEK/ERK signal transduc-
35. Olaus sen KA, Commo F, Tait er M, Lacroix L, V lante I, Raza SQ, et al. Synergistic proapoptotic effects of the two tyrosine kinase inhibitors


Pazopanib Reveals a Role for Tumor Cell B-Raf in the Prevention of HER2+ Breast Cancer Brain Metastasis

Brunilde Gril, Diane Palmieri, Yong Qian, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-10-1603

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2011/01/03/1078-0432.CCR-10-1603.DC1

Cited articles
This article cites 42 articles, 15 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/17/1/142.full.html#ref-list-1

Citing articles
This article has been cited by 10 HighWire-hosted articles. Access the articles at:
/content/17/1/142.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.