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Pazopanib reveals a role for tumor cell B-Raf in the prevention of HER2+ breast cancer brain metastasis

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Abstract

Purpose—Brain metastases of breast cancer contribute significantly to patient morbidity and mortality. We have tested pazopanib, a recently approved anti-angiogenic drug that targets VEGFR1-3, PDGFR β , PDGFR α 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 post-injection. Brain metastases were counted histologically, imaged and immunostained.

Results—Treatment with 100 mg/kg pazopanib resulted in a 73% decline in large 231-BR-HER2 metastases ($p < 0.0001$) and 39% decline in micrometastases ($p = 0.004$). In vitro, pazopanib was directly anti-proliferative 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 while blood vessel density was unaltered. In the MCF7-HER2-BR3 experimental brain metastasis model, pazopanib reduced overall brain metastasis volume upon MRI imaging 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.

Keywords

Brain; metastases; B-Raf; HER2; breast cancer

Introduction

The majority of cancer patients succumb to metastatic disease or the consequences of its treatment. While metastasis to any site in the body is a devastating event, the brain may represent a “final frontier”. Brain metastases are ten-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 US 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 $\alpha_v\beta_3$ (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 metastases. HER2 transfectants of 231-BR produced comparable numbers of micrometastases as controls, indicating that the ability of tumor cells to arrive in the brain and complete the first few rounds of division was not affected by HER2 overexpression; however, large metastases were 2.5–3 fold more prevalent (10).

The role of angiogenesis in brain metastasis has been controversial. The brain is highly vascularized and several reports describe a co-option of the existing vasculature by metastasizing tumor cells (14) (15); others reported a role of VEGF-induced angiogenesis (11). Pazopanib represents a new addition to the multi-targeted VEGFR inhibitors, inhibiting the ATP binding pockets of VEGFR1, VEGFR2, VEGFR3, PDGFR α , PDGFR β and c-kit in the low nanomolar range. Anti-angiogenic activity was demonstrated in corneal pocket and bFGF plug assays, and anti-tumor activity was demonstrated in numerous xenografts (16). Pazopanib was recently FDA approved for the treatment of advanced renal cell carcinoma, and clinical testing is ongoing in a variety of other cancer histologies (17–20). Here, we report efficacy and mechanistic studies of pazopanib in the 231-BR-HER2 model. We found that pazopanib can directly affect tumor cells in addition to endothelial cells and report a new activity for this drug as a B-Raf inhibitor. Pazopanib efficacy on brain metastasis colonization was confirmed in a second, new model of brain metastasis using a brain seeking clone of the MCF7-HER2 cell line. These data identify pazopanib as a potential new drug for the prevention of brain metastasis from HER2+ breast cancer.

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 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 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, CA) and maintained in RPMI-1640 (Invitrogen) supplemented with 10% 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 Supplemental 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 Supplemental 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 1.75×10^5 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 post-injection for 21 days. Histologic analysis is described in Supplemental Materials and Methods.

For MCF7-HER2-BR3 cells: for establishment of the BR3 cell line see Supplemental Material and Methods. Athymic nude mice were injected with 1×10^6 cells in the left cardiac ventricle. Additionally, mice received a subcutaneous injection of 1.5 mg/kg Depo-estradiol one day before tumor cell injection and once a week throughout the duration of the study. On day 3 post-injection, 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 MRI. After MRI, all mice were euthanized and the brain frozen as described in Supplemental Materials and Methods. MRI imaging of brain metastases is described in Supplemental Materials and Methods.

Statistical analysis

Statistical analyses are described in Supplemental methods.

Results

Pazopanib inhibits human brain microvascular endothelial cell growth

To confirm the anti-angiogenic activity of pazopanib, its effect on human brain microvascular endothelial cells (HBMEC) was analyzed. The IC_{50} for pazopanib for anchorage-dependent growth was 2 μ M and 1 μ M after 48 h and 72 h, respectively (Fig. S1A). In an in vitro tube formation assay, pazopanib disrupted 50% of the network at 1 μ M (Fig. S1B and C). Pazopanib abrogated the phosphorylation of VEGFR2 with disruption of downstream PLC γ 1 (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 IC_{50} of pazopanib was 5 μ M 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 μ M (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) (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, phospho-p70S6K and cyclin D1, and an increase in the cyclin-dependent kinase inhibitor p27.

Cell cycle analysis was performed using vehicle or 5 μ M pazopanib. At 72h of culture, vehicle treated 231-BR-vector and 231-BR-HER2 cells were spread throughout the cycle, with 57.99% and 61.03% of cells in G0/G1, 19.97% and 18.02% in S phase and 22.05% and 20.95% in G2/M, 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 principle effect of pazopanib on tumor cells was the inhibition of the constitutively activated Ras-Raf-ERK1/2 pathway, which 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, PDGFR α , PDGFR β and c-kit), because all of these targets are upstream of Ras and B-Raf, which 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 non-clinical 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 (K_i) of pazopanib for wild type B-Raf was 59.2 nM and for B-Raf V600E was 148 nM, a 2.5 fold difference, confirming the lesser activity toward V600E B-Raf⁵. Consistent with its effects

as a B-Raf inhibitor, the anti-proliferative activity of pazopanib on 231-BR-HER2 cells was similar to that of non-clinical 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)(27–28). 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 (Figure 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 pathway in 231-BR-vector cells. Similar results were observed using MCF7 cells with wild type B-Raf activated by HER2 transfection (Figure 2E). In independent experiments, the dissociation constant (K_i) of pazopanib for wild type C-Raf was 91 nM vs. 59.2 nM for B-Raf, identifying a ~1.5-fold better affinity for B-Raf than for C-Raf⁵. Taken together, the data indicate that pazopanib is a pan-Raf 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 mg/kg or 100 mg/kg 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 H&E-stained brain sections (Table 1 and Fig. S2). A cutoff of 300 microns in a single dimension was chosen to separate large metastases from micrometastases because it is proportional to a ~5mm MRI detectable lesion in a human brain. The 231-BR-HER2 cells produced a mean of 101.9 micrometastasis per brain section in the vehicle treated mice. Treatment with 30 mg/kg pazopanib resulted in a 25% decrease to 76.4 micrometastasis (NS); treatment with 100 mg/kg resulted in a 39% decline in micrometastasis to 61.7 ($p=0.004$).

The efficacy of pazopanib in preventing large brain 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 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 ($p<0.0001$). 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.

Since 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 (Fig. S3).

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

It is unknown if pazopanib targeted anti-proliferative (Raf-MEK-ERK) and/or anti-angiogenic 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

⁵Dr. Rakesh Kumar, GlaxoSmithKline, Personal communication.

and all large metastases per brain section for five mice per treatment group were scored for antibody staining on a 0 – 3+ intensity scale (Fig. 3). In the mice treated with vehicle, the staining for pERK1/2 was approximately equally divided between the low (score 0/1+) and high (2+/3+) intensity 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 3–8% 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 between 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 to the metastases (normal brains of vehicle treated mice: 61.4 ± 6.53 vessels/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: $2.90 \pm 0.398\%$ of area in normal brain vs. $4.15 \pm 0.335\%$ of brain metastases area) (Fig. 4). This difference reflected fewer, but dilated blood vessels in the metastases, 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 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 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 three rounds of intracardiac injection, brain dissection, cell culture and re-injection 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 $\sim 7 \mu\text{M}$ and a Raf inhibitor and MEK inhibitor produced similar effects (Fig. S4A). Pazopanib at low dose induced a slight increase in pERK1/2 but, at higher doses, a decrease was observed (Fig. S4B). This trend was previously described in recent papers (26,28). Cell cycle analysis showed that, after 72h of vehicle treatment, 55.67% of cells were in G0/G1, 30.96% were in S phase and 13.36% are in G2/M. Five μM pazopanib induced a complete cell cycle arrest in G0/G1 (Fig. S4C).

Using this model, mice were treated with vehicle or 100 mg/kg pazopanib, starting 3 days post-injection and extending for six weeks thereafter. MRI analysis was performed on the brains of mice on day 42 post-injection 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 to one mouse from the pazopanib treated arm. For the MRI analysis, seven of ten mice per experimental arm developed brain metastases. Metastasis volume for all lesions calculated and representative MRI images are shown (Figure 5A and B and Supplemental Figure S5). Brain metastases were heterogeneous in size in the vehicle treated group. The average metastatic tumor volume in the vehicle treated group was 45.4 mm³ (95% CI: 18.1–72.7), while the average metastasis size in the pazopanib treated group was 20.5 mm³ (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 OCT a priori, for analysis of blood vessel density and percent of blood vessel coverage (Figs. 5C and D). Remodeling of blood vasculature was also observed in this model. The blood vessel density was slightly decreased in the metastases compared to 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). Figure 5E is representative photographs of normal brain tissues. Representative photographs of blood vessels in metastatic tissue are shown in figure 5F. The data confirm vessel remodeling in brain metastases but fail to demonstrate an anti-angiogenic effect of pazopanib.

Discussion

We report a new activity for the multi-kinase 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, since 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 nM 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 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 melanoma⁶. However almost no inhibitory activity of pazopanib toward the V600E B-Raf form was observed. Independent enzymatic Ki 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.

⁶Dr. Meenhard Herlyn, The Wistar Institute, Personal Communication.

Further biological and biochemical analyses of the B-Raf mutational spectrum sensitive to pazopanib inhibition is ongoing using primary tumor xenografts ⁷.

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 inter-relationship of B- 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 25–39% and the formation of large metastases by 51–73%. We report the development of a new experimental brain metastasis model using the estrogen receptor+ MCF7 cell line transfected with HER2. After three rounds of brain passage, the MCF7-HER2-BR3 line produced one large brain metastasis in approximately 70% of mice 6 weeks post-injection. Pazopanib reduced the size of the brain metastases 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-BR3 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.

⁷B Gril, D Palmieri, Y Qian, L Ileva, P Choyke, M Bernardo, DJ Liewehr, SM Steinberg, and PS Steeg: Pazopanib inhibits primary tumor growth through B-Raf status and angiogenesis. In preparation

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-BR3 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 treatment⁷. 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.

Statement of translational relevance

Brain metastases occur in ~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, PDGFRs and c-kit, reduced brain metastatic colonization in two experimental breast cancer models overexpressing 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Pazopanib directly targets breast tumor cells

A- Effect on cell viability. 231-BR-vector and 231-BR-HER2 transfectants were incubated with increasing concentrations of pazopanib for 96 h. Cells were assayed for viability using MTT. Data are represented as mean $\pm$ standard error of the mean (SEM) of three independent experiments. B- Effect on signaling pathways. 231-BR-vector or 231-BR-HER2 cells were serum starved overnight and subsequently treated with pazopanib or DMSO for 24 hours. After treatment, cells were stimulated with 10 ng/mL VEGF for 10 minutes, and effects on signaling proteins assayed. "p-" refers to phosphorylation of the residue(s) in parentheses. The data shown are representative of three separate experiments. C- Cell Cycle analysis. Cells were treated with vehicle (DMSO) or 5 μ M pazopanib for 72h then analyzed by flow cytometry.

Figure 2. Pazopanib inhibits B-Raf

A- Effect of pazopanib on B-Raf protein. Increasing concentrations (0.022, 0.11, 0.22, 0.44, 2.2 μ M) of pazopanib, lapatinib or a non-clinical Raf inhibitor were incubated with B-Raf for 20 min 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 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, 2.2 μ M. C- Effect on cell viability. 231-BR-vector cells were incubated with increasing concentrations of pazopanib, Raf inhibitor or MEK inhibitor (U0126) for 96 h. Cells were assayed for viability using MTT. Data are represented as mean $\pm$ SEM. D and E- B-Raf siRNA transfection. 231-BR-vector (D) and MCF7-HER2 (E) cell lines were transfected with two different B-Raf siRNA constructs (S1 and S2), with a non targeting siRNA (C), or treated with the transfectant agent alone (T). Cell lysates were collected at 48 and 96h after transfection.

Figure 3. Pazopanib inhibits brain metastatic colonization of 231-BR-HER2 cells targeting Raf-MEK-ERK pathway

Brain sections from five 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 it was significant ($p < 0.01$).

Figure 4. Pazopanib does not affect blood vessel density

A- Top- Mean number of blood vessels per high power field. Bottom- Percentage of area occupied by blood vessels, both determined by image analysis of CD31 staining. P values comparing brain metastasis to normal brain are indicated. Raw data are presented but statistical analysis was performed on square root transformed data. B- Representative photographs of CD31 staining in normal brain and metastases.

Figure 5. Pazopanib decreases the brain metastatic outgrowth of MCF7-HER2-BR3 breast carcinoma cells without an effect on the vasculature

A- Mice were injected with MCF7-HER2-BR3 cells and randomized to receive vehicle or 100 mg/kg pazopanib. MRI analyses were performed on 10 mice per group. Each mouse had a single metastasis. Each dot represents a mouse. B- Representative MRI scans of mouse brains. C and D- Vascular changes. C- Blood vessel density. D- Percentage of brain section covered by blood vessels. Raw data are presented but statistical analysis was performed on square root transformed data. E to F- Representative photographs taken at 100x magnification: CD31 staining in (E) normal brain tissue and (F) in metastatic tissue.

Table 1

Pazopanib prevention of brain metastatic colonization *

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

* Mice were inoculated with 1.75×10⁵ 231-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 were analyzed to count every micro- or large (>300 microns in any single dimension) metastasis in each section. The mean number of metastases is reported.