Suppression and Regression of Choroidal Neovascularization by the Multitargeted Kinase Inhibitor Pazopanib

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Objective: To investigate pazopanib hydrochloride, a multitargeted kinase inhibitor, for treatment of choroidal neovascularization (CNV).

Methods: Choroidal neovascularization was induced in mice by rupture of Bruch membrane with laser photocoagulation. Mice were treated with pazopanib by gavage or periocular injection, and the area of CNV was measured.

Results: Twice-daily gavage of pazopanib, 100 mg/kg, suppressed the development of CNV by 93%. Treatment of established CNV between days 7 and 14 with 8, 40, or 200 mg/kg per day reduced CNV by 0%, 58%, and 71%, respectively. Substantial regression (40%) of CNV was also achieved after periocular injection of pazopanib. A single oral dose of 4 or 100 mg/kg resulted in an area under the curve from time 0 to the last quantifiable concentration of 129.6 and 752.0 µg•h/mL, respectively. After 7 days of 4, 20, or 100 mg/kg twice a day by gavage, plasma levels were 1300, 4900, and 5800 ng/mL and levels in the retina/choroid were 4800, 28800, and 38000 ng/g of tissue.

Conclusions: Orally administered pazopanib has good bioavailability to the retina/choroid and strongly suppresses CNV in mice. Treatment with pazopanib after CNV is established causes dose-dependent regression of CNV.

Clinical Relevance: Pazopanib may be useful for treatment of CNV in humans.


CHOROIDAL NEOVASCULARIZATION (CNV) is the most common cause of severe vision loss in patients with age-related macular degeneration and is responsible for visual disability in a substantial number of young patients with pathological myopia, ocular histoplasmosis, angiod streaks, and several other diseases. Although the pathogenesis of CNV is not completely understood, the demonstration that vascular endothelial growth factor (VEGF) is an important stimulator is a major advance.1,2 Clinical trials have confirmed the importance of VEGF because intraocular injections of ranibizumab, a recombinant humanized antibody antigen fragment that binds all isoforms of VEGF-A, resulted in significant improvement in vision in 34% to 40% of patients with subfoveal CNV due to age-related macular degeneration.3,4 Case series have suggested that bevacizumab, a full-length antibody that binds all isoforms of VEGF-A, also benefits patients with CNV due to age-related macular degeneration or other disease processes.5,11

The major effect of antagonists of VEGF-A such as ranibizumab and bevacizumab is to reduce excessive vascular permeability from CNV, which results in rapid reduction in subretinal and intraretinal fluid and improvement in visual acuity. Monthly injections of ranibizumab stopped growth of CNV but did not cause existing CNV to regress.3 Perhaps there are survival factors other than VEGF-A that allow endothelial cells within CNV to survive and remain quiescent despite blockade of VEGF-A with ranibizumab, and as soon as levels of ranibizumab are reduced beyond a critical level, leakage and growth of CNV resume. Likely candidates for adjunctive survival factors include other VEGF family members and platelet-derived growth factor B (PDGF-B), which promotes survival of pericytes, another source of survival factors for endothelial cells in new vessels.12

An efficient way to target multiple VEGF family members is to block VEGF receptors (VEGFRs) with relatively selective VEGFR kinase inhibitors. Because there is high homology between VEGFRs and PDGF receptors (PDGFRs), many ki-
nase inhibitors block both. Pazopanib is a small-molecule kinase inhibitor that blocks VEGFR1, VEGFR2, and VEGFR3 with 50% inhibitory concentration (IC50) of 10nM, 30nM, and 47nM, respectively.13 Pazopanib also has substantial activity directed against PDGFRα (IC50, 71nM), PDGFRβ (IC50, 84nM), c-Kit (IC50, 74nM), fibroblast growth factor receptor 1 (FGFR1; IC50, 140nM), FGFR3 (IC50, 130nM), and c-fms (IC50, 146nM). Activity is substantially less against many other kinases that were tested, and thus pazopanib has an interesting inhibitory profile with regard to potential effects in angiogenic diseases. An agent with a similar inhibitory profile showed strong antitumor and antiangiogenic activity in mouse xenograft tumor models. In this study, we investigated the effects of pazopanib in mouse models of subretinal neovascularization.

**METHODS**

**MOUSE MODEL OF CNV**

Mice were treated in accordance with the Association for Research in Vision and Ophthalmology guidelines for the use of animals in research. The CNV was created by laser photocoagulation—induced rupture of Bruch membrane as previously described.14 Briefly, 5- to 6-week-old female C57BL/6J mice were anesthetized with ketamine hydrochloride (100 mg/kg of body weight), and pupils were dilated with 1% tropicamide. Three burns of 532-nm diode laser photocoagulation (75-µm spot size, 0.1-second duration, 120 mW) were delivered to each retina with the slitlamp delivery system of a diode laser (OcuLight GL; Iridex, Mountain View, California) with the use of a handheld coverslip as a contact lens to view the retina. Burns were performed in the 9-, 12-, and 3-o’clock positions of the posterior pole of the retina. Production of a bubble at the time of laser application, which indicates rupture of Bruch membrane, is an important factor in obtaining CNV, and therefore only burns in which a bubble was produced were included in the study. In the initial study, mice were treated twice a day by gavage with pazopanib hydrochloride, 100 mg/kg, or vehicle (n=15) for 14 days and then the area of CNV at Bruch membrane rupture sites was measured.

**MEASUREMENT OF THE AREA OF CNV**

Mice were perfused with 1 mL of phosphate-buffered saline containing fluorescein-labeled dextran, 50 mg/mL. (2 × 10^4 average molecular weight; Sigma-Aldrich Corp, St Louis, Missouri), and choroidal flat mounts were prepared as previously described.13 Briefly, eyes were removed and fixed for 1 hour in 10% phosphate-buffered formalin. The cornea, lens, and retina were removed and 4 radial cuts were made in the eye cup, allowing it to be flat mounted in aqueous mounting medium. Flat mounts were examined by fluorescence microscopy, and images were digitized by means of a 3-color charge-coupled device video camera and a frame grabber. Image analysis software (Image-Pro Plus; Media Cybernetics, Silver Spring, Maryland) was used to measure the total area of CNV at each rupture site.

**TREATMENT OF ESTABLISHED CNV**

Seven days after laser-induced rupture of Bruch membrane, 10 mice were perfused with fluorescein-labeled dextran and the baseline area of CNV was measured. The remaining 40 mice were treated twice a day by gavage with vehicle or pazopanib hydrochloride, 4, 20, or 100 mg/kg (n=10 for each). In other experiments, 20 mice had rupture of Bruch membrane and, after 7 days, 3 mice were perfused with fluorescein-labeled dextran and the baseline area of CNV was measured. The remaining 15 mice were treated with daily periocular injections of 100 µg of pazopanib or vehicle in 1 eye. After 7 days, the mice were perfused with fluorescein-labeled dextran and the area of CNV at the Bruch membrane rupture sites was measured.

**PHARMACOKINETIC STUDIES**

Female C57BL/6J mice (n=3 for each time point) weighing 18 to 22 g were given pazopanib hydrochloride, 4, or 10 mg/kg, by gavage, and plasma was collected at 0, 30, 60, 120, 240, 480, and 1440 minutes after dosing. Mice were killed by carbon dioxide asphyxiation and bled for plasma samples at 14, 30, and 60 minutes after dosing. The blood was placed in sodium EDTA–containing tubes on ice. Within 30 minutes of collection, the anticoagulated blood samples were centrifuged at 4500 rpm for 10 minutes at 4°C, and the resultant plasma samples were immediately frozen on dry ice and stored at −80°C until analysis.

Mice (n=3 per dose) were given pazopanib hydrochloride, 4, 20, or 100 mg/kg, twice a day by gavage for 7 days. Mice were humanely killed by carbon dioxide asphyxiation and bled for plasma samples at 14, 30, and 60 minutes after the last dose. Eyes were removed, snap frozen in liquid nitrogen, and stored at −80°C until dissection. The right eye of each mouse was used to prepare eye cups by removing the anterior segment and vitreous, leaving the sclera, choroid, and retina. For the left eyes, the anterior segment and vitreous were removed and then the retina and choroid were removed together to provide retina/choroid. The remaining sclera, which were analyzed separately.

**EXTRACTION OF PAZOPANIB FROM TISSUES**

Isolated tissues (eye cup, sclera, and retina/choroid) were weighed, snap frozen in liquid nitrogen, and placed in a liquid nitrogen–primed lysing matrix system (BioPulverizer, Biospec Products Inc, Bartlesville, Oklahoma). The tissue was pulverized and tissue powder was transferred into polypropylene tubes. Extraction buffer (50% methanol/50% 0.3M hydrochloric acid) was added to tissue powder, followed by 2 cycles of sonication, centrifugation, and supernatant collection. Tissue homogenate supernatant was pooled, frozen on dry ice, and stored at −80°C until analysis. The extraction efficiency of this method was assumed to be 100% for calculation purposes.

**MEASUREMENT OF PAZOPANIB**

Plasma samples and eye tissue extracts were analyzed for pazopanib by an analytical method based on protein precipitation followed by analysis with high-performance liquid chromatography and 2 stages of mass spectrometry. With a 20-µL aliquot of mouse plasma, the lower limit of quantification for pazopanib was 100 ng/mL, and the higher limit of quantification was 50,000 ng/mL. For tissue extracts, with a 50-µL aliquot, the lower limit of quantification was 1.0 ng/mL and the higher limit of quantification was 5000 ng/mL.

**DATA ANALYSIS**

Analyst Version 1.4.1 (Applied Biosystems, Foster City, California) and SMS2000 Version 1.6 (developed at GlaxoSmithKline) were used for calculations. Pharmacokinetic analysis of the plasma concentration-time data was performed by noncompartmental methods to obtain estimates of...
pharmacokinetic factors. Tissue concentrations of pazopanib were determined by the following formula:

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\text{Pazopanib (in Nanograms per Gram of Tissue)} = \frac{(\text{Concentration in Supernatant [in Nanograms per Milliliter]} \times \text{Extract Volume [in Milliliters]})}{\text{Tissue Weight in Grams}}.
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**RESULTS**

**EFFECT OF ORAL ADMINISTRATION OF PAZOPANIB**

Treatment with pazopanib hydrochloride, 100 mg/kg by gavage twice a day, resulted in a 93% decrease compared with vehicle treatment in the mean area of CNV at the Bruch membrane rupture sites (**Figure 1**). This represents almost complete suppression of the development of CNV.

To determine the effect of pazopanib on established CNV, mice had rupture of Bruch membrane and the CNV was allowed to grow for 7 days. Ten mice were used to measure the mean area of CNV at the 7-day time point, which provides the baseline. The remaining 40 mice were treated by gavage twice a day between days 7 and 14 with 4, 20, or 100 mg/kg of pazopanib or vehicle. Mice treated with the lowest dose of pazopanib had a mean area of CNV that was significantly less than that seen in mice treated with vehicle and no different from the baseline area of CNV, indicating that this dose essentially stopped further growth of CNV (**Figure 2**). After 7 days of treatment with 40 or 200 mg/kg per day, the area of CNV was significantly less than the baseline amount, indicating that the CNV had regressed. The regression was substantial, consisting of a 58% reduction from baseline area of CNV in mice treated with a pazopanib hydrochloride dosage of 40 mg/kg per day and a 71% reduction in mice treated with 200 mg/kg per day.

**PHARMACOKINETIC STUDIES**

**Single-Dose Plasma Pharmacokinetics**

Plasma pazopanib concentrations were measured at several time points after a single oral dose of 4 or 100 mg/kg. Peak plasma concentrations occurred at approximately 2 hours after dose administration for both dose groups (**Table**). The systemic exposure to pazopanib (area under the curve from time 0 to the last quantifiable concentration, \(C_{\text{max}}\), maximal concentration; \(T_{\text{max}}\), time after dosing that maximal concentration was achieved).

**Multiple-Dose Plasma Pharmacokinetics**

Plasma pazopanib concentrations were measured after oral administration of 4, 20, or 100 mg/kg twice a day for 7 days. Plasma samples were taken from individual mice 16 hours after the last dose was administered. **Figure 3A** shows the mean (SD) and individual mouse...
values for each dosing group. The average plasma pazopanib levels were 1314, 4893, and 5771 ng/mL after 7 days of twice-daily treatment with 4, 20, and 100 mg/kg, respectively.

Ocular Tissue Concentrations of Pazopanib After Oral Administration for 7 Days

Ocular tissue drug distribution was assumed to be equivalent between the 2 eyes. Right eyes were used to dissect eye cups (posterior sclera, choroid, and retina) and left eyes were used to dissect posterior sclera alone and retina/choroid. The mean concentration of pazopanib extracted from eye cups increased in a dose-dependent manner and measured 12.2, 41.7, and 114.5 µg/g of tissue for twice-daily dosing with 4, 20, and 100 mg/kg, respectively (Figure 3B). These values were statistically different from each other by analysis of variance (ANOVA) (P < .02).

The mean concentration of pazopanib in isolated retina/choroid was 4.8, 28.8, and 38.0 µg/g of tissue after twice-daily dosing for 7 days with 4, 20, and 100 mg/kg, respectively (Figure 3C). There was substantial variability in pazopanib content extracted from the sclera among mice, and the values were not statistically different by ANOVA, but they clearly demonstrate that pazopanib that enters the choroid from the circulation is able to penetrate into the sclera. If pazopanib concentrations in isolated retina/choroid and sclera are meaningful, one would anticipate that their sum should approximate values obtained in eye cups, and this was found to be the case (Figure 3D).

EFFECT OF PERIOCULAR INJECTION OF PAZOPANIB

Because pharmacokinetic studies demonstrated that substantial amounts of pazopanib entering the choroid from
the circulation were able to enter the sclera, we postulated that pericocular injection of pazopanib should gain access to the choroid and have an effect on CNV. Seven days after rupture of Bruch membrane, 5 mice were used to measure the baseline area of CNV and the remaining 15 mice were given pericocular injections of 100 µg of pazopanib in one eye and vehicle in the other eye. After 7 days, the area of CNV at Bruch membrane rupture sites was significantly less (40% reduction) in eyes treated with pazopanib than in those treated with vehicle and significantly less than the baseline area of CNV (Figure 4).

It is clear that VEGF-A is a critical target for treatment of patients with CNV, but the role of other VEGF family members and related proangiogenic molecules such as PDGF is uncertain. One way to inhibit the effects of all VEGF family members, PDGF, and other stimulators that activate receptors with homology to VEGFRs and PDGFRs is to use small molecules that block activating phosphorylation signaling induced by ligand binding to these receptors. Pazopanib is a kinase inhibitor that has an interesting inhibitory profile: VEGFR1 ~ VEGFR2 ~ VEGFR3 > PDGFRβ ~ PDGFRα ~ c-kit > FGFR1 ~ FGFR3 ~ cfms > > other kinases. Pazopanib has been shown to suppress tumor angiogenesis and growth in xenograft tumor models in mice.13 In this study, we found that systemic administration of pazopanib strongly suppressed the development of CNV. The level of suppression is far greater than that of many other drugs that we have tested, and in fact the only other agents that have similar efficacy in this mouse model of CNV are other kinase inhibitors that block VEGFRs and PDGFRs as well as some other related molecules.1 In this study, we also found that treatment of established CNV with sufficient doses of pazopanib, either systemically or by pericocular injection, resulted in regression of the CNV.

The ability to cause regression of CNV may be useful. Monthly injections of ranibizumab suppress leakage and growth of CNV in patients with neovascular age-related macular degeneration, but do not cause regression.3 In most patients, when ranibizumab injections are stopped there is recurrent leakage and growth of CNV, and many patients require frequent injections to maintain stability. It is reasonable to speculate that complete or even partial regression of CNV would substantially set back the clock and possibly allow for less frequent treatments. In a phase I study investigating the effect of a fusion protein consisting of binding domains from VEGFR1 and VEGFR2 (VEGF Trap-Eye; Regeneron, Tarrytown, New York), a single intraocular injection of 2 or 4 mg resulted in partial regression of CNV, and 4 of 6 patients did not require additional treatment of any kind at 12 weeks, the end of the study. Because this fusion protein binds other members of the VEGF family in addition to VEGF-A, this result suggests that some or all of them may contribute to endothelial cell survival in CNV and that, by blocking all of them, endothelial cells of CNV are compromised and partial regression occurs. Recently, we found that intraocular expression of soluble VEGFR1 was not sufficient to cause regression of CNV.16 There should not be substantive differences in isoforms of VEGF bound by VEGF Trap-Eye compared with soluble VEGFR1, so we are uncertain as to the reason for this difference; it may represent a difference in behavior of CNV in the mouse model vs CNV in patients with neovascular age-related macular degeneration.

It is unclear whether the other inhibitory activities of pazopanib contribute to its high level of efficacy, but there is some evidence to suggest that combined blockade of PDGF and VEGF-A may be superior to either alone. Increased expression of PDGF-B in the retina causes severe proliferative retinopathy and retinal detachment similar to the most advanced stages of proliferative diabetic retinopathy.17 Endothelial cells produce PDGF-B, which promotes the recruitment, proliferation, and survival of pericytes. Platelet-derived growth factor B also recruits glial cells and retinal pigment epithelial cells,18 which promote scarring, a complication of ocular neovascularization that is the major cause of permanent loss of vision. Antagonists of PDGFs may help to reduce scarring but may also synergize with VEGF antagonists to reduce neovascularization through their antagonism of pericytes, which provide survival signals for endothelial cells of new vessels.19 Blockade of VEGF and PDGF-B also appears additive in models of ocular neovascularization.20

There is also evidence that stem cell factor and its receptor c-Kit contribute to angiogenesis by increasing nuclear localization of hypoxia-inducible factor 1α, resulting in elevation of VEGF, PDGF-B, angiopoietin 2, and the products of other genes containing a hypoxia response element within their promoter.21 It is not known whether blocking c-Kit provides benefit in the treatment of ocular neovascularization in addition to blocking the receptors for VEGF and PDGF, but it is a reasonable supposition because angiopoietin 2 is an important stimulator.22-24 Thus, pazopanib inhibits several proangiogenic signaling pathways, and many of these inhibitory activities may contribute to its strong suppression.
and ability to cause regression of CNV, which sets it apart from specific VEGF-A antagonists.

The oral doses of pazopanib hydrochloride, 20 or 100 mg/kg twice a day for 7 days, that caused substantial regression of CNV were associated with pazopanib levels in the retina/choroid of 28.8 to 38.0 µg/g of tissue. This is useful information because it provides a target tissue concentration range. The high levels achieved in the sclera after oral dosing suggest that pazopanib from the circulation is able to penetrate sclera and that pazopanib deposited along the outside surface of the sclera should have good access to CNV. This proved to be the case because daily periorcular injection of 100 µg of pazopanib hydrochloride caused significant regression of CNV, suggesting that local administration may be worth considering.

In conclusion, we have shown that either systemic or local administration of pazopanib causes regression of already established CNV, making it a good candidate for clinical trials in patients with neovascular age-related macular degeneration. Such clinical trials are under way.

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