Antiangiogenic effects of tivozanib, an oral VEGF receptor tyrosine kinase inhibitor, on experimental choroidal neovascularization in mice

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Abstract

We investigated the effects of tivozanib, an oral vascular endothelial growth factor (VEGF) receptor tyrosine kinase inhibitor, on experimental choroidal neovascularization (CNV) in mice. C57Bl/6 mice were treated with tivozanib (1 mg/kg/day) or vehicle at the onset (day 0) of the study and experimental CNV was induced by laser photoocoagulation the following day. In the other groups, tivozanib or vehicle was started 7 days after the laser photoocoagulation to determine the effects of the drug on established CNV. To evaluate changes in the CNV lesions, choroidal flat mounts, fluorescein angiography, immunofluorescence staining with isolectin B4, and histological examinations were performed 14 days after CNV induction. Expression of phosphorylated ERK1/2 in choroidal tissues was measured by western blot analysis to demonstrate the inhibitory effect of tivozanib on intracellular signaling pathways involved in CNV development. Compared to vehicle-treatment, tivozanib suppressed the development of CNV lesions and led to a significant regression of established CNV, reducing the affected areas by 80.7% and 67.7%, respectively. On fluorescein angiography, tivozanib-treated mice had significantly less fluorescence leakage than vehicle-treated mice (P < 0.001). On immunofluorescence staining, the isolectin B4-labeled area was smaller in tivozanib-treated mice (P < 0.001). Phosphorylated ERK 1/2 levels increased after CNV induction by laser application and were suppressed by tivozanib treatment. Tivozanib effectively inhibited the progression of CNV in an experimental CNV model. These results suggest that tivozanib may be a therapeutic alternative for the treatment of neovascular age-related macular degeneration.

1. Introduction

In developed countries, neovascular age-related macular degeneration (AMD) is one of the most common causes of severe visual loss in people over 50 years of age (Lee et al., 1998). Although choroidal neovascularization (CNV) occurring with neovascular AMD is known to develop as a result of outgrowth of new blood vessels from the choriocapillaries, the exact pathogenesis of CNV formation has not yet been fully elucidated. Various experiments investigating the causes of CNV, however, have identified a number of growth factor receptor pathways that promote CNV development. One of the main pathways involved in these angiogenic processes is vascular endothelial growth factor (VEGF-A, hereafter referred to as VEGF) and its receptors (Ishibashi et al., 1997; Shen et al., 1998; Yi et al., 1997). VEGF is a cytokine that plays a major role in the development of normal blood vessels as well as the development of vessels in tumors and other tissues undergoing abnormal angiogenesis (Hicklin and Ellis, 2005). Therefore, anti-VEGF therapies are widely used for the treatment of patients with CNV secondary to AMD and other pathologic conditions. In practice, patients with neovascular AMD are treated with antibody-based anti-VEGF therapies, such as ranibizumab (Rosenfeld et al., 2006), an anti-VEGF monoclonal antibody fragment, and bevacizumab (Bashshur et al., 2006), a full-length anti-VEGF monoclonal antibody. In addition, aflibercept (Brown et al., 2011), a recombinant fusion protein for VEGF and placental growth factor (PIGF), has also been used.

The effects of VEGF ligands are mediated by VEGF receptor tyrosine kinases. Three receptors have been identified and characterized as specific tyrosine kinase receptors (Hoeben et al., 2004): VEGF receptor 1 (also referred to as fms-like tyrosine kinase 1; Flt-
1), VEGF receptor 2 (also referred to as fetal liver kinase; Flk-1/KDR), and VEGF receptor 3 (also referred to as fms-like tyrosine kinase 4; Flt-4). Each receptor has 7 immunoglobulin-like domains in the extracellular domain, a single transmembrane region, and a consensus tyrosine kinase sequence interrupted by a kinase insert domain (Hicklin and Ellis, 2005; Hooißen et al., 2004). Among the 3 VEGF receptor tyrosine kinases, VEGF receptor 2 is mainly responsible for the downstream signaling pathway of VEGF-induced angiogenesis, including increased endothelial cell proliferation, invasion, migration, survival, and microvascular permeability (Dvorak, 2002; Millauer et al., 1993; Zeng et al., 2001). Therefore, the inhibition of the VEGF signal transduction pathway using a VEGF receptor tyrosine kinase blocker might be a good strategy for pharmacological treatment of pathologic neovascularization in the eye.

Tivozanib, an investigational new drug, is a small-molecule receptor tyrosine kinase inhibitor that inhibits the tyrosine kinase activity of VEGF receptors 1, 2, and 3 at subnanomolar concentrations (Nakamura et al., 2006). Preclinical data shows that tivozanib blocked the proliferation and migration of endothelial cells in vitro, and displayed anti-angiogenesis and anti-tumor activity against a wide variety of human tumor xenografts in vivo (De Luca and Normanno, 2010; Nakamura et al., 2006; Taguchi et al., 2008).

Therefore, in the present study, we investigated the antiangiogenic effects of tivozanib on experimental CNV in mice and discuss its potential utility for the treatment of neovascular AMD.

2. Materials and methods

2.1. Drug

Tivozanib (AV-951, KRN-951) powder was purchased from Selleck Chemicals, Houston, TX, USA. For all studies, the drug was dissolved using dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO, USA) and was diluted in 0.5% carboxyl methyl cellulose. Solutions (0.1 mg/ml) were prepared and stored at −20 °C and thawed immediately prior to administration to mice. The solutions were administered (1 mg/kg/day) by oral gavage.

2.2. Animals

A total of 108 C57BL/6 female mice (age, 9–10 weeks; weight, 20–22 g) were used in this study. Four mice were used as the normal control group and 104 mice had CNV-induced by laser photocoagulation. All mice were handled in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. All animal experiments were reviewed and approved by the Institutional Animal Case and Use Committee of College of Medicine, the Catholic University of Korea.

2.3. Laser-induced CNV in mice

Experimental CNV was induced by laser photocoagulation, described in detail elsewhere (Takehana et al., 1999). Briefly, after general anesthesia was induced with intraperitoneal pentobarbital (74 mg/kg; Entobar, Hanlim Pharm. Co., Gyeonggi, Korea), mice pupils were dilated with 0.5% tropicamide and 0.5% phenylephrine (Mydhrin-P, Santen Pharmaceutical Co., Osaka, Japan). Laser photocoagulation-induced rupture of Bruch’s membrane was performed on both eyes using the following laser parameters: 100 μm spot size, 200 mW intensity, and 0.1 s duration. Six to seven laser spots were applied between the major retinal vessels adjacent to the optic disc. Only mice that experienced a cavitation bubble, indicating a rupture in the Bruch’s membrane, were included in the study.

2.4. Tivozanib treatment of experimental CNV and measurement of CNV area

To assess the inhibitory effects of tivozanib on CNV development, 70 mice were randomly divided into 3 groups: (1) baseline (untreated) laser group (n = 12), (2) vehicle-treated laser group, (n = 31) and (3) tivozanib-treated laser group (n = 31). Vehicle and tivozanib treatment started 1 day before laser application. Two weeks after laser photocoagulation, 30 mice (10 in the baseline laser group; 10 in the vehicle-treated laser group; and 10 in the tivozanib-treated laser group) were anesthetized with intraperitoneal pentobarbital (74 mg/kg) and perfused through the left ventricle with 1.0 ml of phosphate-buffered saline (PBS) containing 25 mg fluorescein isothiocyanate (FITC)-dextran (2 × 105 average molecular weight, Sigma), as previously described (Tobe et al., 1998). Encuclued eyes were fixed in 4% paraformaldehyde for 1 h. Retinal pigment epithelium (RPE)-choroid-sclera eyecup was obtained by hemisectioning the eye and peeling neural retina away from the underlying RPE. After 4 radial incisions were made from the edge to the equator, the RPE-choroid-sclera eyecup was flat-mounted in Aquamount with the RPE side facing up. CNV lesions in the flat mounts were examined by scanning laser confocal microscopy (LSM5 live configuration Variotto Two VRGB, Zeiss, Jena, Germany). Images of CNV in choroidal flat mounts were digitalized using image capture and analysis program (LSM Image Browser, Zeiss) were quantified in a masked fashion by two of us (Kang, S., Kim, I.B.) using a computer analysis program (LSM Image Browser).

To test the effects of tivozanib on established CNV, an additional 30 mice were used. Laser-induced CNV was generated at the onset (day 0) of experiments by laser photocoagulation. One week after laser photocoagulation, the mice were assigned into 3 groups of 10. One group of mice (n = 10) was euthanized to measure baseline CNV size present on day 7. In the other 2 groups, vehicle (n = 10) or tivozanib (n = 10) treatment began on day 7. On day 14, mice were perfused with FITC-dextran, and the CNV lesion areas were measured on choroidal flat mounts, as described earlier.

2.5. Fluorescence angiography

Each of the 5 CNV-induced mice in both the vehicle-treated and tivozanib-treated laser groups was used for fluorescence angiography. Forty-four days after photocoagulation, the laser lesions were evaluated using fluorescence angiography to analyze the amount of CNV leakage. Ten minutes after intraperitoneal injection of 0.05 mL of 10% sodium fluorescein (Fluorescine, Alcon Japan, Tokyo, Japan), fluorescence angiography was performed, and late-phase angiograms were obtained using confocal scanning laser ophthalmoscopy (Spectralis, Heidelberg Engineering Inc., Heidelberg, Germany). Images were analyzed using Image-J software (National Institutes of Health, Bethesda, MD, USA). The signal intensities, defined as the brightness of the CNV lesion with leakage, were measured as previously described (Takahashi et al., 2006). The signal intensity for each pixel of the image was represented as an arbitrary unit from 0 (darkest) and 1 (brightest). As a reference, the intensity within a non-photocoagulated capillary area was defined as 0 and the intensity at the major branch of the retinal vein was defined as 1.

2.6. Quantification of CNV by immunofluorescence staining

Each of the 10 CNV-induced mice in both the vehicle-treated and tivozanib-treated laser groups was used for immunofluorescence staining. Two weeks after laser photocoagulation, mice were euthanized, and their eyes were enucleated and fixed in 4% paraformaldehyde for 1 h. Previously described immunofluorescence
staining techniques were used to label endothelial cells within CNV lesions (Campos et al., 2006). Briefly, the cornea, crystalline lens, and vitreous were removed, and the retinas were gently peeled and separated from the optic disc. The remaining eyecups were rinsed in the blocking solution containing 0.5% bovine serum albumin (BSA) and 0.2% polysorbate 20 (Tween20, Sigma) diluted in PBS, and then incubated overnight at 4 °C with a 1:1000 dilution of a 10 mg/ml solution of 4,6-diamidino-2-phenylindole (DAPI), a 1:100 dilution of a 1 g/L solution of isolectin B4 (IB4) conjugated with Alexa Fluor 568 (Invitrogen-Molecular Probes, Eugene, OR, USA), and a 1:100 dilution of a 0.2 units/L solution of phalloidin conjugated with Alexa Fluor 488 (Invitrogen-Molecular Probes). The eyecups were washed with cold PBS, and then flat-mounted with the RPE side facing up. The images were taken with a fluorescence microscope (Eclipse TE300, Nikon, Tokyo, Japan) and evaluated with image-analysis software (NIS Elements BR, Nikon).

2.7. CNV histology

Each of the 2 CNV-induced mice in the baseline, vehicle-treated, and tivozanib-treated laser groups was used for histologic examinations. Fourteen days after laser injury, mice in each group were euthanized. The eyes were enucleated, and the cornea, lens, and vitreous were removed. Paraformaldehyde-fixed eyes were embedded in paraffin, and serial sections (4-μm thickness) were cut throughout the entire extent of laser burn and stained with hematoxylin-eosin (HE). We examined the serial sections at 200 magnification using a light microscope and an image analysis program (NIS Elements BR, Nikon).

2.8. Western blot analysis

Twelve mice were used in western blot analyses. The phosphorylation of extracellular signal-regulated kinase (ERK1/2) in RPE/choroid tissues was evaluated by western blot analysis of RPE/choroid tissues from 6 mice (2 in the normal control group without laser; 2 in the vehicle-treated laser group; and 2 in the 10 mg/kg/day tivozanib-treated laser group). The analysis was repeated. Vehicle or tivozanib treatment started 1 day before laser application. At days 1 and 2 after laser application, RPE/choroid tissues were obtained and pooled in 100 μl of lysis buffer (Pro-prep Protein Extraction Solution, iNtRON Biotechnology, Korea), and homogenized using TissueLyser II (Qiagen, Hilden, Germany). Next, the tissue lysates were centrifuged at 13,000 rpm for 15 min at 4 °C, and the supernatants were used for protein concentration using bicinchoninic acid (BCA) protein assay. Equal amounts of protein were electrophoresed on a 10% SDS-polyacrylamide gel and transferred electrophoretically onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). After blocking with a 3% BSA solution, the membrane was incubated overnight at 4 °C with an anti-phospho-ERK 1/2 antibody (Cell Signaling, Danvers, MA, USA) and an anti-ERK1/2 antibody (Cell Signaling) to detect phosphorylated ERK1/2 and total ERK1/2, respectively. The intensity of the signals was recorded and analyzed using a molecular imaging system (Molecular Imager

Fig. 1. Suppression of choroidal neovascularization (CNV) by tivozanib. Representative CNV lesions in choroidal flat mounts were obtained 2 weeks after laser-induced rupture of Bruch’s membrane by perfusion of fluorescein isothiocyanate-dextran. CNV lesions in the baseline laser group (A), vehicle-treated laser group (B), and tivozanib (1 mg/kg/day)-treated laser group (C). Red dotted lines indicate CNV lesions. (D) The areas of CNV lesions in the 3 groups were quantified by digital imaging analysis. * P < 0.001 as compared to both baseline and vehicle-treated laser groups. Scale bar, 100 μm.
Chorioidal phalloidin were shown as a tightly packed hexagonal monolayer in eyes without laser application (Fig. 3A). The expression levels of phosphorylated ERK1/2 were normalized to the corresponding levels of total ERK1/2 and expressed as arbitrary units.

2.9. Statistical analysis

The CNV lesion areas identified using FITC-dextran perfusion were evaluated with one-way analysis of variance (ANOVA) and Scheffe’s multiple comparison tests using SPSS (SPSS version 17.0, Chicago, IL, USA). Results are expressed as mean ± standard deviation, if not indicated otherwise, and boxplots are used to display data graphically between groups. Both the size of the CNV lesions obtained by immunofluorescence staining and the amount of leakage from the CNV on fluorescein angiograms were analyzed using an unpaired t-test. P values less than 0.05 were considered statistically significant.

3. Results

Orally administrated tivozanib for 14 days did not cause any notable side effects in all mice. They maintained good activity and normal appetite and showed no significant changes of body weights in any experimental group.

3.1. Suppression of laser-induced CNV after tivozanib administration

Fig. 1 shows representative images of choroidal flat mount preparations from baseline, vehicle-treated, and tivozanib-treated laser groups on day 14 after laser-induced rupture of Bruch’s membrane. Analysis of the choroidal flat mounts revealed that the area of CNV lesions in tivozanib-treated laser group was much smaller than those in either the baseline or vehicle-treated laser groups (P < 0.001). Quantification of CNV from the 3 groups is summarized in Fig. 1D. The CNV lesion areas in the baseline and vehicle-treated laser groups were 15,648.0 ± 7083.9 μm² and 15,498.1 ± 6167.7 μm², respectively. The CNV area in the tivozanib-treated laser group was 2977.2 ± 1817.1 μm². The tivozanib-treated laser group showed a reduction in CNV area by 80.9% and 80.7% compared to the baseline and vehicle-treated laser group, respectively.

3.2. Angiographic assessment

On angiographic analysis, fluorescein leakage was significantly reduced in the eyes that were treated with tivozanib compared to eyes from mice treated with vehicle. Fluorescein signal intensity scores of the vehicle and tivozanib-treated groups were 0.815 ± 0.445 and 0.487 ± 0.320, respectively (P = 0.002) (Fig. 2A–C). The average intensity scores of eyes treated with tivozanib was 40.2% lower than those treated with vehicle. No abnormalities were found in the normal retinal blood vessels as determined by fluorescein angiography.

3.3. Quantitative assessment of CNV by immunofluorescence staining

The normal morphology of the RPE/choroid tissues was evident in eyes without laser application (Fig. 3A–D). RPE cells stained with phalloidin were shown as a tightly packed hexagonal monolayer (Fig. 3A). There were no IB4-labeled endothelial cells in the RPE/choroidal flat mounts of eyes without laser injury (Fig. 3B). DAPI staining identified the nuclei of RPE cells (Fig. 3C). The images of phalloidin-, IB4-, and DAPI-stained sample were merged (Fig. 3D). Day 14 after laser photocoagulation, IB4-labeled areas were visible in both vehicle and tivozanib-treated groups (Fig. 3F and J). The size of the IB4-labeled area was much smaller in the tivozanib-treated group compared to the vehicle-treated laser group (P < 0.001) (Fig. 3M). The IB4 labeled areas in vehicle and tivozanib-treated laser groups were 15,821.3 ± 7191.6 μm² and 5845.6 ± 3523.7 μm², respectively. The tivozanib-treated laser group showed a 68.1% reduction in IB4-labeled area compared to the vehicle-treated laser group.

3.4. Histologic assessment of CNV

Fig. 4 shows representative images of CNV lesions stained with HE 14 days after laser induction of CNV. In the laser lesions, CNV membranes, consisting of fibrovascular tissue, RPE, and pigment clumps, emerged through the ruptured Bruch’s membrane. The CNV lesions in the tivozanib-treated group were smaller in size and had thinner centers than those in the baseline and vehicle-treated groups.

3.5. Inhibition of ERK1/2 phosphorylation by tivozanib treatment

Activation of the ERK1/2 kinase pathway, a downstream target of VEGF receptor 2, is necessary in the development of CNV. Previous studies have demonstrated that inhibitors of multiple tyrosine kinases inhibited experimental CNV via suppression of ERK1/2 suppression (Chung et al., 2009; Yafai et al., 2011). To test whether tivozanib also inhibits the phosphorylation of ERK1/2 in a CNV mouse model, we performed western blot analysis.

Fig. 5 shows one set of western blot analysis. At days 1 and 2 after laser injury, the expression levels of phosphorylated ERK1/2 relative to total ERK1/2 were shown as a tightly packed hexagonal monolayer in eyes without laser application (Fig. 3A). The expression levels of phosphorylated ERK1/2 were normalized to the corresponding levels of total ERK1/2 and expressed as arbitrary units.

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ERK1/2 increased in the vehicle-treated laser group compared to the control group without laser. However, compared to the vehicle-treated laser group, tivozanib-treated laser group showed relatively decreased expression levels of phosphorylated ERK1/2 (Fig. 5). The repeated analysis showed the same results (data not shown).

3.6. Regression of established CNV after tivozanib administration

In order to investigate the effects of tivozanib on established CNV, vehicle and tivozanib treatments were initiated on day 7 after laser-induced rupture of Bruch’s membrane. Two weeks after laser treatment, the CNV areas on choroidal flat mounts were analyzed in each group. Fig. 6 shows images of choroidal flat mounts representing the regressive effect of tivozanib on established CNV. The CNV lesions in the tivozanib-treated laser group were significantly decreased compared to those seen in the 7-day baseline and vehicle-treated laser groups ($P < 0.001$), indicating that tivozanib caused the regression of established CNV. The CNV areas in the 7-day baseline, vehicle, and tivozanib-treated laser groups were 16,444.9 ± 6219.8 μm², 14,324.5 ± 7046.9 μm², and 4626.6 ± 2640.7 μm², respectively. Compared to the 7-day baseline and vehicle-treated laser groups, the tivozanib-treated laser group showed a 71.8% and 67.7% regression in the CNV area, respectively.

4. Discussion

As previously mentioned, AMD patients with CNV-associated visual impairment are currently treated with antibody-based...
anti-VEGF therapies. A number of recent clinical trials have reported that successful CNV growth arrest can result in vision maintenance or improvement following VEGF inhibition (Bashshur et al., 2006; Brown et al., 2011; Rosenfeld et al., 2006). However, repeated intravitreal injections of anti-VEGF antibodies are required for successful treatment, which raises the chance of serious vision-threatening surgical complications (Sampat and Garg, 2010). In addition, many patients still experience deteriorating vision and disease progression despite these treatments (Lux et al., 2007). Thus, new drug delivery methods and new selective anti-angiogenic drugs are highly desirable in order to avoid potential complications and treat patients with persistent, recurrent, or refractory neovascular AMD.

Tivozanib, an oral quinoline-urea derivative with a small-molecular weight, is a novel tyrosine kinase inhibitor of all 3 VEGF receptors. In cell-based assays, tivozanib shows great selectivity for VEGF receptor kinases, with a 10-fold higher potency than other synthetic VEGF receptor tyrosine kinase inhibitors such as PTK787/ZK222584 (Wood et al., 2000), CP-547,632 (Beebe et al., 2003), sunitinib (Mendel et al., 2003), and KRN633 (Nakamura et al., 2004). This drug inhibits ligand-induced phosphorylation of VEGF receptor 1, 2, and 3 at picomolar concentrations (IC50 values = 0.21, 0.16, and 0.24 nmol/L, respectively), and inhibits phosphorylation of platelet-derived growth factor (PDGF) receptors and tyrosine-protein kinase Kit (c-Kit) (IC50 values = 1.72 and 1.63 nmol/L, respectively) (Nakamura et al., 2006). Because of its potency and specificity, tivozanib may enable optimal inhibition of VEGF pathways that are involved in endothelial cell proliferation, migration, survival, differentiation, vascular permeability, and mobilization of endothelial progenitor cells from the bone marrow into the peripheral circulation (Hoeben et al., 2004). Tivozanib is currently under clinical investigation for first and second-line treatment of renal cell carcinoma, as well as in combination with other chemotherapies in breast, colorectal, and other gastrointestinal cancer patients (De Luca and Normanno, 2010). Data from a recent phase 3 clinical trial of tivozanib showed clinically meaningful superiority over the approved standard therapy and might represent a promising antitumor agent through anti-angiogenesis mechanisms (Robert et al., 2012). However, a direct therapeutic effect of tivozanib in CNV has not yet been proven.

In the present study, we demonstrated the anti-angiogenic activity of tivozanib in an experimental CNV model. Oral administration of tivozanib at a dose of 1 mg/kg/day suppressed fluorescein leakage from CNV and the growth of CNV membranes. Immunofluorescence analysis of choroidal flat mounts revealed that there is a significant decrease in the extent of endothelial cells stained with IBA4 following tivozanib treatment. This drug also caused CNV regression when administered after CNV establishment. All our findings indicate that tivozanib has significant potential as a therapeutic agent for the treatment of CNV, in addition to other ocular pathologic neovascularizations.

From a clinical point of view, the ability to cause CNV regression can be useful. Intravitreal injection of ranibizumab or bevacizumab can reduce active leakage of blood and fluid from CNV membranes and suppress further growth of CNV in patients with neovascular AMD, but do not cause regression (Rosenfeld et al., 2006). Moreover, when ranibizumab or bevacizumab treatment is stopped, CNV may recur. Thus, repeated injections are required to maintain the stability of CNV activity or maintain CNV quiescence. Ranibizumab and bevacizumab are antibodies targeted against all VEGF-A isoforms. However, in addition to VEGF-A, other ligands including VEGF-D (Stacker et al., 2001), VEGF-E (Kiba et al., 2003; Laakkonen et al., 2007), and PlGF (Autiero et al., 2003; Carmeliet et al., 2001; Zheng et al., 2006) are also reported to contribute to angiogenesis by binding to VEGF receptor 3, VEGF receptor 1, VEGF receptor 2, or neuropilin (Whitaker et al., 2001). Furthermore, these growth

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**Fig. 4.** Histology of choroidal neovascularization (CNV) lesions stained with hematoxylin-eosin was obtained 2 weeks after laser-induced rupture of Bruch’s membrane. Dome-like CNV complexes, consisting of fibrovascular tissue, retinal pigment epithelial cells, and pigment clumps, are shown in baseline (untreated) laser group (A), vehicle-treated laser group (B), and tivozanib (1 mg/kg/day)-treated laser group (C). The CNV lesions in the tivozanib-treated group were smaller in size and had a thinner center compared to those in the baseline and vehicle-treated laser groups. Scale bar, 100 μm.

**Fig. 5.** Western blot analysis of tivozanib on ERK 1/2 phosphorylation. (A) Phosphorylated ERK 1/2 expression in RPE/choroid tissues of mice from control (without laser), vehicle-treated laser, and tivozanib (10 mg/kg/day)-treated laser groups at days 1 and 2 after laser injury. (B) Densitometric analysis shows that relative expression levels of phosphorylated ERK 1/2 increased after laser photocoagulation compared with control eyes. However, this increase was suppressed following tivozanib treatment.
factors interact with each other synergistically to promote angiogenesis (Carmeliet et al., 2001; Zheng et al., 2006). In this context, tivozanib may have an advantage over VEGF-A-selective therapies, such as ranibizumab or bevacizumab. Moreover, tivozanib blocks the actions of multiple receptor tyrosine kinases, including PDGF receptors, c-Kit, basic fibroblast growth factor receptor, and insulin-like growth factor-1 receptor (Nakamura et al., 2006). Previous studies have demonstrated that simultaneous inhibition of both VEGF and PDGF receptors may not only prevent angiogenesis but also induce the regression of neovascularization (Bergers et al., 2003; Erber et al., 2004). Therefore, tivozanib's inhibitory effects on downstream signaling of tyrosine kinase activation of multiple angiogenic receptors may lead to more complete inhibition of angiogenesis and subsequently to the regression of established new vessels.

Activated VEGF receptors initiate multiple signaling cascades involved in angiogenesis. Among various signaling pathways, ERK1/2 is implicated in promoting endothelial cell proliferation via activation of Raf-MEK-ERK1/2 kinase (Takahashi et al., 2001). On the other hand, VEGF-induced phosphatidylinositol-3 kinase activation contributes to the survival and vascular permeability of endothelial cells via activation of the serine–threonine kinases Akt/protein kinase B and nitric oxide synthase pathways (Olsson et al., 2006), and VEGF receptor-associated protein pathways have been known to regulate endothelial cell migration (Matsumoto et al., 2005). In this study, we demonstrated here that tivozanib suppressed ERK1/2 phosphorylation which was closely related to suppressed CNV formation in choroidal flat mounts. This result suggests that the inhibitory effects of tivozanib on choroidal angiogenesis includes blocking Raf-MEK-ERK1/2 kinase activation and thus, our results confirm a previous reports showing a critical role of the ERK1/2 pathway in ocular angiogenesis, including oxygen-induced retinopathy, laser-induced CNV, and other retinal ischemia models (Bullard et al., 2003; Chung et al., 2009; Hayashi et al., 1997, 1996).

A recent study reported that intracellular autonomous VEGF/VEGF receptor 2 signaling plays an indispensable role in vascular homeostasis and endothelial cell survival and that the activation of intracellular VEGF receptor 2 was suppressed by a small-molecule VEGF receptor inhibitor, but not by anti-VEGF antibodies (Lee et al., 2007). The implication of intracellular VEGF receptor 2 inhibition in the context of CNV-related angiogenic process remains to be elucidated. However, tivozanib may inhibit intracellular VEGF receptor 2 signaling pathways in addition to inhibiting VEGF receptor phosphorylation mediated by extracellular VEGF, which ultimately induces more potent endothelial cell apoptosis.

In conclusion, oral administration of tivozanib, a potent inhibitor of VEGF receptor tyrosine kinases, significantly inhibits CNV progression in an experimental CNV model. Therefore, it may be a candidate drug for clinical trials in patients with neovascular AMD.

Fig. 6. Regression of established choroidal neovascularization (CNV) by oral administration of tivozanib. Representative CNV lesions (B, C) in choroidal flat mounts were obtained 2 weeks after laser-induced rupture of Bruch’s membrane by perfusion of fluorescein isothiocyanate–dextran. Tivozanib (or vehicle) was delivered by oral gavage beginning 7 days after laser application. (A) Seven-day baseline laser group. (B) Vehicle-treated laser group. (C) Tivozanib (1 mg/kg/day)-treated laser group. Red dotted lines indicate CNV lesions. (D) The areas of CNV lesions in each group were quantified by digital imaging analysis. *P < 0.001 as compared to both 7-day baseline and vehicle-treated laser groups. Scale bar, 100 μm.
Further studies to determine optimal dosages, delivery methods, and therapeutic mechanisms of action are warranted to determine the applicability of tivozanib for treatment of CNV in neovascular AMD.

### Financial interest
None.

### References


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