Apratoxin S4 Inspired by a Marine Natural Product, a New Treatment Option for Ocular Angiogenic Diseases

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PURPOSE. Abnormal blood vessel formation is a defining feature of many blinding eye diseases. Targeting abnormal angiogenesis by inhibiting VEGF has revolutionized the treatment of many ocular angiogenic diseases over the last decade. However, a substantial number of patients are refractory to anti-VEGF treatment or may develop resistance over time. The objective of this study was to determine the efficacy and the mechanism of action of Apratoxin S4 in ocular angiogenesis.

METHODS. Retinal vascular cell proliferation, migration, and the ability to form tube-like structure were studied in vitro. Ex vivo aortic ring, choroid, and metatarsal assays were used to study Apratoxin S4’s impact on vessel outgrowth in a multicellular environment. Apratoxin S4 was also tested in mouse models of oxygen-induced retinopathy (OIR) and laser-induced choroidal neovascularization (CNV), and in a rabbit model of persistent retinal neovascularization (PRNV). Western blot and ELISA were used to determine the expression of key angiogenic regulators after Apratoxin S4 treatment.

RESULTS. Apratoxin S4 strongly inhibits retinal vascular cell activation by suppressing multiple angiogenic pathways. VEGF-activated vascular cells and angiogenic vessels are more susceptible to Apratoxin S4 treatment than quiescent vascular cells and vessels. Both intraperitoneal and intravitreal delivery of Apratoxin S4 are able to impede ocular neovascularization in vivo. Apratoxin S4 specifically attenuates pathological ocular angiogenesis and exhibits a combinatorial inhibitory effect with standard-of-care VEGF inhibitor drug (aflibercept).

CONCLUSIONS. Apratoxin S4 is a potent antiangiogenic drug that inhibits the activation of retinal endothelial cells and pericytes through mediating multiple angiogenic pathways. Keywords: angiogenesis, endothelial cells, pericytes, blood vessel, vascular disease

The precisely localized vascular network is critical for maintaining the transparency of visual axis as well as the structural and functional integrity of ocular tissues. Abnormal blood vessel formation in either the anterior or posterior segment of the eye is a common cause of visual impairment, including neovascular AMD (nAMD), neovascular glaucoma, and proliferative diabetic retinopathy (PDR).1 VEGF is a master regulator in angiogenesis during health and disease,2 and its effect on endothelial cell (EC) proliferation, migration, and organization to form tube-like structure is mediated by EC-specific VEGF receptor 2 (VEGFR2).3 Therapeutics blocking VEGF signaling either by neutralizing antibodies or protein-based strategies have been developed to halt new blood vessel formation in ocular angiogenic diseases.4 Despite its efficacy, a substantial number of patients (15%–20%) are intrinsically refractory to anti-VEGF treatment or may develop
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resistance over time, which is likely due to the compensatory activation of alternative angiogenic pathways. Indeed, hepatocyte growth factor (HGF) and erythropoietin were reported to be upregulated in eyes treated with anti-VEGF agents. Besides its role in angiogenesis, VEGF exerts a potent neuroprotective effect and mediates vascular and organ homeostasis. Long-term treatment with anti-VEGF therapies may result in local and systemic side effects. Agents that target multiple angiogenic pathways simultaneously or alternative angiogenic pathways may offer more effective treatment options.

Marine cyanobacteria are a unique group of marine prokaryotes, which produce a wealth of bioactive metabolites that act on a range of therapeutic targets and sometimes provide novel mechanisms of action, such as in the case of Apratoxins. Apratoxins are cyclopeptide cyclodepsipeptides that potentially inhibit co-translational translocation of secreted or membrane-bound proteins at the level of the Sec61 translocon. Drug design and medicinal chemistry campaigns to improve selectivity of the natural product Apratoxin S4 and reduce toxic side effects led to several novel synthetic analogues, including Apratoxin S4 (Supplementary Fig. S1), which possesses improved anticancer activity and synthetic accessibility. The dual effect of synthetic Apratoxin S4 on membrane receptors and their ligands, including VEGFR2 and VEGF, promises to be effective against tumors that rely on autocrine loops and to effectively control vascularized tumors by targeting both cancer cell growth and angiogenesis. Considering the benefits of synthetic Apratoxins in tumor angiogenesis, we intended to explore the impact of Apratoxin S4 onocular-specific vascular cell activation and angiogenesis.

Materials and Methods

Animals

Male and female C57BL/6J mice were purchased from Invivos (Singapore). Male Dutch Belted pigmented rabbits were obtained from Covance (Covance Research Products Inc., Denver, PA, USA). All animals were kept on a 12-hour light-dark cycle and fed a standard rodent chow (normal chow diet [NCD], 18% kcal from fat; Harlan, Hanlan, Madison, WI, USA). Animal experiments were performed in compliance with the guidelines of the Institutional Animal Care and Use Committee for Science, Technology and Research (A*STAR; 140963), Singapore Eye Research Institute (SERI; 1135 and 2014/SHS/0911), and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Materials, Cells, and Cell Culture

Primary human retinal endothelial cells (HRECs) and primary human retinal pericytes (HRPCs) were purchased from Angio-Proteomic (Boston, MA, USA) and maintained in endothelial growth media (EGM) or pericyte growth media (PGM) according to supplier’s instruction. When treated with VEGF, HRECs were cultured in endothelial basal media (EBM) containing 0.2% fetal bovine serum (FBS). Synthetic Apratoxin S4 was provided by Oceanx Pharmaceuticals, Inc. (Woburn, MA, USA) and dissolved in ethanol to make stock solution. Aflibercept was purchased from Bayer (Leverkusen, Germany). TGFβ1 ELISA kit was purchased from IBL (Hamburg, Germany). VEGF165 was purchased from Peprotech (Rehovot, Israel).

 Persistent Retinal Neovascularization (PRNV) Rabbit Model

PRNV model was established as described. In brief, PRNV was induced by a single intravitreal injection with 50 µL 0.025M DL-alpha-aminoacidic acid (DL-AAA) per eye. The establishment of PRNV was confirmed by fluorescein angiography. Apratoxin S4 or vehicle was delivered intravitreally (1.7 µL per eye) 12 weeks after the first DL-AAA injection. The fluorescein leakage was observed 3 days after the treatment was normalized to the baseline fluorescein levels.

 Mouse Model of Laser-Induced Choroidal Neovascularization (CNV) and RPE Flatmount

CNV was induced as described. Eight- to 12-week-old C57BL/6J mice received an intraperitoneal injection of 100 µL of Apratoxin S4 (0.25 mg/kg) or vehicle on 4 days after laser (regression mode). CNV lesions were imaged by fluorescein angiography and optical coherence tomography (OCT) 4 days after laser (before treatment) and 11 days posttreatment. Images were quantified by ImageJ software (http://imagej.nih.gov/ij/). Mouse Model of Oxygen-Induced Retinopathy (OIR) and Retinal Flatmount

OIR was induced as described. In brief, postnatal day (P) 7 mice and nursing mothers were placed in a 75% oxygen chamber for 5 days before being returned to room air. Mice were subject to intraperitoneal injection of Apratoxin S4 (0.25, 0.125, or 0.0625 mg/kg) or vehicle at P12 and P15. Retinas were collected at P17. To determine the synergic effects between Apratoxin S4 and aflibercept, suboptimal dosages of Apratoxin S4 (0.0625 mg/kg) and aflibercept (3 mg/kg) were delivered separately or in combination to neonatal pups at P12 and P15 through intraperitoneal injection. The isolated retina was fixed in 100% methanol followed by staining with primary antibodies against CD31 (rat monoclonal, 555370; BD Biosciences, San Jose, CA, USA) and identified with Alexa 488 secondary antibodies (A11006; Invitrogen, Carlsbad, CA, USA). Choroidal neovascularization was visualized under confocal microscopy (LSM 810; Carl Zeiss, Jena, Germany) and analyzed using Photoshop CS6 (Adobe Systems, Inc., San Jose, CA, USA) and Imaris 7.5 software (Bitplane AG, Belfast, UK).

Statistical Analysis

Data are presented as mean ± SEM. Statistical analysis was performed using two-tailed, unpaired, Student’s t-test or 1-way ANOVA followed by a Tukey post-test analysis as appropriate using Prism 5 (GraphPad Software Inc., La Jolla, CA, USA) (*P < 0.05; **P < 0.01; ***P < 0.001). Each represents significant statistical comparisons among the listed (x axis) experimental
groups. All other methods are described in the Supplementary Materials and Methods.

**RESULTS**

**Apratoxin S4 Inhibits the Activation of Human Retinal Endothelial Cells (HRECs) Through Regulating Multiple Angiogenic Pathways**

ECs are normally quiescent but can switch to a proliferative and migratory phenotype to form tube-like structures with the presence of angiogenic cues. To evaluate the antiangiogenic effect of Apratoxin S4, we first tested its impact on HREC proliferation. Compared with the vehicle control, 1 nM Apratoxin S4 markedly inhibited both basal and VEGF-induced HREC proliferation (Figs. 1a, 1b). Although 1 nM Apratoxin S4 was not able to suppress spontaneous HREC migration (Fig. 1c), it significantly halted the VEGF-induced HREC migration (Fig. 1d). EC tube formation in Matrigel gel mimics the process of blood vessel formation in vivo. Our study showed that 25 nM but not 1 nM Apratoxin S4 was necessary to block the ability of HREC to form tube-like structure as demonstrated by reduced number of branch points and total tube length (Fig. 1e). Similar as observed in cell migration assay, 1 nM Apratoxin S4 potently suppressed the VEGF-induced HREC tube formation in Matrigel (Fig. 1f). Natural and synthetic Apratoxins were previously reported to control co-translational translocation of a number of receptor tyrosine kinases, including VEGFR2. To further understand the mechanism of action of synthetic Apratoxin S4, we evaluated the expression of key angiogenic regulators, their receptors, and downstream signaling transducers in Apratoxin S4–treated HRECs with aflibercept used as a control. Whereas the expression level of VEGFR1 remained unchanged, VEGFR2 and VEGFR3 levels in HRECs were significantly suppressed by 25 nM Apratoxin S4, which was associated with a concomitant reduction in level of its activated downstream signaling transducer, pERK1/2 (Fig. 1g). The angiogenic function of VEGF is complemented by additional factors, such as TGFβ. Surprisingly, the expression of key angiogenic TGFβ receptor serine threonine kinases, TGFβRII, and activin receptor-like kinase 1 (ALK1), was not affected in HRECs treated with Apratoxin S4, whereas TGFβ1 level was significantly lower, which possibly is responsible for the reduced phosphorylation of proangiogenic TGFβ1 signaling transducer, pSmad1/5/8 under the same condition (Fig. 1h). Consistently, TGFβ1 secretion was also reduced in Apratoxin S4–treated HRECs (Fig. 1i). As expected, aflibercept was only able to affect the VEGF-induced phosphorylation of VEGFR2 (Supplementary Fig. S2). Taking together, these results demonstrated a potent antiangiogenic role of Apratoxin S4 in HREC-based in vitro assays and it exerts its function by regulating multiangiogenic pathways. VEGF-activated ECs are more susceptible to Apratoxin S4 treatment.

**Apratoxin S4 Inhibits HRPC Activation and Its Association With HRECs**

Vascular pericytes are critical players during capillary sprouting and the pericyte dynamics is controlled by platelet derived growth factor B (PDGF-B)/platelet derived growth factor receptor β (PDGFR-β) signaling. Our study showed that Apratoxin S4 but not aflibercept significantly suppresses PDGFR-β expression in HRPCs (Fig. 2a). Consistent with this observation, 10 nM Apratoxin S4 strongly inhibited HRPC proliferation (Fig. 2b). Interestingly, 100 pM Apratoxin S4 was sufficient to suppress HRPC migration in Transwell migration assay (Fig. 2c). We further demonstrate a significant reduction in pericyte-associated EC network in Apratoxin-treated HREC/HRPC co-culture in Matrigel (Fig. 2d). Together, our data showed a strong inhibitory effect of Apratoxin S4 in pericyte proliferation, migration, and recruitment.

**Apratoxin S4 Inhibits Vessel Outgrowth in Ex Vivo Models**

Angiogenesis is a complex process involving multiple cell types and extracellular components. To test Apratoxin S4’s effect on angiogenesis in a multicellular environment, mouse aortic ring was subjected to the treatment with 300 pM Apratoxin S4 in the presence and absence of exogenous VEGF. Our study demonstrated a potent inhibitory effect of Apratoxin S4 on both spontaneous and VEGF-induced vessel outgrowth from aortic ring (Fig. 3a). A similar effect was observed in matetasall assay (Fig. 3b). We next investigated Apratoxin S4’s effect on ocular-specific microvascular behavior, particularly for that relevant to nAMD, using RPE/choroid/sclera fragments isolated from P3 C57BL/6j mice. Apratoxin S4 demonstrated a dose-dependent inhibition on choroidal vessel outgrowth as compared with the vehicle-treated controls (Fig. 3c) and showed an IC50 at 130 ± 22 pM (Fig. 3d). Although 300 pM Apratoxin S4 completely suppressed the vessel outgrowth from choroid explants, it is not effective in destroying existing choroidal vasculature. Instead, 10 nM Apratoxin S4 was necessary to disrupt fully established normal vessels derived from choroid explants (Fig. 3e). These data showed that Apratoxin S4 is able to inhibit neovascularization from different vascular beds at picomolar concentration. Strikingly, the same dosage of Apratoxin S4 does not affect the existing normal choroidal vasculature.

**Apratoxin S4 Inhibits Ocular Angiogenesis In Vivo**

Having established a potent inhibitory effect of Apratoxin S4 on vascular growth in vitro and ex vivo, we next investigated whether it is able to inhibit ocular angiogenesis in vivo. CNV was induced in C57BL/6j mice and Apratoxin S4 was administrated by intraperitoneal injection at a single dose of 0.25 mg/kg 4 days after the laser injury. At 14 days postlaser injury (11 days posttreatment), fundus fluorescein angiography (FFA) at 90 seconds revealed a diminished neovascular response in eyes of Apratoxin S4–treated mice as compared with those in vehicle-treated controls (Fig. 4a), which is consistent with the reduced CNV volume in Apratoxin S4–treated group as demonstrated by RPE flatmount stained with endothelial cell marker, CD31 (Fig. 4b). We further demonstrated by OCT that the mean area of CNV lesions in Apratoxin S4–treated eyes were significantly lower than that in vehicle-treatment controls (Fig. 4a), which is consistent with the reduced CNV volume in Apratoxin S4–treated group as demonstrated by RPE flatmount stained with endothelial cell marker, CD31 (Fig. 4b). We further demonstrated by OCT that the mean area of CNV lesions in Apratoxin S4–treated eyes were significantly lower than that in vehicle-treated eyes at 14 days postlaser (Fig. 4c). To confirm the antiangiogenic effect of Apratoxin S4 in vivo, we investigated its impact on retinal neovascularization and leakage as observed in PDR, in a rabbit model of PRNV. Rabbits with established PRNV received a single intravitreal injection of Apratoxin S4 (1.7 μM per eye in vitreous), which is four times lower than the amount of aflibercept (6.96 μM) used in this model. FFA was taken 3 days later to evaluate retinal neovascularization and leakage, which was compared with baseline FFA of the same eye before the treatment. Our study showed that Apratoxin S4 significantly inhibited the retinal neovascularization and leakage as compared with vehicle-treated control eyes (Fig. 4d). Furthermore, based on clinical observation (daily cage side observation) as well as under slit-lamp biomicroscopy, we did not notice any inflammation or corneal irritation in the Apratoxin S4–treatment group, suggesting that intravitreal delivery of 1.7 μM Apratoxin S4 is safe. These data provided compelling evidence that both
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**FIGURE 1.** Apratoxin S4 inhibits HREC activation by regulating multiple angiogenic pathways. (a) AlamarBlue assay demonstrated a reduced HRECs proliferation after treatment with 1 nM Apratoxin S4 (n = 3) for 24 hours. (b) AlamarBlue assay showed that after treatment with 1 nM Apratoxin S4 for 24 hours, the VEGF-induced HREC proliferation was reduced in the presence of 50 ng/mL VEGF (n = 3) for 24 hours. (c) 4',6-diamidino-2-phenylindole (DAPI) staining (left), and quantitative analysis (right) of the motility of Apratoxin S4-treated HRECs (n = 3). After 4 hours of treatment of Apratoxin S4, HRECs’ motility was reduced in a dose-dependent manner. Scale bar: 50 μM. (d) DAPI staining (left), and quantitative analysis (right) showed that treatment with Apratoxin S4 for 4 hours inhibits VEGF-induced migration of HRECs in the presence of 50 ng/mL VEGF (n = 3). Scale bar: 50 μM. (e) HRECs were treated with Apratoxin S4 for 16 hours. Representative images and quantification of Apratoxin S4’s effect on HREC tube formation in Matrigel (n = 3). Scale bar: 200 μM. (f) Representative images and quantification of Apratoxin S4’s effect on VEGF-induced HREC tube formation in Matrigel in the presence of 50 ng/mL VEGF (n = 3). Scale bar: 200 μM. (g) HRECs were treated with 25 nM Apratoxin S4 or 10 μg/mL aflibercept for 24 hours. Representative western blots of VEGFR2, pErk1/2, tErk1/2, and GAPDH in Apratoxin S4 and vehicle-treated HRECs (n = 3). (h) HRECs were treated with 25 nM Apratoxin S4 or 10 μg/mL aflibercept for 24 hours. Representative Western blots of TGFβR2, ALK1, pSmad1/5, tSmad1/5, TGFβ1, and GAPDH in Apratoxin S4 and vehicle treated HRECs (n = 3). (i) Condition media was collected after treatment with Apratoxin S4 for 24 hours. ELISA for TGFβ1 on Apratoxin S4 and vehicle-treated HRECs (n = 3). All images shown are representative and data are represented as means ± SEM; 1-way ANOVA followed by Tukey’s multiple comparisons test or unpaired, two-tailed Student’s t-test; *P < 0.05 and ***P < 0.001.
intrapertitoneal and intravitreal delivery of Apratoxin S4 are effective in controlling abnormal blood vessel formation in the eye. Apratoxin S4 can prevent abnormal retinal and CNV.

**Apratoxin S4 Specifically Inhibits Pathological Neovascularization in the Eye**

The mouse model of OIR is a widely used mouse model to study pathological and physiological neovascularization in the retina. Following intraperitoneal injections of Apratoxin S4 at different dosages, 0.25, 0.125, or 0.0625 mg/kg, at P12 and P15, the retinae were harvested at P17 for analysis of ordered blood vessel formation and pathological neovascularization tufts. Our study showed a dose-dependent inhibition of Apratoxin in the formation of disorganized neovascularization tufts, whereas the size of the avascular region was not affected by Apratoxin S4 treatment (Fig. 5a). Interestingly, pericyte coverage in neovascular tufts was significantly suppressed in

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**Figure 2.** Apratoxin S4 inhibits HRPC activation. (a) HRPCs were treated with 25 nM Apratoxin S4 or 10 μg/ml aflibercept for 24 hours. Representative Western blot demonstrated a reduced level of PDGFRβ in 10 nM Apratoxin S4–treated HRPCs (n = 3). (b) AlamarBlue assay demonstrated reduced HRPC proliferation after treatment with 1 and 10 nM Apratoxin S4 for 24 hours (n = 3). (c) DAPI staining (left) and quantitative analysis (right) of the motility of Apratoxin S4–treated HRPCs (n = 5). After 4 hours of treatment of Apratoxin S4, HRPCs motility was reduced in a dose-dependent manner. Scale bar: 50 μM. (d) HRECs and HRPCs were co-cultured and treated with Apratoxin S4 for 16 hours. Representative images (left) and quantification of Apratoxin S4’s impact on HRPC (red) to HREC (green) ratio and total tube length (right) in Matrigel (n = 3). Scale bar, 200 μM. All images shown are representative and data are represented as means ± SEM; 1-way ANOVA followed by Tukey’s multiple comparisons test. *P < 0.05, **P < 0.01, ***P < 0.001.
the retina of Apratoxin S4–treated mice (Fig. 5b). Furthermore, there is a significant suppression of the proangiogenic TGFβ1 signaling transducer, pSmad1/5/8, downstream mediator of VEGF signaling pathway, pERK, as well as, to a lesser extent, PDGFR-β expression in the retina of Apratoxin S4–treated mice (Fig. 5c). Having demonstrated a potent inhibitory effect of Apratoxin S4 in pathological neovascularization, we investigated the effects of combination therapy with aflibercept, one of
the most commonly used anti-VEGF agents in treatment. By using suboptimal dosage of Apratoxin S4 (0.0625 mg/kg), aflibercept (3 mg/kg), or both Apratoxin S4 (0.0625 mg/kg) and aflibercept (3 mg/kg), we observed inhibition in the formation of hypoxia-induced pathological neovascularization tufts, with the combination treatment giving the most significant inhibition (Fig. 5d). As demonstrated before, physiological vessel regrowth was not affected in all treatment groups (Fig. 5d). These data demonstrated that Apratoxin S4 specifically affects pathological angiogenesis and predominantly acts on retinal pericytes. Finally, acute and chronic toxicity studies were performed in neonatal and adult mice received intraperitoneal injection of Apratoxin S4 at the dosage of 0.25 mg/kg. Our study demonstrated no toxicity effect of Apratoxin S4 as compared with vehicle indicated by body weight, retinas thickness, as well as gross macroscopic appearance and wet weights of kidneys, spleens, and livers (Supplementary Fig. S3). In summary, Apratoxin S4 has a potential to serve as an ocular antiangiogenic drug, either on its own or in combination with currently available anti-VEGF agents.
Despite the phenomenal success in treating ocular angiogenic diseases, the long-term benefits of anti-VEGF therapy to visual acuity have left much to be desired. Beside VEGF, many other angiogenic pathways are critical for neovascularization and are being targeted to address the treatment ceiling of anti-VEGF therapy. For example, PDGF is a key growth factor involved in pericyte survival, recruitment, and maturation. Stripping the protective coating of pericytes from neovascular complex using PDGF inhibitors was considered an attractive strategy to increase the sensitivity of ECs to anti-VEGF drugs. 

**DISCUSSION**

**FIGURE 5.** Apratoxin S4 specifically inhibits pathological neovascularization in the eye. (a) In OIR, Apratoxin S4 significantly decreased the formation of pathological neovascular tufts (delineated in higher power by white boundary line) without affecting the organized normal revascularization at P17. Scale bar: 500 μM (retina tile images) and 100 μM (higher magnification images). Vehicle, n = 6; Apratoxin S4, n = 6 per dosage. (b) Representative image and volume-rendered examples of NG2 (red) and CD31 (green) stained abnormal neovascular tufts as well as quantitative analysis of pericyte versus EC ratio in P17 OIR flatmount prepared from mice subjected to 0.0625 mg/kg Apratoxin S4 or vehicle treatment. Scale bar: 10 μM. (c) Representative Western blot demonstrating a reduced level of pSmad1, 5, 8 in P17 OIR retinal tissue prepared from Apratoxin S4-treated mice (n = 3). 

Combination of Apratoxin S4 and aflibercept in OIR in C57BL/6J mice resulted in enhanced reduction of neovascular tufts compared with single treatment, whereas organized revascularization is not affect. Scale bar: 500 μM (retina tile images) and 100 μM (higher magnification images), n = 6 per group. All images shown are representative and data are represented as means ± SEM; 1-way ANOVA followed by Tukey’s multiple comparisons test, *P < 0.5, **P < 0.01, ***P < 0.001.
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However, the outcome of 2016 clinical trial on rinucumab/afiblercept and pegpleranib/tanibuzumab brought disappointment to this approach. Recently, another prominent pathway leading to angiogenesis, Angiopoietin (Ang)-Tie2, has attracted a lot of attention. Ang-2 is a competitive antagonist to Ang-1 that activates the Tie2 receptor to limit vascular leakage. Several natural or synthetic Apratoxin drugs have been developed to inhibit Ang-2, therefore, allowing the activation of Ang-1/Tie-2 pathway to limit vascular permeability. It is much anticipated to see the outcome of large, late-stage clinical studies. Most drugs targeting angiogenic pathways function to prevent the ligand binding or the activation of respective receptors. Natural and synthetic Apratoxins are potent antitumor and antiangiogenic macrocyclic depsipeptides derived from marine cyanobacteria.

It was reported that Apratoxins exert their function through inhibiting co-translational translocation of secreted or membrane bound proteins, which may offer an alternative way to control the activation of angiogenic pathways. Here, we report that Apratoxin S4, a novel, synthetic Apratoxin-derived molecule with improved potency, increased selectivity and synthetic accessibility, strongly inhibits the activation of different types of retinal vascular cells by modulating multiple angiogenic pathways. Delivery of Apratoxin S4 by either intravitreal or intraperitoneal route is effective in inhibiting ocular angiogenesis, both in mouse and rabbit neovascularization disease models. Importantly, Apratoxin S4 is capable of preventing and/or regressing pathological neovascularization in the eye, and preferentially acts on immature, disordered vessels. Finally, we showed a superior combined antiangiogenic effect of afiblercept and Apratoxin S4. These findings shed light on the antiangiogenic potency of Apratoxin S4 in retinal angiogenic disorders and confirm its novel and selective mechanism of action, thus providing a new avenue for the prevention and/or treatment of ocular angiogenic diseases.

During angiogenesis, ECs must proliferate, migrate, and finally form new vascular tubes. Consistent with the previously reported role of natural and novel synthetic Apratoxins in human umbilical vein endothelial cells (HUVECs) and human umbilical vein endothelial cells (HUVECs), Apratoxin S4 potently inhibits HREC proliferation and migration, and the ability of HRECs to form tube-like structures. Interestingly, the VEGF-activated HRECs are more susceptible to the treatment of Apratoxin S4, which offers a selectivity advantage for Apratoxin S4 on angiogenic processes over quiescent vasculature. Indeed, a much higher dose of Apratoxin S4 is necessary to disrupt fully established choroidal vasculature derived from explanted murine choroid/sclera fragments in an ex vivo model. Most importantly, our data suggest that immature, disordered blood vessels in a hypoxic environment are preferentially sensitive to Apratoxin S4 treatment in mouse model of OIR in vivo. Treatment with Apratoxin S4 may therefore cause lesser unwanted side effects at therapeutic doses.

Angiogenesis is a complex process mediated by multiple cell types. Besides ECs, vascular pericytes also play critical roles in new blood vessel formation. They migrate and proliferate along the PDGF-B gradient created by angiogenic ECs, and are finally recruited to nascent vessels to provide stabilization and maturation signals. In addition to their important role in maintaining structural and functional integrity of newly formed vessels, pericytes also induce survival signals in ECs, therefore protecting ECs from antiangiogenic therapies. Our study showed that the motility of pericytes is especially sensitive to Apratoxin S4 treatment and that it strongly prevents pericyte association to tube-like structures formed by ECs, as demonstrated in the HREC/HRPC Matrigel coculture assay. Consistent with this observation, pathological neovascularization tufts formed in the mouse model of OIR showed reduced pericyte coverage, which would make pathological vessels more susceptible to anti-VEGF treatment. This may be partially responsible for the combinational effect observed in Apratoxin S4 and afiblercept co-treatment group.

Apratoxins were previously reported to exert its function by inhibiting protein co-translational translocation at the level of Sec61 translocon, leading to downregulation of various receptor tyrosine kinases and growth factors. Consistent with these observations, receptor tyrosine kinase VEGFR2 and VEGFR3 expressions were significantly inhibited in Apratoxin S4-treated HRECs. Similarly, receptor tyrosine kinase PDGFR-β level in HRPCs was also suppressed by Apratoxin S4. Interestingly, the levels of receptor serine threonine kinases, including TGFβR2 and ALK1, were not affected by Apratoxin S4 treatment, despite a strong suppression of the proangiogenic TGFβ1 signaling transducer, Smad1/5, in ECs, which is likely due to the reduced secretion of TGFβ1. These data suggest that Apratoxin S4 offers high target selectivity and it affects the activation of multiple angiogenic pathways through different mechanisms.

Another significant challenge facing current anti-VEGF treatments for ocular angiogenic diseases is repeated intravitreal administration of pharmacologic agents, which is associated with significant ocular side effects, such as cataracts and glaucoma. Our study showed that both intravitreal and intraperitoneal delivery of Apratoxin S4 are effective in inhibiting abnormal blood vessel formation in the eye with no obvious impairment observed in quiescent retinal vasculature, physiological revascularization, or retinal neuronal cells. No change in body weight, gross histopathology of liver, kidney, and spleen was observed in both neonatal and adult mice subjected to intraperitoneal delivery of Apratoxin S4. Furthermore, daily intraperitoneal injections of the same dose of Apratoxin S4 for 2 weeks has been previously shown to have no obvious toxicity in major organs of treated mice in a mouse colon xenograft model. The use of Apratoxin S4 might therefore be beneficial for selected patients who are not suitable for intravitreal drug administration or serve as an adjunctive therapy to current anti-VEGF treatments.

In summary, our findings demonstrate a potent antiangiogenic effect of Apratoxin S4, which functions through inhibiting multiple angiogenic pathways in different types of vascular cells. Apratoxin S4 preferentially acts on immature, pathological vasculature and can be used either on its own or in combination with an anti-VEGF agent for the treatment of ocular angiogenic diseases.

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