Supplementary materials and methods

**Proliferation assay**

4x10^4 HRECs or HPECs were cultured in respective media containing Vehicle, VEGF or Apratoxin S4 for 24 hr. AlamarBlue (Life Technology, USA) solution was incubated with cells for 4 hours to label viable cells. The colorimetric signal was captured by Synergy H1 microplate reader (BioTek, USA). The proliferation rate in treatment group was normalized to vehicle-treated control.

**Matrigel assay**

1x10^4 HRECs in Apratoxin or vehicle-containing EGM2 medium (Lonza) were seeded on top of growth factor reduced (GFR) Matrigel (BD Biosciences, Oxford, UK). For co-culture assay, 6x10^3 HRECs and 6x10^3 HRPCs were stained with Qtracker525 (Green) and Qtracker705 (Red) (Thermo Fisher Scientific, USA), respectively, for 1 hour. HRECs and HRPCs were then washed in EGM2 and PGM medium respectively to remove excessive dye before being resuspended in Apratoxin S4 or vehicle containing EGM2 medium and seeded on top of the GFR Matrigel. Images were taken 16hrs later under the Eclipse Ti-E Inverted Research Microscope (Nikon, Japan) and were analyzed using Image J Angiogenesis Analyzer (Gilles Carpentier). The fluorescence intensity was analyzed by Image J.

**Transwell migration assay**

8x10^4 Cells subjected to respective treatments were seeded onto 8.0 μm pore size Transwell plates (Corning) and were allowed to migrate for 4 hours at 37 °C. Migrated cells were fixed in 1% PFA and stained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma) before being visualized under the Eclipse Ti-E Inverted Research Microscope (Nikon, Japan) and counted manually using ImageJ Cell Counter plugin.
**Western blotting**

Total protein was extracted using Tortex lysis buffer (20 mM HEPES, pH 7.9, 350 mM NaCl, 20% v/v glycerol, 1% NP-40, 1 mM MgCl2, 0.5 mM EDTA, 0.1 mM EGTA) and was separated by SDS-PAGE before being transferred onto a Immobilon-PSQ PVDF Membrane (#ISEQ-00010, Merck Millipore, USA). Blots were probed with Phospho-Smad1/5 antibody (rabbit monoclonal, Cell Signaling Technology, 9516), Smad1/5 antibody (mouse monoclonal, Abcam, ab75273), Phospho- Erk1/2 antibody (rabbit monoclonal, Cell Signaling Technology, 4370), Erk1/2 antibody (rabbit monoclonal, Cell Signaling Technology, 4695), VEGFR1 antibody (rabbit monoclonal, Abcam, ab32152), VEGFR2 antibody (rabbit polyclonal, Abcam, ab39256), VEGFR3 antibody (rabbit polyclonal, Abcam, ab27278), VEGFA antibody (rabbit polyclonal, Abcam, ab46154), Platelet-derived growth factor receptor (PDGFR) β antibody (rabbit monoclonal, Cell Signaling Technology, 3169S), or Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (mouse monoclonal, Santa Cruz Biotechnology, sc32233), followed by horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology).

**Metatarsal assay**

Metatarsal assay was performed as describe\(^1\). In brief, metatarsal bones were isolated from E16.5 C57BL/6J mice and seeded onto gelatin-coated 24-well plates. Vehicle, 300 pM Apratoxin S4, 1.8 nM VEGF or 300 pM Apratoxin S4 plus 1.8 nM VEGF were added to explants 2 days after embedding. Media containing treatments were changed every other day. At day 10 of culture, the explants were fixed in 4% PFA followed by 1hr incubation in blocking buffer (3% Triton X, 1% Tween 20, and 0.5% BSA in PBS). The explants were incubated with CD31 antibody (rat monoclonal, BD Biosciences, 553370) and NG2 antibody (rabbit polyclonal, Millipore, AB5320) for overnight at 4°C. The explants were washed with blocking buffer and incubated with secondary antibodies for 2 hrs at room temperature. Vessel outgrowth from metatarsals were

**Aortic ring assay**

Aortic ring assay was performed as describe 2. Aorta from the postnatal day 3 (P3) C57BL/6J mice was cut into 1mm rings before being embedded in a 96-well plate coated with rat tail collagen I gel (BD). Vehicle, 300 pM Apratoxin S4, 1.8 nM VEGF or 300 pM Apratoxin S4 plus 1.8 nM VEGF were added to explants after collagen I gel was polymerised. Treatment media were changed every other day. At day 10 of culture, the explants were fixed in 4% PFA and stained with Griffonia Simplicifolia Lectin (GSL) isolectin IB4 (Vector Lab, ZB0406) and AN2-PE (Miltenyi Biotec, 130-100-468). Vessel outgrowth was visualized under the Eclipse Ti-E Inverted Research Microscope). The number of spouts was counted manually.

**Choroid sprouting assay**

Choroid sprouting assay was performed as described 3. For prevention study, treatment medium was added to the explants on the day of embedding and media were changed every other day. Images were taken after 4 days of treatment. For regression assay, choroidal explants were allowed to grow for 2 days before being subjected to respective treatments for 2 more days. Vessel outgrowth was visualized under the Eclipse Ti-E Inverted Research Microscope. Sprouting area was quantified by TRI2 software.

**Toxicity study**

To check the toxicity of Apratoxin S4 at tested dosages, 0.25 mg/kg Apratoxin S4 was delivered through intraperitoneal injection at P12 and P15 respectively to neonatal mice or once to 8-week old mice. Body weights were monitored daily. Terminal necropsy was performed 3-day (acute) or 14-day after injection. Liver, Kidney and spleen were weighed before being fixed in 10% neutral buffered formalin (NBF) followed by standard H&E staining and histomorphological evaluation. Whole eye balls were fixed in Davidson's fixative for 24 hours followed by 10% NBF fixation for 24 hours and H&E staining. The thickness of retinæ, outer nuclear layer and Inner nuclear layer was determined by ImageJ.
Supplementary figure legends

Supplementary Figure 1. Structure of Apratoxin S4.

Supplementary Figure 2. The impact of Apratoxin S4 and Aflibercept on VEGFR2 expression and activation in HRECs. Representative western blots of pVEGFR2, VEGFR2 and GAPDH in Apratoxin S4, Aflibercept and vehicle treated HRECs (n = 3).

Supplementary Figure 3. Acute and chronic toxicity study of Apratoxin S4 in neonatal and adult mice.

a. Body weight curve of neonatal mice after intraperitoneal injection of 0.25 mg kg⁻¹ Apratoxin S4 at P12 and P15 respectively. The body weight was recorded from the first day of injection. b. Representative images of hematoxylin and eosin–stained paraffin-embedded retinal sections obtained from neonatal mice 3 days after the second injection of Apratoxin. c. Representative images of hematoxylin and eosin–stained paraffin-embedded retinal sections obtained from neonatal mice 14 days after the second injection of Apratoxin S4. d. Ratio of kidney, spleen and liver weight versus body weight measured 3 days after the second injection of Apratoxin in neonatal mice. e. Ratio of kidney, spleen and liver weight versus body weight measured 14 days after the second injection in neonatal mice. f. Representative images of hematoxylin and eosin–stained paraffin-embedded kidney, spleen and liver sections obtained from neonatal mice 3 days after the second injection of Apratoxin S4. g. Representative images of hematoxylin and eosin–stained paraffin-embedded kidney, spleen and liver sections obtained from neonatal mice 14 days after the second injection of Apratoxin S4. h. Body weight curve of 8-week old mice after intraperitoneal injection of 0.25 mg kg⁻¹ Apratoxin S4. The body weight was recorded from the first day of injection. i. Representative images of hematoxylin and eosin–stained paraffin-embedded retinal sections obtained from 8-week old mice 3 days after the second injection of Apratoxin. j. Representative images of hematoxylin and eosin–stained paraffin-embedded retinal sections obtained from 8-week old mice 14 days after the second injection of Apratoxin. k. Ratio of kidney, spleen and
liver weight versus body weight measured 3 days after the second injection of Apratoxin S4 in 8-week old mice. I. Ratio of kidney, spleen and liver weight versus body weight measured 14 days after the second injection in 8-week old mice. m. Representative images of hematoxylin and eosin–stained paraffin-embedded kidney, spleen and liver sections obtained from 8-week old mice 3 days after the second injection of Apratoxin S4. n. Representative images of hematoxylin and eosin–stained paraffin-embedded kidney, spleen and liver sections obtained from 8-week old mice 14 days after the second injection of Apratoxin.

**Supplementary Figure 1.**

![Apratoxin S4](image)

**Supplementary Figure 2.**

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Supplementary Figure 3.
Supplementary references