Critical role of caveolin-1 in ocular neovascularization and multitargeted antiangiogenic effects of cavtratin via JNK

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Ocular neovascularization is a devastating pathology of numerous ocular diseases and is a major cause of blindness. Caveolin-1 (Cav-1) plays important roles in the vascular system. However, little is known regarding its function and mechanisms in ocular neovascularization. Here, using comprehensive model systems and a cell permeable peptide of Cav-1, cavtratin, we show that Cav-1 is a critical player in ocular neovascularization. The genetic deletion of Cav-1 exacerbated and cavtratin administration inhibited choroidal and retinal neovascularization. Importantly, combined administration of cavtratin and anti–VEGF-A inhibited neovascularization more effectively than monotherapy, suggesting the existence of other pathways inhibited by cavtratin in addition to VEGF-A. Indeed, we found that cavtratin suppressed multiple critical components of pathological angiogenesis, including inflammation, permeability, PDGF-B and endothelial nitric oxide synthase expression (eNOS). Mechanistically, we show that cavtratin inhibits CNV and the survival and migration of microglia and macrophages via JNK. Together, our data demonstrate the unique advantages of cavtratin in antiangiogenic therapy to treat neovascular diseases.

caveolin-1 | ocular neovascularization | cavtratin | angiogenesis | inflammation

Ocular neovascularization is a blinding pathology of numerous ocular diseases, such as wet age-related macular degeneration, retinopathy of prematurity, and diabetic retinopathy. Currently, the premier treatment available for ocular neovascular diseases is anti–VEGF-A therapy, which mainly targets vascular endothelial cells (ECs) (1). However, drug resistance to anti–VEGF-A therapy has emerged as a serious challenge (2) and effects of anti–VEGF-A therapy decline for most patients within the first 4 years of treatment (3). Moreover, not all patients with neovascular diseases are responsive to anti–VEGF-A therapy (4, 5), suggesting the existence of other important angiogenic components. Indeed, tremendous efforts have been made in the field to identify new target of antiangiogenic therapy. One such example has been the development of Fovista, a PDGF-B inhibitor. However, combination therapy of Fovista with anti–VEGF-A fails to improve vision in patients with wet age-related macular degeneration (Ophthotech Corp., December 2016), suggesting a more complex scenario of pathological angiogenesis than anticipated.

In addition to ECs, vascular pericytes and smooth muscle cells (SMCs) contribute significantly to pathological neovascularization. Once newly formed vessels are covered with pericytes and SMCs, they become more stable and less sensitive to anti–VEGF-A therapy (6). It is thus important to target vascular pericytes and SMCs together with ECs in antiangiogenic therapy. PDGF-B is a key factor regulating the proliferation, survival, and migration of vascular pericytes and SMCs (7), and it has attracted much attention in the field as a potential target for antiangiogenic therapy. Apart from vascular cells, pathological angiogenesis comprises many other critical components such as inflammatory cells, vascular permeability, and numerous proangiogenic molecules (8, 9). Studies have shown that microglia and macrophages play critical roles in pathological neovascularization (10, 11). The depletion of microglia or macrophages inhibits ocular angiogenesis, and the intravitreal injection of microglia boosts blood vessel growth (11). Moreover, while being a rich source of VEGF-A, microglia and macrophages can also promote neovascularization in a VEGF-A–independent manner by regulating complement systems, cholesterol metabolism (12), extracellular matrixes, and neuropilin-mediated integrin

Significance

Caveolin-1 (Cav-1) is a major structural protein of caveolae found in cell membranes and is critical for numerous cellular functions. However, it remains unclear whether Cav-1 plays a role in ocular neovascularization, a major cause of blindness. In this study, we found that the gene deletion of Cav-1 exacerbates ocular neovascularization, and cavtratin, a cell permeable peptide mimicking Cav-1 function, inhibits ocular neovascularization by targeting multiple critical components of angiogenesis. Importantly, combined administration of cavtratin and anti–VEGF-A inhibits neovascularization more effectively, suggesting at least a partially VEGF-A–independent effect of cavtratin. Our findings reveal multitargeted effects of caveolin-1 and cavtratin in ocular neovascularization that may be of great therapeutic value for antiangiogenic therapy.

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signaling (13). It is thus pivotal to target microglia/macrophages as well as to improve the inhibition of pathological angiogenesis.

Among ocular neovascular diseases, vascular hyperpermeability is a serious pathology causing edema, retinal detachment, and vision loss (14). Vascular permeability plays a critical role in pathological angiogenesis. Hyperpermeable vessels lead to plasma protein leakages into the extravascular space, producing a proangiogenic matrix that promotes angiogenesis and attracts proangiogenic inflammatory and mesenchymal cells. Thus, vascular permeability precedes the onset of neovascularization and is a determining factor of pathological angiogenesis (15, 16). It is therefore highly desirable to identify strategies that can be used to inhibit vascular permeability together with macrophages, microglia, and PDGF-B.

Caveolin-1 (Cav-1) is a major structural protein of caveolae found in cell membranes that plays critical roles in numerous cellular functions, such as cell survival and migration (17). Cav-1 regulates multiple signaling pathways by interacting with different molecules such as endothelial nitric oxide synthase (eNOS) (18), through its scaffolding domain. Cav-1 is highly expressed in ECs (19), microglia (20), and macrophages (21). The loss of Cav-1 by genetic deletion in mice enhances tumor angiogenesis and abnormal permeability (15, 22–24), and the overexpression of Cav-1 inhibits angiogenesis in a hind limb ischemia model (25). Moreover, the application of cavtratin, a cell-permeable peptide of the scaffolding domain of Cav-1 that mimics Cav-1 functions, inhibits tumor growth (16, 26). However, little is known of the roles of Cav-1 in ocular neovascularization and of the underlying mechanisms.

In this study, using multiple in vitro and in vivo models, we investigated the effects of Cav-1 and cavtratin on ocular neovascularization and the cellular and molecular mechanisms involved. We found that Cav-1 is highly expressed in choroidal neovascularization (CNV). In vivo, a loss of Cav-1 by gene deletion exacerbated CNV, and cavtratin treatment markedly inhibited both choroidal and retinal neovascularization by reducing inflammation, vascular permeability, and PDGF-B and eNOS expression. Importantly, a robust synergistic effect of cavtratin with VEGF-A neutralizing antibody was observed for inhibiting CNV. Mechanistically, we show that cavtratin inhibits the transmigration and survival of macrophages and microglia via the JNK pathway. Together, our data show the unique advantages of cavtratin for antiangiogenic therapy by targeting multiple critical components of pathological angiogenesis. Cavtratin may therefore have great therapeutic value in treating neovascular diseases.

Results

Caveolin-1 Is Expressed and Up-Regulated During Ocular Neovascularization. Cav-1 is highly expressed in the eye (27). However, little is known regarding its role in ocular neovascularization. To investigate this, we examined whether Cav-1 is expressed in the fibrovascular membranes of proliferative diabetic retinopathy (PDR), which contain capillaries and inflammatory cells. We found that Cav-1 is highly expressed in the fibrovascular membranes of PDR (Fig. 1*A*), suggesting that Cav-1 may play a role in ocular neovascular diseases. We next examined whether Cav-1 is expressed in CNV using a laser-induced mouse model. Western blots show that Cav-1 protein levels were higher in the choroids and retinae with CNV at different time points (Fig. 1*B*). This observation is further confirmed by real-time PCR at a RNA level (*SI Appendix*, Fig. S1). Moreover, immunofluoresence staining 3 d after laser injury detected Cav-1 expression in the CNV area colocalized with IB4$^+$ staining, which identifies ECs, microglia, and macrophages (Fig. 1*C*). Indeed, this is further supported by F4/80 staining, which identifies microglia and macrophages but not ECs (Fig. 1*D*). Thus, Cav-1 is up-regulated in CNV and is expressed in ECs, microglia, and macrophages.

Cav-1 Deficiency Exacerbates CNV and Microglia/Macrophage Infiltration. We next performed a loss-of-function assay using *Cav-1* knockout mice, and we investigated whether Cav-1 plays a role in CNV. In a laser-induced CNV model, Cav-1 deficiency markedly increased CNV formation as measured by IB4 staining 7 d after laser treatment (Fig. 2 *A* and *B*). Histological analysis of the eyes with CNV showed no obvious morphological difference between Cav-1–deficient and wild-type mice (*SI Appendix*, Fig. S2). Microglia and macrophages

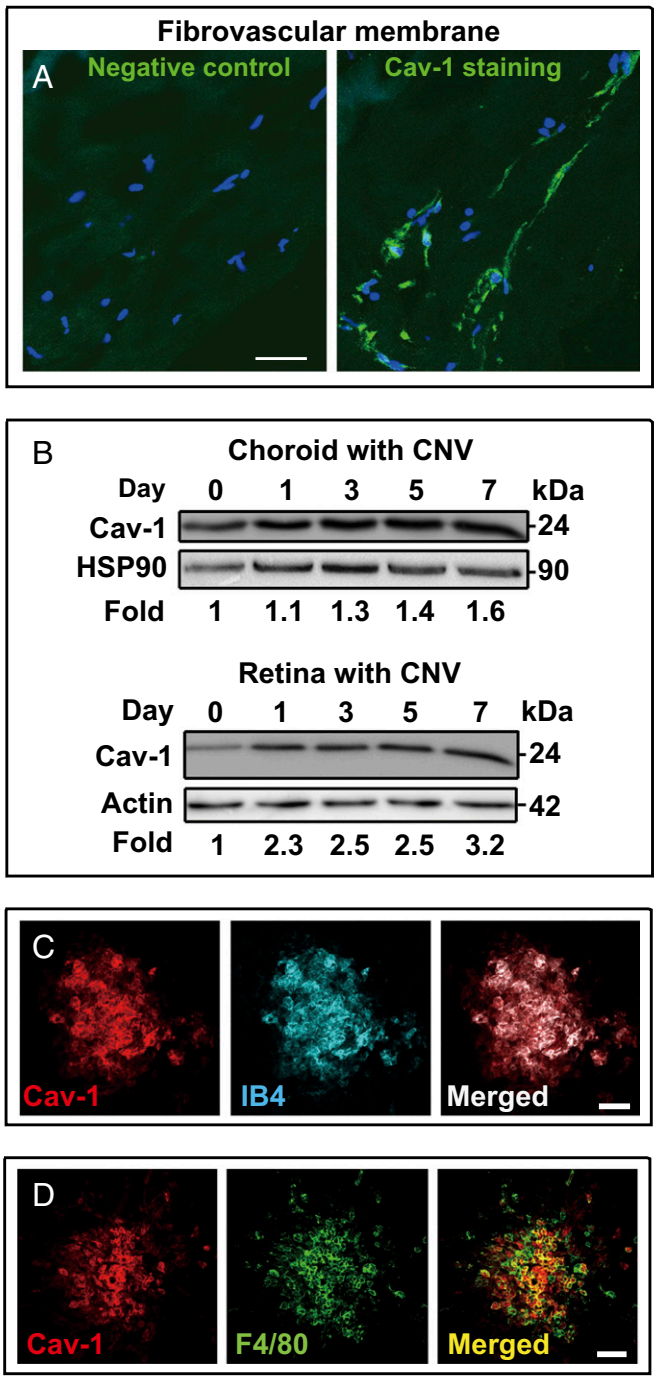

Fig. 1. Caveolin-1 is highly expressed in ocular neovascularization. (*A*) Immunofluoresence staining results show that Cav-1 (green) is abundantly expressed in the fibrovascular membranes of proliferative diabetic retinopathy. (*B*) Western blots show increased Cav-1 protein levels in choroids and retinae with CNV at different time points (n = 8). (*C*) Immunofluoresence staining shows Cav-1 expression in CNV colocalized with IB4 staining, which stains vascular ECs, microglia, and macrophages. (*D*) Immunofluoresence staining shows Cav-1 expression in F4/80$^+$ microglia and macrophages. [Scale bars: (*A*) 25 μm and (*C* and *D*) 50 μm.]

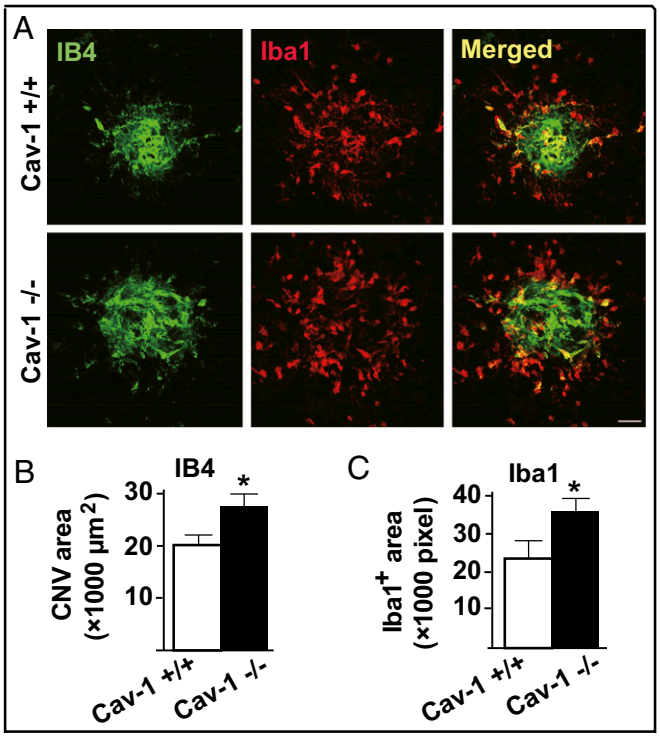

Fig. 2. Cav-1 deficiency exacerbates CNV and increases the number of microglia/macrophages in CNV. (*A* and *B*) Cav-1 deficiency by gene deletion markedly increased CNV shown by IB4 staining (green) 7 d after laser treatment (n = 16, 18; $P < 0.05$). (*A* and *C*) Iba1 staining (red), which identifies microglia and macrophages, shows more Iba1$^+$ cells in Cav-1–deficient CNVs (n = 16, 18; $P < 0.05$). Data are presented as the mean ± SEM values. *$P < 0.05$. (Scale bar: 50 μm.)

are critical for CNV development (11, 28). Iba1 staining, which identifies microglia and macrophages, revealed more Iba1$^+$ cells in Cav-1–deficient CNVs (Fig. 2 *A* and *C*). Thus, a loss of Cav-1 increased CNV formation and microglia/macrophage infiltration.

Cavtratin Inhibits CNV and Has a Synergistic Effect with Anti–VEGF-A Treatment. Cavtratin is a cell-permeable peptide of Cav-1 that mimics Cav-1 function (16, 26, 29). As Cav-1 deficiency increased CNV, we hypothesized that cavtratin might inhibit CNV. Indeed, we found that the intraocular injection of different doses of cavtratin inhibits CNV formation in a dose-dependent manner as measured by IB4 staining 7 d after laser injury using VEGF-A neutralizing antibody (nab) as a positive control (Fig. 3 *A* and *B*). Histological analysis of the eyes with CNV showed no obvious morphological difference between cavtratin- and Antennapedia (AP)-treated eyes (*SI Appendix*, Fig. S3). We next tested whether cavtratin has a synergistic effect with VEGF-A nab, which mainly targets endothelial cells. We found that at suboptimum doses, the intravitreal injection of cavtratin or VEGF-A nab alone before (Fig. 3 *C* and *D*) or after laser injury (*SI Appendix*, Fig. S4) does not inhibit CNV. However, when cavtratin and VEGF-A nab were administered together, CNV was significantly inhibited before (Fig. 3 *C* and *D*) or after laser treatment (*SI Appendix*, Fig. S4), suggesting that cavtratin likely acts independently of the VEGF-A pathway. These data thus reveal synergistic effects of cavtratin with VEGF-A nab in inhibiting CNV.

Cavtratin Inhibits Retinal Neovascularization, Vascular Permeability, and eNOS Expression. Retinal neovascularization is a devastating pathology of many blinding diseases, such as retinopathy of

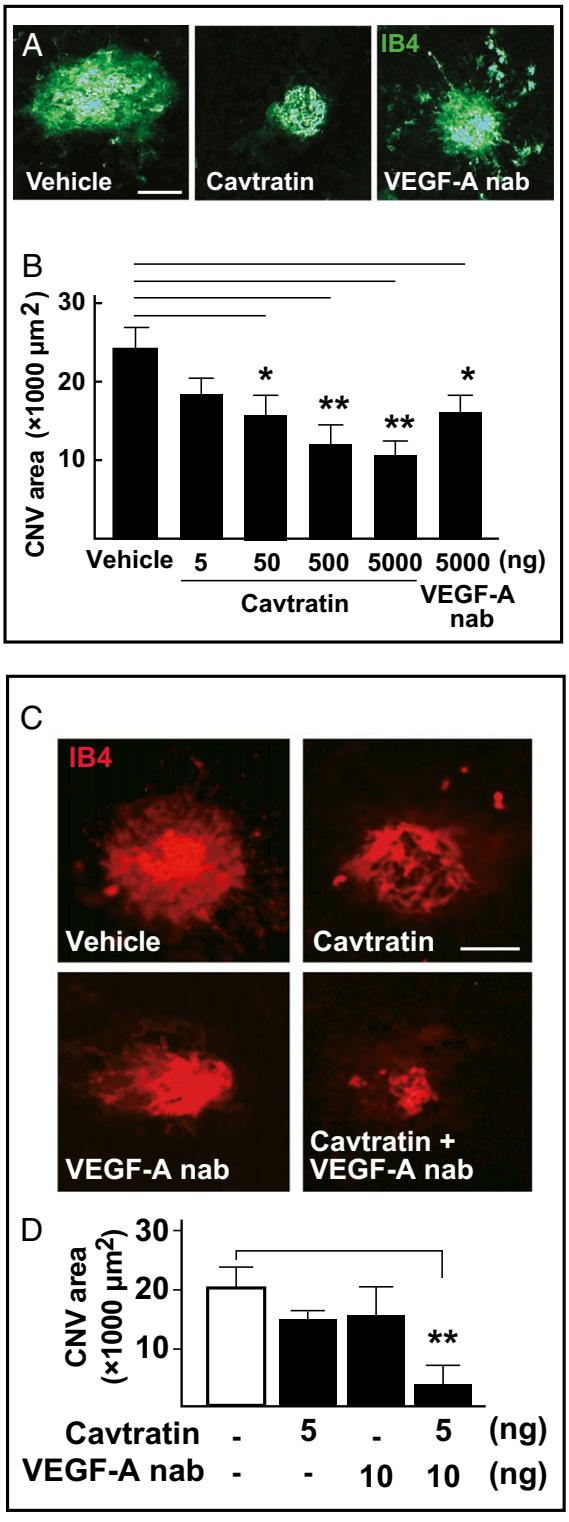

Fig. 3. Cavtratin inhibits CNV and has a synergistic effect with VEGF-A neutralizing antibody (nab). (*A* and *B*) IB4 staining (green) results show that intravitreal injection of cavtratin immediately before laser injury inhibited CNV in a dose-dependent manner (n = 8, $P < 0.01$ or 0.05). VEGF-A nab was used as a positive control. The left image shown in *A* represents a mosaic of several individual images. (*C* and *D*) IB4 staining (red) results show that at suboptimal doses, intravitreal injection of cavtratin or VEGF-A nab alone does not inhibit CNV. However, when cavtratin was coinjected with VEGF-A nab, CNV was significantly inhibited (n = 8, $P < 0.01$). Data are presented as the mean ± SEM values. *$P < 0.05$, **$P < 0.01$. (Scale bar: 100 μm.)

prematurity and diabetic retinopathy. As cavtratin markedly inhibited choroidal neovascularization, we hypothesized that it might suppress retinal neovascularization as well. We tested this using an ischemia-induced retinal angiogenesis model. Indeed, we found that intraocular injection of cavtratin inhibited retinal neovascularization (Fig. 4 *A* and *B*). As increased vascular permeability is a serious pathology of ocular neovascularization, we next investigated whether cavtratin plays a role in this. Using a laser-induced CNV model and an Evans blue permeability assay, we found that the intravitreal injection of cavtratin decreased vascular permeability levels in both retinae and choroids 7 d after laser injury (Fig. 4*C*). As eNOS plays an important role in vascular permeability, we investigated whether cavtratin regulate eNOS levels and phosphorylation. We found that in primary human retinal endothelial cells (HRECs), cavtratin treatment decreased levels of both total and phosphorylated eNOS to a similar extent in a time-dependent manner (Fig. 4*D*), while no significant change was found at a RNA level as shown by real-time PCR (*SI Appendix*, Fig. S5). Thus, cavtratin inhibits retinal neovascularization and vascular permeability and down-regulates eNOS expression.

Cavtratin Inhibits Microglia/Macrophage Infiltration, Transmigration, and Survival. Microglia and macrophages play critical roles in ocular neovascularization (28, 30). We therefore investigated whether cavtratin affects microglia/macrophages in CNV. Immunofluoresence staining using Iba1 as a marker for microglia/macrophages and IB4 for ECs shows that cavtratin treatment reduces the number of Iba1$^+$ cells in CNV lesions 3 d after laser treatment (Fig. 5 *A* and *B*). Indeed, this was confirmed by a real-time PCR assay using F4/80, another marker of microglia/macrophages (*SI Appendix*, Fig. S6*A*). During CNV formation, macrophages are recruited from the bloodstream after transmigration through vascular ECs (28). We next investigated the effects of cavtratin on the transmigration of human monocytic THP-1 cells through a monolayer of HRECs (*SI Appendix*, Fig. S6*B*). Cavtratin treatment markedly reduced the number of transmigrated THP-1 cells (Fig. 5 *C* and *D*). We next investigated whether cavtratin affects microglia/macrophage survival using an MTT assay. We found that cavtratin treatment markedly decreases the survival of mouse macrophage cell line Raw 264.7 cells, mouse microglial cell line N9 cells, and mouse primary bone marrow-derived macrophages (BMDMs) (Fig. 5 *E*–*G*). Consistently, a TUNEL assay confirmed that cavtratin treatment increases apoptosis in BMDMs (Fig. 5 *H* and *I*). Moreover, Western blot shows that cavtratin inhibited lipopolysaccharide (LPS)-induced iNOS expression in cultured Raw 264.7 macrophage cells (*SI Appendix*, Fig. S6*C*), suggesting that cavtratin may suppress macrophage

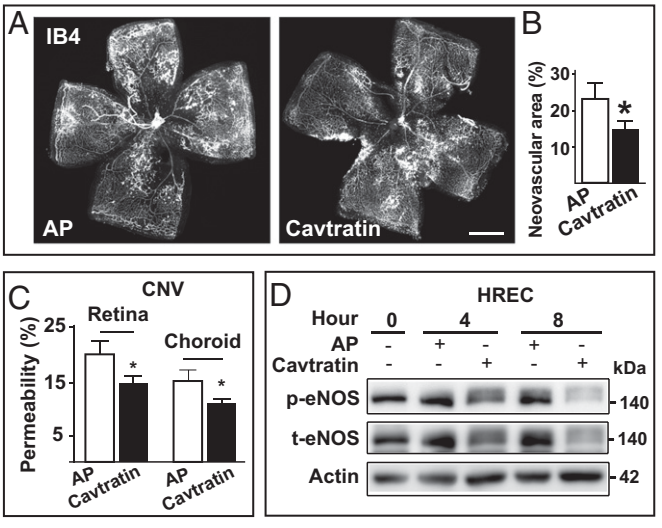

Fig. 4. Cavtratin inhibits retinal neovascularization, reduces vascular permeability, and down-regulates eNOS. (*A* and *B*) IB4 staining (white) results show that the intraocular injection of cavtratin inhibits retinal neovascularization in an ischemia-induced retinal neovascularization mouse model ($n = 8$, $P < 0.05$). (*C*) Evans blue permeability assay results show that the intraocular injection of cavtratin decreased vascular permeability in the retinae and choroids with CNV 7 d after laser treatment ($n = 10$, $P < 0.05$). (*D*) Western blot results show that in primary human retinal endothelial cells (HRECs), cavtratin treatment down-regulated eNOS protein levels in a time-dependent manner. Data are presented as the mean ± SEM values. *$P < 0.05$. (Scale bar: 100 μm.)

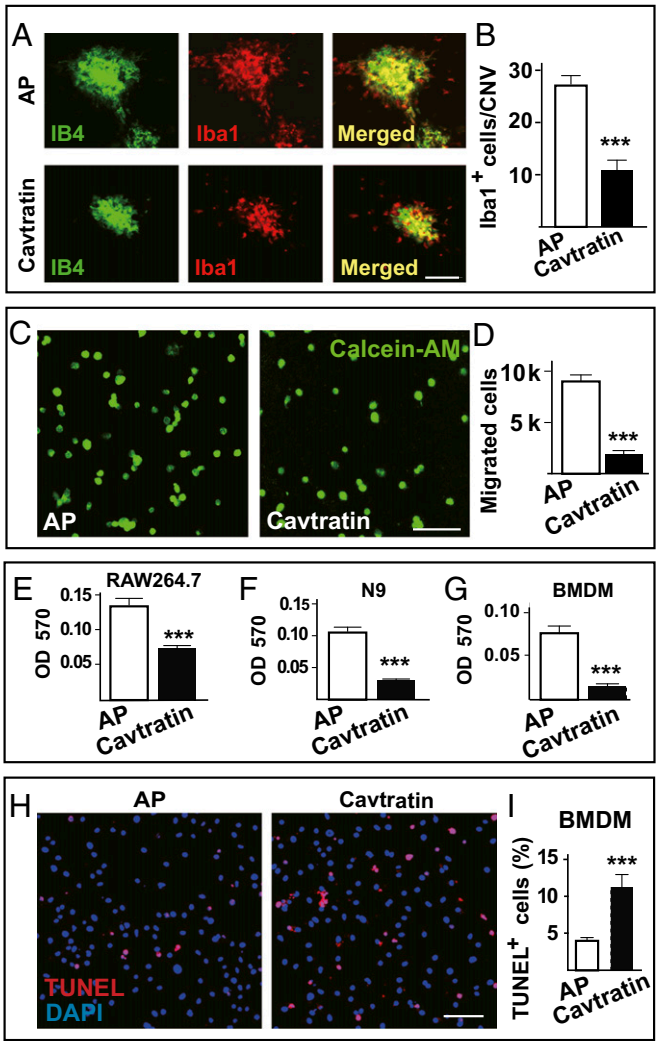

Fig. 5. Cavtratin decreases the number of microglia/macrophages in CNV and inhibits macrophage transmigration and survival. (*A* and *B*) Immunofluoresence staining results using Iba1 as a marker (red) for microglia and macrophages shows fewer Iba1$^+$ cells in the cavtratin-treated CNV lesions (IB4$^+$, green) 3 d after laser treatment ($n = 8$, $P < 0.001$). (*C* and *D*) Transmigration assay using human monocytic THP-1 cells through a monolayer of human retinal vascular endothelial cells (HRECs) shows that cavtratin markedly reduced the transmigration of THP-1 cells through HRECs as identified by Calcein-AM staining (green) ($n = 7$, $P < 0.001$). (*E*–*G*) MTT assay shows that cavtratin markedly inhibited the survival of mouse macrophage cell line Raw 264.7 cells, mouse microglial cell line N9 cells, and mouse primary bone marrow-derived macrophages (BMDMs) ($n = 6$, $P < 0.001$). (*H* and *I*) TUNEL staining (pink) shows that cavtratin treatment increases the apoptosis of BMDMs ($n = 6$, $P < 0.001$). Data are presented as the mean ± SEM values. ***$P < 0.001$. [Scale bars: (*A*) 100 μm, (*C*) 20 μm, and (*H*) 30 μm.]

activation. VEGF-C expression was not affected (*SI Appendix*, Fig. S6*D*). Thus, cavtratin inhibits the migration and survival of microglia/macrophages.

Cavtratin Down-Regulates PDGF-B and JNK Mediates the Effects of Cavtratin. We next investigated genes regulated by cavtratin. We found that cavtratin down-regulates PDGF-B expression in N9 microglia and BMDM cells at a protein level as shown by Western blot (Fig. 6 *A* and *B*) and at a RNA level as shown by a real-time PCR (*SI Appendix*, Fig. S6*E*). This is also true in vivo as the intravitreal injection of cavtratin down-regulates PDGF-B expression in mouse eyes with CNV as shown by a Western blot (Fig. 6*C*) and a real-time PCR (*SI Appendix*, Fig. S6*F*). We next investigated molecular mechanisms underlying the effects of cavtratin. We found that cavtratin induces JNK phosphorylation in both N9 microglia (Fig. 6*D*) and BMDM cells (*SI Appendix*, Fig. S7). We further verified whether JNK plays a role in mediating effects of cavtratin. Using a JNK inhibitor SP600125 and an MTT assay, we found that the JNK inhibitor SP600125 eliminates the antisurvival effect of cavtratin on N9 cells (Fig. 6*E*). In mouse retinal endothelial cells (MRECs) and human umbilical vein endothelial cells (HUVECs), cavtratin also reduced cell proliferation in an MTT assay (*SI Appendix*, Fig. S8 *A* and *B*). However, the JNK inhibitor SP600125 displayed little effect on the antisurvival effect of cavtratin on these cells (*SI Appendix*, Fig. S8 *A* and *B*). Importantly, in a mouse CNV model in vivo, SP600125 attenuated the antiangiogenic effect of cavtratin (Fig. 6 *F* and *G*). By contrast, a p38 inhibitor SB203580 did not eliminate the effect of cavtratin (*SI Appendix*, Fig. S8*C*) even though cavtratin induced p38 phosphorylation in N9 microglia cells (*SI Appendix*, Fig. S8 *D* and *E*) and in primary mouse BMDMs (*SI Appendix*, Fig. S8 *F* and *G*). These data thus suggest that effects of cavtratin are mediated by the JNK pathway.

Discussion

In this study, we found that Cav-1 is highly expressed in ocular neovascularization, and a loss of Cav-1 by gene deletion exacerbates CNV. Consistently, cavtratin, a cell permeable peptide of Cav-1 that mimics Cav-1 functions, inhibited both choroidal and retinal neovascularization. Importantly, cavtratin regulates multiple critical components of pathological angiogenesis, including inflammation, vascular permeability, and PDGF-B and eNOS expression. Noteworthy, the effect of cavtratin appears to be at least partially VEGF-A independent, as combined administration of cavtratin and VEGF-A neutralizing antibody inhibited CNV more efficiently than monotherapy. Mechanistically, we reveal that cavtratin has a direct inhibitory effect on the survival and migration of macrophages and microglia via the JNK pathway. Our data thus show that cavtratin has a multitargeted antiangiogenic effect that may be of promising therapeutic value for the treatment of neovascular diseases.

Over the past several years, combination therapies that target VEGF-A and PDGF-B have attracted much attention in the field. Superior outcomes from such combination therapies in inhibiting pathological angiogenesis were highly expected. However, it was recently announced that the PDGF-B antagonist Fovista

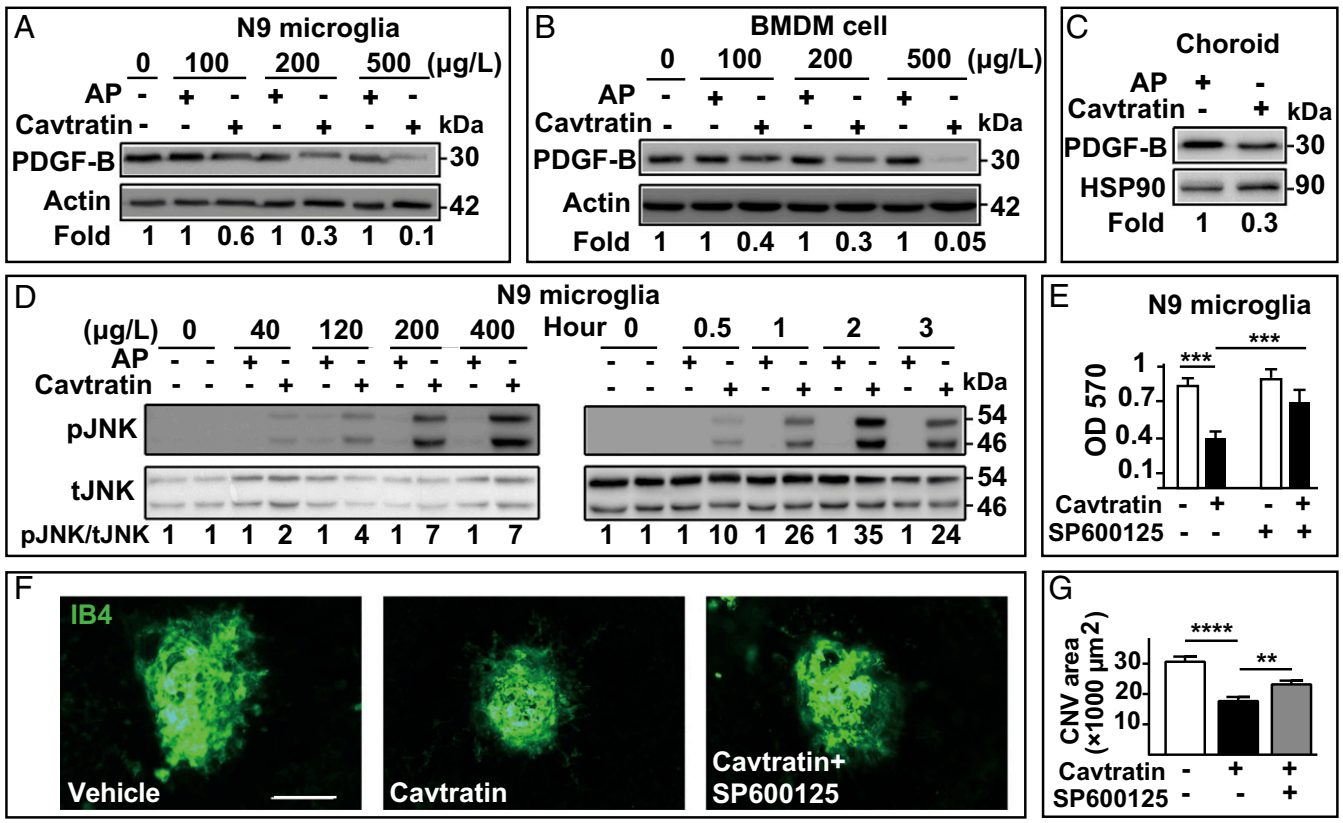

Fig. 6. Cavtratin down-regulates PDGF-B and induces JNK phosphorylation. (*A* and *B*) Western blot results show that cavtratin decreased PDGF-B protein levels in both N9 and BMDM cells in a dose-dependent manner. (*C*) Intravitreal injection of cavtratin decreased PDGF-B levels in mouse choroids with CNV, as is shown by the Western blot results ($n = 8$). (*D*) Western blot results show that in cultured N9 microglia cells, cavtratin induced JNK phosphorylation in a dose- and time-dependent manner. The vertical line in the image on the *Left Bottom* was generated by the image capture device. (*E*) MTT assay shows that in cultured N9 cells, a JNK inhibitor SP600125 eliminated the antisurvival effect of cavtratin ($n = 6$, $P < 0.001$). (*F* and *G*) In a mouse CNV model, SP600125 partially attenuated the antiangiogenic effect of cavtratin ($n = 10$, $P < 0.001$ or 0.01). Data are presented as the mean ± SEM values. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. (Scale bar: 100 μm.)

when combined with Lucentis fails to improve treatment outcomes (Ophthotech Corp., December 2016), suggesting that besides VEGF-A and PDGF-B, other important components of pathological angiogenesis must be considered. Indeed, apart from vascular ECs, pathological angiogenesis involves multiple critical aspects, including inflammatory cells that produce large quantities of angiogenic factors, increased capillary permeability levels that enrich the proangiogenic interstitial compartment, and vascular mural cells and fibroblasts that promote neovessel growth. Thus, inhibiting VEGF-A and PDGF-B alone may not be sufficient to achieve the best antiangiogenic effects. The identification of new and better antiangiogenic reagents that can inhibit multiple critical pathways of pathological angiogenesis remains an urgent task.

In this work, we found that the combined use of cavtratin and VEGF-A neutralizing antibody is more effective in inhibiting pathological angiogenesis than monotherapy, suggesting that the antiangiogenic effects of cavtratin are at least partially VEGF-A independent. Indeed, this finding is substantiated by several lines of evidence. For example, we found that cavtratin has direct effects on inflammatory cells and on PDGF-B and eNOS expression. Cavtratin treatment inhibited the transmigration of macrophages through ECs, thereby limiting their recruitment to the neovascularization site. Cavtratin also markedly decreased the viability of microglia and macrophages, which contribute considerably to pathological angiogenesis. Indeed, in an immunologic mouse model of multiple sclerosis, cavtratin reduces inflammatory cell infiltration and demyelination, leading to the improvement in blood brain barrier function and thus retarding inflammation and subsequent demyelination (31). As microglia and macrophages are not major cellular targets of VEGF-A, these findings may explain the additive antiangiogenic effects of cavtratin on anti–VEGF-A therapy, suggesting that Cav-1–induced signals may represent at least some of the mechanisms of drug resistance or unresponsiveness to anti–VEGF-A therapy.

Apart from the effect of Cav-1 on microglia and macrophages, we found that cavtratin inhibits vascular permeability in CNV. Increased vascular permeability in the eye is a fatal pathology that causes edema, retinal detachment, and when uncontrolled, blindness (1). Given the instrumental role of vascular permeability in early phases of pathological angiogenesis, antileakage effects of cavtratin confer an additional ability to inhibit neovessel growth. In addition, cavtratin down-regulates expressions of PDGF-B and eNOS in vitro and in vivo. PDGF-B is known to be a key growth factor for vascular mural cells and fibroblasts. Furthermore, eNOS plays a critical role in vascular permeability and angiogenesis (16, 26). Thus, by down-regulating PDGF-B and eNOS, cavtratin can suppress vascular mural cell components that play important roles in pathological angiogenesis.

In summary, in this study, we reveal that Cav-1 is a critical player in ocular neovascularization and that cavtratin inhibits pathological angiogenesis by suppressing multiple critical components of pathological angiogenesis. Our findings suggest that cavtratin may have promising therapeutic applications for the treatment of neovascular diseases.

Materials and Methods

All animal experiments were performed according to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals and were approved by the Animal Care and Use Committee at the Zhongshan Ophthalmic Center, Sun Yat-sen University. The Cav-1–deficient mice were obtained from The Jackson Laboratory and described previously (32). More details of materials and methods are provided in *SI Appendix*.

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