Cerebral cavernous malformations (CCMs) are common vascular lesions that can cause hemorrhagic stroke, seizures, and focal neurological deficits. Cases occur sporadically or in an inherited, autosomal-dominant form, the most common of which is caused by mutations in CCM1 (KRIT1).1,2

Our group had shown that KRIT1 is localized to and stabilizes endothelial cell (EC) junctions3 and the loss of KRIT1 leads to increased stress fibers, a hallmark of activation of RhoA and its effector Rho kinase (ROCK).3 Inhibition of ROCK in vitro or in vivo reversed the effect of KRIT1 silencing on endothelial junctional stability,4 suggesting it as a potential therapeutic strategy. Although we demonstrated robust activation of ROCK in ECs lining CCM lesions in humans,4 there has been to date no demonstration of a therapeutic effect on CCM lesion genesis in vivo.

Methods—Our recently generated a model of CCM1 disease (Ccm1+/−/Msh2−/−) was treated with the Rho kinase inhibitor fasudil (100 mg/kg/day administered in drinking water from weaning to 5 months of age), or placebo, and blindly assessed CCM lesion burden by systematic survey of animals’ brains. For comparison, we also assessed therapeutic effect in previously described Ccm2+/−/Trp53−/− mice treated with the same dose and duration of fasudil and placebo.

Results—Fasudil-treated Ccm1+/−/Msh2−/− mice had a significantly decreased prevalence of CCM lesions compared with placebo controls. Lesions in treated animals were smaller and less likely associated with hemorrhage, inflammation, and endothelial proliferation and exhibited decreased expression of Rho kinase activation biomarkers. A therapeutic effect was also documented in Ccm2+/−/Trp53−/− mice.

Conclusions—This represents the first report of therapeutic benefit of pharmacological therapy in development and progression of CCMs and indicates that Rho kinase activation is a critical step in CCM lesion genesis and maturation.

Key Words: cavernous angioma ■ cerebral cavernous malformation ■ fasudil ■ ROCK ■ therapy

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Fasudil Decreases Lesion Burden in a Murine Model of Cerebral Cavernous Malformation Disease

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Background and Purpose—Cerebral cavernous malformations (CCMs) are characterized by grossly dilated capillaries, associated with vascular leak and hemorrhage, and occur in sporadic or inherited (autosomal-dominant) forms with mutations in 1 of 3 gene loci (CCM 1, 2 or 3). We previously reported that the CCM1 protein (KRIT1) localizes to endothelial cell–cell junctions and loss of KRIT1 leads to junctional instability associated with activation of RhoA and its effector Rho kinase. Although Rho kinase inhibition has been proposed as potential therapy for CCM, there has been no demonstration of a therapeutic effect on CCM lesion genesis in vivo.

Methods—Fasudil-treated Ccm1+/−/Msh2−/− mice had a significantly decreased prevalence of CCM lesions compared with placebo controls. Lesions in treated animals were smaller and less likely associated with hemorrhage, inflammation, and endothelial proliferation and exhibited decreased expression of Rho kinase activation biomarkers. A therapeutic effect was also documented in Ccm2+/−/Trp53−/− mice.

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Cerebral cavernous malformations (CCMs) are common vascular lesions that can cause hemorrhagic stroke, seizures, and focal neurological deficits. Cases occur sporadically or in an inherited, autosomal-dominant form, the most common of which is caused by mutations in CCM1 (KRIT1).1,2

Our group had shown that KRIT1 is localized to and stabilizes endothelial cell (EC) junctions3 and the loss of KRIT1 leads to increased stress fibers, a hallmark of activation of RhoA and its effector Rho kinase (ROCK).3 Inhibition of ROCK in vitro or in vivo reversed the effect of KRIT1 silencing on endothelial junctional stability,4 suggesting it as a potential therapeutic strategy. Although we demonstrated robust activation of ROCK in ECs lining CCM lesions in humans,4 there has been to date no demonstration of a therapeutic effect on the pathogenesis of CCM lesions, the hallmark of the disease.

Based on evidence of a 2-hit mutation mechanism demonstrated in CCM,5–7 we recently showed that Ccm1+/− mice bred in a background of homozygous deletion of Msh2, a mismatch repair complex protein, develop CCM lesions recapitulating the human disease.8 We had previously demonstrated similar CCM lesions in Ccm2 heterozygotes sensitized by loss of tumor suppressor gene Trp53.9 Here, we report that treatment with fasudil, a specific ROCK inhibitor,10,11 decreases overall lesion burden and also inhibits the development of the mature CCM lesions.

Methods—The development of Ccm1+/−/Msh2−/− mice has been described previously.8 Immediately after weaning (P21), mice were randomly assigned into a treatment group (N=7), receiving fasudil dissolved in their drinking water at a dose of 100 mg/kg/day, or placebo (N=5), receiving fasudil-free drinking water. We verified water consumption by the treated mice, confirming ingestion of fasudil (see
Supplemental Methods; http://stroke.ahajournals.org). The dose of fasudil administration in this exploratory study was chosen based on that achieving ROCK inhibition and clinical effects in other murine studies\(^9\) and represents the median dose delivered to rodents in >20 published studies. At 5 months of age, the animals were euthanized and their brains harvested and fixed. One animal in the fasudil group and 1 in the placebo group died before completing treatment; their brains were examined postmortem and did not reveal any CCM lesions or tumors. These 2 animals were excluded from analysis. Fasudil-treated animals appeared healthy and did not exhibit overt adverse effects of treatment (see Supplemental Methods).

CCM lesion burden was assessed in the remaining brains of fasudil-treated Ccm1\(^{+/−}\)/Msh2\(^{−/−}\) mice (n=6) and placebo (n=4) as described previously\(^8\) and in the Supplemental Methods. Serial 1-mm-thick coronal slices of each brain were microsectioned at 5 \(\mu\)m and stained with hematoxylin and eosin, and CCM lesions were counted and cataloged blinded to treatment status. A Stage 1 CCM lesion was defined as an isolated dilated capillary with a maximal diameter accommodating at least 25 red blood cells (cavern), whereas a Stage 2 CCM lesion was defined as a cluster of \(\geq 2\) contiguous caverns as described in previous reports.\(^8\) Immunohistochemical stains for iron deposition, B-cell infiltration (the primary inflammatory cell marker in CCM lesions\(^8\)), proliferative index, and phosphorylated myosin light chain as a biomarker of ROCK activation have been described in detail in this model.\(^8\) A second biomarker of ROCK activation, phosphorylated myosin binding subunit (myosin-binding subunit of myosin phosphatase/myosin phosphatase target), was used for verification\(^11\) (see Supplemental Methods).

Another comparison group of Cm2\(^{+/−}\)/Trp53\(^{−/−}\) mice underwent a similar treatment regimen and were assessed for confirmatory effect on lesion burden in the second CCM genotype. This model has been described previously\(^,\)\(^9\) and details about the CCM2 treatment group and the analysis of lesion burden in this group and in the combined cohort (both genotypes) are presented in the Supplemental Methods and Data.

For the statistical assessment of treatment effect on the primary outcome (lesion counts per brain), we tested the assumption of Poisson distribution in each cohort and we used parametric testing (Poisson mean likelihood test) only where Poisson distribution was demonstrated. Results of lesion counts and cavern counts were also analyzed by the more stringent nonparametric Wilcoxon rank-sum test. Results with both parametric and nonparametric comparisons are reported for instances where Poisson distribution was confirmed and only the nonparametric comparison when Poisson distribution was not confirmed. Two-tailed Student t test was used for comparison of lesion size data, where we verified normal distribution. For categorical outcomes, nonparametric comparison tests (Fisher exact) was used. For each comparison, statistical significance was assumed at the 0.05 level. Statistical methods, including justification of Poisson distribution and data normality, where appropriate, are further detailed in the Supplemental Methods.

### Results

Fasudil-treated Ccm1\(^{+/−}\)/Msh2\(^{−/−}\) mice exhibited a significantly lower CCM lesion burden (Figure 1). Twelve CCM lesions were identified in the 4 mice in the placebo group and only 5 CCM lesions in the 6 fasudil-treated mice (\(P=0.01\) by parametric comparison with verified Poisson distribution of lesion counts and 0.06 with the more stringent nonparametric Wilcoxon rank-sum test). The prevalence of single cavern (Stage 1) and multicavernous (Stage 2) CCM lesions was reduced in the treated group as compared with placebo, and the number of total caverns per brain was significantly reduced (\(P=0.030\) by Wilcoxon rank-sum test). The maximal diameter of Stage 2 CCM lesions was significantly smaller in the fasudil group than in the placebo group (142 \(\mu\)m [1 lesion] versus 425±146 \(\mu\)m; \(P=0.03\)) but not the size of individual caverns that comprise the lesions (Figures 1 and 2). There were no brain tumors noted in either cohort on systematic histological screening.

Phenotypic features of lesions in the fasudil and placebo cohorts are presented in the Table and illustrated in Figure 2. Extravascular iron deposits, indicative of chronic hemorrhage, were present in 4 of the 12 lesions (in 3 of the 4 placebo mice) and in none of the lesions in the fasudil group (\(P=0.03\)). Infiltration of B cells was present in the same lesions exhibiting iron deposits, in the placebo group, and in none of the lesions in the fasudil group (\(P=0.03\)). The EC proliferative index (ratio of Ki67-immunopositive EC/total number of ECs lining caverns in CCM lesions) was 7.2% in the placebo group and remarkably 0% in the fasudil group (8 of 111 versus 0 of 59 ECs; Fisher exact test, \(P=0.05\)).

Mice treated with fasudil showed decreased phosphorylated myosin light chain staining in ECs lining CCM lesions (Table; Figure 2), indicating that fasudil inhibited ROCK activity within the lesion proper. The prevalence of phosphorylated myosin light chain staining in ECs in CCM lesions was greater in the placebo group (72.5%/16.5%/11% of cavern-lining ECs with negative/faint/intense staining, respectively).
as compared with the fasudil group (93%/7%/0% of cavern-lining ECs with negative/faint/intense staining, respectively). Fasudil-treated CCM lesions also exhibited less prevalent staining for another biomarker of ROCK activation, phosphorylated myosin binding subunit (phosphatase/myosin phosphatase target 1). The prevalence of phosphorylated myosin binding subunit staining in ECs in CCM lesions was greater in the placebo group (70.6%/17.0%/12.4% of cavern-lining ECs with negative/faint/intense staining, respectively) as compared with the fasudil group (94.5%/5.5%/0% of cavern-lining ECs with negative/faint/intense staining, respectively).

A beneficial effect was also apparent in the blinded analysis of lesion burden in \( Ccm1^{+/+} \) \( Msh2^{-/-} \) \( Msh2^{-/-} \) \( Trp53^{-/-} \) \( Msh2^{-/-} \) \( Msh2^{-/-} \) mice treated with the same dose, treatment onset, and duration of fasudil or placebo. Sixty CCM lesions were identified in the brains of 4 placebo mice (15 lesions per brain) versus 15 lesions in 2 fasudil-treated mice (7.5 lesions per brain). These results are summarized in the Supplemental Data. When combining the 2 cohorts, the difference in the number of CCM lesions and caverns per mouse with both genotypes were significant \((P=0.049\) and \(P=0.022\), respectively) even by the most stringent nonparametric comparisons.

**Discussion**

Fasudil treatment appears to reduce lesion genesis in the CCM1 murine model, as judged by significantly fewer overall CCM lesions in treated animals and fewer individual caverns. Fasudil also appears to prevent lesion maturation

| Table. Iron Deposits, Inflammatory Cell Infiltrates, Endothelial Cell (EC) Proliferative Markers, and ROCK Activity in CCM Lesions in Placebo and Fasudil-Treated \( Ccm1^{+/+} \) \( Msh2^{-/-} \) \( Msh2^{-/-} \) Mice |
|-------------------------------------------------|-------------------|
| Placebo | Fasudil | \( P \) |
| Total no. of mice | 4 | 6 | |
| No. of mice with iron in CCMs | 3 | 0 | 0.03* |
| No. of mice with B cells in CCMs | 3 | 0 | 0.03* |
| No. of K1–67-negative ECs in CCMs | 103 | 59 | |
| No. of K1–67-positive ECs in CCMs | 8 | 0 | 0.05† |
| No. of pMLC-negative ECs in CCMs | 150 | 94 | |
| No. of pMLC faintly positive ECs in CCMs | 34 | 7 | |
| No. of pMLC intensely positive ECs in CCMs | 23 | 0 | 0.000006† |
| No. of pMBS (MYPT1) negative ECs in CCMs | 125 | 69 | |
| No. of pMBS (MYPT1) faintly positive ECs in CCMs | 30 | 4 | |
| No. of pMBS (MYPT1) intensely positive ECs in CCMs | 22 | 0 | 0.00002† |

ROCK indicates Rho kinase; CCM, cerebral cavernous malformation; pMLC, phosphorylated myosin light chain; pMBS, phosphorylated myosin binding subunit; MYPT, myosin phosphatase/myosin phosphatase target.

Nonparametric statistical comparisons were used for each phenotypic feature. For the analysis of iron or inflammatory cell infiltrates, \( P \) is determined by the Fisher exact test comparing the *fraction of mice with iron or B cell infiltration in CCMs in placebo vs fasudil-treated mice. Fisher exact test is also used for \( P \) of *fraction of lesional ECs with staining of Ki-67, pMLC, or pMBS (MYPT1).
into complex clinically significant lesions. The CCM lesions in fasudil-treated mice were smaller and lacked features of hemorrhage, B-cell infiltration, and endothelial cell proliferation present in more mature CCM lesions.8 The inhibition of phosphorylated myosin light chain and phosphorylated myosin binding subunit staining indicated effective blocking of ROCK activity by fasudil in the disease-target ECs lining CCM lesions. The fasudil treatment was well tolerated with both groups exhibiting comparable weights and only 1 death in each group (1 of 5 receiving placebo and 1 of 7 receiving fasudil), an attrition rate not different from that previously reported in larger cohorts of untreated animals in this model8 and a result consistent with the known safety of fasudil in humans.12 Other systemic effects of fasudil, and potential mechanistic impact beyond ROCK inhibition in CCM lesions, were not examined in this preliminary study and will require more careful documentation and specific hypothesis testing in future studies.

Importantly, increased EC RhoA or ROCK activity has also been reported as a consequence of reduced expression of CCM2 or CCM3,4,14,15 the genes mutated in other forms of inherited CCM. Thus, this therapeutic approach may be broadly applicable to the other familial forms of this disease and possibly to sporadic CCM lesions. Indeed, a benefit on lesion burden was also apparent in our CCM2 murine model. Future studies should explore potential differences in the therapeutic effect in various genotypes and with fasudil treatment onset at different ages and for different duration. One or more of the features demonstrated here (lesion burden, size, phenotypic modification) may be more relevant in specific clinical settings. This proof of principle will also need to be confirmed in longitudinal studies that probe the potential therapeutic effect on established CCM lesions. Future research should also explore whether a similar CCM therapeutic effect can be accomplished with other agents such as statins, whose effects independent of lipid-lowering (ie, pleiotropic effects) include ROCK inhibition and which are broadly used chronically in human populations with cardio-vascular disease.12

These data represent the first report of therapeutic benefit of pharmacological intervention in CCM genesis and maturation in vivo and indicate that ROCK inhibition is a potential therapy for CCM disease.

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Disclosures
None.

References
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SUPPLEMENTAL MATERIAL

Fasudil Decreases Lesion Burden in a Murine Model of Cerebral Cavernous Malformation Disease

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Subject Codes: 50, 55, 130, 97
Supplemental Methods

Mice and Treatment Rendered. Animal procedures were approved by the Duke University Institutional Animal Care and Use Committee. The development of Ccm1+/− Msh2−/− mice has been described previously1. All alleles were maintained in a C57BL/6 background and mice are genotyped for Ccm1−/−, Msh2−/− and CRE recombinase (Jackson Laboratories protocol). Immediately after weaning (P 21), mice were randomly assorted into either control or fasudil-treated groups. The fasudil-treated group included 7 animals, received fasudil dissolved in their drinking water at a dose of 100 mg/kg/day while 5 animals in the placebo control received normal drinking water. Mice were aged for four months to allow CCM lesions to develop as in our previous study1. To determine the daily dose of fasudil consumed by the mice, the volume of fasudil containing water that was consumed was measured over the course of a 24-hour period on multiple days. Male mice consumed 4.0mL ± 0.9mL and female mice consumed 4.0mL ± 0.5mL and there was no significant difference in fasudil water consumption between the sexes. A subset of mice was also weighed at various time points during treatment. From these data we calculate that the mice received an average dose of 109 mg fasudil per kilogram body weight per day. This is very close to our attempt to provide 100 mg per kg body weight per day.

Around 5 months of age, the animals were euthanized by carbon dioxide, brains were removed, immersed in 10% formalin and allowed to fix for at least two weeks, and then were sent to the University of Chicago for lesion burden assessment, with an identification number blinded to the treatment status. One animal in the fasudil group and one in the placebo group died prior to completing treatment, and these two animals were excluded from analysis. Their brains were examined postmortem and did not reveal any CCM lesions or tumors. CCM lesion burden was assessed in the remaining brains of fasudil treated mice (n=6) and placebo (n=4). Fasudil dose was overtly well tolerated, as in previously published studies of chronic use at similar dose. No body weight data were gathered for the mice described in this manuscript. We have now analyzed the weight of the identically-treated mice in subsequent experiments. The average weights for mice at two months of age were 21.5g ± 2.1g (n=2) on fasudil and 24.0g ± 2.4g (n=5) on placebo (p = 0.2510, Student’s t-test). At four months of age, the weights were 21.9g ± 2.5g (n=8) on fasudil and 24.7g ± 3.2g (n=3) on placebo (p = 0.1551, Student’s t-test). From these data, there appears to be a trend to lower weight in the fasudil treated mice, but this is not statistically significant.

The development of CCM2 model was described previously3. Three Ccm2+/− Trp53−/− mice were randomized to receive fasudil treatment, at the same onset, duration and dose as CCM1 animals, and 4 mice received placebo treatment. One of the fasudil animals died from systemic malignancy after 94 days of treatment, and was excluded from primary analysis of lesion burden, since it did not complete the prescribed treatment. To assess overall treatment effect with greater statistical power, we also compared lesion burden in the combined cohort with both CCM1 and CCM2 mice.

Sample Preparation and Histology. Putative CCM lesions in the brains were stereotactically located by high-field magnetic resonance with a 14.1 T (600MHz) Bruker Avance imaging spectrometer using a gradient echo protocol described previously3. A mouse brain matrix was used to cut fourteen 1-mm thick coronal slices from the olfactory bulbs at the frontal rostrum and to the most caudal section at the cerebellar hindbrain. Each coronal slice was embedded in paraffin, cut to 5-µm thick sections with a microtome for hematoxylin and eosin (H&E) staining and was independently surveyed for CCM lesions by two observers (CS and RS), and the findings were adjudicated by a third observer (IAA), all blinded to treatment status. Stage 1 CCM lesions were defined as an isolated dilated capillary with a maximal diameter accommodating at least 25 red blood cells (cavern), while Stage 2 CCM lesions were defined as clusters of two or more contiguous caverns as described in previous reports1,3. This sampling method at 1-mm coronal slice intervals was selected in view of the size distribution of
Stage 1 lesions (median about 100 µm) and Stage 2 lesions (median about 400 µm). Few if any Stage 2 lesions would be missed with this technique, and the method allows a consistent assessment of the burden of Stage 1 lesions throughout the brain. As with any morphometric technique, we never imply that the lesion burden index counts every single potential vascular structure that is not evident on sections at the 1-mm sampling interval. Most importantly, the same technique is used in the blinded assessment of lesion burden of treated and untreated animals.

Assessment of Lesion Burden and Phenotypic Features. Lesion burden included all CCM lesions identified by systematic histology on serial 1-mm coronal slices of each brain. Adjacent 5-µm sections from slices harboring CCM lesions were processed for immunohistochemistry. Iron deposits were detected by Perl's Prussian blue. B cell infiltration and proliferative index of endothelial cells in each lesion were assessed using the primary antibodies (with dilutions) anti-B220 (eBioscience, RA3-6B2, 1:500), and anti-Ki67 (Labvision, SP6, 1:300), respectively; biotinylated secondary antibodies (1:1000, Vector Laboratories) and incubation with Elite ABC kit reagents (Vector Laboratories). Staining was independently assessed by two investigators (CS and RS) adjudicated by IAA, as described previously.

ROCK activity was probed by the expression of phosphorylated myosin light chain (pMLC) using rabbit polyclonal anti-pMLC [Thr180/Ser19] antibody (Cell Signaling Technology) at 1:250 dilution and phosphorylated myosin basic subunit (pMBS) using rabbit polyclonal anti-pMBS (MYPT1) [Thr696] antibody (Cell Signaling Technology) at 1:250 dilution, and the immunostaining in ECs lining each cavern was characterized as absent (0), faint (1), or dense (2). Rho GTPase effects are mediated by its interaction with downstream effectors, including the serine/threonine kinases Rho-associated protein kinase -1 and -2, collectively referred to as ROCK. ROCK reorganizes the actin cytoskeleton through phosphorylation of several substrates that contribute to the assembly of actin filaments and contractility. Two central ROCK phosphorylation targets are Myosin Light Chain (MLC) and MLC phosphatase, which is composed of several subunits, one of which is MYPT1 (also referred to as Myosin Binding Subunit, MBS). ROCK phosphorylates MYPT1 at Thr 696 and 850, which inhibits its phosphatase activity and thereby indirectly increases abundance of phosphoMLC. Because phosphorylation of either MLC or MYPT1 is a direct assay of ROCK activity, both are widely used as indices to assay ROCK activity both in vitro and in vivo.

Microscope Image Acquisition. Images were acquired at room temperature, using a bright-field microscope (Olympus BH2) with a camera (Olympus, U-PMTVC) and acquisition software (Magnafire). Objective lenses type, magnification and numerical aperture were DPlanApo 20 UV, 20X, 0.70 and DPlanApo 40 UV, 40X, 0.85, respectively.

Statistical Analysis. For the primary outcome parameter of lesion counts in CCM1 models (number of CCM lesions per animal), we tested the assumption of Poisson distribution within each cohort using the Kolmogorov-Smirnov one-sample test (K-S test) comparing the mouse level data of rate of lesions to a theoretical Poisson distribution with the rate parameter equal to the lesion rate per mouse. The K-S test p value was 0.72 and 0.15 for the placebo and fasudil groups, respectively, indicating that they approximately follow Poisson distribution. Despite justification of Poisson distribution of lesion burden, we also reported a more conservative comparison of lesion rates using the non-parametric Wilcoxon rank-sum test, which does not rely on Poisson distribution.

For cavern rate data (number of caverns per animal), K-S tests demonstrated that these did not follow a Poisson distribution in either the placebo or fasudil group (K-S test p = 0.000003 and 0.016, respectively). Hence the significance level for this outcome is only reported using the non-parametric Wilcoxon rank-sum test.

For lesion and cavern size data, assumption of normal distribution of diameter measures was tested using the Shapiro-Wilk test.
Supplemental Data

Lesion Burden in Ccm2+/-Trp53-/- mice. There were 60 CCM lesions (54 Stage 1 and 6 Stage 2) in 4 placebo mice, and 15 lesions (15 Stage 1 and 0 Stage 2) in 2 fasudil treated mice that completed treatment (15 lesions/mouse versus 7.5 lesions/mouse). Because the numbers were small, the difference was not statistically significant when using non-parametric testing.

Lesion Burden in the Combined Cohort with Both Genotypes. When data from both genotypes were combined, there were 72 CCM lesions in 8 placebo mice and 20 lesions in 8 fasudil treated mice that completed treatment. The lesion burden was significantly reduced from 9 in placebo mice to 2.5 in mice with fasudil treatment (p = 0.049, Wilcoxon rank-sum test). There were 94 caverns in the placebo mice and 22 caverns in the fasudil treated mice that completed treatment. The total number of caverns per mouse was significantly reduced from 11.8 in placebo mice to 2.8 in mice with fasudil treatment (p = 0.022, Wilcoxon rank-sum test). There were 10 Stage 2 lesions in the placebo mice and one Stage 2 lesion in the fasudil treated mice that completed treatment. The total number of Stage 2 lesions per mouse was significantly reduced from 1.2 in placebo mice to 0.1 in mice with fasudil treatment (p = 0.044, Wilcoxon rank-sum test). The maximal diameter of Stage 2 CCM lesions was significantly smaller in the fasudil group than in placebo (142 ± 0 µm vs. 384 ± 108 µm; p = 0.00006, T-test; the data follows a normal distribution by the Shapiro-Wilk test, p = 0.64).

Supplemental References

