RhoA Kinase Inhibition With Fasudil Versus Simvastatin in Murine Models of Cerebral Cavernous Malformations

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Methods—Two heterozygous murine models, Ccm1+/−Msh2−/− and Ccm2+/−Trp53−/−, were treated from weaning to 4 to 5 months of age with Fasudil (100 mg/kg per day), simvastatin (40 mg/kg per day) or with placebo. Mouse brains were blindly assessed for CCM lesion burden, nonheme iron deposition (as a quantitative measure of chronic lesional hemorrhage), and ROCK activity.

Results—Fasudil, but not simvastatin, significantly decreased mature CCM lesion burden in Ccm1+/−Msh2−/− mice, and in meta-analysis of both models combined, when compared with mice receiving placebo. Fasudil and simvastatin both significantly decreased the integrated iron density per mature lesion area in Ccm1+/−Msh2−/− mice, and in both models combined, compared with mice given placebo. ROCK activity in mature lesions of Ccm1+/−Msh2−/− mice was similar with both treatments. Fasudil, but not simvastatin, improved survival in Ccm1+/−Msh2−/− mice. Fasudil and simvastatin treatment did not affect survival or lesion development significantly in Ccm2+/−Trp53−/− mice alone, and Fasudil benefit seemed limited to males.

Conclusions—ROCK inhibitor Fasudil was more efficacious than simvastatin in improving survival and blunting the development of mature CCM lesions. Both drugs significantly decreased chronic hemorrhage in CCM lesions. These findings justify the development of ROCK inhibitors and the clinical testing of commonly used statin agents in CCM.

Key Words: Fasudil □ hemangioma, cavernous □ hemangioma, cavernous, central nervous system □ rho-associated kinases □ simvastatin □ therapeutics

Several therapies1–5 have already been proposed to treat cerebral cavernous malformations (CCMs), a disease that often leads to hemorrhagic stroke and seizures. These have largely arisen from the study of impact of downregulation of CCM genes on cultured endothelial cells. A recent study6 demonstrated that treatments targeting Rho or the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) proteases may be more efficacious in treating CCM disease than other therapies. In a previous report,2 using a limited number of animals, we showed that the Rho kinase (ROCK) inhibitor, Fasudil, decreased CCM lesion burden in a heterozygous murine model where lesions develop stochastically during life, with all phenotypic signatures recapitulating the human disease.7

We now aimed to replicate our previous results with Fasudil, using a larger cohort of animals with 2 heterozygous Ccm genotypes. We also treated the murine models with simvastatin, commonly used in humans, with weak ROCK inhibitor properties.8–11 Unlike specific ROCK inhibitors, statins have broader pleiotropic effects,8 and concerns have been raised whether they might increase brain hemorrhage.12–14 Hence, besides assessing the effect of treatment on the number, stage, and size of CCM lesions in each animal brain, we also

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investigated quantitative nonheme iron deposition within the mature CCM lesions and carefully analyzed animal attrition to address safety and hemorrhagic propensity. A portion of the results from this work was presented in poster form at the 2015 International Stroke Conference held in Nashville, TN.15

Materials and Methods

Ccm Murine Models

Animal procedures were approved by the Duke University Institutional Animal Care and Use Committee. The Ccm1+/-Msh2+/- and Ccm2-/-Trp53-/- murine models for CCM disease were developed as previously reported.16 Based on previously demonstrated Knudsson 2-hit mechanism (heterozygosity in the germ-line and biallelic somatic mutations in lesions), these models create Ccm heterozygous mice in p53- or Msh2-deficient backgrounds, predisposing to somatic mutations and enhancing CCM lesion genesis.3,16,17

Immediately after weaning (P21), mice were randomly assigned to the respective treatment groups. One hundred forty-two Ccm1+/-Msh2+/- mice (71 males and 71 females) and 72 Ccm2-/-Trp53-/- mice (61 males and 11 females) were included in the experiments. The breeding program for creating mice with the appropriate genotype has been described previously.3,16,17 This is detailed along with a treatment assignment scheme in the Methods and Figure I in the online-only Data Supplement.

Randomized Assignment and Treatment Groups

For all groups, the National Institute of Neurological Disorders and Stroke guidelines for objectivity in preclinical research were followed including randomization, blinding of outcome assessment, appropriate sample-size estimation based on the primary outcome, and pre-specified data analyses.18 Ccm1+/-Msh2+/- mice were randomized at weaning to receive Fasudil (100 mg/kg per day in the drinking water) or placebo with drug-free drinking water until 4 to 5 months of age. In a separate study, mice were randomly assigned to receive simvastatin (40 mg/kg per day in the chow) or placebo controls with the same drug free-diet from weaning to 4 to 5 months of age. Ccm2-/-Trp53-/- mice similarly assigned to receive the same regimen of Fasudil or placebo and simvastatin or placebo. Late, short-term studies were completed for Ccm1+/-Msh2+/- mice (15 mice) of the Ccm1+/-Msh2+/- model were used as placebos for the long-term studies with simvastatin and for the late, short-term studies with Fasudil. Notably, the body weights of the CCM models were not affected by Fasudil or simvastatin treatment (Table I in the online-only Data Supplement).

CCM Lesion Analysis

After treatment, brains were removed and assessed for lesion burden on serial histological sections by a procedure previously described.2,3 Briefly, formalin-fixed brains were cut into 1-mm-thick coronal slices, placed into cassettes, and processed. After embedding the slices in paraffin, the slices were cut into 5-µm-thick sections with a microtome. After staining with hematoxylin and eosin, the sections were scored for the number of stage 1 prelesions (single ballooned capillaries >100 µm in diameter) and stage 2 lesions (mature, multicavernous) by 2 observers (R.S., and C.S., C.A., or T.M.), with adjudication by a third observer (I.A.A.), as was done previously.2,19-21 All assessors were blinded to the treatment assignment. The lesional area was measured using the polygon area function of a microscope digital camera DP21 (Olympus) as described previously.19

Hematoxylin and eosin–stained sections from brains of mice that either died or euthanized because of a debilitating illness were examined for hemorrhage or tumors (Table II in the online-only Data Supplement). Tumors on sections were confirmed by University of Chicago neuropathologist IP.

Blank sections from brains with stage 2 CCM lesions were processed for quantitative assessment of nonheme iron deposition by Perl's Prussian blue and for ROCK activity by the intensity of phosphorylated myosin light chain through methods previously described.2,19 We had previously shown that stage 1 prelesions do not manifest iron deposition,1 and this was also true in the present study. Figure 1 illustrates typical stage 1 and 2 lesions and associated iron deposition in the latter.

Primary and secondary outcomes are defined, and the statistical methods are presented in the Methods in the online-only Data Supplement.

Results

Fasudil, but Not Simvastatin, Decreases Lesion Burden in CCM Models

The number of mature multicavernous stage 2 lesions per mouse decreased significantly (P=0.02) by 74% from 0.68±0.30 in placebos to 0.18±0.24 in Fasudil-treated Ccm1+/-Msh2+/- animals (Figure 2A). Stage 2 lesion burden decreased, but not significantly (P=0.25), by 58% from 0.78±0.33 in placebos to 0.33±0.41 in Fasudil-treated Ccm2-/-Trp53-/- animals (Figure 2B). There were significant increases in stage 1 lesion counts, and no impact on total lesion counts, as the proportion of stage 2 lesions among total lesions decreased from 65% in placebos to 22% in the Fasudil-treated Ccm1+/-Msh2+/- mice (P=0.02) and decreased from 64% in placebos to 21% in the Fasudil-treated Ccm2-/-Trp53-/- murine model (P=0.002), indicating an effect of treatment on lesion maturation.

In contrast, the number of stage 2 lesions per mouse and other lesion counts were not decreased by simvastatin in either of the genotypes compared with placebo. A meta-analysis of combined effect in the 2 genotypes showed a significantly decreased incidence of stage 2 lesions favoring Fasudil (P=0.017; Figure 2C). A similar meta-analysis showed no significant effect of simvastatin on the incidence of stage 2 lesions in the 2 genotypes (Figure 2D). Neither Fasudil nor simvastatin significantly affected lesional cross-sectional area in either genotype. But there was a trend of a smaller stage 2 lesional area in Fasudil-treated mice,

Figure 1. Illustrative photomicrographs of stage 1 and 2 lesions, and typical nonheme iron deposition in stage 2, but not in stage 1, lesions. Bar, 50 µm.
approaching statistical significance in meta-analysis of effect in combined genotypes (Figure II in the online-only Data Supplement).

A few significant observations arose in the prespecified subgroup analysis of results per the animal’s sex (Table III in the online-only Data Supplement). In Ccm2<sup>+/−</sup>Trp53<sup>−/−</sup> male brains, there were significantly less stage 2 lesions/animal in Fasudil-treated mice compared with those in placebos (P=0.04), an effect not present in the cohort as a whole. Among Ccm2<sup>+/−</sup>Trp53<sup>−/−</sup> females, there were significantly fewer total (stage 1 and 2) lesions/animal in simvastatin-treated mice than in placebos (P=0.02), an effect not present in the cohort as a whole. But we did note an imbalance in treatment assignment between sexes.

Fasudil given later in life for 1 month (at age 3–4 months) to Ccm1<sup>+/−</sup>Msh2<sup>+/−</sup> mice did not affect lesion burden when compared with placebo in the overall treated cohort (Figure III in the online-only Data Supplement). However, in male mice, the total number of lesions per mouse was significantly lower, 2.89±0.93 in 9 placebos versus 1.29±0.68 in 7 Fasudil-treated animals (P=0.036). An opposite effect (greater lesion burden) was observed in female mice, but did not reach statistical significance. There was an imbalance in the placebo group, with females harboring fewer lesions.

![Figure 2](image)

**Figure 2.** Lesion burden in Ccm models with Fasudil (F) and simvastatin (S) treatment. A, F (n=16 placebo [P], n=22 F-treated mice), but not S (n=20 P, n=28 S-treated mice), decreased the multicavernous stage 2 lesion burden in the Ccm1<sup>+/−</sup>Msh2<sup>+/−</sup> murine model. B, Neither F (n=18 P, n=18 F-treated mice) nor S (n=11 P, n=12 S-treated mice), significantly affected lesion burden in Ccm2<sup>+/−</sup>Trp53<sup>−/−</sup> mice. Meta-analysis of combined effect in the 2 models showed that incidence of stage 2 lesions is decreased with F (C), but not with S (D). The DerSimonian and Laird method<sup>22</sup> used for the meta-analyses considers the relative contribution of effect by the 2 models (weight %) and their different penetrance (incidence rate ratio). CCM indicates cerebral cavernous malformation; and CI, confidence interval.
Fasudil and Simvastatin Decrease Nonheme Iron in Mature CCM Lesions

Both Fasudil (P<0.01) and simvastatin (P<0.05) both significantly decreased nonheme iron in the multicavernous stage 2 lesions of the Ccm1+/-Msh2-/- murine model, when compared with placebo-treated animals (Figure 3). Simvastatin (P<0.05), but not Fasudil, decreased nonheme iron in the multicavernous lesions of the Ccm2+/-Trp53-/- murine model, when compared with placebo-treated animals. Meta-analyses of effect on nonheme iron density per stage 2 lesional area in the 2 genotypes favored both Fasudil and simvastatin over placebo (P=0.017 and P<0.001, respectively). There was no differential sex effect of either treatment on iron deposit in lesions (Table IV in the online-only Data Supplement).

Fasudil and Simvastatin Decrease ROCK Activity in Mature CCM Lesions

Stage 2 lesional endothelial cell ROCK activity in Ccm1+/− Msh2−/− murine brains (measured by the percentage of cells that were phosphorylated myosin light chain positive) was

![Image](http://stroke.ahajournals.org/)

**Figure 3.** Nonheme iron deposition in multicavernous lesions with Fasudil and simvastatin treatment. **A,** Both Fasudil (n=11 and n=4 stage 2 lesions in placebo and Fasudil-treated mice, respectively) and simvastatin (n=6 and n=7 stage 2 lesions in placebo and simvastatin-treated mice, respectively) decreased nonheme iron deposition per lesion area in the multicavernous stage 2 lesions in the Ccm1+/- Msh2-/- murine model. **B,** Simvastatin (n=7 and n=4 stage 2 lesions in placebo and simvastatin-treated mice, respectively), but not Fasudil (n=14 and n=6 stage 2 lesions in placebo and Fasudil-treated mice, respectively), significantly decreased nonheme iron deposition per lesion area in stage 2 lesions in the less penetrant Ccm2+/-Trp53-/- murine model. Meta-analysis of combined effect in the 2 models showed that nonheme iron deposition per lesion area in stage 2 lesions is decreased with Fasudil (C) and with simvastatin (D). The DerSimonian and Laird method was used for the meta-analyses considers the relative contribution of effect by the 2 models (weight %) and their difference in iron deposition per lesion. CCM indicates cerebral cavernous malformation; and CI, confidence interval.
significantly decreased from 68.7% in placebos to 51.9% in Fasudil-treated mice ($P=0.01$ versus placebo) and 57.0% in simvastatin-treated mice ($P=0.007$ versus placebo). There was no significant difference of percent endothelial cell immunopositivity among individual lesions or mice in the placebo, Fasudil, or simvastatin cohorts (Figure IV in the online-only Data Supplement).

**Fasudil Increases Survival in Ccm1 Models**
The proportion of $Ccm1^{+/−}Msh2^{−/−}$ mice that survived to complete the long-term treatment was higher ($P=0.05$) with Fasudil than with placebo (Figure 4). No significant difference on survival was observed between females and male animals. There was no effect on survival in this model with simvastatin treatment compared with placebos. For the $Ccm2^{+/−}Trp53^{−/−}$ murine model, no effect on survival was observed with either Fasudil or simvastatin treatment compared with placebos. In the $Ccm1^{+/−}Msh2^{−/−}$ murine model treated from 3 to 4 months, there was a nonsignificant trend for increased survival with Fasudil treatment ($17/18$ compared with placebos $16/20$). No increase in brain hemorrhage in association with animal attrition was observed with either Fasudil or simvastatin treatment compared with placebos in either genotype (Table I in the online-only Data Supplement).

**Discussion**
Two classes of murine models of CCM disease are available to test potential experimental therapies. Acute models with postnatal-induced homozygous $Ccm$ loss have been used to investigate mechanisms of CCM pathogenesis, including endothelial mesenchymal transition, sulindac therapy, δ-notch signaling, and MEKK3-KLF2/4 (MAP [mitogen-activated protein]/ERK [extracellular signal-regulated kinase] kinase kinase 3 - Krüppel-like factor 2/4) signaling. These models result in high lesion burden in the developing hindbrain and retina, and animal attrition before the first month of age. Although allowing examination of signaling mechanisms involved in lesion genesis, these models assess neither CCM lesion maturation during life nor the hemorrhage burden from lesions, cardinal features of the human disease. In contrast, the chronic heterozygous models we used here$^{−,16,17,20}$ develop CCM lesions during the animal’s lifespan, resembling the human disease more closely than the shorter lived acute models.

Our results provide evidence that long-term Fasudil treatment, but not simvastatin, prevents the development of mature, multicavernous CCM lesions. We also noted that both drugs are more efficacious in the $Ccm1^{+/−}Msh2^{−/−}$ compared with the $Ccm2^{+/−}Trp53^{−/−}$ murine model. Meta-analysis of results from both genotypes confirmed a beneficial effect on the development of mature CCM lesions, favoring Fasudil treatment, but not simvastatin, over placebo. Although both drugs at the chosen doses seemed to affect endothelial ROCK activity similarly in mature murine lesions, there is evidence that statins result in weaker systemic ROCK inhibition when compared with specific ROCK inhibitor drugs, and this could explain the differential benefit favoring Fasudil.

Fasudil and simvastatin both significantly decreased non-heme iron deposition in mature CCM lesions in 2 murine models. This effect was present even when adjusting for lesion area and occurred with simvastatin in the absence of reduction in lesion burden. Simvastatin effect on lesional iron deposition was even greater than Fasudil in the $Ccm2^{+/−}Trp53^{−/−}$ mice. Previous studies$^{20,23,24}$ indicated that ROCK inhibition blocks endothelial hyperpermeability and actin stress fibers resulting from the heterozygous loss of $CCM$ genes, both in cultured endothelial cells in vitro and in murine models in vivo, and simvastatin reverses dermal hyperpermeability in one of these models.$^{25}$ Our group had previously demonstrated a correlation of quantitative vascular permeability with iron content in human CCM lesions evaluated by advanced magnetic resonance imaging.

![Figure 4](http://stroke.ahajournals.org/)

**Figure 4.** Kaplan–Meier plots of survival in cerebral cavernous malformation (CCM) models with Fasudil and simvastatin treatment. Fasudil treatment increased ($P=0.05$) the survival of the $Ccm1^{+/−}Msh2^{−/−}$ murine models (A: $n=25$ per group), whereas simvastatin did not affect the survival of these animals (B: $n=27$ placebo, $n=42$ simvastatin). Both Fasudil treatment (C: $n=24$ per group) and simvastatin treatment (D: $n=12$ per group) did not affect the survival of $Ccm2^{+/−}Trp53^{−/−}$ murine models.
techniques. Blood leak from CCM lesions produces the most debilitating features of CCM disease, including hemorrhagic stroke and seizures. A favorable effect of either drug on lesional iron deposit is hence relevant clinically.

Long-term (≥4 months) experiments in rodents treated with Fasudil in the drinking water or by gavage have used doses ranging from 10 to 228 mg/kg per day without any reported attrition or complications. In our proposed experiments, we used the midrange dose of 100 mg/kg per day, already shown to reduce CCM lesion burden in pilot studies. A lower dose of 10 mg/kg per day has been reported to be effective in suppressing cardiac allograft vasculopathy in mice. An effect at a lower dose was not investigated herein, but would be particularly encouraging for translating this therapy to humans, where oral doses as high as 240 mg/d have been well tolerated in trials on angina, and an intravenous dose of 90 mg/d is approved and has long been used in Japan for the prevention of cerebral vasospasm after subarachnoid hemorrhage. Although treatment of transgenic mice indicates a potential therapeutic efficacy in CCM, Fasudil is a Rock1 and Rock2 inhibitor that was first approved in Japan in 1995 by Asahi Kasei Pharma Corporation for the treatment of cerebral vasospasm, and it has never been approved in the United States. Reported toxicity with Fasudil includes subcutaneous hemorrhage, subarachnoid hemorrhage, nausea, pyrexia, kidney failure, and hypotension. Long-term safety of Fasudil has not been assessed, in particular with concerns about hypotension and systemic side effects. Rock1 has widespread tissue distribution, but there is relatively little Rock1 in brain and skeletal muscle. Rock2 is mainly expressed in the central nervous system and is relatively low in liver, stomach, spleen, kidney, and testis (BioAxone US patent 7572913). Inhibitors that target Rock2 are currently being developed and would be expected to have much less systemic toxicity than inhibitors that target both kinases. For treatment of CCM, Rock2 inhibition promises to target cerebral microvessels and CCM vessels more selectively and could increase targeted effectiveness and minimize systemic side effect.

We have chosen simvastatin as our statin test agent because this drug was shown to reverse dermal hyperpermeability in CCM1+/- murine lesions. Simvastatin has been administered orally in the animals’ chow, drinking water, or by gavage in doses ranging from 2 to 100 mg/kg per day in chronic (≥4 months) studies without adverse effects. The target dose of simvastatin of 40 mg/kg per day is at the midrange used by other investigators and is thought to be equivalent to adult human doses of 20 to 40 mg/d widely used for cardiovascular- and cholesterol-lowering effects. We should nevertheless be cautious about statin dose–effect, as statins have been reported to exhibit biphasic dose effects on inflammation-induced angiogenesis and higher doses are typically needed to achieve greater ROCK inhibition pleiotropic effect.

Because simvastatin at 40 mg/kg per day in the chow was less effective in reducing lesion burden than Fasudil, we are currently treating the Ccm1+/- Msh2+/- murine model with atorvastatin at 80 mg/kg per day in the chow (equivalent to simvastatin at 160 mg/kg per day in mice and thought to be equivalent to the high-dose atorvastatin 80 mg/d approved for clinical use in humans). A putative benefit on lesion burden and blunting CCM hemorrhage with higher dose statin will have implications on dose escalation in clinical trials that may be planned in humans. We are also in the process of treating Ccm3+/- murine models with Fasudil, simvastatin, and atorvastatin, to determine whether ROCK inhibitors can reduce lesion burden in another model with a more penetrant genotype.

The benefit of Fasudil on lesion burden in the current study primarily impacted the development of mature multicavernous stage 2 lesions. Only stage 2 lesions have been associated with bleeding and inflammatory cell infiltrate in previous studies, and we also noted no hemorrhage in stage 1 lesions in our current experiments. There was no effect on total lesion counts, and in fact we noted increases in stage 1 prelesion counts, and a greater prevalence of stage 1 lesions among total lesions. These results are all consistent with a therapeutic effect blunting lesion maturation into clinically relevant multicavernous phenotype, rather than lesion genesis. A trend was also noted of Fasudil on cross-sectional area of mature stage 2 lesions, as assessed by histological morphometry. The combined effect of therapy on lesional count and cross-sectional area promises to be more easily and accurately assessed in ongoing experiments using novel microcomputed tomographic technique recently introduced and validated by our team, with volumetric quantification of CCM lesion burden in murine brains.

We cannot comment on the relative benefits of ROCK inhibition versus B-cell depletion therapy, also demonstrated to be effective in chronic heterozygous Ccm murine models. Either therapy may have a role in the clinical setting, and it is possible that ROCK inhibition effect is part mediated by modulation of the immune response in lesions. Indeed, we had previously shown that ROCK inhibition decreases B-cell infiltration in murine CCM lesions, and B-cell depletion decreases ROCK activity in lesional endothelium. Other compelling therapeutic targets have been identified in CCM, based on aberrant signaling in conjunction with acute postnatally induced Ccm homozygocity. These therapeutic strategies have not been examined in chronic heterozygous models, and a recently identified primary CCM signaling effector was shown to act via downstream RhoA/ROCK activation.

Because of the small numbers of animals, imbalance of treatment assignment and different phenotype severity by sex, definitive conclusions cannot be determined from subgroup analyses by sex. Nevertheless, we documented a greater benefit of Fasudil on lesion burden in males in both short-term and long-term treatments, a potential negative effect of short-term Fasudil treatment, and a weaker phenotype in female mice receiving placebo. These may reflect mechanistic biological effects and will need to be confirmed in future studies. A weaker effect of Fasudil on lesion development in Ccm2 -/- Trp53+/- mice, and a greater benefit of simvastatin than of Fasudil on lesional iron in this model, may also represent differential biological mechanisms. If these observations are confirmed in future studies, they may indicate a complex biology of ROCK inhibition requiring careful calibration.

Our experiments do not exclude pleiotropic effects of either drug, other than ROCK inhibition, as the mechanism of therapeutic effect. Animal weights were not affected by treatment in any cohort. And although we did not document blood
pressure measurements, similar dose and duration of Fasudil treatment in mice did not significantly impact blood pressure in other studies.\textsuperscript{30,52}

Duration of therapy in mice cannot be easily translated into a prescribed course of treatment in humans. Beyond dose–effect questions, it is unclear when or how long to administer the treatment to blunt lesion development and maturation. Clinical trials of ROCK inhibition with specific drugs and or statins may target lesion formation and progression to larger clinically more relevant lesions. CCM hemorrhage is the most compelling outcome parameter, associated with neurological sequelae,\textsuperscript{53} and for the first time this report identifies an effect of a drug on bleeding in CCM lesions. Yet it is unclear at what stage, and for how long a human lesion should be treated to prevent bleeding or rebleeding. It may be compelling to consider drug therapy for a lesion that has recently bled and is hence most likely to rebleed in the next 2 to 3 years,\textsuperscript{34,55} so ROCK inhibition might be conceived as lesion stabilization therapy. Dose escalation in clinical trials may be calibrated using dynamic contrast-enhanced quantitative perfusion on magnetic resonance imaging that can reflect ROCK-induced hyperpermeability in vivo.\textsuperscript{26,56,57} Another magnetic resonance imaging biomarker, quantitative susceptibility mapping, may be useful in clinical trials, to assess CCM lesional hemorrhage as a therapeutic outcome in humans,\textsuperscript{26,57–59} as it was herein shown in mice.

### Summary

ROCK inhibitor Fasudil, but not simvastatin, blunted CCM lesion development into mature clinically significant forms. Both drugs significantly decreased lesional bleeding, the first such effect ever reported in CCM. These results justify the development of ROCK inhibitors as therapeutic agents for CCM, and the testing of statins, with cautious dose escalation, in humans.

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### Disclosures

Dr Liao has consultancy for Asahi-Kasei Pharmaceuticals, Inc., Celgene, Pfiant, and Aimgen. The other authors report no conflicts.

### References


Biol independent of plasma cholesterol lowering.


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Supplemental Methods

Vertebrate Animals

The breeding of mice required for all experiments was conducted at the Duke University site. Mice (*Mus musculus*) with the respective genotypes listed below were used. Mouse lines carrying knockout alleles of *Ccm1* and *Ccm2* were generated and are currently maintained at the Duke site. Mice containing the knockout alleles of *Msh2* were also generated at the Duke site from other lines in the following manner. Mice with an exon of *Msh2* flanked by LoxP sites (obtained from Dr. Raju Kucherlapati under Material Transfer Agreement with Harvard Medical School) were crossed with mice carrying the EIIa-Cre transgene (available from the Jackson Laboratory) to generate the knockout allele of *Msh2*. Mice with a knockout allele of *Trp53* were obtained from the Jackson Laboratory. The genotypes of the animals that underwent the experimental drug treatments were *Ccm1*^+/−*Msh2*^-/- and *Ccm2*^+/−*Trp53*^-/-.

In order to generate these final experimental genotypes, animals of intermediate genotypes were generated in the breeding funnels, as discussed below. These intermediate genotypes were merely used as breeders and did not undergo treatments or procedures. All mutant lines were maintained in the C57BL/6J inbred strain background, also obtained from the Jackson Laboratory. Mice of both sexes were used. Experimental animals were bred and aged to up to 6 months before being sacrificed. Breeders were kept for up to 8 months before being retired, whereupon they were sacrificed. A two-generation breeding scheme is required to produce mice with the final experimental genotypes (*Ccm1*^+/−*Msh2*^-/- and *Ccm2*^+/−*Trp53*^-/-). In the final cross, due to Mendelian segregation of the two mutant alleles, 1 in 8 animals produced the desired genotype. In both the first and second crosses, animals of undesired genotypic combinations were identified before weaning by PCR genotyping, and euthanized.

Figure I presents the schema of concurrent random assignment of animals to placebo or treatment groups.

Primary and Secondary Outcomes and Statistical Analysis

Groups of mice to be compared were raised and treated contemporaneously. Fasudil or simvastatin treatment did not affect the body weight of the animals when compared to placebos in any of the experimental groups (Table I). We noted when the mice either died before completing treatment, or were euthanized after completing treatment or suffering from a debilitating illness before completion of treatment (Table II). As recommended by NINDS guidelines, subgroup analyses were planned to glean any treatment effect related to the animal’s sex (Tables III and IV).

For primary outcome assessment, we hypothesized that the prevalence of mature stage 2 CCM lesions at the conclusion of treatment will be decreased by 50% in each respective drug-treated group as compared to control mice receiving the same drug-free diet and drinking water. Power calculations assumed that 50% of mice will harbor one or more mature stage 2 CCM lesions (defined below) after age 4 months in the placebo group, with a median of 3 lesions per brain, which are conservative estimates based on our preliminary studies.\(^1\) Based on data from preliminary studies with a much smaller number of animals,\(^1\) initial sample size was calculated to be 20 per group using Poisson maximum likelihood to test the significance between the drug-treated and placebo groups (\(\alpha=0.05, 1-\beta=0.88, 2\text{-tailed}\)). We hence commenced randomized assignment to treatment groups in the first experiment with a larger group of mice. This accounts for the larger samples sizes in the *Ccm1*^+/−*Msh2*^-/- Fasudil and simvastatin comparisons. Once
animals were included in a study, they were allowed to complete their treatment arm. Based on results of the $Ccm1^{+/+}$-$Msh2^{+/+}$ Fasudil experiments, we calculated new sample size estimates to be 12 per group within even greater significance between the drug-treated and placebo groups ($\alpha=0.01$, $1-\beta=0.94$, 2-tailed). We hence used the smaller sample size for the later $Ccm2^{+/+}$-$Trp53^{−/−}$ simvastatin comparison to placebo.

Since the data had fit the Poisson model well in our preliminary studies, we used the Poisson maximum likelihood test to assess for statistical differences in lesion number per brain between drug-treated and placebo mice in early- and late-treated groups. For assessment of primary outcome, the number of stage 2 lesions per animal between ROCK inhibited and placebo groups were compared using Negative Binominal Regression if the outcome was over-dispersed and Poisson regression analysis if the mean and variance were equal.

For other prespecified secondary outcome analyses, lesional areas were compared among the different treatment groups. The F test was used to evaluate the variances between two unpaired groups. The differences between the two groups were compared using Student’s t-test with equal variances and Welch’s t test with unequal variances. The Mann-Whitney test was used to compare integrated density of iron per lesion and integrated density of iron per lesional area between treatment groups. The chi-square test was conducted to compare the prevalence of stage 2 lesions among all lesions, and the proportion of endothelial cells with ROCK activity in stage 2 lesions between treatment groups. And the log-rank (Mantel-Cox) test was used to compare the survival of animals between treatment groups.

The DerSimonian and Laird method was used for the meta-analyses for lesion burden, lesional area and iron deposition incorporating effect in the two genotypes. The weights of the $Ccm1$ or $Ccm2$ groups were calculated with the inverse of respective variance (including within each individual study variance and between studies variance). Heterogeneity was assessed by the chi-square test and quantified by the inconsistency index $I^2$, which is the percentage of variance across the $Ccm1$ and $Ccm2$ groups.

Statistical analyses were performed using SAS9.4 (SAS Institute Inc., Cary, NC), R v3.2.3 (R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism 4.0 (GraphPad Software Inc., La Jolla, CA). All probability ($P$) values were considered to be statistically significant at $P<0.05$. 
## Supplemental Tables

### Table I. Weights of Murine Models Receiving Placebo vs. Fasudil or Simvastatin

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Treatment (duration)</th>
<th>Age at weighing</th>
<th>Mice given placebo</th>
<th>Treated mice</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>n</td>
<td>weight*</td>
<td>SD</td>
<td>n</td>
</tr>
<tr>
<td>Ccm1+/Msh2-</td>
<td>Fasudil (weaning - 5 mo)</td>
<td>2 mo</td>
<td>7</td>
<td>22.9</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 mo</td>
<td>8</td>
<td>25.6</td>
<td>5.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 mo</td>
<td>12</td>
<td>27.1</td>
<td>6.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 mo</td>
<td>12</td>
<td>25.7</td>
<td>6.0</td>
</tr>
<tr>
<td>Ccm1+/Msh2-</td>
<td>simvastatin (weaning - 5 mo)</td>
<td>2 mo</td>
<td>11</td>
<td>19.7</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 mo</td>
<td>14</td>
<td>22.2</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 mo</td>
<td>16</td>
<td>25.2</td>
<td>5.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 mo</td>
<td>15</td>
<td>23.6</td>
<td>4.7</td>
</tr>
<tr>
<td>Ccm1+/Msh2-</td>
<td>Fasudil (3 mo - 4 mo)</td>
<td>2 mo</td>
<td>9</td>
<td>19.9</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 mo</td>
<td>9</td>
<td>22.8</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 mo</td>
<td>13</td>
<td>25.5</td>
<td>5.8</td>
</tr>
<tr>
<td>Ccm2+/Trp53-</td>
<td>Fasudil (weaning - 5 mo)</td>
<td>2 mo</td>
<td>17</td>
<td>25.3</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 mo</td>
<td>11</td>
<td>27.4</td>
<td>6.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 mo</td>
<td>15</td>
<td>28.8</td>
<td>6.5</td>
</tr>
<tr>
<td>Ccm2+/Trp53-</td>
<td>simvastatin (weaning - 5 mo)</td>
<td>2 mo</td>
<td>11</td>
<td>22.1</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 mo</td>
<td>11</td>
<td>24.6</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 mo</td>
<td>11</td>
<td>25.5</td>
<td>3.2</td>
</tr>
</tbody>
</table>

n indicates the number of animals; SD, standard deviation; mo, months
*mean weight in grams
Table II: Number of Mice Not Surviving the Complete Treatment with the Indicated Features of Attrition

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>( Ccm1^{+/+}/Msh2^{--} )</th>
<th>( Ccm2^{+/+}/Trp53^{--} )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Treatment groups</strong></td>
<td>Placebo vs. Fasudil</td>
<td>Placebo vs. simvastatin</td>
</tr>
<tr>
<td><strong>Duration of treatment</strong></td>
<td>Weaning to 4-5 months</td>
<td>Weaning to 4-5 months</td>
</tr>
<tr>
<td><strong>Total number of mice that started the placebo treatment</strong></td>
<td>25</td>
<td>27</td>
</tr>
<tr>
<td><strong>Total attrition (placebo)</strong></td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>Brain hemorrhage</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Systemic illness/tumor</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>No information/other</td>
<td>5*</td>
<td>3†</td>
</tr>
<tr>
<td><strong>Total number of mice that started the drug treatment</strong></td>
<td>25</td>
<td>42</td>
</tr>
<tr>
<td><strong>Total attrition (drug-treated)</strong></td>
<td>3</td>
<td>14</td>
</tr>
<tr>
<td>Brain hemorrhage</td>
<td>2</td>
<td>4†</td>
</tr>
<tr>
<td>Systemic illness/tumor</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>No information/other</td>
<td>1</td>
<td>7‡</td>
</tr>
</tbody>
</table>

*One/‡three mice with overgrowth of teeth (malocclusion).
†One mouse with hydrocephalus.
§One mouse with terminal brain pathology, including dilated cerebral vessels, possible CCM
Table III. Number of Lesions Per Animal in Both Sexes

<table>
<thead>
<tr>
<th>CCM Stage</th>
<th>Genotype</th>
<th>Sex</th>
<th>Placebo</th>
<th>Treated</th>
<th>p-value</th>
<th>Placebo</th>
<th>Treated</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>n</td>
<td>mean ± SEM</td>
<td>n</td>
<td>mean ± SEM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage 2</td>
<td>Ccm1⁺/⁻ Msh2⁻/⁻</td>
<td>Male</td>
<td>9</td>
<td>0.56 ± 0.24</td>
<td>9</td>
<td>0.11 ± 0.11</td>
<td>0.14</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Female</td>
<td>7</td>
<td>0.86 ± 0.34</td>
<td>13</td>
<td>0.23 ± 0.12</td>
<td>0.06</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Ccm2⁺/⁻ Trp53⁻/⁻</td>
<td>Male</td>
<td>14</td>
<td>0.93 ± 0.44</td>
<td>13</td>
<td>0.33 ± 0.18</td>
<td>*0.04</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Female</td>
<td>4</td>
<td>0.25 ± 0.25</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>2</td>
</tr>
</tbody>
</table>

All lesions (Stage 1&2)

<table>
<thead>
<tr>
<th>CCM Stage</th>
<th>Genotype</th>
<th>Sex</th>
<th>Placebo</th>
<th>Treated</th>
<th>p-value</th>
<th>Placebo</th>
<th>Treated</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ccm1⁺/⁻ Msh2⁻/⁻</td>
<td>Male</td>
<td>9</td>
<td>0.67 ± 0.33</td>
<td>9</td>
<td>0.22 ± 0.15</td>
<td>0.18</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Female</td>
<td>7</td>
<td>1.29 ± 1.38</td>
<td>13</td>
<td>1.15 ± 1.34</td>
<td>0.8</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Ccm2⁺/⁻ Trp53⁻/⁻</td>
<td>Male</td>
<td>14</td>
<td>1.43 ± 0.40</td>
<td>18</td>
<td>1.61 ± 0.41</td>
<td>0.68</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Female</td>
<td>4</td>
<td>0.50 ± 0.29</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>2</td>
</tr>
</tbody>
</table>

n indicates the number of animals; SEM, standard error of the mean; ND, not determined, since no females were included in the Fasudil group
*p<0.05
†from Wilcoxon rank sum test, since a p value cannot be calculated from a Poisson distribution assumption
Table IV. Integrated Density of Iron Per Stage 2 Lesional Area in Both Sexes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Sex</th>
<th>Placebo</th>
<th>Treated</th>
<th>p-value</th>
<th>Placebo</th>
<th>Treated</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>n</td>
<td>mean ± SEM</td>
<td>n</td>
<td>mean ± SEM</td>
<td>n</td>
<td>mean ± SEM</td>
</tr>
<tr>
<td>Ccm1+/−Msh2−/−</td>
<td>Male</td>
<td>5</td>
<td>12.7 ± 6.6</td>
<td>1</td>
<td>7.5 ± 0.0</td>
<td>†0.76</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>6</td>
<td>172.8 ± 109.5</td>
<td>3</td>
<td>0.005 ± 0.003</td>
<td>*0.02</td>
<td>3</td>
</tr>
<tr>
<td>Ccm2+/−Trp53−/−</td>
<td>Male</td>
<td>13</td>
<td>95.2 ± 40.2</td>
<td>6</td>
<td>79.5 ± 37.8</td>
<td>0.61</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>1</td>
<td>161.9 ± 0.0</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>5</td>
</tr>
</tbody>
</table>

n indicates the number of animals; SEM, standard error of the mean; ND, not determined, since no stage 2 lesions were found in female Ccm2+/−Trp53−/−mice for both treatment groups

* p<0.05

† from the general linear model, since a p value cannot be calculated from the Wilcoxon rank sum test
Supplemental Figures and Figure Legends

Treatment Groups in Ccm1+/− Msh2−/− models
(long term Fasudil, long term Simvastatin and late short term Fasudil)

↓ Wean and begin treatment (~20 days) ↓ End treatment (4-5 months)

\[\begin{array}{c}
\text{n=25} \\
\text{Fasudil (n=22)}
\end{array}\]  \[\begin{array}{c}
\text{n=25} \\
\text{placebo (n=16)}
\end{array}\]

↓ Wean and begin treatment (~20 days) ↓ End treatment (4-5 months)

\[\begin{array}{c}
\text{n=42} \\
\text{simvastatin (n=28)}
\end{array}\]  \[\begin{array}{c}
\text{n=12} \\
\text{placebo (n=20)}
\end{array}\]

↓ Wean (~20 days) ↓

\[\begin{array}{c}
\text{n=20} \\
\text{placebo (n=16)}
\end{array}\]  \[\begin{array}{c}
\text{n=18} \\
\text{Fasudil (n=17)}
\end{array}\]

Begin treatment (~3 months) ↑ ↑ End treatment (~4 months)

Treatment Groups in Ccm2+/− Trp53−/− models
(long term Fasudil and long term Simvastatin)

↓ Wean and begin treatment (~20 days) ↓ End treatment (4-5 months)

\[\begin{array}{c}
\text{n=24} \\
\text{Fasudil (n=18)}
\end{array}\]  \[\begin{array}{c}
\text{n=24} \\
\text{placebo (n=18)}
\end{array}\]

↓ Wean and begin treatment (~20 days) ↓ End treatment (4-5 months)

\[\begin{array}{c}
\text{n=12} \\
\text{simvastatin (n=12)}
\end{array}\]  \[\begin{array}{c}
\text{n=12} \\
\text{placebo (n=11)}
\end{array}\]

**Figure I.** Schema summarizing concurrent random assignments of mice with the two genotypes to placebo, Fasudil and simvastatin treatment groups. For the Ccm1+/− Msh2−/− long term simvastatin experiment, contemporaneously raised placebos from the Ccm1+/− Msh2−/− late, short term Fasudil group were added to balance the treatment arms.
Figure II. Stage 2 lesion area per animal in Ccm models with Fasudil and simvastatin treatment. The stage 2 lesion area in Ccm1+/−Msh2−/− models was decreased non-significantly in Fasudil-treated mice (n=22) compared to placebos (n=16), but not affected in simvastatin-treated mice (n=28) compared to placebos (n=20) (A) Similarly, the stage 2 area in Ccm2+/−Trp53−/− models was decreased non-significantly in Fasudil-treated mice (n=18) compared to placebos (n=18) but not affected in simvastatin-treated mice (n=12) compared to placebos (n=11). Horizontal bars are means (longer lines) and standard error of the mean (shorter lines). Meta analyses of combined effect in the two genotypes show a non-significant trend for reduced stage 2 lesional area per animal treated with Fasudil (C), but not with simvastatin (D) compared to placebo.
Figure III. Lesion burden in after short-term, late Fasudil treatment of $Ccm1^{+/+}Msh2^{-/}$ murine models. The number of stage 2 and total lesions were not affected by 1 month of Fausdil (n=17) treatment in mice between 3 and 4 months of age, when compared to placebos (n=16). Fasudil (n=7) significantly decreased the combined stage 1 and stage 2 CCM lesions in males when compared to placebos (n=9), with an opposite trend in females. A male-female imbalance of lesion burden in placebo mice is noted.
Figure IV. RhoA kinase (ROCK) activity in mature stage 2 lesions with Fasudil and simvastatin treatment. (A) The percentage of all lesional endothelial cells with ROCK activity measured by pMLC staining in multicavernous stage 2 lesions in Ccm1+/−Msh2−/− murine models is decreased by Fasudil treatment (*P=0.003) and by simvastatin treatment (**P=0.0007). The total number of endothelial cells counted, as well as the number of stage 2 lesions and the number of mice used for the cell counts, are indicated. Comparison of the prevalence of immunopositive cells when analyzed per lesion (B) and per mouse (C) are not significantly different.
Supplemental References
