Strategy for Identifying Repurposed Drugs for the Treatment of Cerebral Cavernous Malformation

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Background—Cerebral cavernous malformation (CCM) is a hemorrhagic stroke disease affecting up to 0.5% of North Americans that has no approved nonsurgical treatment. A subset of patients have a hereditary form of the disease due primarily to loss-of-function mutations in KRIT1, CCM2, or PDCD10. We sought to identify known drugs that could be repurposed to treat CCM.

Methods and Results—We developed an unbiased screening platform based on both cellular and animal models of loss of function of CCM2. Our discovery strategy consisted of 4 steps: an automated immunofluorescence and machine-learning–based primary screen of structural phenotypes in human endothelial cells deficient in CCM2, a secondary screen of functional changes in endothelial stability in these same cells, a rapid in vivo tertiary screen of dermal microvascular leak in mice lacking endothelial Ccm2, and finally a quaternary screen of CCM lesion burden in these same mice. We screened 2100 known drugs and bioactive compounds and identified 2 candidates, cholecalciferol (vitamin D₃) and tempol (a scavenger of superoxide), for further study. Each drug decreased lesion burden in a mouse model of CCM vascular disease by ≥50%.

Conclusions—By identifying known drugs as potential therapeutics for CCM, we have decreased the time, cost, and risk of bringing treatments to patients. Each drug also prompts additional exploration of biomarkers of CCM disease. We further suggest that the structure-function screening platform presented here may be adapted and scaled to facilitate drug discovery for diverse loss-of-function genetic vascular disease. (Circulation. 2015;131:289-299.)

Key Words: cerebrovascular disorders ◼ endothelium ◼ genetics ◼ hemorrhage ◼ stroke

There are at least 7000 diseases designated as rare in the United States.¹ With these diseases taken as a whole, at least 25 million Americans are affected by a rare disease.¹² Disease-causing mutations in a single gene account for as many as 5000 rare diseases, the largest subset.¹² The often arduous quest for proper diagnosis and treatment by highly specialized practitioners is costly to our healthcare system and for families faced with a rare disease. Furthermore, the high cost of drug development, combined with relatively small markets, exerts a negative effect on commercial interest in treatment of these diseases. The Orphan Drug Act of 1983 and its subsequent amendments have created regulatory and economic incentives for drug development in the rare-disease space, and there have been major successes both for pharmaceutical companies and for patients with rare diseases.³ However, >95% of rare diseases still have no approved treatment.¹

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An area of current excitement and challenge in the pharmaceutical industry is drug repurposing (sometimes referred to as 5000 rare diseases, the largest subset.¹² The often arduous quest for proper diagnosis and treatment by highly specialized practitioners is costly to our healthcare system and for families faced with a rare disease. Furthermore, the high cost of drug development, combined with relatively small markets, exerts a negative effect on commercial interest in treatment of these diseases. The Orphan Drug Act of 1983 and its subsequent amendments have created regulatory and economic incentives for drug development in the rare-disease space, and there have been major successes both for pharmaceutical companies and for patients with rare diseases.³ However, >95% of rare diseases still have no approved treatment.¹

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as drug rediscovery or drug repositioning) whereby existing drugs are used for additional or alternative indications from those for which they were originally designed or intended.4–6 The classic example of drug repurposing is that of sildenafil (Viagra), intended originally to treat angina but attaining “blockbuster” status for the treatment of erectile dysfunction.7 The vast majority of drug-repurposing successes can be attributed to serendipity or to a reasoned approach based on a deep understanding of a specific disease mechanism.8,9 Although these reasoned approaches often work for well-studied diseases, a comprehensive picture of the molecular mechanisms underlying many rare diseases is lacking. A scalable platform for drug repurposing across many rare diseases would thus have considerable impact.

Over the past decade, we have studied a monogenic loss-of-function stroke disease, cerebral cavernous malformation (CCM), in an attempt to learn more about the molecular mechanisms underlying vascular stability and with an emphasis on translational research. CCM is a hemorrhagic stroke syndrome characterized by vascular malformations in the central nervous system. CCM lesions are leaky and unstable, with chronic and acute bleeding leading to inflammation and stroke, respectively.10

The only treatment for CCM is neurosurgical resection,11 CCM occurs in 2 forms: sporadic and familial, which together affect as many as 1 in 200 to 500 individuals in the United States.12,13 The familial form of CCM accounts for 20% of cases and is most often associated with heterozygous loss-of-function mutations in 1 of 3 genes, KRIT1, CCM2, or PDCD10.14 Although a very limited number of missense mutations have been reported, >90% of the mutations identified in all CCM genes are null (loss-of-function) frameshift, deletion, or splicing mutations.15–27 A somatic second-hit loss-of-heterozygosity mechanism has been proposed, and limited experimental results from human samples are sofar consistent with that model.28–34 We have previously reported a reductionist, target-based, hypothesis-driven drug-repurposing strategy for CCM, and we show here results that caused us to re-evaluate that general approach. As a result, we developed an unbiased drug discovery platform for the identification of an effective therapeutic for CCM. Here, we report that this platform has identified 2 known drugs, cholesterol-lowering agents (statins), which inhibit RHOA activation through antagonistic activity on HMG-CoA reductase, might be repurposed to treat CCM disease.35–37 Because of that approach, we suggested that statins, which inhibit RHOA activation through antagonistic activity on HMG-CoA reductase, might be repurposed to treat CCM disease.38 Although missense mutations have been described in KRIT1 and CCM2 patients, the vast majority of patients with disease harbor frameshift, deletion, or splicing mutations in a single CCM allele.39–27 Even though the exact mechanism by which heterozygosity for a null allele dramatically increases predisposition to disease is incompletely understood, the limited evidence available in humans and from animal models supports a knudsonian second-hit loss-of-heterozygosity mechanism.14,28,29,31–34,38 Thus, we created mice that are compound heterozygous for a loss-of-function allele in Ccm2 with the cognate allele flanked by loxp sites permitting its postnatal, endothelium-specific deletion (PDGFb-iCreERT2).39 Strikingly, these mice develop CCM lesions that reproduce all of the pathological aspects of human CCM disease.40 To our disappointment, 4 months of simvastatin treatment in this model did not result in a decreased burden of disease, as measured by small-animal MRI (Figure I in the online-only Data Supplement). Potential explanations for this result are myriad, do not exclude a role of RHO activation in CCM pathophysiology, and certainly do not exclude the use of statins or other modulators of RHO activity as an effective treatment of CCM disease. However, the effect of this result was to prompt us to critically re-examine the reductionist, target-centric approach to drug identification we had used.

Methods

The complete, detailed methods are given in the online-only Data Supplement.

Primary Screen

Primary human adult dermal microvascular endothelial cells and cell culture reagents were from Lonza (Basel, Switzerland); small interfering RNA (siRNA), from Dharmacon Thermo Scientific (Waltham, MA); and the Spectrum Collection compound library, from MicroSource Discovery Systems (Gaylordsville, CT). Cells were subjected to 2 rounds of transfection (48 hours apart) with siRNA targeting CCM2 or a scrambled control and seeded into 96-well plates. After 72 hours, cells were fixed, stained with probes, and imaged with a high-throughput microscope. For screening, compounds were added to each at a final concentration of 10 μmol/L in 0.5% dimethyl sulfoxide for 24 hours.

Secondary Screen (Electric Cell Substrate Impedance Sensing)

After the second siRNA transfection, human adult dermal microvascular endothelial cells were seeded at 4×10⁴ cells per well onto a 96-well ECIS plate (96W10E+, Applied Biophysics). Plates were monitored by use of an ECIS Z0 system to measure resistance of an alternating current (4000 Hz) across the cell monolayer. Resistance was normalized for each well to just before treatment and was plotted in real time. Seventy-two hours after the second siRNA transfection, compounds were added to each well at a final concentration of 10 μmol/L in 0.5% dimethyl sulfoxide.

In Vivo Screens

The CCM2 mouse model, dermal permeability, and magnetic resonance imaging (MRI) analyses were performed as previously described. Endothelium-specific CCM2 knockout mice were fed standard chow, chow enhanced with vitamin D₃, or water enhanced with tempol for 5 months before MRI analysis. All mouse experiments were approved by the University of Utah Institutional Animal Care and Use Committee or the George E. Wahlen Department of Veterans Affairs Medical Center Institutional Animal Care and Use Committee.

Results

Motivation for a New Approach

We and others have identified dysregulation of a small GTPase, transforming protein RhoA (RHOA), in cells lacking either KRIT1 or CCM2 as a possible pathogenic mechanism of CCM disease. This identification was based on reasoned probing of the molecular mechanisms associated with the disease and a reductionist methodology to target identification.35–37 Because of that approach, we suggested that statins, which inhibit RHOA activation through antagonistic activity on HMG-CoA reductase, might be repurposed to treat CCM disease.38 Although missense mutations have been described in KRIT1 and CCM2 patients, the vast majority of patients with disease harbor frameshift, deletion, or splicing mutations in a single CCM allele.39–27 Even though the exact mechanism by which heterozygosity for a null allele dramatically increases predisposition to disease is incompletely understood, the limited evidence available in humans and from animal models supports a knudsonian second-hit loss-of-heterozygosity mechanism.14,28,29,31–34,38 Thus, we created mice that are compound heterozygous for a loss-of-function allele in Ccm2 with the cognate allele flanked by loxp sites permitting its postnatal, endothelium-specific deletion (PDGFb-iCreERT²).39 Strikingly, these mice develop CCM lesions that reproduce all of the pathological aspects of human CCM disease.40 To our disappointment, 4 months of simvastatin treatment in this model did not result in a decreased burden of disease, as measured by small-animal MRI (Figure I in the online-only Data Supplement). Potential explanations for this result are myriad, do not exclude a role of RHO activation in CCM pathophysiology, and certainly do not exclude the use of statins or other modulators of RHO activity as an effective treatment of CCM disease. However, the effect of this result was to prompt us to critically re-examine the reductionist, target-centric approach to drug identification we had used.
Development, Evaluation, and Execution of the Screen

Endothelial cells deficient in CCM2 have obvious structural and functional phenotypes. We hypothesized that we could use these phenotypes for unbiased drug discovery. Our strategy was to develop a multistage screen, taking advantage of in vitro structural phenotypes (primary screen), in vitro functional phenotypes (secondary screen), acute in vivo functional phenotypes (tertiary screen), and chronic in vivo disease phenotypes (quaternary screen; Figure 1). We chose fluorescent microscopy and automated cellular quantification and profiling as our primary screen, transendothelial resistance as our secondary screen, measurement of the leakiness of dermal vasculature in a CCM mouse model as our tertiary screen, and measurement of lesion burden with small-animal MRI as our quaternary and final screen. The specific assays were chosen on the basis of throughput and the quantity and quality of data provided. In general, each successive step in our platform exchanged decreased throughput and increased associated time and effort with increased predictive ability. Another important aspect of our strategy was to use a library of 2100 small molecules composed of known drugs and bioactive compounds on the basis of our hypothesis that hits from this library could more quickly be translated to the bedside.

Primary Imaging Screen

A primary imaging screen was chosen because of the richness of data available relative to the ease with which an academic laboratory can perform such medium- or high-throughput assays. Because the vast majority of patients with CCM disease have mutations hypothesized to result in loss of function or amounts of CCM protein, we chose to model disease in human cells using well-validated siRNA to knock down CCM2. Human dermal microvascular endothelial cells were treated with well-validated CCM2 mRNA-targeting siRNA or a scrambled control and then seeded into 96-well imaging plates (Figure 2A). Large immunofluorescence images composed of 16 adjacent fields of view stitched together automatically were captured from each well of a 96-well plate in 3 channels sufficient to give an impression of the cell structure including the nucleus, actin stress fibers, and VE-cadherin cell-cell junctions (Figure 2B). A high-throughput microscope developed for phenotypic drug discovery allowed automated imaging of an entire 96-well plate in ≈60 minutes. We used CellProfiler, an open-source, high-content imaging analysis tool developed and overseen by Dr Anne Carpenter of the Broad Institute, to import images, to identify the borders of each cell, and to create a database of a multitude of mathematical descriptors of every cell in every image collected (Figure 2C and Figure IIA and IIB in the online-only.

![Figure 1. Project workflow. An overview of the 4 different screening assays used to identify 2 promising compounds from among 2100 initial candidates. CCM indicates cerebral cavernous malformation; EBD, Evans blue dye; ECIS, Electric Cell Substrate Impedance Sensing; HMVEC-D, human dermal microvascular endothelial cells; and MRI, magnetic resonance imaging.](http://circ.ahajournals.org/attachment/2017-09-22-09.22.17-s1.png)
We then used CellProfiler Analyst, a machine-learning tool, to develop rules that could be used to distinguish whether each cell in an image was more likely to have been treated with scrambled control siRNA or siCCM2 (Table I in the online-only Data Supplement). The software was able to accurately categorize images (based on the proportion of individual cells in each image scored as siCTRL or siCCM2) as siCTRL treated or siCCM2 treated as calculated by a Z' of 0.7, a statistical test for evaluating assays for high-throughput screening for which any value between 0.5 and 1 is considered amenable to high-throughput screening (Figure 2D).

We then screened 2100 known drugs to identify those that could rescue the structural phenotype associated with loss of CCM2. We analyzed the resulting images to identify rescue using CellProfiler and CellProfiler Analyst and using qualitative scoring by 2 blinded reviewers as a comparison. The 2 reviewers who performed qualitative analysis identified 38 compounds in common that, when added to siCCM2-treated cells, resulted in what they perceived was rescue of structural phenotypes. We simultaneously used the CellProfiler software system to prioritize compounds, and we selected the top 38 compounds to provide a direct numeric comparison of the performance of qualitative analysis (38 compounds) and our automated analysis. Interestingly, there was no overlap between the compounds selected by human analysis and those selected by the automated computational scoring system.

### Secondary Transcellular Resistance Screen

To validate our hits and to prioritize future analysis, we developed a secondary orthogonal screen using transcellular resistance based on the functional defect in monolayer stability in cells deficient in CCM2 (Figure 3A). Transcellular resistance was chosen because of its relatively high throughput, its real-time nature, the quality and quantity of data generated, and its label-free, functional output, which is highly orthogonal to the primary imaging screen. This assay uses real-time measurements of the resistance encountered when an electric current is passed between electrodes on which a monolayer of cells are growing, and we used it to simultaneously screen the 2 sets of 38 compounds identified in the primary screen by our manual and automated image analysis. Of the 38 compounds selected by human analysis, only 1 compound (simvastatin) showed rescue of the defect in monolayer stability of cells treated with siCCM2 (Figure 3B and Figure IIIA in the online-only Data Supplement). However, of the 38 compounds identified with the CellProfiler automated machine-learning analysis, 7 showed full or partial rescue of the functional phenotype (Figure 3B). The 7 compounds selected from our automated analysis and validated by our secondary screen include...

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**Figure 2.** Primary screen: rescue of structural phenotypes associated with loss of CCM2. A, Western blot analysis of siCCM2 knockdown. B, Immunofluorescence images of endothelial cells treated with siCTRL or siCCM2 stained for DNA (blue), actin (green), and VE-cadherin (red). C, DNA (top) and VE-cadherin (bottom) raw images segmented into nuclei and cell objects, respectively. D, Result of scoring positive and negative control images using rules generated by machine-learning algorithms in CellProfiler Analyst software. Scale bars, 50 μm. CCM indicates cerebral cavernous malformation.

**Figure 3.** Secondary screen: rescue of functional phenotypes associated with loss of CCM2. A, Baseline transendothelial resistance of unperturbed siCTRL and siCCM2 monolayers (n=6 [siCTRL] and n=75 [siCCM2]). Graph depicts each value, the mean, and the SEM. **P<0.001 as evaluated by t test. B, When added to siCCM2-treated cell monolayers, 7 compounds identified with the use of automated analysis in the primary screen partially or fully rescued resistance measures after 24 hours of treatment. Each compound was tested individually with 2 replicates. A representative replicate is plotted. CCM indicates cerebral cavernous malformation; and DMSO, dimethyl sulfoxide.
compounds from classes previously connected to CCM disease and compounds without any previously described association with the disease (Table II and Figure IIIB–IIIH in the online-only Data Supplement).47–50

**Compound Prioritization in an Acute Animal Model of CCM**

Because of the time and cost associated with chronic treatment trials in our CCM mouse model, we next took advantage of a relatively high-throughput microvascular leak assay in our inducible endothelium-specific Ccm2 knockout mice (Ccm2f/f; +/Tg(Pdgfb-iCreER) as a tertiary screen to prioritize hits for chronic treatment models. When we injected small intradermal wheals of a subset of our hit compounds into these mice, we found that both tempol and cholecalciferol significantly reduced peri-injection microvascular leakiness (multiplicity-adjusted comparisons, \(P=0.04\) and \(P=0.04\), respectively; Figure 4A and 4B). We do not exclude the possibility that the other compounds could be relevant therapeutic candidates for the treatment of CCM disease, but this assay allowed us to immediately prioritize tempol and cholecalciferol for further study.

**Compound Validation in a Chronic Animal Model of CCM**

Next, we performed chronic treatment studies of the effects of tempol and cholecalciferol in our endothelium-specific Ccm2 knockout mice.40 These studies serve not just to evaluate the potential of these treatments for CCM disease but also as a proof of principle for the potential usefulness of our screening platform. Nursing mothers were treated with standard chow (1.5 IU/g cholecalciferol), an identical chow enhanced with cholecalciferol (25 IU/g), or standard chow plus tempol dissolved in drinking water (1 mmol/L) starting 5 days after delivery of their pups. After weaning, mice were fed the same diet as their mother until 5 months of age, a point at which 100% of untreated endothelium-specific Ccm2 knockout mice have cerebrovascular lesions detectable by MRI (Figure 5A). Because progression of human disease is followed up by MRI, mice were also evaluated for lesion status by MRI. Two reviewers with experience reading murine and human MRI were provided MRI files from all mice in the study but were totally blinded to treatment. The reviewers manually outlined each lesion in every MRI, and their results were tabulated. Mice receiving the diet enriched with cholecalciferol had a significant reduction in lesions compared with those receiving standard chow, whereas mice receiving tempol had a marginally significant reduction in lesions (\(P=0.052\); Figure 5B). When lesion size distributions were compared, cholecalciferol appeared to significantly reduce the number of small lesions, and tempol appeared to marginally reduce the number of small lesions, whereas for both treatments, there was a trend toward reduction of most lesion sizes (Figure 5C). The effect of cholecalciferol and tempol supplementation was qualitatively obvious in comparisons of MRI-based 3-dimensional reconstructions of mouse brains (the brain with the median number of lesions from each treatment group is shown; Figure 5D).

**Novel Treatments Inform CCM Pathophysiology**

A major advantage of screening a library of known drugs and bioactive factors is that a large body of research is generally available on the effects of any one of the compounds. Such was the case with both cholecalciferol and tempol. With knowledge of the various biological roles of cholecalciferol and other forms of vitamin D at hand, we assessed the timing of the effects of cholecalciferol on the endothelium. We evaluated the effect of knockdown of CCM2 and subsequent treatment with cholecalciferol on a panel of signaling pathways commonly associated with endothelial instability, including ADP-ribosylation factor 6 (ARF6), cell division control protein 42 homolog, RHOA, phosphorylation of myosin light chain, Ras-related C3 botulinum toxin substrate 1, or Ras-related protein R-Ras (Figure 6A–6C and Figure IVA–IVC in the online-only Data Supplement).51–57 Treatment of monolayers with cholecalciferol, even at a physiological dose 100 times lower than what was used in the screen (100 nmol/L), inhibited the CCM2 knockdown-induced activation of ARF6, RHOA, and phosphorylation of myosin light chain (Figure 6A–6C). Knockdown of CCM2 did not affect activation of cell division control protein 42 homolog, Ras-related C3 botulinum toxin substrate 1, or Ras-related protein R-Ras, nor did treatment of up to 10 μmol/L cholecalciferol basally inhibit activation of these markers (Figure IVA–IVC in the online-only Data Supplement). Because of a strong role of ARF6 as a central modulator of endothelial permeability, we further examined the timing of the effects of cholecalciferol...
on ARF6 activation and found inhibition to occur within 5 minutes (Figure 6D and 6E). Taken together, these data sug-
ggest that cholecalciferol, even at physiological doses, can rap-
idity and directly inhibit multiple key intracellular signaling
pathways that play a role in endothelial activation and stability
in the context of mutation-induced destabilization. These data
shed light on CCM and inform the study of cholecalciferol,
which has not previously been shown to have a direct and
immediate stabilizing effect on the endothelium.

Similarly, identification of tempol as a result of our use of
a library of known drugs and bioactive molecules enabled us
to immediately investigate the known target of tempol, super-
oxide.58 Indeed, a role of oxidative stress in the pathophysiol-
ogy of CCM disease resulting from 2 other causative genes,
KRIT1 and PDCD10, has been reported.48,59–62 In cell culture,
loss of CCM2 induced increased reactive oxygen species and
decreased FOXO1 expression, suggesting a potential com-
mon mechanism of CCM pathophysiology as has been pro-
posed for KRIT1 and PDCD10 (Figure 6F and 6G).48 These
results and previous work in the field led us to hypothesize
that oxidative stress may be a driving force in CCM at the
level of individual cells and may have broader effects on the
physiology of small vessels through effects on endothelial
vasodilation.63 To test this hypothesis, we isolated murine
middle cerebral arteries from our endothelium-specific CCM2
knockout mice and measured both endothelium-dependent
and endothelium-independent vasodilation using acetylchlo-
rine and sodium nitroprusside, respectively.64 We found that
endothelium-specific loss of CCM2 resulted in a significant
defect in endothelium-dependent vasodilation in our model,
which could be completely rescued by tempol (Figure 7A–
7D). Additionally, both systolic and diastolic blood pressures
trended toward an increase in the endothelial knockout mice
(Figure 7E–7G), consistent with constricted, nonresponsive
vessels. Interestingly, the heart rate of the endothelial knock-
outs trended lower than in control mice (Figure 7H). These
data suggest that oxidative stress may play an even more criti-
cal role in CCM disease than previously described because of
systemic effects.

Discussion
We have conceived and implemented a high-content screen
querying both structural and functional cellular phenotypes to
discover novel factors active in the CCM2 pathway. We com-
combined high-content imaging, machine-learning software, and
transcellular electric resistance measurements as efficient and
powerful tools for drug screening. We specifically focused on
known drugs and bioactive compounds because we believe
such drugs have a much higher chance of making a rapid
impact on patient outcomes. By focusing on such molecules,
our high-tech screening method, which combines advances in molecular biology, imaging, and computing, has led to a low-tech solution: the potential for a modulatory role of 2 known drugs on CCM disease. Chief among these is cholecalciferol, which is inexpensive and widely available in supplement form, is freely available outdoors to those with exposed skin, and has a wide safety margin.

One of the most exciting aspects of these data was that the drug candidates chosen with automated software analysis outperformed those chosen by human analysis in our secondary screen. We hypothesize that this is due to a well-described human shortcoming called inattentional blindness in which humans often fail to notice an unexpected stimulus when other attention-demanding tasks are being performed. The actin stress fiber phenotype, which was previously quantified and published by our laboratory and known to both qualitative reviewers, may have overwhelmed or obscured more subtle or unexpected aspects of the phenotype that may have as much or more biological relevance to the disease. Quantitative software analysis, however, is not unduly weighted by the stress fibers, so rescue of many subtle changes becomes important.

We believe it is likely that well-tuned automated analysis will have the most significant gains compared with reviewers who are highly informed or educated, because they are likely to be most susceptible to the preconceived notions and inattentive blindness. This is a major benefit of the automated software analysis, as demonstrated by its considerable success compared with the human analysis in this screen. We would be interested in whether other future high-content screening studies that directly compare human and automated analysis support our findings.

Also of particular excitement to us are recent advances by other groups who have expanded our understanding of the pathophysiology of CCM disease and who have, in some cases, put forth potential treatments for the disease. Recent evidence suggests that endothelial-to-mesenchymal transition plays a critical role in the pathophysiology of CCM disease. Through inhibition of transforming growth factor-β, a key driver of endothelial-to-mesenchymal transition, reduction in severity of disease in a mouse model of CCM disease was identified. There is evidence that the target of tempol, oxidative stress, drives and is driven by increased transforming growth factor-β signaling and endothelial-to-mesenchymal transition, offering a potential mechanistic link. Additionally, metabolites of cholecalciferol have been shown to strongly inhibit transforming growth factor-β signaling and epithelial-to-mesenchymal transition. Our laboratory and others have also proposed a critical role for RHOA activation in CCM disease, which has served as the basis of a recently initiated phase zero clinical trial of the effects of simvastatin in patients with familial CCM caused by mutations in KRIT1. Although our attempt at RHOA inhibition in our murine CCM model with simvastatin failed, we await with excitement the results of a clinical trial currently underway and note that others have had success using fasudil, a downstream inhibitor of RHOA activation. We have also shown here that RHOA activation in combination with ARF6 and phosphorylation of myosin light chain activation are inhibited by cholecalciferol. Taken
Figure 7. Cerebral cavernous malformation (CCM) mice display endothelium-dependent vascular dysfunction. A, Middle cerebral arteries isolated from endothelium-specific CCM2 knockout mice (ecKO) subjected to acetylcholine-induced vasodilation. B, Sodium nitroprusside treatment (a nitric oxide [NO] donor) of middle cerebral arteries of both mouse genotypes demonstrates the normal function of the smooth muscle in both wild-type (WT) and knockout mice. C, L-NAME, nitroarginine methyl ester (LNAME), a specific inhibitor of endothelial NO synthase, eliminates the ability of endothelial cells to produce NO. D, Tempol completely rescues acetylcholine-induced vasodilation in ecKO mice. For A through D, n = 9 to 19. Graphs depict mean and SEM. Two-way ANOVA, with 1 factor being genotype and the second factor being acetylcholine dose as a repeated-measures factor, and the Sidak multiple-comparison test were used to evaluate significance. E, Systolic blood pressure (P = 0.093), F) diastolic blood pressure (P = 0.051), G) and mean blood pressure (P = 0.032) trended toward an increase in ecKO mice, consistent with decreased vasodilatory function. H, Heart rate trended toward a reduction (P = 0.051) in the ecKO mice. For E through H, n = 5 to 10. Graphs depict each value, median, and interquartile range. Wilcoxon Mann-Whitney exact analysis was used to evaluate significance.

together, these studies suggest that both compounds identified in an unbiased manner in our screen could intersect in unique combinations with pathways previously shown to be critical for CCM pathogenesis. The accessibility of cholecalciferol at trivial cost to patients and the various clinical trials underway in the United States for tempol in a variety of conditions suggest that 1 or both of these drugs could be rapidly applied to the treatment of CCM disease. No study has yet shown a complete inhibition of CCM lesion formation in mice with any treatment, and our work, along with the work of others, opens the possibility that multiple treatments could be used synergistically to treat patients with CCM vascular disease. Moreover, identification of these new treatments provides an avenue toward discovery of additional molecular mechanisms relevant to CCM disease.

Future work will determine whether our findings, obtained in both human endothelial cells and a mouse model of CCM disease, will translate to humans with CCM disease. Our findings underline major priorities on which the field must agree. Specifically, success of any clinical trial, especially in rare-disease research, hinges on well-studied natural history and clinically meaningful end points. The long-term sustainability of treatment often also relies on the establishment of relevant biomarkers. In advance of, or in conjunction with, human clinical trials for cholecalciferol or tempol treatment, our work strongly supports immediate amendment of research protocols on human patients with CCM to include measurements of both 25(OH)D₃ and an agreeable marker of oxidative stress. There has even been anecdotal evidence among CCM patients and clinicians that CCM-related symptoms increase during the winter months, which would be consistent with seasonal variation in vitamin D or the increased oxidative stress associated with inflammation resulting from typical winter infections such as influenza. We must also develop widely acceptable standards for tracking the natural history of CCM, including specific imaging modalities, lesion evaluation, and symptom assessment. We must use these standards as a platform by which to perform both observational and interventional studies of CCM disease and any proposed treatment, including simvastatin, fasudil, tempol, cholecalciferol, and others. As with any rare condition, the probability of obtaining meaningful results for patients is exponentially enhanced by agreement and adherence of the field to specific protocols so that data can be aggregated across clinical sites.

Our success in identifying candidates for future study in CCM disease was based not only on an unbiased screen centered on structural and functional phenotypes but also on the generation and characterization of animal models that faithfully recapitulate the genotype and phenotype most often found in the human disease. The adaptation of clinical imaging technology used in the human disease for these mouse models allowed us to evaluate lesions in much the same way as would be done in a clinical setting. The success of our approach has led us to wonder whether we could apply this same methodology to other vascular diseases for which a
genetic basis is known such as supravalvular aortic stenosis or hereditary hemorrhagic telangiectasia or even for a broader set of all genetic loss-of-function diseases.72,73 Other analyses of structural changes in a whole-genome screen using imaging and analysis methods similar to those we have reported here found that at least 10% of all gene knockdowns resulted in a quantifiable structural phenotype.74 Taken together, the aforementioned whole-genome study and our drug-repurposing study highlight the possibility that there may be a substantial number of phenotypes associated with human disease-related genes that are amenable to chemical suppressor screening in a systematic and automated manner. Thus, there may be additional opportunity for the rapid and efficient identification of candidate compounds for the treatment of other human genetic diseases.

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Disclosures
The authors declare competing financial interests. The University of Utah has licensed “know how” related to this article to Recursion Pharmaceuticals, a biotechnology company owned in part by the University of Utah. Utah has licensed “know how” related to this article to Recursion Pharmaceuticals. Drs Gibson, Thomas, and Li have real or optional ownership interest in Recursion Pharmaceuticals. The co-founders of Recursion Pharmaceuticals. Drs Gibson, Thomas, and Li have real or optional ownership interest in Recursion Pharmaceuticals. The other authors report no conflicts.

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analysis software.


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**CLINICAL PERSPECTIVE**

Cerebral cavernous malformation is a hereditary stroke syndrome affecting as many as 1 in 200 to 500 Americans. The majority of patients have a sporadic form of the disease with a single vascular malformation in the central nervous system. However, ≈20% of patients have a familial form of the disease in which multiple, sometimes even thousands, of malformations occur in the central nervous system. The only treatment of this disease is surgical resection, which has risks and is impractical for many patients with familial disease. Furthermore, multiplicity of lesions has been shown to be associated with clinically significant events, including focal neurological deficit and intracerebral hemorrhage. We used an innovative combination of experimental biology and bioinformatics studies in both human cells and animal models of familial cerebral cavernous malformation disease to identify 2 potential medical treatments for the disease. We focused our efforts on known drugs and bioactive compounds in an effort to accelerate transition to the clinic. We found that both vitamin D₃ and a strong superoxide scavenger, tempol, mitigated important phenotypes associated with the disease in both cellular and animal models. Future work will determine whether our findings, obtained in both human endothelial cells and a mouse model of cerebral cavernous malformation disease, will translate to humans with cerebral cavernous malformation disease. Our work illustrates a roadmap for repurposing known drugs for the treatment of loss-of-function genetic diseases.
Strategies for Identifying Repurposed Drugs for the Treatment of Cerebral Cavernous Malformation

Christopher C. Gibson, Weiquan Zhu, Chadwick T. Davis, Jay A. Bowman-Kirigin, Aubrey C. Chan, Jing Ling, Ashley E. Walker, Luca Goitre, Simona Delle Monache, Saverio Francesco Retta, Yan-Ting E. Shiu, Allie H. Grossmann, Kirk R. Thomas, Anthony J. Donato, Lisa A. Lesniewski, Kevin J. Whitehead and Dean Y. Li

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Supplemental Methods:

Reagents.

Primary human adult dermal microvascular endothelial cells (HMVEC-D) and cell culture reagents were obtained from Lonza (Basel, Switzerland). Small interfering RNA (siRNA) were obtained from Dharmacon Thermo Scientific (Waltham, MA). The Spectrum Collection compound library was obtained from MicroSource Discovery Systems (Gaylordsville, CT), via the University of Utah Drug Screening Resource. All remaining reagents were obtained from Tocris Biosciences (Bristol, U.K.) unless otherwise stated.

Cell culture.

Four individual lots of Adult Dermal Human Microvascular Endothelial Cells (HMVEC-D) were pooled at passage 0 and expanded according to manufacturer’s instructions in EBM-2 media (Lonza) supplemented with EGM-2 MV Bulletkits (Lonza). After two expansions, cells were frozen in identical lots at 1x10^6 cells/mL for use in all screening experiments. All in vitro experiments were completed using endothelial cells between passage 3 and 6. The continuous human endothelial cell line EA-hy926 (Fig. 6F,G) was maintained according to the protocol provided by the distributor (ATCC cat. CRL 2922, Manassas, Virginia, USA). Specifically, cells were cultured in DMEM medium with 25 mM glucose (HG) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 2% HAT supplement.

Endothelial transfection.

A well validated ON-TARGETplus siRNA targeting CCM2 (5’ GGAAUUGUCUCGCCAUUUUA 3’) and a scrambled control was obtained from Dharmacon.
Endothelial cell transfection with siRNA was carried out as previously described\(^7\). Briefly, cells were passaged into 25nM siRNA with 1:166 HiPerFect reagent (Qiagen) in 4:1 EGM-2 MV to EBM-2 respectively and plated. After an overnight incubation in the transfection mix, cells were washed and fed with EGM-2 MV. After an additional 48 hours, the transfection process was repeated to achieve more complete knockdown. During the second transfection, cells were seeded into assay plates as described below. Cells were again fed with EGM-2 MV after overnight incubation with the transfection mix. After an additional 48-72 hours, cells were subjected to experimental conditions.

*EA-hy926 transfection.*

EA-hy926 endothelial cells (1x10\(^6\)) were plated in 10 cm culture dishes in 8 ml antibiotic-free standard growth medium supplemented with FBS. Cells were grown to 60% confluence and then transfected for 5 h at 37°C with either CCM2, CCM3 or control siRNAs (final concentration: 100 nmol/L). Specifically, silencing experiments were performed using CCM2 and CCM3 siRNAs from Dharmacon (Dharmacon ON Target Plus J-014728-05-0050 and J-004436-05-0050, respectively). Cell transfections were performed using INTERFERin kit (Polyplus transfection, New York, NY, USA) according to the manufacturer’s protocol. Cells were cultured with siRNAs for 24 h before treatments and analysis.

*Immunofluorescence imaging.*

96-well imaging plates (BD Falcon) were used for immunofluorescent imaging screening. Cells were fixed with 4% formaldehyde in PBS for 10 minutes, washed 3x in ultrasaline (Lonza), then
incubated overnight with mouse anti-human CD144 antibody (BD Biosciences). Cells were washed 3x with ultrasaline, then a mixture of Hoechst 33342, Alexa-Fluor 488 Phalloidin (Invitrogen), and Alexa-fluor 594 goat anti-mouse antibody in ultrasaline was added for 6 hours. After an additional 3 washes, 200 µl ultrasaline was added to each well, and then the plates were covered with adhesive foil and immediately imaged. 96-well imaging plates were imaged using a high-throughput BD Pathway 855 Bioimager. A custom macro was written using the Attovision software (BD) encompassing imaging autofocus at each well, followed by acquisition of each channel. Each image consisted of a 4x4 montage using a 40x objective, covering a total area of 810 x 600 µm in each well. Images were automatically saved in files appended with plate and well name meta-data.

Drug treatment.

Compounds were obtained in 100% DMSO at 2 mM concentration in 96-well polypropylene assay plates from the University of Utah Drug Screening Resource Core and kept frozen at -20°C. Libraries acquired included the Microsource Spectrum Library of exactly 2000 known drugs and bioactive compounds as well as 100 additional known drugs or bioactive compounds available to us in our own or a collaborator’s laboratory. At the time of treatment, drugs were added to 96-well imaging plates containing confluent siCCM2 treated HMVEC-D endothelial monolayers using a 12-channel electronic pipette (Eppendorf Xplorer) to a final concentration of 10µM, 0.5% DMSO (vehicle) in starvation media (EBM-2 + 0.2% bovine serum albumin) and gently mixed. Positive (siCCM2) and negative (siCTRL) control wells received DMSO to 0.5% in starvation media without any compound treatment. Follow-up experiments were performed using the highest available grade of reagent from Tocris or Sigma.
Automated image analysis.

Images were analyzed using CellProfiler and CellProfiler Analyst\textsuperscript{35-37}. A custom CellProfiler pipeline imported all images, applied a light correction filter, and down-sampled the size of the images by a factor of 4 to reduce processing time. Loss of authenticity of each image was guaranteed by extracting meta-data from all original files including both plate and well names, and connecting this data digitally to the MySQL output database created by CellProfiler. The Hoechst channel was first utilized to identify nuclei using an Otsu Global thresholding method. The VE-cadherin channel was utilized next to identify cell-cell borders again using a Global thresholding method minimizing entropy. A threshold correction factor of 0.7 and a regularization factor of 0.001 were determined based on trial and error to give the best cell segmentation. Cells not completely enclosed within the image were automatically excluded from analysis. Several hundred parameters were calculated and recorded for each cell including co-localization of each channel, radial localization of actin and VE-cadherin, texture of actin and VE-cadherin, and nuclear and cell shape and size characteristics. The hundreds of quantifications calculated for each cell in every image were stored in a database accessible by CellProfiler Analyst. CellProfiler Analyst was subsequently used to open 100 control images (siCTRL or siCCM2 treated). Using a random cell selection tool available in CellProfiler Analyst software, approximately 20 cells were selected from each control image and added to categorization bins if appearing qualitatively to be representative of the typical positive or negative control cells. Cells that appeared to be dead or dying (characterized by a small, rounded cell not adhering to its neighbors), for example, were not included into sorting bins. Subsequently, rules were automatically developed using the GentleBoosting machine-learning
algorithm applied to regression stumps (as built into the CellProfiler Analyst software). The rules generated were then used to classify images of cells from additional control images, and the author manually corrected any obvious errors in classification (e.g. a prototypic siCCM2 treated cell in a positive control image being classified as siCTRL-treated). This process was repeated until approximately 2,000 cells comprised each bin; positive (siCCM2) and negative (siCTRL). The rules generated were not further tuned by hand. Previously unused control images (siCTRL or siCCM2 treated), as well as images of siCCM2 treated wells treated with individual drugs, were then evaluated using the rules. To directly compare the capability of qualitative human analysis, in which 38 compounds were identified, and automated machine-analysis, we selected the top 38 compounds identified using CellProfiler Analyst for further study without a specific statistical cut-off.

**Qualitative image analysis**

Two reviewers familiar with immunofluorescence imaging were shown dozens of images of labeled positive and negative controls (siCCM2- and siCTRL-treated, respectively), and were asked to ‘learn’ the phenotype. When each reviewer felt comfortable with identifying the positive and negative controls (with no more specific instructions given about what aspect of the phenotype to focus on), they were then asked to look at each image (blinded to treatment) from all 2,100 compound treatments for both independent replicates of the treatment (4,200 total images). They were instructed to mark any image in which the phenotype associated with siCCM2 treatment seemed to revert back (even partially) to siCTRL (or ‘healthy’). No rating or ranking was given – reviewers were asked to identify any image which appeared ‘improved’. Those images which were identified by both reviewers in both replicates as ‘improved’, were
then noted as primary hits and prioritized for subsequent follow-up in secondary assays (38 compounds).

*Electric cell substrate impedance sensing (ECIS)*.

A 96-well ECIS plate (96W10E+, Applied Biophysics) was used. At the second siRNA transfection, HMVEC-D were seeded at 4x10^4 cell/well. Plates were monitored using an ECIS Zθ system (Applied Biophysics) to measure resistance of an alternating current (4000 Hz) across the cell monolayer. Resistance was normalized for each well to just before treatment, and was plotted in real time. Cells were fed according to the same schedule as in the transfection protocol. 72 hours after the second siRNA transfection, compounds were added to each well at a final concentration of 10 µM in 0.5% DMSO.

*Mouse strains.*

Mice used have been previously reported\(^38,39\). Tamoxifen (Sigma-Aldrich) was re-suspended in corn oil (Sigma-Aldrich) to a concentration of 1 mg/ml, and 40 µl was injected into the peritoneum of pups on perinatal day 1.

*Dermal permeability.*

We performed a modification of a previously described Miles Assay\(^40\). Male mice between 2 and 4 months of age were anesthetized with isoflurane and their backs gently shaved and depilated using a hair-removal cream (Nair). The mice were then gently washed with warm water and allowed to recover. The following day, the mice were again anesthetized with isoflurane and 20 µL intradermal injections of treatments (50 µM in 0.5% DMSO in sterile
saline) in various positions on the back (the drugs were added to syringes by a person not performing the assay and labeled “a”, “b”, etc. therefore blinding the primary experimenter. 90 minutes later, Evans blue dye (100 µL of 1% dye in sterile saline, Sigma) was injected into the tail vein. After 30 minutes, mice were sacrificed and equal-sized skin biopsies from each injection site were collected and placed in formamide (Invitrogen) overnight at 60˚C. Absorbance at 620 nm (measurement of blue dye) and 740 nm (to correct for any blood in the sample) was used to quantify the amount of Evans blue dye in each sample (reading at 620 nm – reading at 740 nm) and the results tabulated. The absorbance is reported with the standard error of the mean.

_Murine CCM lesion formation and MRI analysis._

5 days after birth (P5), litters were assigned to a standard chow (Harlan 2018, 1.5 IU/g D_3_), a standard chow plus tempol in drinking water (1mM), or a cholecalciferol-enhanced chow (Harlan 2018 + 25 IU/g D_3_). The chow was provided to the mother of each litter until the mice were weaned at P21. Mice from each litter continued on their respective diets until 5 months of age. At 5 months of age, mice were sacrificed by exsanguination (blood was collected for later analysis), and subsequent perfusion with saline and then 4% formaldehyde. Brains were dissected from the skull, and postmortem MRI scanning was performed. A gradient recalled echo sequence was used to acquire coronal slices spanning the whole brain. Sequence parameters were as follows: repetition time, 328 ms; echo time, 5.4 ms; flip-angle, 40°; 12 averages, in-plane-resolution, 125 µm × 125 µm; and slice thickness, 0.5 mm. For a representative subset of brains (for use in 3D reconstructions), high-resolution 3D gradient echo was acquired using the following parameters: isotropic voxel size of 78 µm × 78 µm × 78 µm over 9 hours. Other
sequence parameters were as follows: repetition time, 250 ms; echo time, 7.5 ms; flip angle, 30°; and 2 averages. Lesion area and number were quantified by multiple blind reviewers using ImageJ and Osirix software. Specifically, each reviewer was provided with all MRIs which had been relabeled randomly. Each reviewer then used software to circle all ‘lesions’ of any size in every slice of every MRI from all mice. Contiguous lesions were outlined as one large lesion. The results of all reviewers were tabulated. 3D reconstructions were assembled using Osirix software by a blinded reviewer. For murine simvastatin treatment analysis (Supplementary Figure 1), mice were fed a standard diet (Harlan 8640) or a simvastatin-enhanced diet starting at weaning (Harlan 8640, 311 mg/kg simvastatin). Lesions were imaged in live mice on a monthly basis (using the same parameters for non high-resolution MRI as described above). Images from mice at 4.5 or 5 months were scored by blinded reviewers as described as above.

Mouse experiments.

All mouse experiments were approved by the University of Utah Institutional Animal Care and Use Committee or the George E. Wahlen Department of Veterans Affairs Medical Center Institutional Animal Care and Use Committee.

Middle cerebral artery endothelial function.

Endothelial function assays are based upon previously published methods[41]. Middle cerebral artery endothelial function was performed as previously described. Mice (N=7-14/group) were euthanized via exsanguinations by cardiac puncture while under isoflurane anesthesia. Right and left middle cerebral arteries approximately 100-120 micrometers in luminal diameter were excised from the brain and placed in myograph chambers (DMT A/S, Aarhus, Denmark)
containing EDTA-buffered physiological saline solution (PSS), cannulated onto glass micropipettes and secured with nylon (11–0) suture. Once cannulated, the middle cerebral arteries were warmed to 37°C and pressurized to 60 mmHg intraluminal pressure and allowed to equilibrate for 1 h. All arteries were then submaximally preconstricted with phenylephrine (2 μM). Increases in luminal diameter in response to increasing concentrations of the endothelium-dependent dilator acetylcholine (Ach; 1×10−9 to 1×10−4 M) in the absence or presence of the nitric oxide synthase inhibitor, L-NAME (0.1 mmol/L, 30 min) or the superoxide scavenger, TEMPOL (1 mmol/L, 60 min) was assessed. Endothelium-independent dilation to sodium nitroprusside (SNP: 1x10-10 to 1x10-4 mol/L) also was determined (5). Arterial segments were imaged and diameters measured by an automated edge detection; VAS software (DMT A/S, Aarhus, Denmark). All dose response data are presented as a percent of possible dilation after phenylephrine preconstriction.

Non-invasive blood pressure measurements in conscious mice.

Murine blood pressures were non-invasively measured by determining the tail blood volume with a volume pressure recording (VPR) sensor and an occlusion tail-cuff (CODA System, Kent Scientific, Torrington, CT). Blood pressure and heart rate recordings were made in a quiet and warm (24 C) environment. Mice were placed in restrainers on a heating unit and given 15-20 minutes to acclimate and reach a steady body temperature (30-35 C). Each session consisted of 5-10 acclimatization measurements; if those measures were stable, they were followed by 20 experimental measures. Measures with aberrant movement/behavior or inadequate volume pressure measures were treated as outliers. Averages from each session were used for systolic
blood pressure, diastolic blood pressure, mean blood pressure and heart rate for each individual mouse.

**Biochemical assays.**

Rabbit polyclonal antibody against CCM2 was from Sigma (Rabbit HPA020273). α-actinin antibody (clone H-2) was from Santa Cruz Biotechnology. HRP-conjugated secondary antibodies were from Jackson Immunoresearch. Western blots were developed using the ECL Plus Western blotting reagent (GE Healthcare) and Kodak Biomax MR film. Wild-type, siCTRL or siCCM2 treated HMVEC-D cells were incubated with either 100 nM or 10μM cholecalciferol (Tocris), 7-DHC (Sigma) or vehicle (0.5% DMSO) for 60 min (pMLC, ARF6, RAC, CDC42, RRAS) or 24 hours (RHOA), unless otherwise indicated. After treatment, the cells were washed with ice-cold PBS and lysed in 50mM Tris pH 7.4, 150mM NaCl, 10mM MgCl2, 10% Glycerol, 1% NP-40, 1X protease inhibitors, and 1X phosphatase inhibitors. For RhoA, ARF6, Rac1/cdc42 and R-Ras activation assays, crude total cell lysate were generated and GTP-RhoA, ARF6, Rac1/cdc42 and R-Ras were precipitated with Rhotekin-RBD (Millipore), GGA3-PBD (Cell Biolabs), PAK-1-PBD (Millipore) and Raf-1 RBD respectively. Following three washes with lysis buffer, bound proteins were eluted with 2X sample buffer. RhoA, ARF6, Rac1/cdc42 and R-Ras was detected by western blotting with antibodies (RhoA, Rac1 and R-Ras antibody are from Cell Signaling, ARF6 and cad42 antibody are from Millipore). Each blot is representative of at least three independent experiments, for which quantification is shown.

**Fluorimetric intracellular ROS detection.**
(Fig. 4f) Briefly, EA-hy926 cells grown to confluence in complete medium were washed twice with PBS, incubated with 2',7'-dichlorfluorescein-diacetate (DCFH-DA) at a final concentration of 5µM in PBS at 37°C for 30 min and analysed by Tali image based cytometer (Invitrogen). Raw data were elaborated by Flowing software (v. 2.5.0, by Perttu Terho, University of Turku, Finland).

Statistics.

Data presented are mean ± SEM unless otherwise indicated. Statistical significance is indicated generally by a single symbol (i.e. *) to indicate P < 0.05, two symbols (i.e. **) to indicate P < 0.01, three symbols (i.e. ***) to indicate P < 0.001. P<0.01 was considered marginal significance.

Figure 3A: Individual data, mean and SEM are shown for N = 6-75 samples per group. A Student’s t-test was used to evaluate significance.

Figure 4B: Individual data and mean are shown for N = 4-12 samples per group. For dermal permeability analysis, where we screened four compounds to determine which, if any, could inhibit vascular leak in endothelial specific knockout mice, we performed a mixed effects linear model with Absorption (OD) as the outcome and a single predictor variable (drug treatment), with the Vehicle group as the reference category. The mixed model included a random effect for the mice. The wild-type control mice (open circles) were shown descriptively for interested readers but not included in the statistical analysis. This provided four comparisons to the control. The mixed effects model accounted for clustering due to testing multiple treatments within the same mouse. Given that this was a screening experiment, for multiplicity adjustment we used the Benjamini-Hochberg procedure.
**Figure 5B:** Individual data, mean and SEM are shown for N = 8-12 samples per group. For chronic treatment in mice, as evaluated by MRI, we compared the number of lesions as shown in the dot plot using a one-way ANOVA with Dunnett’s multiple comparison test. The data were sufficiently normally distributed to allow for parametric analysis with the given sample sizes.

**Figure 5C:** The number of lesions within each mouse was subdivided into successive intervals reflecting 2-fold increases (starting with the minimum resolution of the MRI analysis) to define a sequence of bins defined on the horizontal axis of the figure. For each of the resulting bins, the number of lesions within the designated size range was computed for each mouse, and compared between diet groups using separate exact Wilcoxon Mann-Whitney tests to account for non-normality and differences in the amount of variability for the numbers of lesions between different bins. P-values were adjusted to account for 2 comparisons within each bin. Medians are shown for N = 8-12 samples per group.

**Figures 6A-C:** Individual data, mean and SEM are shown for N = 3 samples per group. For evaluation of the effect of D3 on activation of various biochemical factors, ordinary one-way ANOVA was utilized with Dunnett’s multiple comparison analysis.

**Figure 6E:** Individual data, mean and SEM are shown for N = 5-8 samples per group. The effect of either D3 or 7DHC were evaluated on the timing of inhibition of the activation of ARF6 in cells deficient in CCM2. Control RNAi-treated cells with 7-DHC are shown as a control at t=1 and t=60 minutes. Multiple t-tests were used to evaluate significance with the Sidak-Bonferroni method of multiple comparison.

**Figures 7A and D:** Individual data, mean and SEM are shown for N = 9-19 samples per group. For evaluation of vasodilation, we utilized a two-way ANOVA with one factor being genotype.
and the second factor being acetylcholine dose as a repeated measures factor with Sidak’s multiple comparison test.

Figures 7E-H: Individual data, medians and interquartile ranges are shown for \( N = 5-10 \) samples per group. The exact Wilcoxon Mann-Whitney test was used due to the presence of apparent outliers.
Supplemental Figures

Fig. S1: Simvastatin treatment has no effect on lesion burden in a murine model of CCM.

Mice were treated with a diet enhanced with simvastatin for 4 months. At 5 months of age, the lesions were quantified by blinded reviewers using MRI. These data were normalized due to variations in the analysis software used by the reviewers.
Fig. S2: Software analysis of immunofluorescence images. Calculations are made for each cell in each image for a wide variety of mathematical descriptors including, for example, (A) the radial distribution of VE-cadherin, and (B) the number of neighbors and percent of cell borders touching other cells.
Fig. S3: Compounds of interest after primary and secondary screens. Immunofluorescence images of siCCM2-treated endothelial cells stained for DNA (blue), actin (green), and VE-cadherin (red) after treatment with (A) simvastatin, (B) pindolol, (C) apomorphine hydrochloride, (D) aloin, (E) cholecalciferol, (F) dimercaprol, (G) gedunin, or (H) tempol. Scale bars represent 50 µm.
Cholecalciferol has no effect on the activation of certain destabilizing pathways in the endothelium. 60 minute treatment with 10 μM cholecalciferol had no effect on activation of (A) RAC1, (B) CDC-42, or (C) R-RAS in endothelial cells. All data, mean and SEM are plotted and significance was evaluated using ordinary one-way ANOVA was utilized with Dunnett’s multiple comparison analysis.
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Supplementary Table 1. Rules generated by Machine-Learning Analysis.
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</tr>
<tr>
<td>Cholecalciferol (vitamin D3)</td>
<td></td>
<td><img src="image3" alt="Cholecalciferol Structure" /></td>
<td></td>
</tr>
<tr>
<td>Dimercaprol</td>
<td>Chelating agent</td>
<td><img src="image4" alt="Dimercaprol Structure" /></td>
<td></td>
</tr>
<tr>
<td>Gedunin</td>
<td>Inhibitor of Heat Shock Protein 90</td>
<td><img src="image5" alt="Gedunin Structure" /></td>
<td>HSP90 selectively binds CCM2&lt;sup&gt;47&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pindolol</td>
<td>Non-selective beta-adrenergic receptor antagonist</td>
<td><img src="image6" alt="Pindolol Structure" /></td>
<td>Role for beta blockers in clinical improvement of CCMs and other vascular malformations&lt;sup&gt;50&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tempol</td>
<td>Superoxide scavenger</td>
<td><img src="image7" alt="Tempol Structure" /></td>
<td>Krit1 (CCM1) and PDCD10 (CCM3) linked to oxidative stress&lt;sup&gt;48, 49, 60-62&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

**Supplementary Table 2. Compounds of interest after primary and secondary screens.**