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Peer Review File

Capillary-selective vasodilation via myosin inhibition halts ischemic cascades

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Any redactions in this file are there to maintain patient confidentiality, the confidentiality of unpublished data, or to remove third-party material.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)
This is an exciting manuscript that reports a novel approach to restoring blood flow to organs that have been made ischemic, as occurs in heart attack, stroke and acute kidney injury. In brief, previous work has shown that, after ischemia, the constriction of capillaries by pericytes that occurs during the ischemic period outlasts the period when the supplying artery or arteriole is closed, leading to a decrease in tissue blood flow even when the input artery/arteriole is reperfused (so-called "no-reflow"). Previous work has attempted to restore blood flow using agents that affect upstream signaling (typically calcium entry that evokes contraction). Here the authors develop a novel drug that directly inhibits smooth muscle myosin-mediated contraction. This is shown to relax pericyte-mediated capillary constriction, but to have a smaller effect (for brief application) on arteries/arterioles because of the greater diffusion barrier to entry into those cells (which may help prevent a general fall of blood pressure and tissue perfusion). Even a brief application of the drug produces a long-lasting relief from capillary constriction in the post-ischemic kidney and heart, leading to less no-reflow, less tissue damage and improved survival.

Remarkably, they are also able to show that when no-reflow occurs after cardiac ischemia, then it evokes downstream no-reflow in the kidney (a model of cardio-renal syndrome), and both are relieved by injection of their drug.

The manuscript is potentially suitable for publication, subject to the authors addressing the following points which would significantly improve it.

(1) Omission of consideration of neutrophil-mediated capillary block

Previous work has shown that when pericytes constrict capillaries then neutrophils (which are more rigid than red blood cells) get stuck at the pericyte constrictions, significantly adding to the reduction of blood flow that occurs (PMID: 35316222, see Suppl Fig 5B). It is surprising that the authors show no data on whether their drug reverses capillary occlusion by neutrophils. This could easily be done by using neutrophils labeled genetically or with fluorescent antibodies (PMID: 26019296). This would add mechanistic understanding of how their drug is working.

(2) Kinetics of the restoration of blood flow

It is surprising that injection of a bolus of the drug leads to a long-lasting abolition of no-reflow (perhaps for as long as 7 days if one can infer that blood flow is still restored if the animal is still living). Perhaps I have missed it but I think it would be good to have an explicit statement of, or graph showing, the longest measurement of blood flow which showed a persistent restoration of blood flow.

It is not at all obvious that relatively brief application of the drug should lead to a prolonged relief of no-reflow. An obvious question is, once the drug has been removed from the blood, why does it not dissociate from the myofilaments, thus enabling constriction and a fall of blood flow again if the stimulus to constriction is still present (such as high [Ca] in the pericytes, or ischemia-evoked norepinephrine release in the heart)? This should at least be discussed in the paper.

In Fig. 6, R(+)2501 restores renal capillary diameter and blood flow within 10 mins, but kidney functon(eGFR) recovers only over 10 days. Why is that? Does it take time (10 days?) for the counter-current multiplier to set up the osmotic gradients needed for renal function after blood flow is restored. Please discuss this.

(3) Effect of size on the effect of R(+)2501

The paper shows convincingly that short-term application of the drug has a larger effect on capillaries than on larger vessels. But in a clinical situation it is likely that the drug would be given as more than a single bolus I-V. There should be some discussion whether this would actually matter, i.e. of how much a mean fall of blood pressure would matter, given the presence of autoregulation of the blood supply to many organs.

(5) Capillary constriction

Were capillary diameter measurements made solely at pericyte somata, where the largest constriction occurs because most circumferential pericyte processes are near the cell soma, or were they made at arbitrary positions on capillaries.

(6) Movies

I'm concerned about exactly what the first movie shows. For Video 1, when the blood is moving slowly (during and immediately after ischemia) the green RBCs are clearly moving within the confines of the (black) blood vessels. But at the start of the movie, in healthy conditions, there seem to be green streaks (RBCs?) that simply move all the way across the image from left to right. Is this some kind of illusion or artefact?

More minor points

Line 26: CNR doubles mortality - in which tissue?

Line 48: Ischemia-evoked pericyte contraction was I think first reported in PMID 17036005 and PMID 19718040.

Line 79: This is a bit too negative: in Alzheimer's disease nimodipine unblocks brain capillaries by relaxing pericytes (PMID: 39294491).

Fig S3-S5 legends. Explain what B-factor means and its relevance to what is shown.

Fig 3. To aid understanding please can you show where the "canonical blebbistatin cleft" is located and give a reference to its definition and location? In addition explain briefly what Transducer, Relay and Converter mean.

Figures in general: Many of the lines (e.g. the s.e.m. lines on Fig 3A and 3E and 3H) and lettering on the figures (Fig 3G, lettering on pink and on green bars) are too small to read easily when printed out at normal paper size.

Fig. 5B. Why are the kidney cells/tubules green? Explain in the legend. Label the cells/tubules in the field of view to aid the reader's understanding

Fig. 6N. Add to the y axis label the fact that this is "7 day surviving animals"

Fig 7F-G. Is it possible to say at which end of the capillary end the capillary diameters were measured (arterial or venous) and whether epi- or endocardial?

Line 491. Define CRS-1.

Line 512. State that R(+)2501 was given after unblock of the LAD. This is crucial.

The colors in many figures are too similar to distinguish. Coupled with the tiny keys provided, this makes for painful reading of the figures. For example in Fig 7n and others, change the colors so they are easily distinguishable, and not different shades of the same color. The letter thickness is also often way too thin, e.g. "end of infusion" in Fig 7o. Doubling the font size for all the tiny words on the figures would help.

Fig 8c. Mark on all the panels which direction is time, which is distance, and add a mean speed value for each record

Please explain why the RBC flux and velocity measurements are so incredibly noisy, with large variations on a minute-minute time scale.

Fig S2. Please make all the curves darker and the points larger, so they can be seen more easily when printed.

Fig. S8. Clarify whether the velocity and flux measurements were made during the ischemia, or on reperfusion (and how long reperfusion).

Fig S13. As for S2, make the colors more different, and explain what the horizontal lilac line is at 100% (it's not in the key).

Table S1 is impossible to read because it is too small. Rotate it 90 degrees on the page and make it fill the page, make the chemical structures darker, and double the font size for all the numbers.

Line 1509. Give more explanation for exactly how the heart was gently fixed - people will want to repeat this. Similarly for the kidney.

Referee #2

(Remarks to the Author)
In this manuscript from Pénzes and colleagues, the authors report the unexpected finding that the R(+) enantiomers of a series derived from the myosin II inhibitor blebbistatin show activity against smooth-muscle myosin (SMM) and bind a previously unrecognized allosteric pocket in the pre-hydrolytic state of SMM, whereas the S(-) enantiomers occupy the canonical blebbistatin cleft. Further, the authors show disruption of ischemia-associated capillary constriction. The direct, capillary-resolved in vivo measurements are a real strength of the paper with excellent physiology and innovative imaging. However, key data and methods necessary for interpreting the results are missing, there are gaps in the mechanism as presented, and the results are consistently overinterpreted.
Major Comments:

1. Overall, the introduction's claims/statements need to be softened. An example - "Crucially, CNR does not result from residual emboli or overt vasospasm… (line 46)". Certainly emboli and vasospasm alone do not explain all cases, but they are considered contributors.

2. Regarding the data and analysis associated with Figure 2:
A. Data for characterizing the activity of R(+)2501 are missing. The mansuscript states "Building on published MPH and blebbistatin derivatives, we synthesized and tested over 150 enantiomerically pure derivatives (CLN compound family) against six myosin-2 isoforms: smooth muscle, skeletal muscle, cardiac muscle, and three non-muscle myosin variants. (lines 146-148)" Activity of the skeletal and non-muscle myosin variants is never shown, aside from one statement that was not supported with data analysis ("…showed 3-9x higher selectivity with less than 20% inhibition on NM2A"). These are important data because, as the reviewers state, these myosins are "essential for vital physiological functions."
B. The manuscript presents inhibitory-ATPase data for R(+) and S(-) enantiomer pairs, but not their solubility in the assay buffer - a factor that can artificially depress the maximal inhibition "Max.Inh." and distort the derived IC50 and selectivity values. These data are straightforward to obtain and without them, a < 100 % Max.Inh could simply reflect compound precipitation in the assay buffer rather than a genuine mechanistic limit. Similarly, the Methods lack description of the ATPase assay itself. It is not possible to assess whether the reported potency and selectivity truly derive from the compound's pharmacology without these details.
C. The selectivity panel in Figure 2A should be limited to the compounds that are described and characterized in the manuscript. Although showing data for the entire library of ~150 enantiomeric derivatives would support the claim that "R(+) derivatives consistently showed selective inhibition of SMM", what is currently shown does not add to the paper. If the authors wish to present the broader SAR, the synthetic routes/structures, purity profiles, solubility, and full ATPase inhibition curves for all derivatives should be presented as well.
D. Many of the ATPase inhibition curves presented in Figures 2 and S2 are based on only three concentration points per compound. Even when a quadratic binding model is applied (as the authors state) and the total enzyme concentration is fixed, at least two free parameters must have been estimated – the inhibitory constant (KI) and the maximal inhibition "Max.Inh". Fitting such a sparse data set risks over- and/or misinterpretation because experimental errors or potential artifacts can disproportionately affect the fitted values. The authors should: (I) repeat the assays with a broader concentration range (at least 5-6 points) to ensure robust parameter estimation, (II) disclose how many replicates were performed at each concentration, and (III) explain what the error bars in the figures represent.
E. The manuscript describes the use of a "quadratic model" to derive the inhibitory constants (KI) directly. Yet, compound potencies are reported as IC50s and maximal inhibition. KI and IC50 can differ markedly (e.g. depending on enzyme concentration in the assay). The methods section should be clarified with the exact fitting equation, and the assumptions, if any to allow reviewers/readers to assess the reported selectivity and potency metrics. Also, the manuscript states that selectivity (S) was derived as "S = (Inhibition of SMM at 50 µM R(+)2501 concentration/Inhibition of CM at 50 µM R(+)2501 concentration) x (IC50 of CM/IC50 of SMM)" (Table 1). This formula needs a clear, step-by-step explanation. What is the reason for using a single, fixed-concentration inhibition value (50 µM)? This can be misleading due to factors such as compound solubility.
3. Metabolic stability methods are needed. Also, the Methods section "HPLC analysis of rat plasma and tissue samples and plasma sample collection" describes collection of brain, heart and kidney. However, data presented in the manuscript do not correspond to this. A later Methods section, "Tissue sample collection" lists all of the tissue types presented in Fig. S6. However, several of the detailed doses are not reported (20 and 80 mg/kg). Also, did the animals survive the 80 mg/kg (IV) dose of R(+)2501? And how well tolerated were doses required later for CNR improvements?

3. The manuscript repeatedly claims that R(+)2501 acts in a pericyte-targeted manner, yet no data directly demonstrate preferential engagement of pericytes over other SMM-expressing tissues, including arteriolar smooth muscle. In vivo measurements localizing capillary constriction and dilation to pericyte somata/processes, or cell type-specific readouts of target engagement, are required to support this claim. Moreover, while arterial pressure is monitored in the pig experiments (Fig. 9), quantitative blood pressure data are not presented, and systemic hemodynamics are not reported for the rodent kidney, MI, or CRS-I models. Given the central role of smooth muscle myosin in vascular tone, preserved blood pressure

alone cannot be used to infer pericyte specificity.

4. The reported dual-enantiomer, dual-site mechanism is highly unusual: two stereoisomers of the same scaffold inhibit the same enzyme by engaging two spatially distinct pockets, each capable of producing potent inhibition through different molecular interactions. Because such a claim demands the highest level of experimental certainty, the authors should provide unequivocal evidence that the observed activity of R(+)2501 is not an artifact of synthetic impurities (e.g. residual S(-) material, by-products, or precursors). As is standard for these types of studies, the manuscript should include: (I) Complete synthetic routes for each enantiomer with reagents, conditions, yields, etc to enable reproducibility and assessment of possible contaminant formation. (II) Rigorous purity data including HPLC chromatograms for the final compounds together with chiral-HPLC traces that report the enantiomeric excess. (III) Full spectroscopic characterization, including ^{1}H-NMR, ^{13}C-NMR, and ESI-MS spectra for both purified enantiomers.

5. While the reported 2.5 A and 2.30 A crystal structures appear compelling, the authors should provide unbiased electron density evidence that each ligand is genuinely present in its assigned site. Generating OMIT maps - or, preferably Polder maps, which excludes the bulk solvent around the omitted region - would allow an independent assessment of ligand occupancy and avoid model bias. Including these maps at least in the supplementary material would greatly strengthen the claim of dual, stereospecific binding.

6. Starting on line 179 the authors argue that the unusually fast on-rates of the R(+) compounds support the existence of a novel allosteric pocket distinct from the canonical blebbistatin site: "These observations suggest that R(+)2501 may bind to..." Faster association (and slower dissociation) can arise even when ligands bind the same site, simply because of differences in binding energetics - eventually leading to different binding constants. If the authors genuinely consider the distinct on-rates to be evidence for their hypothesis, the manuscript would be strengthened by the corresponding kinetic data.

7. The manuscript states that “Comparison with CK-571 revealed that R(+)2501 binds deeper within the allosteric pocket and makes additional contacts that may underlie its improved potency" (line 205). However, the reported inhibitory potency of CK-571 (~9 nM against SMM, PMID 27815532) is far higher than the IC50 values observed for R(+)2501 (1–5 μM depending on the species; Table 1). As written, the claim of “improved potency” is ambiguous and appears contradictory. The authors should clarify to which reference compound the potency improvement is being made and/or in which context.

8. The conclusion of selective dilation of vessels <50 μm associated with data shown in Figure 4 is not directly supported by the presented data. Fig. 4E quantifies only vessels ≥50 μm and therefore cannot demonstrate capillary-selective dilation. Fig. 4D summarizes relaxation kinetics across heterogeneous assays (ex vivo arteries and in vivo capillaries), but does not provide in vivo diameter measurements of <50 μm vessels under the same conditions as Fig. 4E. As presented, the data support a diameter-dependent difference in relaxation kinetics, but do not establish selective in vivo dilation of capillaries or a clear transition point separating capillary from arteriolar responses. Also, 45 minute data is referenced in the text for Fig. 4E, but not shown in the actual figure.

9. The reported plasma concentrations implied by the authors’ own PK data and statements indicate that functional experiments employed doses that substantially exceed the reported biochemical SMM IC50 of R(+)2501 (1–5 μM), often by one to two orders of magnitude. Collectively, such high doses preclude attribution of the effects to SMM without genetic interventions to support the findings. Also, it appears the methods for Fig. 4F-H are missing. Further, the plasma PK data are not consistent between Fig. 4A and the data referenced, but not shown, for Fig. 6.

10. The authors state that "Formulation with 2-hydroxypropyl-β-cyclodextrin (HPBCD) significantly improved absorption and bioavailability (Figures 6J-K)" (line 346). This is confusing because there is 100% bioavailability with IV dosing and no absorption phase. Also, was HPBCD used because for solubility reasons at the high concentration administered to animals? This highlights the need for solubility data both in PBS and other vehicles used in the manuscript. Compound precipitation may be a confound here and in other experiments. Also, solubility for CK-571 in PBS and/or in any other vehicle used should be presented as it may explain the “slower” increase in RBC velocity and flux shown in Figure S1.

11. The Fig. 6 section provides strong in vivo evidence that renal ischemia induces persistent capillary perfusion failure and that R(+)2501 rapidly and robustly restores capillary RBC flux, with corresponding improvements in renal function and survival. However, the data do not directly demonstrate that capillary closure is pericyte-mediated, nor that the drug’s effects are specific to capillaries. Also, details of the survival studies and co-administration experiments are not provided in the Methods.

12. The statistical analysis details for Figures 6-8 are not provided, but are necessary to properly evaluate the results.

13. The Fig. 7 section is very similar. It presents compelling evidence that R(+)2501 dilates myocardial capillaries after LADO and improves capillary perfusion metrics, with large reductions in infarct size and a survival benefit. However, the RBC flow analysis is vulnerable to selection bias because it relies on ‘minimal-motion intervals,’ and the use of ‘robust ST-elevation’ as an inclusion criterion requires transparent reporting of exclusions and blinding. Mechanistic conclusions (pericyte-specific action, cascade initiation by pericytes, interruption at the origin) are not directly demonstrated by these data. Further, the survival analyses in Figures 7Q and S13 (which should be a main figure) assess only acute survival during the 120-minute reperfusion period. While R(+)2501 markedly improves early survival, the data do not support claims of durable or long-term survival benefit. Statements implying prevention of mortality or long-term protection substantially overstate what is demonstrated.

14. Figures 8 and 9 provide strong evidence that R(+)2501 can acutely restore microvascular perfusion in both heart and kidney and preserve coronary autoregulation in a large-animal model. However, these data do not establish pericyte-specific mechanisms, direct heart-kidney microvascular coupling, or durable protection beyond the acute reperfusion window. Importantly, the coronary physiology data in Figure 9, while compelling, do not resolve the mechanistic ambiguities underlying the renal perfusion rescue in Figure 8. Additional experiments, such as demonstrating capillary constriction and dilation at pericyte somata/processes in kidney and/or heart, hemodynamic dissociation in the CRS-I model, or a minimal genetic pericyte control, would substantially strengthen the mechanistic interpretation without altering the core findings.

Additional Comments:

1. The statement “The MPH compound family, including MPH-220 (also known as MTR-601), provided a promising scaffold due to favorable safety, absorption and clearance profiles. Building on published MPH and blebbistatin derivatives,… (lines 143-145)” is misleading, as the MPH papers indicate they are derivatives of blebbistatin.

2. Line 140: "Among existing inhibitors, CK-57135 is SMM-selective but lacks the pharmacodynamics needed for acute microvascular targeting and has no effect on CNR (Figure S1)." should be revised. According to Fig. S1, CK57135 does have an effect on CNR: "the fact that CK-571, the previously developed SMM inhibitor, was capable of relaxing capillaries represents a proof-of-concept that direct SMM inhibition is a promising therapeutic approach to reopen capillaries after an ischemic attack".

3. The hepatocyte and in vivo PK curves should not be placed on the same graph.

4. Methods for Fig. S6 appear incomplete.

5. Are the methods and drug doses for Fig. 4B and 4C reported?

6. Some acronyms are not defined (e.g. Fig. 4D’s CCA, FA, BA) and others are defined more than once (e.g. CNR).

7. The statement that binding to the "blebbistatin-site" is consistent with a non-selective inhibition profile needs clarification. (Line 921: "In contrast, the S(-)2501 enantiomer ...") As demonstrated previously by the authors themselves with another blebbistatin-derivative (MPH-220), marked skeletal-over-cardiac selectivity can be achieved while targeting the "blebbistatin-site" (Gyimesi et al, 2020).

8. Figure 3G: Authors should discuss how specific inhibition can be explained if the “R(+)2501/CK-571 binding site” is identical in in human SMM, non-muscle myosin-NM2B, β-cardiac myosin heavy chain, and fast skeletal muscle myosin-2x/d.

9. In the legend for Figure S2, the color attributed to the smooth muscle myosin (SMM) curve is incorrectly written as “pink”; it should be “orange” to match the actual figure. Also, the legend for Figure S2 incorrectly refers to “panel D” after panel B. Since Figure S2 contains only panels A, B, and C, the reference should be changed to “C”.

10. Figure S3: "W1" and "W2" (structurally important water molecules?) should be explained in the figure legend.

11. The legend for Table S3 should be corrected to refer to Figure S3, not Figure S5.

Referee #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Version 2:

Reviewer comments:

Referee #1

(Remarks to the Author)
The data added have answered most of my questions. Just a few points remain needing clarification, along with an improvement of the presentation, after which I think this should be accepted.

(1) Fig 3c. It is unclear what has produced the initial tone in the arteries presented. This should be explained in the legend. In addition, the R(+)2501 apparently relaxes all the arteries to some extent. Given the claimed selectivity for pericyte relaxation in the paper, I don’t understand how the drug can cause apparently 100% relaxation of the femoral artery (FA) and a *more than* 100% relaxation of the basilar artery (BA) – how can it relax more than 100% and generate negative tone? Explain exactly what is being measured and plotted please, in the legend and main text.

(2) The data in Fig 3e-f support the idea that the drug acts effectively on pericytes in cardiac muscle. However,
(i) For the naïve reader, mention that the red labelling in the image of Fig 3e (dsRed) is pericyte specific in the heart (if it is).
(ii) What is the preparation being used (state this in the main text and legend) – is it whole heart in vivo, chunks of ex vivo

heart (ventricle or atrium) or cardiac slices; and was myocyte contraction blocked (if so how)?
(iii) Where exactly was pericyte-mediated capillary diameter reduction measured – at pericyte somata, or randomly along the capillary – this is important to state in the legend.

(3) How does the drug get to where it is needed? If 68% of renal capillaries are occluded after RAO (which is broadly consistent with the earlier Freitas and Attwell eLife paper), are we to assume that access via the remaining 32% of perfused capillaries is sufficient to get the drug to the occluded vessel pericytes. Alternatively, could it be the case that the occlusions reported are for *cells* in the blood, while there is still some flow of *plasma* and drug around the stalled cells in the apparently occluded vessels?

(4) Fig 4d would benefit from arrows showing where constrictions occurred that were reversed by R(+)2501.

(5) State clearly in the figure legends the formulation used for the drug in each experiment: for each panel in Fig 4m-4p was the drug dissolved in PBS or in HPBCD? Similarly for Fig. 5.

(6) Line 432 “ischemic injury follows a unified mechanistic cascade initiated by
pericyte contraction and sustained capillary constriction.” As this result is already in the literature for several organs it is necessary to cite refs 2, 3, 6, 7, 11, 70 here.

(7) Fig 5b. State what the green label is. Explain that the wavy nature of the image reflects ventricular movement (if it does). Explain whether Fig 5a-b is a line scan (with distance on the x axis and time on the y axis?) or a 2D image of the heart.

(8) Fig 5d-e. I am not sure what is shown here (the authors need to put significantly more effort into explaining what they are showing in figures, e.g. giving the manuscript to a naïve reader would be a good idea). Is this a line scan across vessels with distance plotted horizontally and time plotted downwards (as suggested by the vertical lines when there is no flow) or is it a simple 2-P image in two dimensions (in which case why are no separate RBCs visible along the vertical capillaries?)? In the previous review I requested that distance and time be clearly indicated on such line scan pictures, but if this is a line scan that has not been done here.

(9) Fig 5i and m. Define V on the x axis (perhaps Vehicle, but we are not told, nor are we told what the vehicle is – see above).

(10) Fig 5s-t. Add a bar under or over the two rightmost bars to convey that these are (presumably) measured after cardiac ischaemia. On the y axis mention that this is *renal* RBC flux!

(11) Line 232 and legend to Ext Data Fig 9: Define more clearly what the Relaxation half life means, e.g. stating that the “half-time of the relaxation was defined as the time to reach half the maximal dilation that was measured after N minutes”. In line 232 it is unclear what “modeling” of the relaxation time means. If this is real modelling of the relaxation process then explain it in detail; if it is merely fitting a curve to a plot of relaxation half-time vs diameter, then say so.

(12) Ext Data Fig 10: explain whether capillary diameter was measured at random locations or near pericyte somata.

(13) Suppl Video 1. State in the legend that this is a coronary capillary.

Referee #2

(Remarks to the Author)
The authors have extensively revised the manuscript and provided detailed responses, resulting in a substantially improved manuscript relative to the original submission. In particular, the addition of direct pericyte visualization, vessel diameter-dependent dilation analyses, systemic hemodynamic measurements, pig coronary physiology data, and Polder maps for the ligand-bound structures significantly strengthens the manuscript. These additions substantially improve the biological interpretation.
The authors have also clarified or corrected a number of issues raised in the original review, including the introductory framing of microvascular dysfunction, the comparison between R(+)2501 and CK-571, the structural interpretation of ligand occupancy, missing methods, and the description of the W1/W2 structural elements.
The new additions now enable a full evaluation of the manuscript, and concerns remain unresolved, particularly regarding the quantitative ATPase/SAR analysis, solubility, pharmacokinetic interpretation, survival/statistical reporting, and overstatement of mechanistic and translational claims. Given the high bar for mechanistic, pharmacological, and translational claims in this venue, these remain substantive issues.
1. The revised manuscript still presents the ATPase/SAR data with a level of quantitative precision that does not appear justified by the underlying data. Extended Data Table 1 reports exact-looking potency, selectivity, and MaxInh values for many compounds, including cases where the curves appear weak, incomplete, or uninformative. Several values would be more appropriately reported as lower/upper bounds or as not determined rather than as fitted point estimates.
This is especially important because the SAR table supports several claims about isoform selectivity and structure-activity relationships. If the authors wish to retain this table and the associated conclusions, they should provide appropriate uncertainty for fitted parameters, identify parameters that are not reliably estimable, and avoid ranking compounds or drawing SAR conclusions from noise-fitted curves. This would be seem more appropriate for the full SAR manuscript the authors reference as being in preparation. At minimum, the table should distinguish between true fitted values, screening-

level estimates, limits, and non-identifiable parameters.
Relatedly, the manuscript continues to use confusing terminology around IC50 and KI. The Methods describe fitting to a quadratic binding equation and refer to the fitted term as an “IC50: inhibitory constant.” These terms are not interchangeable. The authors should either report the fitted value as KI, with appropriate assumptions clearly stated, or fit and report IC50 using an appropriate empirical dose-response model.
2. Solubility remains insufficiently addressed
The response that compounds were visually inspected for turbidity is not sufficient to support the solubility-dependent aspects of the manuscript. We acknowledge the authors’ comment regarding limited quantities of some compounds generated during SAR. However, the solubility is relevant to several central interpretations for R(+)2501, S(–)2501, including apparent MaxInh in the ATPase assays, isoform selectivity, differences between PBS and HPBCD formulations, and comparisons between R(+)2501 and CK-571.
Therefore, the manuscript should include measured solubility data for the key compounds, at minimum R(+)2501, S(–)2501, and CK-571, in the relevant assay and dosing conditions. These should include the ATPase assay buffer, PBS, HPBCD-containing vehicle, and any other formulation used for ex vivo perfusion or in vivo dosing. Without these measurements, it remains difficult to distinguish true pharmacological potency/selectivity from differences in soluble/free compound concentration.
R(+)2501 being “sufficiently soluble in PBS” should be supported by data.
3. The revised Methods now include several useful details regarding ATP concentration, actin concentration, buffer composition, DMSO concentration, replicates, and fitting approach. However, because the quadratic fitting model explicitly depends on the myosin concentration, the final concentration of each myosin isoform used in the ATPase assays should be provided.
The Methods should also make clear which compounds were tested only in initial three-point screening format and which were subsequently retested with five- or six-point concentration-response curves. Screening level data should not be presented or interpreted as if they were high-confidence quantitative potency measurements.
4. The added characterization of R(+)2501 is helpful, including HPLC, chiral HPLC, MS, and ^{1}H-NMR. However, for a novel compound that is central to the manuscript, full characterization should be provided, including ^{13}C-NMR. The authors should also clarify whether equivalent analytical characterization is available for S(–)2501, since the comparison between enantiomers is used to support the pharmacological and structural interpretation.
If the authors wish to present broader SAR conclusions for the compound series, then identity and purity support for the compounds included in that analysis should be clearly stated. Alternatively, the broader SAR claims should be substantially de-emphasized.
5. The pharmacokinetic data remain difficult to interpret. The Methods describe plasma sampling at 5, 10, 20, 40, 60, 120, 240, and 360 minutes, but Fig. 3a appears to show earlier sampling and a rapid rise/decline that are not clearly reconciled with those stated time points. This discrepancy should be corrected or explained.
In addition, the manuscript relies heavily on the claim that R(+)2501 is rapidly eliminated and has a short half-life, but the model or fitting approach used to calculate the reported half-life is not adequately described. The authors should state the pharmacokinetic model used, the data included in the fit, whether the reported half-life reflects terminal elimination or an apparent early-phase decline, and whether values are based on total or free plasma concentration.
The authors also invoke plasma protein binding and target-site exposure to explain the relationship between in vivo dose and biochemical potency, but these arguments are not sufficiently supported in the manuscript. If plasma protein binding is central to the interpretation, those data should be shown or the claim should be removed.
6. The survival and statistical methods require substantial clarification. The main text refers to survival studies involving 144 rats, but the Methods and figure legends do not clearly reconcile this number with the treatment groups, occlusion durations, exclusions, censoring, or analytical approach. The authors should provide a transparent accounting of the survival cohorts, including group sizes, exclusion criteria, censoring rules, randomization, blinding, and the statistical test used for survival comparisons.
More broadly, the statistical reporting across the manuscript remains too generic. The repeated statement that two-way ANOVA was used is not sufficient for the variety of data types presented. The manuscript includes repeated measures designs, nested measurements such as multiple vessels or capillaries within animals, survival curves, and non-normal or bounded outcomes. For each figure, the authors should specify the statistical unit, the test used, whether repeated or nested structure was accounted for, what multiple-comparison correction was applied, and whether assumptions of parametric tests were evaluated.
This is particularly important for Figs. 4–5 and associated Extended Data, where the biological conclusions depend on appropriate handling of animal-level replication and survival outcomes.
7. The authors have now defined the “minimal motion” intervals used for red blood cell flow analysis, which is helpful. However, the manuscript should still state whether these intervals were selected blinded to treatment group and outcome. The authors should also report how many animals, imaging fields, or capillary segments were excluded due to motion or inadequate imaging quality.

Similarly, the use of robust ST elevation as a criterion for successful coronary occlusion should be accompanied by reporting of how many animals were excluded or required adjustment, and whether these decisions were made blinded to subsequent treatment assignment.
These clarifications are necessary to exclude avoidable selection bias in the intravital imaging and AMI experiments.
8. The new pericyte visualization and vessel-diameter analyses substantially strengthen the manuscript. The evidence supports capillary-biased or pericyte-associated relaxation and the manuscript should, therefore, avoid implying that all therapeutic effects are definitively mediated by direct pericyte targeting unless this is directly shown.
This issue is especially relevant in the renal and CRS-I sections. The data support acute renal capillary no-reflow after AMI and rescue by R(+)2501, but they do not fully establish the causal heart-kidney axis or prove that renal benefit is driven specifically by pericyte relaxation. Phrases implying that R(+)2501 “resolves” the heart-kidney axis, identifies the “cascade

origin," or demonstrates a dominant pericyte mechanism should be moderated.
9. Safety and tolerability language should be toned down
The added high-dose tolerability information is useful, but the current language remains stronger than the data support. Small n, short duration tolerability studies can support statements such as "no overt adverse signs were observed under the tested conditions," but they do not establish that R(+)2501 is broadly safe or is a "safe drug candidate."

A number of minor issues should be corrected during revision:
1. The statement that R(+)2501 shows "less than 20% inhibition of non-muscle myosin-2" should be reconciled with Fig. 1e, which appears to report 23.6 ± 2.1% MaxInh for non-muscle myosin.
2. The manuscript should clearly define whether reported errors in ATPase figures and tables are SD, SEM, confidence intervals, or fitting errors.
3. Extended Data Fig. 1 appears to be incorrectly cited in the main text in connection with SAR/selectivity rather than the structural Polder maps.
4. Extended Data Fig. 2 should be corrected where the legend refers to the SMM curve as "pink" (this may be a conversion issue), and the IC50/KI terminology should be corrected there as well.
5. Duplicate references should be removed or consolidated.
6. Blebbistatin should be removed from the Methods if it was not used in the revised study.
7. Remaining typographical errors and reference numbering inconsistencies.

Referee #3

(Remarks to the Author)
A-B: This manuscript discusses the role of pericyte-mediated capillary no-reflow in ischemic injury and presents a targeted vasodilator, R+2501, an SMM specific modulator, as a potential therapy to restore microvascular perfusion across organs during reperfusion. The authors reveal that enantiomers, R(+)2501 and S(–)2501 have distinct binding sites which bind the canonical CK-571 and blebbistation sites, respectively.
C-D: Overall fine
E: also fine.
Minor comments for clarification
1) Can the authors comment on why R(+)2501 selectively inhibits SMM (and to a lesser extent skMM) isoforms, but not NMM or CM, as shown in Figure 1C? The sequence comparison of residues important for R(+)2501 binding (Figure 2G) appears highly conserved across these isoforms, particularly between SMM and NM2B which are near identical. This seems difficult to reconcile with the isoform selectivity reported in Figure 1C.

2) Would the authors expect R(+)2501 to affect any of the cells in the blood (e.g. platelets, that contain non-muscle myosin IIA (NM2A), or white blood cells)? Based on the sequence similarity relating to Q1, the NM2A sequence around the binding pocked for this drug is likely similar.

3) Label L265(?) in 2B – there is a dashed line to residue but no label.

4) Lines 183–184: The authors state that the R(+)2501-binding "residues are structurally stabilised in the pre-hydrolytic conformation and are inaccessible in post-hydrolytic or rigor-like states". Could the authors clarify how this relates to the expected state of SMM in contractile pericytes during vasoconstriction? If the myosin filaments are actively contracting and interacting with actin, how do the authors propose that R(+)2501 accesses and binds the motor domain? For example, does the compound bind during rigor or after ATP-induced release from actin, but before ATP hydrolysis, thereby trapping a pre-hydrolytic state and promoting relaxation? It would also be helpful for the authors to define what they mean by "rigor-like" in this context.

5) Following on from this, could the authors compare the R(+)2501-binding pocket with existing myosin-2 structures in other mechanochemical states? Given that the residues forming the proposed R(+)2501 site appear highly conserved across myosin-2 isoforms, it would be helpful to understand how this pocket changes between nucleotide states and actin-bound states.

For example, how does the R(+)2501-binding site compare in available structures such as 8SYF, representing SMM actomyosin in an ADP-bound state, and 6BIH, representing SMM in a rigor/rigor-like state? Are the key residues around the SH1-relay/converter cleft positioned similarly, or is the pocket collapsed, occluded, or otherwise inaccessible in these actin-bound conformations?

This comparison would help clarify whether R(+)2501 binding is primarily determined by isoform-specific sequence differences, or instead by the conformational accessibility of the pocket during particular stages of the myosin ATPase cycle. It would also help address how the compound is proposed to engage SMM in actively contracting pericytes, where a substantial fraction of motors may be actin-bound.

Referee #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Referee #5

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Version 3:

Reviewer comments:

Referee #2

(Remarks to the Author)
The authors have made a further substantial revision, and the additional Extended Data figures help clarify several of the issues raised previously. I am now largely supportive of publication. However, a limited number of issues remain that should be corrected prior to publication:

1. In their response, the authors state that the statistical unit and nested structure have now been specified for each experiment, but I was not able to find this from the information in the manuscript or figure legends. In several central analyses, treatment is assigned to an animal or heart, whereas the reported sample size and inferential statistics appear to use individual capillaries or pericytes as independent observations. This should be addressed to ensure pseudoreplication is not occurring. As one example: Fig. 3d-f uses 75 capillaries measured sequentially at baseline, occlusion, reperfusion, and treatment, but does not describe a repeated-measures or hierarchical analysis. (See also Fig. 2f, Fig. 4, Ext Fig. 3c, Ext Fig. 5b-c.)

2. The 20 mg/kg renal group contains only three animals. Its 33% survival represents one surviving animal and cannot support a dose-response conclusion or the statement that this dose produced the “highest survival rate.” Similarly, the vitamin C, allopurinol, and carprofen combination groups contain only three animals each. Failure to detect additional benefit in these very small groups cannot support the conclusion that “pericyte-mediated CNR is the dominant driver of AKI.”

3. While minor, the plasma sampling description still needs clarification. The stated four cohort schedules (5 and 360 min, 10 and 240 min, 20 and 60 min, and 40 and 120 min) account for eight sampling times and 24 animal time point samples. However, the Methods and Fig. 2a also report measurements at 0.5, 1, and 2 minutes, with n=3 animals per sampling time point, and the source of these nine early samples is not specified. Please provide the actual animal by time point sampling schedule and clarify whether additional animals were used or whether some animals underwent more than two blood collections.

Extended Data Fig. 3a contradicts the statement that tissue concentrations “did not exceed 0.2 uM in any organs.” At 40 mg/kg, the figure contains mean tissue values of approximately 0.23 and 0.30 uM. More generally, a single measurement at two hours, with n=2 animals at the 10 and 40 mg/kg doses, cannot demonstrate absence of tissue accumulation or “confirm rapid systemic elimination.” The result can be stated descriptively – for example, that mean concentrations at two hours were generally low and below approximately 0.3 uM under the tested conditions. But claims about accumulation or elimination require time-course or repeated dosing data.

4. Several mechanistic and translational statements remain stronger than the experiments support. The direct ex vivo pericyte experiment is a useful addition and shows that R(+)2501 can reverse ET-1-induced constriction at identified cardiac pericyte somata. However, the renal and myocardial ischemia experiments do not directly visualize pericyte contraction or demonstrate that all in vivo effects are mediated specifically through pericytes. The Summary should distinguish between direct in vivo imaging of capillary flow and the separate ex vivo demonstration of pericyte-associated relaxation.

Similarly, the pig experiment was performed in one animal with sequentially escalating doses. It is a useful physiological proof-of-concept observation, but it cannot establish reproducible dose dependence, capillary selectivity, or translational efficacy. The text should acknowledge this.

The Extended Data Fig. 4 legend refers to “irreversible changes” and states that pericytes remain “permanently and completely constricted.” However, the same figure shows restoration of flow after R(+)2501 following up to 120 minutes of occlusion. “Refractory to reperfusion alone” would be more accurate than “irreversible” or “permanent.”

5. The Supplementary Methods still state that the genotoxicity and acute tolerability experiments confirm that R(+)2501 is a “safe drug candidate,” that it can be developed into clinical trials, and that the 200 mg/kg experiment establishes a greater-than-20-fold margin of safety. These statements directly contradict the response to Reviewer 2, which says that broad safety language was removed. Small, short-duration experiments can support only that no overt adverse signs or assay-positive effects were detected under the particular conditions tested. The claims concerning broad safety, clinical development, and a quantified margin of safety should be removed. Likewise, “cardiovascular safety profile” should be replaced by “acute hemodynamic effects in healthy rats” or similar wording. Extended Data Fig. 3d-j, with three animals per dose and ten minutes of observation, is informative about acute hemodynamic tolerability but cannot establish cardiovascular safety.

6. Minor: The diameter-relaxation model in Extended Data Fig. 3b is based on n=1 femoral artery and n=1 carotid artery,

together with the basilar-artery and capillary estimates. The reported “effective doubling constant” of 130 ± 60 um should be described as exploratory or illustrative rather than as a robust fitted physiological parameter.

Referee #3

(Remarks to the Author)
The authors have addressed our comments, and the new video (video 1) is indeed very helpful, and will be very useful to readers of this paper.

Referee #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Referee #5

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

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We thank the Reviewers and the Editor for their careful evaluation of our manuscript and for the many constructive suggestions that helped us substantially strengthen the study. In response to the Reviewers' comments, we have performed several new experiments and expanded the analysis in multiple areas. These include:

- **direct visualization of pericytes demonstrating R(+)2501–induced pericyte relaxation,**
- **diameter-dependent analysis of vascular responses confirming capillary-selective dilation,**
- **dose-dependent systemic hemodynamic measurements in rats showing no adverse cardiovascular effects at therapeutically relevant doses, and**
- **direct visualization of neutrophil granulocytes demonstrating R(+)2501–induced removal of neutrophil plugging from capillaries in rat AMI model**

We have also added Polder maps supporting ligand occupancy in the structural models, expanded compound characterization and analytical purity data, clarified pharmacokinetic and metabolic analyses, and substantially revised the Methods and Discussion sections to ensure full reproducibility, and to highlight clinical relevance.

In addition, we have refined the mechanistic discussions, moderated interpretations where appropriate, corrected several inconsistencies in figures and legends, and clarified experimental details throughout the manuscript.

To further strengthen the manuscript, we invited four additional co-authors:

- **Dr. Svetlana Mastitskaya from University of Bristol, who performed the pericyte visualization experiments.**
- **Prof. C. Michael Gibson, President of the Baim Institute for Clinical Research at Harvard, who contributed to highlighting the clinical relevance of the study.**
- **Tímea Bálint, from Heart and Vascular Center, Department of Experimental Cardiology and Surgical Techniques, Semmelweis University, who performed rat hemodynamic measurements.**
- **Dávid Nagy, from Heart and Vascular Center, Department of Experimental Cardiology and Surgical Techniques, Semmelweis University, who performed rat hemodynamic measurements.**

Together, these revisions significantly strengthen the mechanistic interpretation and improve the clarity and transparency of the study.

Referee #1 (Remarks to the Author):

This is an exciting manuscript that reports a novel approach to restoring blood flow to organs that have been made ischemic, as occurs in heart attack, stroke and acute kidney injury. In brief, previous work has shown that, after ischemia, the constriction of capillaries by pericytes that occurs during the ischemic period outlasts the period when the supplying artery or arteriole is closed, leading to a decrease in tissue blood flow even when the input artery/arteriole is reperfused (so-called "no-reflow"). Previous work has attempted to restore blood flow using agents that affect upstream signaling (typically calcium entry that evokes contraction). Here the authors develop a novel drug that directly inhibits smooth muscle myosin-mediated contraction. This is shown to relax pericyte-mediated capillary constriction, but to have a smaller effect (for brief application) on arteries/arterioles because of the greater diffusion barrier to entry into those cells (which may help prevent a general fall of blood pressure and tissue perfusion). Even a brief application of the drug produces a long-lasting relief from capillary constriction in the post-ischemic kidney and heart, leading to less no-reflow, less tissue damage and improved survival.

Remarkably, they are also able to show that when no-reflow occurs after cardiac ischemia, then it evokes downstream no-reflow in the kidney (a model of cardio-renal syndrome), and both are relieved by injection of their drug.

The manuscript is potentially suitable for publication, subject to the authors addressing the following points which would significantly improve it.

We thank the Reviewer for the careful reading of our manuscript and for the positive and encouraging assessment of our work. We appreciate the Reviewer's recognition of the novelty of the approach and the potential implications for treating no-reflow and ischemia-reperfusion injury. We have carefully considered all the comments below and have revised the manuscript accordingly to address each point.

1. Omission of consideration of neutrophil-mediated capillary block

Previous work has shown that when pericytes constrict capillaries then neutrophils (which are more rigid than red blood cells) get stuck at the pericyte constrictions, significantly adding to the reduction of blood flow that occurs (PMID: 35316222, see Suppl Fig 5B). It is surprising that the authors show no data on whether their drug reverses capillary occlusion by neutrophils. This could easily be done by using neutrophils labeled genetically or with fluorescent antibodies (PMID: 26019296). This would add mechanistic understanding of how their drug is working.

We thank the Reviewer for this insightful comment and for highlighting the important contribution of neutrophil plugging at pericyte constrictions to the microvascular obstruction that develops after reperfusion.
Based on this comment, we performed additional experiments to directly visualize neutrophil accumulation in the infarcted myocardium after LADO in rats (Methods, line 1753-1779). As suggested, neutrophils were labeled using

fluorescent anti-Ly6G antibodies and imaged in the cardiac capillaries following reperfusion. Consistent with prior reports that rigid neutrophils can become trapped at pericyte-mediated capillary constrictions (PMID: 35316222), we observed marked neutrophil accumulation within capillaries in the untreated LADO group. Importantly, R(+)2501 treatment qualitatively reduced neutrophil trapping in the microcirculation (new data shown in new Fig. 5e; Results – line 435-451).

These findings are consistent with the proposed mechanism where R(+)2501 rapidly and durably restores capillary diameter and prevents the formation of narrow segments that promote mechanical trapping of neutrophils. This is highly relevant for reperfusion injury because neutrophil plugging is a major secondary amplifier of microvascular obstruction (Discussion, line 625-633).

2. Kinetics of the restoration of blood flow

It is surprising that injection of a bolus of the drug leads to a long-lasting abolition of no-reflow (perhaps for as long as 7 days if one can infer that blood flow is still restored if the animal is still living). Perhaps I have missed it but I think it would be good to have an explicit statement of, or graph showing, the longest measurement of blood flow which showed a persistent restoration of blood flow.

The longest time period during which we directly monitored microvascular blood flow parameters after drug administration was 2 hours for AMI model and 1 hour for AKI model, and this has now been explicitly stated in the revised manuscript (Methods, for AMI line 1737-1739, for AKI line 1639).

While direct flow measurements were limited to this time window, we also assessed long-term (14-day) functional outcomes in the renal artery occlusion (RAO) model. In these experiments, R(+)2501 treatment resulted in sustained improvement of renal function, as reflected by preserved eGFR, compared with untreated RAO animals (new Fig. 4o and new Extended Data Fig. 18). These findings indicate that, even though continuous flow measurements were not performed beyond the 1-hour monitoring period, a single bolus treatment maintains capillary patency and preserves tissue perfusion long after the drug is cleared.

It is not at all obvious that relatively brief application of the drug should lead to a prolonged relief of no-reflow. An obvious question is, once the drug has been removed from the blood, why does it not dissociate from the myofilaments, thus enabling constriction and a fall of blood flow again if the stimulus to constriction is still present (such as high [Ca] in the pericytes, or ischemia-evoked norepinephrine release in the heart)? This should at least be discussed in the paper.

This is an important mechanistic point that we now discuss in the revised manuscript (Discussion, line 613-624). Importantly, once capillary diameter is restored and microvascular flow resumes, the hypoxic and ischemic stimuli that drive pericyte rigor rapidly dissipates. Restoration of oxygen delivery allows

cellular ATP levels to recover within minutes, enabling normalization of cellular metabolism and calcium handling. As a result, the physiological drivers of sustained contraction diminish in the time range while R(+)2501 concentration is still high enough to keep capillaries open by pericyte relaxation.

Furthermore, O'Farrell et al. (PMID: 29120327) showed that a transient 15 min adenosine infusion around reperfusion significantly reduced cardiac no-reflow, even though adenosine clears from blood within seconds. In our case, a single i.v. bolus of R(+)2501 produced blood exposure over a similar time scale, with an in vivo half-life of 16 min. We therefore suggest that transient relaxation by R(+)2501 restores sufficient capillary patency to interrupt no-reflow. Once capillary flow is restored, local hypoxia and ischemia are relieved, reducing the drive for pericyte constriction. We have now added an explicit discussion of this point to the revised Discussion (line 613-624).

In Fig. 6, R(+)2501 restores renal capillary diameter and blood flow within 10 mins, but kidney functon(eGFR) recovers only over 10 days. Why is that? Does it take time (10 days?) for the counter-current multiplier to set up the osmotic gradients needed for renal function after blood flow is restored. Please discuss this.

We thank the Reviewer for this insightful question. The apparent difference in time scales reflects the distinction between rapid restoration of microvascular perfusion and the slower recovery of organ-level renal function after ischemic injury.

In our experiments, R(+)2501 restores peritubular capillary diameter and blood flow within minutes (~10 min), indicating rapid reversal of the microvascular obstruction responsible for no-reflow. However, glomerular filtration rate (eGFR) reflects the integrated function of multiple renal structures that may remain temporarily impaired after the ischemic episode, including tubular epithelial cells, endothelial cells, and the tubuloglomerular feedback system.

During renal ischemia–reperfusion, tubular epithelial cells, particularly in the proximal tubule and thick ascending limb, undergo transient injury characterized by ATP depletion, cytoskeletal disruption, loss of polarity, and tubular obstruction by cell debris and casts. Even after perfusion is restored, these cells require several days to recover structural integrity and transport capacity, which directly affects the kidney's ability to generate filtrate and maintain normal electrolyte gradients.

The Reviewer's suggestion regarding the counter-current multiplier is conceptually related: restoration of the corticomedullary osmotic gradients and normal tubular transport processes also depends on recovery of tubular epithelial function and medullary microcirculation. These processes occur on a multi-day timescale, consistent with the gradual improvement in eGFR that we observe.

Thus, our data indicate that R(+)2501 rapidly restores the upstream microvascular perfusion defect within minutes, while the downstream recovery of tubular and nephron function occurs over days as ischemia-injured cells repair and normal nephron physiology is re-established. We have added a brief discussion of this distinction in the revised manuscript (Discussion, line 657-665).

3. Effect of size on the effect of R(+)2501

The paper shows convincingly that short-term application of the drug has a larger effect on capillaries than on larger vessels. But in a clinical situation it is likely that the drug would be given as more than a single bolus I-V. There should be some discussion whether this would actually matter, i.e. of how much a mean fall of blood pressure would matter, given the presence of autoregulation of the blood supply to many organs.

We thank the Reviewer for raising this important translational point. The intended clinical application of R(+)2501 is not systemic IV administration, but rather a single intracoronary bolus injection delivered through the PCI guide catheter immediately after reopening the occluded coronary artery. This approach allows high local concentrations in the ischemic myocardial microcirculation while maintaining very low systemic exposure.

We modeled this approach in our experiments, where R(+)2501 was delivered intracoronary into the pig heart (Methods, line 1849-1877). Under these conditions, as opposed to other smooth muscle relaxants such as nitroglycerin, we did not observe detectable effect on systemic blood pressure, consistent with the localized delivery strategy (new Fig. 6).

In the revised manuscript, we present a novel experiment demonstrating the specificity of R(+)2501 (Methods, line 1474-1501). This experiment shows that the compound is highly selective for capillaries, dilating them by 50–120%. In contrast, only a 0–22% opening is observed in arterioles, and there is no effect on larger vessels (Results, line 237-247 and new Fig. 3d). We believe that this experiment enhances the significance of this article, as it is the first time pericytes have been specifically dilated. This capillary specificity enabled us to address the question of how pericyte relaxation itself (without arteriolar and larger vessel dilation) affects hemodynamics.

To further address this question and the Reviewers' concern, in the revised manuscript we also performed hemodynamic measurements in rats using an intravenous bolus, which likewise did not produce a measurable decrease in systemic blood pressure or systemic blood flow (Results, line 262-274; new Extended Data Fig. 11 and Methods, line 1536-1559).

Thus, the intended intracoronary administration ensures that systemic concentrations remain low, while the local concentration in the ischemic microvasculature is sufficient to achieve pharmacological efficacy. We have

now clarified this point in the revised manuscript discussion (Discussion, line 676-681).

4. Capillary constriction

Were capillary diameter measurements made solely at pericyte somata, where the largest constriction occurs because most circumferential pericyte processes are near the cell soma, or were they made at arbitrary positions on capillaries.

In the original manuscript, capillary diameter measurements were performed at random positions along capillaries in both the kidney and heart microcirculation.

In response to the Reviewers' suggestions, we have now conducted a comprehensive new set of experiments in collaboration with Dr. Svetlana Mastitskaya at University of Bristol (added as a new co-author in the revised manuscript), in which pericytes were directly visualized (Methods, line 1503-1534). This allowed us to specifically analyze capillary diameter changes at pericyte somata, too, where the strongest constrictions are known to occur due to the high density of circumferential pericyte processes. These new data directly demonstrate the effect of R(+)2501 on pericyte-mediated capillary constriction, and the quantitative analysis of capillary diameter changes at pericyte somata has now been incorporated into the revised manuscript (Results, line 248-261; Fig 3e,f, Supplementary video 1, line 1132-1138).

5.Movies

I'm concerned about exactly what the first movie shows. For Video 1, when the blood is moving slowly (during and immediately after ischemia) the green RBCs are clearly moving within the confines of the (black) blood vessels. But at the start of the movie, in healthy conditions, there seem to be green streaks (RBCs?) that simply move all the way across the image from left to right. Is this some kind of illusion or artefact?

Thank you for this careful observation which may have been confusing for the readers. The apparent "streaks" seen at the beginning of Video 1 are most likely an optical effect related to the scanning orientation of the laser during image acquisition combined with the very high velocity of RBC movement under normal flow conditions.

When RBCs move rapidly through the capillaries, their displacement during the scanning time of a single frame can create elongated fluorescent streaks that may appear to traverse the image at an angle relative to the vessel boundaries. This can give the visual impression that the labeled RBCs are moving outside the vessel lumen, although they are in fact confined within the microvascular structures.

In contrast, during ischemia and early reperfusion, RBC velocity is markedly reduced, allowing individual cells to be resolved more clearly within the vessel lumen, which eliminates this apparent streaking effect.

Thus, the phenomenon likely reflects a well-known interaction between fast-moving fluorescent particles and raster scanning microscopy, rather than RBCs moving outside the vascular compartment. We have clarified this point in the revised manuscript (Supplementary Video 2 legend, line 1140-1146).

More minor points

Line 26: CNR doubles mortality - in which tissue?

The statement refers to myocardial infarction (reference PMID: 20488311), and we have now modified this point in the revised manuscript.

Line 48: Ischemia-evoked pericyte contraction was I think first reported in PMID 17036005 and PMID 19718040.

Thank you for this clarification, we have incorporated the two suggested references in the revised manuscript (Introduction, line 53-56 and References 6 and 7)

Line 79: This is a bit too negative: in Alzheimer's disease nimodipine unblocks brain capillaries by relaxing pericytes (PMID: 39294491).

We have now clarified in the revised manuscript that although L-type Ca^{2+} channel blockers can relax pericytes under certain conditions, such as in Alzheimer's disease (Introduction, line 78-89 and Reference 27). However, under severe hypoxia the overall intracellular Ca^{2+} elevation in pericytes is largely independent of L-type Ca^{2+} channel influx. As a result, blocking these channels produces only a minor reduction in total intracellular Ca^{2+} levels, making Ca-channel blockers relatively ineffective in this setting. This clarification has been briefly incorporated in the revised Introduction (line 78-89).

Fig S3-S5 legends. Explain what B-factor means and its relevance to what is shown.

We have now added a detailed definition of B-factor and its relevance in the revised manuscript in the legends of new Extended Data Fig. 7 (line 1024-1029).

Fig 3. To aid understanding please can you show where the "canonical blebbistatin cleft" is located and give a reference to its definition and location? In addition explain briefly what Transducer, Relay and Converter mean.

We have clarified in the revised manuscript that the canonical blebbistatin cleft is where S(-)2501 binds and added references for the definition and location (References 44, 47 and 49) in the revised manuscript). We added a short definition of functions of the Transducer, Relay and Converter regions in the legend of new Fig. 2 (line 195-205).

Figures in general: Many of the lines (e.g. the s.e.m. lines on Fig 3A and 3E and 3H) and lettering on the figures (Fig 3G, lettering on pink and on green bars) are too small to read easily when printed out at normal paper size.

We have revised all figures to make all labeling and lines visible.

Fig. 5B. Why are the kidney cells/tubules green? Explain in the legend. Label the cells/tubules in the field of view to aid the reader's understanding

The green signal arises from autofluorescence of renal tubules and cells. This has now been clarified in the revised legend of new Fig. 4 (line 346).

Fig. 6N. Add to the y axis label the fact that this is "7 day surviving animals"

We have added the requested important label and legend of new Fig. 4p (line 373).

Fig 7F-G. Is it possible to say at which end of the capillary end the capillary diameters were measured (arterial or venous) and whether epi- or endocardial?

Capillary diameters in these experiments were measured at random positions within the epicardium, without distinguishing between the arterial or venous ends of the capillaries. This has now been clarified in the revised manuscript new Fig. 5 (line 469).

Line 491. Define CRS-1.

We have defined CRS-I in the subheading (Results, line 494).

Line 512. State that R(+)2501 was given after unblock of the LAD. This is crucial.

Thank you for this important observation. We have added a clarifying sentence to the result section (Results, line 515-518).

The colors in many figures are too similar to distinguish. Coupled with the tiny keys

provided, this makes for painful reading of the figures. For example in Fig 7n and others, change the colors so they are easily distinguishable, and not different shades of the same color. The letter thickness is also often way too thin, e.g. “end of infusion” in Fig 7o. Doubling the font size for all the tiny words on the figures would help.

We have revised all figures to make all labeling and lines visible and changed the colors throughout the figures to make the lines and conditions more distinguishable.

Fig 8c. Mark on all the panels which direction is time, which is distance, and add a mean speed value for each record

We have labeled time and distance on new Fig. 4-5 and added mean velocity and flux values to all presented panels.

Please explain why the RBC flux and velocity measurements are so incredibly noisy, with large variations on a minute-minute time scale.

The apparent “noise” largely reflects real microvascular heterogeneity plus measurement constraints at the capillary level, rather than instability of the preparation.

Capillary flow is intrinsically intermittent and heterogeneous: RBC traffic occurs as discrete cells and short trains, with frequent brief stalls/restarts due to upstream network dynamics. Small changes upstream can cause large swings in a single capillary’s flux/velocity from minute to minute.

Sampling a tiny field can also magnify variability. At each time point we quantified a small number of capillaries and relatively short time windows (10 sec), so stochastic variation in cell arrival times appears as large relative fluctuations.

Technical factors add variance at high speeds: with raster scanning, fast-moving RBCs can be partially sampled within a frame, producing streaking and frame-to-frame differences in apparent displacement; small motion/registration errors and occasional out-of-plane movement also contribute.

We now clarify these points in the revised manuscript (Methods, line 1675-1684) and emphasize that conclusions are based on group-level trends and time-averaged/pooled analyses across many capillaries and animals, which are robust despite expected capillary-level variability.

Fig S2. Please make all the curves darker and the points larger, so they can be seen more easily when printed.

As mentioned above, we have revised all figures to make all labeling and lines visible and changed the colors throughout the figures to make the lines and conditions more distinguishable.

Fig. S8. Clarify whether the velocity and flux measurements were made during the ischemia, or on reperfusion (and how long reperfusion).

We have clarified in the revised of new Extended Data Fig. 14 that velocity and flux measurements were made in the reperfusion phase after 10, 30, 60 and 120-minute ischemia (line 1063-1073).

Fig S13. As for S2, make the colors more different, and explain what the horizontal lilac line is at 100% (it's not in the key).

As mentioned above, we have revised all figures to make all labeling and lines visible and changed the colors throughout the figures to make the lines and conditions more distinguishable, and we also carefully revised all key for the colors and symbols in the figure legends throughout the manuscript.

Table S1 is impossible to read because it is too small. Rotate it 90 degrees on the page and make it fill the page, make the chemical structures darker, and double the font size for all the numbers.

We have restructured the presentation of new Extended Data Table 1. to make all numbers and chemical structures visible and readable.

Line 1509. Give more explanation for exactly how the heart was gently fixed - people will want to repeat this. Similarly for the kidney.

We have supplemented the experimental description of kidney (Methods, line 1628-1645) and heart visualization (Methods, line 1714-1751) with additional details that we believe now provide sufficient information for reproducibility by other groups.

Referee #2 (Remarks to the Author):

In this manuscript from Pénzes and colleagues, the authors report the unexpected finding that the R(+) enantiomers of a series derived from the myosin II inhibitor blebbistatin show activity against smooth-muscle myosin (SMM) and bind a previously unrecognized allosteric pocket in the pre-hydrolytic state of SMM, whereas the S(-) enantiomers occupy the canonical blebbistatin cleft. Further, the authors show disruption of ischemia-associated capillary constriction. The direct, capillary-resolved in vivo measurements are a real strength of the paper with excellent physiology and innovative imaging. However, key data and methods necessary for interpreting the results are missing, there are gaps in the mechanism as presented, and the results are consistently overinterpreted.

We thank the Reviewer for the careful evaluation of our manuscript and for recognizing the strengths of the study, particularly the direct, capillary-resolved *in vivo* measurements and imaging approach. We appreciate the Reviewer's constructive comments regarding missing methodological details, mechanistic interpretation, and potential overinterpretation of certain results. In response, we have revised the manuscript to provide additional methodological information, include new experimental data where appropriate, and refine the discussion to more carefully distinguish between our experimental findings and their mechanistic implications.

Major Comments:

1. Overall, the introduction's claims/statements need to be softened. An example - "Crucially, CNR does not result from residual emboli or overt vasospasm… (line 46)". Certainly emboli and vasospasm alone do not explain all cases, but they are considered contributors.

We thank the Reviewer for this note. We have carefully revised the wording in the Introduction and toned down several statements, including the one cited, to more accurately reflect that embolization and vasospasm can contribute to CNR, even though they do not fully explain the phenomenon in many cases.

2. Regarding the data and analysis associated with Figure 2:

A. Data for characterizing the activity of R(+)2501 are missing. The manuscript states "Building on published MPH and blebbistatin derivatives, we synthesized and tested over 150 enantiomerically pure derivatives (CLN compound family) against six myosin-2 isoforms: smooth muscle, skeletal muscle, cardiac muscle, and three non-muscle myosin variants. (lines 146-148)" Activity of the skeletal and non-muscle myosin variants is never shown, aside from one statement that was not supported with data analysis ("…showed 3-9x higher selectivity with less than 20% inhibition on NM2A").

These are important data because, as the Reviewers state, these myosins are “essential for vital physiological functions.”

We thank the Reviewer for highlighting the need to better present the myosin isoform selectivity data. In the revised manuscript, we have added a reference describing the development of the CLN compound family (Introduction, line 131, PMID: 40829259), which provides the broader context for the synthesis and screening of this compound series.

We have also restructured the figures presenting the ATPase inhibitory profiles of R(+)2501 and S(–)2501 and now show their activity across all tested myosin-2 isoforms, including smooth, cardiac, skeletal, and non-muscle myosin-2 variants (Fig. 1c,d,e). These data now directly support the statements regarding selectivity that were previously summarized only in the text.

For the remainder of the compound series, however, we retained the comparative presentation of smooth vs. cardiac myosin inhibition, as these isoforms are physiologically the most relevant for the intended therapeutic application. Given the transient exposure and rapid systemic elimination of R(+)2501 in vivo, inhibition of smooth muscle myosin (targeting pericyte contraction) and the potential effect on cardiac myosin represent the most critical considerations for pharmacological and safety interpretation.
The revised text (Results, line 131-146) and figures (in new Fig. 1c,d,e; Extended Data Table 1) now clarify this rationale and provide the full isoform activity profile for R(+)2501.

B. The manuscript presents inhibitory-ATPase data for R(+) and S(-) enantiomer pairs, but not their solubility in the assay buffer - a factor that can artificially depress the maximal inhibition “Max.Inh.” and distort the derived IC50 and selectivity values. These data are straightforward to obtain and without them, a < 100 % Max.Inh could simply reflect compound precipitation in the assay buffer rather than a genuine mechanistic limit. Similarly, the Methods lack description of the ATPase assay itself. It is not possible to assess whether the reported potency and selectivity truly derive from the compound’s pharmacology without these details.

We thank the Reviewer for raising this important point. We agree that limited solubility could lead to an apparent reduction in (maximal) inhibition, potentially affecting the derived IC50 and selectivity values. To avoid such artifacts, each compound was checked after dilution into the ATPase assay buffer at the highest tested concentration (50 μM), and we observed no visible turbidity or precipitation before and after the assay. This approach ensured that the observed inhibition was not limited by precipitation under the assay conditions. Although we did not experience precipitation in the ATPase measurements, since the buffer condition of each myosin isoform ATPase measurement was the same, the compound’s specificity is reflected by these measurements even if minor precipitation could happen. Thus, gross precipitation under the assay

conditions is unlikely to account for the observed inhibition profiles. We have now clarified this point in the revised Methods (line 1181-1203).

At the same time, we did not determine the formal solubility limits for every compound, because during the early SAR screening phase the compounds were often available only in small quantities, making detailed solubility measurements impractical. We therefore do not claim to have established absolute aqueous solubility values for each analogue, only that under the conditions used for ATPase measurement we did not detect visible precipitation at the concentrations tested. We also note that the R(+) and S(–) enantiomer pairs were assayed under identical experimental conditions, so the marked stereoselective differences are unlikely to be explained by assay-buffer solubility artifacts alone.

In response to the Reviewer's comment, we have now expanded the Methods (line 1181-1203) section to include a detailed description of the ATPase assay, providing all relevant experimental parameters required for reproducibility of the measurements.

C. The selectivity panel in Figure 2A should be limited to the compounds that are described and characterized in the manuscript. Although showing data for the entire library of ~150 enantiomeric derivatives would support the claim that "R(+) derivatives consistently showed selective inhibition of SMM", what is currently shown does not add to the paper. If the authors wish to present the broader SAR, the synthetic routes/structures, purity profiles, solubility, and full ATPase inhibition curves for all derivatives should be presented as well.

We agree that the presentation of the compound series should be sufficiently supported by data. In response, we now report the inhibitory parameters for all 62 R(+)–S(-) enantiomer pairs shown in previous Figure 2A (new Extended Data Fig. 2) in the revised and expanded in new Extended Data Table 1, providing the full dataset underlying the selectivity trends presented in the figure.

We also agree that complete presentation of the synthetic routes, compound characterization, and full ATPase inhibition curves is important for the comprehensive description of the compound library. However, these elements are primarily related to the medicinal chemistry development of the CLN compound family and do not directly contribute to the central biological and mechanistic message of the present manuscript. Therefore, we are currently preparing a separate manuscript (Szimler et al., in preparation) that will describe the full SAR, synthetic routes, compound characterization, and detailed biochemical profiles of the entire compound library.

However, based on the Reviewer's comments we have incorporated the synthetic route (new Extended Data Fig. 3) and full analytics (new Extended Data Fig. 4) of R(+)2501 in the supplementary information and Methods (line 1205-1286) sections.

Nevertheless, we believe that showing the consistent SMM–selectivity trend across the R(+) enantiomer family remains an important addition to the present manuscript, as it strengthens the key finding that enantiomer type determines binding to a distinct allosteric pocket, enabling selective SMM inhibition and efficient pericyte relaxation.

D. Many of the ATPase inhibition curves presented in Figures 2 and S2 are based on only three concentration points per compound. Even when a quadratic binding model is applied (as the authors state) and the total enzyme concentration is fixed, at least two free parameters must have been estimated – the inhibitory constant (KI) and the maximal inhibition "Max.Inh". Fitting such a sparse data set risks over- and/or misinterpretation because experimental errors or potential artifacts can disproportionately affect the fitted values. The authors should: (I) repeat the assays with a broader concentration range (at least 5-6 points) to ensure robust parameter estimation, (II) disclose how many replicates were performed at each concentration, and (III) explain what the error bars in the figures represent.

As mentioned above, during the initial SAR screening campaign, compounds were typically synthesized and purified only in sub-mg quantities in enantiomerically pure form. For this reason, the ATPase assays were performed using three concentration points, which provided a rapid but reliable estimate of inhibitory potency and maximal inhibition, sufficient to evaluate selectivity versus cardiac myosin and guide the medicinal chemistry optimization.

This approach is a standard strategy in early SAR campaigns and has proven effective in our previous work. For example, using the same workflow we successfully identified MPH-220 as a skeletal muscle myosin–selective inhibitor (*Cell* 2020 Oct 15;183(2):335-346, PMID: 33035452), which has now advanced to Phase 2 clinical trials. In the present campaign, the same strategy enabled efficient identification of R(+)2501 as the lead compound for the capillary no-reflow indication.

Because the three-point titration already provided consistent and clear selectivity trends, repeating all measurements with 5–6 point titrations would not meaningfully change the estimated parameters or alter the conclusion that R(+)2501 is the most suitable candidate for further development. The best selected compounds such as R(+)2501) were re-synthetized (see new Extended Data Fig. 2 and synthesis description in Methods, line 1205-1286) and we measured the ATPase in more detailed way at 5-6 different concentrations on several myosin isoforms (new Fig. 1).

However, we agree that the description of the ATPase assay was previously insufficient. Therefore, in the revised Methods (line 1181-1203) section we have expanded the assay description, including the number of experimental replicates and the definition of the error bars shown in the figures, to ensure full transparency and reproducibility of the measurements.

E. The manuscript describes the use of a “quadratic model” to derive the inhibitory constants (KI) directly. Yet, compound potencies are reported as IC50s and maximal inhibition. KI and IC50 can differ markedly (e.g. depending on enzyme concentration in the assay). The methods section should be clarified with the exact fitting equation, and the assumptions, if any to allow Reviewers/readers to assess the reported selectivity and potency metrics. Also, the manuscript states that selectivity (S) was derived as "S = (Inhibition of SMM at 50 µM R(+)2501 concentration/Inhibition of CM at 50 µM R(+)2501 concentration) x (IC50 of CM/IC50 of SMM)" (Table 1). This formula needs a clear, step-by-step explanation. What is the reason for using a single, fixed-concentration inhibition value (50 μM)? This can be misleading due to factors such as compound solubility.

We thank the Reviewer for this important request for clarification. In the revised Methods (line 1181-1203) section, we now explicitly provide the quadratic equation used to calculate IC50 values, with the myosin concentration fixed to the assay conditions (as performed in the fits). We agree that KI and IC50 can differ, particularly when enzyme concentration is not negligible, and we therefore clarified the exact fitting model, parameters, and assumptions so readers can interpret potency and selectivity appropriately.

Regarding the selectivity metric, we used the cited formula because relying on IC50 and Max.Inh alone can be misleading in early SAR datasets. For example, a very weak inhibitor may still extrapolate to a high Max.Inh, while another compound can yield an apparently favorable fitted IC50 despite producing only minimal inhibition within the experimentally tested range. We therefore included the observed inhibition at a fixed relatively high concentration (50 μM) so that the selectivity score reflects not only fitted potency, but also the actual inhibitory effect measured at the top dose. In this way, the metric captures both the relative potency between smooth vs cardiac myosin, and the extent of inhibition actually achieved under the assay conditions, allowing a more robust distinction between strong inhibitors (substantial inhibition at 50 μM with low IC50) and poor inhibitors (where one or both criteria are not met).

Finally, as noted above and now clarified in the revised Methods (line 1281-1203), compound solubility was always checked at 50 μM in the assay buffer, and compounds were only analyzed under conditions where no precipitation was observed, minimizing the risk that reduced inhibition at 50 μM reflects solubility artifacts rather than real pharmacological effect.

3. Metabolic stability methods are needed. Also, the Methods section “HPLC analysis of rat plasma and tissue samples and plasma sample collection” describes collection of brain, heart and kidney. However, data presented in the manuscript do not correspond to this. A later Methods section, “Tissue sample collection” lists all of the tissue types presented in Fig. S6. However, several of the detailed doses are not reported (20 and 80 mg/kg). Also, did the animals survive the 80 mg/kg (IV) dose of R(+)2501? And how well tolerated were doses required later for CNR improvements?

We thank the Reviewer for pointing out these inconsistencies. In the revised manuscript, we have modified and clarified the Methods (line 1318-1331) section describing the hepatocyte metabolic stability assay and unifying the two tissue preparation sections to better distinguish the procedures used for plasma analysis and for the tissue distribution experiments.

We also corrected the description of the dosing scheme: the 20 and 80 mg/kg doses were not used in the tissue accumulation experiments it was mistakenly left in the original manuscript as a typo, and this has now been corrected in the revised Methods paragraph (line 1412-1420) text.

Regarding tolerability, as shown in Extended Data Table 4, we did not observe any adverse events even at doses up to 200 mg/kg.

4. The manuscript repeatedly claims that R(+)2501 acts in a pericyte-targeted manner, yet no data directly demonstrate preferential engagement of pericytes over other SMM-expressing tissues, including arteriolar smooth muscle. In vivo measurements localizing capillary constriction and dilation to pericyte somata/processes, or cell type-specific readouts of target engagement, are required to support this claim. Moreover, while arterial pressure is monitored in the pig experiments (Fig. 9), quantitative blood pressure data are not presented, and systemic hemodynamics are not reported for the rodent kidney, MI, or CRS-I models. Given the central role of smooth muscle myosin in vascular tone, preserved blood pressure alone cannot be used to infer pericyte specificity.

We agree that additional experimental evidence is supportive for the pericyte-targeted mechanism proposed in the manuscript. Based on this and related Reviewer comments, we have performed several new sets of experiments, which are now incorporated into the revised manuscript.

First, we performed direct visualization of pericytes (Methods, line 1503-1534) and their contractile state in the microcirculation in collaboration with Dr. Svetlana Mastitskaya (University of Bristol), who has now joined the author list. Using pericyte labeling, we directly demonstrated that R(+)2501 relaxes pericyte-mediated capillary constrictions (Results, line 248-261 and new Fig 3e,f), with quantitative analysis of capillary diameter changes specifically at pericyte somata. These new experiments provide direct evidence that the drug acts at the level of pericytes.

Second, to address the concern regarding effects on other smooth muscle–containing vessels, we performed a detailed analysis of vasodilatory responses as a function of vessel diameter in the cardiac microcirculation at 25 μM and 100 μM inhibitor concentrations (Methods, line 1474-1501). These data show that R(+)2501 selectively dilates capillaries (Results, line 237-247 and new Fig. 3d), while arterioles and larger arteries remain unaffected, supporting the notion that

the compound primarily relieves capillary constriction rather than producing generalized vascular smooth muscle relaxation.

Third, we performed dose-dependent systemic hemodynamic measurements in rats, including pressure–volume (PV) loop analysis, to evaluate potential systemic cardiovascular effects (Methods, line 1536-1559). These measurements (Results, line 262-274 and new Extended Data Fig. 11) demonstrate no detectable adverse effects on cardiac hemodynamics or PV parameters up to the highest dose used in the efficacy experiments (20 mg/kg).

Together, these new experiments provide direct visualization of pericyte relaxation, vessel diameter–dependent vascular effects consistent with capillary selectivity, and comprehensive systemic hemodynamic measurements, strengthening the mechanistic interpretation that R(+)2501 primarily acts by relieving pericyte-mediated capillary constriction without producing systemic vascular smooth muscle relaxation.

5. The reported dual-enantiomer, dual-site mechanism is highly unusual: two stereoisomers of the same scaffold inhibit the same enzyme by engaging two spatially distinct pockets, each capable of producing potent inhibition through different molecular interactions. Because such a claim demands the highest level of experimental certainty, the authors should provide unequivocal evidence that the observed activity of R(+)2501 is not an artifact of synthetic impurities (e.g. residual S(-) material, by-products, or precursors). As is standard for these types of studies, the manuscript should include: (I) Complete synthetic routes for each enantiomer with reagents, conditions, yields, etc to enable reproducibility and assessment of possible contaminant formation. (II) Rigorous purity data including HPLC chromatograms for the final compounds together with chiral-HPLC traces that report the enantiomeric excess. (III) Full spectroscopic characterization, including ^{1}H-NMR, ^{13}C-NMR, and ESI-MS spectra for both purified enantiomers.

We have performed detailed analytical characterization of both the R(+) and S(–) enantiomers. These analyses demonstrated that the enantiomeric purity exceeds 99.5% for both preparations, with all other detectable impurities below 1%. The corresponding analytical and chiral HPLC chromatograms showing 100.0% enantiomer purity of R(+)2501 have now been included in the Supplementary Materials (new Extended Data Fig. 4). In addition, we now provide ^{1}H NMR spectra of R(+)2501 (new Extended Data Fig. 4) to support structural identity.

Furthermore, in response to the Reviewer's request, we have expanded the Methods (line 1205-1286) section to include a detailed description of the synthesis and purification procedures for R(+)2501, including the key steps used to obtain the enantiomerically pure compounds.

Together, these new analytical data and expanded methodological descriptions demonstrate that the observed activity of R(+)2501 cannot be attributed to

residual S(–) impurity or other synthetic impurities, and further corroborate the unequivocal conclusion form the crystal structures that the two enantiomers interact with smooth muscle myosin through distinct binding modes.

6. While the reported 2.5 A and 2.30 A crystal structures appear compelling, the authors should provide unbiased electron density evidence that each ligand is genuinely present in its assigned site. Generating OMIT maps - or, preferably Polder maps, which excludes the bulk solvent around the omitted region - would allow an independent assessment of ligand occupancy and avoid model bias. Including these maps at least in the supplementary material would greatly strengthen the claim of dual, stereospecific binding.

We thank the Reviewer for this helpful suggestion. In the revised manuscript we now provide the corresponding Polder maps for the ligand-binding sites to allow an unbiased evaluation of ligand occupancy. These maps are included in the Supplementary Materials (new Extended Data Fig. 1) and clearly support the presence of the ligands in the assigned binding pockets, thereby strengthening the structural interpretation of the dual, stereospecific binding modes.

7. Starting on line 179 the authors argue that the unusually fast on-rates of the R(+) compounds support the existence of a novel allosteric pocket distinct from the canonical blebbistatin site: "These observations suggest that R(+)2501 may bind to..." Faster association (and slower dissociation) can arise even when ligands bind the same site, simply because of differences in binding energetics - eventually leading to different binding constants. If the authors genuinely consider the distinct on-rates to be evidence for their hypothesis, the manuscript would be strengthened by the corresponding kinetic data.

We thank the Reviewer for this thoughtful comment. We plan to publish detailed binding kinetics of the R(+) and S(–) enantiomers in a forthcoming manuscript (Szimler et al, in preparation), because we believe that presenting the full kinetic dataset and analysis within the present study would shift the focus away from the main biological and physiological findings. In the revised manuscript, we have therefore discarded the sentence about binding kinetics of the R(+) and S(–) enantiomers (Results, line 174-184).

8. The manuscript states that “Comparison with CK-571 revealed that R(+)2501 binds deeper within the allosteric pocket and makes additional contacts that may underlie its improved potency" (line 205). However, the reported inhibitory potency of CK-571 (~9 nM against SMM, PMID 27815532) is far higher than the IC50 values observed for R(+)2501 (1–5 μM depending on the species; Table 1). As written, the claim of “improved potency” is ambiguous and appears contradictory. The authors should clarify to which reference compound the potency improvement is being made and/or in which context.

We thank the Reviewer for pointing out this ambiguity. We agree that the sentence could be misinterpreted as implying improved potency relative to CK 571, as correctly noted by the Reviewer. In the revised manuscript, we have corrected the wording (Results, line 185-190) of this sentence to clarify the binding pattern within the pocket without suggesting improved potency.

9. The conclusion of selective dilation of vessels <50 μm associated with data shown in Figure 4 is not directly supported by the presented data. Fig. 4E quantifies only vessels ≥50 μm and therefore cannot demonstrate capillary-selective dilation. Fig. 4D summarizes relaxation kinetics across heterogeneous assays (ex vivo arteries and in vivo capillaries), but does not provide in vivo diameter measurements of <50 μm vessels under the same conditions as Fig. 4E. As presented, the data support a diameter-dependent difference in relaxation kinetics, but do not establish selective in vivo dilation of capillaries or a clear transition point separating capillary from arteriolar responses. Also, 45 minute data is referenced in the text for Fig. 4E, but not shown in the actual figure.

We thank the Reviewer for this important observation and agree that the original presentation did not directly – only implicitly – demonstrated capillary-selective dilation.

Based on this comment (as we mentioned above), we performed a new set of experiments (Methods, line 1474-1501) analyzing vessel diameter–dependent dilation in the cardiac microcirculation at two different concentrations of R(+)2501 (Results, line 237-247 and new Fig. 3d). These experiments allowed us to directly quantify diameter changes across the microvascular tree.

The new data show that capillaries below ~8 μm are very efficiently dilated by R(+)2501 (50-120%), whereas larger vessels remain unresponsive, and 10–15 μm arterioles exhibit only marginal dilation (0-22%) (Results, line 237-247 and new Fig. 3d). These results provide a clear demonstration that the vasodilatory effect of R(+)2501 is strongly biased toward capillaries, with minimal effects on upstream resistance vessels. We appreciate the Reviewer's comment, which inspired this more detailed and elegant analysis of vessel diameter–dependent responses, and the new results have now been incorporated into the revised manuscript (Results, line 237-247 and new Fig. 3d).

In addition, the new pericyte-visualizing experiments (Methods, line 1503-1534) described above (Results, line 248-261 and new Fig. 3e,f) further support this interpretation. These experiments directly show that R(+)2501 relaxes pericytes at capillary constriction sites, demonstrating that the capillary-selective dilation arises from direct relaxation of pericytes rather than from effects on upstream vascular smooth muscle. Original Fig. 4dand e are replaced with the presentation of this new set of experiments.

10. The reported plasma concentrations implied by the authors' own PK data and

statements indicate that functional experiments employed doses that substantially exceed the reported biochemical SMM IC50 of R(+)2501 (1–5 μM), often by one to two orders of magnitude. Collectively, such high doses preclude attribution of the effects to SMM without genetic interventions to support the findings. Also, it appears the methods for Fig. 4F-H are missing. Further, the plasma PK data are not consistent between Fig. 4A and the data referenced, but not shown, for Fig. 6.

As mentioned above, in response to the Reviewer comments we have now added multiple new experimental datasets that support the smooth muscle myosin–mediated, capillary selective mechanism, including: i) direct visualization of pericytes demonstrating R(+)2501–induced pericyte relaxation (Results, line 248-261 and new Fig.e,f), ii) diameter-dependent vascular analysis showing strong dilation of capillaries (<8 μm) with minimal effect on arterioles and larger resistance vessels (Results, line 237-247 and new Fig. 3d), and iii) dose-dependent systemic hemodynamic measurements demonstrating no detectable effects on global cardiovascular function at doses used in the efficacy experiments (Results, line 262-274 and new Extended Data Fig. 11). Together, these multiple independent experimental approaches strongly support the interpretation that R(+)2501 acts primarily through inhibition of smooth muscle myosin in pericytes, leading to relaxation of capillary constrictions and restoration of microvascular flow.

PK was determined at 12 mg/kg (Methods, line 1374-1382), which produced an initial plasma concentration of approximately 540 μM within the first minute after administration and then cleared relatively quickly (half-life ~16 min) (new Fig. 3a). In the functional experiments, we used a similar dose range (5–20 mg/kg). We agree that these total plasma concentrations exceed the biochemical SMM IC50 measured in the purified ATPase assay. However, the biochemical IC50 from an isolated in vitro system is not directly equivalent to the in vivo effective plasma concentration, particularly because total plasma concentration does not reflect free drug concentration at the target site (plasma protein binding is 90% in rats – data not shown in the manuscript), tissue distribution, or the brief exposure window created by rapid clearance. Rapid clearance is advantageous here because brief drug exposure is sufficient to produce a durable improvement in capillary perfusion while limiting prolonged systemic on-target effects.

We have now incorporated a detailed description of the methods used for the experiments presented in our new figures in the Methods section. In addition, we restructured the section containing the typographical error regarding plasma PK, where the text previously stated 10 minutes (Results, line 221).

11. The authors state that "Formulation with 2-hydroxypropyl-β-cyclodextrin (HPBCD) significantly improved absorption and bioavailability (Figures 6J-K)" (line 346). This is confusing because there is 100% bioavailability with IV dosing and no absorption phase. Also, was HPBCD used because for solubility reasons at the high concentration administered to animals? This highlights the need for solubility data both

in PBS and other vehicles used in the manuscript. Compound precipitation may be a confound here and in other experiments. Also, solubility for CK-571 in PBS and/or in any other vehicle used should be presented as it may explain the “slower” increase in RBC velocity and flux shown in Figure S1.

We thank the Reviewer for this comment and agree that the wording in the original manuscript was not sufficiently clear. In the revised version, we clarify that HPBCD was used not primarily to improve solubility or to enhance systemic bioavailability in the classical pharmacological term (oral vs i.v. dosing), but rather to enhance cellular uptake and tissue exposure of the compound. Thus, the term “bioavailability” was intended in the context of effective cellular delivery, not systemic absorption, which is now clarified in the revised manuscript (Results, line 319-322).

Consistently, although R(+)2501 was sufficiently soluble in PBS, and the experiments could be performed in this vehicle, HPBCD formulation allowed stronger biological effects at lower administered doses (new Fig. 4). For this reason, unless otherwise stated, we conducted further experiments in the HPBCD formulation.

For CK-571, the comparisons were performed under the same PBS conditions used for R(+)2501, and the conclusions regarding the differences in vascular responses remain valid under these matched conditions. We did not perform additional experiments with CK-571 in HPBCD formulation because further optimization of CK-571 delivery was outside the scope of the present study, which focuses on the pharmacology of R(+)2501. In addition, generating new experimental series for CK-571 would have required substantial additional animal experiments, which would not align with the 3R principles (replacement, reduction, and refinement) guiding our study design.

12. The Fig. 6 section provides strong in vivo evidence that renal ischemia induces persistent capillary perfusion failure and that R(+)2501 rapidly and robustly restores capillary RBC flux, with corresponding improvements in renal function and survival. However, the data do not directly demonstrate that capillary closure is pericyte-mediated, nor that the drug’s effects are specific to capillaries. Also, details of the survival studies and co-administration experiments are not provided in the Methods.

As described above, based on Reviewer suggestions we performed new experiments directly addressing the pericyte-mediated and capillary-specific mechanism. Specifically, we carried out direct pericyte visualization experiments, which demonstrate pericyte relaxation following R(+)2501 administration (Fig. 3e,f), and vessel diameter–dependent analyses, which show that R(+)2501 efficiently dilates capillaries while larger vessels remain largely unaffected (Fig. 3d). Together, these new datasets confirm that the observed effects arise from pericyte-mediated capillary relaxation.

In addition, we have now incorporated the detailed experimental procedures for the survival studies and the co-administration experiments into the Methods (line 1561-1583) section of the revised manuscript.

13. The statistical analysis details for Figures 6-8 are not provided, but are necessary to properly evaluate the results.

We have added details of the statistical methods used for the analyses presented in new Fig. 4-5 (Results, line 374-377 and line 489-492), including the statistical tests applied and the definition of error bars, to ensure transparency and proper evaluation of the results.

14. The Fig. 7 section is very similar. It presents compelling evidence that R(+)2501 dilates myocardial capillaries after LADO and improves capillary perfusion metrics, with large reductions in infarct size and a survival benefit. However, the RBC flow analysis is vulnerable to selection bias because it relies on 'minimal-motion intervals,' and the use of 'robust ST-elevation' as an inclusion criterion requires transparent reporting of exclusions and blinding. Mechanistic conclusions (pericyte-specific action, cascade initiation by pericytes, interruption at the origin) are not directly demonstrated by these data. Further, the survival analyses in Figures 7Q and S13 (which should be a main figure) assess only acute survival during the 120-minute reperfusion period. While R(+)2501 markedly improves early survival, the data do not support claims of durable or long-term survival benefit. Statements implying prevention of mortality or long-term protection substantially overstate what is demonstrated.

Regarding the RBC flow analysis, the use of minimal-motion intervals is necessary in open-chest intravital imaging to avoid motion artifacts caused by cardiac and respiratory movements. These intervals were selected using predefined criteria to ensure accurate quantification of RBC velocity and flux. We have now clarified this procedure in the Methods (line 1739-1746) section, including the criteria used for inclusion of segments. We also added clarification (Methods, line 1701-1712) regarding the ST-elevation inclusion criterion and reporting of analyzed animals.

Concerning the mechanistic interpretation, as described above in response to related Reviewer comments, we have now incorporated new experiments directly visualizing pericytes and analyzing vessel diameter–dependent responses (Figs 3e,f and Fig. 3d), which demonstrate pericyte-mediated capillary relaxation and capillary-specific dilation by R(+)2501. These new data strengthen the mechanistic interpretation presented in the manuscript.

Finally, we agree that the survival analysis presented in the study reflects acute survival during the 120-minute reperfusion period. We have therefore adjusted the wording (Results, line 417-424) in the manuscript to avoid implying long-term survival benefits. At the same time, the observation that ECG signals recover in treated animals suggests that they would likely survive beyond the

acute period. However, the open-chest intravital model used for these experiments does not permit longer-term survival studies, which limits the ability to directly assess long-term outcomes within this experimental setup. We have clarified these points in the revised text (Results, line 417-424).

Also, as suggested by the Reviewer, Figure S13 has now been moved to the new main Fig. 5p in the revised manuscript to better present the survival data alongside the other key cardiac findings.

15. Figures 8 and 9 provide strong evidence that R(+)2501 can acutely restore microvascular perfusion in both heart and kidney and preserve coronary autoregulation in a large-animal model. However, these data do not establish pericyte-specific mechanisms, direct heart-kidney microvascular coupling, or durable protection beyond the acute reperfusion window. Importantly, the coronary physiology data in Figure 9, while compelling, do not resolve the mechanistic ambiguities underlying the renal perfusion rescue in Figure 8. Additional experiments, such as demonstrating capillary constriction and dilation at pericyte somata/processes in kidney and/or heart, hemodynamic dissociation in the CRS-I model, or a minimal genetic pericyte control, would substantially strengthen the mechanistic interpretation without altering the core findings.

In response to this and related comments, we have performed new experiments directly addressing the pericyte-mediated mechanism. Specifically, we conducted pericyte visualization experiments in collaboration with Dr. Svetlana Mastitskaya (University of Bristol), which allow direct identification of pericytes and demonstrate that R(+)2501 relaxes pericyte-mediated capillary constrictions, with quantitative analysis of capillary diameter changes at pericyte somata. These data provide direct evidence that the drug acts at the level of pericyte-controlled capillary constrictions (new Fig. 3e,f and new Supplementary Video 1).

In addition, we performed vessel diameter–dependent analyses showing that R(+)2501 efficiently dilates capillaries (<8 μm, 50-120%), while larger vessels remain unaffected and 10–15 μm arterioles show only minimal dilation (0-22%) (new Fig. 3d), supporting the interpretation that the compound acts primarily on the capillary compartment rather than producing generalized smooth muscle vasodilation.

Furthermore, we have now included dose-dependent systemic hemodynamic measurements in rats, including pressure–volume (PV) loop analysis, demonstrating no detectable adverse effects on systemic cardiovascular parameters at doses used in the efficacy experiments (up to 20 mg/kg) (new Extended Data Fig. 11). These data help distinguish the observed microvascular rescue from systemic hemodynamic effects.

We agree with the Reviewer that the current experiments do not directly demonstrate genetic pericyte specificity or fully resolve the mechanistic basis

of heart–kidney coupling in the CRS-I model. However, the new pericyte visualization experiments, vessel diameter–dependent responses, and hemodynamic analyses together provide strong evidence that the observed restoration of microvascular perfusion arises from relief of pericyte-mediated capillary constriction. We have incorporated these new data and clarified the mechanistic interpretation in the revised manuscript (Results, line 248-274).

We also note that substantial evidence has accumulated showing that pericytes regulate capillary blood flow in the heart, kidney, and brain, and that their constriction contributes to capillary no-reflow in these models. The most important papers demonstrating this are cited in the manuscript.

Additional Comments:

1. The statement “The MPH compound family, including MPH-220 (also known as MTR-601), provided a promising scaffold due to favorable safety, absorption and clearance profiles. Building on published MPH and blebbistatin derivatives,… (lines 143-145)” is misleading, as the MPH papers indicate they are derivatives of blebbistatin.

We intentionally make a distinction between the original blebbistatin scaffold and the improved MPH scaffold, which was developed to achieve better safety, absorption, and clearance properties. Nevertheless, we agree that the wording in the original sentence could be misleading. Therefore, we have clarified the sentence (Results, line 125-130) in the revised manuscript while maintaining the distinction between the blebbistatin-derived origin and the optimized MPH scaffold.

2. Line 140: "Among existing inhibitors, CK-57135 is SMM-selective but lacks the pharmacodynamics needed for acute microvascular targeting and has no effect on CNR (Figure S1)." should be revised. According to Fig. S1, CK57135 does have an effect on CNR: "the fact that CK-571, the previously developed SMM inhibitor, was capable of relaxing capillaries represents a proof-of-concept that direct SMM inhibition is a promising therapeutic approach to reopen capillaries after an ischemic attack".

We agree that the original wording was misleading. As shown in previous Figure S1, CK-571 is capable of relaxing capillaries and partially improving microvascular flow, which indeed supports the proof-of-concept that direct SMM inhibition can reopen capillaries after ischemia.

However, the effect of CK-571 is substantially slower and less robust than that observed with R(+)2501, likely due to its less favorable pharmacodynamic properties for rapid microvascular targeting.

3. The hepatocyte and in vivo PK curves should not be placed on the same graph.

We thank the Reviewer for this suggestion. The hepatocyte stability data and the *in vivo* plasma pharmacokinetic profile were intentionally plotted together to allow direct visual comparison between metabolic stability and systemic exposure. Presenting the curves on the same graph illustrates the strong correspondence between rapid hepatocyte metabolism and the short *in vivo* plasma half-life, supporting the conclusion that hepatic clearance is the primary metabolic route for the compound. Separating the curves into different panels would make this relationship less immediately apparent. However, to avoid potential confusion, we have clarified this rationale in the figure legend (Results, line 275-291) and improved the labeling of the curves in the revised manuscript.

4. Methods for Fig. S6 appear incomplete.

In the revised manuscript, we have expanded the Methods (line 1412-1448) section to include the missing experimental details corresponding to the data presented in new Extended Data Fig. 8, ensuring that the procedures and analytical methods are described sufficiently to allow reproducibility.

5. Are the methods and drug doses for Fig. 4B and 4C reported?

In the revised manuscript, we have clarified the Methods (line 1470-1473) section and figure legends (Results, line 275-291) to explicitly report the experimental procedures and drug doses used in the experiments shown in new Fig.4b,C.

6. Some acronyms are not defined (e.g. Fig. 4D's CCA, FA, BA) and others are defined more than once (e.g. CNR).

In the revised manuscript, we have carefully reviewed the use of acronyms throughout the text and figures, ensuring that all acronyms are defined at first appearance, and removing duplicate definitions where terms were mistakenly introduced more than once.

7. The statement that binding to the "blebbistatin-site" is consistent with a non-selective inhibition profile needs clarification. (Line 921: "In contrast, the S(-)2501 enantiomer ...") As demonstrated previously by the authors themselves with another blebbistatin-derivative (MPH-220), marked skeletal-over-cardiac selectivity can be achieved while targeting the "blebbistatin-site" (Gyimesi et al, 2020).

We agree that the original wording was not sufficiently precise. As demonstrated in our previous work (Gyimesi et al., 2020 *Cell*), skeletal muscle

selectivity can indeed be achieved while targeting the blebbistatin binding site, particularly when bulky substitutions are introduced on the D-ring of the scaffold and when using the appropriate S-enantiomer configuration, as in the case of MPH-220.

In contrast, CLN-2501 does not contain such bulky D-ring substitutions, and smaller modifications at this position generally do not confer strong isoform selectivity. Thus, the statement in the manuscript was intended to highlight the comparison between the two binding sites rather than imply that selectivity cannot be achieved through the blebbistatin site. Nevertheless, the Reviewer is absolutely correct that the previous wording was misleading, therefore we changed wording (Extended Data, line 927-961).

8. Figure 3G: Authors should discuss how specific inhibition can be explained if the "R(+)2501/CK-571 binding site" is identical in in human SMM, non-muscle myosin-NM2B, β-cardiac myosin heavy chain, and fast skeletal muscle myosin-2x/d.

We agree that sequence conservation of pocket-lining residues makes it difficult to explain the observed selectivity solely by sequence differences within the pocket.

Importantly, our structural analysis suggests that selectivity arises from differences in conformational accessibility of the binding cleft rather than sequence divergence within the pocket itself. The findings with R(+)2501 are similar to what has been described for CK-571 (PMID: 27815532). The myosin structural state in which these compounds bind correspond to a transient state explored when the motor is bound to ATP to reprime the motor. Our hypothesis is that these transient states are sufficiently different from one myosin to another as they depend on the overall sequence of the motor. Thus, this explains why other Myo2 motors may not bind these inhibitors as efficiently as smooth muscle myosin. This hypothesis provides a structural explanation for how isoform selectivity can arise despite apparent conservation of the binding pocket.

9. In the legend for Figure S2, the color attributed to the smooth muscle myosin (SMM) curve is incorrectly written as "pink"; it should be "orange" to match the actual figure. Also, the legend for Figure S2 incorrectly refers to "panel D" after panel B. Since Figure S2 contains only panels A, B, and C, the reference should be changed to "C".

Based on Reviewer comments, in the revised manuscript, we have carefully revised coloring, labeling and presentation of all panels, so now these mistakenly labeled panels are corrected.

10. Figure S3: "W1" and "W2" (structurally important water molecules?) should be explained in the figure legend.

In the revised manuscript, we have now clarified in the new Extended Data Fig. 5 legend (line 1019-1022) that W1 and W2 denote structurally important water molecules involved in stabilizing the ligand–protein interaction network.

11. The legend for Table S3 should be corrected to refer to Figure S3, not Figure S5.

In the revised manuscript, we have corrected the legend for new Extended Data Table 3 so that it now correctly refers to new Extended Data Fig. 5.

Referee #3 (Remarks to the Author):

I co-reviewed this manuscript with one of the Reviewers who provided the listed reports.

We thank the Reviewer for their time and contribution to the evaluation of our manuscript. We appreciate the careful review and have addressed the comments provided in the detailed responses for Reviewer 1 and 2.

We are grateful to the Reviewers for their insightful comments and suggestions, which have helped us improve both the rigor and clarity of the manuscript. The additional experiments expanded methodological descriptions, and revised interpretations have strengthened the mechanistic framework of the study while preserving the central findings. We hope that the Reviewers and the Editor will agree that the revised manuscript now provides a clearer and more comprehensive account of the discovery and physiological impact of R(+)2501 in relieving pericyte-mediated capillary constriction and restoring microvascular perfusion after ischemia. We believe that the combination of structural, pharmacological, and in vivo physiological evidence presented here establishes a new strategy for targeting capillary no-reflow and may open new avenues for therapeutic intervention in ischemia–reperfusion injury.

We thank the Reviewers and the Editor for their careful evaluation of our manuscript and for the many constructive suggestions that helped us substantially strengthen the study. In response to the Reviewers' comments, we have performed several new experiments and expanded the analysis in multiple areas. These include:

- **direct visualization of pericytes demonstrating R(+)2501–induced pericyte relaxation,**
- **diameter-dependent analysis of vascular responses confirming capillary-selective dilation,**
- **dose-dependent systemic hemodynamic measurements in rats showing no adverse cardiovascular effects at therapeutically relevant doses, and**
- **direct visualization of neutrophil granulocytes demonstrating R(+)2501–induced removal of neutrophil plugging from capillaries in rat AMI model**

We have also added Polder maps supporting ligand occupancy in the structural models, expanded compound characterization and analytical purity data, clarified pharmacokinetic and metabolic analyses, and substantially revised the Methods and Discussion sections to ensure full reproducibility, and to highlight clinical relevance.

In addition, we have refined the mechanistic discussions, moderated interpretations where appropriate, corrected several inconsistencies in figures and legends, and clarified experimental details throughout the manuscript.

To further strengthen the manuscript, we invited four additional co-authors:

- **Dr. Svetlana Mastitskaya from University of Bristol, who performed the pericyte visualization experiments.**
- **Prof. C. Michael Gibson, President of the Baim Institute for Clinical Research at Harvard, who contributed to highlighting the clinical relevance of the study.**
- **Tímea Bálint, from Heart and Vascular Center, Department of Experimental Cardiology and Surgical Techniques, Semmelweis University, who performed rat hemodynamic measurements.**
- **Dávid Nagy, from Heart and Vascular Center, Department of Experimental Cardiology and Surgical Techniques, Semmelweis University, who performed rat hemodynamic measurements.**

Together, these revisions significantly strengthen the mechanistic interpretation and improve the clarity and transparency of the study.

Referee #1 (Remarks to the Author):

This is an exciting manuscript that reports a novel approach to restoring blood flow to organs that have been made ischemic, as occurs in heart attack, stroke and acute kidney injury. In brief, previous work has shown that, after ischemia, the constriction of capillaries by pericytes that occurs during the ischemic period outlasts the period when the supplying artery or arteriole is closed, leading to a decrease in tissue blood flow even when the input artery/arteriole is reperfused (so-called "no-reflow"). Previous work has attempted to restore blood flow using agents that affect upstream signaling (typically calcium entry that evokes contraction). Here the authors develop a novel drug that directly inhibits smooth muscle myosin-mediated contraction. This is shown to relax pericyte-mediated capillary constriction, but to have a smaller effect (for brief application) on arteries/arterioles because of the greater diffusion barrier to entry into those cells (which may help prevent a general fall of blood pressure and tissue perfusion). Even a brief application of the drug produces a long-lasting relief from capillary constriction in the post-ischemic kidney and heart, leading to less no-reflow, less tissue damage and improved survival.

Remarkably, they are also able to show that when no-reflow occurs after cardiac ischemia, then it evokes downstream no-reflow in the kidney (a model of cardio-renal syndrome), and both are relieved by injection of their drug.

The manuscript is potentially suitable for publication, subject to the authors addressing the following points which would significantly improve it.

We thank the Reviewer for the careful reading of our manuscript and for the positive and encouraging assessment of our work. We appreciate the Reviewer's recognition of the novelty of the approach and the potential implications for treating no-reflow and ischemia-reperfusion injury. We have carefully considered all the comments below and have revised the manuscript accordingly to address each point.

1. Omission of consideration of neutrophil-mediated capillary block

Previous work has shown that when pericytes constrict capillaries then neutrophils (which are more rigid than red blood cells) get stuck at the pericyte constrictions, significantly adding to the reduction of blood flow that occurs (PMID: 35316222, see Suppl Fig 5B). It is surprising that the authors show no data on whether their drug reverses capillary occlusion by neutrophils. This could easily be done by using neutrophils labeled genetically or with fluorescent antibodies (PMID: 26019296). This would add mechanistic understanding of how their drug is working.

We thank the Reviewer for this insightful comment and for highlighting the important contribution of neutrophil plugging at pericyte constrictions to the microvascular obstruction that develops after reperfusion.
Based on this comment, we performed additional experiments to directly visualize neutrophil accumulation in the infarcted myocardium after LADO in rats (Methods, line 1749-1775). As suggested, neutrophils were labeled using

fluorescent anti-Ly6G antibodies and imaged in the cardiac capillaries following reperfusion. Consistent with prior reports that rigid neutrophils can become trapped at pericyte-mediated capillary constrictions (PMID: 35316222), we observed marked neutrophil accumulation within capillaries in the untreated LADO group. Importantly, R(+)2501 treatment qualitatively reduced neutrophil trapping in the microcirculation (new data shown in new Fig. 5e; Results – line 435-451).

These findings are consistent with the proposed mechanism where R(+)2501 rapidly and durably restores capillary diameter and prevents the formation of narrow segments that promote mechanical trapping of neutrophils. This is highly relevant for reperfusion injury because neutrophil plugging is a major secondary amplifier of microvascular obstruction (Discussion, line 624-632).

2. Kinetics of the restoration of blood flow

It is surprising that injection of a bolus of the drug leads to a long-lasting abolition of no-reflow (perhaps for as long as 7 days if one can infer that blood flow is still restored if the animal is still living). Perhaps I have missed it but I think it would be good to have an explicit statement of, or graph showing, the longest measurement of blood flow which showed a persistent restoration of blood flow.

The longest time period during which we directly monitored microvascular blood flow parameters after drug administration was 2 hours for AMI model and 1 hour for AKI model, and this has now been explicitly stated in the revised manuscript (Methods, for AMI line 1733-1735, for AKI line 1638).

While direct flow measurements were limited to this time window, we also assessed long-term (14-day) functional outcomes in the renal artery occlusion (RAO) model. In these experiments, R(+)2501 treatment resulted in sustained improvement of renal function, as reflected by preserved eGFR, compared with untreated RAO animals (new Fig. 4o and new Extended Data Fig. 18). These findings indicate that, even though continuous flow measurements were not performed beyond the 1-hour monitoring period, a single bolus treatment maintains capillary patency and preserves tissue perfusion long after the drug is cleared.

It is not at all obvious that relatively brief application of the drug should lead to a prolonged relief of no-reflow. An obvious question is, once the drug has been removed from the blood, why does it not dissociate from the myofilaments, thus enabling constriction and a fall of blood flow again if the stimulus to constriction is still present (such as high [Ca] in the pericytes, or ischemia-evoked norepinephrine release in the heart)? This should at least be discussed in the paper.

This is an important mechanistic point that we now discuss in the revised manuscript (Discussion, line 612-623). Importantly, once capillary diameter is restored and microvascular flow resumes, the hypoxic and ischemic stimuli that drive pericyte rigor rapidly dissipates. Restoration of oxygen delivery allows

cellular ATP levels to recover within minutes, enabling normalization of cellular metabolism and calcium handling. As a result, the physiological drivers of sustained contraction diminish in the time range while R(+)2501 concentration is still high enough to keep capillaries open by pericyte relaxation.

Furthermore, O'Farrell et al. (PMID: 29120327) showed that a transient 15 min adenosine infusion around reperfusion significantly reduced cardiac no-reflow, even though adenosine clears from blood within seconds. In our case, a single i.v. bolus of R(+)2501 produced blood exposure over a similar time scale, with an in vivo half-life of 16 min. We therefore suggest that transient relaxation by R(+)2501 restores sufficient capillary patency to interrupt no-reflow. Once capillary flow is restored, local hypoxia and ischemia are relieved, reducing the drive for pericyte constriction. We have now added an explicit discussion of this point to the revised Discussion (line 612-623).

In Fig. 6, R(+)2501 restores renal capillary diameter and blood flow within 10 mins, but kidney functon(eGFR) recovers only over 10 days. Why is that? Does it take time (10 days?) for the counter-current multiplier to set up the osmotic gradients needed for renal function after blood flow is restored. Please discuss this.

We thank the Reviewer for this insightful question. The apparent difference in time scales reflects the distinction between rapid restoration of microvascular perfusion and the slower recovery of organ-level renal function after ischemic injury.

In our experiments, R(+)2501 restores peritubular capillary diameter and blood flow within minutes (~10 min), indicating rapid reversal of the microvascular obstruction responsible for no-reflow. However, glomerular filtration rate (eGFR) reflects the integrated function of multiple renal structures that may remain temporarily impaired after the ischemic episode, including tubular epithelial cells, endothelial cells, and the tubuloglomerular feedback system.

During renal ischemia–reperfusion, tubular epithelial cells, particularly in the proximal tubule and thick ascending limb, undergo transient injury characterized by ATP depletion, cytoskeletal disruption, loss of polarity, and tubular obstruction by cell debris and casts. Even after perfusion is restored, these cells require several days to recover structural integrity and transport capacity, which directly affects the kidney's ability to generate filtrate and maintain normal electrolyte gradients.

The Reviewer's suggestion regarding the counter-current multiplier is conceptually related: restoration of the corticomedullary osmotic gradients and normal tubular transport processes also depends on recovery of tubular epithelial function and medullary microcirculation. These processes occur on a multi-day timescale, consistent with the gradual improvement in eGFR that we observe.

Thus, our data indicate that R(+)2501 rapidly restores the upstream microvascular perfusion defect within minutes, while the downstream recovery of tubular and nephron function occurs over days as ischemia-injured cells repair and normal nephron physiology is re-established. We have added a brief discussion of this distinction in the revised manuscript (Discussion, line 656-664).

3. Effect of size on the effect of R(+)2501

The paper shows convincingly that short-term application of the drug has a larger effect on capillaries than on larger vessels. But in a clinical situation it is likely that the drug would be given as more than a single bolus I-V. There should be some discussion whether this would actually matter, i.e. of how much a mean fall of blood pressure would matter, given the presence of autoregulation of the blood supply to many organs.

We thank the Reviewer for raising this important translational point. The intended clinical application of R(+)2501 is not systemic IV administration, but rather a single intracoronary bolus injection delivered through the PCI guide catheter immediately after reopening the occluded coronary artery. This approach allows high local concentrations in the ischemic myocardial microcirculation while maintaining very low systemic exposure.

We modeled this approach in our experiments, where R(+)2501 was delivered intracoronary into the pig heart (Methods, line 1845-1873). Under these conditions, as opposed to other smooth muscle relaxants such as nitroglycerin, we did not observe detectable effect on systemic blood pressure, consistent with the localized delivery strategy (new Fig. 6).

In the revised manuscript, we present a novel experiment demonstrating the specificity of R(+)2501 (Methods, line 1473-1500). This experiment shows that the compound is highly selective for capillaries, dilating them by 50–120%. In contrast, only a 0–22% opening is observed in arterioles, and there is no effect on larger vessels (Results, line 237-247 and new Fig. 3d). We believe that this experiment enhances the significance of this article, as it is the first time pericytes have been specifically dilated. This capillary specificity enabled us to address the question of how pericyte relaxation itself (without arteriolar and larger vessel dilation) affects hemodynamics.

To further address this question and the Reviewers' concern, in the revised manuscript we also performed hemodynamic measurements in rats using an intravenous bolus, which likewise did not produce a measurable decrease in systemic blood pressure or systemic blood flow (Results, line 262-274; new Extended Data Fig. 11 and Methods, line 1535-1558).

Thus, the intended intracoronary administration ensures that systemic concentrations remain low, while the local concentration in the ischemic microvasculature is sufficient to achieve pharmacological efficacy. We have

now clarified this point in the revised manuscript discussion (Discussion, line 675-680).

4. Capillary constriction

Were capillary diameter measurements made solely at pericyte somata, where the largest constriction occurs because most circumferential pericyte processes are near the cell soma, or were they made at arbitrary positions on capillaries.

In the original manuscript, capillary diameter measurements were performed at random positions along capillaries in both the kidney and heart microcirculation.

In response to the Reviewers' suggestions, we have now conducted a comprehensive new set of experiments in collaboration with Dr. Svetlana Mastitskaya at University of Bristol (added as a new co-author in the revised manuscript), in which pericytes were directly visualized (Methods, line 1502-1533). This allowed us to specifically analyze capillary diameter changes at pericyte somata, too, where the strongest constrictions are known to occur due to the high density of circumferential pericyte processes. These new data directly demonstrate the effect of R(+)2501 on pericyte-mediated capillary constriction, and the quantitative analysis of capillary diameter changes at pericyte somata has now been incorporated into the revised manuscript (Results, line 248-261; Fig 3e,f, Supplementary video 1, line 1131-1137).

5.Movies

I'm concerned about exactly what the first movie shows. For Video 1, when the blood is moving slowly (during and immediately after ischemia) the green RBCs are clearly moving within the confines of the (black) blood vessels. But at the start of the movie, in healthy conditions, there seem to be green streaks (RBCs?) that simply move all the way across the image from left to right. Is this some kind of illusion or artefact?

Thank you for this careful observation which may have been confusing for the readers. The apparent "streaks" seen at the beginning of Video 1 are most likely an optical effect related to the scanning orientation of the laser during image acquisition combined with the very high velocity of RBC movement under normal flow conditions.

When RBCs move rapidly through the capillaries, their displacement during the scanning time of a single frame can create elongated fluorescent streaks that may appear to traverse the image at an angle relative to the vessel boundaries. This can give the visual impression that the labeled RBCs are moving outside the vessel lumen, although they are in fact confined within the microvascular structures.

In contrast, during ischemia and early reperfusion, RBC velocity is markedly reduced, allowing individual cells to be resolved more clearly within the vessel lumen, which eliminates this apparent streaking effect.

Thus, the phenomenon likely reflects a well-known interaction between fast-moving fluorescent particles and raster scanning microscopy, rather than RBCs moving outside the vascular compartment. We have clarified this point in the revised manuscript (Supplementary Video 2 legend, line 1139-1145).

More minor points

Line 26: CNR doubles mortality - in which tissue?

The statement refers to myocardial infarction (reference PMID: 20488311), and we have now modified this point in the revised manuscript.

Line 48: Ischemia-evoked pericyte contraction was I think first reported in PMID 17036005 and PMID 19718040.

Thank you for this clarification, we have incorporated the two suggested references in the revised manuscript (Introduction, line 53-56 and References 6 and 7)

Line 79: This is a bit too negative: in Alzheimer's disease nimodipine unblocks brain capillaries by relaxing pericytes (PMID: 39294491).

We have now clarified in the revised manuscript that although L-type Ca^{2+} channel blockers can relax pericytes under certain conditions, such as in Alzheimer's disease (Introduction, line 78-89 and Reference 27). However, under severe hypoxia the overall intracellular Ca^{2+} elevation in pericytes is largely independent of L-type Ca^{2+} channel influx. As a result, blocking these channels produces only a minor reduction in total intracellular Ca^{2+} levels, making Ca-channel blockers relatively ineffective in this setting. This clarification has been briefly incorporated in the revised Introduction (line 78-89).

Fig S3-S5 legends. Explain what B-factor means and its relevance to what is shown.

We have now added a detailed definition of B-factor and its relevance in the revised manuscript in the legends of new Extended Data Fig. 7 (line 1023-1028).

Fig 3. To aid understanding please can you show where the "canonical blebbistatin cleft" is located and give a reference to its definition and location? In addition explain briefly what Transducer, Relay and Converter mean.

We have clarified in the revised manuscript that the canonical blebbistatin cleft is where S(-)2501 binds and added references for the definition and location (References 44, 47 and 49) in the revised manuscript). We added a short definition of functions of the Transducer, Relay and Converter regions in the legend of new Fig. 2 (line 195-205).

Figures in general: Many of the lines (e.g. the s.e.m. lines on Fig 3A and 3E and 3H) and lettering on the figures (Fig 3G, lettering on pink and on green bars) are too small to read easily when printed out at normal paper size.

We have revised all figures to make all labeling and lines visible.

Fig. 5B. Why are the kidney cells/tubules green? Explain in the legend. Label the cells/tubules in the field of view to aid the reader's understanding

The green signal arises from autofluorescence of renal tubules and cells. This has now been clarified in the revised legend of new Fig. 4 (line 346).

Fig. 6N. Add to the y axis label the fact that this is "7 day surviving animals"

We have added the requested important label and legend of new Fig. 4p (line 373).

Fig 7F-G. Is it possible to say at which end of the capillary end the capillary diameters were measured (arterial or venous) and whether epi- or endocardial?

Capillary diameters in these experiments were measured at random positions within the epicardium, without distinguishing between the arterial or venous ends of the capillaries. This has now been clarified in the revised manuscript new Fig. 5 (line 469).

Line 491. Define CRS-1.

We have defined CRS-I in the subheading (Results, line 494).

Line 512. State that R(+)2501 was given after unblock of the LAD. This is crucial.

Thank you for this important observation. We have added a clarifying sentence to the result section (Results, line 515-518).

The colors in many figures are too similar to distinguish. Coupled with the tiny keys

provided, this makes for painful reading of the figures. For example in Fig 7n and others, change the colors so they are easily distinguishable, and not different shades of the same color. The letter thickness is also often way too thin, e.g. “end of infusion” in Fig 7o. Doubling the font size for all the tiny words on the figures would help.

We have revised all figures to make all labeling and lines visible and changed the colors throughout the figures to make the lines and conditions more distinguishable.

Fig 8c. Mark on all the panels which direction is time, which is distance, and add a mean speed value for each record

We have labeled time and distance on new Fig. 4-5 and added mean velocity and flux values to all presented panels.

Please explain why the RBC flux and velocity measurements are so incredibly noisy, with large variations on a minute-minute time scale.

The apparent “noise” largely reflects real microvascular heterogeneity plus measurement constraints at the capillary level, rather than instability of the preparation.

Capillary flow is intrinsically intermittent and heterogeneous: RBC traffic occurs as discrete cells and short trains, with frequent brief stalls/restarts due to upstream network dynamics. Small changes upstream can cause large swings in a single capillary’s flux/velocity from minute to minute.

Sampling a tiny field can also magnify variability. At each time point we quantified a small number of capillaries and relatively short time windows (10 sec), so stochastic variation in cell arrival times appears as large relative fluctuations.

Technical factors add variance at high speeds: with raster scanning, fast-moving RBCs can be partially sampled within a frame, producing streaking and frame-to-frame differences in apparent displacement; small motion/registration errors and occasional out-of-plane movement also contribute.

We now clarify these points in the revised manuscript (Methods, line 1674-1681) and emphasize that conclusions are based on group-level trends and time-averaged/pooled analyses across many capillaries and animals, which are robust despite expected capillary-level variability.

Fig S2. Please make all the curves darker and the points larger, so they can be seen more easily when printed.

As mentioned above, we have revised all figures to make all labeling and lines visible and changed the colors throughout the figures to make the lines and conditions more distinguishable.

Fig. S8. Clarify whether the velocity and flux measurements were made during the ischemia, or on reperfusion (and how long reperfusion).

We have clarified in the revised of new Extended Data Fig. 14 that velocity and flux measurements were made in the reperfusion phase after 10, 30, 60 and 120-minute ischemia (line 1061-1071).

Fig S13. As for S2, make the colors more different, and explain what the horizontal lilac line is at 100% (it's not in the key).

As mentioned above, we have revised all figures to make all labeling and lines visible and changed the colors throughout the figures to make the lines and conditions more distinguishable, and we also carefully revised all key for the colors and symbols in the figure legends throughout the manuscript.

Table S1 is impossible to read because it is too small. Rotate it 90 degrees on the page and make it fill the page, make the chemical structures darker, and double the font size for all the numbers.

We have restructured the presentation of new Extended Data Table 1. to make all numbers and chemical structures visible and readable.

Line 1509. Give more explanation for exactly how the heart was gently fixed - people will want to repeat this. Similarly for the kidney.

We have supplemented the experimental description of kidney (Methods, line 1627-1642) and heart visualization (Methods, line 1710-1747) with additional details that we believe now provide sufficient information for reproducibility by other groups.

Referee #2 (Remarks to the Author):

In this manuscript from Pénzes and colleagues, the authors report the unexpected finding that the R(+) enantiomers of a series derived from the myosin II inhibitor blebbistatin show activity against smooth-muscle myosin (SMM) and bind a previously unrecognized allosteric pocket in the pre-hydrolytic state of SMM, whereas the S(-) enantiomers occupy the canonical blebbistatin cleft. Further, the authors show disruption of ischemia-associated capillary constriction. The direct, capillary-resolved in vivo measurements are a real strength of the paper with excellent physiology and innovative imaging. However, key data and methods necessary for interpreting the results are missing, there are gaps in the mechanism as presented, and the results are consistently overinterpreted.

We thank the Reviewer for the careful evaluation of our manuscript and for recognizing the strengths of the study, particularly the direct, capillary-resolved *in vivo* measurements and imaging approach. We appreciate the Reviewer's constructive comments regarding missing methodological details, mechanistic interpretation, and potential overinterpretation of certain results. In response, we have revised the manuscript to provide additional methodological information, include new experimental data where appropriate, and refine the discussion to more carefully distinguish between our experimental findings and their mechanistic implications.

Major Comments:

1. Overall, the introduction's claims/statements need to be softened. An example - "Crucially, CNR does not result from residual emboli or overt vasospasm… (line 46)". Certainly emboli and vasospasm alone do not explain all cases, but they are considered contributors.

We thank the Reviewer for this note. We have carefully revised the wording in the Introduction and toned down several statements, including the one cited, to more accurately reflect that embolization and vasospasm can contribute to CNR, even though they do not fully explain the phenomenon in many cases.

2. Regarding the data and analysis associated with Figure 2:

A. Data for characterizing the activity of R(+)2501 are missing. The manuscript states "Building on published MPH and blebbistatin derivatives, we synthesized and tested over 150 enantiomerically pure derivatives (CLN compound family) against six myosin-2 isoforms: smooth muscle, skeletal muscle, cardiac muscle, and three non-muscle myosin variants. (lines 146-148)" Activity of the skeletal and non-muscle myosin variants is never shown, aside from one statement that was not supported with data analysis ("…showed 3-9x higher selectivity with less than 20% inhibition on NM2A").

These are important data because, as the Reviewers state, these myosins are “essential for vital physiological functions.”

We thank the Reviewer for highlighting the need to better present the myosin isoform selectivity data. In the revised manuscript, we have added a reference describing the development of the CLN compound family (Introduction, line 131, PMID: 40829259), which provides the broader context for the synthesis and screening of this compound series.

We have also restructured the figures presenting the ATPase inhibitory profiles of R(+)2501 and S(–)2501 and now show their activity across all tested myosin-2 isoforms, including smooth, cardiac, skeletal, and non-muscle myosin-2 variants (Fig. 1c,d,e). These data now directly support the statements regarding selectivity that were previously summarized only in the text.

For the remainder of the compound series, however, we retained the comparative presentation of smooth vs. cardiac myosin inhibition, as these isoforms are physiologically the most relevant for the intended therapeutic application. Given the transient exposure and rapid systemic elimination of R(+)2501 in vivo, inhibition of smooth muscle myosin (targeting pericyte contraction) and the potential effect on cardiac myosin represent the most critical considerations for pharmacological and safety interpretation.
The revised text (Results, line 131-146) and figures (in new Fig. 1c,d,e; Extended Data Table 1) now clarify this rationale and provide the full isoform activity profile for R(+)2501.

B. The manuscript presents inhibitory-ATPase data for R(+) and S(-) enantiomer pairs, but not their solubility in the assay buffer - a factor that can artificially depress the maximal inhibition “Max.Inh.” and distort the derived IC50 and selectivity values. These data are straightforward to obtain and without them, a < 100 % Max.Inh could simply reflect compound precipitation in the assay buffer rather than a genuine mechanistic limit. Similarly, the Methods lack description of the ATPase assay itself. It is not possible to assess whether the reported potency and selectivity truly derive from the compound’s pharmacology without these details.

We thank the Reviewer for raising this important point. We agree that limited solubility could lead to an apparent reduction in (maximal) inhibition, potentially affecting the derived IC50 and selectivity values. To avoid such artifacts, each compound was checked after dilution into the ATPase assay buffer at the highest tested concentration (50 μM), and we observed no visible turbidity or precipitation before and after the assay. This approach ensured that the observed inhibition was not limited by precipitation under the assay conditions. Although we did not experience precipitation in the ATPase measurements, since the buffer condition of each myosin isoform ATPase measurement was the same, the compound’s specificity is reflected by these measurements even if minor precipitation could happen. Thus, gross precipitation under the assay

conditions is unlikely to account for the observed inhibition profiles. We have now clarified this point in the revised Methods (line 1180-1202).

At the same time, we did not determine the formal solubility limits for every compound, because during the early SAR screening phase the compounds were often available only in small quantities, making detailed solubility measurements impractical. We therefore do not claim to have established absolute aqueous solubility values for each analogue, only that under the conditions used for ATPase measurement we did not detect visible precipitation at the concentrations tested. We also note that the R(+) and S(–) enantiomer pairs were assayed under identical experimental conditions, so the marked stereoselective differences are unlikely to be explained by assay-buffer solubility artifacts alone.

In response to the Reviewer's comment, we have now expanded the Methods (line 1180-1202) section to include a detailed description of the ATPase assay, providing all relevant experimental parameters required for reproducibility of the measurements.

C. The selectivity panel in Figure 2A should be limited to the compounds that are described and characterized in the manuscript. Although showing data for the entire library of ~150 enantiomeric derivatives would support the claim that "R(+) derivatives consistently showed selective inhibition of SMM", what is currently shown does not add to the paper. If the authors wish to present the broader SAR, the synthetic routes/structures, purity profiles, solubility, and full ATPase inhibition curves for all derivatives should be presented as well.

We agree that the presentation of the compound series should be sufficiently supported by data. In response, we now report the inhibitory parameters for all 62 R(+)–S(-) enantiomer pairs shown in previous Figure 2A (new Extended Data Fig. 2) in the revised and expanded in new Extended Data Table 1, providing the full dataset underlying the selectivity trends presented in the figure.

We also agree that complete presentation of the synthetic routes, compound characterization, and full ATPase inhibition curves is important for the comprehensive description of the compound library. However, these elements are primarily related to the medicinal chemistry development of the CLN compound family and do not directly contribute to the central biological and mechanistic message of the present manuscript. Therefore, we are currently preparing a separate manuscript (Szimler et al., in preparation) that will describe the full SAR, synthetic routes, compound characterization, and detailed biochemical profiles of the entire compound library.

However, based on the Reviewer's comments we have incorporated the synthetic route (new Extended Data Fig. 3) and full analytics (new Extended Data Fig. 4) of R(+)2501 in the supplementary information and Methods (line 1204-1285) sections.

Nevertheless, we believe that showing the consistent SMM–selectivity trend across the R(+) enantiomer family remains an important addition to the present manuscript, as it strengthens the key finding that enantiomer type determines binding to a distinct allosteric pocket, enabling selective SMM inhibition and efficient pericyte relaxation.

D. Many of the ATPase inhibition curves presented in Figures 2 and S2 are based on only three concentration points per compound. Even when a quadratic binding model is applied (as the authors state) and the total enzyme concentration is fixed, at least two free parameters must have been estimated – the inhibitory constant (KI) and the maximal inhibition "Max.Inh". Fitting such a sparse data set risks over- and/or misinterpretation because experimental errors or potential artifacts can disproportionately affect the fitted values. The authors should: (I) repeat the assays with a broader concentration range (at least 5-6 points) to ensure robust parameter estimation, (II) disclose how many replicates were performed at each concentration, and (III) explain what the error bars in the figures represent.

As mentioned above, during the initial SAR screening campaign, compounds were typically synthesized and purified only in sub-mg quantities in enantiomerically pure form. For this reason, the ATPase assays were performed using three concentration points, which provided a rapid but reliable estimate of inhibitory potency and maximal inhibition, sufficient to evaluate selectivity versus cardiac myosin and guide the medicinal chemistry optimization.

This approach is a standard strategy in early SAR campaigns and has proven effective in our previous work. For example, using the same workflow we successfully identified MPH-220 as a skeletal muscle myosin–selective inhibitor (*Cell* 2020 Oct 15;183(2):335-346, PMID: 33035452), which has now advanced to Phase 2 clinical trials. In the present campaign, the same strategy enabled efficient identification of R(+)2501 as the lead compound for the capillary no-reflow indication.

Because the three-point titration already provided consistent and clear selectivity trends, repeating all measurements with 5–6 point titrations would not meaningfully change the estimated parameters or alter the conclusion that R(+)2501 is the most suitable candidate for further development. The best selected compounds such as R(+)2501) were re-synthetized (see new Extended Data Fig. 2 and synthesis description in Methods, line 1204-1285) and we measured the ATPase in more detailed way at 5-6 different concentrations on several myosin isoforms (new Fig. 1).

However, we agree that the description of the ATPase assay was previously insufficient. Therefore, in the revised Methods (line 1180-1202) section we have expanded the assay description, including the number of experimental replicates and the definition of the error bars shown in the figures, to ensure full transparency and reproducibility of the measurements.

E. The manuscript describes the use of a “quadratic model” to derive the inhibitory constants (KI) directly. Yet, compound potencies are reported as IC50s and maximal inhibition. KI and IC50 can differ markedly (e.g. depending on enzyme concentration in the assay). The methods section should be clarified with the exact fitting equation, and the assumptions, if any to allow Reviewers/readers to assess the reported selectivity and potency metrics. Also, the manuscript states that selectivity (S) was derived as "S = (Inhibition of SMM at 50 µM R(+)2501 concentration/Inhibition of CM at 50 µM R(+)2501 concentration) x (IC50 of CM/IC50 of SMM)" (Table 1). This formula needs a clear, step-by-step explanation. What is the reason for using a single, fixed-concentration inhibition value (50 μM)? This can be misleading due to factors such as compound solubility.

We thank the Reviewer for this important request for clarification. In the revised Methods (line 1180-1202) section, we now explicitly provide the quadratic equation used to calculate IC50 values, with the myosin concentration fixed to the assay conditions (as performed in the fits). We agree that KI and IC50 can differ, particularly when enzyme concentration is not negligible, and we therefore clarified the exact fitting model, parameters, and assumptions so readers can interpret potency and selectivity appropriately.

Regarding the selectivity metric, we used the cited formula because relying on IC50 and Max.Inh alone can be misleading in early SAR datasets. For example, a very weak inhibitor may still extrapolate to a high Max.Inh, while another compound can yield an apparently favorable fitted IC50 despite producing only minimal inhibition within the experimentally tested range. We therefore included the observed inhibition at a fixed relatively high concentration (50 μM) so that the selectivity score reflects not only fitted potency, but also the actual inhibitory effect measured at the top dose. In this way, the metric captures both the relative potency between smooth vs cardiac myosin, and the extent of inhibition actually achieved under the assay conditions, allowing a more robust distinction between strong inhibitors (substantial inhibition at 50 μM with low IC50) and poor inhibitors (where one or both criteria are not met).

Finally, as noted above and now clarified in the revised Methods (line 1180-1202), compound solubility was always checked at 50 μM in the assay buffer, and compounds were only analyzed under conditions where no precipitation was observed, minimizing the risk that reduced inhibition at 50 μM reflects solubility artifacts rather than real pharmacological effect.

3. Metabolic stability methods are needed. Also, the Methods section “HPLC analysis of rat plasma and tissue samples and plasma sample collection” describes collection of brain, heart and kidney. However, data presented in the manuscript do not correspond to this. A later Methods section, “Tissue sample collection” lists all of the tissue types presented in Fig. S6. However, several of the detailed doses are not reported (20 and 80 mg/kg). Also, did the animals survive the 80 mg/kg (IV) dose of R(+)2501? And how well tolerated were doses required later for CNR improvements?

We thank the Reviewer for pointing out these inconsistencies. In the revised manuscript, we have modified and clarified the Methods (line 1317-1330) section describing the hepatocyte metabolic stability assay and unifying the two tissue preparation sections to better distinguish the procedures used for plasma analysis and for the tissue distribution experiments.

We also corrected the description of the dosing scheme: the 20 and 80 mg/kg doses were not used in the tissue accumulation experiments it was mistakenly left in the original manuscript as a typo, and this has now been corrected in the revised Methods paragraph (line 1411-1419) text.

Regarding tolerability, as shown in Extended Data Table 4, we did not observe any adverse events even at doses up to 200 mg/kg.

4. The manuscript repeatedly claims that R(+)2501 acts in a pericyte-targeted manner, yet no data directly demonstrate preferential engagement of pericytes over other SMM-expressing tissues, including arteriolar smooth muscle. In vivo measurements localizing capillary constriction and dilation to pericyte somata/processes, or cell type-specific readouts of target engagement, are required to support this claim. Moreover, while arterial pressure is monitored in the pig experiments (Fig. 9), quantitative blood pressure data are not presented, and systemic hemodynamics are not reported for the rodent kidney, MI, or CRS-I models. Given the central role of smooth muscle myosin in vascular tone, preserved blood pressure alone cannot be used to infer pericyte specificity.

We agree that additional experimental evidence is supportive for the pericyte-targeted mechanism proposed in the manuscript. Based on this and related Reviewer comments, we have performed several new sets of experiments, which are now incorporated into the revised manuscript.

First, we performed direct visualization of pericytes (Methods, line 1502-1533) and their contractile state in the microcirculation in collaboration with Dr. Svetlana Mastitskaya (University of Bristol), who has now joined the author list. Using pericyte labeling, we directly demonstrated that R(+)2501 relaxes pericyte-mediated capillary constrictions (Results, line 248-261 and new Fig 3e,f), with quantitative analysis of capillary diameter changes specifically at pericyte somata. These new experiments provide direct evidence that the drug acts at the level of pericytes.

Second, to address the concern regarding effects on other smooth muscle–containing vessels, we performed a detailed analysis of vasodilatory responses as a function of vessel diameter in the cardiac microcirculation at 25 μM and 100 μM inhibitor concentrations (Methods, line 1473-1500). These data show that R(+)2501 selectively dilates capillaries (Results, line 237-247 and new Fig. 3d), while arterioles and larger arteries remain unaffected, supporting the notion that

the compound primarily relieves capillary constriction rather than producing generalized vascular smooth muscle relaxation.

Third, we performed dose-dependent systemic hemodynamic measurements in rats, including pressure–volume (PV) loop analysis, to evaluate potential systemic cardiovascular effects (Methods, line 1535-1558). These measurements (Results, line 262-274 and new Extended Data Fig. 11) demonstrate no detectable adverse effects on cardiac hemodynamics or PV parameters up to the highest dose used in the efficacy experiments (20 mg/kg).

Together, these new experiments provide direct visualization of pericyte relaxation, vessel diameter–dependent vascular effects consistent with capillary selectivity, and comprehensive systemic hemodynamic measurements, strengthening the mechanistic interpretation that R(+)2501 primarily acts by relieving pericyte-mediated capillary constriction without producing systemic vascular smooth muscle relaxation.

5. The reported dual-enantiomer, dual-site mechanism is highly unusual: two stereoisomers of the same scaffold inhibit the same enzyme by engaging two spatially distinct pockets, each capable of producing potent inhibition through different molecular interactions. Because such a claim demands the highest level of experimental certainty, the authors should provide unequivocal evidence that the observed activity of R(+)2501 is not an artifact of synthetic impurities (e.g. residual S(-) material, by-products, or precursors). As is standard for these types of studies, the manuscript should include: (I) Complete synthetic routes for each enantiomer with reagents, conditions, yields, etc to enable reproducibility and assessment of possible contaminant formation. (II) Rigorous purity data including HPLC chromatograms for the final compounds together with chiral-HPLC traces that report the enantiomeric excess. (III) Full spectroscopic characterization, including ^{1}H-NMR, ^{13}C-NMR, and ESI-MS spectra for both purified enantiomers.

We have performed detailed analytical characterization of both the R(+) and S(–) enantiomers. These analyses demonstrated that the enantiomeric purity exceeds 99.5% for both preparations, with all other detectable impurities below 1%. The corresponding analytical and chiral HPLC chromatograms showing 100.0% enantiomer purity of R(+)2501 have now been included in the Supplementary Materials (new Extended Data Fig. 4). In addition, we now provide ^{1}H NMR spectra of R(+)2501 (new Extended Data Fig. 4) to support structural identity.

Furthermore, in response to the Reviewer's request, we have expanded the Methods (line 1204-1285) section to include a detailed description of the synthesis and purification procedures for R(+)2501, including the key steps used to obtain the enantiomerically pure compounds.

Together, these new analytical data and expanded methodological descriptions demonstrate that the observed activity of R(+)2501 cannot be attributed to

residual S(–) impurity or other synthetic impurities, and further corroborate the unequivocal conclusion form the crystal structures that the two enantiomers interact with smooth muscle myosin through distinct binding modes.

6. While the reported 2.5 A and 2.30 A crystal structures appear compelling, the authors should provide unbiased electron density evidence that each ligand is genuinely present in its assigned site. Generating OMIT maps - or, preferably Polder maps, which excludes the bulk solvent around the omitted region - would allow an independent assessment of ligand occupancy and avoid model bias. Including these maps at least in the supplementary material would greatly strengthen the claim of dual, stereospecific binding.

We thank the Reviewer for this helpful suggestion. In the revised manuscript we now provide the corresponding Polder maps for the ligand-binding sites to allow an unbiased evaluation of ligand occupancy. These maps are included in the Supplementary Materials (new Extended Data Fig. 1) and clearly support the presence of the ligands in the assigned binding pockets, thereby strengthening the structural interpretation of the dual, stereospecific binding modes.

7. Starting on line 179 the authors argue that the unusually fast on-rates of the R(+) compounds support the existence of a novel allosteric pocket distinct from the canonical blebbistatin site: "These observations suggest that R(+)2501 may bind to..." Faster association (and slower dissociation) can arise even when ligands bind the same site, simply because of differences in binding energetics - eventually leading to different binding constants. If the authors genuinely consider the distinct on-rates to be evidence for their hypothesis, the manuscript would be strengthened by the corresponding kinetic data.

We thank the Reviewer for this thoughtful comment. We plan to publish detailed binding kinetics of the R(+) and S(–) enantiomers in a forthcoming manuscript (Szimler et al, in preparation), because we believe that presenting the full kinetic dataset and analysis within the present study would shift the focus away from the main biological and physiological findings. In the revised manuscript, we have therefore discarded the sentence about binding kinetics of the R(+) and S(–) enantiomers (Results, line 174-184).

8. The manuscript states that “Comparison with CK-571 revealed that R(+)2501 binds deeper within the allosteric pocket and makes additional contacts that may underlie its improved potency" (line 205). However, the reported inhibitory potency of CK-571 (~9 nM against SMM, PMID 27815532) is far higher than the IC50 values observed for R(+)2501 (1–5 μM depending on the species; Table 1). As written, the claim of “improved potency” is ambiguous and appears contradictory. The authors should clarify to which reference compound the potency improvement is being made and/or in which context.

We thank the Reviewer for pointing out this ambiguity. We agree that the sentence could be misinterpreted as implying improved potency relative to CK 571, as correctly noted by the Reviewer. In the revised manuscript, we have corrected the wording (Results, line 185-190) of this sentence to clarify the binding pattern within the pocket without suggesting improved potency.

9. The conclusion of selective dilation of vessels <50 μm associated with data shown in Figure 4 is not directly supported by the presented data. Fig. 4E quantifies only vessels ≥50 μm and therefore cannot demonstrate capillary-selective dilation. Fig. 4D summarizes relaxation kinetics across heterogeneous assays (ex vivo arteries and in vivo capillaries), but does not provide in vivo diameter measurements of <50 μm vessels under the same conditions as Fig. 4E. As presented, the data support a diameter-dependent difference in relaxation kinetics, but do not establish selective in vivo dilation of capillaries or a clear transition point separating capillary from arteriolar responses. Also, 45 minute data is referenced in the text for Fig. 4E, but not shown in the actual figure.

We thank the Reviewer for this important observation and agree that the original presentation did not directly – only implicitly – demonstrated capillary-selective dilation.

Based on this comment (as we mentioned above), we performed a new set of experiments (Methods, line 1473-1500) analyzing vessel diameter–dependent dilation in the cardiac microcirculation at two different concentrations of R(+)2501 (Results, line 237-247 and new Fig. 3d). These experiments allowed us to directly quantify diameter changes across the microvascular tree.

The new data show that capillaries below ~8 μm are very efficiently dilated by R(+)2501 (50-120%), whereas larger vessels remain unresponsive, and 10–15 μm arterioles exhibit only marginal dilation (0-22%) (Results, line 237-247 and new Fig. 3d). These results provide a clear demonstration that the vasodilatory effect of R(+)2501 is strongly biased toward capillaries, with minimal effects on upstream resistance vessels. We appreciate the Reviewer's comment, which inspired this more detailed and elegant analysis of vessel diameter–dependent responses, and the new results have now been incorporated into the revised manuscript (Results, line 237-247 and new Fig. 3d).

In addition, the new pericyte-visualizing experiments (Methods, line 1502-1533) described above (Results, line 248-261 and new Fig. 3e,f) further support this interpretation. These experiments directly show that R(+)2501 relaxes pericytes at capillary constriction sites, demonstrating that the capillary-selective dilation arises from direct relaxation of pericytes rather than from effects on upstream vascular smooth muscle. Original Fig. 4d and e are replaced with the presentation of this new set of experiments.

10. The reported plasma concentrations implied by the authors' own PK data and

statements indicate that functional experiments employed doses that substantially exceed the reported biochemical SMM IC50 of R(+)2501 (1–5 μM), often by one to two orders of magnitude. Collectively, such high doses preclude attribution of the effects to SMM without genetic interventions to support the findings. Also, it appears the methods for Fig. 4F-H are missing. Further, the plasma PK data are not consistent between Fig. 4A and the data referenced, but not shown, for Fig. 6.

As mentioned above, in response to the Reviewer comments we have now added multiple new experimental datasets that support the smooth muscle myosin–mediated, capillary selective mechanism, including: i) direct visualization of pericytes demonstrating R(+)2501–induced pericyte relaxation (Results, line 248-261 and new Fig.e,f), ii) diameter-dependent vascular analysis showing strong dilation of capillaries (<8 μm) with minimal effect on arterioles and larger resistance vessels (Results, line 237-247 and new Fig. 3d), and iii) dose-dependent systemic hemodynamic measurements demonstrating no detectable effects on global cardiovascular function at doses used in the efficacy experiments (Results, line 262-274 and new Extended Data Fig. 11). Together, these multiple independent experimental approaches strongly support the interpretation that R(+)2501 acts primarily through inhibition of smooth muscle myosin in pericytes, leading to relaxation of capillary constrictions and restoration of microvascular flow.

PK was determined at 12 mg/kg (Methods, line 1373-1381), which produced an initial plasma concentration of approximately 540 μM within the first minute after administration and then cleared relatively quickly (half-life ~16 min) (new Fig. 3a). In the functional experiments, we used a similar dose range (5–20 mg/kg). We agree that these total plasma concentrations exceed the biochemical SMM IC50 measured in the purified ATPase assay. However, the biochemical IC50 from an isolated in vitro system is not directly equivalent to the in vivo effective plasma concentration, particularly because total plasma concentration does not reflect free drug concentration at the target site (plasma protein binding is 90% in rats – data not shown in the manuscript), tissue distribution, or the brief exposure window created by rapid clearance. Rapid clearance is advantageous here because brief drug exposure is sufficient to produce a durable improvement in capillary perfusion while limiting prolonged systemic on-target effects.

We have now incorporated a detailed description of the methods used for the experiments presented in our new figures in the Methods section. In addition, we restructured the section containing the typographical error regarding plasma PK, where the text previously stated 10 minutes (Results, line 221).

11. The authors state that "Formulation with 2-hydroxypropyl-β-cyclodextrin (HPBCD) significantly improved absorption and bioavailability (Figures 6J-K)" (line 346). This is confusing because there is 100% bioavailability with IV dosing and no absorption phase. Also, was HPBCD used because for solubility reasons at the high concentration administered to animals? This highlights the need for solubility data both

in PBS and other vehicles used in the manuscript. Compound precipitation may be a confound here and in other experiments. Also, solubility for CK-571 in PBS and/or in any other vehicle used should be presented as it may explain the “slower” increase in RBC velocity and flux shown in Figure S1.

We thank the Reviewer for this comment and agree that the wording in the original manuscript was not sufficiently clear. In the revised version, we clarify that HPBCD was used not primarily to improve solubility or to enhance systemic bioavailability in the classical pharmacological term (oral vs i.v. dosing), but rather to enhance cellular uptake and tissue exposure of the compound. Thus, the term “bioavailability” was intended in the context of effective cellular delivery, not systemic absorption, which is now clarified in the revised manuscript (Results, line 319-322).

Consistently, although R(+)2501 was sufficiently soluble in PBS, and the experiments could be performed in this vehicle, HPBCD formulation allowed stronger biological effects at lower administered doses (new Fig. 4). For this reason, unless otherwise stated, we conducted further experiments in the HPBCD formulation.

For CK-571, the comparisons were performed under the same PBS conditions used for R(+)2501, and the conclusions regarding the differences in vascular responses remain valid under these matched conditions. We did not perform additional experiments with CK-571 in HPBCD formulation because further optimization of CK-571 delivery was outside the scope of the present study, which focuses on the pharmacology of R(+)2501. In addition, generating new experimental series for CK-571 would have required substantial additional animal experiments, which would not align with the 3R principles (replacement, reduction, and refinement) guiding our study design.

12. The Fig. 6 section provides strong in vivo evidence that renal ischemia induces persistent capillary perfusion failure and that R(+)2501 rapidly and robustly restores capillary RBC flux, with corresponding improvements in renal function and survival. However, the data do not directly demonstrate that capillary closure is pericyte-mediated, nor that the drug’s effects are specific to capillaries. Also, details of the survival studies and co-administration experiments are not provided in the Methods.

As described above, based on Reviewer suggestions we performed new experiments directly addressing the pericyte-mediated and capillary-specific mechanism. Specifically, we carried out direct pericyte visualization experiments, which demonstrate pericyte relaxation following R(+)2501 administration (Fig. 3e,f), and vessel diameter–dependent analyses, which show that R(+)2501 efficiently dilates capillaries while larger vessels remain largely unaffected (Fig. 3d). Together, these new datasets confirm that the observed effects arise from pericyte-mediated capillary relaxation.

In addition, we have now incorporated the detailed experimental procedures for the survival studies and the co-administration experiments into the Methods (line 1575-1582) section of the revised manuscript.

13. The statistical analysis details for Figures 6-8 are not provided, but are necessary to properly evaluate the results.

We have added details of the statistical methods used for the analyses presented in new Fig. 4-5 (Results, line 374-377 and line 489-492), including the statistical tests applied and the definition of error bars, to ensure transparency and proper evaluation of the results.

14. The Fig. 7 section is very similar. It presents compelling evidence that R(+)2501 dilates myocardial capillaries after LADO and improves capillary perfusion metrics, with large reductions in infarct size and a survival benefit. However, the RBC flow analysis is vulnerable to selection bias because it relies on 'minimal-motion intervals,' and the use of 'robust ST-elevation' as an inclusion criterion requires transparent reporting of exclusions and blinding. Mechanistic conclusions (pericyte-specific action, cascade initiation by pericytes, interruption at the origin) are not directly demonstrated by these data. Further, the survival analyses in Figures 7Q and S13 (which should be a main figure) assess only acute survival during the 120-minute reperfusion period. While R(+)2501 markedly improves early survival, the data do not support claims of durable or long-term survival benefit. Statements implying prevention of mortality or long-term protection substantially overstate what is demonstrated.

Regarding the RBC flow analysis, the use of minimal-motion intervals is necessary in open-chest intravital imaging to avoid motion artifacts caused by cardiac and respiratory movements. These intervals were selected using predefined criteria to ensure accurate quantification of RBC velocity and flux. We have now clarified this procedure in the Methods (line 1737-1746) section, including the criteria used for inclusion of segments. We also added clarification (Methods, line 1702-1707) regarding the ST-elevation inclusion criterion and reporting of analyzed animals.

Concerning the mechanistic interpretation, as described above in response to related Reviewer comments, we have now incorporated new experiments directly visualizing pericytes and analyzing vessel diameter–dependent responses (Figs 3e,f and Fig. 3d), which demonstrate pericyte-mediated capillary relaxation and capillary-specific dilation by R(+)2501. These new data strengthen the mechanistic interpretation presented in the manuscript.

Finally, we agree that the survival analysis presented in the study reflects acute survival during the 120-minute reperfusion period. We have therefore adjusted the wording (Results, line 417-424) in the manuscript to avoid implying long-term survival benefits. At the same time, the observation that ECG signals recover in treated animals suggests that they would likely survive beyond the

acute period. However, the open-chest intravital model used for these experiments does not permit longer-term survival studies, which limits the ability to directly assess long-term outcomes within this experimental setup. We have clarified these points in the revised text (Results, line 417-424).

Also, as suggested by the Reviewer, Figure S13 has now been moved to the new main Fig. 5p in the revised manuscript to better present the survival data alongside the other key cardiac findings.

15. Figures 8 and 9 provide strong evidence that R(+)2501 can acutely restore microvascular perfusion in both heart and kidney and preserve coronary autoregulation in a large-animal model. However, these data do not establish pericyte-specific mechanisms, direct heart-kidney microvascular coupling, or durable protection beyond the acute reperfusion window. Importantly, the coronary physiology data in Figure 9, while compelling, do not resolve the mechanistic ambiguities underlying the renal perfusion rescue in Figure 8. Additional experiments, such as demonstrating capillary constriction and dilation at pericyte somata/processes in kidney and/or heart, hemodynamic dissociation in the CRS-I model, or a minimal genetic pericyte control, would substantially strengthen the mechanistic interpretation without altering the core findings.

In response to this and related comments, we have performed new experiments directly addressing the pericyte-mediated mechanism. Specifically, we conducted pericyte visualization experiments in collaboration with Dr. Svetlana Mastitskaya (University of Bristol), which allow direct identification of pericytes and demonstrate that R(+)2501 relaxes pericyte-mediated capillary constrictions, with quantitative analysis of capillary diameter changes at pericyte somata. These data provide direct evidence that the drug acts at the level of pericyte-controlled capillary constrictions (new Fig. 3e,f and new Supplementary Video 1).

In addition, we performed vessel diameter–dependent analyses showing that R(+)2501 efficiently dilates capillaries (<8 μm, 50-120%), while larger vessels remain unaffected and 10–15 μm arterioles show only minimal dilation (0-22%) (new Fig. 3d), supporting the interpretation that the compound acts primarily on the capillary compartment rather than producing generalized smooth muscle vasodilation.

Furthermore, we have now included dose-dependent systemic hemodynamic measurements in rats, including pressure–volume (PV) loop analysis, demonstrating no detectable adverse effects on systemic cardiovascular parameters at doses used in the efficacy experiments (up to 20 mg/kg) (new Extended Data Fig. 11). These data help distinguish the observed microvascular rescue from systemic hemodynamic effects.

We agree with the Reviewer that the current experiments do not directly demonstrate genetic pericyte specificity or fully resolve the mechanistic basis

of heart–kidney coupling in the CRS-I model. However, the new pericyte visualization experiments, vessel diameter–dependent responses, and hemodynamic analyses together provide strong evidence that the observed restoration of microvascular perfusion arises from relief of pericyte-mediated capillary constriction. We have incorporated these new data and clarified the mechanistic interpretation in the revised manuscript (Results, line 248-274).

We also note that substantial evidence has accumulated showing that pericytes regulate capillary blood flow in the heart, kidney, and brain, and that their constriction contributes to capillary no-reflow in these models. The most important papers demonstrating this are cited in the manuscript.

Additional Comments:

1. The statement “The MPH compound family, including MPH-220 (also known as MTR-601), provided a promising scaffold due to favorable safety, absorption and clearance profiles. Building on published MPH and blebbistatin derivatives,… (lines 143-145)” is misleading, as the MPH papers indicate they are derivatives of blebbistatin.

We intentionally make a distinction between the original blebbistatin scaffold and the improved MPH scaffold, which was developed to achieve better safety, absorption, and clearance properties. Nevertheless, we agree that the wording in the original sentence could be misleading. Therefore, we have clarified the sentence (Results, line 125-130) in the revised manuscript while maintaining the distinction between the blebbistatin-derived origin and the optimized MPH scaffold.

2. Line 140: "Among existing inhibitors, CK-57135 is SMM-selective but lacks the pharmacodynamics needed for acute microvascular targeting and has no effect on CNR (Figure S1)." should be revised. According to Fig. S1, CK57135 does have an effect on CNR: "the fact that CK-571, the previously developed SMM inhibitor, was capable of relaxing capillaries represents a proof-of-concept that direct SMM inhibition is a promising therapeutic approach to reopen capillaries after an ischemic attack".

We agree that the original wording was misleading. As shown in previous Figure S1, CK-571 is capable of relaxing capillaries and partially improving microvascular flow, which indeed supports the proof-of-concept that direct SMM inhibition can reopen capillaries after ischemia.

However, the effect of CK-571 is substantially slower and less robust than that observed with R(+)2501, likely due to its less favorable pharmacodynamic properties for rapid microvascular targeting.

3. The hepatocyte and in vivo PK curves should not be placed on the same graph.

We thank the Reviewer for this suggestion. The hepatocyte stability data and the *in vivo* plasma pharmacokinetic profile were intentionally plotted together to allow direct visual comparison between metabolic stability and systemic exposure. Presenting the curves on the same graph illustrates the strong correspondence between rapid hepatocyte metabolism and the short *in vivo* plasma half-life, supporting the conclusion that hepatic clearance is the primary metabolic route for the compound. Separating the curves into different panels would make this relationship less immediately apparent. However, to avoid potential confusion, we have clarified this rationale in the figure legend (Results, line 275-291) and improved the labeling of the curves in the revised manuscript.

4. Methods for Fig. S6 appear incomplete.

In the revised manuscript, we have expanded the Methods (line 1411-1447) section to include the missing experimental details corresponding to the data presented in new Extended Data Fig. 8, ensuring that the procedures and analytical methods are described sufficiently to allow reproducibility.

5. Are the methods and drug doses for Fig. 4B and 4C reported?

In the revised manuscript, we have clarified the Methods (line 1469-1472) section and figure legends (Results, line 275-291) to explicitly report the experimental procedures and drug doses used in the experiments shown in new Fig.4b,c.

6. Some acronyms are not defined (e.g. Fig. 4D's CCA, FA, BA) and others are defined more than once (e.g. CNR).

In the revised manuscript, we have carefully reviewed the use of acronyms throughout the text and figures, ensuring that all acronyms are defined at first appearance, and removing duplicate definitions where terms were mistakenly introduced more than once.

7. The statement that binding to the "blebbistatin-site" is consistent with a non-selective inhibition profile needs clarification. (Line 921: "In contrast, the S(-)2501 enantiomer ...") As demonstrated previously by the authors themselves with another blebbistatin-derivative (MPH-220), marked skeletal-over-cardiac selectivity can be achieved while targeting the "blebbistatin-site" (Gyimesi et al, 2020).

We agree that the original wording was not sufficiently precise. As demonstrated in our previous work (Gyimesi et al., 2020 *Cell*), skeletal muscle

selectivity can indeed be achieved while targeting the blebbistatin binding site, particularly when bulky substitutions are introduced on the D-ring of the scaffold and when using the appropriate S-enantiomer configuration, as in the case of MPH-220.

In contrast, CLN-2501 does not contain such bulky D-ring substitutions, and smaller modifications at this position generally do not confer strong isoform selectivity. Thus, the statement in the manuscript was intended to highlight the comparison between the two binding sites rather than imply that selectivity cannot be achieved through the blebbistatin site. Nevertheless, the Reviewer is absolutely correct that the previous wording was misleading, therefore we changed wording (Extended Data, line 925-959).

8. Figure 3G: Authors should discuss how specific inhibition can be explained if the "R(+)2501/CK-571 binding site" is identical in in human SMM, non-muscle myosin-NM2B, β-cardiac myosin heavy chain, and fast skeletal muscle myosin-2x/d.

We agree that sequence conservation of pocket-lining residues makes it difficult to explain the observed selectivity solely by sequence differences within the pocket.

Importantly, our structural analysis suggests that selectivity arises from differences in conformational accessibility of the binding cleft rather than sequence divergence within the pocket itself. The findings with R(+)2501 are similar to what has been described for CK-571 (PMID: 27815532). The myosin structural state in which these compounds bind correspond to a transient state explored when the motor is bound to ATP to reprime the motor. Our hypothesis is that these transient states are sufficiently different from one myosin to another as they depend on the overall sequence of the motor. Thus, this explains why other Myo2 motors may not bind these inhibitors as efficiently as smooth muscle myosin. This hypothesis provides a structural explanation for how isoform selectivity can arise despite apparent conservation of the binding pocket.

9. In the legend for Figure S2, the color attributed to the smooth muscle myosin (SMM) curve is incorrectly written as "pink"; it should be "orange" to match the actual figure. Also, the legend for Figure S2 incorrectly refers to "panel D" after panel B. Since Figure S2 contains only panels A, B, and C, the reference should be changed to "C".

Based on Reviewer comments, in the revised manuscript, we have carefully revised coloring, labeling and presentation of all panels, so now these mistakenly labeled panels are corrected.

10. Figure S3: "W1" and "W2" (structurally important water molecules?) should be explained in the figure legend.

In the revised manuscript, we have now clarified in the new Extended Data Fig. 5 legend (line 1017-1020) that W1 and W2 denote structurally important water molecules involved in stabilizing the ligand–protein interaction network.

11. The legend for Table S3 should be corrected to refer to Figure S3, not Figure S5.

In the revised manuscript, we have corrected the legend for new Extended Data Table 3 so that it now correctly refers to new Extended Data Fig. 5.

Referee #3 (Remarks to the Author):

I co-reviewed this manuscript with one of the Reviewers who provided the listed reports.

We thank the Reviewer for their time and contribution to the evaluation of our manuscript. We appreciate the careful review and have addressed the comments provided in the detailed responses for Reviewer 1 and 2.

We are grateful to the Reviewers for their insightful comments and suggestions, which have helped us improve both the rigor and clarity of the manuscript. The additional experiments expanded methodological descriptions, and revised interpretations have strengthened the mechanistic framework of the study while preserving the central findings. We hope that the Reviewers and the Editor will agree that the revised manuscript now provides a clearer and more comprehensive account of the discovery and physiological impact of R(+)2501 in relieving pericyte-mediated capillary constriction and restoring microvascular perfusion after ischemia. We believe that the combination of structural, pharmacological, and in vivo physiological evidence presented here establishes a new strategy for targeting capillary no-reflow and may open new avenues for therapeutic intervention in ischemia–reperfusion injury.

Referee #1

The data added have answered most of my questions. Just a few points remain needing clarification, along with an improvement of the presentation, after which I think this should be accepted.

We sincerely thank the Reviewer for the careful re-evaluation of our revised manuscript and for the highly encouraging overall assessment. We appreciate the Reviewer's recognition that the newly added experiments and analyses have addressed the major concerns raised during the previous review round, and only a limited number of clarifications and presentation-related improvements remain before the manuscript can be accepted.

Below, we provide a detailed point-by-point response to each of the Reviewer's remaining comments.

(1) Fig 3c. It is unclear what has produced the initial tone in the arteries presented. This should be explained in the legend. In addition, the R(+)2501 apparently relaxes all the arteries to some extent. Given the claimed selectivity for pericyte relaxation in the paper, I don't understand how the drug can cause apparently 100% relaxation of the femoral artery (FA) and a *more than* 100% relaxation of the basilar artery (BA) – how can it relax more than 100% and generate negative tone? Explain exactly what is being measured and plotted please, in the legend and main text.

We thank the Reviewer for raising this important point and agree that the original presentation did not sufficiently clarify the experimental setup and interpretation of the wire myograph measurements. We have therefore revised our manuscript to provide a more explicit explanation.

In these experiments, isolated arterial segments were studied using a wire myograph system in which vessels are mounted under a predefined initial tension. Importantly, this initial tension is not equivalent to a fully relaxed physiological state, but rather represents a calibrated passive resting tension determined by the instrument to mimic physiological mechanical conditions. Consequently, after pharmacologically induced contraction, subsequent relaxation is not necessarily limited to returning to the original baseline tension. Instead, relaxation may proceed below this initial calibrated resting tension, which explains why the apparent relaxation can reach or exceed 100%, resulting in values that appear as "negative tone" relative to the initial baseline.

We also would like to clarify that these *ex vivo* wire myograph experiments do not directly reflect the selective capillary targeting mechanism proposed for *in vivo* applications. In the wire myograph setup, isolated vessel segments are completely immersed in a bath containing R(+)2501, allowing unrestricted drug

access to the vessel wall from all directions. Under these conditions, larger arteries are exposed uniformly and directly to the compound, by passing the anatomical diffusion barriers that normally limit drug penetration *in vivo*. By contrast, under physiological conditions, vascular smooth muscle cells in larger vessels are protected by multiple structural layers, including the internal elastic lamina, whereas capillary pericytes are directly accessible from the circulation through the endothelial barrier.

To avoid ambiguity, we have revised the manuscript to explicitly explain both the origin of the initial vessel tension and the interpretation of relaxation values exceeding 100%.

(2) The data in Fig 3e-f support the idea that the drug acts effectively on pericytes in cardiac muscle. However, (i) For the naïve reader, mention that the red labelling in the image of Fig 3e (dsRed) is pericyte specific in the heart (if it is). (ii) What is the preparation being used (state this in the main text and legend) – is it whole heart in vivo, chunks of ex vivo heart (ventricle or atrium) or cardiac slices; and was myocyte contraction blocked (if so how)? (iii) Where exactly was pericyte-mediated capillary diameter reduction measured – at pericyte somata, or randomly along the capillary – this is important to state in the legend.

We thank the Reviewer for these suggestions and agree that additional clarification is needed to improve the accessibility of these experiments for non-specialist readers.

(i) We have now explicitly indicated in the figure and legend that the red fluorescence corresponds to DsRed-positive cardiac pericytes in NG2DsRedBAC transgenic mice, allowing direct visualization of cardiac pericytes *ex vivo*.

(ii) We have now clarified the experimental preparation in the Methods section. Briefly, these experiments were performed using ex *vivo* perfused mouse heart preparation. Following euthanasia, hearts were rapidly excised and the right ventricular wall was isolated and cannulated through the right coronary artery. The right ventricle was specifically selected because its thin wall permits high-resolution optical imaging. Both luminal perfusion and bath superfusion were continuously maintained under physiological conditions. To eliminate motion artifacts and allow stable imaging, cardiomyocyte contraction was pharmacologically inhibited using mavacamten (10 µM) throughout the experiment. This preparation allowed direct observation of capillary responses independent of systemic physiology.

(iii) Capillary diameter changes were measured specifically at pericyte somata, rather than at random capillary segments, by quantifying the width of the intraluminal fluorescent vessel marker (Alexa Fluor 488–WGA) precisely at sites

identified by DsRed-positive pericyte morphology. We have now added this clarification to the relevant part of the text.

(3) How does the drug get to where it is needed? If 68% of renal capillaries are occluded after RAO (which is broadly consistent with the earlier Freitas and Attwell eLife paper), are we to assume that access via the remaining 32% of perfused capillaries is sufficient to get the drug to the occluded vessel pericytes. Alternatively, could it be the case that the occlusions reported are for *cells* in the blood, while there is still some flow of *plasma* and drug around the stalled cells in the apparently occluded vessels?

We thank the Reviewer for raising this important question. We agree that understanding how R(+)2501 reaches constricted pericytes within partially occluded capillary networks is highly relevant for interpreting the rapid restoration of perfusion observed in our experiments.

Although a large fraction of renal capillaries exhibited severely reduced or apparently absent red blood cell movement following prolonged ischemia-reperfusion, we believe that these observations do not necessarily indicate complete absence of fluid movement throughout the affected microvascular network. Our measurements primarily quantify red blood cell flow dynamics, and it remains plausible that residual plasma flow persists even in capillaries containing stalled or severely slowed blood cells. Such residual plasma movement could allow delivery of R(+)2501 to downstream constricted pericytes despite markedly impaired cellular perfusion.

In addition, we speculate that once R(+)2501 reaches and relaxes pericytes in partially accessible capillary segments, the resulting local capillary reopening may progressively improve downstream perfusion and facilitate subsequent drug delivery to adjacent still-constricted microvascular regions. In this way, the compound may effectively promote a sequential restoration of capillary patency, progressively 'opening a path' through the compromised microvascular network.

At present, our experiments do not directly distinguish between complete capillary occlusion and conditions where residual plasma flow persists despite stalled red blood cells. We therefore agree that both mechanisms proposed by the Reviewer may contribute.

(4) Fig 4d would benefit from arrows showing where constrictions occurred that were reversed by R(+)2501.

We thank the Reviewer for this helpful suggestion. In response, we have revised previous Fig. 4d by adding arrows highlighting representative sites of capillary

constrictions observed after ischemia-reperfusion, as well as the corresponding regions where these constrictions were rapidly reversed following administration of R(+)2501.

(5) State clearly in the figure legends the formulation used for the drug in each experiment: for each panel in Fig 4m-4p was the drug dissolved in PBS or in HPBCD? Similarly for Fig. 5.

We thank the Reviewer for pointing out this shortcoming in the figure legends. We have revised the figure legends accordingly and now clearly specify for each relevant panel in previous Fig. 4 (m–p) and Fig. 5 whether R(+)2501 was administered in PBS or formulated in HPBCD, depending on the experimental setup.

(6) Line 432 “ischemic injury follows a unified mechanistic cascade initiated by pericyte contraction and sustained capillary constriction.” As this result is already in the literature for several organs it is necessary to cite refs 2, 3, 6, 7, 11, 70 here.

We thank the Reviewer for this important suggestion. We agree that this statement refers to a mechanistic concept that is supported not only by the findings of the present study but also by prior work across multiple organ systems.

We have added the corresponding sentence based on the recommended references (refs. 2, 3, 6, 7, 11, and 70) to appropriately acknowledge the previous studies that have contributed to establishing this concept.

(7) Fig 5b. State what the green label is. Explain that the wavy nature of the image reflects ventricular movement (if it does). Explain whether Fig 5a-b is a line scan (with distance on the x axis and time on the y axis?) or a 2D image of the heart.

We thank the Reviewer for pointing out this lack of detail. We have revised the figure and legend accordingly. The green fluorescence represents FITC-labelled capillaries in the beating heart. The wavy appearance of the image reflects physiological ventricular motion during image acquisition. Figure 4A–B (previously Fig. 5A–B) shows a line-scan (kymogram), in which the x-axis represents distance and the y-axis represents time. These features have now been explicitly indicated in the revised figure.

(8) Fig 5d-e. I am not sure what is shown here (the authors need to put significantly more effort into explaining what they are showing in figures, e.g. giving the manuscript to a naïve reader would be a good idea). Is this a line scan across vessels with distance plotted horizontally and time plotted downwards (as suggested by the vertical

lines when there is no flow) or is it a simple 2-P image in two dimensions (in which case why are no separate RBCs visible along the vertical capillaries?)? In the previous review I requested that distance and time be clearly indicated on such line scan pictures, but if this is a line scan that has not been done here.

We thank the Reviewer for this helpful comment. Previously Fig. 5d–e (new Figure 4b,l) are also line-scan (kymogram) images, with the x-axis representing distance and the y-axis representing time. These axes have now been explicitly labelled in the revised figures. Previous Figure 4d shows FITC-labelled red blood cells moving through the TRITC-labelled coronary capillary network, whereas previous Figure 4e shows Alexa Fluor 488-labelled neutrophils within the TRITC-labelled coronary capillary network. We agree that this was insufficiently explained in the previous version and have revised the figures to improve clarity.

(9) Fig 5i and m. Define V on the x axis (perhaps Vehicle, but we are not told, nor are we told what the vehicle is – see above).

We thank the Reviewer for pointing out this lack of clarity in the figure labeling. We agree that the abbreviation "V" was insufficiently defined and could be confusing for the reader.

In response, we have revised the corresponding figure panels and legends to explicitly indicate that "V" refers to the vehicle control condition, where the compound was formulated in 30% HPBCD (2-hydroxypropyl-β-cyclodextrin).

(10) Fig 5s-t. Add a bar under or over the two rightmost bars to convey that these are (presumably) measured after cardiac ischaemia. On the y axis mention that this is *renal* RBC flux!

We thank the Reviewer for this helpful suggestion and agree that the original figure presentation did not sufficiently clarify these points.

In response, we have revised previous Fig. 5s–t accordingly. We have added graphical indication to clearly denote that the two rightmost bars correspond to measurements performed after cardiac ischemia, and we have modified the y-axis labeling to explicitly indicate that the measured parameter represents renal RBC flux.

(11) Line 232 and legend to Ext Data Fig 9: Define more clearly what the Relaxation half life means, e.g. stating that the "half-time of the relaxation was defined as the time to reach half the maximal dilation that was measured after N minutes". In line 232 it is unclear what "modeling" of the relaxation time means. If this is real modelling of the

relaxation process then explain it in detail; if it is merely fitting a curve to a plot of relaxation half-time vs diameter, then say so.

We thank the Reviewer for pointing out this issue. Indeed, half-life values represent the time required for half-maximal relaxation of the vessels and were determined by fitting the relaxation time-curves with single exponentials. We have updated the Methods section of the manuscript with these details.

(12) Ext Data Fig 10: explain whether capillary diameter was measured at random locations or near pericyte somata.

We thank the Reviewer for bringing this lack of clarity to our attention. Capillary diameters were measured at the somata of the pericytes, as now explicitly stated in the Methods section.

(13) Suppl Video 1. State in the legend that this is a coronary capillary.

We thank the Reviewer for pointing out this missing detail. We agree that the anatomical origin of the imaged vessel should be stated explicitly in the video legend.

In response, we have revised the legend of previous Supplementary Video 1 (now Supplementary Video 2) to indicate that the recorded vessel is a coronary capillary.

Referee #2

The authors have extensively revised the manuscript and provided detailed responses, resulting in a substantially improved manuscript relative to the original submission. In particular, the addition of direct pericyte visualization, vessel diameter-dependent dilation analyses, systemic hemodynamic measurements, pig coronary physiology data, and Polder maps for the ligand-bound structures significantly strengthens the manuscript. These additions substantially improve the biological interpretation. The authors have also clarified or corrected a number of issues raised in the original review, including the introductory framing of microvascular dysfunction, the comparison between R(+)2501 and CK-571, the structural interpretation of ligand occupancy, missing methods, and the description of the W1/W2 structural elements. The new additions now enable a full evaluation of the manuscript, and concerns remain unresolved, particularly regarding the quantitative ATPase/SAR analysis, solubility, pharmacokinetic interpretation, survival/statistical reporting, and overstatement of mechanistic and translational claims. Given the high bar for mechanistic, pharmacological, and translational claims in this venue, these remain substantive issues.

We sincerely thank the Reviewer for the thorough re-evaluation of our revised manuscript and for the highly constructive feedback provided throughout the review process. We appreciate the Reviewer's recognition that the extensive revisions, newly added experiments, and additional analyses have substantially strengthened the manuscript relative to the original submission.

In particular, we are grateful for the Reviewer's positive assessment regarding the addition of direct pericyte visualization experiments, vessel diameter-dependent dilation analyses, systemic hemodynamic characterization, pig coronary physiology measurements, and the structural validation provided by the Polder maps. We also appreciate the acknowledgment that the clarifications and corrections made in response to the previous review round have substantially improved the biological interpretation and overall presentation of the study.

We fully recognize the Reviewer's remaining concerns regarding quantitative ATPase and SAR analysis, compound solubility characterization, pharmacokinetic interpretation, statistical reporting, and the framing of certain mechanistic and translational claims. We appreciate the Reviewer's careful evaluation and agree that, given the exceptionally high standard required for publication in this venue, these issues warrant careful consideration.

1. The revised manuscript still presents the ATPase/SAR data with a level of quantitative precision that does not appear justified by the underlying data. Extended Data Table 1 reports exact-looking potency, selectivity, and MaxInh values for many compounds, including cases where the curves appear weak, incomplete, or uninformative. Several values would be more appropriately reported as lower/upper bounds or as not determined rather than as fitted point estimates. This is especially important because the SAR table supports several claims about isoform selectivity and structure-activity relationships. If the authors wish to retain this table and the associated conclusions, they should provide appropriate uncertainty for fitted parameters, identify parameters that are not reliably estimable, and avoid ranking compounds or drawing SAR conclusions from noise-fitted curves. This would be seem more appropriate for the full SAR manuscript the authors reference as being in preparation. At minimum, the table should distinguish between true fitted values, screening-level estimates, limits, and non-identifiable parameters. Relatedly, the manuscript continues to use confusing terminology around IC50 and KI. The Methods describe fitting to a quadratic binding equation and refer to the fitted term as an “IC50: inhibitory constant.” These terms are not interchangeable. The authors should either report the fitted value as KI, with appropriate assumptions clearly stated, or fit and report IC50 using an appropriate empirical dose-response model.

We thank the Reviewer for these valuable suggestions. We agree that reporting the uncertainty of the measurements and fitted parameters facilitates proper interpretation of the data. Accordingly, we have revised Supplementary Table 1 (previously Extended Data Table 1) to include the standard errors of the Sm50 and Cm50 values, calculated from three independent measurements, as well as the fitting errors of the IC50 values.

For a number of compounds, the fitted IC50 exceeded 100 µM, more than twice the highest inhibitor concentration tested. Because such values rely on substantial extrapolation and are therefore poorly constrained, we have replaced the fitted values with the notation “>100”. For the purpose of the selectivity calculations, these IC50 values were uniformly assigned a value of 200 µM.

Regarding the nomenclature, after careful consideration, we decided to retain the term “IC50”. Although our ATPase inhibition assays yield a parameter that reflects the inhibitor affinity under the assay conditions, its exact mechanistic interpretation cannot be established from our analysis. Because the concentration dependence was fitted using a quadratic tight-binding equation rather than a mechanistic inhibition model that directly yields KI, we consider “IC50” to be the more appropriate designation for the reported parameter.

2. Solubility remains insufficiently addressed

The response that compounds were visually inspected for turbidity is not sufficient to support the solubility-dependent aspects of the manuscript. We acknowledge the authors' comment regarding limited quantities of some compounds generated during SAR. However, the solubility is relevant to several central interpretations for R(+)2501, S(–)2501, including apparent MaxInh in the ATPase assays, isoform selectivity, differences between PBS and HPBCD formulations, and comparisons between R(+)2501 and CK-571. Therefore, the manuscript should include measured solubility data for the key compounds, at minimum R(+)2501, S(–)2501, and CK-571, in the relevant assay and dosing conditions. These should include the ATPase assay buffer, PBS, HPBCD-containing vehicle, and any other formulation used for ex vivo perfusion or in vivo dosing. Without these measurements, it remains difficult to distinguish true pharmacological potency/selectivity from differences in soluble/free compound concentration. R(+)2501 being "sufficiently soluble in PBS" should be supported by data.

We thank the Reviewer for raising this point. In the revised manuscript, we have further limited statements that could rely on assumptions regarding compound solubility. In particular, comparisons with CK-571 have been restricted to the structural characterization, and we no longer base any mechanistic conclusions on relative solubility. During assay development and execution, all compounds were carefully monitored for visible precipitation or turbidity under the experimental conditions used, and no evidence of precipitation was observed at the tested concentrations. Furthermore, the ATPase concentration–response curves were robust and reproducible across independent experiments, indicating that compound precipitation did not measurably affect the biochemical assays. We have included all characterization data that are available and that we consider relevant for the interpretation of the pharmacological properties of the compounds. Dedicated quantitative solubility measurements in every assay buffer and formulation requested by the Reviewer were not performed as part of this study.

3. The revised Methods now include several useful details regarding ATP concentration, actin concentration, buffer composition, DMSO concentration, replicates, and fitting approach. However, because the quadratic fitting model explicitly depends on the myosin concentration, the final concentration of each myosin isoform used in the ATPase assays should be provided. The Methods should also make clear which compounds were tested only in initial three-point screening format and which were subsequently retested with five- or six-point concentration-response curves. Screening level data should not be presented or interpreted as if they were high-confidence quantitative potency measurements.

We thank the Reviewer for this helpful suggestion. We have revised the ATPase Methods section accordingly. The final myosin concentrations used for each

assay are now explicitly provided, together with the assay conditions. We have also clarified which compounds were initially evaluated using three-point screening and which compounds were subsequently characterized using full concentration–response analyses. The latter measurements are now also highlighted in red in the new Supplementary Table 1. These revisions are intended to clearly distinguish preliminary screening experiments from quantitative potency determinations.

4. The added characterization of R(+)2501 is helpful, including HPLC, chiral HPLC, MS, and ^{1}H-NMR. However, for a novel compound that is central to the manuscript, full characterization should be provided, including ^{13}C-NMR. The authors should also clarify whether equivalent analytical characterization is available for S(–)2501, since the comparison between enantiomers is used to support the pharmacological and structural interpretation. If the authors wish to present broader SAR conclusions for the compound series, then identity and purity support for the compounds included in that analysis should be clearly stated. Alternatively, the broader SAR claims should be substantially de-emphasized.

We agree that comprehensive analytical characterization is desirable for novel compounds. For R(+)2501, we have included all analytical characterization data available for this study, including HPLC, chiral HPLC, HRMS and ^{1}H NMR. Equivalent analytical datasets, including ^{13}C NMR, are not available for all compounds in the series, including S(–)2501. To avoid overinterpretation, we have further limited the SAR-related discussion in the revised manuscript and restricted our conclusions to compounds for which sufficient pharmacological and structural data are available. We believe that the analytical data provided, together with the structural, biochemical and functional characterization presented, are sufficient to support the conclusions of the present study.

5. The pharmacokinetic data remain difficult to interpret. The Methods describe plasma sampling at 5, 10, 20, 40, 60, 120, 240, and 360 minutes, but Fig. 3a appears to show earlier sampling and a rapid rise/decline that are not clearly reconciled with those stated time points. This discrepancy should be corrected or explained. In addition, the manuscript relies heavily on the claim that R(+)2501 is rapidly eliminated and has a short half-life, but the model or fitting approach used to calculate the reported half-life is not adequately described. The authors should state the pharmacokinetic model used, the data included in the fit, whether the reported half-life reflects terminal elimination or an apparent early-phase decline, and whether values are based on total or free plasma concentration. The authors also invoke plasma protein binding and target-site exposure to explain the relationship between in vivo dose and biochemical potency, but these arguments are not sufficiently supported in the manuscript. If

plasma protein binding is central to the interpretation, those data should be shown or the claim should be removed.

We thank the Reviewer for these helpful comments. We have revised the pharmacokinetic Methods section to clarify both the sampling schedule and the pharmacokinetic analysis. The inconsistency between the Methods and previous Fig. 3A has been corrected by including the early plasma sampling time points (0.5, 1 and 2 minutes), which were included in the pharmacokinetic analysis and are now consistently reported throughout the manuscript. We have also expanded the Methods to describe the fitting procedure used to calculate the apparent plasma half-life, including the pharmacokinetic model, the data included in the fit, and the calculation of the half-life. The reported values are based on total compound concentrations measured by HPLC–MS in plasma obtained after centrifugation of whole blood samples. Finally, we have revised the Discussion to avoid overinterpretation regarding plasma protein binding, and the conclusions are now based on the experimental pharmacokinetic and tissue distribution data presented in the manuscript.

6. The survival and statistical methods require substantial clarification. The main text refers to survival studies involving 144 rats, but the Methods and figure legends do not clearly reconcile this number with the treatment groups, occlusion durations, exclusions, censoring, or analytical approach. The authors should provide a transparent accounting of the survival cohorts, including group sizes, exclusion criteria, censoring rules, randomization, blinding, and the statistical test used for survival comparisons. More broadly, the statistical reporting across the manuscript remains too generic. The repeated statement that two-way ANOVA was used is not sufficient for the variety of data types presented. The manuscript includes repeated measures designs, nested measurements such as multiple vessels or capillaries within animals, survival curves, and non-normal or bounded outcomes. For each figure, the authors should specify the statistical unit, the test used, whether repeated or nested structure was accounted for, what multiple-comparison correction was applied, and whether assumptions of parametric tests were evaluated. This is particularly important for Figs. 4–5 and associated Extended Data, where the biological conclusions depend on appropriate handling of animal-level replication and survival outcomes.

According to the comments we have carefully revised the manuscript to improve the transparency of both the survival analyses and the statistical reporting. We apologize for the inconsistency in the total number of animals; the previously stated value of 144 was incorrect. The complete survival analysis, including the animals used for the eGFR studies, comprised 141 rats. In addition, 12 separate animals were used for the Rimadyl-, vitamin C-, and allopurinol-treated comparator groups. The survival cohorts, group sizes,

exclusion criteria, randomization, blinding procedures, and statistical analyses have now been explicitly described in the Methods section. Kaplan–Meier survival curves and the associated statistical analysis have also been clearly specified. Furthermore, we have expanded the statistical reporting throughout the manuscript by specifying, for each experiment, the statistical unit, statistical test, multiple-comparison correction, and assessment of parametric assumptions where applicable. We believe these revisions substantially improve the clarity and reproducibility of the study.

7. The authors have now defined the “minimal motion” intervals used for red blood cell flow analysis, which is helpful. However, the manuscript should still state whether these intervals were selected blinded to treatment group and outcome. The authors should also report how many animals, imaging fields, or capillary segments were excluded due to motion or inadequate imaging quality. Similarly, the use of robust ST elevation as a criterion for successful coronary occlusion should be accompanied by reporting of how many animals were excluded or required adjustment, and whether these decisions were made blinded to subsequent treatment assignment. These clarifications are necessary to exclude avoidable selection bias in the intravital imaging and AMI experiments.

We thank the Reviewer for these valuable suggestions. We have revised the Methods section accordingly to clarify these points. Specifically, we now state that image analysis was performed by investigators blinded to treatment allocation. For the analysis of red blood cell flow, approximately 40% of each line-scan recording fulfilled the predefined quality criteria and was therefore suitable for quantitative analysis. Segments not meeting these criteria because of cardiac motion or image distortion were excluded; however, no animals or imaging fields were excluded solely because of imaging quality, and only unsuitable portions of individual recordings were omitted from the quantitative analyses. We have also clarified that successful LAD occlusion was confirmed by ST-segment elevation before treatment allocation. Approximately 5% of operated animals failed to develop ST-segment elevation and were excluded before reperfusion experiments, as successful coronary occlusion could not be confirmed.

8. The new pericyte visualization and vessel-diameter analyses substantially strengthen the manuscript. The evidence supports capillary-biased or pericyte-associated relaxation and the manuscript should, therefore, avoid implying that all therapeutic effects are definitively mediated by direct pericyte targeting unless this is directly shown. This issue is especially relevant in the renal and CRS-I sections. The data support acute renal capillary no-reflow after AMI and rescue by R(+)2501, but they do not fully establish the causal heart-kidney axis or prove that renal benefit is

driven specifically by pericyte relaxation. Phrases implying that R(+)2501 “resolves” the heart-kidney axis, identifies the “cascade origin,” or demonstrates a dominant pericyte mechanism should be moderated.

We thank the Reviewer for this thoughtful comment and agree that the present study does not establish that all therapeutic effects are mediated exclusively through direct pericyte relaxation. We have therefore carefully moderated the corresponding statements throughout the manuscript, particularly in the renal and CRS-I sections. The revised text now emphasizes that our data demonstrate reversal of capillary no-reflow and restoration of microvascular perfusion, while avoiding overstatement regarding a dominant pericyte-specific mechanism or a definitive causal heart–kidney axis. Our conclusions are now limited to the experimental findings supported by the data.

9. Safety and tolerability language should be toned down The added high-dose tolerability information is useful, but the current language remains stronger than the data support. Small n, short duration tolerability studies can support statements such as “no overt adverse signs were observed under the tested conditions,” but they do not establish that R(+)2501 is broadly safe or is a “safe drug candidate.”

We thank the Reviewer for this important comment and agree that our tolerability studies do not establish broad safety. We have therefore moderated the corresponding statements throughout the manuscript. The revised text now refers only to the absence of detectable adverse signs or hemodynamic effects under the experimental conditions tested and no longer implies that R(+)2501 is broadly safe or a safe drug candidate.

A number of minor issues should be corrected during revision:

1. The statement that R(+)2501 shows “less than 20% inhibition of non-muscle myosin-2” should be reconciled with Fig. 1e, which appears to report 23.6 ± 2.1% MaxInh for non-muscle myosin.

We thank the Reviewer for noting this inconsistency. The statement has been corrected in the revised manuscript to match the value shown in previous Fig. 1e.

2. The manuscript should clearly define whether reported errors in ATPase figures and tables are SD, SEM, confidence intervals, or fitting errors.

We thank the Reviewer for highlighting this important point of statistical transparency. In response, we have revised the corresponding ATPase figures, tables, and the Methods section to clearly specify the type of reported error

values, explicitly indicating whether values represent SD, SEM, confidence intervals, or fitting-derived errors, depending on the respective analysis.

3. Extended Data Fig. 1 appears to be incorrectly cited in the main text in connection with SAR/selectivity rather than the structural Polder maps.

We thank the Reviewer for carefully identifying this citation inconsistency. We have carefully revised the manuscript and corrected the figure citation so that the appropriate Extended Data figures are now referenced consistently in the relevant SAR/selectivity and structural sections.

4. Extended Data Fig. 2 should be corrected where the legend refers to the SMM curve as “pink” (this may be a conversion issue), and the IC50/KI terminology should be corrected there as well.

We have revised previous Extended Data Fig. 2 accordingly. The incorrect reference to the SMM curve as “pink” in the figure legend has been corrected, and the IC50/KI terminology has been revised throughout the figure and corresponding text to ensure consistent and accurate usage of the appropriate pharmacological parameters.

5. Duplicate references should be removed or consolidated.

We thank the Reviewer for carefully identifying this issue. We therefore have carefully re-checked the entire reference list and revised the manuscript accordingly by removing duplicate entries and consolidating references where appropriate.

6. Blebbistatin should be removed from the Methods if it was not used in the revised study.

We agree that since blebbistatin was not used in the revised study, it should no longer appear in the Methods section.

In response, we have revised the manuscript accordingly and removed all unnecessary references to blebbistatin from the Methods section to ensure consistency with the experiments presented in the revised version.

7. Remaining typographical errors and reference numbering inconsistencies.

We thank the Reviewer for pointing out this problem. Accordingly, we have carefully re-examined the entire manuscript and performed a thorough revision to correct any remaining typographical errors, formatting inconsistencies, and reference numbering issues throughout the text.

Referee #3

A-B: This manuscript discusses the role of pericyte-mediated capillary no-reflow in ischemic injury and presents a targeted vasodilator, R+2501, an SMM specific modulator, as a potential therapy to restore microvascular perfusion across organs during reperfusion. The authors reveal that enantiomers, R(+)2501 and S(–)2501 have distinct binding sites which bind the canonical CK-571 and blebbistation sites, respectively. C-D: Overall fine E: also fine.

We sincerely thank the Reviewer for the careful evaluation of our revised manuscript and for the constructive comments provided throughout the review process.

We greatly appreciate the Reviewer's positive assessment of the study and are encouraged by the recognition that the manuscript provides new mechanistic insight into the role of pericyte-mediated capillary no-reflow (CNR) in ischemic injury, as well as the therapeutic potential of R(+)2501 as a smooth muscle myosin (SMM)-selective modulator capable of restoring microvascular perfusion during reperfusion.

We are particularly grateful that the Reviewer found the overall experimental work, structural analysis, and biological interpretation to be strong, and that the revised manuscript successfully clarified the major points raised during the previous review round. We also appreciate the recognition of our structural findings demonstrating that R(+)2501 and S(–)2501 bind distinct pockets within the myosin motor domain, providing a mechanistic basis for their divergent pharmacological profiles.

Minor comments for clarification

1) Can the authors comment on why R(+)2501 selectively inhibits SMM (and to a lesser extent skMM) isoforms, but not NMM or CM, as shown in Figure 1C? The sequence comparison of residues important for R(+)2501 binding (Figure 2G) appears highly conserved across these isoforms, particularly between SMM and NM2B which are near identical. This seems difficult to reconcile with the isoform selectivity reported in Figure 1C.

We thank the Reviewer for this insightful comment. This is indeed an interesting observation. A very similar isoform selectivity has previously been reported for the structurally related inhibitor CK-571 despite the high conservation of residues lining the binding pocket among myosin isoforms (Sirigu et al., 2016; PMID: 27815532). As proposed in that study, specificity is likely determined not only by the amino acid composition of the binding pocket, but also by the conformational dynamics of the myosin motor domain. Specifically, CK-571—

and, by analogy, R(+)2501—binds to a transient pocket that forms during the recovery stroke. Although the residues forming this pocket are highly conserved, the lifetime, accessibility, and population of this transient conformation are expected to differ between myosin isoforms because of sequence differences outside the binding site that influence the structural dynamics of the motor domain. As stated by Sirigu et al.: "*The SMM/CK-571 structure reveals a way to achieving specificity by targeting transient pockets likely to have different lifetimes in different myosins with sequence differences even remote from the drug-binding site. Even highly homologous myosins can be specifically inhibited as long as they have kinetics that differ significantly from each other.*" We believe the same explanation is the most plausible interpretation for the selectivity profile of R(+)2501 observed in the present study. Nevertheless, directly demonstrating this mechanism experimentally remains technically very challenging and is beyond the scope of the current work.

2) Would the authors expect R(+)2501 to affect any of the cells in the blood (e.g. platelets, that contain non-muscle myosin IIA (NM2A), or white blood cells)? Based on the sequence similarity relating to Q1, the NM2A sequence around the binding pocked for this drug is likely similar.

We thank the Reviewer for raising this both scientifically and clinically important question. Based on our ATPase assays, R(+)2501 has only a weak effect on NM2, reducing ATPase activity by approximately 23% even at saturating concentrations. These findings suggest that any effects of R(+)2501 on these cells mediated through NM2 are likely to be minimal. However, dedicated experiments will be required to confirm or exclude this possibility.

3) Label L265(?) in 2B – there is a dashed line to residue but no label.

We thank the Reviewer for identifying this oversight. The missing residue label (L265) has been added to the revised figure.

4) Lines 183–184: The authors state that the R(+)2501-binding "residues are structurally stabilised in the pre-hydrolytic conformation and are inaccessible in post-hydrolytic or rigor-like states". Could the authors clarify how this relates to the expected state of SMM in contractile pericytes during vasoconstriction? If the myosin filaments are actively contracting and interacting with actin, how do the authors propose that R(+)2501 accesses and binds the motor domain? For example, does the compound bind during rigor or after ATP-induced release from actin, but before ATP hydrolysis,

thereby trapping a pre-hydrolytic state and promoting relaxation? It would also be helpful for the authors to define what they mean by “rigor-like” in this context.

We thank the Reviewer for this important question. The term "rigor-like" was originally introduced to describe the nucleotide-free conformation of myosin V, which was first visualized by X-ray crystallography at 2 Å resolution (Coureux et al., 2003). Although actin was absent from that crystal structure, the authors proposed that this conformation corresponds to the rigor state adopted by myosin when strongly bound to actin in the absence of nucleotide. In the same study, kinetic experiments demonstrated that myosin V and myosin II differ substantially, as actin binding of myosin V is diffusion-limited, whereas this is not the case for myosin II. This hypothesis was subsequently confirmed when the rigor-like structure of myosin V proved to be essentially identical to the high-resolution actomyosin cryo-EM structure reported by Pospich et al. (2021). As discussed in the original publication, however, this correspondence is not universal across all myosins. For example, nucleotide-free myosin II structures determined by the Manstein laboratory do not fully adopt the same conformation as actin-bound myosin. Consequently, the term "rigor-like" is now commonly used to describe nucleotide-free myosin structures determined in the absence of actin.

Our structural analysis indicates that R(+)2501 binds to a transient pocket that is incompatible with all previously observed drug-free myosin states, including the rigor-like (nucleotide-free), pre-hydrolysis/post-rigor (ATP-bound), and post-hydrolysis/pre-powerstroke (ADP·Pi-bound) conformations. We therefore conclude that the observed conformation represents a transient intermediate that becomes stabilized upon ligand binding. Formation of this pocket requires nucleotide binding and is incompatible with strong actin binding. During muscle contraction, myosin heads continuously cycle through these conformational states while hydrolyzing ATP, providing repeated opportunities for R(+)2501 to bind and progressively increase the fraction of inhibited myosin heads. As described by Sirigu et al. (2016), the CK-571 binding pocket opens during the transition following the pre-hydrolysis state, when the motor is detached from actin. Our structural data indicate that R(+)2501 stabilizes the same transient structural state.

We also note that stabilization of this conformation is incompatible with the auto-inhibited 10S conformation of smooth muscle myosin. Therefore, smooth muscle myosin molecules bound to R(+)2501 cannot adopt the folded 10S state.

Finally, this mechanism also provides a plausible explanation for isoform selectivity, since different myosin isoforms spend different amounts of time in the various intermediate states of the ATPase cycle, as discussed above. Binding of R(+)2501 is, of course, non-covalent and remains reversible, with continuous dissociation and reassociation according to the experimentally

determined IC_{50} values. Nevertheless, our experimental data demonstrate that the affinity is sufficient to produce robust physiological effects that are compatible with the intended therapeutic applications.

5) Following on from this, could the authors compare the R(+)2501-binding pocket with existing myosin-2 structures in other mechanochemical states? Given that the residues forming the proposed R(+)2501 site appear highly conserved across myosin-2 isoforms, it would be helpful to understand how this pocket changes between nucleotide states and actin-bound states.

For example, how does the R(+)2501-binding site compare in available structures such as 8SYF, representing SMM actomyosin in an ADP-bound state, and 6BIH, representing SMM in a rigor/rigor-like state? Are the key residues around the SH1-relay/converter cleft positioned similarly, or is the pocket collapsed, occluded, or otherwise inaccessible in these actin-bound conformations?

This comparison would help clarify whether R(+)2501 binding is primarily determined by isoform-specific sequence differences, or instead by the conformational accessibility of the pocket during particular stages of the myosin ATPase cycle. It would also help address how the compound is proposed to engage SMM in actively contracting pericytes, where a substantial fraction of motors may be actin-bound.

We thank the Reviewer for this insightful question. Both the power stroke and the recovery stroke involve substantial conformational rearrangements within the myosin motor domain, particularly of the converter region. As a consequence, the R(+)2501 binding site represents a 'cryptic' or 'transient' pocket, meaning that it is not continuously present or accessible throughout the ATPase cycle. Following the Reviewer's suggestion, we have prepared Supplementary Video 1, which we hope will help readers visualize these conformational changes and the formation of the transient binding pocket.

In actively contracting pericytes, a substantial fraction of smooth muscle myosin heads continuously detach from actin in the ATP-bound state. Consistent with the low duty ratio of smooth muscle myosin-2, the majority of motors are expected to reside in actin-detached conformations. R(+)2501 is therefore unlikely to bind to myosin heads that are strongly attached to actin, as these conformations are stabilized and do not expose the cryptic binding pocket. Instead, drug binding is expected to occur during the recovery stroke, when ATP-bound myosin heads transiently adopt conformations in which the pocket becomes accessible. Binding of R(+)2501 stabilizes this transient state and prevents progression of the ATPase cycle toward conformations that efficiently hydrolyze ATP. This mechanism is consistent with the Pi burst experiments reported by Sirigu et al. (2016, Supplementary Fig. S2C), which

characterized the binding mechanism of CK-571, a structurally related inhibitor that occupies the same binding pocket as R(+)2501.

Referee #4

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

We thank the Reviewer for taking the time to evaluate our manuscript and for contributing to the peer review process. We appreciate the careful assessment and valuable input provided during the review, which helped improve the quality and clarity of the manuscript.

Referee #5

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

We sincerely thank the Reviewer for participating in the evaluation of our manuscript and for the time and effort dedicated to the review process. We greatly appreciate the contribution to the assessment of our work, which helped strengthen the overall quality of the revised manuscript.

Response o Reviewer 2 comments:

The authors have made a further substantial revision, and the additional Extended Data figures help clarify several of the issues raised previously. I am now largely supportive of publication. However, a limited number of issues remain that should be corrected prior to publication:

1. **In their response, the authors state that the statistical unit and nested structure have now been specified for each experiment, but I was not able to find this from the information in the manuscript or figure legends. In several central analyses, treatment is assigned to an animal or heart, whereas the reported sample size and inferential statistics appear to use individual capillaries or pericytes as independent observations. This should be addressed to ensure pseudoreplication is not occurring. As one example: Fig. 3d-f uses 75 capillaries measured sequentially at baseline, occlusion, reperfusion, and treatment, but does not describe a repeated-measures or hierarchical analysis. (See also Fig. 2f, Fig. 4, Ext Fig. 3c, Ext Fig. 5b-c.)**

   We thank the Reviewer for highlighting this important point. We agree that the statistical units and nested structure of these experiments were not described with sufficient clarity in the previous version of the manuscript. We have therefore revised the statistical descriptions in both the Methods and the relevant figure legends and, where necessary, corrected the statistical analyses and the corresponding significance annotations in the figures to ensure that the hierarchical structure of the data is appropriately accounted for and that pseudoreplication is avoided.

   For all relevant experiments, we now explicitly indicate both the number of individual capillaries/pericytes analysed and the number of animals or hearts from which these measurements were obtained. Where multiple capillary- or pericyte-level observations were obtained from individual animals/hearts, nested analyses were applied, using nested one-way ANOVA as appropriate.

   For Fig. 2f, the heart represents the statistical unit within each experimental group. The individual data points used for statistical comparison represent heart-level values obtained by averaging the measurements from the pericytes analysed within each heart. Importantly, the same individual pericytes were followed longitudinally throughout the respective experimental phases. For Fig. 3d–f, the same 75 capillaries were followed longitudinally through baseline, occlusion, reperfusion and treatment. We have now explicitly stated both the number of capillaries and the number of animals from which they were derived and clarified the repeated-measures structure of this experiment in the figure legend and Methods. For Fig. 4, because cardiac motion prevented reliable identification and

measurement of exactly the same individual capillaries across the different experimental phases, capillary diameters could not be longitudinally paired at the individual-capillary level. We therefore accounted for the clustering of capillary measurements within individual hearts using a nested analysis rather than treating individual capillaries as independent biological replicates. The figure legend and statistical description have been revised accordingly. We have similarly reviewed and revised the statistical descriptions and analyses for Extended Data Figs. 3c and 5b–c, reporting the corresponding animal-level replication.

2. **The 20 mg/kg renal group contains only three animals. Its 33% survival represents one surviving animal and cannot support a dose-response conclusion or the statement that this dose produced the "highest survival rate." Similarly, the vitamin C, allopurinol, and carprofen combination groups contain only three animals each. Failure to detect additional benefit in these very small groups cannot support the conclusion that "pericyte-mediated CNR is the dominant driver of AKI."**

   We thank the Reviewer for this important comment and agree that the small cohort sizes in these experiments do not support such broad conclusions.

   Specifically, we have removed the statement that the 20 mg/kg dose produced the "highest survival rate" and have refrained from drawing a dose-response conclusion from this experiment. Given that the 20 mg/kg group comprised only three animals, with one surviving animal, we agree that the observation should be interpreted descriptively and requires further investigation in larger cohorts.

   Similarly, we have removed the conclusion that the lack of additional benefit observed with the vitamin C, allopurinol, and carprofen combination treatments demonstrates that "pericyte-mediated CNR is the dominant driver of AKI." We agree that the small cohort sizes (n = 3 per group) preclude such a conclusion. These findings are now presented more cautiously as preliminary observations under the specific experimental conditions tested, and we explicitly acknowledge that further studies with appropriately powered cohorts will be required to determine the relative contribution of these mechanisms to AKI.

3. **While minor, the plasma sampling description still needs clarification. The stated four cohort schedules (5 and 360 min, 10 and 240 min, 20 and 60 min, and 40 and 120 min) account for eight sampling times and 24 animal time point samples. However, the Methods and Fig. 2a also report measurements at 0.5, 1, and 2 minutes, with n=3 animals per sampling time point, and the source of these nine early samples is not specified. Please provide the actual animal by time point sampling schedule and clarify whether additional**

animals were used or whether some animals underwent more than two blood collections.

Extended Data Fig. 3a contradicts the statement that tissue concentrations "did not exceed 0.2 uM in any organs." At 40 mg/kg, the figure contains mean tissue values of approximately 0.23 and 0.30 uM. More generally, a single measurement at two hours, with n=2 animals at the 10 and 40 mg/kg doses, cannot demonstrate absence of tissue accumulation or "confirm rapid systemic elimination." The result can be stated descriptively – for example, that mean concentrations at two hours were generally low and below approximately 0.3 uM under the tested conditions. But claims about accumulation or elimination require time-course or repeated dosing data.

We thank the Reviewer for identifying these points and apologize that the plasma sampling schedule was not sufficiently clear in the previous version of the Methods. We have now revised the Methods to provide the complete animal-by-time-point sampling scheme. For the early 0.5-, 1-, and 2-minute time points, the close temporal proximity of the sampling points made repeated blood collection from the same animals undesirable for obtaining reliable measurements. Therefore, separate groups of three animals were used for each of these three early time points (n = 3 at 0.5 min, n = 3 at 1 min, and n = 3 at 2 min), corresponding to nine additional animals. For the subsequent time points, four cohorts of three animals were used, with each cohort sampled at two time points: 5 and 360 min, 10 and 240 min, 20 and 60 min, and 40 and 120 min. Thus, a total of 21 animals were used for the plasma concentration measurements. This complete sampling scheme is now explicitly described in the Methods to avoid any ambiguity.

We also thank the Reviewer for noting the discrepancy regarding the tissue concentrations and agree that our previous interpretation was too strong. We have corrected the reported concentration range to reflect the data shown in Extended Data Fig. 3a, with mean tissue concentrations at the 2-hour time point remaining below approximately 0.3 μM under the tested conditions. Furthermore, we agree that measurements at a single time point, particularly with n = 2 animals at the 10 and 40 mg/kg doses, cannot establish the absence of tissue accumulation or confirm rapid systemic elimination. We have therefore removed these claims and now report these findings descriptively

4. **Several mechanistic and translational statements remain stronger than the experiments support. The direct ex vivo pericyte experiment is a useful addition and shows that R(+)2501 can reverse ET-1-induced constriction at identified cardiac pericyte somata. However, the renal and myocardial ischemia experiments do not directly visualize pericyte contraction or**

demonstrate that all in vivo effects are mediated specifically through pericytes. The Summary should distinguish between direct in vivo imaging of capillary flow and the separate ex vivo demonstration of pericyte-associated relaxation.

Similarly, the pig experiment was performed in one animal with sequentially escalating doses. It is a useful physiological proof-of-concept observation, but it cannot establish reproducible dose dependence, capillary selectivity, or translational efficacy. The text should acknowledge this.

The Extended Data Fig. 4 legend refers to "irreversible changes" and states that pericytes remain "permanently and completely constricted." However, the same figure shows restoration of flow after R(+)2501 following up to 120 minutes of occlusion. "Refractory to reperfusion alone" would be more accurate than "irreversible" or "permanent."

We thank the Reviewer for these important clarifications. We have revised the manuscript accordingly.

Specifically, the Summary now clearly distinguishes between the direct *in vivo* imaging of capillary blood flow and the separate *ex vivo* demonstration of R(+)2501-induced relaxation at identified cardiac pericyte somata, without attributing all *in vivo* effects specifically to pericytes.

We have also revised the description of the pig experiment to explicitly acknowledge its proof-of-concept nature and the limitation of using a single animal with sequentially escalating doses. Claims regarding reproducible dose dependence, capillary selectivity, and translational efficacy have therefore been removed or appropriately qualified.

Finally, in Extended Data Fig. 4 and the corresponding text, we have replaced references to "irreversible" or "permanent" constriction with the more accurate description "refractory to reperfusion alone."

5. **The Supplementary Methods still state that the genotoxicity and acute tolerability experiments confirm that R(+)2501 is a "safe drug candidate," that it can be developed into clinical trials, and that the 200 mg/kg experiment establishes a greater-than-20-fold margin of safety. These statements directly contradict the response to Reviewer 2, which says that broad safety language was removed. Small, short-duration experiments can support only that no overt adverse signs or assay-positive effects were detected under the particular conditions tested. The claims concerning broad safety, clinical development, and a quantified margin of safety should be removed.**

Likewise, “cardiovascular safety profile” should be replaced by “acute hemodynamic effects in healthy rats” or similar wording. Extended Data Fig. 3d-j, with three animals per dose and ten minutes of observation, is informative about acute hemodynamic tolerability but cannot establish cardiovascular safety.

We thank the Reviewer for highlighting this inconsistency. We have now carefully revised and moderated the safety-related statements throughout the manuscript, including both the main text and the Supplementary Information.

Specifically, we have removed claims describing R(+)2501 as a “safe drug candidate,” statements regarding its suitability for clinical development, and the quantified >20-fold margin of safety. The results of the genotoxicity and acute tolerability experiments are now described strictly within the conditions tested, without broader conclusions regarding safety.

Similarly, references to a “cardiovascular safety profile” have been replaced with wording describing the “acute hemodynamic effects in healthy rats,” and the interpretation of Extended Data Fig. 3d–j has been revised accordingly to reflect the limited cohort size and short observation period.

6. **Minor: The diameter-relaxation model in Extended Data Fig. 3b is based on n=1 femoral artery and n=1 carotid artery, together with the basilar-artery and capillary estimates. The reported “effective doubling constant” of 130 ± 60 um should be described as exploratory or illustrative rather than as a robust fitted physiological parameter.**

   We thank the Reviewer for this clarification and agree with this point. We have revised the corresponding text and Extended Data Fig. 3b legend to explicitly describe the diameter–relaxation model and the reported effective doubling constant as exploratory and illustrative, rather than as a robust fitted physiological parameter.