

REVIEWER 2 - Comments and Suggestions for Authors

This is an extensive study on the efficacy of rho inhibitors vis-a-vis endothelial health and wound healing. The writing needs to be clarified at various points. For example, some measurements could be better explained with positive/negative controls. In addition, the science behind the pharmacological effects of the Rho kinase inhibitors must be sufficiently well described.

Line 2 - Title – alternative to rho-kinase inhibitors – the word alternative does not make sense without mentioning Y-27632. Please complete the title more effectively.

The title has been amended to “Effects of rho-associated kinase (rock) inhibitors alternative to Y-27632 on corneal endothelial cells” (Line 2)

Lines 21-22 - I would be surprised if others have not examined this in the literature.

Before proceeding with our *in silico* study, our team conducted a thorough literature search of over 800 publications on the *in silico* binding of various classes of rho kinase inhibitors to ROCK-I and ROCK-II. Although our searches revealed a number of papers discussing binding affinity and *in silico* discovery of new inhibitors of rho kinases, they were mainly focused in the context of other systemic diseases. To our knowledge, we are unaware of any studies that have combined *in silico* screening for ROCK-I and ROCK-II, specifically in the corneal endothelium, with subsequent validation through *in vitro* and *ex vivo* experiments, as presented in our manuscript.

Line 23 – how do you distinguish between “optimal” and “effective”? They appear identical to me.

We thank the reviewer for highlighting this. We meant “optimal” as the most effective concentration, hence we have changed the sentence to “most effective”. The line now reads “We then evaluated nine ROCKi for their effects on cultured primary CECs, before narrowing it down to the two most efficacious: AR-13324 (Netarsudil) and its active metabolite AR-13503, and assessed their impact on cellular proliferation *in vitro*.” (Line 22-24)

Line 31 – a typographical error

The error (µm) has been corrected (Line 29)

The abstract is lengthy and can be shortened. However, the abstract also does mention any data on the statistical significance of the results.

The abstract has been shortened, with mention of the statistical findings of the results section (Line 32-35).

Line 51- what is meant by “rich in tight junctions”? The corneal endothelium is a leaky epithelium since the number of tight junctional strands are few.

We have shortened this part of the introduction to be more concise (Line 48-56)

Line 55 – should be “endothelial.” Also, please avoid the abbreviation CED as it is not standard.

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We have corrected the mistake (Line 53) and have removed the use of the abbreviation CED from the manuscript.

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Line 101-105 – Provide references.

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The sentence has been rephrased with 2 references added (Line 92-98).

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Line 47-84: It would be good to abridge these lines. The Introduction is very long.

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We have re-written these three paragraphs in a more concise and objective manner (Line 48-79).

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Line 167 – Are we sure that In silico experiments described here have not been performed before? Explain if your computations/simulations are new or different. I am also surprised that there are citations in the paragraph.

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From our earlier reply, the experimental design we presented in this manuscript is unique in the context of the cornea endothelium where *in vitro* as well as *ex vivo* validations were performed.

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Line 198 – Describe xCelligence in 3-4 lines before detailing the experiments. Otherwise, the reader will not understand what was measured here. You talk about impedance in Line 216. Also, provide positive and negative control for cell index. What drugs/agents increase/decrease cell index?

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We have briefly described xCelligence as follows and have include it within the sub-section 2.4.2: Cellular viability assay using xCelligence – “The experiments involved the use of E-Plates (ACEA Biosciences, San Diego, CA, US) with gold-microelectrodes fused to their culture surface. As the CECs adhered, presence of cells at the electrode-solution interface will impedes electron flow. The impedance is measured by the integrated software, and is converted into arbitrary cell indexes for comparative analysis” (Line 195-199). For xCelligence experiments, the positive control was the inclusion of ROCKi Y-27632; whereas negative control was the exclusion of ROCKi (see Supplementary Figure 4). In the first 24 hours, the inclusion of ROCKi positive enhanced the attachment of CECs, resulting in a higher raw cell index (5.16 ± 1.02) as compared to the no ROCKi control (3.22 ± 0.66). This is in agreement with our previous observation (Peh et al., 2015).

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Line 221: The details of the xCELLigence real-time cell analyzer (ACEA Biosciences) are repeated.

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The repetition has been removed.

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Line 226 –manufacturer’s name is repeated in the line. Not necessary.

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This has been removed.

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Lines 234-236 – Description of Click-iT Cell Proliferation assay should be Line 231 before discussing the two ROCK inhibitors.

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We have corrected this.

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Line 233 – Cell index does NOT reach 1.0 when AR-13503 was 1 micromolar (fig. 1). What is the relevance?

For Figure 1, the concentrations used for the experiments were 100 nM, 10 μ M and 30 μ M. Assuming the reviewer meant 10 micromolar instead of 1 micromolar, where the cell index did not reach 1.0 at 48 h; this indicated that following the decreased CI at the first 24 h, the effect is not cytotoxic, and this is shown by the partial re-establishment of the respective CI at 48 h following the removal of AR-13501. This also suggest that, the working concentration for AR-13503 may be better for the CECs at concentrations lower than 10 μ M, as at this concentration or higher, the CECs viability, as gauged by xCelligence, become suboptimal.

Could you remove the subheadings in lines 270 and 278?

We have removed these sub-headers.

Correct units for concentration are in Fig. 2. Statistics are not indicated.

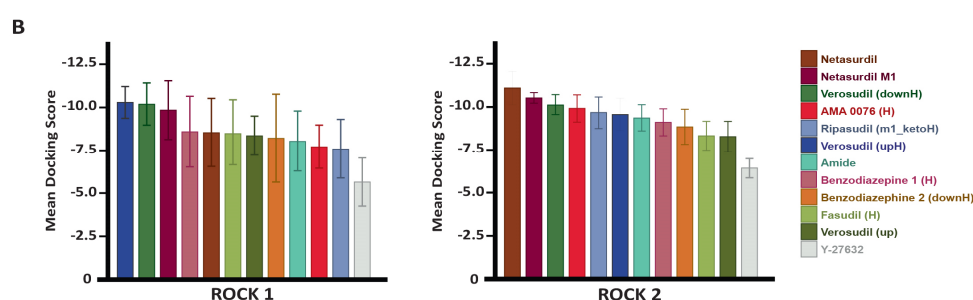
Statistical analysis using Tukey's multiple comparisons have been included for Figure 2 (Line 301-304 and Line 319-321).

Line 333: Table 2 shows less than 10 drugs but not 46 compounds.

Table 2 (added below) refers to the compounds that were used for the *in vitro* and *ex vivo* assays. The compounds used in the *in silico* experiments were presented within supplementary materials (Supplementary Figure 2B; see below where the bar chart plotted showed the mean docking scores and standard deviation of the top 10 compounds (from 46 compounds) that had the highest docking scores for both ROCK-1 and ROCK-2 and Y-27632.

Table 2. Summary of ROCKi used in this study

ROCK inhibitor Compounds	Compound Class	Concentration Range (optimal)
Y-27632	Pyridine Carboxamide	10 μ M
AR-13324 (Netasurdil)	Isoquinoline	100 nM to 1 μ M
AR-13503	Isoquinoline	100 nM to 10 μ M
AR-12286 (Verosudil)	Isoquinoline	100 nM to 10 μ M
Y-39983	Pyrrolopyridine	100 nM
K-115 (Ripasudil)	Isoquinoline	30 μ M
G-1	Indazole	30 μ M
G-2	Aminofurazan	10 μ M
G-3	Amide	30 μ M
G-4	Benzodiazapiene	10 μ M



Supplementary Figure 2. *In silico* docking scores of ROCK inhibitors used in this study. Histogram showing the clustered frequency distribution of compounds from ROCK inhibitors from different classes within ranges of docking score for (A) ROCK-1 and ROCK-2. (B) Bar chart showing the mean docking scores and standard deviation of the top 10 compounds (out of 46 compounds evaluated) that had the highest docking scores for both ROCK-1 and ROCK-2 and Y-27632.

Line 342-343. I cannot understand the sentence. A typical reader would need help understanding the docking score. Could you define/explain the term before using it? How does it relate to affinity?

We thank the reviewer for this comment and agree that these terms are complex and can be confusing.

Molecular docking matches structural and energetics of a ligand and a target at the molecular level. The molecular interactions are evaluated by means of scoring function. Classical scoring functions contains the physicochemical terms of the interactions, i.e. bonded interactions (bond stretching, angle bending and torsion angles) and non-bonded interactions (van der Waals and electrostatic interactions). Ideally, docking score can be correlated with the experimentally measured binding affinity, but sometimes this correlation might be limited, for example protein flexibility is not taken into account in rigid docking (Stanzione, et al., 2021).

In order to avoid the confusion with these terms, we have decided to not use terms related to “affinity” and replaced them by docking score or binding strength, which are more appropriate (Line 328 – Line350).

Fig. 3 – In the legend, indicate the number of biological repeats. Also, show statistical comparisons.

We have included these details in the legends (Line 412-418).

Line 352. What determines the impedance? Explain before using the term. Also, note that the Y axis in Fig. 2 is in terms of cell index. Importantly, you have yet to show control agents that can increase or decrease cell index.

As defined above, and added into sub-section 2.4.2 (Line 195-199), the xCELLigence system uses a unique E-plate containing interdigitated gold-microelectrodes fused to its culture surface. The baseline voltage without any CECs attached, and following CECs adherence are measured at the stipulated time points. The impedance is determined by the difference in baseline voltage and the voltage after cellular attachment. We have also included a Supplementary Figure 3 showing the treatment of primary CECs with 10 μ M of AR-13324, resulting in massive cell death at T=24h. Following refreshing of medium, at T=48h, only a small number of cells that survived the treatment were left (enlarged). This can be recapitulated by the graph as shown in Figure 1B. Additionally, we have included Supplementary Figure 4, which showed the adherence of primary CECs in the presence of Y-27632 (showing increased CI) and negative control

where primary CECs were left to adhere without the addition of Y-27632 (decreased CI). This observation is consistent with our previous report (Peh et al., 2015).

Fig. 4 – lacks a significant number of biological repeats.

Unfortunately, obtaining a proper number of corneal pairs to achieve statistical relevance of this age/regeneration correlation was not feasible due to the fact of the limited donor corneas that meet the inclusion criteria for our study. Majority of the “good quality” corneas are destined to clinical purposes.

The main objective in the inclusion of this figure is to point out a trend which we had described previously (Soh et al, 2016), and also found relevant in this particular study.

Lines 462-464 – Please double-check for accuracy. Does not seem right to me.

Line 462-464 discussed the cytotoxicity of AR-13324 to the cultured primary CECs when exposed at a concentration of 10 μ M and above. We have now substantiated this observation with additional study and has included it as Supplementary Figure 3 (Line 497-501).

Lines 455-468 – Are you correlated to cell adhesion to cell index/impedance?

Yes, as outlined in the schematic of Figure 1A, we have used the CI provided by xCELLigence during the first 24h as a measurement of adhesion. From this point on, increases to the CI might be attributed to cell-spreading and strengthening of cellular attachment as well as cellular expansion through cell proliferation; while a decrease in the CI can be caused by sub-optimal culture condition resulting in retraction of cells, weakening of adherence strength, and/or cellular toxicity leading to cell death. Specifically, as seen in Figure 1B, we believe that exposure to 10 μ M of AR-13324 lead to cell death where a fresh media change do not induce the recovery of cell index as the CECs had become non-viable and had detached (also see Supplementary Figure 3). Evidently, for Figure 1C, the first 24 hours showed signs of detrimental effects to the CECs based on the decrease of cell index in a dose dependent manner. With the change of fresh media, cell index rebounded back suggesting that the CECs remain viable. We have included Supplementary Figure 3 to illustrate this occurrence.

REFERENCES

1. Peh, G.S.L.; Adnan, K.; George, B.L.; Ang, H.-P.; Seah, X.-Y.; Tan, D.T.; Mehta, J.S. The effects of Rho-associated kinase inhibitor Y-27632 on primary human corneal endothelial cells propagated using a dual media approach. *Scientific Reports* 2015, 5, 9167, doi:10.1038/srep09167.
2. Stanzione, F.; Giangreco, I.; Cole, J.C. Use of molecular docking computational tools in drug discovery. *Prog Med Chem* 2021, 60, 273-343, doi:10.1016/bs.pmch.2021.01.004.
3. Soh, Y.Q.; Peh, G.; George, B.L.; Seah, X.Y.; Primalani, N.K.; Adnan, K.; Mehta, J.S. Predicative Factors for Corneal Endothelial Cell Migration. *Invest Ophthalmol Vis Sci* 2016, 57, 338-348, doi:10.1167/iovs.15-18300.

Change of Figures 2

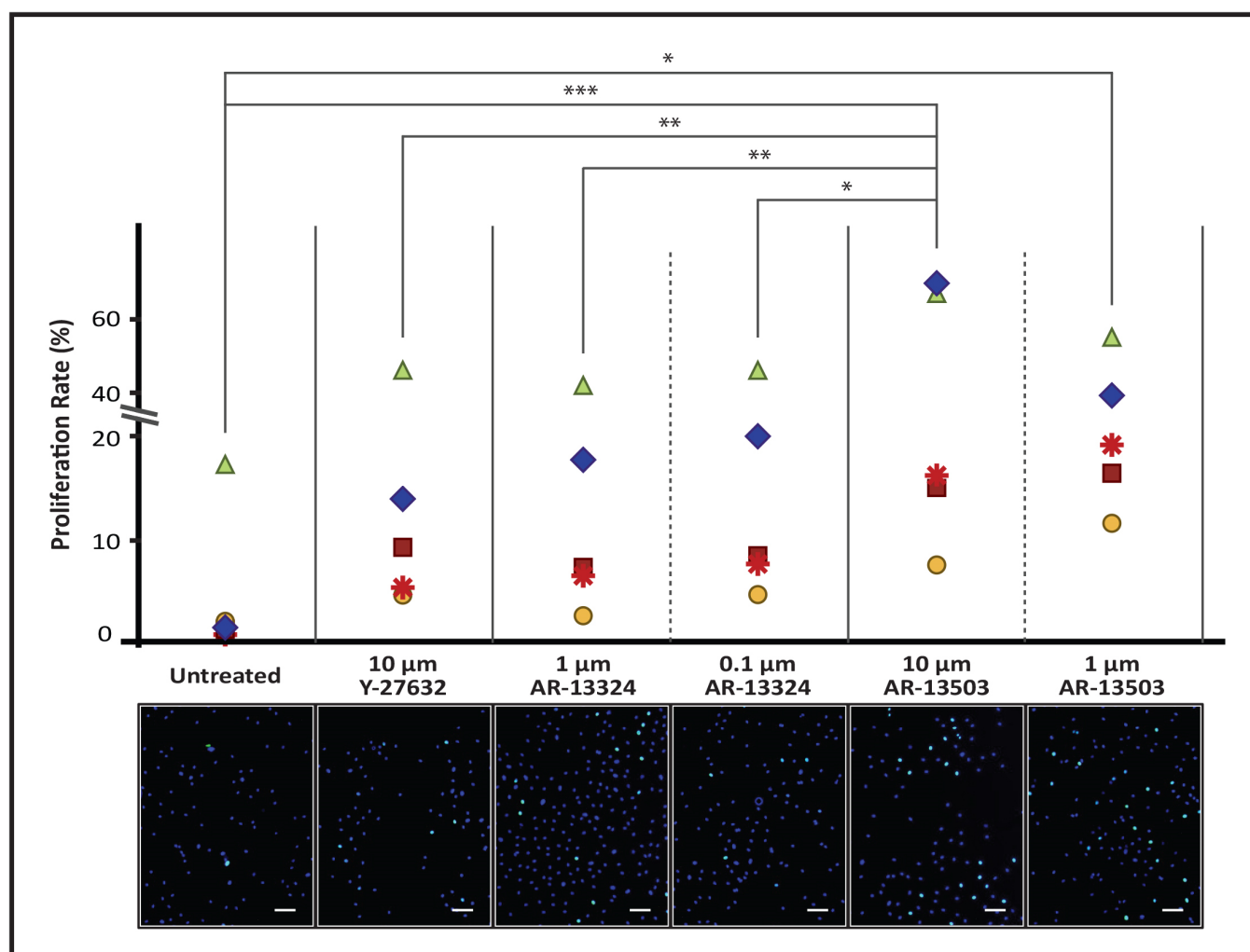
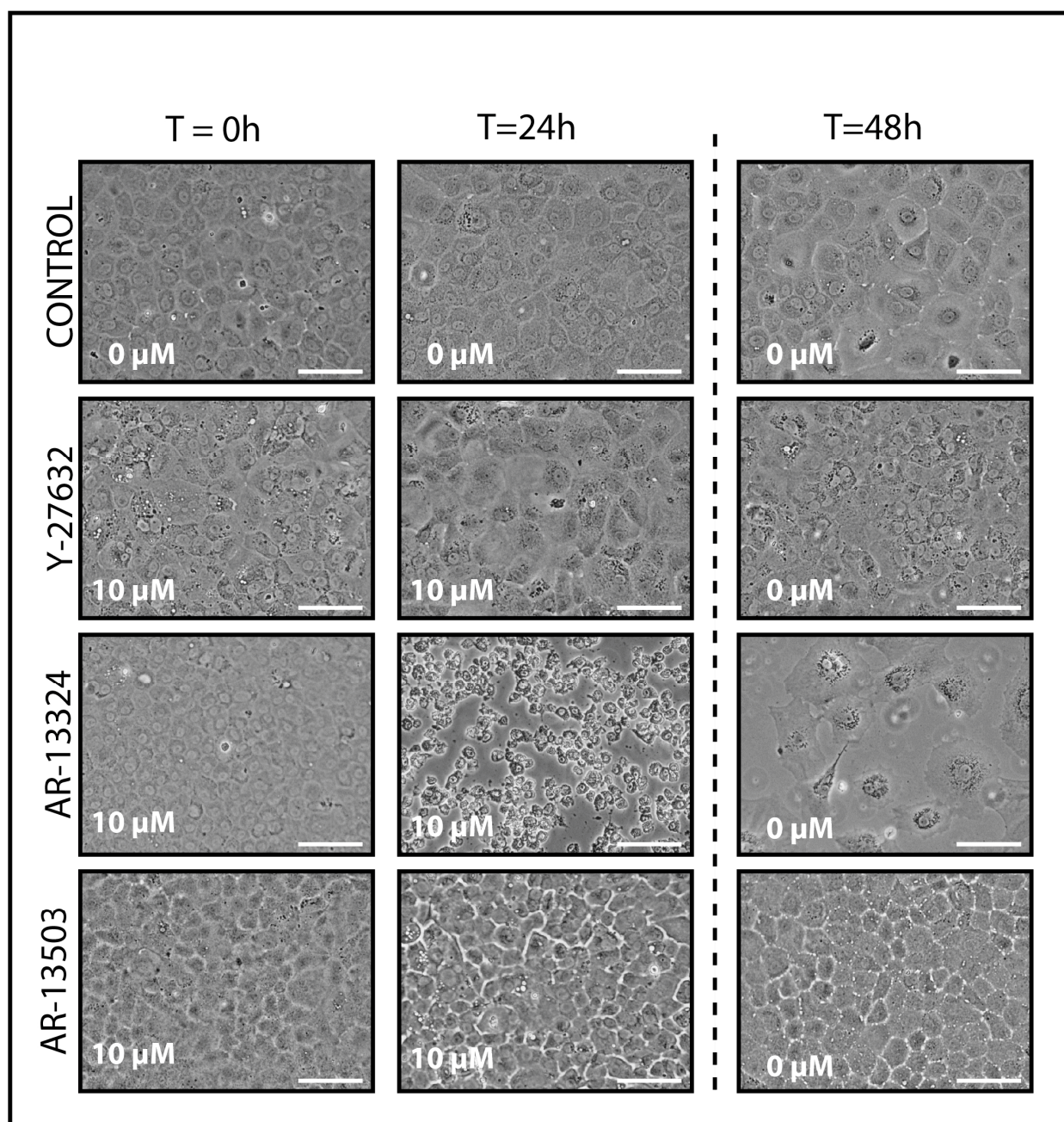


Figure 2. Proliferation of primary CECs treated with ROCK inhibitors: The dot plot represents the proliferation rate (%) of expanded primary CECs that were donor-matched, comparing between CECs that were untreated (negative control), as well as supplementation of Y-27632 (10 μM; positive control); AR-13324 (1 μM and 0.1 μM); and AR-13503 (10 μM and 1 μM). The corresponding images below each dot plot are representative images of the Click-IT Edu proliferation assay. The merged images showed nuclei of the primary CECs counter-stained blue (DAPI), while proliferating cells were labelled with fluorescent green (Click-IT Edu-GFP). Tukey's Test for multiple comparisons found the proliferation rates were significantly different between (i) untreated and AR-13503 (1 μM; $*p < 0.05$); (ii) untreated and AR-13503 (10 μM; $***p < 0.001$); (iii) Y-27632 and AR-13503 (10 μM; $**p < 0.005$); (iv) AR-13324 (1 μM) and AR-13503 (10 μM; $**p < 0.005$); as well as (v) AR-13324 (0.1 μM) and AR-13503 (10 μM; $*p < 0.05$).

Supplementary Figure 3

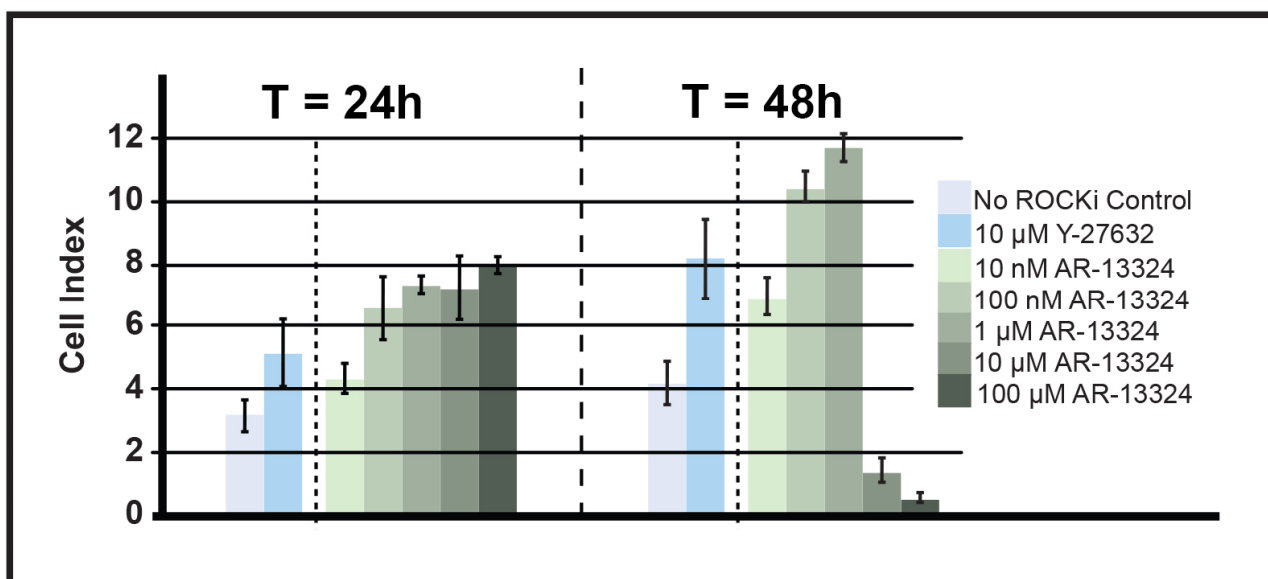


Supplementary Figure 3. Cytotoxic effects of ROCK inhibitor AR-13324 on primary CECs. Dissociated primary CECs (second passage) were seeded and left to attach to establish a monolayer (Time = 0h) before being treated with ROCK inhibitors Y-27632, AR-13324, or AR-13503 at the concentration of 10 μ M for 24 hours (Time = 24h). Subsequently, ROCK inhibitors were removed with a change of fresh culture medium (depicted by the dotted line) and left for an additional 24 hours (Time = 48h) Scale bars: 100 μ m.

Supplementary Figure 4

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Supplementary Figure 4. Impedance readings of cellular attachment of CECs in AR-13324. Representative figure showing the effects of the extended range of ROCK inhibitors AR-13324 on primary CECs as recorded by impedance reading with the xCelligence at Time = 24h when seeded without the presence of any ROCKi (No ROCKi Control (light blue)), and in the presence of 10 μ M of Y-27632 (blue), 10 nM AR-13324 (light green); and 100 nM AR-13324 (remaining 4 CECs populations) for the first 24 hours. On the following day, media were refreshed with the respective concentrations of ROCKi, and its corresponding impedance read-out were subsequently recorded following 24 hours (Time = 48h) of exposure.

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