nature portfolio

Peer Review File

Small Molecule APOL1 Inhibitors as a Precision Medicine Approach for APOL1-mediated Kidney Disease

Corresponding Author: Dr Brandon Zimmerman

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
General
The authors follow up their 2023 New Eng J Med report of a Phase 2 clinical trial of inaxaplin in APOL1-mediated proteinuric kidney disease. The current report includes a brief summary of the APOL1 cation channel inhibitor screen, and demonstration that small molecule inhibition of APOL1 blocks cell death of clonal APOL1-expressing cell lines, blocks trypanolysis, prevents development of proteinuric kidney disease in APOL1 transgenic mice and reverses established proteinuric kidney disease in those mice. The clinical candidate inhibitor inaxaplin also was shown to block APOL1 cation permeability as measured by thallium influx and patch clamp assays.

The work is very well done and concisely presented.

Several relevant earlier publications have not been cited, or cited but not credited for particular prior findings.

Major points

Methods:
APOL1 G0, G1, and G2 constructs.
Please specify if these AAV constructs encode “original reference sequences” or “physiological haplotype sequences.”
The APOL1 G2 multicopy transgenic mouse expressed the physiological G2 haplotype. The APOL1 G2 single copy homozygous and G2 multicopy transgenic mouse strains greatly resemble those described in ref 26 but not referenced here. Please clarify the origin of these two mouse strains. Please also note whether these mice express the physiological G2 haplotype.

Electrophysiology Line 384: Note if NMDG is as the hydrochloride or gluconate?

Trypanosome assay: Provide citation for Alamar Blue assay here or in Results.

Compound 3 assessment in mice Lines 516-7.
Use of murine IFN in CpG-free plasmid should be referenced to ref. 26.
Mouse plasma IFN levels should be presented in supplement or Methods.

Results:
Presentation is very sparse. Some methodological details as mentioned below should be provided in text or figures/legends for ease of reading, or reader should more often be referred to Methods for details.
Fig. 2 should include exemplar whole cell current traces across the range of tested potentials.
Fig. 2A. Legend should include bath and pipette solution compositions. Text (line 121) mention of “when isolating outward currents” should be described in greater detail.
Fig 2B. Legend should include holding potential at which reported current amplitude measured.
Fig 2C. Legend or Methods should identify anti-APOL1 antibody and dilution used in FACS assay and the targeted antigenic component of APOL1. Note “N” of measurements in panel or legend.
Fig 2D Legend should define media compositions (beyond the brief designations in the key) and the number of hours of Tet

induction preceeding measurement of the dead/live ratio. Note “N” of measurements in panel or legend.

Text describing Fig 2 should note that panel A reversal potentials are considerably more electronegative than previously reported, likely due to the choice of permeant ions in the current experiments, with Cs substituting for internal K, and NMDG substituting for external Na,
Text describing Fig 2 should note that results of panel B are similar to previous reports of
O’Toole et al, JASN 2018, and Vandorpe et al Pflugers 2023.
Text describing Fig 2C should note that G0 surface expression 2.5-3-fold higher than G1 or G2 has not been previously reported – previous reports were of roughly equivalent surface expression.
Text describing Fig 3 should note that high K medium’s near-total prevention of cell death is more protective than most recently reported by ref 21.
The conclusion that cell death is associated with increased K+ efflux rather than Na+ influx should be noted as supporting the conclusion presented by ref. 21.

Fig 3A legend should note that whole cell currents were measured.
Fig 3 all panels should present error bars as SD rather than SEM. “N” of measurements should be noted in panels or in legend.

Fig S5A should note identity of anti-APOL1 antibody used for IHC.
Fig S5B key should change G2hom to G2mc/G2mc or G2mc/hom. (for uniformity of presentation, if I understand correctly)
Fig S5C Y axis label should change G2hom to G2mc/G2mc or G2mc/hom
Fig S5 all panels should present error bars as SD rather than SEM

Lines 153-156: Please clarify if transgenic APOL1 G2mc mouse should be referenced to ref 26.
Line 170 – Clarify process of randomization based on albuminuria. Were mice without albuminuria excluded from the experiment? Or were mice merely randomized between treatment and vehicle groups?
Lines 179-80 – synechiae and vacuolation/reduplication of PEC should be annotated in panels B or E

Fig 5A-F should include H&E low power panels for each condition to allow visualization of multiple glomeruli.
Fig 5 legend line 837-8 – change to “Control nephrin immunostaining intensity was significantly reduced in vehicle-treated mice, and was rescued to control levels by compound 3 treatment.”
Fig. 5G should be accompanied by images of nephrin immunofluorescence staining in all three conditions.
Fig 5 legend line 841: “kidney filtration slit diaphragm.”
Fig 5 H/I/J: MIP image is pseudocolored for depth. The podocin and integrin a3 immunofluorescence images must also be presented, either here or in supplemental data.

Discussion:
Line 221: Note that VX-147 binding affinity was 50-100x lower than inhibitory activity in thallium influx assay (ref 25) or patch clamp currents (this paper).
Lines 228-231: Ref 32 should be cited in Results when appropriate.

Minor points
Line 57 – “explanation both as to why not all people…..”

Lines 116-118 – inaxaplin as inhibitor of APOL1-mediated thallium influx needs citation of ref 25.
Lines 182: change “no findings” to “no abnormalities”
Line 184. Is ref 28 an intended citation? Better to use an article on nephrin rather than a textbook chapter. If as intended, please complete the ref.
Line 185 – glomerular
Line 221 – VX-147 was previously shown…
Line 226 – There seem to be

Reviewer #2

(Remarks to the Author)
General Feedback: The authors have contributed valuable information to the existing scientific literature on APOL1 inhibitors, specifically focusing on VX-147 and three analogs. Promising Phase 2 results have recently been published in NEJM (Ref 25) with VX-147. The authors have described the origins of the chemical matter for clinical candidate, VX-147 and presented compelling data demonstrating the inhibitory effects of these molecules on APOL1, thereby reducing damage in both in vitro and in vivo systems by targeting the ion channel activity of APOL1. It should be noted that certain data presented in this manucrsipt overlaps with a previously published article in the New England Journal of Medicine (NEJM). Therefore, we recommend that the editor assess whether this publication contains sufficiently novel material to justify its publication in Nat. Comm., or if it would be more appropriate for another journal. Nevertheless, the inclusion of the treatment mode in vivo experiments and the additional characterization of the molecules' mode of action contribute to the overall scientific understanding of VX-147 which is progressing in the clinic for the treatment of kidney disease associated with genetic variants of ApoL1. While we believe that this study enhances the knowledge of these molecules and their mechanism of action, we bring this to the attention of the editor for consideration.

Specific Feedback:
General: APOL1 italics is mostly correct regarding when it is a gene versus when the authors refer to the APOL1 protein, but there are some cases that look to be incorrect formatting. E.g. Line 35: reads “two APOL1 alleles” but should be italicized “two APOL1 alleles”
Lines 202 and 207: “two APOL1 risk variants” one it italicized, not the other.

Lines 36-37 wording consideration: reads “Patients with AMKD progress more rapidly than people with similar clinical features who lack APOL1 genotype, and have higher rates of..” Feedback: the disease progresses, not the patients. Also specify the variants of the gene in the sentence.

Line 85 & Lines 221-222 The authors state that these are specific inhibitors of APOL1 channel activity and broad profiling data is available. It isn’t clear in this manuscript that the authors are referring to a list of proteins in the supplemental of Ref 25 for VX-147. Please provide additional data in the supplemental to show specificity of compound 3 in the supplemental material for this manuscript, since it is being tested in animal models. In Reference 25 the authors show a list of off-target proteins assessed, but we didn’t see data reported such as drug concentrations tested and whether any marginal activity was observed against other channels. To put in perspective the in vivo data reported in this manuscript the specificity data for compound 3 would be valuable.

Line 109 reads “across the plasma membrane”. Suggested edit: “across the cell membrane”

Line 114: refer to Table 1 with in-vitro and in-vivo PK data for 4 compounds

Page 4, Line 136, Figure 3a: Provide a reference for the Thallium flux data since this information was previous reported for VX-147 in the NEJM reference #25.
Figure 3a: The curve and data points presented for VX-147 is different than was reported in NEJM Ref 25 Figure 1a. There are several discrepancies between the data presented in the two figures for the same assay and same compound. Please either provide an explanation for why there are these discrepancies (e.g. number of test points are different, curve fitting is different, different data set etc) or remove Fig.3a from this paper and just reference Figure 1 in Ref 25 and show the IC50 values reported in the text and Table 1. From a curve fitting perspective adding a 100% point is not appropriate for graphical purposes as was done in Figure 3a but not Fig1 -Ref 25.
Line 139-140, 161/Table 1: Since compound 3 was studied in an ApoL1 G2 mouse, provide the in vitro data for compound 3 in the G2 cell line. There is data for VX-147 in both G1 and G2 cell lines (considering Table 1 and Figure 3), but not for compound 3, which was studied in this paper. The reader doesn’t have the context to understand if/how the Methyl to Fluorine change in structure impacts activity at ApoL1 G2.
Line 158-162: As readers of drug discovery literature, we were interested in the report that VX-147 showed efficacy at 3 mpk (REF 25) but compound 3 was tested at 30 mpk in the G2 in-vivo efficacy model in this report. The authors state the molecules have “comparable potency”. Please provide a brief statement of why a 10 fold higher dose was studied for compound 3. This will add to the understanding of the pharmacology and PK/PD relationships for the inhibitors.
Figure 4: Please provide drug exposures in the 30 mpk bid study treatment study if data is available. The data would be valuable to the community to understand the drug concentrations needed to achieve treatment efficacy.
Table 1: Consistent with Nature Journal guidelines provide statistical analysis and number of replicates for the in vitro assays on both APOL1 G1 assays on lines 2 and 3.
Line 185: reads “glomerulal” suggestion: glomerular

Lines 206-207: “relative to those without two APOL1 risk variants” . For the reader who isn’t as familiar with APOL1 biology, it would be helpful if the authors can briefly discuss whether people with one APOL1 variant have evidence of disease as well under some circumstances.

Line 221: It is confusing to the reader that the authors are referring to binding data in NEJM reference 25. Please be more specific than just providing the reference. Both reviewers went back to look for direct binding data in this paper before realizing you were referring to ref 25 Figure 1 data.

Figure 2D: It is hard to see what is different in the bar graphs when printed in black and white. Consider making the bars a different texture and color so it is clear.

Line 672: As requested for Nature Journals please provide the synthesis of compounds 2, 3 and 4 rather than refer the reader to the patent. We recommend that additional characterization data is provided for 3 and 4 (VX-147) consistent with medicinal chemistry literature and Nature Journals requirements for lead molecules. C13 NMR for both molecules as well as an optical rotation and single molecule crystal structure for either Compound 3 or VX-147.

Line 687 (characterization table): Please check the formatting and data in the characterization table.
Discrepancies in NMR between submission and patent were noted in NMR for compound 2.

-Compound 1: parenthesis missing, J should be italicized
Compound 2: Two HNMR signals are missing from the NMR report. Possibly obscured by solvent. If so please indicate that “peak obscured by solvent”.

Reviewer #3

(Remarks to the Author)
The manuscript by Zimmerman et al. demonstrates that inhibiting APOL1 ion channels is beneficial in a mouse model of APOL1 associated nephropathy. Mark E. Bunnage was previously an author on a publication in NEJM that showed that inhibiting APOL1 channels in a mouse model of APOL1 associated nephropathy before the induction of proteinuria was beneficial in reducing proteinuria compared to untreated mice. This paper is significant as it highlights that the detrimental effects of APOL1 G1/G2 variants is due to potassium efflux and shows that inhibiting APOL-1 channels after the induction of proteinuria is beneficial in reducing kidney failure outcomes.

The original article is interesting and addresses an important area of research. However, there are a few key points that need to be considered:

a) The paper discusses the drug discovery process of identifying inaxaplin as a clinical drug targeting ApoL1, however the drug used in the manuscript is compound 3 which was a previous APOL1 inhibitor that was then developed into inaxaplin. The paper should discuss why they utilized compound 3 in this study instead of inaxaplin, or at least in comparison to inaxaplin.
b) Since the paper highlights G1/G2 risk alleles being the cause of APOL1 associated nephropathy over G0 risk alleles, the in vivo data should also include G0 transgenic mice.
c) Fig S5. should show significance in B and E.
d) Data in figure 3 should also be normalized to the G0 group if the groups are on the same graph to show the difference between the risk variants. The Trypanosoma viability is higher in the G2 group at baseline compared to the G0 group, which is opposite of what is described.
e) Significance should be shown in Fig. 4A.
f) Are there representative images for Fig. 5G?
g) The amount of male vs female mice used in the study should be displayed.

Reviewer #4

(Remarks to the Author)
Very interesting study with major translational potential. It appears that the presented drug has been moving to clinical trials.

I cannot see the original electrophysiology data. I/V curves or single channel recording if available should be shown and channel selectivity data etc must be shown. It is hard to fully understand what is presented on figure2.

From the figure it seems that the compound prevented the cell surface expression of APOL1, if this is the case it should be validated. In addition we would need to know whether total APOL1 expression showed differences (like a western blot). While surface reduction of apol1 is interesting this information is not consistent with channel blocking function.

The mouse model should be fully characterized.

Fig2d should have a comparison to wild type or G0 cells.

Figure3. we would need to have surface apol1 expression for these cells to understand whether this is a channel blocker or a molecule that inhibits surface expression of apol1.

Figure4. Was there a difference in IFN expression? Was there a difference in APOL1 expression?
Panel b is not consistent with panel A

Please share toxicology data? How about liver enzymes, blood pressure, and CBC?

Figure5. was the nephrin score different because podocytes died? Please share podocyte count?

PAS stain should be quantified.

How does the channel function lead to cell death?

Overall interesting work and the new chemistry is interesting but we do not understand whether or not the compound truly inhibit channel function and the mechanism this compound protect mice from kidney disease development.

Reviewer #5

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #6

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This revision of NCOMMS-24-09345 has responded conscientiously to the critical points raised by the reviewers. Several issues remain, however.

APOL1 transgenic mice.
In Rebuttal the authors note: "The mice used in the study are consistent with those in ref 26 (now 28)."
This unsual wording is ambiguous, and is reinforced by inconsistent ambiguities in both Rebuttal and revised Methods and revised Results/Discussion.
The first paragraph of Revised Results states "we generated mice homozygous for the APOL1 G2 variant" without citation, leaving the reader to believe the mice are newly generated for this paper.
The first sentence of the Supplemental Methods section "Mouse Model Methods" now declares a transgenic mouse was "procured" (meaning generated?) Please specify if the CHORI BAC clone was G0, G1 or G2. Please clarify uniformly throughout the paper if this mouse is that described in ref 26 (now 28). which describes construction of the mouse strains with which the strains described in the current manuscript "are consistent?").
G2-multicopy mice are referenced to CRL. Please clarify it these G2 multicopy mice are those described in (revised) ref 28. Please clarify if CRL was used for strain maintenance and propagation.
The rebuttal notes that the G2 multicopy mice expressed APOL1 K150. This information must still be added to Supplemental methods on p 13.

In the Rebuttal response to Reviewer #3, the authors state "we have clarified in the manuscript that the mouse models used are described in more detail in ref 26 (now 28)." However, this clarification appears to be missing from the revised manuscript in several places. On p.2 of revised Results the authors write in the section entitled "In vivo characterization of APOL1 channel inhibitors:

"Since model organisms lack endogenous APOL1 expression, we established transgenic mice expressing human APOL1 G2. We determined the APOL1 G2 transgene inserted multiple times in tandem, and therefore classify these mice as APOL1 G2 multicopy mice (APOL1 G2mc). We found that homozygous APOL1 G2mc were viable, healthy, lived a normal lifespan, and had no baseline phenotype. However, a single injection of interferon-γ (IFNγ), which induces APOL1 kidney expression (Supplementary Fig. 9A), led to significant APOL1 dependent proteinuria in APOL1 G2mc compared to control Friend leukemia B virus (FVB) mice (Supplementary Fig. 9B, 9C and 10)."

Average readers will interpret the above quoted section to understand that the manuscript authors created and characterized the mouse as part of the current paper. But these mice and all attendant results summarized in the bolded paragraph appear to have been reported in print 3 years ago in ref. 26 (now 28). If the latter understanding is correct, then the wording in the above paragraph and throughout the paper should reflect this.

Supplemental Mouse model methods lines 14-16: Was the "recombineering" done in vivo to generate the G2 single copy mouse? How was G2 derived from G1 by recombineering into a G0 BAC?. Citation and description are needed.

Please clarify if both multi-copy and single-copy transgenic mice were bred to homozygosity.

Electrophysiological studies:
The electrophysiology solution compositions have been more fully explained in revision, but can be further clarified.
Rebuttal states that "NMDG replaced Na+ in the extracellular solution to isolate K+ efflux." But the intracellular solution also completely replaced K+ with nominally impermeant Cs+.
The revised results and supplemental methods sections claim that APOL1 is permeable to Cs+, but data testing Cs+ substitution as a single experimental maneuver is not presented. To this reviewer's awareness APOL1 Cs+ permeability has not been previously reported. Authors should relate or add to the Supplemental Methods data documenting VX-147-sensitive APOL1 Cs+ permeability and the permeability of Cs+ relative to Na+ or K+ before presenting data obtained in conditions of simultaneous CsF substitution for intracellular KCl and NMDG for extracellular Na.

Revised Fig 2C legend describes peak current at (presumably intracellular) holding potential of -80 mV, while presenting outward currents. Was the holding potential intended to be written as +80 mV?

Cytoxicity experiments
Revised Fig 2E: requirement of K+ efflux for cell death.

Change “necessity for K+ triggering cell death” to “necessity for cell K+ efflux to trigger cell death.”
The revised text currently lacks and should make additional reference to the earlier work from Raper and colleagues proposing a more significant role for Na+ entry than for K+ efflux in APOL1 cytoxicity, note whether that data was obtained in the same or different cell types, and briefly discuss the difference in their and the current conclusion.

Drug binding:
Rebuttal states that Discussion was modified to reflect 50-100-fold lower affinity for VX-147 binding than for channel or flux inhibition. But this reviewer finds no such modification in the Discussion. Rather, revised Discussion claims “VX-147 was previously shown to bind directly to purified, recombinant APOL1 with high affinity,” without further explanation. Please clarify this statement, noting difference between binding affinity and affinity based on inhibition of conductance.
Please also clarify the phrase “potential off-targets” by specifying what was measured (inhibition of other ion channels?) and by what method (can be reference or notation of CRO or panel product).

Revised discussion: change “We demonstrate that efflux of K+, and not influx of Na+, is responsible for APOL1-mediated cell death,” to indicate that the conclusion applies “in our recombinant cell system.”

Minor points:
Please paginate and add line numbers.
Please refer to original manuscript line numbers for the several changes made without reference to line numbers in either original or revised manuscript.

Fig. 4A. Please specify if APOL1-G2 transgenic mice used for this figure were multi-copy or single-copy.
Fig 5B legend. Change “black asterisk” to “gray star”.

Multiple supplemental figures are incorrectly numbered in the rebuttal
Rebuttal description of changes to Fig S5A and S5B are found in S9A and S9B
Rebuttal discusses INFg levels in Fig S11, but revised paper presents the data as Fig S13.
Rebuttal Fig S12 is manuscript Fig. S14.
Rebuttal Fig S13 is manuscript Fig. S15.

Reviewer #2

(Remarks to the Author)
Our reviewer comments were satisfactorily addressed, and complementary data were incorporated to the manuscript and Supplemental materials. Syntheses of all compounds and intermediates are reported, and initially observed discrepencies have been addressed. 13C, 19F and 1H NMR data for lead molecules are reported. We also appreciate that HPLC purity trace and single molecule crystal structure for VX-147 were included.

Minor suggestions:
Data in table 1 show activity toward ApoL1 G1 and G2 variants are comparable, bridging the HTS screen and in vivo data. This key finding could be highlighted more strongly in the text. As per the author’s response to the reviewer: “in vitro characterizations of Compound 3 and VX-147 in both thallium-flux assay and HEK cell rescue assay indicate this series of APOL1 inhibitors display comparable inhibitory potential across the APOL1 variants.”

Minor corrections in synthesis section:
- “J” should italicized in NMR reports
- Line 755 reads: “7.21 (t, 2H), either J coupling is missing or a multiplet was observed
- Line 833 reads “Mg2SO4”: should it read MgSO4 or Na2SO4?
- Line 846: structure of S14 should be corrected to the carboxylic acid (the ester is displayed)
- Lines 984/985: if possible, clarify the column used for HPLC trace, in particular, it would be more informative to indicate if a chiral column was used for the purity

Reviewer #3

(Remarks to the Author)
The authors of the manuscript “Small Molecule APOL1 Inhibitors as a Precision Medicine Approach for APOL1-mediated Kidney Disease” included revisions/improvements or addressed our concerns and others. This included adding additional supplemental figures. Some revisions and improvements should still be addressed, however. In particular, all experiments should be presented with a sufficient number of biological replicates. In addition, measurement of podocyte number should be included.

1)Supplementary figures contain titles above and below the figure. Please include just one unless this is the correct format for the journal.

2)The supplementary figure 13 (serum levels of IFN) is not mentioned in the main text of the manuscript.

3)Supplementary figure 14 says the images are 40x, however; the images do not seem to be at the magnification of 40x based on the size of the glomeruli. Please include a scale bar.

4)Pease include a scale bar on Supplementary fig 15.

5)Please remove the bold for Fig. 2 (A) caption. “Representative voltage ramp-current response after 16 h tetracycline induction in APOL1 G1 expressing HEK293 cells.”

6)Two of the groups in Fig 3B, C are done in one biological sample (N=1). This should be increased to multiple biological groups.

7)It appears some data points in Fig 4A are missing SEM bars. Please include these.

8)Please include a scale bar/magnification for figure 5.

9)In response to the critiques of the reviewers, the authors stated that the channel inhibitor does not affect expression of APOL1 without showing data supporting this or providing the appropriate citation. Data showing how the channel inhibitor affects APOL1 expression would be interesting regardless of the result.

10)It remains unclear if the nephrin score is due to decreased nephrin expression or to a different rate of podocyte loss. As recommended in the first round of review, podocyte number should be evaluated by performing a WT1 staining.

Reviewer #4

(Remarks to the Author)
The team has responded to my concerns.
I have no additional concerns.

Reviewer #5

(Remarks to the Author)
"I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts."

Reviewer #6

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have responded to almost all the points raised.
Only minor points remain.

Fig 2A and B legend (or panel inset) should restate the predominant ionic composition of extracellular and intracellular (pipette) solutions to aid reader understanding, just as panel E legend presents the cell growth media compositions rather than referring reader back to Methods.

Fig 2C legend still states that the outward currents presented are at “holding potential = -80 mV”. The rebuttal states “the holding potential was indeed -80 mV followed by the ramp.” But the holding potential information doesn’t address the maximal currents presented in Fig 2C. Fig 2C presents outward currents of a magnitude consistent with current measured at the end of the ramp, i.e. +80 mV. Indeed, the Methods section lines 447-448 states that “peak outward current was measured at the end of the 200 s test pulse at +80 mV.
So Fig 2C legend should add that the outward currents presented are those measured at +80 mV at the end of the voltage ramp.
Note (line 447) that the test pulse duration should be 200 ms rather than the current 200 s.

Specify in Fig 2B and 2C legend if mean+/-SEM is shown, as for later panels.

Supplementary Fig 9B legend Line 1015: delete “outward.”

Electrophysiology methods:
Line 437-8: we identified APOL1 channels to permeable to Cs+. Cs+ was selected also to block endogenous voltage-gated K+ channels.
Line 440: supporting nonspecific cation flow
Line 440: Resistance
Lines 443-4: to each concentration, 5 min after initial current recording (control).
Line 451: if the peak current amplitude was ….”
Lines 450-452: specify if these parameters used to remove cells from analysis were applied to all analyses, or only to inhibitor IC50 analyses (since a small number of values shown in Figs 2B and 2C would not meet the criteria for inclusion.

Reviewer #3

(Remarks to the Author)
The authors have properly addressed all my concerns.

Reviewer #5

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

We thank the reviewers for their thorough review of the manuscript. Please see below a point by point response to the comments from the four reviewers.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

General
The authors follow up their 2023 New Eng J Med report of a Phase 2 clinical trial of inaxaplin in APOL1-mediated proteinuric kidney disease. The current report includes a brief summary of the APOL1 cation channel inhibitor screen, and demonstration that small molecule inhibition of APOL1 blocks cell death of clonal APOL1-expressing cell lines, blocks trypanolysis, prevents development of proteinuric kidney disease in APOL1 transgenic mice and reverses established proteinuric kidney disease in those mice. The clinical candidate inhibitor inaxaplin also was shown to block APOL1 cation permeability as measured by thallium influx and patch clamp assays.

The work is very well done and concisely presented.

Several relevant earlier publications have not been cited, or cited but not credited for particular prior findings.

Thank you for pointing this out. We have cited additional manuscripts according to your requests and credited them appropriately.

Major points

Methods:
APOL1 G0, G1, and G2 constructs.
Please specify if these AAV constructs encode “original reference sequences” or “physiological haplotype sequences.”

We have updated the methods section to reference the sequence of APOL1 used. The 3 AAV vectors, all use the physiological haplotype sequence with E150/I228/K255.

The APOL1 G2 multicopy transgenic mouse expressed the physiological G2 haplotype. The APOL1 G2 single copy homozygous and G2 multicopy transgenic mouse strains greatly resemble those described in ref 26 but not referenced here. Please clarify the origin of these two mouse strains. Please also note whether these mice express the physiological G2 haplotype.

We have clarified the manuscript to indicate that the APOL1 G2 multicopy mice have a lysine at position 150 (K150), whereas the single copy mice have a glutamate at position 150 (E150). The mice used in the study are consistent with those in reference 26 (now 28).

Electrophysiology Line 384: Note if NMDG is as the hydrochloride or gluconate?

We have clarified the manuscript to list the catalog number of the NMDG from Sigma. The NMDG is the free base. The extracellular solution used for the electrophysiology experiments was acidified using hydrochloric acid to obtain a final pH of 7.2

Trypanosome assay: Provide citation for Alamar Blue assay here or in Results.

Thank you for calling this out. We have added the following reference to the methods section; Rampersad SN (2012). Sensors. 12(9):12347-12360; which is found on the ThermoFisher scientific website.

Compound 3 assessment in mice Lines 516-7.
Use of murine IFNγ in CpG-free plasmid should be referenced to ref. 26.
Mouse plasma IFNγ levels should be presented in supplement or Methods.

Thank you for highlighting this. We have added a reference to ref. 26 (now 28) in the methods on line 517. We have added a figure in the supplemental section (Supplementary Fig. 11) presenting the plasma levels of IFNγ from this study.

Results:
Presentation is very sparse. Some methodological details as mentioned below should be provided in text or figures/legends for ease of reading, or reader should more often be referred to Methods for details.

We thank the reviewer for this guidance. We have either increased details present in the figure legends or directed the reader to the Methods for further details.

Fig. 2 should include exemplar whole cell current traces across the range of tested potentials.

We have added an example APOL1-mediated current with voltage protocol also included, showing activation of this current.

Fig. 2A. Legend should include bath and pipette solution compositions. Text (line 121) mention of “when isolating outward currents” should be described in greater detail.

The same solutions were used for all the electrophysiological recordings. These are included in the methods sections under electrophysiology. For your reference “*Cells were perfused with extracellular solution containing: 137 mM NMDG, 5.4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Glucose, 10 mM HEPES. The pH was adjusted to 7.2 with NaOH and adjusted to 300-305 mOsm with sucrose. Ouabain (100 µM) was added in the extracellular solution for blockade of Na^+/K^+ ATP-ase. NMDG replaced Na^+ in the extracellular solution to*

isolate K^+ efflux. The intracellular solution contained: 124 mM CsF, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 11 mM EGTA, 10 mM HEPES, 1 mM ATP pH 7.2, 285-290 mOsm."

Fig 2B. Legend should include holding potential at which reported current amplitude measured.
Thank you for the comment. The holding potential in these experiments is -80V. We have added this detail in the legend for Fig 2C.

Fig 2C. Legend or Methods should identify anti-APOL1 antibody and dilution used in FACS assay and the targeted antigenic component of APOL1. Note "N" of measurements in panel or legend.

We thank the reviewer for this comment. The antibody used and the dilution for the FACS assay are present in the manuscript in the methods section. "*Cells were stained for surface APOL1 expression in 50 µL of a 1:100 dilution of anti-APOL1 rabbit monoclonal antibody (Abcam, ab252218) in FACS buffer on ice for 30 mins*." **We have updated the legend to also reflect that APOL1 staining was performed with the indicated antibody. The targeted antigenic component is not revealed by Abcam, nor was the immunogen used for its generation. We have updated the legend to reflect the "N" of measurements with the following statement: "***Three independent experiments were performed with 16 replicates per experiment. Representative experiment is shown."*

Fig 2D Legend should define media compositions (beyond the brief designations in the key) and the number of hours of Tet induction preceeding measurement of the dead/live ratio. Note "N" of measurements in panel or legend.

We thank the reviewer for this comment. We have added the detailed media composition, the time of induction and the number of measurements to the legend. This is now figure 2E.

Text describing Fig 2 should note that panel A reversal potentials are considerably more electronegative than previously reported, likely due to the choice of permeant ions in the current experiments, with Cs substituting for internal K, and NMDG substituting for external Na,
Thank you for noticing this difference and the comment. Replacement of Na^+ with NMDG, prevents the influx of Na^+ ions shiftinh the reversal potential to a more negative value. In the presence of only Na^+ and Cs^+ ions, the reversal potential is close to ~0mV, given Na^+ ions are flowing inward and cesium ions flowing outward. Cesium substitution with for K^+ was included in order to isolate the APOL1-mediated current, by blocking endogenous voltage gated K^+ channels. We have added some text to clarify the differences with previous reports.

Text describing Fig 2 should note that results of panel B are similar to previous reports

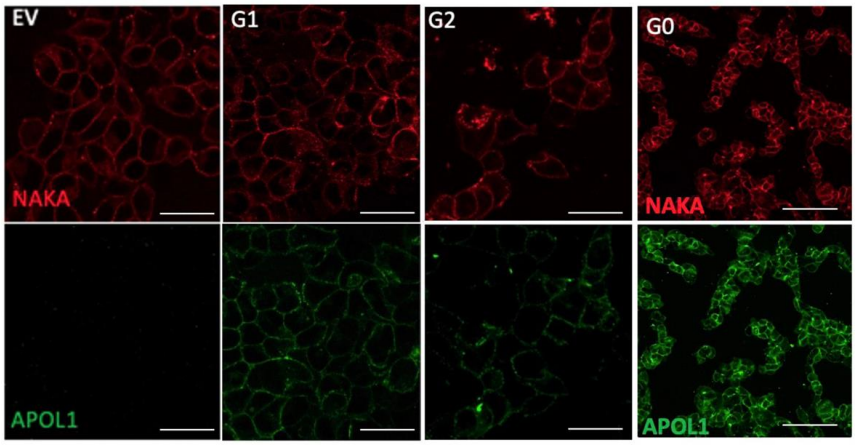
of
O'Toole et al, JASN 2018, and Vandorpe et al Pflugers 2023.

Thank you for this comment. We have updated the text to reflect that the results are similar to previous reports and added citations to both works.

Text describing Fig 2C should note that G0 surface expression 2.5-3-fold higher than G1 or G2 has not been previously reported – previous reports were of roughly equivalent surface expression.

We thank the reviewer for this comment. Some reports have shown similar levels on the surface like Olabisi *et al.* PNAS 2016 using cell surface biotinylation. A more recent report from Vandorpe et al. Pflugers 2023, presents immunocytochemistry data of the APOL1 G0, G1 and G2 cells. While they don't comment on relative expression, visual inspection of the data shown in figure 1C (pasted below) appears to show greater levels of APOL1 G0 relative to G1 or G2. We are not making a claim here about the expression levels of APOL1 with this data but are just saying that the increased ionic flux observed for APOL1 G1 and G2 is not due to increased surface expression.

C



Text describing Fig 3 should note that high K medium's near-total prevention of cell death is more protective than most recently reported by ref 21.

Thank you for the comment. We have updated the text describing figure 2D to note that the impact of our prevention of cell death was greater than reported in ref 21. It also indicates that our data is supported by the conclusion that K+ efflux is necessary for cell death.

The conclusion that cell death is associated with increased K+ efflux rather than Na+ influx should be noted as supporting the conclusion presented by ref. 21.

Thank you for noting this. We have added an additional sentence here to reflect the importance of ref 21 in supporting our findings. We did not add a reference to

the conclusion as ref 21 does not present data demonstrating that APOL1 itself is the channel responsible for this ion flux.

Fig 3A legend should note that whole cell currents were measured.

We thank the reviewer for their comment. We have updated the legend of Fig 3A to note that whole cell currents were measured.

Fig 3 all panels should present error bars as SD rather than SEM. “N” of measurements should be noted in panels or in legend.

We thank the reviewer for their comment. Nature Communications guidelines do not specify SD over SEM. We will continue to display SEM for the totality of the paper. The number of measurements was added to the legend.

Fig S5A should note identity of anti-APOL1 antibody used for IHC.

We have updated the legend with the anti-apol1 antibody and the dilution used.

Fig S5B key should change G2hom to G2mc/G2mc or G2mc/hom. (for uniformity of presentation, if I understand correctly)

Thank you for noticing this inconsistency. We have updated the figure to $G2_{mc}$.

Fig S5C Y axis label should change G2hom to G2mc/G2mc or G2mc/hom

Thank you for noticing this inconsistency. We have updated the figure to $G2_{mc}$.

Fig S5 all panels should present error bars as SD rather than SEM

We thank the reviewer for their comment. Nature Communications guidelines do not specify SD over SEM. We will continue to display SEM for the totality of the paper.

Lines 153-156: Please clarify if transgenic APOL1 G2mc mouse should be referenced to ref 26.

We thank the reviewer for their comment. We have clarified the manuscript to reflect that the mice used are consistent with those from reference 26 (now 28).

Line 170 – Clarify process of randomization based on albuminuria. Were mice without albuminuria excluded from the experiment? Or were mice merely randomized between treatment and vehicle groups?

Thank you for highlighting the lack of clarity around our method of randomization. Details around the randomization were added into the methods section. All mice developed albuminuria.

Lines 179-80 – synechiae and vacuolation/reduplication of PEC should be annotated in panels B or E

Thank you for the comment. We have added annotation using a star to point the vacuolation and a bracket to highlight the glomerular synechia and duplication of PECs in the panels of figure 5.

Fig 5A-F should include H&E low power panels for each condition to allow visualization of multiple glomeruli.

Thank you for the comment. We have added Supplementary Fig. 12 allowing for the visualization of multiple glomeruli.

Fig 5 legend line 837-8 – change to "Control nephrin immunostaining intensity was significantly reduced in vehicle-treated mice, and was rescued to control levels by compound 3 treatment."

Thank you for the comment and noticing the confusing phrasing. We have revised the text as follows: "Nephrin immunostaining intensity was significantly reduced in vehicle-treated mice compared to control and whereas the Compound 3 treated group was statistically comparable to control."

Fig. 5G should be accompanied by images of nephrin immunofluorescence staining in all three conditions.

We have added the nephrin staining to fig. 5A-F and added panels for nephrin from each glomerulus shown. We have also included the zoom out of more glomeruli in the Supplementary Fig. 12.

Fig 5 legend line 841: "kidney filtration slit diaphragm."

We have updated the text to add the word "diaphragm" as indicated.

Fig 5 H/I/J: MIP image is pseudocolored for depth. The podocin and integrin a3 immunofluorescence images must also be presented, either here or in supplemental data.

We have added Supplementary Fig. 13 presenting the podocin and integrin alpha3 data as indicated.

Discussion:
Line 221: Note that VX-147 binding affinity was 50-100x lower than inhibitory activity in thallium influx assay (ref 25) or patch clamp currents (this paper).

Thank you for the comment. We have introduced a modification to the text noting that the binding was to purified, recombinant APOL1. We believe that extracting APOL1 from the membrane and placing it in detergent likely accounts for this disconnect.

Lines 228-231: Ref 32 should be cited in Results when appropriate.

Thank you for the comment. We feel that while similar experiments were performed in this reference and this manuscript, the conclusions made from the data or use of VX-147 is not the same.

Minor points
Line 57 – "explanation both as to why not all people….."

We have made the requested edit to the text.

Lines 116-118 – inaxaplin as inhibitor of APOL1-mediated thallium influx needs citation of ref 25.

We have added ref 25 to line 118 as requested.

Lines 182: change "no findings" to "no abnormalities"

Thank you for the comment. We edited the text to say "no microscopic abnormalities".

Line 184. Is ref 28 an intended citation? Better to use an article on nephrin rather than a textbook chapter. If as intended, please complete the ref.

Thank you for the comment. We have replaced this reference with Ruotsalainen, V. *et al. PNAS. 1999,* which is now reference 30.

Line 185 – glomerular

We have made the requested edit to the text.

Line 221 – VX-147 was previously shown…

We have made the requested edit to the text.

Line 226 – There seem to be

We have made the requested edit to the text.

Reviewer #2 (Remarks to the Author):

General Feedback: The authors have contributed valuable information to the existing scientific literature on APOL1 inhibitors, specifically focusing on VX-147 and three analogs. Promising Phase 2 results have recently been published in NEJM (Ref 25) with VX-147. The authors have described the origins of the chemical matter for clinical candidate, VX-147 and presented compelling data demonstrating the inhibitory effects of these molecules on APOL1, thereby reducing damage in both in vitro and in vivo systems by targeting the ion channel activity of APOL1. It should be noted that certain data presented in this manucrsipt overlaps with a previously published article in the New England Journal of Medicine (NEJM). Therefore, we recommend that the editor assess whether this publication contains sufficiently novel material to justify its publication in Nat. Comm., or if it would be more appropriate for another journal. Nevertheless, the inclusion of the treatment mode in vivo experiments and the additional characterization of the molecules' mode of action contribute to the overall scientific understanding of VX-147 which is progressing in the clinic for the treatment of kidney disease associated with genetic variants of ApoL1. While we believe that this study enhances the knowledge of these molecules and their mechanism of action, we bring this to the attention of the editor for consideration.

We thank the reviewer for this feedback. There is no overlapping data presented between the NEJM paper and the data contained here. The reviewer identified the thallium flux assay data presented in the NEJM paper as being the same as the electrophysiology data presented here. The only overlap between the two manuscripts is the inclusion of the IC_{50} values of VX-147 in table 1 and in the NEJM paper.

Specific Feedback:

General: APOL1 italics is mostly correct regarding when it is a gene versus when the authors refer to the APOL1 protein, but there are some cases that look to be incorrect formatting. E.g. Line 35: reads “two APOL1 alleles” but should be italicized “two APOL1 alleles”

Thank you for noticing this inconsistency. We have made this edit to the text.

Lines 202 and 207: “two APOL1 risk variants” one it italicized, not the other.

Thank you for noticing this inconsistency. We have italicized both instances in the text.

Lines 36-37 wording consideration: reads “Patients with AMKD progress more rapidly than people with similar clinical features who lack APOL1 genotype, and have higher rates of..” Feedback: the disease progresses, not the patients. Also specify the variants of the gene in the sentence.

Thank you for the feedback. We have updated the sentence as follows: “Patients with AMKD have accelerated disease progression relative to patients with similar clinical features who lack APOL1 risk variants, and have higher rates of end stage renal disease, transplant, dialysis and death.”

Line 85 & Lines 221-222 The authors state that these are specific inhibitors of APOL1 channel activity and broad profiling data is available. It isn’t clear in this manuscript that the authors are referring to a list of proteins in the supplemental of Ref 25 for VX-147. Please provide additional data in the supplemental to show specificity of compound 3 in the supplemental material for this manuscript, since it is being tested in animal models. In Reference 25 the authors show a list of off-target proteins assessed, but we didn’t see data reported such as drug concentrations tested and whether any marginal activity was observed against other channels. To put in perspective the in vivo data reported in this manuscript the specificity data for compound 3 would be valuable.

Thank you for this comment. Unfortunately, large off-target panels are only performed for potential clinical candidates. We do have limited selectivity profiling for compound 3, which is now included in Supplementary table 2.

Line 109 reads “across the plasma membrane”. Suggested edit: “across the cell membrane”

We have made the requested edit to the text.

Line 114: refer to Table 1 with in-vitro and in-vivo PK data for 4 compounds

We have made the requested edit to the text.

Page 4, Line 136, Figure 3a: Provide a reference for the Thallium flux data since this information was previous reported for VX-147 in the NEJM reference #25.
Thank you for this comment. This data was not previously reported in reference 25. The data presented here is electrophysiology data and not thallium flux data. The only thallium flux data reported here is found in table 1.

Figure 3a: The curve and data points presented for VX-147 is different than was reported in NEJM Ref 25 Figure 1a. There are several discrepancies between the data presented in the two figures for the same assay and same compound. Please either provide an explanation for why there are these discrepancies (e.g. number of test points are different, curve fitting is different, different data set etc) or remove Fig.3a from this

paper and just reference Figure 1 in Ref 25 and show the IC50 values reported in the text and Table 1. From a curve fitting perspective adding a 100% point is not appropriate for graphical purposes as was done in Figure 3a but not Fig1 -Ref 25.

Thank you for the comment. This data is not thallium flux data but electrophysiology data as indicated. We have made some changes and clarifications based on feedback from some of the other reviewers. We hope this addresses the concern with the different curve appearance. We are confident with the methodology used to fit the electrophysiology data implemented here.

Line 139-140, 161/Table 1: Since compound 3 was studied in an ApoL1 G2 mouse, provide the in vitro data for compound 3 in the G2 cell line. There is data for VX-147 in both G1 and G2 cell lines (considering Table 1 and Figure 3), but not for compound 3, which was studied in this paper. The reader doesn't have the context to understand if/how the Methyl to Fluorine change in structure impacts activity at ApoL1 G2.

Thank you for the comment. We will add the G2 values into table 1 for all 4 compounds. This series of APOL1 inhibitors fortunately do not display differential inhibitory potential across the APOL1 variants.

Line 158-162: As readers of drug discovery literature, we were interested in the report that VX-147 showed efficacy at 3 mpk (REF 25) but compound 3 was tested at 30 mpk in the G2 in-vivo efficacy model in this report. The authors state the molecules have "comparable potency". Please provide a brief statement of why a 10 fold higher dose was studied for compound 3. This will add to the understanding of the pharmacology and PK/PD relationships for the inhibitors.

Thank you for this comment. In ref 25, VX-147 is used a dose of 3 mg/kg three times a day, every 8 hours (tid), while here we are using a dose of 30 mg/kg twice daily every 12 hours (bid). The pharmacokinetic properties of these molecules in mice are poor requiring an elevated dose to maintain therapeutic levels through the dosing interval. While we are not trying to make comparisons between the molecules in the mouse model, we added a sentence to clarify the discrepancy.

Figure 4: Please provide drug exposures in the 30 mpk bid study treatment study if data is available. The data would be valuable to the community to understand the drug concentrations needed to achieve treatment efficacy.

Thank you for raising this important consideration. We have added in the exposure data from these studies in the supplemental section, in Supplementary Table 3.

Table 1: Consistent with Nature Journal guidelines provide statistical analysis and number of replicates for the in vitro assays on both APOL1 G1 assays on lines 2 and 3.

Thank you for bringing this to our attention. We added the replicates to the in vitro assays for APOL1 G1 and G2, and added it into the legend for figure 3.

Line 185: reads “glomerulal” suggestion: glomerular

We have made the requested edit to the text.

Lines 206-207: “relative to those without two APOL1 risk variants”. For the reader who isn’t as familiar with APOL1 biology, it would be helpful if the authors can briefly discuss whether people with one APOL1 variant have evidence of disease as well under some circumstances.

Thank you for this insight. We modified the sentence to “those with one or no *APOL1* risk variants”. We hope this addresses the gap.

Line 221: It is confusing to the reader that the authors are referring to binding data in NEJM reference 25. Please be more specific than just providing the reference. Both reviewers went back to look for direct binding data in this paper before realizing you were referring to ref 25 Figure 1 data.

Thank you for this comment. We added the word “previously” to the sentence to indicate it was not part of this manuscript. We apologize for the confusion.

Figure 2D: It is hard to see what is different in the bar graphs when printed in black and white. Consider making the bars a different texture and color so it is clear.

Thank you for this comment. We will make the requested changes to Fig. 2D as indicated.

Line 672: As requested for Nature Journals please provide the synthesis of compounds 2, 3 and 4 rather than refer the reader to the patent. We recommend that additional characterization data is provided for 3 and 4 (VX-147) consistent with medicinal chemistry literature and Nature Journals requirements for lead molecules. C13 NMR for both molecules as well as an optical rotation and single molecule crystal structure for either Compound 3 or VX-147.

Thank you for the comment. We have added the experimental procedures for the synthesis of compounds 2, 3, and 4 to the supplemental information. We have provided additional characterization data, including ^{13}C and ^{19}F NMR, and ^{1}H NMR traces for lead molecules, as well as HPLC purity trace and single molecule crystal structure for VX-147.

Line 687 (characterization table): Please check the formatting and data in the characterization table.
Discrepancies in NMR between submission and patent were noted in NMR for

compound 2.

Thank you for the comment and noticing the inconsistency. The data characterization table was replaced with the more thorough analysis described above.

-Compound 1: parenthesis missing, J should be italicized
Compound 2: Two HNMR signals are missing from the NMR report. Possibly obscured by solvent. If so please indicate that “peak obscured by solvent”.

Thank you for the comment. We have made the recommended changes in the manuscript.

Reviewer #3 (Remarks to the Author):

The manuscript by Zimmerman et al. demonstrates that inhibiting APOL1 ion channels is beneficial in a mouse model of APOL1 associated nephropathy. Mark E. Bunnage was previously an author on a publication in NEJM that showed that inhibiting APOL1 channels in a mouse model of APOL1 associated nephropathy before the induction of proteinuria was beneficial in reducing proteinuria compared to untreated mice. This paper is significant as it highlights that the detrimental effects of APOL1 G1/G2 variants is due to potassium efflux and shows that inhibiting APOL-1 channels after the induction of proteinuria is beneficial in reducing kidney failure outcomes.

The original article is interesting and addresses an important area of research. However, there are a few key points that need to be considered:

a) The paper discusses the drug discovery process of identifying inaxaplin as a clinical drug targeting ApoL1, however the drug used in the manuscript is compound 3 which was a previous APOL1 inhibitor that was then developed into inaxaplin. The paper should discuss why they utilized compound 3 in this study instead of inaxaplin, or at least in comparison to inaxaplin.

Thank you for raising this important issue. Internally, we do not perform experimentation on clinical candidates once they have progressed to the clinic. Therefore, in an effort to show the potential impact of inaxaplin in newly established models, we used a structurally similar compound, Compound 3, to represent the potential impact inaxaplin could have in these models. A sentence was added to the manuscript to explain this reasoning.

b) Since the paper highlights G1/G2 risk alleles being the cause of APOL1 associated

nephropathy over G0 risk alleles, the in vivo data should also include G0 transgenic mice.

Thank you for the comment. Unfortunately, APOL1 G0 mice have no phenotype in this model, or any model we have tested to date. We can provide reference 26 (now reference 28) as a point of guidance.

c) Fig S5. should show significance in B and E.

Thank you for your comment. In fig S5 C and F depicts the area under the curve (AUC) of the UACR over the study time course that is shown in fig S5 B and E. We included the statistical analysis of AUC UACR to integrate the binary measure of time out of the longitudinal UACR measures which is a more accurate representation of the results compared to evaluation at a single timepoint.

d) Data in figure 3 should also be normalized to the G0 group if the groups are on the same graph to show the difference between the risk variants. The Trypanosoma viability is higher in the G2 group at baseline compared to the G0 group, which is opposite of what is described.

Thank you for this comment. The individual data is already normalized to controls for each APOL1 variant; therefore an additional normalization step would be challenging since each curve is already fit to a 100% maximum, 0% minimum value. In regard to the trypanosoma viability, while the potency of VX-147/inaxaplin is reported to be slightly more potent on APOL1 G2 compared to APOL1 G0 or G1, we do not described or attribute any difference to this. Recombinant APOL1 G2 protein is slightly less effective at killing trypanosomes relative to APOL1 G1 or APOL1 G0, explaining the slight difference in the baseline.

e) Significance should be shown in Fig. 4A.

Thank you for your comment. Fig 4B depicts the area under the curve (AUC) of the UACR over the study time course that is shown in fig 4A. We included the statistical analysis of AUC UACR to integrate the binary measure of time out of the longitudinal UACR measures which is a more accurate representation of the results compared to evaluation at a single timepoint.

f) Are there representative images for Fig. 5G?

Thank you for the comment. We have added in a row of representative nephrin images for the glomeruli depicted in fig. 5.

g) The amount of male vs female mice used in the study should be displayed.

Thank you for your comment. We have added the amount of males vs females to

the figure legend. We have also included multiple new supplementary figures, Supplementary Figs. 8-11, which displays the data for all in vivo pharmacology studies separated by sex. Additionally where appropriate, dots were differentiated by sex in the main text figures.

Very interesting study with major translational potential. It appears that the presented drug has been moving to clinical trials.

I cannot see the original electrophysiology data. I/V curves or single channel recording if available should be shown and channel selectivity data etc must be shown. It is hard to fully understand what is presented on figure2.

Thank you for the comment. We have added in the I/V curve as figure 2A for the electrophysiology data. We also added in a limited off-target panel we had available for Compound 3. As it is not a clinical candidate, limited profiling is done on compounds in this regard. A more comprehensive off-target panel for this family of APOL1 inhibitors is available for inaxaplin in reference 28.

From the figure it seems that the compound prevented the cell surface expression of APOL1, if this is the case it should be validated. In addition we would need to know whether total APOL1 expression showed differences (like a western blot). While surface reduction of apol1 is interesting this information is not consistent with channel blocking function.

We apologize for the confusion related to the flow cytometry figure. The APOL1 surface levels shown are in cells without exposure to compound, and solely represents the baseline membrane expression of each of these proteins in our cell line. The compound is applied acutely for the electrophysiology experiments and therefore it blocks the APOL1-mediated current via the channel but does not impact cell surface expression.

The mouse model should be fully characterized.

Thank you for your comment. We have clarified in the manuscript that the mouse models used are described in more detail in reference 26 (now 28).

Fig2d should have a comparison to wild type or G0 cells.

We thank the reviewer for this comment. The goal of this panel is to highlight that APOL1 G1 mediated cytotoxicity is dependent on the flux of potassium ions, not sodium ions, as APOL1 is a monovalent cation channel that allows ions in both directions. The data is normalized to the amount of death observed with no APOL1 expressed.

Figure3. we would need to have surface apol1 expression for these cells to understand whether this is a channel blocker or a molecule that inhibits surface expression of apol1.

Thank you again for this comment. As indicated above, the compound is applied acutely for the electrophysiology experiments and therefore it blocks the APOL1-mediated current via the channel but does not affect cell surface expression.

Figure4. Was there a difference in IFN expression? Was there a difference in APOL1 expression?
Panel b is not consistent with panel A

We thank the reviewer for this comment. We have now included the serum levels of IFN in the Supplementary Figure 11. The mice were randomized according to their proteinuria levels. Panel B is consistent with Panel A, as the data is presented in log-scale and we are looking at the area under the curve from day 6-14 for both groups.

Please share toxicology data? How about liver enzymes, blood pressure, and CBC?

We thank the reviewer for this comment. Compound 3 is a tool molecule used for studying the impact of an APOL1 inhibitor in preclinical models relevant to APOL1 function. Toxicology data is not available for Compound 3 (Supplementary Table 2). We have now included a limited off-target panel that we have for compound 3, and a broader panel for VX-147/inaxaplin can be found in the NEJM paper, reference 28.

Figure5. was the nephrin score different because podocytes died? Please share podocyte count?

We thank the reviewer for this comment. We agree that this could be a result of podocyte cell death. Nephrin being an integral component of filtration slit diaphragm is adversely affected by the morphological changes in the podocytes (degeneration and/or cell death). The significant degenerative changes noted in the vehicle-treated glomeruli as evidenced by the presence of collapsed capillaries, synechiae, and presence of foamy vacuolated cells in the Bowman's space is consistent with podocyte injury and subsequent glomerular remodeling as is shown in multiple previous studies. While we do not have a direct metric to count podocytes in these samples, there is significant histological evidence to support the loss of podocytes by day 14 in this animal model. Based upon the lack of histopathological damage and evidence of normal nephrin expression levels and filtration slit density in the Compound 3 treated animal group, we strongly believe that the data supports a protective role of APOL1 inhibition for podocyte effacement and death and preservation of filtration barrier morphology in this model.

PAS stain should be quantified.

We thank the reviewer for this comment. PAS stains for complex mucopolysachharides and hence, an essential diagnostic aid for evaluating changes to glomerular capillary basement membrane and mesangial architecture. Though theoretically, the PAS staining of the mesangial matrix should increase with severity of disease in this animal model, however, due to considerable glomerular remodeling occurring in the mice such that some glomeruli do not have PAS-positive matrix despite the significant damage making the PAS quantification an unreliable metric. We strongly believe that the quantification of nephrin immunostaining and the filtration slit diaphragm density data together profile a more complete picture of the damage that is occurring.

How does the channel function lead to cell death?

We thank the reviewer for this comment. We feel the data presented here and supported by other works in the literature demonstrate that channel function is the initiation event leading to cell death through potassium ion flux. A recent publication explored all the pathways downstream of APOL1 ion flux, and used inaxaplin/VX-147 to show that these pathways were abrogated (Datta *et al.* JCI 2024). We do not believe there is a sole pathway downstream that is entirely responsible for cell death, thus targeting at the source of the issue, the ion flux itself is critical for a therapeutic targeting APOL1.

Overall interesting work and the new chemistry is interesting but we do not understand whether or not the compound truly inhibit channel function and the mechanism this compound protect mice from kidney disease development.

We hope given the information provided above and the clarification of the compounds as direct inhibitors of APOL1-mediated ion flux and not cell surface expression allows for better understanding of the mechanism and how it protects mice from kidney disease development.

Reviewer #1 (Remarks to the Author)

This revision has responded conscientiously to the critical points raised by the reviewers. Several issues remain, however.

APOL1 transgenic mice.
In Rebuttal the authors note: “The mice used in the study are consistent with those in ref 26 (now 28).”
This unusual wording is ambiguous and is reinforced by inconsistent ambiguities in both Rebuttal and revised Methods and revised Results/Discussion.
The first paragraph of Revised Results states “we generated mice homozygous for the APOL1 G2 variant” without citation, leaving the reader to believe the mice are newly generated for this paper.
The first sentence of the Supplemental Methods section “Mouse Model Methods” now declares a transgenic mouse was “procured” (meaning generated?) Please specify if the CHORI BAC clone was G0, G1 or G2. Please clarify uniformly throughout the paper if this mouse is that described in ref 26 (now 28). which describes construction of the mouse strains with which the strains described in the current manuscript “are consistent?”).

G2-multicopy mice are referenced to CRL. Please clarify it these G2 multicopy mice are those described in (revised) ref 28. Please clarify if CRL was used for strain maintenance and propagation.

Thank you for these comments. We have revised the text to reflect that both strains of mice were licensed from BIDMC, and that CRL was used for strain maintenance and propagation. We have referenced ref 28 (back to ref 26) appropriately.

The rebuttal notes that the G2 multicopy mice expressed APOL1 K150. This information must still be added to Supplemental methods on p 13.
Thank you for the comment. We corrected the methods to say lysine instead of glutamate. We have also removed most of the model generation details and just reference Mccarthy et al 2021 where the mice were generated.

In the Rebuttal response to Reviewer #3, the authors state “we have clarified in the manuscript that the mouse models used are described in more detail in ref 26 (now 28).” However, this clarification appears to be missing from the revised manuscript in several places. On p.2 of revised Results the authors write in the section entitled “In vivo characterization of APOL1 channel inhibitors:

“Since model organisms lack endogenous APOL1 expression, we established transgenic mice expressing human APOL1 G2. We determined the APOL1 G2 transgene inserted multiple times in tandem, and therefore classify these mice as APOL1 G2 multicopy mice (APOL1 G2mc). We found that homozygous APOL1 G2mc were viable, healthy, lived a normal lifespan, and had no baseline phenotype. However, a single injection of interferon-γ (IFNγ), which induces APOL1

kidney expression (Supplementary Fig. 9A), led to significant APOL1 dependent proteinuria in APOL1 G2mc compared to control Friend leukemia B virus (FVB) mice (Supplementary Fig. 9B, 9C and 10)."

Average readers will interpret the above quoted section to understand that the manuscript authors created and characterized the mouse as part of the current paper. But these mice and all attendant results summarized in the bolded paragraph appear to have been reported in print 3 years ago in ref. 26 (now 28). If the latter understanding is correct, then the wording in the above paragraph and throughout the paper should reflect this.

Thank you for the comment. We have added the reference (ref 28 now 26) for the multicopy mice to the text.

Supplemental Mouse model methods lines 14-16: Was the "recombineering" done in vivo to generate the G2 single copy mouse? How was G2 derived from G1 by recombineering into a G0 BAC?. Citation and description are needed.

Thank you for the comment. We have removed the details of the mouse model generation from the text and cited the reference exclusively.

Please clarify if both multi-copy and single-copy transgenic mice were bred to homozygosity.

Thank you for the comment. In the methods section, it already indicates that both mice are homozygous for APOL1 G2.

Electrophysiological studies:
The electrophysiology solution compositions have been more fully explained in revision, but can be further clarified.
Rebuttal states that "NMDG replaced Na+ in the extracellular solution to isolate K+ efflux." But the intracellular solution also completely replaced K+ with nominally impermeant Cs+.
The revised results and supplemental methods sections claim that APOL1 is permeable to Cs+, but data testing Cs+ substitution as a single experimental maneuver is not presented. To this reviewer's awareness APOL1 Cs+ permeability has not been previously reported. Authors should relate or add to the Supplemental Methods data documenting VX-147-sensitive APOL1 Cs+ permeability and the permeability of Cs+ relative to Na+ or K+ before presenting data obtained in conditions of simultaneous CsF substitution for intracellular KCl and NMDG for extracellular Na.

Thank you for the comment. We have added an additional supplemental figure showing APOL1-mediated current with extracellular NaCl and intracellular CsF. In this case, the reversal potential is close to 0 mV demonstrating that Cs^+ ions are still able to permeate to the APOL1 ion channel. These results in combination with our previous results showing efflux of Cs^+ ions in the presence of NMDG, and shift of the reversal potential to more negative potential,

support that Cs^+ is acting as a surrogate for K^+ mediating the efflux of APOL1 current. We have added supplementary figure 9 to show our reversal potential is near 0 mV.

Revised Fig 2C legend describes peak current at (presumably intracellular) holding potential of -80 mV, while presenting outward currents. Was the holding potential intended to be written as +80 mV?

Thank you for the comment. The holding potential was indeed -80mV followed by the ramp. This is visible in the figure where you can see the voltage held at -80mV prior to ramp initiation.

Cytoxicity experiments
Revised Fig 2E: requirement of K+ efflux for cell death.
Change "necessity for K+ triggering cell death" to "necessity for cell K+ efflux to trigger cell death."

Thank you for the comment. We have revised the sentence accordingly.

The revised text currently lacks and should make additional reference to the earlier work from Raper and colleagues proposing a more significant role for Na+ entry than for K+ efflux in APOL1 cytotoxicity, note whether that data was obtained in the same or different cell types, and briefly discuss the difference in their and the current conclusion.

Thank you for the comment. Most of the work done by Raper and colleagues in Giovinazzo et al. eLife 2020 is done using recombinant protein. Their experiments in HEK293 cells express modified APOL1 RUSH constructs, which could potentially alter the functionality relative to non-modified forms of APOL1. Additionally, we feel that their methodology allowing for an intermediate level of potassium and sodium ions makes it hard to be sure that it wasn't an impact on potassium ion efflux and not sodium influx. We have added two sentences in the discussion discussing this data.

Drug binding:
Rebuttal states that Discussion was modified to reflect 50-100-fold lower affinity for VX-147 binding than for channel or flux inhibition. But this reviewer finds no such modification in the Discussion. Rather, revised Discussion claims "VX-147 was previously shown to bind directly to purified, recombinant APOL1 with high affinity," without further explanation. Please clarify this statement, noting difference between binding affinity and affinity based on inhibition of conductance.

Thank you for the comment. We have removed the terminology high affinity. The substrate for the binding assays is purified APOL1 in detergent which may impact the overall fold of the APOL1 protein.

Please also clarify the phrase "potential off-targets" by specifying what was measured

(inhibition of other ion channels?) and by what method (can be reference or notation of CRO or panel product).
Thank you for the comment. Off-target activity across a panel of 174 targets including a diverse panel of receptors and ions channels was used in our NEJM paper (ref 25) and all targets had a margin >8500× to the IC50 of inaxaplin on APOL1. A smaller panel is available in this manuscript for Compound 3 in supplementary table 2 and uses a similar methodology to that performed in reference 25. We added text in the legend for supplementary table 2 explaining this.

Revised discussion: change "We demonstrate that efflux of K+, and not influx of Na+, is responsible for APOL1-mediated cell death," to indicate that the conclusion applies "in our recombinant cell system."

Thank you for the comment. We have made the requested change.

Minor points:
Please paginate and add line numbers.
Please refer to original manuscript line numbers for the several changes made without reference to line numbers in either original or revised manuscript.

Fig. 4A. Please specify if APOL1-G2 transgenic mice used for this figure were multi-copy or single-copy.

Thank you for the comment. We have clarified that single copy mice were used in the figure legend.

Fig 5B legend. Change "black asterisk" to "gray star".
Thank you for the comment. We have made the requested change.

Multiple supplemental figures are incorrectly numbered in the rebuttal
Rebuttal description of changes to Fig S5A and S5B are found in S9A and S9B
Rebuttal discusses INFg levels in Fig S11, but revised paper presents the data as Fig S13.
Rebuttal Fig S12 is manuscript Fig. S14.
Rebuttal Fig S13 is manuscript Fig. S15.
Thank you for this feedback. We apologize for this error as we added two supplemental figures late in our revision process and did not catch this.

Reviewer #2 (Remarks to the Author)

Our reviewer comments were satisfactorily addressed, and complementary data were incorporated to the manuscript and Supplemental materials. Syntheses of all compounds and intermediates are reported, and initially observed discrepencies have been addressed. 13C, 19F and 1H NMR data for lead molecules are reported. We also appreciate that HPLC purity trace and single molecule crystal structure for VX-147 were included.

Minor suggestions:
Data in table 1 show activity toward ApoL1 G1 and G2 variants are comparable, bridging the HTS screen and in vivo data. This key finding could be highlighted more strongly in the text. As per the author's response to the reviewer: "in vitro characterizations of Compound 3 and VX-147 in both thallium-flux assay and HEK cell rescue assay indicate this series of APOL1 inhibitors display comparable inhibitory potential across the APOL1 variants."

Thank you for the feedback. We have added an additional sentence at the end of the "Discovery of small molecule inhibitors of APOL1 channel activity" section outlining this finding.

Minor corrections in synthesis section:

- "J" should italicized in NMR reports
We have made the requested change and italicized "J" where applicable.

- Line 755 reads: "7.21 (t, 2H), either J coupling is missing or a multiplet was observed
We corrected this typo to include the J coupling.

- Line 833 reads "Mg2SO4": should it read MgSO4 or Na2SO4?
The typo was corrected to read as "$MgSO_4$"

- Line 846: structure of S14 should be corrected to the carboxylic acid (the ester is displayed)
Thank you for the comment. We have corrected the structure of S14.

- Lines 984/985: if possible, clarify the column used for HPLC trace, in particular, it would be more informative to indicate if a chiral column was used for the purity
Thank you for the comment. The specifications and identity of the column used have been added to the HPLC trace supplementary figure legend.

Reviewer #3 (Remarks to the Author)

The authors of the manuscript "Small Molecule APOL1 Inhibitors as a Precision Medicine Approach for APOL1-mediated Kidney Disease" included revisions/improvements or addressed our concerns and others. This included adding additional supplemental figures. Some revisions and improvements should still be addressed, however. In particular, all experiments should be presented with a sufficient number of biological replicates. In addition, measurement of

podocyte number should be included.

1)Supplementary figures contain titles above and below the figure. Please include just one unless this is the correct format for the journal.

Thank you for the comment. We have made this revision.

2)The supplementary figure 13 (serum levels of IFN) is not mentioned in the main text of the manuscript.

Thank you for the comment. We have now called out supplementary figure 13 in the text.

3)Supplementary figure 14 says the images are 40x, however; the images do not seem to be at the magnification of 40x based on the size of the glomeruli. Please include a scale bar.

Thank you for the comment. We have added a scale bar for this figure and removed the magnification comment.

4)Please include a scale bar on Supplementary fig 15.

Thank you for the comment. We have added a scale bar for this figure.

5)Please remove the bold for Fig. 2 (A) caption. “Representative voltage ramp-current response after 16 h tetracycline induction in APOL1 G1 expressing HEK293 cells.”

Thank you for the comment. We have removed the font formatting.

6)Two of the groups in Fig 3B, C are done in one biological sample (N=1). This should be increased to multiple biological groups.

Thank you for the comment. While we appreciate that increased replicates for APOL1 G0 and G2 in both assays would be preferable, they are unavailable for VX-147. Members of this compound family show no intrinsic potency difference between APOL1 forms, therefore we anchored on the sufficient replicates on APOL1 G1. This can be noted in table 2, where Compound 3 shows no significant difference in potency for APOL1 G1 and APOL1 G2 in the two assays presented.

7)It appears some data points in Fig 4A are missing SEM bars. Please include these.

Thank you for the comment. Error bars are presented on all of the dots but in some cases the error is smaller than can be presented due to the scale on the graph.

8)Please include a scale bar/magnification for figure 5.

Thank you for the comment. We have added a scale bar for figure 5.

9)In response to the critiques of the reviewers, the authors stated that the channel inhibitor does not affect expression of APOL1 without showing data supporting this or providing the appropriate citation. Data showing how the channel inhibitor affects APOL1 expression would be interesting regardless of the result.

Thank you for the comment. The context of the flow cytometry data is to demonstrate that the cell surface levels of APOL1 protein during the electrophysiology experiments is not the source of the increased flux for APOL1 G1 and APOL1 G2 variant forms.

10)It remains unclear if the nephrin score is due to decreased nephrin expression or to a different rate of podocyte loss. As recommended in the first round of review, podocyte number should be evaluated by performing a WT1 staining.

Thank you for the comment. As we indicated in the first round if review, we agree that the loss of nephrin could be due to decreased nephrin expression or to a loss of podocytes. However, we do not feel that this affects our stance that the data supports a protective role of APOL1 inhibition for podocyte damage, effacement and death and preservation of filtration barrier morphology in this mouse model.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have responded to almost all the points raised.
Only minor points remain.

Fig 2A and B legend (or panel inset) should restate the predominant ionic composition of extracellular and intracellular (pipette) solutions to aid reader understanding, just as panel E legend presents the cell growth media compositions rather than referring reader back to Methods.

Due to length limits in the manuscript, we were unfortunately not able to add these solutions. The detailed solutions are included in the methods.

Fig 2C legend still states that the outward currents presented are at “holding potential = -80 mV”. The rebuttal states “the holding potential was indeed -80 mV followed by the ramp.” But the holding potential information doesn’t address the maximal currents presented in Fig 2C. Fig 2C presents outward currents of a magnitude consistent with current measured at the end of the ramp, i.e. +80 mV. Indeed, the Methods section lines 447-448 states that “peak outward current was measured at the end of the 200 s test pulse at +80 mV.
So Fig 2C legend should add that the outward currents presented are those measured at +80 mV at the end of the voltage ramp.

We have edited the legend to reflect that the current amplitude is measured at the end of the ramp.

Note (line 447) that the test pulse duration should be 200 ms rather than the current 200 s.

We have made this change.

Specify in Fig 2B and 2C legend if mean+/-SEM is shown, as for later panels.
We have made this change.

Supplementary Fig 9B legend Line 1015: delete “outward.”
We have made this change.

Electrophysiology methods:
Line 437-8: we identified APOL1 channels to permeable to Cs+. Cs+ was selected also to block endogenous voltage-gated K+ channels.
We have made this change.

Line 440: supporting nonspecific cation flow
We have made this change.

Line 440: Resistance
We have made this change.

Lines 443-4: to each concentration, 5 min after initial current recording (control).
We have made this change.

Line 451: if the peak current amplitude was ….”
We have made this change.

Lines 450-452: specify if these parameters used to remove cells from analysis were applied to all analyses, or only to inhibitor IC50 analyses (since a small number of values shown in Figs 2B and 2C would not meet the criteria for inclusion.

We clarified that the parameters described are applied to compound analysis.

Reviewer #3 (Remarks to the Author):

The authors have properly addressed all my concerns.

Reviewer #5 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communicationsinitiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.