nature portfolio

Peer Review File

CYP5122A1 encodes an essential sterol C4-methyl oxidase in *Leishmania donovani* and determines the antileishmanial activity of antifungal azoles

Corresponding Author: Professor Michael Wang

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)
Upon meticulous scrutiny of the article bearing the title "CYP5122A1 encodes an indispensable sterol C4-methyl oxidase in Leishmania donovani and governs the antileishmanial potency of antifungal azoles," it is with a sense of regret that I articulate the observation that the 1D and 2D NMR spectra illustrating the purified sterol metabolite fall short of achieving a satisfactory standard of graphical depiction (Figure 4). Foremost, the 2D spectrum is conspicuously devoid of any carbon or proton labeling dimensions. The carbon resolution is markedly deficient. The genesis of the cross-peaks ostensibly corresponding to C29 (Figure 4 E) remains enigmatic, as no concomitant signal aligning with the carbon dimension is discernible. Concomitantly, conspicuous resonant lines, expected to manifest distinctly within both the 13C and 1H dimensions in tandem with each cross-peak, are conspicuously absent from Figure 4. Evidently, the procedural protocol employed is fraught with errors. The NMR spectrum at this juncture holds minimal potential for informative extraction, rendering confounding the means by which the authors purportedly gleaned pertinent insights from their NMR investigations.

The originality inherent in the present work appears to be conspicuously lacking, and the elucidations sought by the authors remain obfuscated within the content of manuscript. The pursuit undertaken by the authors, regrettably, exhibits an insufficiency to provide a substantial cornerstone for the advancement of novel antileishmanial therapeutics. Consequently, it is incumbent to state that the subject article falls short of the exacting standards warranted by publications such as Nature Communications.

Reviewer #2

(Remarks to the Author)
This manuscript by Wang et al describes a detailed investigation into the oxidative enzymes responsible for key steps in sterol biosynthesis in Leishmania, in particular exploring the function of CYP5122A1. In recent work they have reported that, in contrast to other organisms, leishmania parasites use these two enzymes to work in parallel. This is also seems to be observed in other trypanosomatid parasites. In this work they build on this observation to establish its functon(a C-4 demethylase) and role in sterol biosynthesis inhibition by azoles.
Although first reported in 2011, and shown to be essential, the precise of CYP5122A1 fnction remained to be established. In the current study binding studies reveal a preference for C4- methylated substrates and, using recombinant protein, strong evidence is supplied for the formation of the various intermediates consistent with a role as a C4 demethylase. This appears to be an important (essential) activity for stress response etc although why is not fully established
The authors then use this information to better understand the function of azole CYP inhibitos as potential antileishmanial agents. Although azoles have long been recognized as antileishmanial the efficacy did not correlate well with the projected role as CYP51 inhibitors. In this report they provide compelling evidence to show that sterol inhibition is a combination of activities. Compounds with good levels of antileishmanial activity correlated with good levels of inhibition of CYP5122A1 although there is no attempt to correlate this with azole and protein structure e.g. the strongest inhibitors all have an imidazole – is this significant. Maybe with alphafold this should not be impossible???.
Overall the work appears to be rigorous and the findings are interesting and merit publication. Where I struggle and it could help if this was more strongly articulated in the discussion is what this all means. Shaha Ref 26 had previously reported that

that combinations of compounds that are effective CYP51 (posoconazole) and CP5122A1 (DB766) inhibitors have a synergistic antileishmanial effect albeit without clear understanding why. This paper gives that mechanistic understanding of this observation but not necessarily the molecular insight to build upon (nor the exploitation?). Interestingly most of the more effective CYP5521A1 inhibitors are (I think) examples of azole antifungal which are not used in an oral context (too toxic / metabolically vulnerable) but do have topical applications. Have the authors looked at a CL species - it might make the work more directly applicable?

minor typographical suggestions
P20 line 17 albeit is unable
P31 line 5 Sentence starting These results showed … does not make sense
P31 line 16 Fig 10A

Reviewer #3

(Remarks to the Author)
This manuscript reports what I believe is seminal work potentially solving a frustrating conundrum that has plagued efforts to repurpose azole drugs for use in leishmaniasis (and American trypanosomiasis).

Given Leishmania, like fungi, use ergosterol as a main membrane lipid there has been a long held view that inhibitors of Cyp51 that have been developed successfully as anti-fungals should also be effective against leishmaniasis (and Chagas disease).

And yet clinical trials of those azoles has invariably led to failure.

The fundamental advance made in this paper is the identification of a C4 sterol demethylase (Cy5221A1) working alongside the C14 sterol demethylase in a branched pathway, both branches of which can lead to sustained sterol production.

In addition to explaining the past failures to cure these diseases with azoles, it is also of note that current screens against Chagas disease, in particular, and to a lesser extend Leishmania reveal an extraordinary number of compounds that inhibit Cyp51. Given the failure of Cyp51 inhibitors in the clinic, currently new inhibitors of the enzyme are actually removed from further development through the risk of wasting time and resource. However, given that dual Cyp51/Cyp5221A1 inhibitors might be effective, an opportunity to revisit some of these high throughput screens to seek such dual inhibitors is an opportunity.

The manuscript is data rich and I am not qualified to assess the analytical chemistry aspects, although understanding the mechanisms and enzyme binding will be of value as others will aim to generate specific inhibitors of this key enzyme.

The work showing that the enzyme is essential in both promastigotes and (probably) amastigotes was necessary in spite of earlier work (referred to appropriately) that had already demonstrated likely essentiality given the inability to make a null mutant. The more sophisticated approach here verifies essentiality in promastigotes at least.

There are some points, however, that need to be addressed.

For the mouse work on essentiality, once gancyclovir is removed there is a bounce back after seven weeks. This is likely (and likely rather than probably as they suggest (line 18, page 26)). What was the reason for not sustaining that GCV pressure longer? The bounce back, however, indicates that there were parasites not killed in the absence of CYP5221A1. In fact very few drugs (maybe even none) give sterile cure in mice hence that is not surprising in itself. However, it is not known from the data whether the recrudescence (measured in spleen enlargement) comes as quiescent parasites that can survive without the enzyme re-emerge, or could secondary mutations that compensate for loss of the enzyme allow recrudescence. Or simply due to a small number not losing the plasmid over the time frame studied. In the absence of confirmatory data, some qualifying words are required.

Given the key impact of this work on therapeutic strategies for leishmaniasis, it was surprising not to see some more mouse work on efficacy of dual inhibitors or combinations in comparison to Cyp51 specific inhibitors. Given difficulties of the mouse work and interpretation in light of the difficulty to cure, comparative efficacies in a macrophage model could take us further towards verifying the likelihood of better pharmaceutical efficacy without needing to scale to the mouse at this time.

Promastigote growth was assessed using a colorimetric test. Were parasites also viewed microscopically to distinguish leishmanicidal and leishmanistatic activity?

C4 substrate binding assays (P19-20) only use zymosterol as a C4 methyl lacking species, which is not very comprehensive. The later data all corroborates C4 demethylase activity but conclusions should be tempered based on the n=1 SAR in that section.

Page 27 line 6 talks about downregulating sterols whereas it is not regulation per se, just reduced abundance because of the lost gene

In that section, the data point to the C4 demethylation reaction being dominant over the C14 demethylation reaction. How

does that reconcile with other work where Cyp51 is lost or mutated? (are there species-specific differences across the Leishmania genus?). Moreover, some more consideration should be given on the branched pathway proposed in the scheme of figure 1. A priori without Cyp51 then later sterols including ergosterol can be produced via the C4 demethylation pathway with C14 demethylation occurring due to the “Sterol C14-demethylase” later in the branched part. (presumably this is a separate enzyme from Cyp51 = lanosterol 14a demethylase?), and this is what happens. Cell lines lacking Cyp51 should, therefore, make ergosterol. However, this doesn’t reconcile with observations e.g. from McCall et al., 2015 (PMID: 25768284) or Mwenechanya et al. 2017 (PMID: 28622334). In addition to dominant versus lesser pathways, can the prospect of a multitude of potential inhibitory regulations made by different intermediates accumulating etc. be invoked? Some reasoning on how to reconcile the scheme in figure 1 with other data is desirable.

In considering the situation vis-à-vis LPG/PPG on the one hand (the molecules themselves have altered levels and altered distribution) and SHERP on the other hand (RNA levels are altered) thought should be given to the differences between these two types of observation. The former could be explained by changes to lipid architecture altering where these molecules go. The latter, however, implies the parasites do have a change in gene expression in response to their changed lipid architecture and this difference in interpretation of changed macromolecule distribution vs regulated gene expression needs to be highlighted. There is reference in the discussion to differentiation defects in the over-expressors, but this isn’t clearly explained and a global transcriptomic analysis could be more informative than focusing on a single gene (although not necessary here as the core message of the paper offers an explanation for failure of azole therapy in leishmania and considerations on broader system wide changes in biology are less important in that regard).

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
None of my comments are addressed properly. Resolution of 2D-spectrum is very low. It is not possible to get any information from this low-resolution spectrum.
The cross-peaks corresponding to C29 (Figure 4 E)
remains enigmatic, as no concomitant signal aligning with the carbon dimension is discernible.
Concomitantly, conspicuous resonant lines, expected to manifest distinctly within both the 13C
and 1H dimensions in tandem with each cross-peak, are conspicuously absent from Figure 4.

Reviewer #3

(Remarks to the Author)
The authors have addressed questions I set, but a couple of questions remain from my end.

Specifically the reasoning for not lengthening GCV pressure is clear and reasonable and also the case that it was necessary for the parasites to retain the plasmid expressing the ectopic copy of the gene is also reasonable evidence for essentiality in mice.

Clarifying a static effect on promastigotes and including new supplementary information addresses that query.

Adding an additional C4 methyl lacking sterol addresses that query.

I agree that reporting the PG/PPG and SHERP changes is of interest but a minor part of the overall narrative and additional transcriptome analysis etc. can be the subject of a later study. Its okay to keep it as there is possibly something around differentiation and cell architecture that others might glean from seeing the data

There are a couple of points, however, that I think require further clarification.

Firstly, supplementary scheme 1 shows that that branched pathway (red arrows) requires Sterol C-14 demethylase to allow those C14 methylated sterols to enter the rest of the pathway. Since those reactions labelled as Sterol C14-demethylase are carried out by CYP51, it should be shown that they are indeed the same enzyme as lanosterol 14a-demethylase. (perhaps add CYP51 in parenthesis to clarify that on the figure, and reiterate in the legend).

Since Cyp51 is, therefore, necessary to produce ergostane sterols either via the classical linear pathway, or through the alternative pathway following the C4-oxidase reaction, why aren't Cyp51 specific inhibitors more effective than they are?

This also plays alongside another important point. If Cyp5122A1 is essential, why do we need to contemplate dual inhibition of Cyp51 and Cyp5122A1 when just inhibiting the latter should kill the parasites?

Its also not clear, based on the scheme, why Cyp5122A1 is essential since the classical pathway can provide ergostane sterols without the alternative pathway. The authors do point to differences in accumulation of C4 vs C4,14 methylated sterols depending on whether Cyp51 or Cyp51/Cyp5122A1 inhibitors are used and this leads to speculation on possible impacts of C4 methylated species on the cell. I presume that flux-kinetic effects of the different pathways plays a role as

alluded to by the authors. However, although it would be nice to have a more satisfactory handle on the reason for essentiality I think the authors have had a reasonable attempt to think of one and are left accepting that this is a topic for further work.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
My concern regarding the resolution of NOESY, HSQC, HMBC and 13C spectrum are addressed properly by the authors by repeating all of the NMR experiments. It can be accepted in its current form.

Reviewer #3

(Remarks to the Author)
I was pleased to see the authors have resubmitted their article and I hope that the improved NMR spectra will now satisfy other referees. The revised manuscript is also improved in terms of its emphasis on what I consider to be the main breakthrough, i.e. a rationale as to why anti-fungal azole inhibitors of Cyp51 have not be effective against Leishmania (or T. cruzi). The alternative pathway to ergostane sterol production via Cyp51221A yields the answer. Another recent publication, not picked up by the authors, is very important to their narrative and its inclusion, along with appropriate reconsideration of their findings is desirable. The original conclusion that Cyp51 is essential to L. donovani was probably made in error. Ironically, the error probably arose due to the fact that production of null mutants in that original study was taken as evidence for essentiality. The authors here have spent time explaining why their approach with Cyp5122A1 is more meticulous and thus more reliable and the confusion sowed by the earlier Cyp51 essentiality conclusion now serves to strongly support taking that more meticulous approach. The key addition to the literature is: Tulloch et al. (2024) Sterol 14-alpha demethylase (CYP51) activity in Leishmania donovani is likely dependent upon cytochrome P450 reductase 1. PLoS Pathog. 20, e1012382. PMID: 38991025). Including its findings in a revised version will clarifying some of the key issues. It likely means that it is inhibition of Cyp5122A1 that is key to the activity of the dual inhibitors and opens the door to screening for more selective and specific inhibitors of that enzyme. In this regard, the detailed functional enzymology reported in the manuscript also takes additional relevance as it may support inhibitor discovery/design.

Point-by-point responses to reviewers' comments:

Reviewer #1:

Comment: "… that 1D and 2D NMR spectra illustrating the purified sterol metabolite fall short of achieving a satisfactory standard of graphical depiction (Figure 4). 2D spectrum is conspicuously devoid of any carbon or proton labeling dimensions."

Response: We have now included proper labels for carbon and proton dimensions in the NMR spectral data (new Fig. 4). They now appear as "^{13}C chemical shift (ppm)" or "^{1}H chemical shift (ppm)" next to the X- or Y-axis. We have also cleaned up the spectra to remove unnecessary labels, e.g., data filenames.

Comment: "The carbon resolution is markedly deficient."

Response: We agree that 1D ^{13}C signal strength was weak, primarily due to a limited quantity of the oxidation metabolite purified from the large scale CYP5122A1/lanosterol reconstitution reaction. It was also pointed out in our original (and revised) manuscript that stereochemistry of the unknown compound at the C-4 position could not be ascertained due to the low signal intensities in the NOESY spectrum. However, despite the weak ^{13}C signal, there were several lines of indisputable evidence to support our structural assignment of the unknown aldehyde metabolite of lanosterol formed by CYP5122A1, which include aldehyde signals in the ^{1}H and ^{13}C NMR spectra, location of the aldehyde revealed by the HMBC correlations, missing H-29 methyl signal at C-4 position, intact H-30 methyl signal at C-14 position, and downfield shift of nearby H-19 and H-28 methyl signals (Fig. 4 and Supplementary Table 2). These NMR results strongly point towards a structure containing an aldehyde group that replaces one of the methyl groups at the C-4 position in lanosterol, which was the main conclusion regarding the biochemical function of leishmanial CYP5122A1. We have now revised the NMR result section on pages 24-26 to clarify our rationale for the structural assignment of the unknown aldehyde metabolite.

Comment: "The genesis of the cross-peaks ostensibly corresponding to C29 (Figure 4 E) remains enigmatic, as no concomitant signal aligning with the carbon dimension is discernible. Concomitantly, conspicuous resonant lines, expected to manifest distinctly within both the 13C and 1H dimensions in tandem with each cross-peak, are conspicuously absent from Figure 4."

Response: To improve clarity, we have now included dotted resonant lines to mark the HMBC correlation between C-29 (207.2 ppm) and H-28 (1.09 ppm) methyl signals in the new Fig. 4C (originally Fig. 4E). Similarly, we have now included dotted resonant lines to other 2D NMR spectra to clearly mark correlation peaks (Fig. 4B-E). The indiscernible ^{13}C signals mentioned by the reviewer are those of C-8 (135.0 ppm), C-9 (134.1 ppm), C-24 (125.3 ppm), and C-25 (131.2 ppm) in the new Fig. 4C (originally Fig. 4E). Although it is true that they were not discernible during the 2D HMBC experiment, their chemical shifts were verified by comparison to those of the lanosterol standard (Supplemental Table 2) since these olefinic carbons are also present in lanosterol and relatively farther away from the aldehyde group at C-4 position.

Comment: “The procedural protocol employed is fraught with errors. The NMR spectrum at this juncture holds minimal potential for informative extraction, rendering confounding the means by which the authors purportedly gleaned pertinent insights from their NMR investigations”

Response: As addressed above, despite the weak ^{13}C NMR signal, there were several lines of indisputable evidence to support our structural assignment of the unknown aldehyde metabolite of lanosterol formed by CYP5122A1. In the main text, we have now revised the NMR result section (pages 24-26) to clearly present these lines of evidence obtained from 1D and 2D NMR spectra data of the unknown and the lanosterol standard. In addition, per reviewer’s suggestions, we have now improved the NMR data presentation in the new Fig. 4 and included a table (Supplementary Table 2) comparing selected ^{1}H and ^{13}C NMR chemical shifts of the lanosterol standard and the unknown. We strongly believe that these NMR data, along with LC-MS/MS sterol analysis data, support the proposed biochemical function of CYP5122A1 as sterol C4-methyl oxidase. We certainly hope that the revised manuscript, significantly improved based on your and other reviewers’ suggestions, will provide a compelling case for a more favorable consideration of the work for publication in Nature Communications.

Reviewer #2:

Comment: “In this report they provide compelling evidence to show that sterol inhibition is a combination of activities. Compounds with good levels of antileishmanial activity correlated with good levels of inhibition of CYP5122A1 although there is no attempt to correlate this with azole and protein structure e.g. the strongest inhibitors all have an imidazole – is this significant. Maybe with alphafold this should not be impossible???”

Response: With only one exception (oteseconazole, a tetrazole approved by the FDA in 2022), all the CYP51 inhibitors used clinically for the treatment of fungal infections contain either an imidazole or triazole ring. The nitrogen atom at N3 of imidazole or N4 of triazole coordinates with the heme iron atom at the active site of the enzyme (for example, PMID:24613931 or doi.org/10.1073/pnas.1324245111). We have measured the activity of twenty clinical antifungal agents for their activity against *L. donovani* promastigotes (this manuscript and PMID:35994895 or doi.org/10.1016/j.ijpddr.2022.07.003) and against recombinant CYP51 and CYP5122A1 (this manuscript). Protein modeling studies, particularly those that also involve the heme prosthetic group, are important for understanding the structural basis underlying different biochemical functions of these two CYP enzymes. Due to similar overall structural folds of all CYP proteins, we believe that this is best studied with X-ray crystal structures of the proteins (rather than Alphafold), which is currently underway in the author’s laboratory. However, CYP protein crystallization and X-ray diffraction are not trivial and hence are beyond the scope of this manuscript that focuses on biochemical function, essentiality, and antileishmanial activity of azole antifungals.

Comment: “Overall the work appears to be rigorous and the findings are interesting and merit publication. Where I struggle and it could help if this was more strongly articulated in the discussion is what this all means. Shaha Ref 26 had previously reported that that combinations of compounds that are effective CYP51 (posoconazole) and CP5122A1 (DB766) inhibitors have a synergistic antileishmanial effect albeit without clear understanding why. This paper gives that mechanistic understanding of this observation but not necessarily the molecular insight to build upon (nor the exploitation?).”

Response: We previously investigated the combination of azoles (e.g., posaconazole) and DB766 against leishmaniasis (PMID: 29061761 or doi.org/10.1128/AAC.01129-17) and showed that DB766 and posaconazole had an additive antileishmanial effect in vitro, although the combination displayed a mild synergistic effect in a murine VL model. DB766 most likely involved other unknown mechanism(s) of action besides CYP5122A1 inhibition as its antileishmanial activity (IC_{50} values ranging from 0.004 to 0.5 μM) was markedly more potent than its CYP5122A1 inhibitory activity (IC_{50} of 1.0 μM). This point was discussed in the original manuscript and revised manuscript (page 40). We believe that it would necessitate the determination of CYP5122A1 protein structure and the identification of the unknown DB766 mechanisms of action to further build upon or exploit the discovery described herein, e.g., CYP5122A1 structure-based drug design and screening new hits against targets involved in the antileishmanial action of DB766. These new discussion points have now been included in the revised manuscript (page 39 and page 40).

Comment: “Interestingly most of the more effective CYP5521A1 inhibitors are (I think) examples of azole antifungal which are not used in an oral context (too toxic / metabolically vulnerable) but do have topical applications. Have the authors looked at a CL species - it might make the work more directly applicable?”

Response: Table 2 indicates that the systemic azole antifungal ketoconazole inhibits CYP5122A1 with an IC_{50} value of 0.42 μM, making it one of the strongest CYP5122A1 inhibitors from among the antifungal azole drugs evaluated. Most of the systemic azole antifungals in clinical use are triazoles (itraconazole, posaconazole, fluconazole, voriconazole, and isavuconazole). Itraconazole, posaconazole, and isavuconazole are slightly weaker inhibitors of CYP5122A1 than ketoconazole but nonetheless inhibit this enzyme in the low micromolar range. Interestingly, fluconazole and voriconazole are very poor inhibitors of CYP5122A1 as shown in Table 2 (IC_{50}>100 μM). The data summarized in Table 2 indicate that dual inhibitors of CYP51 and CYP5122A1 are more effective against *L. donovani* than selective CYP51 inhibitors like fluconazole and voriconazole as described on pages 32-33 and elsewhere in the manuscript. Our labs are in the process of identifying new inhibitors of CYP5122A1 that will hopefully overcome some of the shortcomings of the existing drugs shown in Table 2, although a description of this work is beyond the scope of the current manuscript. Regarding the activity against CL species, other labs have reported on the activity of azole antifungal drugs against CL species (new ref #15 [PMID: 30569856 or doi:10.2174/1568026619666181220114627] and ref #17 [PMID:24376670 or doi:10.1371/journal.pone.0083247]). As mentioned in the Introduction (page 6), selective CYP51 inhibitors like fluconazole and voriconazole were ineffective against CL species, while dual inhibitors like miconazole, clotrimazole, itraconazole, and posaconazole were effective. We agree that the biochemical role and essentiality of CYP5122A1 in CL species warrant additional investigations, which have been underway in our labs. Our unpublished data indicate that CYP5122A1 is also essential in both promastigote and amastigote stages of *L. major* (manuscript in preparation), in contrast to the nonessential role of CYP51 in *L. major* previously reported by Zhang lab (ref #11 [PMID: 25340392 or doi:10.1371/journal.ppat.1004427).

Comment: “Minor typographical suggestions. P20 line 17 albeit is unable”

Corrected.

Comment: “P31 line 5 Sentence starting These results showed … does not make sense”

Corrected.

Comment: “P31 line 16 Fig 10A”

Corrected.

Reviewer #3:

Comment: “For the mouse work on essentiality, once gancyclovir is removed there is a bounce back after seven weeks. This is likely (and likely rather than probably as they suggest (line 18, page 26)). What was the reason for not sustaining that GCV pressure longer? The bounce back, however, indicates that there were parasites not killed in the absence of CYP5221A1. In fact very few drugs (maybe even none) give sterile cure in mice hence that is not surprising in itself. However, it is not known from the data whether the recrudescence (measured in spleen enlargement) comes as quiescent parasites that can survive without the enzyme re-emerge, or could secondary mutations that compensate for loss of the enzyme allow recrudescence. Or simply due to a small number not losing the plasmid over the time frame studied. In the absence of confirmatory data, some qualifying words are required.”

Response: We used i.p. injection to deliver GCV at 0.5 ml/day for 14 consecutive days which could lead to external trauma, internal injury, and misplaced injection into an organ. Prolonged treatment will increase the risk of these complications. A previous study on chronic *L. major* infection in mice revealed a mixture of rapidly replicating parasites and dormant persisters (PMID: 28096392 or DOI: 10.1073/pnas.1619265114). The latter is more likely to survive the GCV treatment and resume replication after GCV is removed. While we cannot rule out the possibility of compensatory mutations, both the amastigote and promastigote forms of Ld22A1- +pXNG4-22A1 from GCV-treated mice retained pXNG4-22A1 and were still chromosomal-null for 22A1. In contrast, our previous work on leishmanial CEPT (an essential gene for promastigotes but not amastigotes) showed that cept- +pXNG4-CEPT amastigotes rapidly lost pXNG4-CEPT and proliferated normally in mice (PMID: 33777852 or DOI: 10.3389/fcimb.2021.647870). Together, these findings support CYP5122A1 as an essential gene in *L. donovani* amastigotes.

Comment: “Given the key impact of this work on therapeutic strategies for leishmaniasis, it was surprising not to see some more mouse work on efficacy of dual inhibitors or combinations in comparison to Cyp51 specific inhibitors. Given difficulties of the mouse work and interpretation in light of the difficulty to cure, comparative efficacies in a macrophage model could take us further towards verifying the likelihood of better pharmaceutical efficacy without needing to scale to the mouse at this time.”

Response: In an earlier paper cited in this manuscript, we examined the efficacy of posaconazole and fluconazole in an intracellular *L. donovani* model (ref #38, Joice et al., 2018, PMID 29061761). At that time, we were unaware of the biochemical function of CYP5122A1 or of the inhibition profile of these azole antifungals against this enzyme. We

reported that fluconazole showed little activity in the intracellular model, while we were able to observe modest activity with posaconazole (Fig. 2 legend and Fig. S3 in the ref #38). We also indicated that “precise IC50s were difficult to determine with posaconazole because of its low in vitro selectivity for the parasites”. While we referred to these experiments in the legend of Fig. 2 in ref #38 and showed the results of single representative experiments with posaconazole and fluconazole in Supplementary Fig. 3B and 3D, respectively, we have now plotted the complete data examining the effect of fluconazole and posaconazole against intracellular *L. donovani* from four different experiments (biological replicates) conducted at that time and have included these results in Supplementary Fig. 9 and on Page 33 of this revised manuscript for consideration by the reviewers and the editor. In ref #38, we noted that parasite numbers within macrophages were static over the three-day course of our experiment measuring intracellular parasite burdens in peritoneal macrophages, which may help to explain why the potency of posaconazole in our intracellular assay is modest. Others have reported good efficacy with posaconazole against intracellular *L. amazonensis* (ref #17, PMID 24376670) and low efficacy with fluconazole against intracellular *L. amazonensis* and *L. major* (ref #16, PMID 15891135).

No CYP51 inhibitors have been approved for clinical use against kinetoplastid parasites despite the extensive study of CYP51 inhibitors against kinetoplastid parasites, suggesting that better inhibitors of *Leishmania* CYPs are needed. With our current efforts to identify dual inhibitors of CYP51 and CYP5122A1, we hope to pair inhibition of these parasite CYPs with lower toxicity to identify promising compounds for antileishmanial drug discovery efforts.

Comment: “Promastigote growth was assessed using a colorimetric test. Were parasites also viewed microscopically to distinguish leishmanicidal and leishmanistatic activity?”

Response: We viewed promastigote cultures treated with three different concentrations of posaconazole and butoconazole by light microscopy and observed a static effect with these azoles. The results of this experiment are shown in the new Supplementary Fig. 10 in the revised manuscript and on Page 33.

Comment: “C4 substrate binding assays (P19-20) only use zymosterol as a C4 methyl lacking species, which is not very comprehensive. The later data all corroborates C4 demethylase activity but conclusions should be tempered based on the n=1 SAR in that section.”

Response: We have now added an additional C4 methyl-lacking sterol, 7-dehydrodesmosterol, to establish a more robust spectral binding SAR that CYP5122A1 binds specifically with C4-methylated sterols (lanosterol, 4,14-DMZ, FF-MAS, and T-MAS; Fig. 2A), whereas CYP51 binds indiscriminately with both C4-methylated sterols (lanosterol and 4,14-DMZ) and C4-demethylated sterols (zymosterol and 7-dehydrodesmosterol) (Fig. 2B). In addition, 7-dehydrodesmosterol was incubated with CYP51 and CYP5122A1 to determine if this sterol serves as a substrate to produce oxidation metabolites. LC-MS/MS sterol analysis of these incubations has now been added as Fig. 3F.

Comment: “Page 27 line 6 talks about downregulating sterols whereas it is not regulation per se, just reduced abundance because of the lost gene”

Corrected.

Comment: “In that section, the data point to the C4 demethylation reaction being dominant over the C14 demethylation reaction. How does that reconcile with other work where Cyp51 is lost or mutated? (are there species-specific differences across the Leishmania genus?). Moreover, some more consideration should be given on the branched pathway proposed in the scheme of figure 1. A priori without Cyp51 then later sterols including ergosterol can be produced via the C4 demethylation pathway with C14 demethylation occurring due to the “Sterol C14-demethylase” later in the branched part. (presumably this is a separate enzyme from Cyp51 = lanosterol 14a demethylase?), and this is what happens. Cell lines lacking Cyp51 should, therefore, make ergosterol. However, this doesn’t reconcile with observations e.g. from McCall et al., 2015 (PMID: 25768284) or Mwenechanya et al. 2017 (PMID: 28622334). In addition to dominant versus lesser pathways, can the prospect of a multitude of potential inhibitory regulations made by different intermediates accumulating etc. be invoked? Some reasoning on how to reconcile the scheme in figure 1 with other data is desirable.

Response: The sterol C14-demethylase in Scheme 1 is CYP51 (lanosterol 14-demethylase). There are indeed species-specific differences among *Leishmania* parasites regarding sterol biosynthesis. For example, CYP51 (sterol C14-demethylase) is essential in *L. donovani* but not in *L. major* (PMID: 25768284 and PMID: 25340392). However, genetic or chemical inhibition of CYP51 resulted in depletion of ergosterol (and other ergostane-based sterols) and accumulation of 14-methyl sterol intermediates, indicating there is no separate sterol C14-demethylase enzyme.

Comment: “In considering the situation vis-à-vis LPG/PPG on the one hand (the molecules themselves have altered levels and altered distribution) and SHERP on the other hand (RNA levels are altered) thought should be given to the differences between these two types of observation. The former could be explained by changes to lipid architecture altering where these molecules go. The latter, however, implies the parasites do have a change in gene expression in response to their changed lipid architecture and this difference in interpretation of changed macromolecule distribution vs regulated gene expression needs to be highlighted. There is reference in the discussion to differentiation defects in the over-expressors, but this isn’t clearly explained and a global transcriptomic analysis could be more informative than focusing on a single gene (although not necessary here as the core message of the paper offers an explanation for failure of azole therapy in leishmania and considerations on broader system wide changes in biology are less important in that regard).”

Response: We have now provided more context in page 31. Because LPG/PPG structure and abundance are developmentally regulated in *Leishmania*, we examined whether the altered expression of LPG/PPG in CYP5122A1 mutants reflected defects in metacyclogenesis (Fig. 8) and stress response (Fig. 9). We agree that a transcriptomic analysis would generate more information, although the impact of such a study on CYP5122A1 function in *Leishmania* may be limited.

Point-by-point responses to reviewers' comments:

Reviewer #1:

Comment: "None of my comments are addressed properly. Resolution of 2D-spectrum is very low. It is not possible to get any information from this low-resolution spectrum. The cross-peaks corresponding to C29 (Figure 4 E) remains enigmatic, as no concomitant signal aligning with the carbon dimension is discernible. Concomitantly, conspicuous resonant lines, expected to manifest distinctly within both the 13C and 1H dimensions in tandem with each cross-peak, are conspicuously absent from Figure 4."

Response: We appreciate the astute comment and agreed with the reviewer on the low resolution of our original 2D NMR spectra. To properly address this comment, we decided to redo the scale-up biosynthesis of the aldehyde metabolite of lanosterol formed by recombinant CYP5122A1 and re-collected NMR data on the newly purified metabolite, which has taken us over seven months beginning with expressing and purifying sufficient amount of recombinant CYP5122A1 and TbCPR proteins to the completion of NMR data analysis. Overall, our new NMR data were of markedly higher resolution and quality to clearly show the 1H/13C peaks and cross peaks (Fig. 4A-D) that were missing from original 2D NMR spectra and allowed us to further determine the stereochemistry of the aldehyde metabolite at the C4 position based on the new NOESY data (Fig. 4E), which could not be ascertained in our original analysis. To improve clarity, we have now added blowup insets showing cognate cross points of 2D NMR. It is of note that the numbering of the oxidized C4 methyl group has now been assigned to C28 based on the newly obtained stereochemistry information on the aldehyde metabolite (it was C29 in our original manuscript) and we have revised all relevant figures and texts accordingly. We now believe that the new NMR data unequivocally elucidated the structure of the lanosterol metabolite formed by leishmanial CYP5122A1, supporting our conclusion that CYP5122A1 is a sterol C4-methyl oxidase.

Reviewer #3:

Comment: "The authors have addressed questions I set, but a couple of questions remain from my end. … There are a couple of points, however, that I think require further clarification. Firstly, supplementary scheme 1 shows that that branched pathway (red arrows) requires Sterol C-14 demethylase to allow those C14 methylated sterols to enter the rest of the pathway. Since those reactions labelled as Sterol C14-demethylase are carried out by CYP51, it should be shown that they are indeed the same enzyme as lanosterol 14a-demethylase. (perhaps add CYP51 in parenthesis to clarify that on the figure, and reiterate in the legend)."

Response: We agree with the reviewer and have now added "CYP51" to downstream reactions in the Supplementary Scheme 1 and reiterated it in the legend.

Comment: "Since Cyp51 is, therefore, necessary to produce ergostane sterols either via the classical linear pathway, or through the alternative pathway following the C4-oxidase reaction, why aren't Cyp51 specific inhibitors more effective than they are? This also plays alongside another important point. If Cyp5122A1 is essential, why do we need to contemplate dual inhibition of Cyp51 and Cyp5122A1 when just inhibiting the latter should kill the parasites?"

Response: Initially, we were baffled by our observation that dual inhibitory antifungal azoles (inhibiting both CYP51 and CYP5122A1) showed much greater antileishmanial activities than those of selective CYP51 inhibitors, even though CYP51 has been shown to be essential to *L. donovani* by independent studies. Later, our sterol analysis of the parasites treated with either dual inhibitor or selective CYP51 inhibitor (clotrimazole vs. voriconazole in Table 3 and posaconazole vs. fluconazole in Table 1 of our previous report [Feng et al. 2022; DOI:10.1016/j.ijpddr.2022.07.003) shed some light on this as dual inhibitors led to the accumulation of 4,14-methylated sterols (e.g., lanosterol or 4,14-Dimethylzymosterol) and 4-methylated sterols, whereas selective CYP51 inhibitors did not. This suggests that the accumulation of 4-methylated sterols has detrimental effects on *Leishmania* parasites and we have discussed this in the Discussion (pp.40 line 14-20 and pp.41, line 5-19). In theory, selective CYP5122A1 inhibitors should also lead to the accumulation of 4-methylated sterols like the dual inhibitors, but it has been difficult to identify any selective CYP5122A1 inhibitors to prove this. As such, to address the comment, we added "selective CYP5122A1 inhibitors" as a potential strategy and called for future studies to identify selective CYP5122A1 inhibitors (pp. 40, line 4 and 12-14).

Comment: "Its also not clear, based on the scheme, why Cyp5122A1 is essential since the classical pathway can provide ergostane sterols without the alternative pathway. The authors do point to differences in accumulation of C4 vs C4,14 methylated sterols depending on whether Cyp51 or Cyp51/Cyp5122A1 inhibitors are used and this leads to speculation on possible impacts of C4 methylated species on the cell. I presume that flux-kinetic effects of the different pathways plays a role as alluded to by the authors. However, although it would be nice to have a more satisfactory handle on the reason for essentiality I think the authors have had a reasonable attempt to think of one and are left accepting that this is a topic for further work."

Response: We agree that more studies are needed in the future to fully elucidate the ergosterol biosynthetic pathway in *Leishmania* (and other trypanosomatids), especially those reactions following the initial metabolism of lanosterol, as most reactions have been assumed to take place as such and be catalyzed by enzymes orthologous to fungi or mammalian species. Our recent work on characterizing sterol C4-methyl oxidase in *Leishmania major* revealed that leishmanial ERG25 (orthologous to the yeast ERG25 or C4 methylsterol oxidase [SMO]) is not required for C4 demethylation in *L. major* and CYP5122A1 is possibly only sterol C-4 methyl oxidase in *Leishmania* (manuscript submitted for publication; DOI: 10.20944/preprints202408.0195.v1). As such, inhibiting CYP5122A1 alone is expected to shut down the synthesis of ergostane sterols, supporting its essential role in *Leishmania* as described in the current manuscript. Furthermore, the accumulation of 4,14-methylated sterols (e.g., lanosterol) and 4-methylated sterols may have detrimental effects on *Leishmania* parasites, and we have previously raised this point in the Discussion (pp. 41, lines 9-21), as the reviewer pointed out.