nature portfolio

Peer Review File

Towards clinically relevant dose ratios for Cabamiquine and Pyronaridine combination using immediate ex-vivo/in-silico P. falciparum field isolate data

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
The manuscript is highly relevant as it is addressing the current need for improving antimalaria treatments. The authors outline a novel workflow to evaluates such treatments through the use of modelling and simulation. The work is of good quality. However, there are several major and minor points that need to be addressed.

Major points:
1. Line 152-153 (Figure 2): Due to the inclusion of the no very informative static EC50 of pyronaridine the scale makes it hard to see the early differences of EC50 for cabamiquine. Consider instead to include the static EC50 of cabamiquine to illustrate the importance of including the adaptive resistance.
2. Figure 4: Could you clarify how the red isobole relates to the EC50 cabamiquine as it is changing with concertation
3. Line 216-218 and Tabe 2: If the EC50 is beyond the tested concentration, an sig. emax curve cannot be properly estimated and you should investigate other models such as a linear slope model.
4. Line 232-241:
a. Please elaborate on the tested dosing regimen and their clinical relevance
b. Include how you produced your virtual populations, did you consider correlations in your covariates?
c.
5. Line 232-278: For the clinical trials simulations there is a lack of how kill rate relates to clinical success. Although is give insights into how different gives different kill-rates, it is not clear how it should be interpreted. How was the rate of >90% selected as target?
6. Line 511-526: Please include the route of administration and the structure of the PK model used. Also briefly discuss on the relevance of the population the models were built on and your target population.

Minor points
7. Line 107, define EC50
8. Line 125-126: “parasites were exposed to different concentrations of cabamiquine and pyronaridine covering the EC50, concentration required to reach Emax”, please specify if you refer to the range of the reference strain 3D7 here. If not, elaborate on how you determined the concentrations to be included (e.g. were multiple experiments needed?)
9. Line 136: please state to what value you fixed Emax and how it was determined
10. Line 171-178: Please clarify the difference between N0 and baseline
11. Figure 3, The facet label of the plot on in the first column, third row is not fully readable. Also, consider using facet_grid to increase readability of concentration gradient

Reviewer #2:
Remarks to the Author:
DDD107498 is the most promising compound in the global malaria pipeline. I am excited to see it progressing from Phase 1 into patients, and I agree with the ex vivo methodology used here to select a combination dose to take forward into Phase 2.

I support publication of this manuscript, but have the following comments which I hope can be addressed, because they will help the reader to better interpret the manuscript.

Parasite strains. How were the 7 parasite strains characterised? I assume that they were sequenced, because the authors looked for resistance mutations. How closely related are they to each other and to 3D7? Since the parasites were all collected from the same site from Mali, I would expect them to be closely related to each other. If the seven strains were all closely related to each other, this would affect my interpretation of the results and how well the results would generalise to other parts of the world.

Emax and EC50. The experimental design correctly allowed for Emax and EC50 to vary and therefore be estimated from the model fit to the data. However, the data had important limitations which meant this original plan could not be followed exactly.
1. Emax could not be estimated because of rapid killing between baseline and the first timepoint (12 hours?). The explanation given by the authors is unclear (lines 135, 137). The authors state that "Emax was subsequently fixed in the model, as it was not estimable precisely within the model..." This is a vague statement that could be improved by explaining that parasite reduction was much faster than expected given the experimental design: which is to say, if the experiment had included timepoints at shorter timepoints (for example, 1, 2 and 6 hours), it is possible that Emax could have been estimated. This is a key limitation of the experimental design which must be called out explicitly in the main body of the manuscript.
2. Figure 3. The vertical axis is labelled "Read-out", which not informative. The reader needs to know if this is parasite concentration or PCR or killing rate or arbitrary fluorescence units...what? Please add units.
3. Given the lack of precision in Emax, the reader needs to know how this could have affected the final conclusions. What is the slowest rate of parasite killing consistent with the data? What is this rate relative to the Emax that the model was eventually fixed at? How sensitive is the model to this assumption? I cannot tell from this manuscript how conservative the Emax chosen was, relative to the data observed.
4. I note that the Hill slope is extremely large (>20). This is not surprising for infectious diseases where time-above-threshold is a common PD driver. Was this observed in previous in vitro experiments? is this value consistent with previous in vitro experiments with 3D7 or other "standard" strains of Pf? Given the extremely large Hill slope, is an Emax model even the right model to use? Was the model fitting stable? How many methods were tried? Did the authors consider using a threshold model instead? (e.g., threshold autoregressive models are available in R)
5. What does the high Hill slope mean for the estimate of the EC50? How does this relate to the concentration-time dependency of cabamiquine observed? How sensitive is the model to "high"

Hill slope? Do the conclusions change if the Hill slope is 20 or if it is 50?

Synergy and dose selection. The authors' key conclusions are laid out in 343 to 351. One of the rationales for using fixed dose combinations is to prevent selection for resistance. Please consider this scenario: What happens if the parasite has baseline resistance to pyronairidine? This is not implausible, since artemesinin-pyronaridine combinations are in use. Is the cabamiquine dose recommended sufficient to clear the parasite on its own? Please also consider the converse scenario, where there is cabamiquine monoresistance. Would the low dose of pyronaridine and the long half-life then result in selection of pyronaridine resistance? I would expect to see these two scenarios explored explicitly in the discussion section of the manuscript.

Lines 421 to 436. Please list the actual time points used in the cabamiquine-pyronaridine interaction assay. They are difficult to read in Figure 3.

Line 490. The Hill is set to 1. I am not clear what is happening here, since the Hill for cabamiquine is set to 20 in other analyses?

Lines 521–523. The Lean body mass formula is from James 1976. Please add the appropriate literature reference, since there are at least three different formulae for lean body mass in common use.

Reviewer #3:
Remarks to the Author:
The authors employed immediate ex vivo P. falciparum field isolates to evaluate the mono and combination effects of cabamiquine and pyronaridine. Subsequently, they utilized a pharmacometric model to simulate and identify the optimal clinical dose ratios. The manuscript is well-written, and the findings could facilitate early dosage selection for the development of new drug combinations. Specific comments are as follows:

1.The authors mentioned the 3Rs principle several times throughout the manuscript. Giving a short explanation of this principle in the introduction could be helpful for readers who are not familiar with the term.

2.The authors explained that the Emax was derived from the reduction of the first to the second time point at the highest concentration of each drug and the growth data (Line 134-135). In Table 1, the Emax for both cabamiquine and pyronaridine were the same. It is unclear whether this was a coincidence or if there is a specific reason behind it. Could the authors elaborate on this?

3.Regarding the estimated growth rate of 0.015 (h^{-1}), would it be possible to compare this estimate with the growth rate reported in previously published work from the volunteer infection study (3D7 strain) (https://doi.org/10.1093/infdis/jiz557)? Are the parasite multiplication rates per 48 hours (PMR48) similar? And would this impact the estimation of other parameters in the developed

model?

4.Figures 5 and 6 are a bit difficult to follow because most of the killing rates are high, and initially, there is some confusion between the killing rate and the proportion of virtual patients with a killing rate of >90%. The authors might consider adding a horizontal line representing the 90% killing rate to aid visualization.

5.Line 272: The author mentioned that for the worst-case scenario, the simulations were conducted using PK parameters reported in patients instead of healthy volunteers, as the patients had reduced pyronaridine exposure. It could be helpful for the reader if the authors explicitly mention earlier that the PK data used for both cabamiquine and pyronaridine were from healthy volunteers for the results presented in the main manuscript.

6.Line 246-278: The authors mentioned that when using pyronaridine PK parameters from patients, the slower killing rate of pyronaridine was only evident when it was given alone, and when combined with 200mg cabamiquine, the results were similar for both wild-type and cabamiquine-resistant parasites. However, the results for the cabamiquine-resistant parasites in Supplementary Figure 12 (page 13) are somewhat different from those in Figure 6, especially at 96 hours. This discrepancy was not discussed in the manuscript.

7.Line 481: Ecomb was presented using the Bliss independence equation and was scaled by Emax. Could the authors also explicitly explain the rationale for this scaling in the manuscript?

8.The pharmacometric model developed by the authors is valuable. For the sake of reproducibility, the authors could consider providing the NONMEM code in the supplementary materials. This would also be beneficial for future studies that aim to use the same framework.

9.Please double-check that the equation numbers are presented in chronological order.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The manuscript is highly relevant as it is addressing the current need for improving antimalaria treatments. The authors outline a novel workflow to evaluates such treatments through the use of modelling and simulation. The work is of good quality. However, there are several major and minor points that need to be addressed.

Authors' reply: We appreciate the acknowledgment of the manuscript's relevance in addressing the pressing need for advancing antimalarial treatments. We extend our sincere gratitude to the reviewer for providing invaluable and constructive feedback. Below, we address his suggestions/comments which helped to enhancing the quality of our manuscript.

Major points:

1. Line 152-153 (Figure 2): Due to the inclusion of the no very informative static EC50 of pyronaridine the scale makes it hard to see the early differences of EC50 for cabamiquine. Consider instead to include the static EC50 of cabamiquine to illustrate the importance of including the adaptive resistance.

Authors' reply: We have replaced the static EC50 of pyronaridine with the static EC50 of cabamiquine from one of the inferior models compared to the model that considered adaptive resistance development.

2. Figure 4: Could you clarify how the red isobole relates to the EC50 cabamiquine as it is changing with concertation

Authors' reply: The red line represents an isobole of 50% effect. The shift of this isobole due to the altered EC50 of cabamiquine over time can be seen in the different facets of the plot and are in line with Figure 2.

3. Line 216-218 and Tabe 2: If the EC50 is beyond the tested concentration, an sig. emax curve cannot be properly estimated and you should investigate other models such as a linear slope model.

Authors' reply: We agree that these data stemming from the cabamiquine-resistant strains can be also described with a simpler slope model. Indeed, this was done during the modelling process. Nonetheless, as the slope cannot be directly compared to an EC50, we intent to keep the (uncertain) but high EC50 estimates to allow for a better comparison with the non-resistant strains. We added a clarifying statement to the manuscript and comment on the use of the slope model.

Changes to the manuscript:

"Hence, the effect of cabamiquine against the resistant isolates could be described by a slope model; nonetheless the EC50 model was kept for comparability with the susceptible strain. In summary, the drug effect of cabamiquine…."

4. Line 232-241:
a. Please elaborate on the tested dosing regimen and their clinical relevance

Authors' reply: For cabamiquine, doses from 0 to 660 mg were simulated. These doses were selected based on observed clinical efficacy from 2 volunteer infection studies (McCarthy JS et al. Safety, pharmacokinetics, and antimalarial activity of the novel plasmodium eukaryotic translation elongation factor 2 inhibitor M5717: a first-in-human, randomised, placebo-controlled, double-blind, single ascending dose study and volunteer infection study. Lancet Infect Dis. 2021 Dec;21(12):1713-1724. doi: 10.1016/S1473-3099(21)00252-8. and, an der Plas JL et al. Causal chemoprophylactic activity of cabamiquine against Plasmodium falciparum in a controlled human malaria infection: a randomised, double-blind, placebo-controlled study in the Netherlands. Lancet Infect Dis. 2023 Oct;23(10):1164-1174. doi: 10.1016/S1473-3099(23)00212-8.). In the induced-blood stage malaria challenge model, single doses of 660 mg of free cabamiquine (= 800 mg cabamiquine succinate) were shown to be efficacious in monotherapy. In the sporozoite challenge model, single 100 mg and 200 mg doses of cabamiquine provided complete protection when treating both early and late liver stages. In addition, alternative doses were simulated to provide a large picture of dose-exposure-response relationship, and to match the doses that are currently tested in ongoing phase 2 trials. Pyronaridine doses have been selected based on the label (pyramax, INN-pyronaridine tetraphosphate / artesunate (europa.eu)). Since Pyramax tablets contains 180 mg of pyronaridine tetraphosphate, multiple of 180 mg were used in model-based simulations, up to the highest dose of 720 mg administered in clinical practice.

Changes in the text addressing reviewer's comment: "doses were selected to offer a comprehensive view of the dose-exposure-response relationship, and to cover clinical relevant doses"

b. Include how you produced your virtual populations, did you consider correlations in your covariates?

Authors' reply: As outlined in the methods, covariates were sampled from a database that includes these covariates for a population of patients with uncomplicated malaria across clinical trials phases 2 to 3. This approach guarantees the preservation of correlations between the covariates.

5. Line 232-278: For the clinical trials simulations there is a lack of how kill rate relates to clinical success. Although is give insights into how different gives different kill-rates, it is not clear how it should be interpreted. How was the rate of >90% selected as target?

Authors' reply: First, we wanted to assure that the killing potential of cabamiquine and pyronaridine is maximized; therefore, we set the threshold for the observed killing rate to >90% in the respective time frames as a target at 24 and 96 h. To define probability of target attainment, since no standard approach is in place to define targets for antimalarial combination therapies, we have taken the threshold of >95% from defining sufficient target attainment for antibiotics (Mouton JW et al. The role of pharmacokinetics/pharmacodynamics in setting clinical MIC breakpoints: the EUCAST approach. Clin Microbiol Infect. 2012 Mar;18(3):E37-45. doi: 10.1111/j.1469-0691.2011.03752.x.). Here, 95% probability target attainment is a very common target and usually deemed adequate and used for determination of clinical breakpoints.

Changes to the manuscript:

"A killing rate of >90% was deemed adequate. Since no standard approach is in place to define the probability of target attainment for antimalarial combinations, the probability threshold of >95% was used in accordance with probability of target attainment assessment for antibiotics."

6. Line 511-526: Please include the route of administration and the structure of the PK model used. Also briefly discuss on the relevance of the population the models were built on and your target population.

Authors' reply: The structures of the popPK models are described in references 27 and 28. Given the complexity of the popPK for cabamiquine and in order to avoid adding more complexity, we think that referencing to these modelling publications adequately serves the purpose of this analysis. In addition, the selection of the pyronaridine popPK model with regards to the target population is described lines 272-275.

The section has been adjusted as follows based on the reviewer's comment:

"Due to the lack of cabamiquine PK information in the target malaria population at the time of the analysis, the popPK model developed in healthy volunteers was utilized. For pyronaridine, different popPK models have been reported to characterize PK profile in healthy volunteers vs malaria patients. Due to the lack of understanding of these differences and the absence of pyronaridine PK data in our target population receiving a combination of cabamiquine and pyronaridine, the two popPK models have been utilized in clinical trial simulations. The main manuscript presents results based on the popPK model in healthy volunteers, while results based on the popPK model in malaria patients are included in the supplementary material. A simulation dataset was developed in which single doses of 0-660 mg or 0-720 mg of cabamiquine or pyronaridine tetraphosphate, alone or combined, respectively, were orally administered to a virtual patient population of 40,000 patients (1,000 virtual patients for each dosing scenario). Doses were selected to offer a comprehensive view of the dose-exposure-response relationship, and to cover clinically relevant doses."

Minor points:

7. Line 107, define EC50

Authors' reply: As suggested, we added the definition into the text.

8. Line 125-126: "parasites were exposed to different concentrations of cabamiquine and pyronaridine covering the EC50, concentration required to reach Emax", please specify if you refer to the range of the reference strain 3D7 here. If not, elaborate on how you determined the concentrations to be included (e.g. were multiple experiments needed?)

Authors' reply: As suggested, we added in the text the reference strain we relied on to select the concentrations. Consequently, we have changed the text as follow:

"Once 1% parasitemia was reached, parasites were exposed to different concentrations of cabamiquine and pyronaridine covering the EC50, concentration required to reach Emax, as

well as the clinically relevant concentration range based on previous data obtained with the reference strain 3D7."

9. Line 136: please state to what value you fixed Emax and how it was determined

Authors' reply: Emax was not estimable precisely within the model due to the reduction of the assay signal to baseline from the first to the second time point at the highest concentration studied, as described in the manuscript. The estimate hence represents the full reduction of the assay signal from 0 to 12 h. Indeed, it cannot be excluded, that this reduction might already occur earlier. Hence, the estimate given in the manuscript represents a conservative estimate of Emax. Of note, the precise value is not used in the dosing simulations as these are given relative to the value of Emax. Consequently, the simulation results and interpretation of the dosing rationale would be similar even if a different value of Emax was quantified. We added this aspect to the manuscript:

"[…] E_{max} was subsequently fixed in the model, as it was not estimable precisely within the model due to the reduction of the assay signal to baseline from the first to the second time point, hence representing a conservative estimate of E_{max}."

10. Line 171-178: Please clarify the difference between N0 and baseline

Authors' reply: N0 represents the assay signal at the beginning of the experiment, whereas baseline represents the minimum assay signal, which is observed after full killing. Both were further explained in the Table caption.

11. Figure 3, The facet label of the plot on in the first column, third row is not fully readable. Also, consider using facet_grid to increase readability of concentration gradient

Authors' reply: Many thanks for spotting! We have updated Figure 3 such that all captions are now fully readable. Facet_grid is unfortunately not possible given the experimental design (see below). We hence would keep the arrangement using facet_wrap in ggplot2.

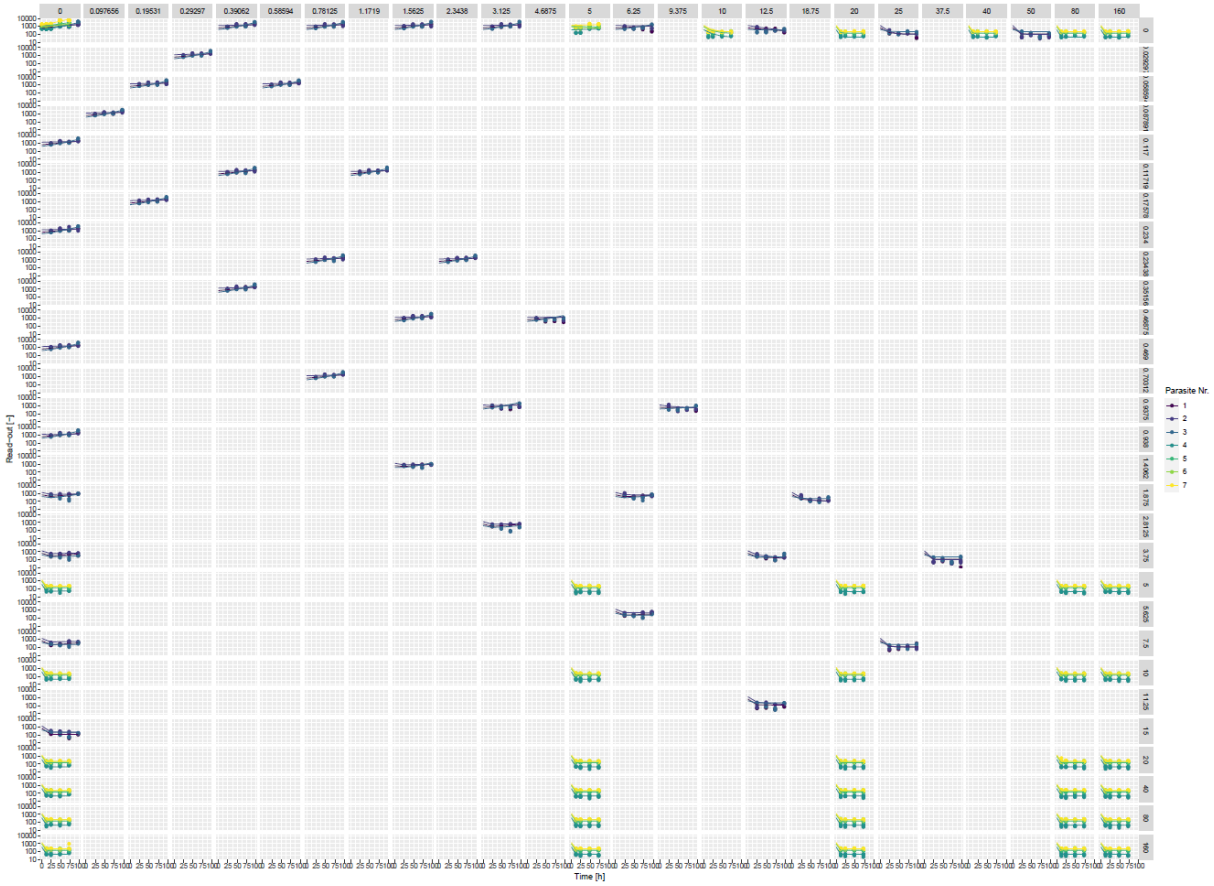


Figure 3 using facet_grid: Due to the experimental design chosen, we intend to keep facet_wrap.

Reviewer #2 (Remarks to the Author):

DDD107498 is the most promising compound in the global malaria pipeline. I am excited to see it progressing from Phase 1 into patients, and I agree with the ex vivo methodology used here to select a combination dose to take forward into Phase 2.

I support publication of this manuscript, but have the following comments which I hope can be addressed, because they will help the reader to better interpret the manuscript.

Authors' reply: We are encouraged by the positive assessment of our work. We acknowledge the presence of both major and minor points that require attention. Thank you for bringing these points to our attention. We are committed to thoroughly addressing each of these points to ensure the robustness and clarity of our work.

- Parasite strains. How were the 7 parasite strains characterised? I assume that they were sequenced, because the authors looked for resistance mutations. How closely related are they to each other and to 3D7? Since the parasites were all collected from the same site from Mali, I would expect them to be closely related to each other. If the seven strains were all closely related to each other, this would affect my interpretation of the results and how well the results would generalise to other parts of the world.

Authors' reply: Since this was not the purpose of the article the 7 different *P. falciparum* parasites isolated directly from Malian patients (referred as IEV: EEF210, EFF211, EFF223,

EEF209, EEF192, EFF204 and EFF269) were not genetically characterized and compared to 3D7 strain. By conducting this study, we aimed to characterize the pharmacodynamic interactions between cabamiquine and pyronaridine and to determine the contribution of each compound when used in combination using real world data, thus confirming that the preliminary findings obtained *in vivo* with 3D7 strain. This follows up on our recent study (Stadler E et al. Propensity of selecting mutant parasites for the antimalarial drug cabamiquine. Nat Commun. 2023 Aug 25;14(1):5205. doi: 10.1038/s41467-023-40974-8.) that showed differences in the selection of mutant's parasites between field and laboratory strains. To note, that the two cabamiquine resistant parasites used in the study (isolate 1: parasite strain ID EFF192 with E134V mutation and isolate 2: parasite stain ID EFF209 with Y186C mutation), and for which we have characterized the mutations, come from this previously study in which we investigated the translation of cabamiquine drug resistance from laboratory parasites to clinical isolates.

Regarding the generalization of the results obtained to other parts of the world, we agree that parasites may have different genetic backgrounds that could influence the outcomes. Nonetheless, a recent study done in Uganda (Kreutzfeld O at al. Susceptibility of Ugandan Plasmodium falciparum Isolates to the Antimalarial Drug Pipeline. Microbiol Spectr. 2023 Jun 15;11(3):e0523622. doi: 10.1128/spectrum.05236-22.) reported similar results to those obtained in Mali, showing that despite the possible genetic difference, anti-malarial susceptibility is similar between *P. falciparum* parasites coming from various sites/countries. Also as mentioned above the purpose of the study is to support the choice of the combination and inform clinical trials (which will be conducted in different countries) on the choice of initial doses to be used and not on the analysis of parasite genetic.

- Emax and EC50. The experimental design correctly allowed for Emax and EC50 to vary and therefore be estimated from the model fit to the data. However, the data had important limitations which meant this original plan could not be followed exactly.

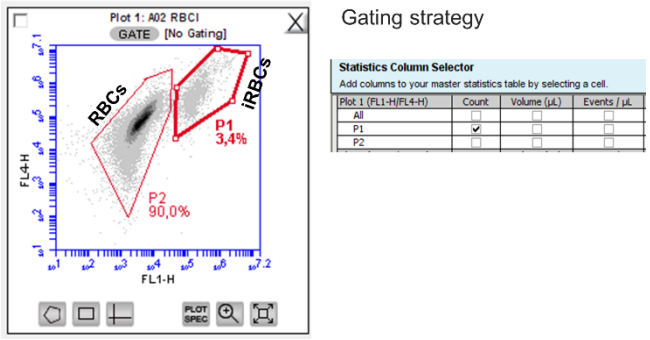
1) Emax could not be estimated because of rapid killing between baseline and the first timepoint (12 hours?). The explanation given by the authors is unclear (lines 135, 137). The authors state that "Emax was subsequently fixed in the model, as it was not estimable precisely within the model..." This is a vague statement that could be improved by explaining that parasite reduction was much faster than expected given the experimental design: which is to say, if the experiment had included timepoints at shorter timepoints (for example, 1, 2 and 6 hours), it is possible that Emax could have been estimated. This is a key limitation of the experimental design which must be called out explicitly in the main body of the manuscript.

Authors' reply: Indeed, the reviewer is correct and this is a limitation of the experimental design. Emax was not estimable precisely within the model due to the reduction of the assay signal to baseline from the first to the second time point at the highest concentration studied, as described in the manuscript. The estimate hence represents the full reduction of the assay signal from 0 to 12 h. Indeed, it cannot be excluded, that this reduction might already occur earlier. Hence, the estimate given in the manuscript represents a conservative estimate of Emax. We added this aspect to the manuscript:

"E_{max} was subsequently fixed in the model, as it was not estimable precisely within the model due to the reduction of the assay signal to baseline from the first to the second time point, hence representing a conservative estimate of E_{max}."

2) Figure 3. The vertical axis is labelled "Read-out", which not informative. The reader needs to know if this is parasite concentration or PCR or killing rate or arbitrary fluorescence units...what? Please add units.

Authors' reply: We assessed parasite viability by flow cytometry using SYBR Green and MitoTracker Dyes technique by obtaining, at the same time, the parasite positive red blood cell count (iRBCs). Cells gating analysis was performed using a gating strategy shown below (Figure below). SYBR Green (FL1 see figure below), which detect the parasite nuclei DNA, and MitoTracker (FL4 see figure below), that bind on viable parasite mitochondrion, fluorescence signals were collected for individual parasites. Red blood cells (RBCs) are lacking the cell organelle such as nuclei and mitochondrion and they appeared negative for both dyes while the infected red blood cells (iRBCs) containing the viable parasites (SYBR Green and MitoTracker positive) are observed into distinct plot (See. Flow cytometry analysis was applied to individual cells count, parasite raw count is displayed on the vertical axis). Consequently, to we added a description of the labelled "Read-out" in the figure 3 legend as follow: "Read-out: total SYBR Green and MitoTracker positive parasite count".



3) Given the lack of precision in Emax, the reader needs to know how this could have affected the final conclusions. What is the slowest rate of parasite killing consistent with the data? What is this rate relative to the Emax that the model was eventually fixed at? How sensitive is the model to this assumption? I cannot tell from this manuscript how conservative the Emax chosen was, relative to the data observed.

Authors' reply: As outlined above, the value of Emax represents a conservative estimate and the true value might be higher as the full reduction to baseline assay signal could have occurred before 12 h. As pointed out correctly by the reviewer, this is a limitation of the experimental design. However, the precise value of Emax is not used in the dosing simulations as these are given relative to the value of Emax. Consequently, the simulation results and interpretation of the dosing rationale would be similar even if a different value of Emax was quantified. We added this aspect to the manuscript:

"It is a minor limitation, that the experimental design did not allow to precisely estimate Emax. Nonetheless, the here determined value quantifies the full reduction of the assay signal until 12h and hence represents a conservative estimate of maximum effect, as the maximum effect may have occurred even earlier. Moreover, since the dosing simulations were evaluated relative to Emax, the same conclusion would have been drawn even for a higher value of Emax."

4) I note that the Hill slope is extremely large (>20). This is not surprising for infectious

diseases where time-above-threshold is a common PD driver. Was this observed in previous in vitro experiments? is this value consistent with previous in vitro experiments with 3D7 or other "standard" strains of Pf? Given the extremely large Hill slope, is an Emax model even the right model to use? Was the model fitting stable? How many methods were tried? Did the authors consider using a threshold model instead? (e.g., threshold autoregressive models are available in R)

Authors' reply: We agree that finding a high Hill factor is not uncommon. For example, a Hill factor of around 20 very well resembles an on-off behavior if the concentrations pass the EC50, e.g. as observed with vancomycin (Wicha et al. doi:10.1002/psp4.12197, Nielsen et al. doi:10.1128/AAC.00604-06.). Since the estimate in question here is part of a time-kill model with a complex model structure, the estimate cannot be directly compared to previous simpler experiments. During estimation, the estimate of Hill tended towards a value around 20, but a standard error could not be calculated given that the on-off behavior at high Hill factors. It was hence fixed to 20.

5) What does the high Hill slope mean for the estimate of the EC50? How does this relate to the concentration-time dependency of cabamiquine observed? How sensitive is the model to "high" Hill slope? Do the conclusions change if the Hill slope is 20 or if it is 50?

Authors' reply: The model is insensitive to changes of the Hill slope above 20. A Hill slope of 50 gives a very similar estimate of the EC50 due to the resulting on-off behavior.

- Synergy and dose selection. The authors' key conclusions are laid out in 343 to 351. One of the rationales for using fixed dose combinations is to prevent selection for resistance. Please consider this scenario: What happens if the parasite has baseline resistance to pyronairidine? This is not implausible, since artemesinin-pyronaridine combinations are in use. Is the cabamiquine dose recommended sufficient to clear the parasite on its own? Please also consider the converse scenario, where there is cabamiquine monoresistance. Would the low dose of pyronaridine and the long half-life then result in selection of pyronaridine resistance? I would expect to see these two scenarios explored explicitly in the discussion section of the manuscript.

Authors' reply: Based on the rationale (e.g., preventing selection of resistant parasites) regarding the development and use of fixed doses combinations mentioned by the reviewer and, considering the results obtained, it would seem that combining cabamiquine with pyronaridine, even a low doses, will protect both compounds from resistance. In fact, we have demonstrated, using susceptible parasites, that although an adaptation to cabamiquine in monotherapy has been observed, the latter is absent when the two compounds are used (starting from the EC50 of pyronaridine of 28.3 nM), showing the ability of pyronaridine to control the selection of possible cabamiquine-resistant parasites. In addition, when cabamiquine-resistant parasites are used, pyronaridine alone controls them. Interestingly, although cabamiquine alone does not appear to have any effect on these parasites, it appears to enhance the effect of pyronaridine even at low doses (Table 2).

Regarding pyronaridine resistance, although we understand the rationale behind the reviewer's questions, to our knowledge it has not yet been possible to select a parasite that has become resistant *in vitro* or *in vivo* to pyronaridine (Henrich PP, O'Brien C, Sáenz FE, Cremers S, Kyle DE, Fidock DA. 2014. Evidence for pyronaridine as a highly effective partner drug for

treatment of artemisinin-resistant malaria in a rodent model. Antimicrob Agents Chemother 58:183–195. https://doi.org/10.1128/AAC.01466-13.), making it difficult to assess the effect of cabamiquine on pyronaridine-resistant parasites. Nevertheless, since all scenarios must be taken into consideration, if a parasite became resistant to pyronaridine it would seem that cabamiquine can control its growth since data show that low doses (200 mg) of cabamiquine is able to control (>90% killing rate) in >95.3% patient (Figure 5).

- Lines 421 to 436. Please list the actual time points used in the cabamiquine-pyronaridine interaction assay. They are difficult to read in Figure 3.

Authors' reply: As suggested, we added the timepoints into the text and we have changed the text as follow:

"The parasites viability was assessed at different timepoints (12, 24, 48 and 72 hours post drug exposure) using flow cytometry (BD Accuri™ C6 Flow Cytometer®)…"

- Line 490. The Hill is set to 1. I am not clear what is happening here, since the Hill for cabamiquine is set to 20 in other analyses?

Authors' reply: The Hill here refers to the Hill of the interaction, not of the drug effect itself. Assuming a simpler sigmoidal model is common practice when using the GPDI model to quantify the interaction as sigmoidal models are more difficult to identify and more unstable in estimation (here as well). Cf. Wicha S. at al. A General Pharmacodynamic Interaction Model Identifies Perpetrators and Victims in Drug Interactions', Nature Communications, 8.1 (2017), p. 2129, doi:10.1038/s41467-017-01929-y.

- Lines 521–523. The Lean body mass formula is from James 1976. Please add the appropriate literature reference, since there are at least three different formulae for lean body mass in common use.

Authors' reply: As suggested, we have changed the text as follow:

"Lean body weight (LBW) was calculated according to the formula used for the development of pyronaridine popPK model [28], as follows: "

Reviewer #3 (Remarks to the Author):

The authors employed immediate ex vivo P. falciparum field isolates to evaluate the mono and combination effects of cabamiquine and pyronaridine. Subsequently, they utilized a pharmacometric model to simulate and identify the optimal clinical dose ratios. The manuscript is well-written, and the findings could facilitate early dosage selection for the development of new drug combinations.

Authors' reply: It was great to receive insightful and constructive feedback from the reviewer. In the following sections, we will discuss the suggestions we used to improve our manuscript.

Specific comments are as follows:

1.The authors mentioned the 3Rs principle several times throughout the manuscript. Giving a short explanation of this principle in the introduction could be helpful for readers who are not familiar with the term.

Authors' reply: We agree that for those unfamiliar with the term 3R principle a small description should be included. Consequently, we have changed the text as follow:

Changes in the text addressing reviewer's comment: "This will allow us to: (i) test the combination in a reasonable time and cost under the 3Rs principle by replacing and/or reducing the use of animals, thus providing a basis for alternative methods to reduce animal testing,…"

2.The authors explained that the Emax was derived from the reduction of the first to the second time point at the highest concentration of each drug and the growth data (Line 134-135). In Table 1, the Emax for both cabamiquine and pyronaridine were the same. It is unclear whether this was a coincidence or if there is a specific reason behind it. Could the authors elaborate on this?

Authors' reply: Indeed, Emax was not estimable precisely within the model due to the reduction of the assay signal to baseline from the first to the second time point at the highest concentration studied, as described in the manuscript. The estimate hence represents the full reduction of the assay signal from 0 to 12 h. Therefore, the same estimate appears for both drugs. Indeed, it cannot be excluded, that this reduction might already occur earlier. Hence, the estimate given in the manuscript represents a conservative estimate of Emax. We added this aspect to the manuscript (Line 139-141):

Results:
"Emax, i.e. the maximum possible killing rate was derived from the reduction of the first to the second time point at the highest concentration of each drug and the growth data. E_{max} was subsequently fixed in the model, as it was not estimable precisely within the model due to the reduction of the assay signal to baseline from the first to the second time point, hence representing a conservative estimate of E_{max}."

3.Regarding the estimated growth rate of 0.015 (h^{-1}), would it be possible to compare this estimate with the growth rate reported in previously published work from the volunteer infection study (3D7 strain) (https://doi.org/10.1093/infdis/jiz557)? Are the parasite multiplication rates per 48 hours (PMR48) similar? And would this impact the estimation of other parameters in the developed model?

Authors' reply: The growth rate in patients as given in the reference by Wockner et al ranges between 0.3 to 0.7 /day, i.e. 0.0125 to 0.029 /h. Hence the determined growth rate in our assay of 0.015 /his in line with those observed in the volunteer infection studies.

Changes to the manuscript:
"Using a pharmacometric modelling approach, we characterized the time-kill kinetics of cabamiquine and pyronaridine, alone and combined, respectively, and the model allowed to characterize the typical pharmacodynamics as well as inter-parasite variability. Parasite killing was quantified in relation to parasite growth. The quantified growth rates ex vivo were very similar compared to those in volunteer infection studies."

4.Figures 5 and 6 are a bit difficult to follow because most of the killing rates are high, and initially, there is some confusion between the killing rate and the proportion of virtual patients with a killing rate of >90%. The authors might consider adding a horizontal line representing the 90% killing rate to aid visualization.

Authors' reply: Thank you for this suggestion. The horizontal line at 90% was added.

5.Line 272: The author mentioned that for the worst-case scenario, the simulations were conducted using PK parameters reported in patients instead of healthy volunteers, as the patients had reduced pyronaridine exposure. It could be helpful for the reader if the authors explicitly mention earlier that the PK data used for both cabamiquine and pyronaridine were from healthy volunteers for the results presented in the main manuscript.

Authors' reply: We recognize the confusion and have revised the manuscript to ensure clarity.

The section has been adjusted as follows based on the reviewer's comment: The final in vitro-based PD models described above were linked to PK models of cabamiquine[27] and pyronaridine[28] developed in humans in healthy volunteers and/or patients. Due to the lack of cabamiquine PK information in the target malaria population at the time of the analysis, the popPK model developed in healthy volunteers was utilized. For pyronaridine, different popPK models have been reported to characterize PK profile in healthy volunteers vs malaria patients. Due to the lack of understanding of these differences and the absence of pyronaridine PK data in our target population receiving a combination of cabamiquine and pyronaridine, the two popPK models have been utilized in clinical trial simulations. The main manuscript presents results based on the popPK model in healthy volunteers, while results based on the popPK model in malaria patients are included in the supplementary material. A simulation dataset was developed in which single doses of 0-660 mg or 0-720 mg of cabamiquine or pyronaridine tetraphosphate, alone or combined, respectively, were orally administered to a virtual patient population of 40,000 patients (1,000 virtual patients for each dosing scenario)."

6.Line 246-278: The authors mentioned that when using pyronaridine PK parameters from patients, the slower killing rate of pyronaridine was only evident when it was given alone, and when combined with 200mg cabamiquine, the results were similar for both wild-type and cabamiquine-resistant parasites. However, the results for the cabamiquine-resistant parasites in Supplementary Figure 12 (page 13) are somewhat different from those in Figure 6, especially at 96 hours. This discrepancy was not discussed in the manuscript.

Authors' reply: Thanks for pointing this out. We point out these differences now more clearly.

Changes to the manuscript:
"However, the difference was only visible when pyronaridine was given in monotherapy or at 96 h. When combined with 400 mg of cabamiquine, the results were similar in both scenarios for doses of pyronaridine ≥360 mg, also at 96 h."

7. Line 481: Ecomb was presented using the Bliss independence equation and was scaled by Emax. Could the authors also explicitly explain the rationale for this scaling in the manuscript?

Authors' reply: Since the Bliss Independence criterion was derived from probability theory, it is bound between 0 and 1. Effects are thus scaled relative to the highest Emax, which is set to 1, when calculating Bliss Independence and then scaled back to the observed effect scale. This is common practice when using the Bliss Independence criterion (see Pearson et al doi:10.1002/jcph.2128).

Changes to the manuscript:
"Drug effects were scaled by the highest Emax as a prerequisite for Bliss Independence 24."

8.The pharmacometric model developed by the authors is valuable. For the sake of reproducibility, the authors could consider providing the NONMEM code in the supplementary materials. This would also be beneficial for future studies that aim to use the same framework.

Authors' reply: We understand the importance to share it with the community and consequently, we have changed the text as follow:

"Any requests for data by qualified scientific and medical researchers for legitimate research purposes will be subject to the Data Sharing Policy of the healthcare business of Merck KGaA, Darmstadt, Germany. All requests should be submitted in writing to the data sharing portal for the healthcare business of Merck KGaA, Darmstadt, Germany (Germany https://www.emdgroup.com/en/research/our-approach-to-research-and-development/healthcare/clinical-trials/commitment-responsible-data-sharing.html.) When the healthcare business of Merck KGaA has a co-research, co-development, or co-marketing or co-promotion agreement, or when the product has been out-licensed, the responsibility for disclosure might be dependent on the agreement between parties. Under these circumstances, the healthcare business of Merck KGaA will endeavor to gain agreement to share data in response to requests."

9. Please double-check that the equation numbers are presented in chronological order.

Authors' reply: We apologize for the typo and adjusted it consequently.

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
All comments have been addressed and I have no further comments and think the manuscript should be accepted for publication.

Reviewer #2:
Remarks to the Author:
You acknowledge that you did not sequence the seven strains used in your study: this is a limitation because if the strains all turned out to be identical, then you have simply got seven technical replicates and not seven biological replicates. You cannot say that this is outside the scope of your study, since this affects the technical interpretation of your results. If you did not sequence the parasites, then I request that you state this explicitly as an omission in the text of the manuscript, please.
I remain concerned about the generalisability of the study to areas outside Mali. In your rebuttal, your cite results from Uganda being translatable to Mali (Kreutzfeld 2023). I am more concerned about translatability to Southeast Asia (e.g., Thailand, Vietnam), for example. I strongly request that you include this as a limitation in the Discussion section of the manuscript.

Thank you for your rationale in fixing the Hill slope at 20, and for stating that the model is insensitive to higher values of the Hill slope. Please include this text in the Methods section of the manuscript.

Reviewer #3:
Remarks to the Author:
The authors have addressed all of my questions and concerns. I have no further comments.

REVIEWER COMMENTS 2

Reviewer #1 (Remarks to the Author):

All comments have been addressed and I have no further comments and think the manuscript should be accepted for publication.

Reviewer #1 (Remarks on code availability):

I was able to run the provided code within the environment and reproduce the figures. I did not re-estimate the model in NONMEM but the provided code would allow for that and to use the model on other data.

Reviewer #2 (Remarks to the Author):

You acknowledge that you did not sequence the seven strains used in your study: this is a limitation because if the strains all turned out to be identical, then you have simply got seven technical replicates and not seven biological replicates. You cannot say that this is outside the scope of your study, since this affects the technical interpretation of your results. If you did not sequence the parasites, then I request that you state this explicitly as an omission in the text of the manuscript, please.

I remain concerned about the generalisability of the study to areas outside Mali. In your rebuttal, your cite results from Uganda being translatable to Mali (Kreutzfeld 2023). I am more concerned about translatability to Southeast Asia (e.g., Thailand, Vietnam), for example. I strongly request that you include this as a limitation in the Discussion section of the manuscript.

Authors' reply: Thank you for your valuable feedback. We acknowledge the importance of genetic diversity in interpreting the results of our study. While the primary aim of our research was to demonstrate the efficacy of the combination therapy using non-culture adapted parasites in a real-world setting, we understand the added value of your comments/concerns.

To address this, we have explicitly stated in the Methods section that the field isolated parasites were not sequenced. Additionally, regarding the generalizability of our findings to regions outside Mali, such as Southeast Asia (e.g., Thailand, Vietnam), we have added a detailed limitation in the Discussion section as you suggested.

Changes to the manuscript:

"In addition, to ensure the universality of the observed outcomes, field isolates from various countries and continents should be used. As a result of this broader approach, we will be able to determine whether the findings are applicable across different geographical and demographic contexts."

"To note that the parasites used in this study were not sequenced, and therefore, the genetic diversity among the strains was not assessed."

Thank you for your rationale in fixing the Hill slope at 20, and for stating that the model is insensitive to higher values of the Hill slope. Please include this text in the Methods section of the manuscript.

Authors' reply: As suggested we added this information into the text in the results section.

Changes to the manuscript:

"Consequently, we fixed the Hill factor to 20 as the model was insensitive to higher values."

Reviewer #3 (Remarks to the Author):

The authors have addressed all of my questions and concerns. I have no further comments.

Reviewer #3 (Remarks on code availability):

The code provides a README file explaining the details of each file. Both the NONMEM and R code run properly. The authors might consider adding comments to the NONMEM code to explain the definition of each parameter in $PK and $DES, which could facilitate interpretation for other modellers. Additionally, the code provided by the authors uses individual concentrations of each drug directly from the input dataset, which were probably derived from a separate pharmacokinetic model. It might be helpful mentioning in the code how these concentrations were derived.