nature portfolio

Peer Review File

Structural and Mechanistic Insights into the Inhibition of Plasmodium falciparum MDR1

Corresponding Author: Professor Xin Jiang

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)
The manuscript titled “Structural and Mechanistic Insights into the Inhibition of Plasmodium falciparum MDR1” by Zhao et al. determined the cryo-EM structure of PfMDR1 in complex with the clinical-stage antimalarial compound ACT-451840 at 3.42 Å resolution. Combined with biochemical assays and mutagenesis, the study elucidates the molecular mechanism of PfMDR1 inhibition, reveals the structural basis of resistance-conferring mutations, and rationalizes the selective inhibition over human ABCB1. Overall, the work is technically rigorous and conceptually important, providing a valuable structural framework for understanding multidrug resistance in Plasmodium and guiding the design of next-generation antimalarial agents. The manuscript is clearly written, and the data are convincing. I recommend publication after minor revision.

Major Comments:
1. The discussion on ACT-451840’s pharmacokinetic limitations and metabolic instability is brief. Given the compound’s clinical background, a more detailed structural rationale for improving its metabolic stability (e.g., potential chemical modifications without disturbing binding) would be valuable.
2. PfMDR1 functions in concert with PfCRT in mediating drug resistance. Although beyond the main scope, a short discussion on possible interplay between PfMDR1 inhibition and PfCRT-mediated drug efflux would make the work more comprehensive.

Minor Comments:
1. The transmembrane (TM) helix numbers should be clearly labeled in Figure 2b to facilitate structural interpretation.
2. The MST data shown in Figure 2e could be combined or summarized in a single panel for improved readability.
3. The density map of ACT-451840 should be displayed from two different orientations to better illustrate the ligand placement and improve clarity.

Reviewer #2

(Remarks to the Author)
This manuscript describes the structure of the Plasmodium falciparum multidrug resistance transporter 1 (PfMDR1) in complex with ACT-451840, an anti-malarial drug in Phase I clinical trials. The authors demonstrate that ACT-451840 binds directly to PfMDR1 and inhibits its ATPase activity. A high-resolution cryo-EM structure clearly delineates the interactions between the drug and the transporter, which are further validated by structure-guided mutagenesis and functional assays. Structural comparison and mutagenesis-based functional analyses elucidate the differential effects of mutations on ACT-451840 versus another antimalarial drug, MFQ. In addition, the authors provide a rationale for the selectivity of ACT-451840 toward PfMDR1 over its human homolog ABCB1. Overall, this work offers valuable insights into the inhibition mechanism of PfMDR1 and provides a foundation for the development of new anti-malarial agents. Given the global threat of malaria and the urgent need for novel therapeutics, I recommend publication of this manuscript in Nature Communications, provided the following points are adequately addressed.

Major points
1. The validity of comparing IC_{50} values between wild-type and mutant PfMDR1 relies on an accurate baseline. The reported IC_{50} for the wild-type protein (376.1 ± 225.5 nM) has an excessively wide confidence interval, making it an unreliable reference. This measurement should be repeated to obtain a more precise value. IC_{50} of other mutations such as F331A

(407 ± 234.5 nM) should be re-measured as well.

2. The authors propose a structural mechanism for the selectivity of ACT-451840 towards PfMDR1 over its human homolog, ABCB1. This claim is based on the fundamental premise that ACT-451840 potently inhibits PfMDR1 but has minimal activity against ABCB1. However, direct functional data to validate this premise is lacking. The reported safety and tolerability from clinical studies cannot serve as direct biochemical evidence for a lack of inhibition against ABCB1 at the cellular or molecular level. The authors should provide in vitro assays measuring the IC_{50} of ACT-451840 against human ABCB1.

3. The claim of competitive inhibition between MFQ and ACT-451840, while structurally plausible, requires further validation. The presented data showing that ACT-451840 reduces MFQ-induced ATPase activity are only preliminary indications. To robustly support this claim, the authors should provide definitive enzyme kinetic profiles, such as a Dixon plot. Alternatively, if such data cannot be obtained, the language in the main text and figure legends should be revised to more cautious phrasing (e.g., "suggests a potential competitive interaction") that is appropriate for the preliminary nature of the evidence.

Minor points

1. L139: “(citation PNAS)“ should be replaced with a complete and formally formatted citation

2. L144-L146: the observed inhibitory effect may be mediated by alternative mechanisms, such as direct action on the ATPase domains. This possiblity should be explicitly acknowledged and discussed.

3. Fig 1a: the reported ATPase Vmax and Km values differ notably from those in reference 32. It is crucial that the authors discuss potential reasons for this discrepancy. Possible factors to consider include differences in experimental conditions, such as the detergents used during protein purification.

4. Fig 1a: to rule out the possibility of non-specific effects or background signal, a definitive control experiment should be included. It is recommended to measure the ATPase activity curve across varying ATP concentrations for a catalytically inactive mutant (e.g., the canonical EQ mutant common in ABC transporters)

5. L163-L165: the method details that duplicate content in the Methods section should be removed from the main text.

6. L191-L194: The authors should carefully verify the positioning of the drug moieties within the PfMDR1 binding pocket. Fig. 2a shows that moiety 1 and 2 appear to be located in the helix bundle comprising TM4-5, TM7-9, and TM12, while moiety 3 and 4 seem to be positioned in the opposing bundle of TM1-3, TM6, and TM10-11. The manuscript should clearly define what TMD1 and TMD2 refer to in the structural context.

7. L195: “almost fully occupies the translocation path” is an overstatement.

8. L204: “involves in” replaced by “ is mediated by”

9. L209: Leu324 and Ile328 should be added as resides providing hydrophobic interactions

10. L211-L212: the claimed hydrogen bonds between Gln1038/Asn1042 and ACT-451840 are not well supported by the atomic coordinates, as the measured distances are suboptimal. The authors should either revise the structural model or explicitly propose water-mediated hydrogen bonding as the more likely interaction.

11. L214-L215: “which is stabilized by Asn283 on TM4” should be rephrased to clearly reflect the role of Asn283

12. L216-L227: to improve the logical flow, this paragraph should be re-ordered to reflect the functional significance of the residues. The discussion should begin with Tyr290 and Tyr1076, which—as indicated by the most substantial increases in Km—are the most critical. The residues Phe331, Tyr803, and Asn1042, which showed less pronounced effects, should be addressed lastly.

13. L227: the IC50 of Tyr290 and Tyr1076 should be significantly “higher” according to Fig 2e.

14. Fig 2a: the color scheme of the TMs is confusing and inconsistant to the figure legend (TMD1 is shown in blue, and TMD2 is shown in red).

15. Fig 2c: the “key residues” indicated in this panel are much less than described in the maintext.

16. Fig 2d: I282 should be correctly indicated to interact with moiety 2 (5.4Å), instead of moiety 1 (7.3 Å)

17. L271: The description "Met841, shielded by the phenol group of Tyr1076" does not accurately reflect the structural evidence. The authors should re-analyze the side chain conformations and spatial arrangement to provide a more precise description.

18. L295: the nonspecific description "A807P variant's protein behavior" should be revised to align with the specific experimental observations, such as "protein stability" or "expression level".

19. L297-L299: The increased expression level of the A807P variant (described in L296) was observed in the HEK293F heterologous expression system. Here, the hypothesis "Consequently, a higher concentration of ACT-451840 is needed to inhibit the A807P mutation-mediated high expression of PfMDR1, which ultimately results in the resistant phenotype" is extrapolated to the Plasmodium falciparum parasite. However, as no direct correlation in expression levels has been established between these two systems, this proposed mechanistic link remains speculative and requires further experimental support.
Furthermore, the underlying assumption that elevated expression of a target protein necessarily requires a higher inhibitor concentration is oversimplified. This relationship is not universally conclusive and depends on multiple factors, including the binding affinity, the turnover rate of the protein, and the overall cellular context. A more nuanced discussion of the inhibition mechanism is warranted.

20. Fig S4c: the meaning of “F” “W” “E” should be clearly defined in the figure legend.

21. L334-L336 and Fig 4b: the conclusion that "MFQ-induced ATPase activity is reduced by the addition of ACT-451840" is not fully substantiated by the presented data. Fig 4b must include the ATPase activity profile for MFQ in the absence of ACT-451840 [i.e., the MFQ(+)/ACT(-) condition] to serve as a baseline for this comparison. Furthermore, the concentration of both drug should be provided.

22. Fig 4f: Statistically significant differences between experimental groups should be clearly indicated on the bar graph, preferably using asterisks or connecting lines with corresponding p-values.

23. L340-L344: The terms "Pocket 1" and "Pocket 2" have been defined in the text. To improve readability and avoid confusion, these definitions should be provided in the initial description of ACT-451840 binding to PfMDR1 (lines 187-203) rather than later in the manuscript.

24. L359-L364: Given that G316 is solvent-exposed in the previously reported outward-facing structure of PfMDR1, the G316R mutation could plausibly stabilize this conformation. Including of such discuss would enhance the statement made here.

25. Fig 5a-b: description of structure comparison is done should be provided.

26. Fig 5c-f: The legends for Figures 5c-f are too long and should be condensed. Figure legends must be limited to a factual description of the experimental data presented. All interpretive commentary and broader context should be relocated to the main body of the text.

27. L478-L479: “Insights from this study are now guiding structure-guided drug discovery efforts to improve the metabolic stability of ACT-21365.” this sentence should be rephrased to convey its meaning more clearly to the reader.

28. L480-L481: ”Our studies provide key insights by characterizing the direct binding of ACT-451840 through the inhibition of the basal ATPase activity of PfMDR132” should be rephrased to convey its meaning more clearly to the reader. Reference 32 should not be cited here.

29. L497-L498: as pointed previously, the description "Met841, shielded by the phenol group of Tyr1076" does not accurately reflect the structural evidence. The authors should re-analyze the side chain conformations and spatial arrangement to provide a more precise description.

30. L513: The citation of references 33-38 is not appropriate here, as these studies are unrelated to ACT-451840.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The editors have addressed all my concerns and I recommend it for publication.

Reviewer #2

(Remarks to the Author)
Thank you for addressing my comments. The improvement of the manuscript is satisfactory. I have no further comments.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

"The manuscript titled "Structural and Mechanistic Insights into the Inhibition of *Plasmodium falciparum* MDR1" by Zhao et al. determined the cryo-EM structure of PfMDR1 in complex with the clinical-stage antimalarial compound ACT-451840 at 3.42 Å resolution. Combined with biochemical assays and mutagenesis, the study elucidates the molecular mechanism of PfMDR1 inhibition, reveals the structural basis of resistance-conferring mutations, and rationalizes the selective inhibition over human ABCB1. Overall, the work is technically rigorous and conceptually important, providing a valuable structural framework for understanding multidrug resistance in *Plasmodium* and guiding the design of next-generation antimalarial agents. The manuscript is clearly written, and the data are convincing. I recommend publication after minor revision."

Response:

We sincerely thank the reviewer for the highly positive assessment of our work. We are gratified that the reviewer found our study to be "technically rigorous," "conceptually important," and "clearly written." We appreciate the recognition of the value our structural framework provides for understanding multidrug resistance and for future antimalarial drug design.

In the revised manuscript, we have carefully addressed all the suggestions, which we believe have further strengthened the clarity and impact of our study.

Major comments:

1.The discussion on ACT-451840's pharmacokinetic limitations and metabolic instability is brief. Given the compound's clinical background, a more detailed structural rationale for improving its metabolic stability (e.g., potential chemical modifications without disturbing binding) would be valuable.

We thank the reviewer for this constructive suggestion. We agree that providing a structural rationale for overcoming the metabolic liabilities of ACT-451840 adds significant value to the study.

In the revised manuscript, we have expanded the **Discussion** (Lines 544–552) to include a brief analysis of potential optimization strategies. Specifically, we noted that the *tert*-butyl and piperazine moieties, which are known primary sites of metabolic attack, are positioned in regions of the binding pocket that may tolerate specific modifications. We discuss how these sites could potentially be optimized through bioisosteric replacements (e.g., to stabilize the *tert*-butyl group) or structural rigidification (e.g., to protect the piperazine linkers) to improve metabolic stability while preserving essential drug-target interactions.

2. PfMDR1 functions in concert with PfCRT in mediating drug resistance. Although beyond the main scope, a short discussion on possible interplay between PfMDR1 inhibition and PfCRT-mediated drug efflux would make the work more comprehensive.

Response:

We appreciate the reviewer's suggestion to discuss the interplay between PfMDR1 and PfCRT. Recent mechanistic studies (e.g., Summers et al., PLoS Biol 2022) have demonstrated that mutant isoforms of PfCRT possess a Gain-of-Function ability to transport LMF and MFQ out of the digestive vacuole (DV) and back into the cytosol. This contrasts with PfMDR1, which acts as an importer into the DV.

As the primary targets for LMF and MFQ (such as the Pf80S ribosome) reside in the cytosol, PfMDR1-mediated sequestration into the DV serves as a resistance mechanism. By inhibiting PfMDR1 with ACT-451840, we effectively block this sequestration route. When combined with the fact that many resistant strains harbor PfCRT mutations that further augment cytosolic drug levels, PfMDR1 inhibition represents a synergistic strategy to "trap" antimalarials in their active compartment. We have added a concise discussion on this synergistic redistribution in the revised

manuscript

Minor Comments:

1.The transmembrane (TM) helix numbers should be clearly labeled in Figure 2b to facilitate structural interpretation.

Response:

We agree with the reviewer that explicit labeling of the transmembrane helices is essential for clarity. As suggested, we have added the TM helix numbers to **Figure 2b** in the revised manuscript.

2. The MST data shown in Figure 2e could be combined or summarized in a single panel for improved readability.

Response:

We thank the reviewer for this helpful suggestion. To facilitate a more direct comparison of the affinities across different PfMDR1 variants, we have integrated the individual inhibition curves into a single, summarized panel in **Figure 2e**. The IC_{50} values are also clearly tabulated within the figure to ensure clarity and accessibility for the reader.

3. The density map of ACT-451840 should be displayed from two different orientations to better illustrate the ligand placement and improve clarity.

Response:

We thank the reviewer for this suggestion to enhance the transparency of our structural modeling. In the revised manuscript, we have provided the electron density maps for ACT-451840 from two orthogonal orientations to clearly demonstrate the ligand's fit within the binding pocket. These detailed views have been added to **Supplementary Figure 2b**, providing robust evidence for the ligand placement.

Reviewer #2 (Remarks to the Author):

“This manuscript describes the structure of the *Plasmodium falciparum* multidrug resistance transporter 1 (PfMDR1) in complex with ACT-451840, an anti-malarial drug in Phase I clinical trials. The authors demonstrate that ACT-451840 binds directly to PfMDR1 and inhibits its ATPase activity. A high-resolution cryo-EM structure clearly delineates the interactions between the drug and the transporter, which are further validated by structure-guided mutagenesis and functional assays. Structural comparison and mutagenesis-based functional analyses elucidate the differential effects of mutations on ACT-451840 versus another antimalarial drug, MFQ. In addition, the authors provide a rationale for the selectivity of ACT-451840 toward PfMDR1 over its human homolog ABCB1. Overall, this work offers valuable insights into the inhibition mechanism of PfMDR1 and provides a foundation for the development of new anti-malarial agents. Given the global threat of malaria and the urgent need for novel therapeutics, I recommend publication of this manuscript in Nature Communications, provided the following points are adequately addressed.”

Response:

We sincerely thank the reviewer for the supportive comments and for recognizing the significance of our work in the context of global malaria therapeutics. We are encouraged by the assessment that our study provides "valuable insights" and a "foundation for the development of new anti-malarial agents."

We have carefully considered all of the reviewer’s suggestions and have revised the manuscript accordingly. In particular, we have performed additional analyses and included necessary data to strengthen our mechanistic conclusions. We believe these revisions have significantly improved the clarity and impact of our study.

Major Comments:

1. The validity of comparing IC_{50} values between wild-type and mutant PfMDR1 relies on an accurate baseline. The reported IC_{50} for the wild-type protein (376.1 ± 225.5 nM) has an excessively wide confidence interval, making it an unreliable

reference. This measurement should be repeated to obtain a more precise value. IC_{50} of other mutations such as F331A (407 ± 234.5 nM) should be re-measured as well.

Response:

We fully agree with the reviewer that a precise baseline is critical for evaluating the effects of mutations. We apologize for the initial variability in the biochemical assays. In response to this concern, we have meticulously optimized our experimental conditions and repeated the IC_{50} measurements for both the wild-type PfMDR1 and the F331A variant. By improving assay precision, we successfully obtained much more robust values:

Wild-type IC_{50}: 352.0 ± 16.7 nM (previously 376.1 ± 225.5 nM)

F331A IC_{50}: 874.1 ± 246.6 nM (previously 407.0 ± 234.5 nM)

The significantly reduced confidence intervals provide a more reliable reference for our comparative analysis. We have updated the corresponding data in the main text and **Figure 1b, 2e, 3f, 5f** (and **Supplementary figures**) to reflect these new, high-quality measurements.

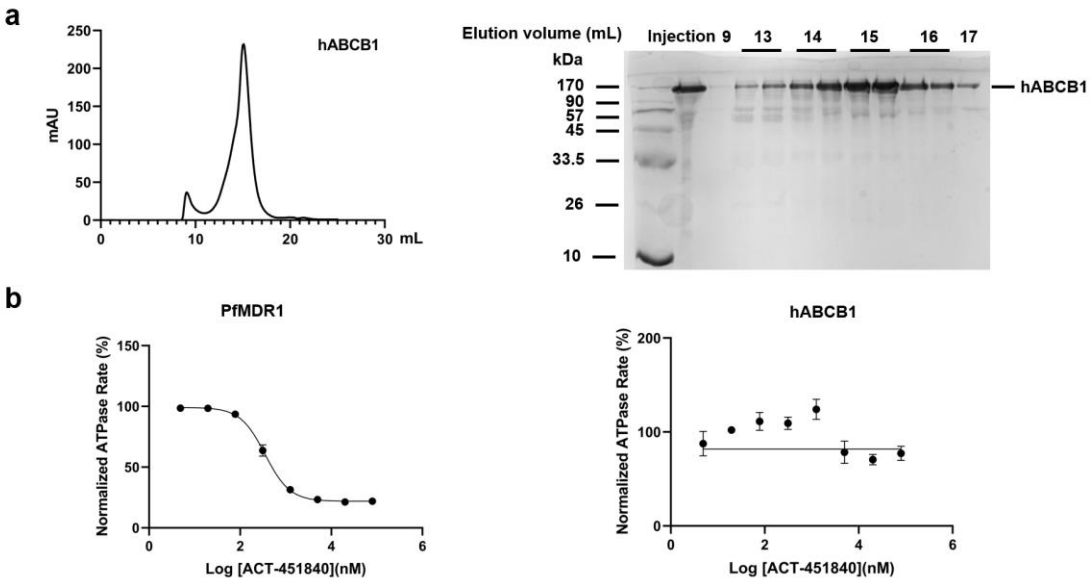
2. The authors propose a structural mechanism for the selectivity of ACT-451840 towards PfMDR1 over its human homolog, ABCB1. This claim is based on the fundamental premise that ACT-451840 potently inhibits PfMDR1 but has minimal activity against ABCB1. However, direct functional data to validate this premise is lacking. The reported safety and tolerability from clinical studies cannot serve as direct biochemical evidence for a lack of inhibition against ABCB1 at the cellular or molecular level. The authors should provide *in vitro* assays measuring the IC_{50} of ACT-451840 against human ABCB1.

Response:

We completely agree with the reviewer that direct biochemical evidence is essential to support our claims of selectivity. To address this, we have successfully expressed and

purified the human ABCB1 homolog with high purity, ensuring its stability and activity are comparable to our PfMDR1 preparations **(Respond Fig. 1a)**.

We then performed *in vitro* ATPase inhibition assays for hABCB1 under the same experimental conditions used for PfMDR1. Our results show that ACT-451840 exhibits negligible inhibition of hABCB1 ATPase activity **(Respond Fig. 1b)**. These data have been integrated into **Supplementary Figure 5a** of the revised manuscript. We believe this direct functional comparison significantly strengthens our structural rationale for the compound's selectivity.



Response Figure 1| Characterization and Functional Analysis of human ABCB1 (hABCB1). **a.** Size-exclusion chromatography (SEC) profile of purified hABCB1. **b**. ATPase inhibition assay of hABCB1 and PfMDR1 by ACT-451840.

3. The claim of competitive inhibition between MFQ and ACT-451840, while structurally plausible, requires further validation. The presented data showing that ACT-451840 reduces MFQ-induced ATPase activity are only preliminary indications. To robustly support this claim, the authors should provide definitive enzyme kinetic profiles, such as a Dixon plot. Alternatively, if such data cannot be obtained, the language in the main text and figure legends should be revised to more cautious phrasing (e.g., "suggests a potential competitive interaction")

that is appropriate for the preliminary nature of the evidence.

Response:

We appreciate the reviewer's rigorous attention to our mechanistic claims. We agree that while our structural data strongly suggest a competitive binding mode, definitive kinetic classification would require more extensive enzymatic profiling.

Following the reviewer's second suggestion, we have revised the manuscript and figure legend to adopt a more cautious tone. We now state that the data "**suggests a potential competitive interaction**" rather than making a definitive claim of competitive inhibition. Specifically, the main text has been revised as follows (Lines 347-350): "*This structural finding is supported by functional data showing that the MFQ-stimulated ATPase activity is reduced by the addition of ACT-451840, suggesting a potential competitive binding between these two molecules*." We have also updated the Figure Legend **(Figure 4b)** to reflect this more cautious interpretation.

We believe this revised phrasing accurately reflects the nature of our biochemical evidence while maintaining the structural context of the study.

Minor Comments:

1. L139: "(citation PNAS)" should be replaced with a complete and formally formatted citation

Response:

We apologize for this oversight in the draft. The placeholder has been replaced with the formal citation (Line 138). The reference list has also been updated accordingly.

2. L144-L146: the observed inhibitory effect may be mediated by alternative mechanisms, such as direct action on the ATPase domains. This possibility should be explicitly acknowledged and discussed.

Response:

We appreciate the reviewer's insight regarding the inhibition mechanism. We have revised the manuscript (Lines 145-150) to acknowledge that while the observed ATPase inhibition could potentially involve direct action on the ATPase domains or TMD-NBD coupling, we have provided a definitive structural basis for this effect. Our cryo-EM structure of the PfMDR1-ACT-451840 complex elucidates how ligand binding at the TMDs physically prevents the conformational transition to the NBD-closed state.

3. Fig 1a: the reported ATPase Vmax and Km values differ notably from those in reference 32. It is crucial that the authors discuss potential reasons for this discrepancy. Possible factors to consider include differences in experimental conditions, such as the detergents used during protein purification.

Response:

We thank the reviewer for this astute observation. We agree that the choice of detergent can significantly impact the enzymatic kinetics of membrane proteins.

Initially, our PfMDR1 samples were purified using glyco-diosgenin **(GDN)**. To investigate whether this accounted for the discrepancy with the literature, we re-evaluated the ATPase assay using lauryl maltose neopentyl glycol **(LMNG)**, the detergent employed in **Reference 31** (previously Ref. 32). Under these synchronized conditions, we obtained a Vmax of 127.50 ± 3.65 nmol/mg/min and a Km of 0.1618 ± 0.0198 mM, which are in agreement with the values reported values (Vmax ~198.3 nmol/mg/min and Km ~0.33 mM).

To maintain consistency with the existing literature and ensure the robustness of our baseline, we have updated **Figure 1a** in the revised manuscript using the LMNG-based data. This alignment ensures that our functional assays are directly comparable to previous structural and biochemical studies of PfMDR1.

4. Fig 1a: to rule out the possibility of non-specific effects or background signal, a definitive control experiment should be included. It is recommended to measure the ATPase activity curve across varying ATP concentrations for a catalytically inactive mutant (e.g., the canonical EQ mutant common in ABC

transporters)

Response:

We thank the reviewer for this constructive suggestion to further validate the specificity of our ATPase assays. As recommended, we have generated and purified the catalytically inactive variant of PfMDR1, containing the canonical mutations in the Walker B motifs of both NBDs (**E588Q/E1337Q**, hereafter referred to as the **EQ mutant**).

We measured the ATPase activity of the EQ mutant under the same experimental conditions as the wild-type protein. Our results show that the EQ variant exhibits negligible ATPase activity, which is consistent with the essential role of these glutamate residues in ATP hydrolysis and aligns with previously reported data **(Ref. 31)**. This lack of activity effectively rules out potential interference from non-specific background signals or co-purified contaminants. We have updated **Figure 1a** to include the ATPase curve of the EQ mutant as a definitive control.

5. L163-L165: the method details that duplicate content in the Methods section should be removed from the main text.

Response:

We appreciate this suggestion to improve the conciseness of our manuscript. As requested, we have removed the redundant experimental details from the main text (Lines 169-171) and ensured that all such technical descriptions are appropriately placed in the **Methods** section.

6. L191-L194: The authors should carefully verify the positioning of the drug moieties within the PfMDR1 binding pocket. Fig. 2a shows that moiety 1 and 2 appear to be located in the helix bundle comprising TM4-5, TM7-9, and TM12, while moiety 3 and 4 seem to be positioned in the opposing bundle of TM1-3, TM6, and TM10-11. The manuscript should clearly define what TMD1 and TMD2 refer to in the structural context.

Response:

We thank the reviewer for this careful examination of our structural model. We apologize for the imprecise description of the drug's orientation and the ambiguity regarding TMD definitions.

In the revised manuscript, we have redefined these sections to ensure clarity:

1. Definition of TMDs (Lines 176): We now explicitly define TMD1 as comprising TM1–6 and TMD2 as comprising TM7–12, following the canonical nomenclature for PfMDR1.

2. Corrected Positioning (Lines 195-201): We have carefully re-verified the density and updated the description of ACT-451840's orientation. As the reviewer correctly noted, moieties 1 and 2 are primarily coordinated by the helical bundle consisting of TM4-5, TM7-9, and TM12. Conversely, moieties 3 and 4 are positioned toward the opposing bundle, involving TM1-3, TM6, and TM10-11.

We have revised the corresponding text to reflect these specific structural relationships accurately, which now perfectly aligns with the updated **Figure 2**.

7. L195: “almost fully occupies the translocation path” is an overstatement.

Response:

We agree with the reviewer that our original phrasing was somewhat imprecise. To provide a more accurate description of the inhibition mechanism based on our structural data, we have revised the sentence (Line 201-203).

Instead of claiming full occupancy, we now describe ACT-451840 as acting as a molecular "wedge" that physically obstructs the translocation pathway. This binding prevents the necessary conformational transitions (from inward-open to outward-open) required for substrate transport, thereby disabling the transport activity of PfMDR1.

8. L204: “involves in” replaced by “is mediated by”

Response:

We thank the reviewer for this stylistic suggestion. We have replaced “involves in” with “is mediated by” in the revised manuscript (Line 211) to more accurately describe the interaction.

9. L209: Leu324 and Ile328 should be added as resides providing hydrophobic interactions

Response:

We thank the reviewer for the careful inspection of our structural model. We agree that Leu324 and Ile328 contribute to the hydrophobic environment of the binding pocket. In the revised manuscript (Lines 214-215), we have included these two residues in the description of the protein-ligand interactions. We have also updated **Figure 2d** and **Supplementary figure 3a** to clearly display the side chains of Leu324 and Ile328 and their spatial proximity to ACT-451840.

10. L211-L212: the claimed hydrogen bonds between Gln1038/Asn1042 and ACT-451840 are not well supported by the atomic coordinates, as the measured distances are suboptimal. The authors should either revise the structural model or explicitly propose water-mediated hydrogen bonding as the more likely interaction.

Response:

We thank the reviewer for this meticulous check of our atomic model. Upon re-evaluating the coordination distances and geometry, we agree that direct hydrogen bonding between Gln1038/Asn1042 and ACT-451840 appears suboptimal. Following the reviewer's constructive suggestion, we have revised the manuscript (Lines 218-219). We now propose that these residues likely interact with the drug molecule through water-mediated hydrogen bonds.

11. L214-L215: “which is stabilized by Asn283 on TM4” should be rephrased to clearly reflect the role of Asn283.

Response:

We thank the reviewer for this insightful comment. We have rephrased this description to more accurately reflect the structural role of Asn283. Our model suggests that the amide group of Asn283 forms a hydrogen bond with the phenolic hydroxyl group of Tyr1076. This interaction effectively stabilizes the side-chain orientation of Tyr1076, thereby facilitating its pi-pi stacking interaction with the phenylalanine moiety (moiety 1) of ACT-451840. We have updated the manuscript (Lines 221-226) to clarify this indirect but essential stabilization mechanism.

12. L216-L227: to improve the logical flow, this paragraph should be re-ordered to reflect the functional significance of the residues. The discussion should begin with Tyr290 and Tyr1076, which—as indicated by the most substantial increases in Km—are the most critical. The residues Phe331, Tyr803, and Asn1042, which showed less pronounced effects, should be addressed lastly.

Response:

We thank the reviewer for this constructive suggestion regarding the logical flow of our functional analysis. We agree that prioritizing residues with the most significant impact on binding kinetics enhances the clarity of the discussion. As suggested, we have reorganized this paragraph (Lines 227-231) to follow a "significance-first" order. This restructuring more effectively aligns our structural observations with the biochemical data.

13. L227: the IC50 of Tyr290 and Tyr1076 should be significantly "higher" according to Fig 2e.

Response:

We apologize for this oversight in our original manuscript. As the reviewer correctly noted, the mutations of Tyr290 and Tyr1076 result in a significant loss of inhibitory potency for ACT-451840, which is reflected by a substantial increase in the IC_{50} values. We have corrected the text in the revised manuscript (Lines 229-231) to accurately state that the IC_{50} values for these mutants are significantly higher than that of the wild-type protein, consistent with the data presented in **Figure 2e**.

14. Fig 2a: the color scheme of the TMs is confusing and inconsistant to the figure legend (TMD1 is shown in blue, and TMD2 is shown in red).

Response:

We apologize for the confusion caused by the inconsistent color scheme in **Figure 2a**. Our original intention was to highlight the specific transmembrane helices (TMs) forming the binding pocket; however, we agree that this led to a discrepancy with the overall TMD color coding. As suggested, we have revised **Figure 2a** to strictly adhere to a consistent color scheme: TMD1 is now shown in blue and TMD2 in red throughout the figure and its sub-panels.

15. Fig 2c: the "key residues" indicated in this panel are much less than described in the maintext.

Response:

We thank the reviewer for this observation. Our intention in **Figure 2c** was to highlight the most critical interactions—specifically the polar contacts and pi-pi stacking—to maintain visual clarity and avoid an overly cluttered panel. To address the reviewer's concern and ensure consistency between the text and figures, we have explicitly stated in the revised **Figure 2** legend that **Figure 2c** focuses on these primary interactions, while a comprehensive view of all participating residues, including those contributing to the hydrophobic environment (such as the newly added Leu324 and Ile328), is provided in **Supplementary Figure 3a**. We believe this hierarchical presentation effectively guides the reader through the most significant binding determinants without overwhelming the main figure.

16. Fig 2d: I282 should be correctly indicated to interact with moiety 2 (5.4Å), instead of moiety 1 (7.3 Å)

Response:

We sincerely thank the reviewer for this meticulous observation. Upon re-examining the atomic distances in our model, we confirm that the side chain of Ile282 is indeed

positioned within 5.4Å of moiety 2, whereas its distance to moiety 1 is 7.3 Å, making it a more likely contributor to the binding environment of moiety 2.

We have corrected this in **Figure 2d** and **supplementary Figure 3a** to reflect this accurate spatial relationship. We appreciate the reviewer's attention to detail, which has helped us ensure the structural accuracy of our manuscript.

17. L271: The description "Met841, shielded by the phenol group of Tyr1076" does not accurately reflect the structural evidence. The authors should re-analyze the side chain conformations and spatial arrangement to provide a more precise description.

Response:

We thank the reviewer for this critical evaluation of the spatial relationship between Met841 and Tyr1076. Upon re-analyzing the side-chain conformations and the local structural environment, we agree that the term "shielded" was imprecise.

In the revised manuscript (Lines 283-286), we have updated the description to characterize Met841 as a second-shell residue. While it does not establish direct contact with ACT-451840, it is situated in close proximity to Tyr1076. We propose that Met841 may indirectly influence drug binding by modulating the positioning or side-chain dynamics of Tyr1076, which is a primary binding determinant. This revised interpretation provides a more accurate reflection of the structural arrangement in this region of the binding pocket.

18. L295: the nonspecific description "A807P variant's protein behavior" should be revised to align with the specific experimental observations, such as "protein stability" or "expression level".

Response:

We appreciate this suggestion for improved precision. We agree that "protein behavior" is too vague. We have replaced this phrase with "protein stability" in the revised manuscript (Lines 305-316).

19. L297-L299: The increased expression level of the A807P variant (described in L296) was observed in the HEK293F heterologous expression system. Here, the hypothesis "Consequently, a higher concentration of ACT-451840 is needed to inhibit the A807P mutation-mediated high expression of PfMDR1, which ultimately results in the resistant phenotype" is extrapolated to the *Plasmodium falciparum* parasite. However, as no direct correlation in expression levels has been established between these two systems, this proposed mechanistic link remains speculative and requires further experimental support.

Furthermore, the underlying assumption that elevated expression of a target protein necessarily requires a higher inhibitor concentration is oversimplified. This relationship is not universally conclusive and depends on multiple factors, including the binding affinity, the turnover rate of the protein, and the overall cellular context. A more nuanced discussion of the inhibition mechanism is warranted.

Response:

We sincerely thank the reviewer for this insightful critique. We agree that extrapolating protein expression data from a HEK293F heterologous system directly to *P. falciparum* involves a degree of speculation. We also acknowledge that the relationship between target abundance and inhibitor potency is complex and influenced by various biochemical factors beyond simple expression levels.

Following the reviewer's suggestion, we rephrased the sentence to "*Interestingly, the A807P variant exhibited significantly increased expression levels compared to the wild-type protein in our HEK293F expression system, while maintaining similar protein stability (Supplementary Fig. 4c). While the correlation in expression profiles between heterologous systems and P. falciparum requires further validation, this observation raises the possibility that the resistant phenotype may be linked to altered protein abundance rather than a direct loss of drug affinity. It is conceivable that elevated PfMDR1 levels could potentially necessitate higher concentrations of ACT-451840 for*

effective inhibition. Such a mechanism, if confirmed in vivo, would be reminiscent of the decreased susceptibility to ACT-213615 (the parent compound of ACT-451840) observed in the FCB strain, which carries two copies of pfmdr1."

20. Fig S4c: the meaning of "F" "W" "E" should be clearly defined in the figure legend.

Response:

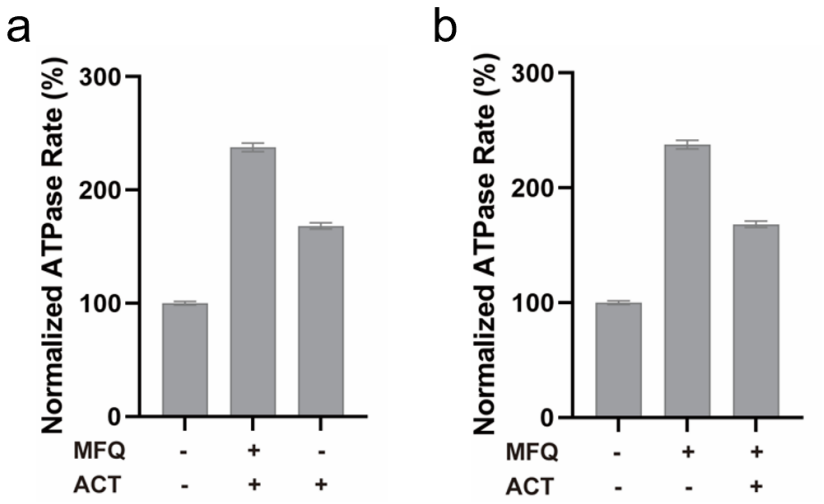
We thank the reviewer for pointing out this omission. We have now clearly defined these abbreviations in the revised figure legend for **Supplementary Figure 4c**. Specifically, "F", "W", and "E" represent Flow-through, Wash, and Elution fractions, respectively, collected during the protein purification process.

21. L334-L336 and Fig 4b: the conclusion that "MFQ-induced ATPase activity is reduced by the addition of ACT-451840" is not fully substantiated by the presented data. Fig 4b must include the ATPase activity profile for MFQ in the absence of ACT-451840 [i.e., the MFQ(+)/ACT(-) condition] to serve as a baseline for this comparison. Furthermore, the concentration of both drug should be provided.

Response:

We apologize for the oversight and the confusion caused by the mislabeling in **Figure 4b**. In the original figure, the baseline condition [MFQ(+) / ACT(-)] was mislabeled **(Response Fig. 2a)**. We have now corrected **Figure 4b (Response Fig. 2b)** to explicitly include the ATPase activity profile for MFQ in the absence of ACT-451840. This baseline clearly demonstrates that MFQ stimulates PfMDR1 ATPase activity, and the subsequent addition of ACT-451840 results in a reduction of this induced activity.

Furthermore, as requested, the specific concentrations used for both MFQ and ACT-451840 in these assays have been added to the **Figure 4** legend in the revised manuscript to ensure full transparency of the experimental conditions.



Reponses Figure 2| Comparison of original and revised ATPase assay data. **a.** original mislabeled figure. **b.** revised figure

22. Fig 4f: Statistically significant differences between experimental groups should be clearly indicated on the bar graph, preferably using asterisks or connecting lines with corresponding p-values.

Response:

We fully agree that statistical significance is essential for a rigorous interpretation of our quantitative data. As suggested, we have updated **Figure 4f** to include statistical analysis using one-way ANOVA. We have added the corresponding *p-values* directly above the bars to clearly indicate significant differences between the experimental groups. Furthermore, we have updated the figure legend to specify the statistical parameters and the number of independent replicates (n) used. We believe these additions significantly enhance the clarity and reliability of our comparative analysis.

23. L340-L344: The terms "Pocket 1" and "Pocket 2" have been defined in the text. To improve readability and avoid confusion, these definitions should be provided in the initial description of ACT-451840 binding to PfMDR1 (lines 187-203) rather than later in the manuscript.

Response:

We sincerely appreciate the reviewer's suggestion to streamline the definitions of the binding pockets. We have carefully re-evaluated the narrative flow in the initial description of ACT-451840 binding (original manuscript, Lines 187-203).

However, we have opted to retain the original placement of these definitions. Our primary concern is that **Figure 2**, which accompanies the initial description, is intended to provide a global overview of the complex and does not yet feature the specific sub-pocket segmentations. Formally defining "Pocket 1" and "Pocket 2" at that stage, without the necessary visual annotations, might impose an unnecessary cognitive load on the reader and lead to confusion.

These terms are essential for the detailed mechanistic discussions and comparative analysis presented later in the context of **Figure 4**, where they are explicitly labeled and functionally defined. To ensure maximum clarity, we believe it is more intuitive for the reader to encounter these specific definitions only when they can be directly cross-referenced with the corresponding structural illustrations. We hope the reviewer understands this choice to maintain a tight alignment between the text and the visual evidence.

24. L359-L364: Given that G316 is solvent-exposed in the previously reported outward-facing structure of PfMDR1, the G316R mutation could plausibly stabilize this conformation. Including of such discuss would enhance the statement made here.

Response:

We sincerely thank the reviewer for this insightful suggestion. We agree that contextualizing the G316R mutation within the previously reported outward-facing **(OF)** structure of PfMDR1 provides a more comprehensive understanding of its role in modulating drug sensitivity.

In the revised manuscript (Lines 376-381), we have incorporated this comparative analysis. We discuss how G316 is part of a hydrophobic gating region on the DV lumen side in our inward-facing **(IF)** structure, where it is relatively buried. The introduction of

a charged arginine side chain in the G316R variant would be energetically unfavorable in this hydrophobic environment. Conversely, as the reviewer noted, G316 becomes solvent-exposed in the OF state. This transition-from a buried hydrophobic environment in the IF state to a solvent-exposed environment in the OF state-suggests that the G316R mutation imposes a significant energetic penalty during the conformational transition, thereby perturbing the transport cycle.

25. Fig 5a-b: description of structure comparison is done should be provided.

Response:

We thank the reviewer for this suggestion to clarify the structural comparison methodology. We have now provided a detailed description of how the structural alignment was performed in the revised figure legend of **Figure 5**.

26. Fig 5c-f: The legends for Figures 5c-f are too long and should be condensed. Figure legends must be limited to a factual description of the experimental data presented. All interpretive commentary and broader context should be relocated to the main body of the text.

Response:

We thank the reviewer for this constructive suggestion regarding manuscript conciseness and formatting. We agree that figure legends should be restricted to factual descriptions of the data. In the revised manuscript, we have substantially condensed the legends for **Figures 5c-f**. All interpretive statements and broader mechanistic discussions have been moved to the corresponding **Result** section.

27. L478-L479: "Insights from this study are now guiding structure-guided drug discovery efforts to improve the metabolic stability of ACT-21365." this sentence should be rephrased to convey its meaning more clearly to the reader.

Response:

We thank the reviewer for this suggestion. We have rephrased this transition to more

clearly delineate the gap between previous SAR-based optimization and our current structure-guided approach.

In the revised manuscript (Lines 495-497), we have clarified that while previous efforts were limited by the lack of structural information, leading to metabolic stability issues in clinical trials. Our current study provides the necessary molecular blueprint. The revised sentence now reads: "*The structural insights from this study provide a precise molecular blueprint to overcome these pharmacological limitations, facilitating the structure-guided optimization of ACT-451840 and its derivatives to enhance their metabolic stability*." This revision more effectively bridges the historical context of the ACT series with the structural mechanisms described in the following sections.

28. L480-L481: "Our studies provide key insights by characterizing the direct binding of ACT-451840 through the inhibition of the basal ATPase activity of PfMDR1[32]" should be rephrased to convey its meaning more clearly to the reader. Reference 32 should not be cited here.

Response:

We appreciate the reviewer's suggestion for clarity. We have rephrased this sentence to more accurately describe the biochemical relationship between ATPase inhibition and drug binding. We have also removed the redundant citation **(Ref 32)** as these insights are derived from the data presented in this manuscript. The revised sentence now reads: "*Our results suggest that ACT-451840 directly engages PfMDR1, as demonstrated by the inhibition of its basal ATPase activity*." (Lines 498-499).

29. L497-L498: as pointed previously, the description "Met841, shielded by the phenol group of Tyr1076" does not accurately reflect the structural evidence. The authors should re-analyze the side chain conformations and spatial arrangement to provide a more precise description.

Response:

We appreciate the reviewer's suggestion. We have revised the main text to provide a

more precise spatial description as requested. We now clarify that while Ala807 is behind Phe1072, Met841 is adjacent to Tyr1076. We have also rephrased the conclusion to maintain conciseness while emphasizing the indirect mechanism: "*Mutations at Ala807 and Met841 are positioned behind Phe1072 or adjacent to Tyr1076, respectively. These structural arrangements suggest that they compromise inhibitor potency through an indirect, allosteric manner, likely by perturbing the position or dynamics of the primary binding residues*." (Lines 515-519)

30. L513: The citation of references 33-38 is not appropriate here, as these studies are unrelated to ACT-451840.

Response:

We apologize for the lack of clarity that led to this misunderstanding. **References 33–38** were cited to support the description of the multiple conformational states of hABCB1, rather than to suggest that these studies involved ACT-451840. We used these structural coordinates of hABCB1 to perform our comparative docking and modeling analysis.

To prevent further confusion, we have rephrased the sentence in the revised manuscript to explicitly clarify that these citations refer to the structural studies of hABCB1. (Line 541).