nature portfolio

Peer Review File

Cryo-EM structures of *Candida albicans* Cdr1 reveal azole-substrate recognition and inhibitor blocking mechanisms

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This is a well-written manuscript that describes the cryo-EM structure of the Cdr1 transporter of Candida albicans in the abo state, in the fluconazole bound state, and the milbemycin oxime-inhibited state. Cdr1 is an ABC transporter that plays a central role in fluconazole resistance in Candida species much like the Pdr5 transporter in Saccharomyces cerevisiae. In Candida albicans, overexpression of CDR1 and CDR2 occur due to activating mutations in the transcriptional regulator gene TAC1. This constitutive overexpression results in increased fluconazole efflux and reduced susceptibility to fluconazole as well as other clinically important azole antifungals. Moreover, fluconazole exposure in wild-type Candida albcians induces expression of CDR1, and loss of CDR1 confers hypersusceptibility. Orthologs of Cdr1 have proven important to fluconazole resistance in other often multi-drug resistant Candida species such as C. glabrata, C. parapsilosis, and C. auris. Understanding how Cdr1 functions as a transporter, how it differs from Pdr5 (whose stricture was recently solved), and how Cdr1 activity might be inhibited are all important for the ultimate development of therapeutic strategies to impede CDR1 and enhance the activity of the azole antifungals against both resistant and susceptible Candida isolates.

This work used the veterinary antiparasitic agent milbemycin as an inhibitor of Cdr1 activity. It is a reasonable chemical tool to employ here to visualize how a competitive inhibitor might interact with Cdr1. The authors describe a functional and structural characterization of Cdr1 and the recognition of fluconazole by Cdr1, and make comparisons with published data for Pdr5 in S. cereviase. The also clearly demonstrate recognition of milbemycin by Cdr1 and describe the mechanism by which milbemycin competitively inhibits Cdr1 activity. Milbemycin is one of several known inhibitors of Cdr1 and work towards finding viable strategies to inhibit Cdr1 therapeutically to restore or enhance fluconazole activity have been ongoing for well over a decade. The significant challenges presented more recently by MDR Candida (such as C. glabrata, C. parapsilosis, and C. auris) make this study timely and I share in the hope that it will reinvigorate work in this specific area.

The introduction provides sufficient background, results are clearly presented with excellent use of figures, methods appear to be described in sufficient detail, and I appreciate the discussion which is written with brevity but substance. My only critique is that the paper is written in a way that suggests milbemycin is an FDA approved drug that could be used immediately in the clinic, when to my knowledge this is not the case. It should be made clear that this is a veterinary agent and much work remains to be done before a viable Cdr1 inhibitor might be found or developed that could be used clinically.

Reviewer #2 (Remarks to the Author):

In this manuscript Peng et. al, present cryo-EM structures of Cdr1 in three distinct states：the apo state (Cdr1Apo), fluconazole-bound state (Cdr1Flu), and milbemycin oxime-bound state (Cdr1Mil). This study shows that:
1) While both Flu and Mil locate within the same central cavity of Cdr1, Flu binds the top but Mil occupies the entire cavity.
2) Mil occupies the central cavity of Cdr1 with hydrophobic interactions and these interactions may constrain conformational changes after ATP binding, thus inhibits ATP hydrolysis which is required to export cargos.
3) The relatively rigid entrance region and closed conformation of Cdr1Mil hinder substrates from entering the central cavity.
Therefore, this study represents a major advancement in understanding the mechanism of Cdr1-mediated azole resistance and inhibition of milbemycin oxime.

Minor concerns:
1. The binding modes of compounds with Cdr1 and ATPase assay suggest that Mil exhibits a higher binding affinity toward Cdr1 compared to fluconazole. It would be nice to measure the binding affinities of the two compounds.
2. The authors didn't mention what type of interaction N1359 is involved in Flu binding.
3. Line 202: "Figs. 2a, 4a" should be "Fig. 2a, 4a"
4. Fig 1.d: pip2 should be uppercase and identified in three structures.
5. Residues should be labeled in Extended Data Fig. 9 c-d.
6. The density maps and coordinates should be deposited in the Electron Microscopy Data Bank (EMDB) and Protein Data Bank (PDB).
7. Please remove exaggerated language such as "for the first time" (Line 280), "the first structure" (Line 290)

Reviewer #3 (Remarks to the Author):

Peng et al. present three cryo-EM structures in apo, substrate-bound, and inhibitor-bound states of an ABC transporter, Cdr1, from Candida albicans, a clinically significant opportunistic pathogenic fungus. These structures detail the long-awaited structural basis of drug recognition and the inhibitory mechanism of the transporter, which is of great interest due to the prevalence of drug resistance. While the structures of Pdr5 from S. cerevisiae in apo, substrate-bound, ADP-bound, and vanadate-trapped states have been previously reported, the main findings—particularly the inhibitory mechanism by milbemycin oxime—will likely appeal to a broad audience, including scientists in the medical field. Certainly, many in the ABC transporter field will find these results particularly interesting. The manuscript is written in a polite manner, and the methodology is generally sound, with a few exceptions. Addressing the following corrections would significantly improve the quality of the article, making it suitable for publication.

Major points:

1. The evaluation of protein expression levels in Cdr1 mutants in S. cerevisiae BY4741 is necessary to determine whether drug sensitivity depends on these mutations.

2. Additionally, assessing the ATP hydrolysis activity of these mutants is crucial for an effective functional evaluation.

3. The ATP hydrolysis activity of Cdr1 should be evaluated not by using detergent-solubilized Cdr1 but by using membrane-embedded Cdr1, such as liposome- or lipid nanodisc-reconstituted Cdr1.

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2. In the legend for Fig. 5, Line 622-623, (d) and (e) should be placed after Cdr1Flu and Cdr1Mil, respectively.

d-e, Stability analysis of the cytoplasmic (d) and inner-leaflet (e) entrances of Cdr1Flu and Cdr1Mil.

3. The maps and PDB coordinates should be deposited.

We sincerely appreciate the reviewers for their time and efforts in providing positive and constructive feedback on this work. Our point-by-point response is provided below (highlighted in yellow in revised manuscript).

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might be found or developed that could be used clinically.

Thank you for the high evaluation of our work. We sincerely appreciate your constructive suggestions to improve our manuscript. In the revised manuscript, we now emphasize that milbemycin oxime is a veterinary agent. We agree that significant clinical research is needed before it can be considered for use in human medicine.

Reviewer #2 (Remarks to the Author):

In this manuscript Peng et. al, present cryo-EM structures of Cdr1 in three distinct states: the apo state (Cdr1Apo), fluconazole-bound state (Cdr1Flu), and milbemycin oxime-bound state (Cdr1Mil). This study shows that:

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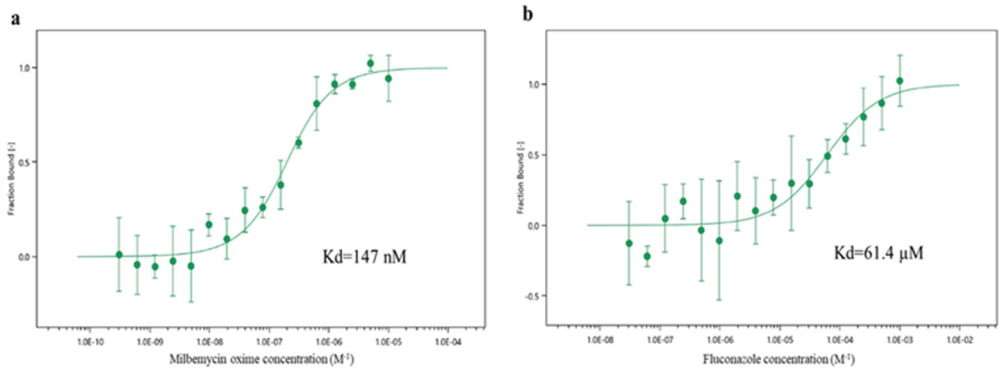

Thank you for the constructive suggestion. We measured the binding affinities of fluconazole and milbemycin oxime for Cdr1 using microscale thermophoresis (MST) (see the figure above). The dissociation constants (Kd) for milbemycin oxime and fluconazole were found to be 147 nM (left figure) and 61.4 µM (right figure), respectively, indicating that milbemycin oxime has a much higher binding affinity for Cdr1 compared to fluconazole. We have incorporated these

results into the revised manuscript.

2. The authors didn't mention what type of interaction N1359 is involved in Flu binding.
Thank you for the constructive suggestion. We added the van der Waals interaction of N1359 in the revised manuscript.

3. Line 202: "Figs. 2a, 4a" should be "Fig. 2a, 4a"
Thank you for pointing out the error. We have made the necessary corrections in the revised manuscript.

4. Fig 1.d: pip2 should be uppercase and identified in three structures.
Thank you for pointing out the error. We have made the necessary corrections in the revised manuscript.

5. Residues should be labeled in Extended Data Fig. 9 c-d.
Thank you for the constructive suggestion. We have labeled the residues in Extended Data Fig. 9 c-d in the revised manuscript.

6. The density maps and coordinates should be deposited in the Electron Microscopy Data Bank (EMDB) and Protein Data Bank (PDB).
Thank you for the constructive suggestion. We have deposited the density maps and coordinates and added the entries of EMDB and PDB in the Extended Data Table 1 of the revised manuscript.

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Reviewer #3 (Remarks to the Author):
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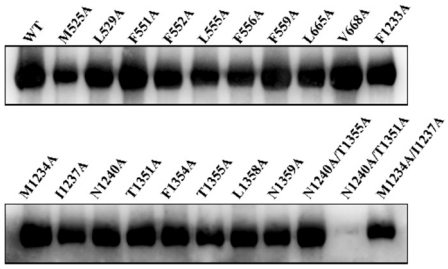
Thank you for your high evaluation of our work. We sincerely appreciate your constructive suggestions to improve our manuscript.

Major points:

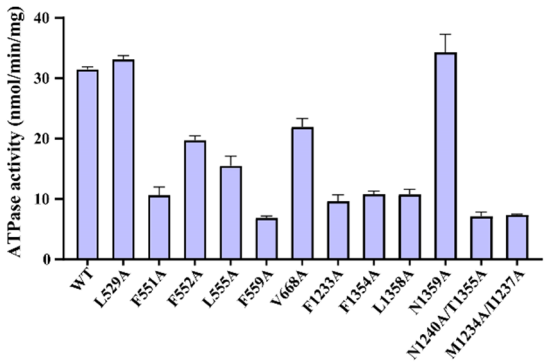
1. The evaluation of protein expression levels in Cdr1 mutants in S. cerevisiae BY4741 is necessary to determine whether drug sensitivity depends on these mutations.

Thank you for the constructive suggestion. We performed western blotting assay to evaluate the protein expression levels of Cdr1 mutants in S. cerevisiae BY4741 ΔPdr5 strain (below). The results show that the double mutation N1240A/T1351A results in nearly undetectable expression, while other mutations exhibited expression levels similar to the wild type. The hyper-drug sensitivity observed in the N1240A/T1351A mutant is likely due to this lack of expression. We have incorporated these results into the revised manuscript.



2. Additionally, assessing the ATP hydrolysis activity of these mutants is crucial for an effective functional evaluation.

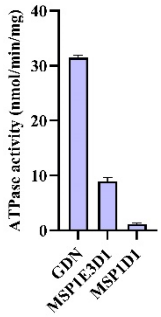
Thank you for your constructive suggestion. We have assessed the ATP hydrolysis activity of these mutants that decreased fluconazole drug resistance, in GDN detergent (the reason is explained in response to major point 3 below). We observed that group 1 mutants (F551A, F552A, L555A, F559A, V668A, F1233A, F1354A, L1358A, M1234A/I1237A, and N1240A/T1355A) exhibited reduced ATP hydrolysis activity, while group 2 mutants (L529A and N1359A) did not. For group 1 mutants, the reduced drug resistance may result from impaired fluconazole recognition as well as decreased ATP hydrolysis activity. For group 2 mutants, the reduced drug resistance appears to result primarily from impaired fluconazole recognition. We have incorporated these results into the revised manuscript.



3. The ATP hydrolysis activity of Cdr1 should be evaluated not by using detergent-solubilized Cdr1 but by using membrane-embedded Cdr1, such as liposome- or lipid nanodisc-

reconstituted Cdr1.

Thank you for your constructive suggestion. We reconstituted the wild-type Cdr1 into nanodiscs with scaffold proteins MSP1D1 or MSP1E3D1 and lipids (POPC: POPG = 2:1). The ATPase activities in both MSP1D1 and MSP1E3D1 nanodiscs were significantly lower than that in GDN detergent. However, testing two other fungal ABC transporters with our nanodisc reconstitution system showed high ATPase activities (unpublished data), indicating the system's effectiveness. The low ATPase activity of Cdr1 in nanodiscs might be due to the absence of specific lipids or other unknown factors, requiring further investigation. Given that GDN detergent showed higher ATPase activity and ATP hydrolysis is often evaluated in detergent-solubilized environments[1-6], we used GDN-solubilized environment to assess ATP hydrolysis in these mutants in the revised manuscript.



References:

1. Liu, F., Zhang, Z., Csanády, L., Gadsby, D.C. & Chen, J. Molecular Structure of the Human CFTR Ion Channel. *Cell* **169**, 85-95.e88 (2017).
2. Qian, H. *et al.* Structure of the Human Lipid Exporter ABCA1. *Cell* **169**, 1228-1239.e1210 (2017).
3. Xie, T., Zhang, Z., Fang, Q., Du, B. & Gong, X. Structural basis of substrate recognition and translocation by human ABCA4. *Nature Communications* **12** (2021).
4. Xie, T., Zhang, Z., Yue, J., Fang, Q. & Gong, X. Cryo-EM structures of the human surfactant lipid transporter ABCA3. *Science Advances* **8** (2022).
5. Khandelwal, N.K. *et al.* The structural basis for regulation of the glutathione transporter Ycf1 by regulatory domain phosphorylation. *Nature Communications* **13** (2022).
6. Schleker, E.S.M. *et al.* Structural and functional investigation of ABC transporter STE6-2p from Pichia pastoris reveals unexpected interaction with sterol molecules. *Proceedings of the National Academy of Sciences* **119** (2022).

Minor points:

1. While the structural comparison of the inward-facing conformation of substrate-bound Cdr1 with the published outward-facing conformation of Pdr5 is reasonable, this reviewer wonders whether the authors attempted to obtain a cryo-EM structure in an outward-facing conformation of Cdr1.

Thank you for the constructive suggestion. We attempted to follow the conditions described in the Pdr5 paper, which used ATP/orthovanadate (Vi) to capture outward conformational changes,

but we were unable to obtain an outward-facing conformation of Cdr1 in GDN detergent. Similarly, using hydrolysis-deficient Cdr1^{E1027Q} with 8 mM ATP/Mg^{2+} also failed to achieve the outward-facing conformation. We will continue to explore different conditions to capture outward-facing conformation of Cdr1 in future studies. Given the high similarity between Cdr1 and Pdr5, we utilized the published outward-facing conformation of Pdr5 to hypothesize how fluconazole is exported by Cdr1.

2. In the legend for Fig. 5, Line 622-623, (d) and (e) should be placed after Cdr1Flu and Cdr1Mil, respectively. d-e, Stability analysis of the cytoplasmic (d) and inner-leaflet (e) entrances of Cdr1Flu and Cdr1Mil.

Thank you for pointing out the error. We have made the necessary corrections in the revised manuscript.

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Thank you for the constructive suggestion. We have deposited the density maps and coordinates and added the entries of EMDB and PDB in the Extended Data Table 1 of the revised manuscript.

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Reviewer #2 (Remarks to the Author):

The authors have successfully addressed all the concerns, actually not much, raised during the previous round of revision. As other reviewers mentioned, these findings detail the long-awaited structural basis of drug recognition and the inhibitory mechanism of the transporter, which is of great interest due to the prevalence of drug resistance and therefore are highly significant for the field and thus warranting publication in a journal such as Nature Communications.

Reviewer #3 (Remarks to the Author):

In this revised version of the manuscript, the concerns raised by this reviewer have been thoroughly addressed. Extensive and time-consuming experiments were meticulously conducted, and the in-depth crosstalk between structure and function relationships has been carefully integrated into the paper, resulting in a significant enhancement in the overall quality of the manuscript.

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