nature portfolio

Peer Review File

The discovery and structural basis of two distinct state-dependent inhibitors of BamA

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The work of Sun and colleagues screened two macrocyclic peptides (PTB1 and PTB2) against the bacterial OMP assembly protein BamA. BAM has been extensively studied as an important drug target against Gram-negative pathogens due to its essential function and high conservation across species. The authors aimed to explore novel anti-BAM inhibitors and performed an in vitro screen using a large library of macrocyclic peptides against the purified BamA or whole BAM complex. The authors demonstrated the inhibition efficiency of the two identified peptides PTB1 and PTB2 in vivo, and also determined cryo-EM or crystal structures of peptide bound BAM complex in different membrane-mimetic agents. Their structural analysis of the BAM-PTB complexes and resistant strain mutations revealed distinct binding specificities of PTB1 and PTB2 to different states of the BamA barrel, which are very interesting findings.

This work has important scientific implications, and the experiments are carefully designed and the data are well presented. There are only a few comments I would like to add:

1. It is fascinating that PTB1 binds at the closed lateral gate of the BamA barrel, a position that is new to other known inhibitors. I wonder if PTB1 binding locks the BamA barrel in the close conformation, i.e. whether the inhibition is irreversible? Is it possible to perform dynamic simulations (like what is done for the BAM-PTB2 structure) to confirm that PTB1-bound BamA structure is fixed.

2. Line234 “the PTB2-BAM-SMA and PTB2-BAM-DDM complex structures are both highly similar to the apo-BAM-SMA/DDM complex that we have determined (RMSD<0.7angstrom)”. I wonder why the same conformation was also captured for BAM in the absence of PTB2. Shouldn’t be there any other conformation for an apo-BAM complex?

3. In the PTB2-BAM structure, PTB2-lg-1 and PTB2-lg-2 interacted to each other at the open lateral gate of BamA. Is there a risk of aggregation of this peptide?

4. It says that PTB2 binding in BamA lumen does not restrict widening of the lateral gate. If PTB2 does not restrict conformational changes of the BamA barrel, then what is the inhibition mechanism of PTB2? Is the inhibitory effect of PTB2 observed is due to binding of PTB2-lg-1 to the beta1 strand, not PTB2-lumen?

5. The implications from the crystal structure of the BamA-psBamA complex are not very convincing as it is not a physiological relevant folding intermediate hybrid complex, but more like an artifact formed due to crystal packing of BamA.

Reviewer #2 (Remarks to the Author):

Inhibition of the Bam complex is a new route to antibacterial action. This study identified two macrocyclic peptide inhibitors targeting different states and unique sites with BamA. The authors provide compelling genetic and biochemical data supporting BamA as the target of antibacterial action and the exact modes of binding. These results are important by providing new knowledge of Bam conformations susceptible to inhibitors and lead inhibitors that are amenable to manufacturing. Two clarifying points would help the reader.

1. The content of PTB inhibitors (F,W,R,K) is reminiscent of general membrane disruptive peptides. However, the resistant mutants clearly show that they target BamA. It does seem likely that they can still disrupt (non-lytic) the outer membrane, which may enable BamA access. Ethidum bromide used to measure membrane disruption requires access through both outer and inner membranes. Is outer membrane disruption alone (NPN assay) observed for these peptides in wild type and PTB resistant strains? If yes this could be a mode to reach susceptible BamA sites. It could also suggest a more general route of non-lytic outer membrane disruption that could be used to reach other essential components of the cell envelope.

2. PTB2-1 was shown to bind in the lumen of BamA as well as along the lateral gate. Resistant mutants indicate that lumen binding is the driver of antibacterial activity. Can the authors comment on the importance in lateral gate binding of PTB2-1 such as a precursor stage to lumen entry? Does PTB2-1 still bind to the lateral gate of BamA in PTB2-1 resistant mutants?

Reviewer #3 (Remarks to the Author):

Summary:

The rise of antibiotic resistant Gram-negative bacteria is a pressing worldwide problem. The outer membrane beta-barrel assembly machinery (BAM) complex functions in the outer membrane to

facilitate the folding and insertion of beta-barrel proteins into the membrane. The central component of the BAM complex, BamA, is an attractive target to design novel therapeutics against because it is easily accessible from the extracellular space and it is essential for cell viability. In this manuscript Sun, Storek, Tegunov et al. identify two novel macrocycles, PTB1 and PTB2, that kill Gram-negative bacteria by interacting with BamA and inhibit its function. CryoEM structures of the BAM complex interacting with PTB1 or PTB2 show different binding sites of the peptides to BamA; PTB1 on the extracellular loops above the lateral gate, and PTB2 on the luminal side of the BamA extracellular loops and at the BamA lateral gate.

Overall, this manuscript shares exciting advancements in screening for novel therapeutics and identification of two new peptide inhibitors of BamA. However, this reviewer feels that the manuscript requires serious editing to expand discussion in the context of currently known therapeutics, expand on methods descriptions, and address lack of information pertaining to replicate experiments and descriptions of averaged data. There is a surprising lack of quality in both the data and the presentation of results, and there is simply not enough information provided for someone else to reproduce the work.

Major comments:

1. SPR experiments are used to demonstrate PTB1 and PTB2 interact with the BAM complex, yet the binding affinity of these interactions is not discussed in detail within the text.

a. Line 167 states PTB1-1 binds BamA in the presence of Zn2+ at 50pM. Does BamA bind Zn2+ or other cations in the absence of PTB1-1? Please expand discussion of the binding affinities for both PTB1 and PTB2.

Please provide addition of a summary plot of binding affinities for replicates of each peptide, buffer control, and other relevant conditions (such as BAM+PTB1-1 in the presence of EDTA)

b. Comparison to binding affinities of BAM/BamA with darobactin and dynobactin would also add to the manuscript and further showcase differences between these new peptides and darobactin/dynobactin.

c. Two replicate SPR sensograms are shown in Supplementary Figure S1 for each macrocycle, and the KD values are fairly different. Are those the only replicates for each peptide?

d. I am surprised that the authors haven't done an analysis of PTB1 and PTB2 in mice. Since such data are available for darobactin, the comparison is important.

2. Information on replicates, how many data points are averaged, and what error bars represent are missing from numerous figures and captions. Please add this to figure captions and appropriate methods sections.

a. Figures 1C & 2C how many replicates were completed with similar results?

b. Figure 1I how many replicates with similar results? Suggest generating summary plot with KD values from all replicates, could include one column for each condition tested here.

c. Supplementary Figure S1C how many replicates with similar results? Generate summary plot with all replicates, one column per peptide tested and add a buffer control.

d. Supplementary Figure S2A, S6B how many replicates with similar results?

e. Supplementary Figure S2B, S6C how many times was experiment repeated with similar results? How many replicates went into average shown on plot? What do the error bars represent (standard deviation? Standard error?)

f. Supplementary Figure S2C, S6D how many times was experiment repeated with similar results? How many replicates went into average shown on plot?

3. BAM structure in DDM bound to PTB1-1:

a. Methods state that this structure was obtained with a FAB bound, but main text does not include this information or a discussion of how FAB binding could impact BAM dynamics and/or interactions with PTB1-1. What is the relevance of FAB to this manuscript? These is no discussion and the structural characterization are poor.

b. Given that the map resolution is 3.7Å, is density clear enough to unambiguously assign PTB1-1? Supplementary Figure S4I has one view of the PTB1-1 model within the map, however additional views would be useful. Quantification of the model fit within the map density (such as a CC score) would be useful to add.

4. Lines 193-206 give the impression that the PTB2 molecule in the BamA lumen is solely responsible for locking the BamA barrel in an outward open state. However, this fails to consider the role the other two PTB2 molecules bound at the lateral gate may have. Could the two PTB2 molecules at the lateral gate induce the same conformation by blocking full closure of the lateral gate? What experimental support do you have to show that the luminal PTB2 molecule binds first to open the lateral gate?

5. Inconsistent use of PTB2 and PTB2-1 within the text and figures is confusing. Since these are two different molecules it is important to edit for clarity. Same for PTB1 versus PTB1-1.

a. For example, figure 2b shows structure of PTB2 but 2c is with PTB2-1. It is unclear if the structure solved contains PTB2 or PTB2-1

6. PTB2-lg-1 molecule interacts with the BamA lateral gate beta-strand 1 much like darobactin and dynobactin do, however no discussion on the similarities and differences of these interactions is

included. This is an interesting area of discussion, particularly because darobactin and dynobactin interact with the same portion of BamA however the BamA lateral gate is closed in those structures.

7. Numerous BamA mutations that confer resistance to PTB1 or PTB2 are identified. Are there any that confer resistance to both?

a. Were any of these mutations previously identified to confer resistance to other antibiotics/novel therapeutics, such as darobactin A?

b. May want to consider the recent paper in which MD simulations suggest mutations in BamA that confer darobactin A resistance alter the dynamics of the BamA lateral gate. (doi:10.1021/acs.jpcb.3c04501)

8. Lines 212-215 state that PTB2-lg-1 interacts with BamA beta1 in a similar manner as a beta-signal motif from a native substrate but does not expand further.

a. Does PTB2 have a sequence similar to the beta-signal motif?

b. Are these interactions similar or different than the interactions between BamA and darobactin A or dynobactin?

9. Lines 216-220 describe differences in fold between PTB2-lumen and PTB2-lg-1. How does the fold compare between PTB2-lg-1 and PTB2-lg-2?

10. Suggest additional biophysical experiments be conducted to support three binding sites of PTB2 on BamA.

11. This reviewer strongly disagrees with referring to the second copy of BamA in the crystal structures (lines 247-270) as a “pseudo-substrate” since this is a beta1-beta1 interaction. The substrate BamA beta-signal is on beta16, therefore a BamA(substrate)-BamA(machinery) interaction is between beta16(substrate) and beta1(machinery). The observed arrangement of BamA in the crystal structures is non-physiological, likely a result of crystallization.

a. The statement in line 267 that this conformation is non-physiological needs to be brought up earlier in this section.

b. The non-physiological beta1-beta1 interaction has also been observed in other beta-barrel machinery (doi:10.1038/s41467-020-17144-1), an artifact resulting from the purification process/conditions.

c. Caution should be used in discussing promiscuity of lateral gate of Bam A for different substrates as the structure is not sufficient evidence that BamA interacts with substrates like beta1 in

biologically relevant conditions. Line 267-268 “this observation further highlights the promiscuity of the lateral gate to engage diverse ligands” should be deleted.

12. Additionally, crystallization results section should include description of potential differences in BamA barrel only conformations compared to full length BamA and complete BAM complex. This is particularly important because E. coli BamA POTRA domains 3-5 are essential for functon(doi:10.1038/sj.embor.7401092, doi:10.1126/science.1143993), yet they are missing in the construct used in this crystal structure.

13. Lines 281-282 suggests that PTB1 binding results in inability of substrates to interact at BamA lateral gate. Support of this statement with an activity assay would be beneficial. Either with folding assays (see doi:10.1128/mbio.02286-22) or competition assays for native beta-signal peptides (see doi: 10.1038/s41586-021-03455-w). The rest of that sentence is also misleading, since the open lateral gate being required for BamA function was demonstrated by multiple groups beginning with Noinaj et al, 2013.

a. Similarly, lines 324-325 state that PTB2 could inhibit BAM function sterically or by competition. This should also be investigated by activity assays suggested above.

14. Lines 305-308 state that the lumen binding PTB2 molecule is responsible for the inhibition of BAM function not the molecule at the lateral gate, however there is no experimental evidence for this statement within the manuscript. Add experiments to show that this is the case or remove this statement.

15. Lines 308-310 describe promiscuity of BamA binding to various ligands, however all the biologically relevant examples stated contain a beta-signal sequence motif which is known to be important for association with BamA and BamA facilitated beta-barrel folding (doi:10.1016/0022-2836(91)90880-F, doi:10.1007/BF00332243, doi:10.1371/journal.pbio.0040377, doi:10.1073/pnas.2220762120)

a. It would be beneficial to analyze PTB2 to determine if it also contains a beta-signal sequence motif and include this in the discussion.

16. Important cryoEM data processing details are missing from methods sections and relevant figures. Including:

a. Particle picking parameters

b. particle box size

i. were particles binned during initial steps and later unbinned?

c. non-standard parameters used during processing steps

d. how the masks were made for local/non-uniform refinements

17. The cryoEM workflow figures are lacking important details and are uninterpretable for many figures.

a. Supplementary Figure S4E is a good starting point, though is still missing critical information including:

i. Particle box sizes

ii. Densities for 3D classification in the middle (from cisTEM)

iii. Were there any additional steps to get from the 10.3Å map in relion to the 3.7Å map in cryoSPARC? If so these need to be clearly stated in methods and shown in work flow.

b. Supplementary Figures S7, S8, S9, S10 workflows are not useful in present form, as current images do not show any details of reconstructions. Each row of ab-initio reconstructions or 3D classifications should be shown as densities (like the 3D classification panel in S4E). Re-make these panels to be more similar in design as S4E, add particle box sizes, binning, number of particles at each step, number of particles (or percentage) in each ab-initio/3D class.

18. Supplementary Figures S7, S8, S9, S10 need to be heavily edited/remade with essential details

a. Scale bars need to be added to the representative micrograph image

b. Scale bars or statement of box size needs to be added for the 2D classification panels

c. Workflows need to be edited to be more useful. Current figures are difficult to see anything and do not have all necessary information. (see point 17)

d. FSC curves need to have the loose, tight, and corrected curves shown at minimum. Supplementary Figure 4G does this well, make remaining figures similarly.

e. Colors for the local resolution should be consistent between figures (S4h is blue/2.7Å-red/4.5Å, but all others are red/2.5Å-blue6.5+Å). Also, captions should state how local resolution was calculated (using cryoSPARC, phenix, or other program?).

f. Why do these figures use direction-dependent Fourier-space coverage plots while supplementary figure S4 uses angular distribution plot? Suggest editing figures so that same type of plot is used for all.

19. The methods section fails to describe how the BAM-SMA sample was purified.

a. Additionally, line 482 states that BAM-SMA was copurified with PTB2 but no information was provided for what step the PTB2 was added, or how much.

20. The methods section describes purifying the protein using Superdex200 16/60 column, which is a large prep-grade column. However, all of the size exclusion chromatograms in the supplemental figures are a 3mL elution profile. Please clarify how these SEC profiles were obtained.

Minor comments:

1. Line 70 is missing reference to structures of the BAM complex stalled folding intermediate states solved by the Bernstein and Hinshaw groups (doi:10.1016/j.cell.2022.02.016)

2. Reference to BamA sequence alignment figure (Supplementary Figure S3) is missing from the main text lines 106-110 where it is relevant.

3. Italic font is missing in a number of places

a. Line 276 “in vitro”

b. Lines 966-968 species names

4. Methods section includes centrifugation information in rpm but should be reported in g instead

a. Line 343, 369, etc

5. Lines 355-371 describe BamA-POTRA3-5 was purification and state that it was used in the mRNA-display selections. It was not clear in the main text that a truncated form was used for these experiments. Add to main text along with explanation why this construct was used.

6. Several places in the methods section state the use of 1.5% OG in size exclusion chromatography. This is a somewhat high detergent concentration. If this concentration was indeed used, could PTB1/PTB2 be partitioning into the micelles during binding experiments and impacting results? Please add buffer control replicates for binding experiments.

a. Line 370, 389, 426, etc

7. The size exclusion chromatogram labels imply that the BAM complex was copurified with PTB1 or PTB2 which based on the methods sections (lines 477-483) is not true. Please edit labels and figure captions to clarify this.

8. Is the “Growth conditions” section in methods (line 585) for the growth assays? If so, please specify this in section header as earlier methods section describing purification state use of TB for growth.

9. The use of “schematic of BamA-binding PTB…” in the figure captions for the structures of PTB1 and PTB2 is confusing (lines 866, 891). Suggest using “structure of PTB…” instead.

10. Figures:

a. Figure 1:

i. 1D: color other BAM subunits and FAB density

ii. Suggest adding panel of cryoEM density from 1D zoomed in on PTB1-1 and BamA with different contour level to more clearly show lateral gate is closed and the PTB density better.

iii. 1G&H consider making into 1 panel and adding rotation axis to help reader understand the relationship of the two views.

b. Figure 2:

i. Add panel showing full density of BAM+PTB, including BAM accessory proteins. Color BAM subunits same as in 1D.

ii. 2D: please also color the PTB2 molecules at the lateral gate (same colors as in figure 3)

iii. 2E: please change BamA lateral gate to tan so that it is consistent with figure 1 (pink is already assigned to BamB)

iv. Add another panel with PTB2-BAM-SMA density to compare to DDM density. Also color PTB2 molecules at lateral gate.

v.

c. Figure 3:

i. 3C: add PTB2-lg-2 as it looks quite different than the other two (at least from panel b inset)

ii. Consider adding panel with superposition of all 3 PTB2 models (1 solid color per model) to compare differences between them more easily

iii. 3F: clarify in caption what the shading in the plot represents

d. Figure 4:

i. 4A: add label for 2nd PTB2 molecule in ps-BamA lumen.

ii. 4A-C: change point with line to attach more clearly to panel B versus panel C (right now both attach to panel B which is confusing).

iii. 4B,C: why are atom representations different between these two panels (sphere in B and stick in C)? Suggest changing B to stick representation and adding in hydrogen bonds (if there are any?)

iv. 4D: suggest coloring EspP a different color to emphasize it is a different protein (compared to BamA in left and right most panels).

v. 4D: add row with side view (view from membrane plane) of all four models as well. This will help clearly show that the second copy of Bam from the crystal structure is in a non-physiological orientation.

e. Supplementary Figure S1:

i. S2D,E: what does omega stand for in "Δ494-496ΩS"?

f. Supplementary Figure S3:

i. Add annotations for mutations that confer resistance to PTB2 (use circle of a different color than the red that identifies PTB1 resistance mutations)

ii. Add annotation for extracellular loop 6

g. Supplementary Figure S4:

i. S4A,B: clarify in captions what detergent this protein is purified in.

ii. S4D: details in 2D classes are somewhat difficult to see. Could this panel be made bigger to see features more clearly? Also, please check that the scale bar is correct and/or provide box dimensions.

iii. S4I: add additional view of map & model. Add to figure caption what contour level map is shown at.

h. Supplementary Figure S6:

i. S6A: add comparison to the beta-signal sequence motif?

ii. S6D: what does dotted line on plot represent? Explain in caption or remove.

i. Supplementary Figure S7:

i. Missing purification information (size exclusion chromatogram and SDS-PAGE). Please add. If same protein purification was used as pictured in S4A,B specify in figure caption and in methods.

ii. S7E: add additional views of density and PTB2 models. PTB-lg-1 and PTB-lg-2 are not very convincing with numerous side chains outside of the density.

1. Include in figure caption what contour level map is shown at

j. Supplementary Figure S8:

i. Clarify in caption what program generated the 2D classes shown

k. Supplementary Figure S11:

i. S11A: Was the DDM or SMA cryoEM model used for this? Were the BamB-E subunits also removed during the MD setup? Please clarify these things in the figure, caption, and methods section.

ii. S11C: caption states it shows hydrogen bonding between BamA and three PTB2 molecules, yet only the two at the lateral gate are shown clearly. Either add panels of the figure to show luminal PTB2 hydrogen bond interactions or edit figure caption to clarify only looking at lateral gate PTB2 molecules.

iii. S11C: plot on the right is not described in figure caption. Make into separate panel (ie S11D) and clearly describe plot

l. Supplementary Figure S12:

i. S12A: difficult to see details, please make larger or add inset with zoom of important/interesting area

ii. S12C: at a different contour level is there density for all side chains? Current contour level leaves numerous side chains out. If not, please address this in the text.

m. Supplementary Figure S13:

i. S13A: clarify that these are top views. Add another row showing models viewed from the membrane plane (side view).

ii. S13B: make BAM-darobactin BamA cartoon darker grey so it can be clearly distinguished in the superposition. Include beta-16 from darobactin structure in the superposition panel to emphasize clash this would have with PTB2-lg-1. In caption, please specify what view is shown (ie lateral gate viewed from from membrane plane).

iii. S13C color beta1 and beta16 to be consistent with earlier figures (Figure 1 has these strands in tan). Specify in caption what points are being measured using residue numbers (ie same residues in each model? Or the closest points in each model which could be different residues?)

n. Supplementary Table S1:

i. Apo-BAM-SMA and PTB2-BAM-SMA do not have initial models listed, yet methods state that Apo-BAM-DDM and PTB2-BAM-DDM models were used, respectively. Please put these model names into table S1.

ii. It would be nice to have CC scores added to this table, especially for the ligands. It would also be useful to have main chain CC scores for BAM included.

11. Add supplementary figures/panels showing sample map density around model in stick representation from parts of all chains in each reconstruction. While density and model is shown for PTB1 and PTB2, panels should also show density and model for other areas of the map.

a. At minimum, the lateral gate of BamA and BamA extracellular loops/beta strands near the PTB2 lumen binding sites should be shown for all relevant maps. Include in figure caption what contour level of map is shown.

b. Additional view(s) of the PTB1 and PTB2 densities and models should also be added

Would be very beneficial if CC scores could be calculated and included for the ligands within the density to give the reader a better idea of how well the model matches the map.

Could also calculate and include CC scores for BAM/BamA

20. The methods section describes purifying the protein using Superdex200 16/60 column, which is a large prep-grade column. However, all of the size exclusion chromatograms in the supplemental figures are a 3mL elution profile. Please clarify how these SEC profiles were obtained.

Reviewer #4 (Remarks to the Author):

The paper from Sun et al. describes the discovery of new antibiotic peptide macrocycles that function by inhibiting BamA in the Gram-negative outer membrane. Different macrocycles are found to target distinct conformations of BamA, namely open and closed states. High-resolution structures of BamA with bound compounds validate the proposed mechanisms.

This is a very interesting and promising study. I have a few questions and comments that I hope could further improve the manuscript.

The authors should compare PTB1-1 that binds to the extracellular loops to the peptidomimetic compounds of Luther et al. (2019). Based on NMR, they also bind at the outside. I don't think it's the same site, but maybe there's some overlap worth mentioning (or even if none). A recent computational study also speculated that these peptidomimetics might target the lateral gate as well (Kuo et al. "Drug Binding to BamA Targets Its Lateral Gate" J. Phys. Chem. B, 2023. https://pubs.acs.org/doi/10.1021/acs.jpcb.3c04501).

I think the claim that the compounds actually lock BamA in an open or closed state, while quite reasonable, is still a bit speculative. Can the authors look to their simulations for additional evidence? Are the dynamics around the gate reduced with compounds compared to without? No apo BamA simulation was reported, but I assume it wouldn't take very long to run in both laterally closed and open states.

How reliable are the placements of the compounds in the cryo-EM maps? The local resolution is low (around 4.5 Ang.). The images in Fig. S7e are not entirely convincing, particularly given the compounds' flexibility. Can other orientations be ruled out?

Why was a DMPC membrane chosen for the simulations? I assume in part it was for convenience, but it would be helpful if the authors could justify the choice.

We thank our Reviewers for their positive feedback and thoughtful suggestions. Your constructive comments have helped us to strengthen our study. All significant comments have been thoroughly addressed, as detailed below, and these updates are exemplified in the revised manuscript by:

- the design, engineering, and testing of BamA chimeric proteins and *E. coli* strains that establish a critical role for the luminal PTB2 macrocycle binding site in the mechanism of action for this inhibitor series. We have added a new results section and main-text figure describing this innovative approach.
- performing additional experiments including a red blood cell lysis assay (data included in the revised manuscript) and NPN assay (data included in the response to reviewers) further demonstrating that the activities of our PTB1 and PTB2 inhibitors operate through specific targeting of BamA and do not act by non-specific membrane permeabilization activity at any relevant concentrations.
- significantly updating our cryo-EM methods with detailed descriptions to improve the clarity and accuracy of our analysis.
- significantly updating our methods section and figure legends to further improve clarity and accuracy of our manuscript, including substantial updates to our supplementary figures.

We believe our revised work will advance the study of the β-barrel assembly machine (BAM) and antibiotic discovery efforts targeting BAM, and that this work also provides a generalizable template to discover potent and selective modulators against high value but challenging membrane protein drug targets. We hope that you agree that our revised manuscript is now appropriate for publication in *Nature Communications*.

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The work of Sun and colleagues screened two macrocyclic peptides (PTB1 and PTB2) against the bacterial OMP assembly protein BamA. BAM has been extensively studied as an important drug target against Gram-negative pathogens due to its essential function and high conservation across species. The authors aimed to explore novel anti-BAM inhibitors and performed an in vitro screen using a large library of macrocyclic peptides against the purified BamA or whole BAM complex. The authors demonstrated the inhibition efficiency of the two identified peptides PTB1 and PTB2 in vivo, and also determined cryo-EM or crystal structures of peptide bound BAM complex in different membrane-mimetic agents. Their structural analysis of the BAM-PTB complexes and resistant strain mutations revealed distinct binding specificities of PTB1 and PTB2 to different states of the BamA barrel, which are very interesting findings.

This work has important scientific implications, and the experiments are carefully designed and the data are well presented. There are only a few comments I would like to add:

1. It is fascinating that PTB1 binds at the closed lateral gate of the BamA barrel, a position that is new to other known inhibitors. I wonder if PTB1 binding locks the BamA barrel in the close conformation, i.e. whether the inhibition is irreversible? Is it possible to perform dynamic simulations (like what is done for the BAM-PTB2 structure) to confirm that PTB1-bound BamA structure is fixed.

Our SPR measurements indicate that the disassociation of PTB1 from BamA is very slow. Specifically, the data fitted to a two-state model implies that binding of PTB1 to BamA is fully dependent on the availability of a Zn-bound BamA state as the peptide forms a high affinity complex (KD = 50 pM) with BamA in the presence of Zn while no binding was observed up to 1 μM peptide when BamA was stripped of bound Zn. The high-quality fit of the two-state model supports rapid formation of a lower stability complex which rapidly conformationally shifts to a tightly bound complex with an apparent half-life of ~80 min (notably, longer than the expected doubling time of *E. coli*). Hence, multiple MD simulations would need to be run for an unreasonable duration in order to study this event and is therefore beyond the scope of our intended study.

2. Line234 “the PTB2-BAM-SMA and PTB2-BAM-DDM complex structures are both highly similar to the apo-BAM-

SMA/DDM complex that we have determined (RMSD<0.7angstrom)". I wonder why the same conformation was also captured for BAM in the absence of PTB2. Shouldn't be there any other conformation for an apo-BAM complex?

Our apo-BAM structural model is highly similar to previously reported models from other cryo-EM studies of BAM reconstituted in different detergent and/or membrane environments (e.g., Xiao et al 2020, White at al 2021, Doyle et al 2022, although our structure is determined to higher nominal resolution), and our cryo-EM analysis did not identify any additional conformations of apo-BAM. We speculate that this "open conformation" of apo-BAM may be representative of a population that exists in the native outer membrane, and hence, PTB2 appears to have direct access to its binding site without requiring significant conformational changes in BAM. However, to be more accurate, we have updated the phrasing of this sentence as follows:

"Beyond very slight adjustments in the ECL1 and ECL2 loops that occur to accommodate interactions with PTB2, the PTB2-BAM-SMA and PTB2-BAM-DDM complex structures are both highly similar to the apo-BAM-SMA/DDM complex that we have determined (RMSD<0.7angstrom)."

3. In the PTB2-BAM structure, PTB2-lg-1 and PTB2-lg-2 interacted to each other at the open lateral gate of BamA. Is there a risk of aggregation of this peptide?

At our Reviewer's suggestion, we measured the logD and kinetic solubility of both PTB1-1 and PTB2 to gain insight into their properties. At pH 7.4 PTB1-1 had a logD of 2.9 and PTB2 had a logD of 2.6, indicating that neither macrocycle is extremely lipophilic. The kinetic solubility for PTB1-1 was 297 µg/ml (131 µM) and 320 µg/ml (124 µM) for PTB2 at pH 7.4. Thus, these macrocycles are soluble at the concentrations tested in our activity assays and structural studies. Additionally, we performed a red blood cell lysis assay and found that neither PTB1-1 or PTB2 had any lysis activity at relevant concentrations (see point below for data). Finally, we performed an NPN assay which further demonstrated that neither PTB1-1 or PTB2 permeabilize the bacterial outer membrane at relevant concentrations (see point below for data). Overall, although we are not suggesting these macrocycles have amazing "drug-like properties", it is clear that they have reasonable physiochemical and pharmacological properties.

4. It says that PTB2 binding in BamA lumen does not restrict widening of the lateral gate. If PTB2 does not restrict conformational changes of the BamA barrel, then what is the inhibition mechanism of PTB2? Is the inhibitory effect of PTB2 observed is due to binding of PTB2-lg-1 to the beta1 strand, not PTB2-lumen?

We thank Reviewer 1 for this important comment, and we have updated this statement to clarify our conclusions as follows:

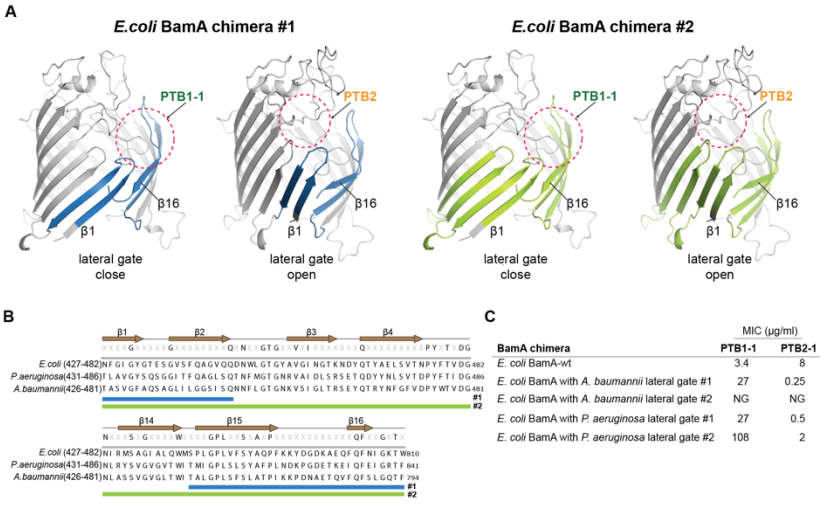
"This observation suggests that PTB2 binding does not formally restrict widening of the lateral gate. However, examination of PTB2-bound structures does demonstrate that the presence of PTB2-luman would sterically clash with the walls of the β-barrel in the closed conformation of BamA, indicating that PTB2-bound structures are not compatible with substantial closure of the BAM lateral gate. Overall, this suggests a mechanism whereby PTB2 prevents conformations of BAM presumably required to complete the OMP folding cycle."

Regarding whether PTB2 exerts its inhibitor effect at the lateral gate, we note that closely related non-PTB2 sensitive species *Klebsiella* BamA and *Enterbacter* BamA, which have identical sequences in this region, are not sensitive to PTB2 inhibition, informing that lateral gate binding is not sufficient for inhibition and substantiating our inference that luminal PTB2 binding is required for inhibition. However, although we further note that all identified resistance mutations to PTB2 are those which the macrocycle makes direct contact with within the lumen of the *E. coli* BamA barrel, and that none are found at or near the beta1 strand of the lateral gate, we wanted to more formally evaluate the mechanism of action of PTB2. To address this important question, we generated BamA-BamA chimeras between more distantly related, non-sensitive BamA proteins (e.g., *E. coli-Acinetobacter* BamA and *E. coli-Pseudomonas* BamA), where significant sequence differences are found at their respective BamA lateral gates. The design of these chimeric constructs substituted the beta1 and beta16 lateral

gate regions of *E. coli* BamA for the equivalent regions in these non-PTB2-sensitive strains, which have substantial sequence differences. Three important observations stem from these new studies (which are highlighted in a new section and new figure added to our updated results section):

- **The chimeras support growth of *E. coli*, demonstrating that they are functionally competent.**
- **Strains with these chimeras lose sensitivity to PTB1-1, which is expected because the lateral gate binding site of PTB1-1 has been substantially altered through replacement of sequences from these non-sensitive species.**
- **Strains with these chimeras remain sensitive to PTB2-1, which maintain all *E. coli* luminal binding residues observed to engage the PTB2 macrocycle, thus substantiating the hypothesis that PTB2 binding within the lumen is required for inhibition (but perhaps not sufficient)**

Altogether, we speculate that restricted motion imparted by PTB2 is likely the MOA for this inhibitor; however, we also recognize in the text the possibility that PTB2 may sterically occlude incoming substrates, and now add the possibility raised here that lateral gate binding may also play a role. Unfortunately, we find this is a difficult question to definitively answer from a technical perspective and look forward to future studies that can shed further light.



5. The implications from the crystal structure of the BamA-psBamA complex are not very convincing as it is not a physiological relevant folding intermediate hybrid complex, but more like an artifact formed due to crystal packing of BamA.

We understand the reviewers concerns here. Accordingly, we have already pointed out in the results that this "complex" must arise due to crystallization artifact and is clearly not physiologically relevant (as also indicated by the "inverted topology" of the psBamA).

Please note, we were particularly motivated to present this crystal structure because it provides novel insights into the chemistry that can interact alongside beta1 of BAM and should in principle inspire new approaches and designs to target this important region (as we have followed up in our own unpublished work). Moreover, we now directly compare this x-ray model with recently "trapped" more physiologically relevant complexes (Shen et al 2023), and find a high structural likeness between these complexes, indicating the BamA (in our BamA-psBamA) complex appears to be adopting a potentially functionally relevant conformation. If this explanation is not sufficient for our reviewer, we are very willing to consider removing this result altogether, as it was not intended to be controversial.

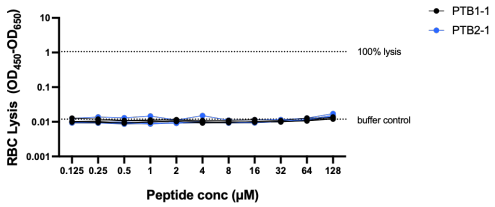
Reviewer #2 (Remarks to the Author):

Inhibition of the Bam complex is a new route to antibacterial action. This study identified two macrocyclic peptide

inhibitors targeting different states and unique sites with BamA. The authors provide compelling genetic and biochemical data supporting BamA as the target of antibacterial action and the exact modes of binding. These results are important by providing new knowledge of Bam conformations susceptible to inhibitors and lead inhibitors that are amenable to manufacturing. Two clarifying points would help the reader.

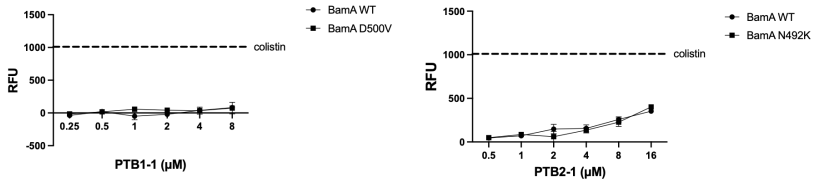
1. The content of PTB inhibitors (F,W,R,K) is reminiscent of general membrane disruptive peptides. However, the resistant mutants clearly show that they target BamA. It does seem likely that they can still disrupt (non-lytic) the outer membrane, which may enable BamA access. Ethidum bromide used to measure membrane disruption requires access through both outer and inner membranes. Is outer membrane disruption alone (NPN assay) observed for these peptides in wild type and PTB resistant strains? If yes this could be a mode to reach susceptible BamA sites. It could also suggest a more general route of non-lytic outer membrane disruption that could be used to reach other essential components of the cell envelope.

We have several lines of evidence to suggest that these peptides are not the result of non-specific activity, including MIC measurements against Gram-positive bacteria which do not contain BamA or an outer membrane (Figure 1A (PTB1) and Figure 2A (PTB2)). To further investigate this important question, we have now performed a red blood cell lysis assay and found no impact of either PTB1 and PTB2 up to 128 uM concentration, demonstrating that these compounds are not simply permeabilizing membranes. These results are now included in Supplementary Figures 2 and 6 as well as below):



Red blood cell (RBC) lysis assay with PTB1-1. Lysis was measured after 4 hours of incubation with indicated concentrations of PTB1-1, 0.5% Triton X-100 (set to 100% lysis), or buffer (set to 0% lysis). Assay was performed in quadruplicate and all for replicates are plotted.

To further investigate this question, we also performed the NPN assay as suggested and found no impact of PTB1-1 at concentrations tested, and only a slight impact of PTB2-1 at concentrations that exceed its effective MIC. This likely rules out the possibility that outer membrane permeabilization plays a role in the inhibitor mechanism of either peptide



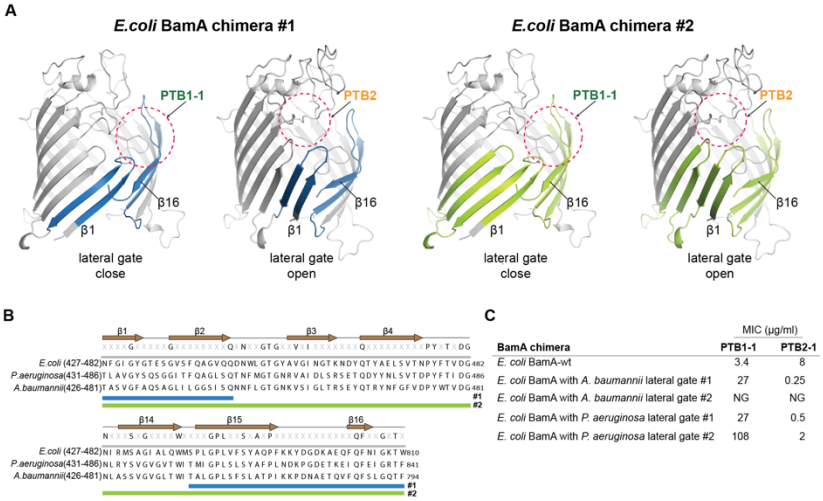
NPN assay with PTB1-1 and PTB2-1 using *E. coli* strains producing wild-type or a macrocycle-resistant BamA variant. Colistin (100 μg/ml) is the positive control. Bacterial cells grown to log phase in LB, washed 2x in HEPES buffer, then NPN reagent and PD compounds were added. The cells, reagent, and compounds are incubated at RT for 1h prior to reading on the plate reader. Note that these conditions are different from the MIC assay, and this was done in triplicate (mean and SEM plotted).

2. PTB2-1 was shown to bind in the lumen of BamA as well as along the lateral gate. Resistant mutants indicate that lumen binding is the driver of antibacterial activity. Can the authors comment on the importance in lateral gate binding of PTB2-1 such as a precursor stage to lumen entry? Does PTB2-1 still bind to the lateral gate of BamA in PTB2-1 resistant mutants?

Regarding whether PTB2 exerts its inhibitor effect at the lateral gate, we note that closely related non-PTB2 sensitive species *Klebsiella* BamA and *Enterbacter* BamA, which have identical sequences in this region, are not sensitive to PTB2 inhibition, informing that lateral gate binding is not sufficient for inhibition and substantiating our inference that luminal PTB2 binding is required for inhibition. However, although we further note that all identified resistance mutations to PTB2 are those which the macrocycle makes direct contact with within the lumen of the *E. coli* BamA barrel, and that none are found at or near the beta1 strand of the lateral gate, we wanted to more formally evaluate the mechanism of action of PTB2. To address this important question, we generated BamA-BamA chimeras between more distantly related, non-sensitive BamA proteins (e.g., *E. coli-Acinetobacter* BamA and *E. coli-Pseudomonas* BamA), where significant sequence differences are found at their respective BamA lateral gates. The design of these chimeric constructs substituted the beta1 and beta16 lateral gate regions of *E. coli* BamA for the equivalent regions in these non-PTB2-sensitive strains, which have substantial sequence differences. Three important observations stem from these new studies (which are highlighted in a new section and new figure added to our updated results section):

- **The chimeras support growth of *E. coli*, demonstrating that they are functionally competent.**
- **Strains with these chimeras lose sensitivity to PTB1-1, which is expected because the lateral gate binding site of PTB1-1 has been substantially altered through replacement of sequences from these non-sensitive species.**
- **Strains with these chimeras remain sensitive to PTB2-1, which maintain all *E. coli* luminal binding residues observed to engage the PTB2 macrocycle, thus substantiating the hypothesis that PTB2 binding within the lumen is required for inhibition (but perhaps not sufficient)**

Altogether, we speculate that restricted motion imparted by PTB2 is likely the MOA for this inhibitor; however, we also recognize in the text the possibility that PTB2 may sterically occlude incoming substrates, and now add the possibility raised here that lateral gate binding may also play a role. Unfortunately, we find this is a difficult question to definitively answer from a technical perspective and look forward to future studies that can shed further light.



Reviewer #3 (Remarks to the Author):

Summary:

The rise of antibiotic resistant Gram-negative bacteria is a pressing worldwide problem. The outer membrane beta-barrel assembly machinery (BAM) complex functions in the outer membrane to facilitate the folding and insertion of beta-barrel proteins into the membrane. The central component of the BAM complex, BamA, is an attractive target to design novel therapeutics against because it is easily accessible from the extracellular space and it is essential for cell viability. In this manuscript Sun, Storek, Tegunov et al. identify two novel macrocycles, PTB1 and PTB2, that kill Gram-negative bacteria by interacting with BamA and inhibit its function. CryoEM structures of the

BAM complex interacting with PTB1 or PTB2 show different binding sites of the peptides to BamA; PTB1 on the extracellular loops above the lateral gate, and PTB2 on the luminal side of the BamA extracellular loops and at the BamA lateral gate.
Overall, this manuscript shares exciting advancements in screening for novel therapeutics and identification of two new peptide inhibitors of BamA. However, this reviewer feels that the manuscript requires serious editing to expand discussion in the context of currently known therapeutics, expand on methods descriptions, and address lack of information pertaining to replicate experiments and descriptions of averaged data. There is a surprising lack of quality in both the data and the presentation of results, and there is simply not enough information provided for someone else to reproduce the work.

Major comments:

1. SPR experiments are used to demonstrate PTB1 and PTB2 interact with the BAM complex, yet the binding affinity of these interactions is not discussed in detail within the text.
a. Line 167 states PTB1-1 binds BamA in the presence of Zn2+ at 50pM. Does BamA bind Zn2+ or other cations in the absence of PTB1-1? Please expand discussion of the binding affinities for both PTB1 and PTB2.

It is technically challenging to detect single metal atom binding to purified proteins by SPR due to signal to noise limitations of this method. On the other hand, native mass spectrometry is an appropriately sensitive methodology to address this important question. In our supplementary data we demonstrate by native mass spec that Zn2+, Cu2+ and Ni2+ can all bind BamA in the absence of PTB1. We look forward to future work that will address additional details of metal binding to BamA.

As part of our screening funnel and cascade, we did characterize by SPR binding of PTB2 to BamA and BAM and found modest binding to BamA (~1.3 uM K_D) and high binding to BAM (~6 nM). We did not endeavor to further characterize PTB2 binding in further detail, as we routinely observe significant potency shifts relative to the measured MICs, and so these experiments were not employed as part of our strategy to improve the cellular potency of this peptide series. However, we did further investigate the binding of PTB1 to BamA by SPR because we later observed the metal-site and metal dependence, as noted above, so wanted to understand this in finer details. Still, we note the significant shift in cellular MIC measured for PTB1-1 relative to the pM affinity measured by SPR so do not feel further characterization helps understand the mechanism in the context of a biological membrane and cell.

Please provide addition of a summary plot of binding affinities for replicates of each peptide, buffer control, and other relevant conditions (such as BAM+PTB1-1 in the presence of EDTA)

These details are now provided in the revised manuscript. Our detailed SPR studies have focused on PTB1 and have been measured in duplicate. No binding was observed in the presence of EDTA.

b. Comparison to binding affinities of BAM/BamA with darobactin and dynobactin would also add to the manuscript and further showcase differences between these new peptides and darobactin/dynobactin.

We respectfully point out that other groups have previously reported these experiments and data, and they are not the focus of our current work (e.g., darobactin's K_D is 1.2 uM by ITC (Imai et al. 2019)). However, our structural and resistance data definitively do demonstrate that our inhibitors are distinct in structure, binding site, and mechanism relative to darobactin and dynobactin. We have now provided a structural overlay in our supplemental material to support this conclusion (Supplementary Figure 13). We hope that reviewer 3 will recognize referencing the published literature and publicly available PDB structures as an acceptable method to demonstrate these differences.

c. Two replicate SPR sensograms are shown in Supplementary Figure S1 for each macrocycle, and the KD values are fairly different. Are those the only replicates for each peptide?

As already described above, as part of our massive screening cascade and screening funnel and cascade, hundreds of peptides were characterized, so n=1 was initially performed at the time those discovery experiments were performed. We point out that these data are provided only in the supplement as a reference and early validation point for the characteristics of these peptides and clearly state in the paper that we are presenting a generalizable discovery method and approach to potentially discover modulators of diverse membrane proteins, and in hopes to demonstrate an appropriate screening triage cascade that will motivate other groups to pursue additional discovery efforts to identify new potential antibacterial agents.

d. I am surprised that the authors haven't done an analysis of PTB1 and PTB2 in mice. Since such data are available for darobactin, the comparison is important.

We respectfully point out that we have only presented and described PTB1 and PTB2 as tool molecules, not potential drug candidates. Our in vitro ADMET profiling of these compounds show that they have inadequate properties to justify in vivo dosing in mice, under any formulation or regiment that we have considered, and it is therefore unethical for us to consider this type of experiment. We look forward to future work inspired by our findings and early tool molecules that may generate derivatives with promising lead-like molecules to test in vivo.

2. Information on replicates, how many data points are averaged, and what error bars represent are missing from numerous figures and captions. Please add this to figure captions and appropriate methods sections.
a. Figures 1C & 2C how many replicates were completed with similar results?

This information is now provided in the figure legends.

b. Figure 1I how many replicates with similar results? Suggest generating summary plot with KD values from all replicates, could include one column for each condition tested here.

This information is now provided in the figure legends.

c. Supplementary Figure S1C how many replicates with similar results? Generate summary plot with all replicates, one column per peptide tested and add a buffer control.

This information is now provided in the figure legends (n=1; rationale discussed in detail above).

d. Supplementary Figure S2A, S6B how many replicates with similar results?

This information is now provided in the figure legends.

e. Supplementary Figure S2B, S6C how many times was experiment repeated with similar results? How many replicates went into average shown on plot? What do the error bars represent (standard deviation? Standard error?)

This information is now provided in the figure legends (error bars represent SEM).

f. Supplementary Figure S2C, S6D how many times was experiment repeated with similar results? How many replicates went into average shown on plot?

This information is now provided in the figure legends.

3. BAM structure in DDM bound to PTB1-1:
a. Methods state that this structure was obtained with a FAB bound, but main text does not include this information or a discussion of how FAB binding could impact BAM dynamics and/or interactions with PTB1-1. What

is the relevance of FAB to this manuscript? These is no discussion and the structural characterization are poor.

As described in our methods, this FAB was discovered in previous work and does not impact the function of BAM [PMID: 29555747]. Our structural comparisons of our FAB-BAM-PTB1 complex shows that the only well resolved structural differences to be PTB1, and the epitope and binding region of the Fab on BAM is very poorly resolved. This sample was generated early on in our studies when it was helpful for us to include FAB as a fudical marker, a point we now clarify in the methods and figure legend. Moreover, we note that the FAB and PTB1-1 bound closed structure of BAM does not differ significantly from previous closed BamA and BAM structures; and we have updated the text accordingly:

"To define the molecular determinants of the PTB1-1 interaction, we determined a co-structure with the BAM complex by cryogenic electron microscopy (cryo-EM) to 3.7 Å resolution in the detergent dodecyl-maltoside (DDM) and a non-functional antibody fragment used as a fiducial marker (Fig. 1d, Supplementary Fig. S4a-i, Supplementary Table S1). In the BAM complex, BamA is observed in a closed lateral gate conformation where PTB1-1 is bound within the extracellular leaflet region of the outer membrane (Fig. 1d, e). PTB1-1 interacts with multiple extracellular loops of BamA that form a composite acidic patch located directly above its closed transmembrane lateral gate (Fig. 1e, f). The PTB1-1 binding site is dramatically remodeled and absent in known open conformations of the BAM complex[11,13,14], defining PTB1-1 as a state-dependent antagonist which targets the closed state of BamA. However, beyond slight local side-chain adjustments, the closed PTB1-1-bound BAM conformation is essentially unchanged from unbound BAM complexes, indicating that PTB1-1 binding does not require substantial structural rearrangements in BAM (Supplementary Fig. 4j)."

b. Given that the map resolution is 3.7Å, is density clear enough to unambiguously assign PTB1-1? Supplementary Figure S4I has one view of the PTB1-1 model within the map, however additional views would be useful. Quantification of the model fit within the map density (such as a CC score) would be useful to add.

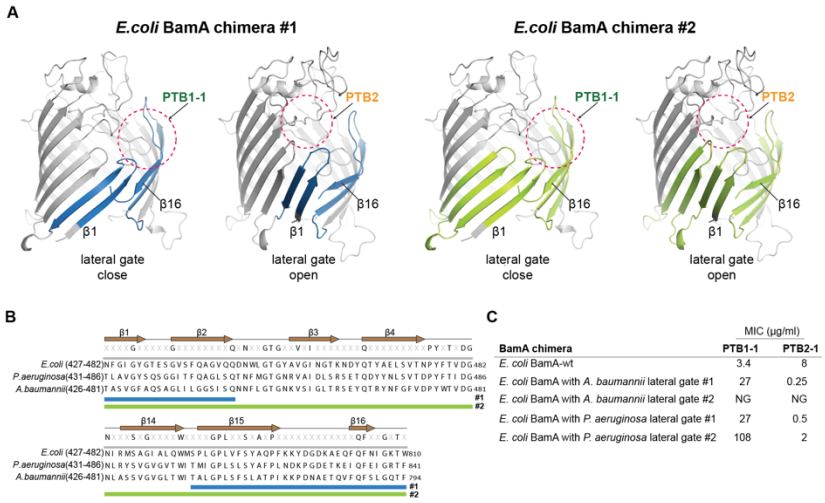
To address Reviewer 3's concerns, we have provided additional views in the revised manuscript and included the CC score in the Supplementary Table S1. We are also happy to provide the experimental maps and structural models to our Reviewers for inspection as well. Additionally, we point out that extensive structure-activity relationships and resistance mutations in BAM further and fully support the structural model generated from the available map.

4. Lines 193-206 give the impression that the PTB2 molecule in the BamA lumen is solely responsible for locking the BamA barrel in an outward open state. However, this fails to consider the role the other two PTB2 molecules bound at the lateral gate may have. Could the two PTB2 molecules at the lateral gate induce the same conformation by blocking full closure of the lateral gate? What experimental support do you have to show that the luminal PTB2 molecule binds first to open the lateral gate?

Regarding whether PTB2 exerts its inhibitor effect at the lateral gate, we note that closely related non-PTB2 sensitive species *Klebsiella* BamA and *Enterbacter* BamA, which have identical sequences in this region, are not sensitive to PTB2 inhibition, informing that lateral gate binding is not sufficient for inhibition and substantiating our inference that luminal PTB2 binding is required for inhibition. However, although we further note that all identified resistance mutations to PTB2 are those which the macrocycle makes direct contact with within the lumen of the *E. coli* BamA barrel, and that none are found at or near the beta1 strand of the lateral gate, we wanted to more formally evaluate the mechanism of action of PTB2. To address this important question, we generated BamA-BamA chimeras between more distantly related, non-sensitive BamA proteins (e.g., *E. coli-Acinetobacter* BamA and *E. coli-Pseudomonas* BamA), where significant sequence differences are found at their respective BamA lateral gates. The design of these chimeric constructs substituted the beta1 and beta16 lateral gate regions of *E. coli* BamA for the equivalent regions in these non-PTB2-sensitive strains, which have substantial sequence differences. Three important observations stem from these new studies (which are highlighted in a new section and new figure added to our updated results section):

- **The chimeras support growth of *E. coli*, demonstrating that they are functionally competent.**
- **Strains with these chimeras lose sensitivity to PTB1-1, which is expected because the lateral gate binding site of PTB1-1 has been substantially altered through replacement of sequences from these non-sensitive species.**
- **Strains with these chimeras remain sensitive to PTB2-1, which maintain all *E. coli* luminal binding residues observed to engage the PTB2 macrocycle, thus substantiating the hypothesis that PTB2 binding within the lumen is required for inhibition (but perhaps not sufficient)**

Altogether, we speculate that restricted motion imparted by PTB2 is likely the MOA for this inhibitor; however, we also recognize in the text the possibility that PTB2 may sterically occlude incoming substrates, and now add the possibility raised here that lateral gate binding may also play a role. Unfortunately, we find this is a difficult question to definitively answer from a technical perspective and look forward to future studies that can shed further light.



5. Inconsistent use of PTB2 and PTB2-1 within the text and figures is confusing. Since these are two different molecules it is important to edit for clarity. Same for PTB1 versus PTB1-1.
a. For example, figure 2b shows structure of PTB2 but 2c is with PTB2-1. It is unclear if the structure solved contains PTB2 or PTB2-1

Thank you, we have carefully addressed Reviewer 3 points here and throughout the manuscript and confirmed that our labeling is appropriate throughout. Here specifically, 2b is with PTB2, 2c is with PTB2-1, and the structure is with PTB2 (2d and 2e).

6. PTB2-lg-1 molecule interacts with the BamA lateral gate beta-strand 1 much like darobactin and dynobactin do, however no discussion on the similarities and differences of these interactions is included. This is an interesting area of discussion, particularly because darobactin and dynobactin interact with the same portion of BamA however the BamA lateral gate is closed in those structures.

Our structural and resistance data definitively demonstrate that our inhibitors are distinct in structure, binding site, and mechanism relative to darobactin and dynobactin, and we have provided a structural overlay in our Supplemental Figure 13 to support this conclusion and discuss the difference in the main text (mainly that PTB2-lg-1 only binds to the open lateral gate whereas these other molecules bind to BAM with a closed lateral gate. The chemical structures of these molecules are very distinct, and their common mode of interaction with the BamA lateral gate is through H-bonding (and favorable van der Waals interactions), as we describe in our results section.

7. Numerous BamA mutations that confer resistance to PTB1 or PTB2 are identified. Are there any that confer resistance to both?

There are no common resistance mutations; all mutations are listed in Supplementary Figure 2 and 6.

a. Were any of these mutations previously identified to confer resistance to other antibiotics/novel therapeutics, such as darobactin A?

All PTB1 and PTB2 resistance mutations are listed in Supplementary Figure 2 and 6. E435K, which provides PTB1 resistance, was identified as part of a triple resistance mutation to darobactin (E435K, G443D, F394V), however, E435K resides far from the darobactin binding site, and its individual contribution to darobactin resistance was not previously characterized (Imai et al.)

b. May want to consider the recent paper in which MD simulations suggest mutations in BamA that confer darobactin A resistance alter the dynamics of the BamA lateral gate. (doi:10.1021/acs.jpcb.3c04501)

We thank Reviewer 3 for this suggestion, but it is unclear how this suggestion would provide further insight into our current inhibitors, especially in light of the additional new chimeric BamA experiments we describe above that indicate a prominent role in the luminal BamA PTB2 molecule. However, we expect this type of question will be of significant interest and future work by many others in the field.

8. Lines 212-215 state that PTB2-lg-1 interacts with BamA beta1 in a similar manner as a beta-signal motif from a native substrate but does not expand further.
a. Does PTB2 have a sequence similar to the beta-signal motif?

No, the structural motif is $_{9}$FRYWF$_{13}$ which has shares similar chemical characteristics (i.e. hydrophobic/aromatic) but no clear sequence motif with the beta-signal.

b. Are these interactions similar or different than the interactions between BamA and darobactin A or dynobactin?

Beyond forming H-bonds to the backbone of BamA, the interactions and structures are very different, PTB2 interacts with the open BAM lateral gate, not the closed.

9. Lines 216-220 describe differences in fold between PTB2-lumen and PTB2-lg-1. How does the fold compare between PTB2-lg-1 and PTB2-lg-2?

These folds are very different, as shown in Fig. 3c and Supplement Figure 7e, but we note the lower confidence of the PTB2-lg-2 model due to the weaker peripheral density.

10. Suggest additional biophysical experiments be conducted to support three binding sites of PTB2 on BamA.

We respectfully point out that we have performed X-ray crystallography, cryo-EM, molecular dynamics, native mass spec and preliminary SPR studies. In the revised manuscript, we have additionally performed red blood cell lysis assays. It is unclear what additional studies would add further information or impact to this work.

11. This reviewer strongly disagrees with referring to the second copy of BamA in the crystal structures (lines 247-270) as a "pseudo-substrate" since this is a beta1-beta1 interaction. The substrate BamA beta-signal is on beta16, therefore a BamA(substrate)-BamA(machinery) interaction is between beta16(substrate) and beta1(machinery). The observed arrangement of BamA in the crystal structures is non-physiological, likely a result of crystallization.
a. The statement in line 267 that this conformation is non-physiological needs to be brought up earlier in this section.
b. The non-physiological beta1-beta1 interaction has also been observed in other beta-barrel machinery

(doi:10.1038/s41467-020-17144-1), an artifact resulting from the purification process/conditions.
c. Caution should be used in discussing promiscuity of lateral gate of Bam A for different substrates as the structure is not sufficient evidence that BamA interacts with substrates like beta1 in biologically relevant conditions. Line 267-268 “this observation further highlights the promiscuity of the lateral gate to engage diverse ligands” should be deleted.

As noted above, we understand the concerns raised by more than one reviewer here. Accordingly, we have further pointed out in the results that this “complex” must arise due to crystallization artifact and is clearly not physiologically relevant (as also indicated by the “inverted topology” of the psBamA).

Please note, we were particularly motivated to present this crystal structure because it provides novel insights into the chemistry that can interact alongside beta1 of BAM and should in principle inspire new approaches and designs to target this important region (as we have followed up in our own unpublished work). Moreover, we now directly compare this x-ray model with recently “trapped” more physiologically relevant complexes (Shen et al. 2023) and find a high structural likeness between these complexes indicating the BamA (in our BamA-psBamA) complex appears to be adopting a potentially functionally relevant conformation. If this explanation is not sufficient to please our reviewer, we are very willing to consider removing this result altogether, as it was not intended to be controversial.

12. Additionally, crystallization results section should include description of potential differences in BamA barrel only conformations compared to full length BamA and complete BAM complex. This is particularly important because E. coli BamA POTRA domains 3-5 are essential for functon(doi:10.1038/sj.embor.7401092, doi:10.1126/science.1143993), yet they are missing in the construct used in this crystal structure.

Existing literature reporting crystal structures of isolated BamA beta-barrel only, +/- POTRA domains, and full-length crystal and cryoEM structures, and MD and NMR studies, have already discussed these points in detail. This is not the focus of our manuscript, and we make no claims to suggest any differences in other BamA regions or BAM components are relevant (in fact, we purposely do not discuss them).

13. Lines 281-282 suggests that PTB1 binding results in inability of substrates to interact at BamA lateral gate. Support of this statement with an activity assay would be beneficial. Either with folding assays (see doi:10.1128/mbio.02286-22) or competition assays for native beta-signal peptides (see doi: 10.1038/s41586-021-03455-w). The rest of that sentence is also misleading, since the open lateral gate being required for BamA function was demonstrated by multiple groups beginning with Noinaj et al, 2013.

We thank Reviewer 3 for raising this point and have updated the text to clarify that PTB1 binding would result in the inability of substrates to access or interact with the *open* lateral gate of BamA. Our structural comparisons and superpositions (Supplementary Figure 13) also clarify that the closed PTB1-bound structure would sterically exclude native beta-signal peptides from binding, negating the need to evaluate this further experimentally (which would bring exceptional technical challenges).

a. Similarly, lines 324-325 state that PTB2 could inhibit BAM function sterically or by competition. This should also be investigated by activity assays suggested above.

We respectfully point out that there is no clear and definitive experiment that will address this very technically challenging question. Importantly, in the revised manuscript, we have included additional chimeric experiments which help to rule out the likely importance of PTB2-lg-1 in the inhibitory mechanism. To further satisfy Reviewer 3’s concerns, in the revised manuscript we have now extended our discussion of all 3 speculative mechanisms of action that we can reasonably justify for PTB2.

14. Lines 305-308 state that the lumen binding PTB2 molecule is responsible for the inhibition of BAM function not the molecule at the lateral gate, however there is no experimental evidence for this statement within the

manuscript. Add experiments to show that this is the case or remove this statement.

Please see response to #4 and additional experiments summarized therein.

15. Lines 308-310 describe promiscuity of BamA binding to various ligands, however all the biologically relevant examples stated contain a beta-signal sequence motif which is known to be important for association with BamA and BamA facilitated beta-barrel folding (doi:10.1016/0022-2836(91)90880-F, doi:10.1007/BF00332243, doi:10.1371/journal.pbio.0040377, doi:10.1073/pnas.2220762120)
a. It would be beneficial to analyze PTB2 to determine if it also contains a beta-signal sequence motif and include this in the discussion.

As discussed above, the structural motif is $_{9}$FRYWF$_{13}$ which shares similar chemical characteristics but no clear sequence motif with the beta-signal.

16. Important cryoEM data processing details are missing from methods sections and relevant figures. Including:
a. Particle picking parameters
b. particle box size

Information about the picking parameters has been added to the text. Particle box sizes have been added to Supplementary Table S1.

i. were particles binned during initial steps and later unbinned?

This has no effect on the processing, as the early sorting steps are limited in resolution by efault in cryoSPARC and cisTEM.

c. non-standard parameters used during processing steps

No non-standard parameters were needed.

d. how the masks were made for local/non-uniform refinements

Information about the preparation of masks for local refinements has been added to the methods section.

17. The cryoEM workflow figures are lacking important details and are uninterpretable for many figures.
a. Supplementary Figure S4E is a good starting point, though is still missing critical information including:
i. Particle box sizes

Information about box sizes has been added to Supplementary Table S1.

ii. Densities for 3D classification in the middle (from cisTEM)

This has been updated in Supplementary Figure S4e.

iii. Were there any additional steps to get from the 10.3Å map in relion to the 3.7Å map in cryoSPARC? If so these need to be clearly stated in methods and shown in work flow.

The only step taken is mentioned in the workflow figure (Supplementary Figure S4): Non-uniform 3D refinement in cryoSPARC. 3D classification using RELION's approach is expected to achieve significantly lower resolution than 3D refinement with a mask.

b. Supplementary Figures S7, S8, S9, S10 workflows are not useful in present form, as current images do not show any details of reconstructions. Each row of ab-initio reconstructions or 3D classifications should be shown as

densities (like the 3D classification panel in S4E). Re-make these panels to be more similar in design as S4E, add particle box sizes, binning, number of particles at each step, number of particles (or percentage) in each ab-initio/3D class.

We believe the density projections used in our figures to be more useful in assessing the map quality. Isosurface renderings hide many artifacts, especially in overfitted maps like those produced during *ab initio* map generation and 3D classification. We assessed the classes based on 2D map slices and projection images. Please note that the presented workflows were employed to clean up the initial particle sets (i.e. to decide between "junk/broken particle" and "particle of interest"), not to assess biologically relevant heterogeneity. The latter was later assessed using 3D PCA, which is more robust for this purpose. As no heterogeneity was found, we do not report these results.

18. Supplementary Figures S7, S8, S9, S10 need to be heavily edited/remade with essential details
a. Scale bars need to be added to the representative micrograph image

Scale bars have been added in Supplementary Figures S7a, S8c and S9c.

b. Scale bars or statement of box size needs to be added for the 2D classification panels

Information about box sizes has been added to Supplementary Table S1.

c. Workflows need to be edited to be more useful. Current figures are difficult to see anything and do not have all necessary information. (see point 17)

Information about box sizes has been added to Supplementary Table S1. Class percentages have been added to Supplementary Figures S7c, S8f and S9f.

d. FSC curves need to have the loose, tight, and corrected curves shown at minimum. Supplementary Figure 4G does this well, make remaining figures similarly.

"Loose" and "tight" masks have some diagnostic utility during cryoSPARC refinements, but they lack any definition in literature or even user manuals and are irrelevant to the resolution of the final map. The FSC curves displayed are corrected through phase randomization, as is customary in modern cryo-EM literature. We have modified the plot in Supplementary Figure S4g to be in line with this.

e. Colors for the local resolution should be consistent between figures (S4h is blue/2.7Å-red/4.5Å, but all others are red/2.5Å-blue6.5+Å). Also, captions should state how local resolution was calculated (using cryoSPARC, phenix, or other program?).

Local resolution was calculated in RELION using its implementation of windowed FSC estimation, as mentioned in the text. Color resolution of supplementary figure S4e has been updated the same as others with red/2.5Å - blue/6.5+Å.

f. Why do these figures use direction-dependent Fourier-space coverage plots while supplementary figure S4 uses angular distribution plot? Suggest editing figures so that same type of plot is used for all.

We believe Fourier-space coverage plots to be more informative for this purpose and have replaced the plot in Supplementary Figure S4f.

19. The methods section fails to describe how the BAM-SMA sample was purified.
a. Additionally, line 482 states that BAM-SMA was copurified with PTB2 but no information was provided for what step the PTB2 was added, or how much.

Thank you for identifying this omission, details have been added in the methods section.

20. The methods section describes purifying the protein using Superdex200 16/60 column, which is a large prep-grade column. However, all of the size exclusion chromatograms in the supplemental figures are a 3mL elution profile. Please clarify how these SEC profiles were obtained.

Thank you for identifying this detail; the indicated SEC were the result of performing a micro-Akta step prior to sample freezing, which has been updated in the methods section.

Minor comments:
1. Line 70 is missing reference to structures of the BAM complex stalled folding intermediate states solved by the Bernstein and Hinshaw groups (doi:10.1016/j.cell.2022.02.016)

Thank you, now added.

2. Reference to BamA sequence alignment figure (Supplementary Figure S3) is missing from the main text lines 106-110 where it is relevant.

Thank you, now added.

3. Italic font is missing in a number of places
a. Line 276 “in vitro”
b. Lines 966-968 species names

Thank you, now added.

4. Methods section includes centrifugation information in rpm but should be reported in g instead
a. Line 343, 369, etc

Thank you, now added.

5. Lines 355-371 describe BamA-POTRA3-5 was purification and state that it was used in the mRNA-display selections. It was not clear in the main text that a truncated form was used for these experiments. Add to main text along with explanation why this construct was used.

Thank you, now added.

6. Several places in the methods section state the use of 1.5% OG in size exclusion chromatography. This is a somewhat high detergent concentration. If this concentration was indeed used, could PTB1/PTB2 be partitioning into the micelles during binding experiments and impacting results? Please add buffer control replicates for binding experiments.
a. Line 370, 389, 426, etc

These are very standard detergent concentrations. OG just has a very low CMC (0.5% wt/v).

7. The size exclusion chromatogram labels imply that the BAM complex was copurified with PTB1 or PTB2 which based on the methods sections (lines 477-483) is not true. Please edit labels and figure captions to clarify this.

Only the SMA sample was co-purified, for all others PTB1/2 were added prior to sample freezing as described.

8. Is the “Growth conditions” section in methods (line 585) for the growth assays? If so, please specify this in section header as earlier methods section describing purification state use of TB for growth.

Thank you, now added.

9. The use of “schematic of BamA-binding PTB…” in the figure captions for the structures of PTB1 and PTB2 is confusing (lines 866, 891). Suggest using “structure of PTB…” instead.

Thank you.

10. Figures:
a. Figure 1:
i. 1D: color other BAM subunits and FAB density

Thank you, but we indicate the FAB in the figure legend and methods section; this is not a relevant part of the paper or discussion as discussed above.

ii. Suggest adding panel of cryoEM density from 1D zoomed in on PTB1-1 and BamA with different contour level to more clearly show lateral gate is closed and the PTB density better.

Thanks for the suggestion.

iii. 1G&H consider making into 1 panel and adding rotation axis to help reader understand the relationship of the two views.

Thanks, this was considered.

b. Figure 2:
i. Add panel showing full density of BAM+PTB, including BAM accessory proteins. Color BAM subunits same as in 1D.

As discussed above, we believe this is not a relevant part of the figure, paper, or discussion.

ii. 2D: please also color the PTB2 molecules at the lateral gate (same colors as in figure 3)

Thanks, this was considered.

iii. 2E: please change BamA lateral gate to tan so that it is consistent with figure 1 (pink is already assigned to BamB)

Thanks, this was considered.

iv. Add another panel with PTB2-BAM-SMA density to compare to DDM density. Also color PTB2 molecules at lateral gate.

Thanks, this was considered.

v.
c. Figure 3:
i. 3C: add PTB2-lg-2 as it looks quite different than the other two (at least from panel b inset)

We’ve omitted a direct comparison in the main figure because this macrocycle is less-well defined relative to the others.

ii. Consider adding panel with superposition of all 3 PTB2 models (1 solid color per model) to compare differences between them more easily

Thanks, this was considered; see comment above.

iii. 3F: clarify in caption what the shading in the plot represents
d.

done

Figure 4:
i. 4A: add label for 2nd PTB2 molecule in ps-BamA lumen.

Updated in Figure 4A.

ii. 4A-C: change point with line to attach more clearly to panel B versus panel C (right now both attach to panel B which is confusing).

done

iii. 4B,C: why are atom representations different between these two panels (sphere in B and stick in C)? Suggest changing B to stick representation and adding in hydrogen bonds (if there are any?)

Thanks, this was considered.

iv. 4D: suggest coloring EspP a different color to emphasize it is a different protein (compared to BamA in left and right most panels).

Thanks, this was considered.

v. 4D: add row with side view (view from membrane plane) of all four models as well. This will help clearly show that the second copy of Bam from the crystal structure is in a non-physiological orientation.

Thanks, this was considered.

e. Supplementary Figure S1:
i. S2D,E: what does omega stand for in "Δ494-496ΩS"?

This is an insertion, has now been clarified by calling is '494-496insS' to avoid confusion.

f. Supplementary Figure S3:
i. Add annotations for mutations that confer resistance to PTB2 (use circle of a different color than the red that identifies PTB1 resistance mutations)

Thanks for the suggestion. Resistance to PTB2 has been update in Supplementary S3.

ii. Add annotation for extracellular loop 6

Thanks for the suggestion. Extracellular loop6 has been labeled in Supplementary S3.

g. Supplementary Figure S4:
i. S4A,B: clarify in captions what detergent this protein is purified in.

Thanks for the suggestions. It has been updated in figure legend of Supplementary S4a.

ii. S4D: details in 2D classes are somewhat difficult to see. Could this panel be made bigger to see features more clearly? Also, please check that the scale bar is correct and/or provide box dimensions.

Thanks for the suggestions. 2D classes and scale bar have been updated in Supplementary Figure 4. The box dimensions have been updated in Supplementary Table S1.

iii. S4I: add additional view of map & model. Add to figure caption what contour level map is shown at.

Thanks for the suggestion. Additional views of PTB1 have been updated in Supplementary Figure S4i.

h. Supplementary Figure S6:
i. S6A: add comparison to the beta-signal sequence motif?

As discussed above, there is no clear or direct homology.

ii. S6D: what does dotted line on plot represent? Explain in caption or remove.

We have now noted in the figure legends that this line denotes the limit of detection. Thank you for catching this omission.

i. Supplementary Figure S7:
i. Missing purification information (size exclusion chromatogram and SDS-PAGE). Please add. If same protein purification was used as pictured in S4A,B specify in figure caption and in methods.

Same protein used, now clarified.

ii. S7E: add additional views of density and PTB2 models. PTB-lg-1 and PTB-lg-2 are not very convincing with numerous side chains outside of the density.

Thanks for the suggestions. Different views of PTB2 with density are updated in Supplementary Figure S7e.

1. Include in figure caption what contour level map is shown at

Thanks for the suggestions. The absolute intensity scale of cryo-EM maps has no meaning beyond the SNR properties of the original micrographs, and there is no agreed-upon convention in the field for expressing it as standard deviations. We do not believe this value would add useful information to the figure or the text. Readers wishing to reproduce the isosurface rendering will download the corresponding EMDB entry, where the value used in the figure is noted.

j. Supplementary Figure S8:
i. Clarify in caption what program generated the 2D classes shown

Updated in figure legend.

k. Supplementary Figure S11:
i. S11A: Was the DDM or SMA cryoEM model used for this? Were the BamB-E subunits also removed during the MD setup? Please clarify these things in the figure, caption, and methods section.

The high resolution DDM structure was used, and BamB-E were removed to reduce the system size.

ii. S11C: caption states it shows hydrogen bonding between BamA and three PTB2 molecules, yet only the two at the lateral gate are shown clearly. Either add panels of the figure to show luminal PTB2 hydrogen bond

interactions or edit figure caption to clarify only looking at lateral gate PTB2 molecules.

Thanks, figure legends have been updated.

iii. S11C: plot on the right is not described in figure caption. Make into separate panel (ie S11D) and clearly describe plot

Thanks, this was considered.

l. Supplementary Figure S12:
i. S12A: difficult to see details, please make larger or add inset with zoom of important/interesting area

Updated in Supplementary Figure S12a.

ii. S12C: at a different contour level is there density for all side chains? Current contour level leaves numerous side chains out. If not, please address this in the text.

Mostly yes, maps and models have been provided and will be released upon publication.

m. Supplementary Figure S13:
i. S13A: clarify that these are top views. Add another row showing models viewed from the membrane plane (side view).

Updated in Supplementary Figure S13a.

ii. S13B: make BAM-darobactin BamA cartoon darker grey so it can be clearly distinguished in the superposition. Include beta-16 from darobactin structure in the superposition panel to emphasize clash this would have with PTB2-lg-1. In caption, please specify what view is shown (ie lateral gate viewed from from membrane plane).

Updated in Supplementary Figure S13b.

iii. S13C color beta1 and beta16 to be consistent with earlier figures (Figure 1 has these strands in tan). Specify in caption what points are being measured using residue numbers (ie same residues in each model? Or the closest points in each model which could be different residues?)

Thanks for the suggestion. In Supplementary Figure S13c, we colored beta1 and beta16 of BAM-PTB2 (CryoEM structure) as same color as beta1 and beta16 of BAM-PTB2 (CryoEM structure) in Figure 2E.

Supplementary Table S1:
i. Apo-BAM-SMA and PTB2-BAM-SMA do not have initial models listed, yet methods state that Apo-BAM-DDM and PTB2-BAM-DDM models were used, respectively. Please put these model names into table S1.

Added to Supplementary Table S1.

ii. It would be nice to have CC scores added to this table, especially for the ligands. It would also be useful to have main chain CC scores for BAM included.

This has been updated and added to Supplementary Table S1

11. Add supplementary figures/panels showing sample map density around model in stick representation from parts of all chains in each reconstruction. While density and model is shown for PTB1 and PTB2, panels should also show density and model for other areas of the map.

Thanks for the suggestion, however, this manuscript already contains 14 supplementary figures. Moreover, all readers will have free access to the deposited EMDB and PDB entries upon publication.

a. At minimum, the lateral gate of BamA and BamA extracellular loops/beta strands near the PTB2 lumen binding sites should be shown for all relevant maps. Include in figure caption what contour level of map is shown.

Thanks for the suggestion, however, this manuscript already contains 14 supplementary figures. Moreover, all readers will have free access to the deposited EMDB and PDB entries upon publication.

b. Additional view(s) of the PTB1 and PTB2 densities and models should also be added

Additional views of PTB1 and PTB2 have been updated in Supplementary Figure S4i and S7e.

Would be very beneficial if CC scores could be calculated and included for the ligands within the density to give the reader a better idea of how well the model matches the map.

This has been updated and added to Supplementary Table S1.

Could also calculate and include CC scores for BAM/BamA

This has been updated and added to Supplementary Table S1.

20. The methods section describes purifying the protein using Superdex200 16/60 column, which is a large prep-grade column. However, all of the size exclusion chromatograms in the supplemental figures are a 3mL elution profile. Please clarify how these SEC profiles were obtained.

Thank you for identifying this detail; the indicated SEC were the result of performing a micro-Akta step prior to sample freezing, which has been updated in the method part.

Reviewer #4 (Remarks to the Author):

The paper from Sun et al. describes the discovery of new antibiotic peptide macrocycles that function by inhibiting BamA in the Gram-negative outer membrane. Different macrocycles are found to target distinct conformations of BamA, namely open and closed states. High-resolution structures of BamA with bound compounds validate the proposed mechanisms.

This is a very interesting and promising study. I have a few questions and comments that I hope could further improve the manuscript.

The authors should compare PTB1-1 that binds to the extracellular loops to the peptidomimetic compounds of Luther et al. (2019). Based on NMR, they also bind at the outside. I don't think it's the same site, but maybe there's some overlap worth mentioning (or even if none). A recent computational study also speculated that these peptidomimetics might target the lateral gate as well (Kuo et al. "Drug Binding to BamA Targets Its Lateral Gate" J. Phys. Chem. B, 2023. https://pubs.acs.org/doi/10.1021/acs.jpcb.3c04501).

We thank the reviewer for this interesting question. In the revised manuscript, we have compared the NMR nuclei reported to shift upon binding of the peptidomimetic compounds (Luther et al.) and find these residues to be completely different from those targeted by PTB1-1. This comparison is now presented in Supplementary Figure 14.

I think the claim that the compounds actually lock BamA in an open or closed state, while quite reasonable, is still a bit speculative. Can the authors look to their simulations for additional evidence? Are the dynamics around the gate reduced with compounds compared to without? No apo BamA simulation was reported, but I assume it

wouldn't take very long to run in both laterally closed and open states.

Our SPR measurements indicate that the disassociation of PTB1 from BamA is very slow. Specifically, the data fitted to a two-state model implies that binding of PTB1 to BamA is fully dependent on the availability of a Zn-bound BamA state as the peptide forms a high affinity complex (K_D =50 pM) with BamA in the presence of Zn while no binding was observed up to 1 μM peptide when BamA was stripped of bound Zn. The high-quality fit of the two-state model supports rapid formation of a lower stability complex which rapidly conformationally shifts to a tightly bound complex with an apparent half-life of ~80 min (notably, longer than the expected doubling time of *E. coli*). Hence, multiple MD simulations would need to be run for an unreasonable duration to study this event and is therefore beyond the scope of our intended study.

How reliable are the placements of the compounds in the cryo-EM maps? The local resolution is low (around 4.5 Ang.). The images in Fig. S7e are not entirely convincing, particularly given the compounds' flexibility. Can other orientations be ruled out?

To address Reviewer 4's concerns, we have provided additional views in the revised manuscript. Reviewer #4 is right to point out that PTB2-lg2 is more ambiguous and difficult to definitively define, and we have more clearly noted this in the manuscript that the confidence of the PTB2-lg2 model is quite low given its very peripheral location. Nevertheless, the maps for PTB2-luman and PTB2-lg1 are sufficiently well-defined to model these peptides and this was independently assigned by the ML modeling program ModelAngelo. We are happy to provide the experimental maps and structural models to our Reviewers for inspection as well.

Why was a DMPC membrane chosen for the simulations? I assume in part it was for convenience, but it would be helpful if the authors could justify the choice.

Yes, DMPC was in fact chosen for computational convenience and has been a standard for MD studies of diverse OMPs [PMID: 14653713 (OmpA); PMID: 15315948 (OmpT)], structural studies of OMPs, [PMID: 32817429 (AlkL)] and functional studies on BamA [PMID: 28919234]. Simulating the complexity of the OM in MD simulations remains a specialization in and of itself, and computationally intensive, and we anticipate will be taken on by other specialized groups in the field.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have fully addressed my concerns and carefully revised the manuscript with added supporting data. I recommend acceptance and publication.

Reviewer #2 (Remarks to the Author):

All comments have been addressed. No further comments.

Reviewer #3 (Remarks to the Author):

The authors have made an exciting discovery regarding BamA peptide inhibitors, significantly advancing the scientific community. Nevertheless, the presentation and writing are subpar, potentially causing comprehension issues for readers. There are many errors.

Major points:

1. We strongly disagree with the use of “pseudo-substrate” to describe the BamA-BamA non-physiological dimer in the crystal structure. While we appreciate the authors being more forthcoming in the text that this is likely a crystallization result, the use of “pseudo-substrate” to refer to the second copy of BamA is not appropriate.

• In addition, the second structure (psBamA) is non-physiological, the authors should limit their analysis in the main text and discussion to the physiological structure, PTB2-BamA.

2. We strongly disagree with the discussion of BAM promiscuity in interacting with substrates. The crystal structure non-physiological dimer is not sufficient evidence of BAM substrate promiscuity. While the conformation it adopts in this crystal structure may be similar to a conformation during folding of a native substrate, you have no evidence to support the promiscuity of binding in physiologically relevant conditions.

• Lines 274-276 (“…this observation further highlights the promiscuity of the lateral gate to engage diverse ligands”) need to be removed.

• Lines 350-351 ("…clearly demonstrate an intrinsic promiscuity of ligand binding and coordination that can occur along B-strand 1 of BamA").

3. The authors state in response to reviewers that the lateral gate PTB2 molecule does not contain a beta-signal. However, the "9FRYWF13" does partly match the latter portion of the prototypical bacterial barrel beta-signal (hydrophobic, glycine, hydrophobic, X, tyrosine, positive, phenylalanine) including the terminal phenylalanine (doi.org/10.1073/pnas.2220762120).

4. Figure 5: while showing the BamA beta-barrel with colored regions replaced in chimera constructs is useful, the figure legend fails to describe where these models came from (E. coli models with regions replaced in chimeras colored? Structural predictions of chimeras? New structures solved of chimera constructs?). Please clarify.

5. The current format of Supplementary figures displaying cryoEM data processing is not useful.

• To enhance clarity, remove Supplementary Figures S7 C, S8 F, S9 F, and S10 E, replacing them with a comprehensive data processing pipeline akin to the one illustrated in Supplementary Figure S4 E.

• Moreover, for FSC curves presented in Supplementary Figures S4 G, S7 D, S8 G, S9 G, S10 F, include labeled curves for masked, unmasked, and corrected data. Include the reported resolution on the curve for the selected curve. This modification is suggested because FSC curves can serve as valuable indicators of map and data quality. Experienced readers can analyze these curves to promptly assess map resolution and potential pathologies with the data. It is advisable to allow readers to make informed decisions about the quality of maps and datasets, distinguishing between reliable and questionable data.

6. The DDM-BAM-PTB1-1 cryoEM structure was determined at 3.5Å resolution. Is there sufficient side chain density to clearly assign residues interacting with the PTB1-1? The discussion of specific hydrogen bonds (lines 153-159) at this resolution seems like a bit of a stretch. Alternatively, provide a snapshot of electron density showing the interactions.

7. S14 introduces "peptide 3" which is not discussed anywhere in the main text, and S14 isn't referenced at all in the main text. What is peptide 3? How is it relevant to this manuscript? Either discuss and reference figure in main text, or replace this figure with something more relevant.

Minor points:

1. Line 69 is missing reference to structures of the BAM complex stalled folding intermediate states solved by the Bernstein and Hinshaw groups (doi:10.1016/j.cell.2022.02.016).

2. It is still unclear in the main text that a truncated form of BamA was used in the mRNA-display experiments, and why.

3. Methods fails to describe how Apo-BAM-SMA was purified. While it is likely a similar scheme as the PTB2 bound sample, it ought to be clearly stated somewhere in the methods section.

4. Figure 4 A-C: dots/lines from B1 and B16 in A both connect to panel B though only B16 view is shown. Please edit this for clarity.

5. S3: nice to have PTB1-1 and PTB2-1 resistance mutations on sequence alignment. However, current red/green color scheme is not colorblind friendly.

6. CryoEM data processing figures:

a. 2D classification panels ought to have box size stated in figure caption or scale bar on figure panel.

7. Information on biological replicates is still missing several figure legends.

a. S2C, S6D…

8. Lines 1131 (S6G) and 1073 (S2F) state that “assay was performed in quadruplicate and all for replicates are plotted” assuming “for” is a typo for “four”, only 2 lines are visible in the plot. Please explain.

9. Line 349 “BamAS4” : is “S4” referring to a figure? Or is it supposed to be a reference?

10. Check S7E figure caption (line 1143), doesn’t seem to fully describe what is in figure panel

Reviewer #4 (Remarks to the Author):

I appreciate the changes the authors have made to the manuscript and now support publication.

We thank all Reviewers for evaluation of our revised work and their recognition that it is ready for publication in Nature Communications. In our final revision, we address Reviewer #3's remaining comments as described point-by-point below.

Reviewer #1 (Remarks to the Author):
The authors have fully addressed my concerns and carefully revised the manuscript with added supporting data. I recommend acceptance and publication.
Amazing, thank you!

Reviewer #2 (Remarks to the Author):
All comments have been addressed. No further comments.
Amazing, thank you!

Reviewer #3 (Remarks to the Author):
The authors have made an exciting discovery regarding BamA peptide inhibitors, significantly advancing the scientific community. Nevertheless, the presentation and writing are subpar, potentially causing comprehension issues for readers. There are many errors.

We would like to address Reviewer #3's remaining concerns but point out that none of our other three Reviewers have come close to suggesting that our presentation or writing are subpar or potentially confusing. With all due respect to Reviewer #3, this comment seems non-constructive at this point in the review process.

Major points:
1. We strongly disagree with the use of "pseudo-substrate" to describe the BamA-BamA non-physiological dimer in the crystal structure. While we appreciate the authors being more forthcoming in the text that this is likely a crystallization result, the use of "pseudo-substrate" to refer to the second copy of BamA is not appropriate.

We appreciate Reviewer #3's continued concerns on this point and as a result we have renamed this as an "inverted non-substrate BamA (ins-BamA)" throughout our revised manuscript. Moreover, we have also renamed the subsection of this portion of the manuscript to reflect Reviewer #3's strong views here, to:

"Crystallization traps an inverted non-physiological BamA substrate"

• In addition, the second structure (psBamA) is non-physiological, the authors should limit their analysis in the main text and discussion to the physiological structure, PTB2-BamA.

We respectfully counter Reviewer #3's point of view here since this unique feature of ins-BamA should directly lead other groups to consider peptide libraries and additional chemical matter to design and screen as novel BAM inhibitors; in fact, based on the structural observation of ins-BamA, we have followed this exact strategy in on-going unpublished discovery work.

Furthermore, we absolutely anticipate that this region along the open lateral gate of BamA will bind a range of different, non-beta-signal chemical matter, including coordination with surrounding lipids, sugars, and water molecules. Moreover, our sentiment here is further exemplified by the non-OMP substrate antibiotics darobactin and dynobactin that also bind at this region (albeit, these later molecules do bind to the beta1 strand of the closed lateral gate), which highlights our motivation to discuss this unexpected observation.

Still, we have renamed the subsection of this portion of the manuscript to help satisfy Reviewer #3's strong views here, to:
"Crystallization traps an inverted non-physiological BamA substrate"

2. We strongly disagree with the discussion of BAM promiscuity in interacting with substrates. The crystal structure non-physiological dimer is not sufficient evidence of BAM substrate promiscuity. While the conformation it adopts in this crystal structure may be similar to a conformation during folding of a native substrate, you have no evidence to support the promiscuity of binding in physiologically relevant conditions.
• Lines 274-276 ("…this observation further highlights the promiscuity of the lateral gate to engage diverse ligands") need to be removed.

To address Reviewer #3's concern on this point, we have revised this section to highlight our aforementioned interest and focus on motivating future inhibitor design strategies; accordingly, to "The PTB2-BamA crystal structure reveals extensive backbone-mediated β1-β1 strand interactions between BamA and ins-BamA at the lateral gate (Fig. 4c). Although the topology of the ins-BamA is inverted with respect to the outer membrane and a product of non-physiological crystallization conditions, this observation demonstrates that a β-signal is not strictly required for peptide binding to the open lateral gate, which, together with the visualization of PTB2-lg-1, suggests avenues for future inhibitor design strategies aimed at targeting the lateral gate of BAM."

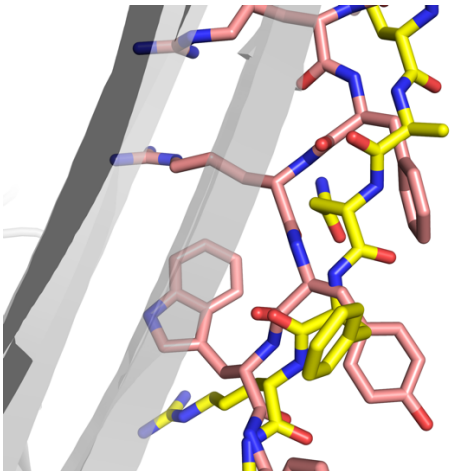
• Lines 350-351 ("…clearly demonstrate an intrinsic promiscuity of ligand binding and coordination that can occur along B-strand 1 of BamA").

We have prefer to retain this language in the discussion section of our paper and respectfully point out to Reviewer #3 that BAM is expected to engage and fold >20 distinct OMPs (i.e. those genomically encoded in E. coli and/or other Gram-negative species) under the full range of physiological conditions. It seems reasonable to us to define this range of substrate recognition and catalytic activity as "promiscuous" compared to most enzyme systems. Moreover, as we discuss above, the open lateral gate undoubtedly interacts with surrounding lipids, sugars and water molecules during various stages of the BAM folding cycle and regular/expected protein dynamics. Additionally, as discussed above, non-OMP substrates like darobactin and dynobactin are known to bind along the beta1-strand of BamA. Additionally, in our current work, we have observed PTB2-lg-1 binding at the open lateral gate of BamA, and we have similarly observed

ins-BamA binding at the open lateral gate of BamA (granted, the latter observation is made in the context of a non-physiologically relevant crystallographic, symmetry related packing interaction). For all these reasons, we believe the wording that we have chosen in the discussion is justified and reasonable.

3. The authors state in response to reviewers that the lateral gate PTB2 molecule does not contain a beta-signal. However, the "9FRYWF13" does partly match the latter portion of the prototypical bacterial barrel beta-signal (hydrophobic, glycine, hydrophobic, X, tyrosine, positive, phenylalanine) including the terminal phenylalanine (doi.org/10.1073/pnas.2220762120).

As Reviewer #3 points out, the sequence features of PTB2 are in fact reminiscent of the beta-signals found in OMPs, which is typical for membrane associating and/or partitioning peptides. The structure of PTB2-lg-1 bound at the lateral gate (in salmon below), however, is not strictly analogous to known structures of native OMP substrates, like EspP (in yellow below), even though side-chain positions roughly correlate, it can be seen that the peptide backbone of these respective "substrates" is dramatically different. In order not to confuse readers, we will not venture towards suggesting that our selection experiments have "discovered" a beta-signal containing peptide, in particular, because our chimera experiments demonstrate the requisite requirement of PTB2-lumen for the mechanism of action, and we've noted, the structure of PTB2-lumen is completely different in tertiary structure compared to PTB2-lg-1.



In this figure, our PTB2-BAM cryo-EM structure (with PTB2 macrocycle colored in salmon) has been superimposed with the EspP-BAM cryo-EM structure (PDB code: 7TT7; with the beta-signal of EspP colored in yellow). Note, remaining regions of PTB2 and EspP have been omitted for clarity. While some side-chain positions roughly overlap, significant differences in the backbone positions of these 'substrates' are observed.

4. Figure 5: while showing the BamA beta-barrel with colored regions replaced in chimera constructs is useful, the figure legend fails to describe where these models came from (E. coli

models with regions replaced in chimeras colored? Structural predictions of chimeras? New structures solved of chimera constructs?). Please clarify.

Thank you for asking for this clarification; we have just colored the regions swapped in the chimeras onto our experimental structures, and have now clarified this point in the figure legend.

"**a.** The lateral gate of the *E. coli* BamA barrel was substituted with the lateral gate sequence from *A. baumannii* or *P. aeruginosa*. As described in the main test, chimera #2 (green, right) has more extensive substitutions compared to chimera #1 (blue, left) **and all swapped regions are highlighted in the context of our PTB1-1-BAM structure (*left*: closed lateral gate) and PTB2-BAM structure (*right*: open lateral gate), respectively**. The approximate binding site location of PTB1-1 and PTB2 are circled with pink dash lines."

5. The current format of Supplementary figures displaying cryoEM data processing is not useful.
• To enhance clarity, remove Supplementary Figures S7 C, S8 F, S9 F, and S10 E, replacing them with a comprehensive data processing pipeline akin to the one illustrated in Supplementary Figure S4 E.

• Moreover, for FSC curves presented in Supplementary Figures S4 G, S7 D, S8 G, S9 G, S10 F, include labeled curves for masked, unmasked, and corrected data. Include the reported resolution on the curve for the selected curve. This modification is suggested because FSC curves can serve as valuable indicators of map and data quality. Experienced readers can analyze these curves to promptly assess map resolution and potential pathologies with the data. It is advisable to allow readers to make informed decisions about the quality of maps and datasets, distinguishing between reliable and questionable data.

We thank Reviewer #3 for raising these points and requiring us to clarify details further. Our choice of map projections over isosurface renderings reflects a specific methodological purpose, as it allows the reader to evaluate potential artifacts, crucial in assessing map quality. The figures are comprehensive, and show every processing step involved. The reason they may seem less comprehensive than Figure S4 E is because there were fewer steps and software tools involved. Furthermore, complying with the requested alteration presents a significant technical challenge. The intermediate processing results required for such changes are not currently available due to our HPC cluster's temporary data retention policy, making the request impractical to complete without a very significant delay from reprocessing all datasets. We appreciate Reviewer #3's understanding of these circumstances, and expect they recognize that our current format accurately and comprehensively presents our data and findings. To clarify these details, we have added the above description into our Materials and Methods section of our paper.

6. The DDM-BAM-PTB1-1 cryoEM structure was determined at 3.5Å resolution. Is there sufficient side chain density to clearly assign residues interacting with the PTB1-1? The discussion of specific hydrogen bonds (lines 153-159) at this resolution seems like a bit of a stretch. Alternatively, provide a snapshot of electron density showing the interactions.

Yes, the map details are sufficient, as shown in Supplementary Figure 4, and we refrain from assigning these as 'putative' interactions because i) we believe that it is well-understood that this is a molecular model, and ii) the interactions are supported by our structure-activity relationships (SAR) of the PTB1 series, and iii) the interactions are supported by the resistance mutations discovered in BamA, and iv) the metal site and importance of coordinating histidine side-chains are supported by our native mass spec experiments and SAR studies. Finally, all coordinates and maps will be released publicly upon publication of this work.

7. S14 introduces "peptide 3" which is not discussed anywhere in the main text, and S14 isn't referenced at all in the main text. What is peptide 3? How is it relevant to this manuscript? Either discuss and reference figure in main text, or replace this figure with something more relevant.

Thank you for asking for this clarification; S14 was prepared in response to prior questions from Reviewer #4 regarding the prior work by Luther et al 2019. 'peptide 3' is the nomenclature of the molecule described in the associated paper by Luther et al, and we have no clarified this in the revised S14; and in the revised discussion as follows:

"Notably, the PTB1-1 binding site on BamA is distinct in location and nature from previously identified closed-state inhibitors darobactin, dynobactin and peptide 3 (Supplementary Fig. 13b and 14)[17,19, 23,24], highlighting the power of our in vitro approach to identify novel modulatory sites and future opportunities for closed-state inhibitor discovery and optimization efforts targeting BAM."

Minor points:
1. Line 69 is missing reference to structures of the BAM complex stalled folding intermediate states solved by the Bernstein and Hinshaw groups (doi:10.1016/j.cell.2022.02.016).

Thanks, done.

2. It is still unclear in the main text that a truncated form of BamA was used in the mRNA-display experiments, and why.

This is described in the Materials and Methods and was done so to minimize the discovery of peptides binding to the POTRA domains, which we expected not to be accessible in the context of an intact OM; so they POTRAs 1-3 were removed in order to minimize futile macrocycle discovery.

3. Methods fails to describe how Apo-BAM-SMA was purified. While it is likely a similar scheme as the PTB2 bound sample, it ought to be clearly stated somewhere in the methods section.

Thanks, done.

4. Figure 4 A-C: dots/lines from B1 and B16 in A both connect to panel B though only B16 view is shown. Please edit this for clarity.

Thanks, done.

5. S3: nice to have PTB1-1 and PTB2-1 resistance mutations on sequence alignment. However, current red/green color scheme is not colorblind friendly.

Thanks, changed to blue/orange.

6. CryoEM data processing figures:
a. 2D classification panels ought to have box size stated in figure caption or scale bar on figure panel.

Thanks, relevant box sizes are provided in Supplementary Table 1, and we've updated into the figure legends.

7. Information on biological replicates is still missing several figure legends.
a. S2C, S6D…

Thanks, clarified.

8. Lines 1131 (S6G) and 1073 (S2F) state that "assay was performed in quadruplicate and all for replicates are plotted" assuming "for" is a typo for "four", only 2 lines are visible in the plot. Please explain.

Thanks, clarified; all four are plotted, they are so similar these are hard to see/distinguish.

9. Line 349 "BamAS4" : is "S4" referring to a figure? Or is it supposed to be a reference?

Thanks, corrected.

10. Check S7E figure caption (line 1143), doesn't seem to fully describe what is in figure panel

Thanks, corrected.

Reviewer #4 (Remarks to the Author):
I appreciate the changes the authors have made to the manuscript and now support publication.
Amazing, thank you!

REVIEWER COMMENTS

Reviewer #3 (Remarks to the Author):

Major points:

1. The authors solve the structure of BAM in complex with PTB2 molecule. However, the biochemical data they present all uses PTB2-1 which differs from PTB2 by 2 unnatural amino acids (S6A). This is not clear within the main text when the authors present the structure and the biochemical data, nor when they use the data to explain other findings (ie use the structure to explain outcome of biochemical experiment).

• This is a critical piece of information that is not addressed in the main text but has huge implications in the conclusions that the authors can draw from the data.

i. Particularly concerning is the "BamA chimeras indicate PTB2-lumen to be required for inhibition" section (starting at line 281) because they are using biochemical data from PTB2-1 to justify their structure with PTB2. These are not the same molecules and they do not present any data to support that PTB2-1 binds the same regions of BamA.

• At minimum, a structure of PTB2-1 needs to be added to Figure 2 (like how PTB2 is displayed in Figure 2B) and the main text needs to explain why PTB2 was used for the structure and not PTB2-1.

• Have the authors either solved a structure with PTB2-1 or predicted the structure of the molecule in complex with BamA?

2. The main text still fails to specify when the BamA truncations (BamA β-barrel, BamA POTRA3-5) were used in experiments. Use of "BamA" in the main text implies that a full length BamA protein was used for these experiments, which according to the methods is not true. The methods describe the engineering and purification of these constructs and state that they were used in the mRNA display experiments, yet it is still unclear which truncation ultimately led to the discovery of the inhibitors presented in this work.

• We have requested this clarification the past two rounds of revision, and still this information is not sufficiently clarified in the main text.

3. Labeled FSC curves for masked, unmasked, and corrected data need to be presented for all cryoEM structures. These data are valuable as experienced readers can use them to assess map quality.

• We have requested these data the past two rounds of revision, and the authors still refuse to provide these. The methods section states that cryoSPARC was used for the final refinements, and therefore providing these plots should not be an issue as cryoSPARC automatically generates them

for each final refinement. In addition, since the authors provided an edited version showing a single FSC curve, it leads us to believe that they have the plots and it's just a matter of retrieving them.

4. We again request the authors to edit the cryoEM data processing workflows. The current schemes for S7, S8, S9, and S10 are not useful, and these portrayals are not in line with standard practices in the field. The author's rebuttal that they cannot remake these figures because they no longer have the data due to HPC system data deletion is not acceptable. In fact, the deletion of data before publication is a huge red flag.

• We have requested these changes in the past two rounds of revision, and the authors refuse to update the figures.

5. The methods section states that local refinements were used for PTB2 structures and ultimately merged for a composite map (lines 592-594). These details are not included in the workflow figure (S8), which is unacceptable.

6. We continue to object to the use of "promiscuous" when describing BamA substrate interactions, as it is not accurate.

• If the authors edit the main text to include the important specifications made in their rebuttal (i.e. that BamA interactions with substrate are more promiscuous than traditional enzyme-substrate interactions, and that the BamA lateral gate is expected to interact with the surrounding lipids, sugars, and water molecules during the folding process), the use of "promiscuous" would be slightly more acceptable.

• The authors rebuttal points out that non-native substrates darobactin and dynobactin have been seen to bind the BamA lateral gate is true, however they fail to acknowledge that these compounds do contain a beta-signal sequence motif like native substrates, as does their PTB2 molecule. It is valuable to point out these shared characteristics as clearly the beta-signal sequence motif contributes to the interaction with the lateral gate of BamA.

7. The authors present no physiologically relevant data to support the statement in lines 275-297 ("this observation demonstrates that a β-signal is not strictly required for peptide binding to the open lateral gate"). This statement must be removed.

8. Lines 115, 124, 182, and 188 need to be edited to specify that LptD OMP levels were reduced, as the authors only present data for LptD reduction in the included Western blots (Figures 1C, 2C). Alternatively, include Western blots for other OMPs to be able to make this generalized statement.

9. The authors provide no physiological data to support the statement in lines 336-339 “Because the PTB2-BAM-SMA-compolymer complex structure is highly similar to the apo-BAM complex structure determined under similar solution conditions (Supplementary Fig. 13a), this establishes that PTB2 targets and traps a natural open lateral gate conformation of BamA that must exist within the native outer membrane.”

• Their apo structures do not display an open lateral gate, nor do they have any data to show that the observed PTB2-bound open lateral gate conformation exists in the absence of PTB2 in a native membrane.

• A statement such as “PTB2 traps an open lateral gate conformation of BamA” would be more appropriate as it only speaks to the data they have presented.

10. If binding affinities of these molecules are going to be presented (such as in lines 168-169, supplementary figure 1c) more than one biological replicate is required.

• While figure 1i presents a “duplicate SPR sensorgram of PTB1-1 interaction with surface-bound BamA…” there is no information on biological replicates nor the range of binding affinities for multiple biological replicates. A single biological replicate of a binding affinity experiment is not enough.

11. There are several alarming inconsistencies in key data reported:

• Line 105 states PTB1 has a MIC of 25μM against wild-type E.coli however the corresponding figures (1A, S1B) list the MIC as 42μg/mL and 50μM respectively. Which is correct value?

• Line 180 states that PTB2 has 2μM MIC against wild type E. coli however the corresponding figures (2A, S1B) list the PTB2 MIC as 4μg/mL and 4μM respectively. Which is the correct value?

12. Questionable data:

• Lines 638-639: Rwork/Rfree is 32.5/36.7, approaching R-values for incorrect model. I’m not sure if this was addressed in the initial review process. The authors mentioned that the crystal they collected on suffers from anisotropy, but they don’t supply a diffraction pattern. At the very least, they should supply an image of a diffraction pattern in the supplement. They did use STARANISO to correct anisotropy but there is no mention if REFMAC’s built in anisotropic scaling algorithm is used during refinement. See https://srv.mbi.ucla.edu/Anisoscale/discussion#:~:text=Diffraction anisotropy is commonly observed,in one direction than another.

Minor Points:

We believe that the manuscript could be significantly improved by incorporating minor corrections provided below. Some of the issues below have been previously raised during revisions, yet they remain inadequately addressed or completely overlooked. It is noteworthy that any of the authors, including the primary author and the PI, could have caught these mistakes but failed to do so. We find it concerning that we are being forced to invest considerable time in identifying and bringing attention to these errors.

1. S14 uses a BamA PDB that contains a C-terminal extension to map the Peptide 3 interactions on. Why did the authors not use the apo structures they solved for this figure (apo BAM-DDM or apo BAM-SMA)? This figure should be remade to use a WT BamA structure (or at least a structure where the beta-barrel has not been mutated)

2. The S14 figure legend incorrectly lists the PDB ID as “6SFU” (line 1236).

3. Figure 1D displays the cryoEM structure of the BAM complex bound to PTB1-1. Two regions on opposite sides of the BamA barrel are colored in pink, and according to the legend the pink density is assigned as BamB. In the current view, this subunit is not consistent with any other BAM structure published to date. There is also density colored in yellow that is not described in the legend.

• Please clarify in legend or remake figure to accurately portray this.

4. Species names in S3 legend ought to be italicized.

• We pointed out this error in previous rounds of revision and still the authors fail to make this change

5. Figure 4 A-C: dots/lines from B1 and B16 in panel A both connect panel B though only B16 view is shown in panel B. This needs to be edited for clarity because it is difficult to tell which view is in panel B vs C.

• We have requested these edits the past two rounds of revision and while the authors claim to have edited (rebuttal 1 says “done”, rebuttal 2 says “Thanks, done.”) no changes have been made to this figure.

6. Supplementary Figure 1c is illegible. Panel needs to be remade so that Kd values can be clearly read. Also, edit the corresponding text in lines 1057-1058 to explain why there are data for 119 presented (it is not discussed in main text).

7. The differences in structure and sequence between PTB1 and PTB1-1 are still unclear. Given that the molecules have drastically different MICs, this information is important to provide. Show the residues for PTB1 and PTB1-1 similar to the one shown for PTB2 vs. PTB2.2 in Supplementary figure 6. Alternatively, only talk about PTB1-1 and delete all data and text referring to PTB1.

8. Supplementary Figure 1 panels and legend must be updated to clarify the BamA truncation used for these experiments. Use of "BamA" in the main text implies that a full length BamA protein was used for these experiments, which according to the methods is not true. The methods describe the engineering and purification of two different BamA truncation constructs and state that they were used in the mRNA display experiments. The main text and S1 fail to clearly describe/show which truncation ultimately lead to the discovery of the inhibitors presented in this work.

9. Supplementary Figure 2d and 6e:

• Legends fail to specify which BamA PDB is shown. Please add this information to legend.

• Furthermore, please clarify why different BamA conformations are used for these figures since both are mapping PTB1-1 (S2d) or PTB2-1 (S6e) resistance mutations onto the structure. Why use a closed conformation of BamA for S2d but an open BamA conformation for S6e? Why isn't one of the apo structures presented in this paper used?

10. Different units are used when reporting MIC values in the text versus in the figures, which is confusing to the reader. Please edit so that MIC values in figures and text so that are all reported in the same units and consistent throughout the manuscript.

• Figures 1A and 2A list the MIC values in μg/mL while the main text and S1B report values in μM.

i. Line 105: MIC unit doesn't match fig.1a, this will confuse the reader.

ii. Line 107: MIC unit doesn't match fig.1a, this will confuse the reader.

iii. Line 178-184: MIC unit doesn't match fig. 2a, this will confuse the reader.

• Fig.S5c shows MIC values in units of μM which is inconsistent with all the previous figures.

11. Line 302 needs to specify that PTB1-1 and PTB2-1 molecules were used in these experiments.

• Clarification within the manuscript on when which molecules (PTB1 vs PTB1-1 or PTB1 vs PTB1-1) were used for certain experiments has been an issue in every revision.

12. Line 33: italicize in vitro

13. Several figure panels look like low-resolution screenshots. Please replace with higher resolution images to improve clarity.

- Fig.S1c
- Fig.S2d
- Fig.S6e
- Fig.S11c

14. Line 120-127: Explain in text why BamA-D500N was chosen (fig.1C) for this experiment over the other mutations identified that confer resistance.

15. Line 184: Explain in text why N492K mutant chosen (fig.2c) for this experiment over the other mutations identified that confer resistance.

16. Lines 303-305: correct the start of the sentence to say “As expected, all the strains lose sensitivity …”

17. Lines 346-349: re-write the sentence to improve clarity (reference specific figs.)

18. Line 356-367: Remove psBamA and replace with the new nomenclature

19. Lines 381-397 and beyond: Change p.s.i to psi (this is the accepted abbreviation for pound per square inch)

20. Lines 390 and beyond (entire methods section): ml or mL were interchangeably used for milliliter. Please choose one and stick to it. We know that different co-authors wrote different sections, but the primary author should edit for consistent abbreviation.

21. Lines 529-548: Clarify the final protein/complex (including peptide concentration when applicable) concentration applied to grids.

22. Line 560: Correct format of cisTEM ref

23. Lines 560-572: Some of the references have underline in them and some don't. Please correct

24. Lines 598-602: Re-write or complete removal is highly recommended. It reads as a response or a letter. It doesn't seem appropriate in the context of the entire manuscript.

25. Line 729, It's not common to start a sentence with a number

26. Line 746: change equilibration to equilibrated

27. Line 762, It's not common to start a sentence with a number

28. Line 1242: supplementary table 1: Add what camera was used for each dataset

29. All CryoEM supplementary figures: Some of the micrographs have boxed particles while others don't; please edit to make consistent.

Reviewer #5 (Remarks to the Author):

I was asked to review the manuscript and provide my comments on the remaining comments from previous rounds of review.

This is a rigorous investigation and with important new knowledge that is potentially translatable into pre-clinical trials. I think it is really quite striking that these inhibitors trap BAM in distinct inward-open and outward-open conformations! The presentation and writing is very good – I could easily comprehend the manuscript. I congratulate the authors on this great article!

I see no barrier to publication at Nature Communications. I would, however, very much like to see the following changes:

Major:

I do agree with reviewer 3 that the crystal structure is over interpreted – particularly the paragraphs on Lines 266-279 really are quite unsubstantiated. The speculation here is needless and the purple copy of BamA in Fig 4 is totally not physiologically relevant. I suggest moving Fig 4 to the sup and deleting lines 266-279 entirely. None of this subsection is necessary when the rest of the manuscript is so excellent!

[A side point is that much of the most interesting and useful data is hidden in the supplementary which is a shame. E.g. Fig S2, S5, S6. should be main figures in my opinion. This is an optional suggestion]

Tid-bits:

Line 52: According to convention. “OMPs typically possess short periplasmic loops and long extracellular loops…” Should be “OMPs typically possess short periplasmic turns and long extracellular loops…”.

Line 65: “ The prevailing model for how BAM folds and inserts OMPs posits that unfolded substrates are paired through β-strand complementation at the lateral gate of BamA…” I’ve never heard of a model in which the substrates are “paired”…as a dimer? Consider changing to: “A favoured model posits that BAM folds and inserts OMPs unfolded substrates through β-strand complementation at the lateral gate of BamA…”.

We thank Reviewer 3 for their continued attention and efforts to help improve the clarity of our work. We similarly thank Reviewer 5 for joining the party and helping to improve the clarity and impact of our work. Point-by-point response to reviewer comments is provided below.

REVIEWER COMMENTS
Reviewer #3 (Remarks to the Author):

Major points:
1. The authors solve the structure of BAM in complex with PTB2 molecule. However, the biochemical data they present all uses PTB2-1 which differs from PTB2 by 2 unnatural amino acids (S6A). This is not clear within the main text when the authors present the structure and the biochemical data, nor when they use the data to explain other findings (ie use the structure to explain outcome of biochemical experiment).
• This is a critical piece of information that is not addressed in the main text but has huge implications in the conclusions that the authors can draw from the data.
i. Particularly concerning is the "BamA chimeras indicate PTB2-lumen to be required for inhibition" section (starting at line 281) because they are using biochemical data from PTB2-1 to justify their structure with PTB2. These are not the same molecules and they do not present any data to support that PTB2-1 binds the same regions of BamA.
• At minimum, a structure of PTB2-1 needs to be added to Figure 2 (like how PTB2 is displayed in Figure 2B) and the main text needs to explain why PTB2 was used for the structure and not PTB2-1.
• Have the authors either solved a structure with PTB2-1 or predicted the structure of the molecule in complex with BamA?

In Figure 2A, we report the MIC of PTB2 and PTB2-1 to be identical against wild-type *E. coli* but, importantly, that PTB2-1 shows no background activity against non-*E. coli* species, leading to the conclusion that PTB2-1 is a more selective *E. coli* BamA-targeting molecule. Therefore, the remaining microbiological experiments focused on characterizing PTB2-1. We have also stated that PTB2-1 is a highly related second-generation derivative of PTB2 which was identified through additional rounds of selection starting from the PTB2 parental scaffold against purified *E. coli*-BAM complex, in a process akin to antibody maturation or engineering experiments where the binding epitope is not expected to change. Consistent with this conclusion, the resistance mutations raised against PTB2 also exhibit resistance to PTB2-1, confirming that the major binding determinants of both PTB2 and PTB2-1 macrocycles are highly similar (these data are now included in Supplementary Fig. S6f). Notably, PTB2 and PTB2-1 differ mainly by side-chain changes with similar properties and sizes (e.g., $R10_{PTB2}$>$K10_{PTB2\text{-}1}$ and $F13_{PTB2}$>$Y13_{PTB2\text{-}1}$ (Supplementary Fig. S6a)) which can be easily accommodated (or modeled) within the PTB2-BAM structure. The additional change ($R8_{PTB2}$>$Aib_{PTB2\text{-}1}$) replaces a solvent exposed sidechain that makes no direct interactions with BamA in the PTB2-BAM structure. Additionally, molecular modeling demonstrates that the remaining sidechain differences ($G2_{PTB2}$>$H3m_{PTB2\text{-}1}$ and

$H5_{PTB2}$>$R5_{PTB2-1}$) can be readily accommodated in the PTB2-BAM structure with only very minor side-chain adjustments. Notably, all anchoring interactions are maintained within the PTB2-1-BAM model, as seen in the PTB2-BAM structure, and as would be expected given their similar resistance profiles. We have revised the main text to summarize these details.

2. The main text still fails to specify when the BamA truncations (BamA β-barrel, BamA POTRA3-5) were used in experiments. Use of "BamA" in the main text implies that a full length BamA protein was used for these experiments, which according to the methods is not true. The methods describe the engineering and purification of these constructs and state that they were used in the mRNA display experiments, yet it is still unclear which truncation ultimately led to the discovery of the inhibitors presented in this work.
• We have requested this clarification the past two rounds of revision, and still this information is not sufficiently clarified in the main text.

We have updated the revised main text to indicate that the BamA protein lacked POTRA1-2 domains and to provide our justification for this. These domains were excluded because at the time our thinking was to try and minimize the identification of macrocycle binders to periplasmic regions of the protein.

3. Labeled FSC curves for masked, unmasked, and corrected data need to be presented for all cryoEM structures. These data are valuable as experienced readers can use them to assess map quality.
• We have requested these data the past two rounds of revision, and the authors still refuse to provide these. The methods section states that cryoSPARC was used for the final refinements, and therefore providing these plots should not be an issue as cryoSPARC automatically generates them for each final refinement. In addition, since the authors provided an edited version showing a single FSC curve, it leads us to believe that they have the plots and it's just a matter of retrieving them.

The complete workflow is now presented for all cryoEM structures with appropriate figures, legends, and methods updated.

4. We again request the authors to edit the cryoEM data processing workflows. The current schemes for S7, S8, S9, and S10 are not useful, and these portrayals are not in line with standard practices in the field. The author's rebuttal that they cannot remake these figures because they no longer have the data due to HPC system data deletion is not acceptable. In fact, the deletion of data before publication is a huge red flag.
• We have requested these changes in the past two rounds of revision, and the authors refuse to update the figures.

The complete workflow is now presented for all cryoEM structures with appropriate figures, legends, and methods updated. We have appreciated the reviewers concerns about this point for the entire review process and have had extensive discussion on how to address it, so appreciate the patience. To clarify, we did not 'delete' our data, but rather given our company's automatic data retention policy and the length of time under

review, we were unable to retain it. We have finally been able to reallocate the substantial resources required to completely redo our analysis as this seems to be the only option. We can now be certain that the reviewer's concerns have been appropriately addressed.

5. The methods section states that local refinements were used for PTB2 structures and ultimately merged for a composite map (lines 592-594). These details are not included in the workflow figure (S8), which is unacceptable.

The complete workflow is now presented for all cryoEM structures with appropriate figures, legends, and methods updated.

6. We continue to object to the use of "promiscuous" when describing BamA substrate interactions, as it is not accurate.
• If the authors edit the main text to include the important specifications made in their rebuttal (i.e. that BamA interactions with substrate are more promiscuous than traditional enzyme-substrate interactions, and that the BamA lateral gate is expected to interact with the surrounding lipids, sugars, and water molecules during the folding process), the use of "promiscuous" would be slightly more acceptable.
• The authors rebuttal points out that non-native substrates darobactin and dynobactin have been seen to bind the BamA lateral gate is true, however they fail to acknowledge that these compounds do contain a beta-signal sequence motif like native substrates, as does their PTB2 molecule. It is valuable to point out these shared characteristics as clearly the beta-signal sequence motif contributes to the interaction with the lateral gate of BamA.

We believe we have finally understood our Reviewer's challenges with our prior wording and have removed the use of 'promiscuous' entirely throughout the revised manuscript and updated the corresponding sections of text to be more specific about our intended meaning.

7. The authors present no physiologically relevant data to support the statement in lines 275-297 ("this observation demonstrates that a β-signal is not strictly required for peptide binding to the open lateral gate"). This statement must be removed.

We believe we have finally understood our Reviewer's challenges with our prior wording and have removed this statement.

8. Lines 115, 124, 182, and 188 need to be edited to specify that LptD OMP levels were reduced, as the authors only present data for LptD reduction in the included Western blots (Figures 1C, 2C). Alternatively, include Western blots for other OMPs to be able to make this generalized statement.

BamA, itself a BAM-dependent OMP, is also shown to be reduced in this figure, thus we maintain that it is a generalizable observation.

9. The authors provide no physiological data to support the statement in lines 336-339 "Because the PTB2-BAM-SMA-compolymer complex structure is highly similar to the apo-BAM complex structure determined under similar solution conditions (Supplementary Fig. 13a), this establishes that PTB2 targets and traps a natural open lateral gate conformation of BamA that must exist within the native outer membrane."
• Their apo structures do not display an open lateral gate, nor do they have any data to show that the observed PTB2-bound open lateral gate conformation exists in the absence of PTB2 in a native membrane.
• A statement such as "PTB2 traps an open lateral gate conformation of BamA" would be more appropriate as it only speaks to the data they have presented.

We believe we have finally understood our Reviewer's challenges with our prior wording and have updated the corresponding sections of text to be more specific about our intention.

10. If binding affinities of these molecules are going to be presented (such as in lines 168-169, supplementary figure 1c) more than one biological replicate is required.
• While figure 1i presents a "duplicate SPR sensorgram of PTB1-1 interaction with surface-bound BamA…" there is no information on biological replicates nor the range of binding affinities for multiple biological replicates. A single biological replicate of a binding affinity experiment is not enough.

The SPR experiments presented in Fig. 1i are not biological, but rather biophysical analyses using purified protein with each experiment utilizing multiple doses of the macrocycles, as is standard for SPR. These experiments were optimized for the reported conditions and have been demonstrated to be reproducible as indicated. We have updated the figure legends to report that the K_D 50 pM +/- 2 pM, which, importantly, was determined from the model that was fit to our data.

The SPR experiments presented in Supplementary Figure 1c were used as part of our high-throughput discovery approach, and as previously explained and noted below, the data are not presented with the expectation that they should represent high-quality SPR data with quantitative outputs (as optimization of SPR parameters and conditions often requires optimization for each molecule under study). This is noted in the methods and now in the figure legend as well. Moreover, we remade this panel so it is easier to read.

11. There are several alarming inconsistencies in key data reported:
• Line 105 states PTB1 has a MIC of 25μM against wild-type E.coli however the corresponding figures (1A, S1B) list the MIC as 42μg/mL and 50μM respectively. Which is correct value?
• Line 180 states that PTB2 has 2μM MIC against wild type E. coli however the corresponding figures (2A, S1B) list the PTB2 MIC as 4μg/mL and 4μM respectively. Which is the correct value?

We apologize for the confusion here. Different reviewers throughout the process have requested the reporting of different assays with different concentrations and we seem to

have missed changing some to match. We believe that we have now consistently reported MICs in µg/mL throughout but have also included µM as well as the peptide MWs so that all readers can interpret the data as needed. Additionally, given the nature of the MICs assay, a 2-fold variation is not an unexpected finding across experiments performed under different conditions.

12. Questionable data:
• Lines 638-639: Rwork/Rfree is 32.5/36.7, approaching R-values for incorrect model. I'm not sure if this was addressed in the initial review process. The authors mentioned that the crystal they collected on suffers from anisotropy, but they don't supply a diffraction pattern. At the very least, they should supply an image of a diffraction pattern in the supplement. They did use STARANISO to correct anisotropy but there is no mention if REFMAC's built in anisotropic scaling algorithm is used during refinement. See https://srv.mbi.ucla.edu/Anisoscale/discussion#:~:text=Diffraction anisotropy is commonly observed,in one direction than another.

We have indicated details of the anisotropy in the Methods section and provide examples of the Autoproc output below, since we feel this is a more appropriate and complete representation of the diffraction data rather than any single image. However, we strongly object with our reviewer's suggestion that our Rwork/Rfree are anywhere close to approaching an incorrect model, and would like to refer this reviewer to a classic paper that sets expectations for the field in this respect.

"Checking your imagination: applications of the free R value"
Gerard J Kleywegt and Axel T Brünger

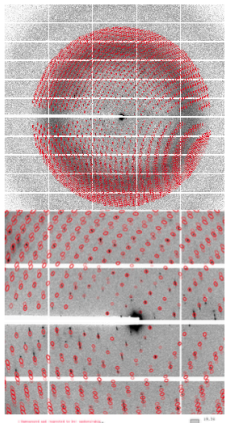
Additionally, we've significantly updated our conclusions in the manuscript related to the crystal structure based on prior Reviewer 3 concerns and comments around our interpretation/speculation of the structure. Nevertheless, we'd like to point out again that we only interpret and discuss high level (or, effectively low-resolution detail) of this structure given the limited resolution and quality of the electron density map. Moreover, we have been clear in the manuscript that we only define the details of side-chain interaction from our much higher quality and resolution cryo-EM maps of PTB2-BAM complexes. Still, we would like to point out that performing molecular replacement with 1, 2, 3 or 4 copies of BamA per AU, where the macrocycle has been removed from all copies of BamA in the MR model, provides the phase information for us to very clearly see in strong positive density for the unassigned PTB2 macrocycle in all cases (as well as for any removed BamA copies, depending on the MR model that we use). Thus, we are certain that the general features of our BamA-PTB2 crystal structure and refined model have been determined within reason and within the limitations of the available diffraction data, and therefore feel that we have interpreted and discussed this model appropriately.

Finally, we are well aware of approaches to assess potential issues related to (mis)spacegroup assignment, as well as other data curation procedures that can impact and/or potentially arbitrarily decrease Rfree, and can assure our reviewer that we have

evaluated spacegroup assignment rigorously and have not undertaken inappropriate truncation of high or low resolution data.

“On the validation of crystallographic symmetry and the quality of structures”
Jimin Wang

“Estimation of the quality of refined protein crystal structures”
Jimin Wang



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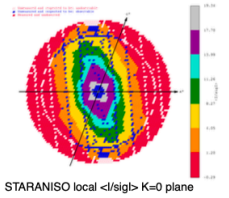

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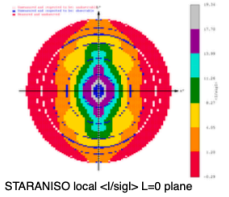

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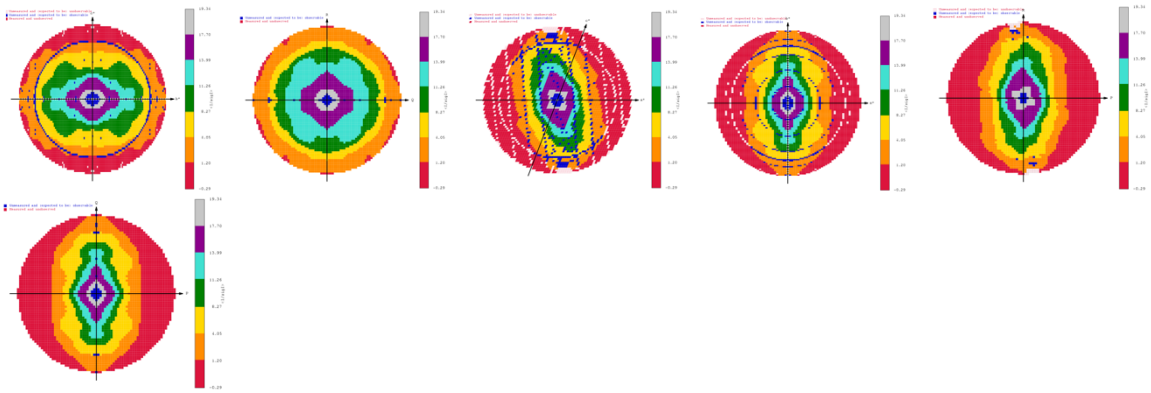
XDS	VERSION Mar 15, 2019 BUILT=20191211
AIMLESS	Version 0.7.4
STARANISO	Version 2.3.36 (11-May-2020)
CCP4	Version 7.0.077
Host	ng007
User	payandej (group = idmgmt)
Date	Tue Jul 6 01:49:52 PDT 2021
autoPROC	/gstore/apps/buster/20200520
	30259_p117x12_1_####.cbf (900 images, 180°)

Anisotropic data analysis with STARANISO:

Spacegroup	P21
Cell parameters	98.768 167.232 104.792
	90.000 110.993 90.000
Wavelength [A]	0.97910
Diffraction limits [A]	4.256 2.731 2.885
Eigenvector-1	0.821 0.000 0.571
Eigenvector-2	0.000 1.000 0.000
Eigenvector-3	-0.571 0.000 0.821
Direction-1	0.955 _a_* + 0.295 _c_*
Direction-2	_b_*
Direction-3	-0.485 _a_* + 0.875 _c_*

	Overall	Inner Shell	Outer Shell
Low resolution limit	31.626	31.626	2.967
High resolution limit	2.731	9.001	2.731
Rmerge (all I+ & I-)	0.121	0.037	0.837
Rmeas (all I+ & I-)	0.144	0.044	0.991
Rpim (all I+ & I-)	0.077	0.023	0.524
Total number of observations	155816	7745	7853
Total number unique	45815	2290	2291
Mean(I)/sd(I)	6.1	15.0	1.5
Completeness (spherical)	54.5	97.3	12.4
Completeness (ellipsoidal)	87.6	97.3	63.5
Multiplicity	3.4	3.4	3.4
CC(1/2)	0.997	0.999	0.712
Anomalous completeness (spherical)	50.4	94.4	11.1
Anomalous completeness (ellipsoidal)	81.1	94.4	56.8
Anomalous multiplicity	1.8	1.8	1.8
CC(ano)	-0.201	-0.383	-0.001
lDANOl/sd(DANO)	0.730	0.568	0.751



Minor Points:
We believe that the manuscript could be significantly improved by incorporating minor corrections provided below. Some of the issues below have been previously raised during revisions, yet they remain inadequately addressed or completely overlooked. It is noteworthy that any of the authors, including the primary author and the PI, could have caught these mistakes but failed to do so. We find it concerning that we are being forced to invest considerable time in identifying and bringing attention to these errors.

1. S14 uses a BamA PDB that contains a C-terminal extension to map the Peptide 3 interactions on. Why did the authors not use the apo structures they solved for this figure (apo BAM-DDM or apo BAM-SMA)? This figure should be remade to use a WT BamA structure (or at least a structure where the beta-barrel has not been mutated)

This figure was made in response to a previous reviewer request to include Peptide 3 and we used the same PDB file that was originally reported (in Luther et al. Chimeric peptidomimetic antibiotics against gram-negative bacteria. *Nature* **576**: 452-458 (2019). In response to the current request, we have now used our Apo-BAM-DDM structure and hope this finally helps address the reviewer's question.

2. The S14 figure legend incorrectly lists the PDB ID as "6SFU" (line 1236).

As noted above, we have changed the structure used in this figure to our Apo-BAM-DDM structure and note this in the figure legend.

3. Figure 1D displays the cryoEM structure of the BAM complex bound to PTB1-1. Two regions on opposite sides of the BamA barrel are colored in pink, and according to the legend the pink density is assigned as BamB. In the current view, this subunit is not consistent with any other BAM structure published to date. There is also density colored in yellow that is not described in the legend.
• Please clarify in legend or remake figure to accurately portray this.

We have remade the figure and adjusted the figure legend and hope the reviewer finds it easier to interpret.

4. Species names in S3 legend ought to be italicized.
• We pointed out this error in previous rounds of revision and still the authors fail to make this change

Thank you for the continued copy-editing. This is finally corrected.

5. Figure 4 A-C: dots/lines from B1 and B16 in panel A both connect panel B though only B16 view is shown in panel B. This needs to be edited for clarity because it is difficult to tell which view is in panel B vs C.
• We have requested these edits the past two rounds of revision and while the authors claim to have edited (rebuttal 1 says “done”, rebuttal 2 says “Thanks, done.”) no changes have been made to this figure.

We had changed this in the previous version so it is unclear to us why the question persists but have remade this figure in a way that we hope much more clearly delineates the panels for the reviewer.

6. Supplementary Figure 1c is illegible. Panel needs to be remade so that Kd values can be clearly read. Also, edit the corresponding text in lines 1057-1058 to explain why there are data for 119 presented (it is not discussed in main text).

As previously explained, the SPR experiments presented in Supplementary Figure 1c were part of our high-throughput discovery approach, and, as previously explained and noted above, the data are not presented with the expectation that they should represent high-quality SPR data with quantitative outputs (as optimization of SPR parameters and conditions often requires optimization for each molecule under study as was done for experiments in Fig. 1i). This is noted in the methods and now in the figure legend as well. Moreover, we have remade this panel, and it should be easier to read in the high-resolution final version.

7. The differences in structure and sequence between PTB1 and PTB1-1 are still unclear. Given that the molecules have drastically different MICs, this information is important to provide. Show the residues for PTB1 and PTB1-1 similar to the one shown for PTB2 vs. PTB2.2 in Supplementary figure 6. Alternatively, only talk about PTB1-1 and delete all data and text referring to PTB1.

We apologize for the oversight. This is now presented in Supplementary Figure 2a.

8. Supplementary Figure 1 panels and legend must be updated to clarify the BamA truncation used for these experiments. Use of “BamA” in the main text implies that a full length BamA protein was used for these experiments, which according to the methods is not true. The methods describe the engineering and purification of two different BamA truncation constructs and state that they were used in the mRNA display experiments. The main text and S1 fail to clearly describe/show which truncation ultimately lead to the discovery of the inhibitors presented in this work.

The figure legend has updated, and we note that the cartoon shows BamA with only POTRA3-5 domains.

9. Supplementary Figure 2d and 6e:
• Legends fail to specify which BamA PDB is shown. Please add this information to legend.
• Furthermore, please clarify why different BamA conformations are used for these figures since both are mapping PTB1-1 (S2d) or PTB2-1 (S6e) resistance mutations onto the structure. Why use a closed conformation of BamA for S2d but an open BamA conformation for S6e? Why isn't one of the apo structures presented in this paper used?

Supplementary Fig. S2d and S6e show BamA from our respective PTB1-1-BAM and PTB2-BAM structures, respectively, with the macrocycles removed for clarity.
We do not detail our apo-BAM structure because it is highly similar to previously reported apo-BAM structures, and therefore not of significant novelty or importance to our conclusions.

10. Different units are used when reporting MIC values in the text versus in the figures, which is confusing to the reader. Please edit so that MIC values in figures and text so that are all reported in the same units and consistent throughout the manuscript.
• Figures 1A and 2A list the MIC values in μg/mL while the main text and S1B report values in μM.
i. Line 105: MIC unit doesn't match fig.1a, this will confuse the reader.
ii. Line 107: MIC unit doesn't match fig.1a, this will confuse the reader.
iii. Line 178-184: MIC unit doesn't match fig. 2a, this will confuse the reader.
• Fig.S5c shows MIC values in units of μM which is inconsistent with all the previous figures.

As noted above, we apologize for the confusion here. Different reviewers throughout the process have requested the reporting of different assays with different concentrations and we seem to have missed changing some to match. We believe that we have now consistently reported MICs in μg/mL throughout but have also included μM as well as the peptide MWs so that all readers can interpret the data as appropriate. Regarding Supplemental Fig. S5c, for the purpose of this experiment, we have opted to leave the concentrations as μM to allow direct molar comparisons as each of these peptides have different molecular weights.

11. Line 302 needs to specify that PTB1-1 and PTB2-1 molecules were used in these experiments.
• Clarification within the manuscript on when which molecules (PTB1 vs PTB1-1 or PTB1 vs PTB1-1) were used for certain experiments has been an issue in every revision.

We have specified the molecules used in the text and note that the figure and legend correctly identify the molecules used in all cases.

12. Line 33: italicize in vitro

We have updated the font.

13. Several figure panels look like low-resolution screenshots. Please replace with higher resolution images to improve clarity.
• Fig.S1c
• Fig.S2d
• Fig.S6e
• Fig.S11c

For the process of peer review, we had to compress our figures to enable uploading through the web portal. Our final submission includes high-resolution figures which do not change any results or conclusions in the manuscript.

14. Line 120-127: Explain in text why BamA-D500N was chosen (fig.1C) for this experiment over the other mutations identified that confer resistance.

We did not aim to characterize the entire suite of resistant mutants but rather chose a representative. Of note, this particular mutant was isolated in multiple independent selections. We have indicated this in the revised text and figure legend.

15. Line 184: Explain in text why N492K mutant chosen (fig.2c) for this experiment over the other mutations identified that confer resistance.

We did not aim to characterize the entire suite of resistant mutants but rather chose a representative. We have noted this in the revised text and figure legend.

16. Lines 303-305: correct the start of the sentence to say “As expected, all the strains lose sensitivity …”

We have changed the text as suggested.

17. Lines 346-349: re-write the sentence to improve clarity (reference specific figs.)

We have now referenced each figure individually in this sentence and hope it improves the clarity.

18. Line 356-367: Remove psBamA and replace with the new nomenclature

Thank you for catching this old nomenclature. We have corrected it here and ensured that it is correct throughout the manuscript.

19. Lines 381-397 and beyond: Change p.s.i to psi (this is the accepted abbreviation for

pound per square inch)

We have changed this throughout the manuscript as suggested.

20. Lines 390 and beyond (entire methods section): ml or mL were interchangeably used for milliliter. Please choose one and stick to it. We know that different co-authors wrote different sections, but the primary author should edit for consistent abbreviation.

We have corrected this to mL throughout the manuscript.

21. Lines 529-548: Clarify the final protein/complex (including peptide concentration when applicable) concentration applied to grids.

This information is already included in the indicated section of the results (see below). Unless there is additional specific guidance, we will leave this section as is:

EM sample preparation and imaging

For the apo structure of BamABCDE (BAM) complex prepared in DDM detergent (apo BAM-DDM), Au substrate Quantifoil (Quantifoil GMBH) cryo-EM grids with hole diameter / spacing of 0.6 / 1.0 um with 25 nm thick Au foil were incubated with a thiol reactive, self-assembling reaction mixture of 4 mM monothiolalkane(C11)PEG6-OH (11-mercaptoundecyl) hexaethyleneglycol (SPT-0011P6, SensoPath Technologies, Inc., Bozeman, MT) to improve sample behavior[38]. Grids were incubated with this self-assembled monolayer (SAM) solution for 24 hours. Prior to grid freezing, grids were removed from the SAM solution and rinsed with EtOH. **3 µL of BAM complex (4-5 mg/mL)** was applied to grids. Grids were then blotted for 3 seconds and plunged into liquid ethane, using the Leica Microsystems automatic plunge freezer (EM GP2, Leica Microsystems, Buffalo Grove, IL). For the PTB1-1-BAM structure, the MAB2 antibody fragment, which has no functional impact on BAM activity[18] or PTB1 series pharmacology (and was included in our initial experiments as a fiducial marker), was included and the **BAM-MAB2 Fab complex (4-5 mg/mL) was incubated with PTB1-1 macrocycle at 1:2 molar ratio** on ice for 30 min. The sample was applied to Ultrafoil R0.6/1.0 (300 mesh) cryo-EM grids (Quantifoil GMBH) which have been plasma cleaned using the Solarus plasma cleaner (Gatan, Pleasanton, CA) and plunge frozen as above. For the **PTB2-BAM-DDM structure, apo BAM-DDM (4-5 mg/mL) was incubated with PTB2 macrocycle at 1:2 molar ratio** on ice for 1h. The sample was applied to grids as above and plunge frozen. **For apo BAM-SMA and PTB2-BAM-SMA structures, the sample of apo BAM-SMA or BAM-SMA co-purified with PTB2 was diluted as a final concentration of 1-4 mg/mL.** The sample was applied to Ultrafoil R1.2/1.3 (300 mesh) cryo-EM grids (Quantifoil GMBH), previously glow discharged in Solarus plasma cleaner (Gatan, Pleasanton, CA). The grids were plunge frozen in liquid ethane using a Vitrobot Mark IV (ThermoFisher).

22. Line 560: Correct format of cisTEM ref

We have not been able to figure out why our reference program did this for some of the in-text references, but they are now formatted correctly.

23. Lines 560-572: Some of the references have underline in them and some don't. Please correct

We have not been able to figure out why our reference program is doing this but have fixed the underlining in this section and are confident that any additional formatting issues can be identified during the editorial process.

24. Lines 598-602: Re-write or complete removal is highly recommended. It reads as a response or a letter. It doesn't seem appropriate in the context of the entire manuscript.

This was written after an editorial discussion to address a reviewer question that was technically unanswerable at the time. We have since reprocessed our data (see above) to provide the standard workflow in its entirety, and thus this section is no longer relevant and has been removed.

25. Line 729, It's not common to start a sentence with a number

We have edited the text as suggested.

26. Line 746: change equilibration to equilibrated

Thank you for catching this typo. We have edited the text as suggested.

27. Line 762, It's not common to start a sentence with a number

We have edited the text as suggested.

28. Line 1242: supplementary table 1: Add what camera was used for each dataset

Camera information has been added to Supplementary Table S1.

29. All CryoEM supplementary figures: Some of the micrographs have boxed particles while others don't; please edit to make consistent.

The boxed particles have been removed to make all of the panels consistent.

Reviewer #5 (Remarks to the Author):

I was asked to review the manuscript and provide my comments on the remaining comments from previous rounds of review.

This is a rigorous investigation and with important new knowledge that is potentially translatable into pre-clinical trials. I think it is really quite striking that these inhibitors trap BAM in distinct inward-open and outward-open conformations! The presentation and writing is very good – I could easily comprehend the manuscript. I congratulate the authors on this great article!

I see no barrier to publication at Nature Communications. I would, however, very much like to see the following changes:

We thank the reviewer for their comments.

Major:
I do agree with reviewer 3 that the crystal structure is over interpreted – particularly the paragraphs on Lines 266-279 really are quite unsubstantiated. The speculation here is needless and the purple copy of BamA in Fig 4 is totally not physiologically relevant. I

suggest moving Fig 4 to the sup and deleting lines 266-279 entirely. None of this subsection is necessary when the rest of the manuscript is so excellent!

We believe we have addressed Reviewer 3's concerns about substrate promiscuity (and have removed this connotation) but feel the interactions observed and discussed in this region of the manuscript are similar to, and extend, those found in a previously described "trapped" BamA-BamA complex (Tomasek et al. Structure of a nascent membrane protein as it folds on the BAM complex. *Nature* **583**: 473-487 (2020)) that the field largely appears to recognize as being physiologically relevant. Because these specific features were not directly addressed in this earlier publication, we feel it is reasonable to describe the interactions here as we expect similar geometry and interactions to be found in physiologically relevant late-stage folding intermediates of BAM with its larger substrates, such as BamA, LptD, FhuA, etc.

[A side point is that much of the most interesting and useful data is hidden in the supplementary which is a shame. E.g. Fig S2, S5, S6. should be main figures in my opinion. This is an optional suggestion]

This has been driven mainly by reviewer requests that have limited the amount of these data we are able to include in the main figures. We agree that there is a lot of interesting and useful data in the SI which we hope colleagues in the field find enabling.

Tid-bits:
Line 52: According to convention. "OMPs typically possess short periplasmic loops and long extracellular loops…" Should be "OMPs typically possess short periplasmic turns and long extracellular loops…".

Thank you for this comment. We have corrected this statement.

Line 65: "The prevailing model for how BAM folds and inserts OMPs posits that unfolded substrates are paired through β-strand complementation at the lateral gate of BamA…" I've never heard of a model in which the substrates are "paired"…as a dimer? Consider changing to: "A favoured model posits that BAM folds and inserts OMPs unfolded substrates through β-strand complementation at the lateral gate of BamA…".

Thank you for your insight here. We have edited this sentence as suggested.