

Conformational hijacking of lipoprotein transporter LolDF enables precision antimicrobial activity against *Acinetobacter baumannii*

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Pang et al present a manuscript on the interaction of a previously discovered small molecule, abaucin that inhibits the action of the *Acinetobacter baumannii* lipoprotein transporter LolDF. The authors present data on its effectiveness against Carbapenem resistant *Acinetobacter baumannii* (CRAB) clinical isolates and demonstrate the effectiveness of abaucin in a murine pneumonia model. They go on to solve the structure of *A. baumannii* LolDF complexed with abaucin, validate the binding with by in vivo analysis of mutants in the related species. *A. baylyi*. Finally, they synthesise and characterize a derivative of abaucin. Bacterial lipoprotein synthesis and transport is a key target for new antibiotics and understanding the mechanism of an inhibitor is important for the further development of small molecule inhibitors of *A. baumannii*. The efficacy and specificity of the drug had been addressed in a previous study, the novelty here is in the structural determination of the *A. baumannii* LolDF protein and biophysical/biochemical assessment of drug interaction with the purified transporter. However, these characterisations lack key data that are required to support the conclusions; these issues are detailed below.

Major points

1. The authors utilise murine infection models to demonstrate in vivo efficacy and demonstrate a specificity of abaucin that spares bacterial species making up the microbiome. Similar studies were carried out by Liu et al who originally described abaucin and these studies should be directly acknowledged in these sections. In addition, Figure 1 is missing the data for the *Klebsiella* and *E.coli* strains referred to in the main text (line 116) and Figure 1 legend.
2. The most significant part of the paper is the structural determination of *A. baumannii* LolDF in complex with abaucin. As the authors note, there is no published structural information for this class of Lol transporter. However, there is little description of how this structure is similar or different from *E. coli* LolCDE. This section should be expanded to clarify this
3. Following on from the previous point, the authors state (line 196) that no lipoprotein density was obtained in the presence of abaucin, but they do not provide a structure of the transporter in the absence of abaucin. This is vital data which is required to understand the mechanism and provide more confidence that the density ascribed to be abaucin is indeed missing from the abaucin structure. Structures of *E.coli* LolCDE, including those previously published by the authors, show co-purified lipoproteins (either endogenous lipoproteins or co-over expressed). An abaucin-free structure would therefore reasonably be expected to contain lipoprotein and comparison of this with the current abaucin-bound structure are required to draw conclusions about the drug mechanism. If the complex does not contain lipoprotein, then structures of the *A. baylyi* LolDF-lipoprotein complex (see point 5) in the presence and absence of abaucin should be solved.
4. *A. baumannii* LolDF is incubated with abaucin in the presence and absence of ATP (line 181), but both structures presented have ATP bound. Was abaucin binding observed in the absence of ATP? The authors do not further discuss the relevance of the two different abaucin bound states. Are these proposed to be sequential states in the inhibitor binding?
4. *E.coli* LolCDE transports triacylated lipoproteins whereas *A. baumannii* is presumed capable of also transporting diacylated lipoproteins since the enzyme transferring the third acyl chain to lipoproteins is not essential in *Acinetobacter* species. The acyl chain binding sites in the *E.coli* and *A. baumannii* proteins are not necessarily identical. A structure of the transporter in complex with lipoprotein would also verify the binding site of the acyl chains in this transporter rather than inferring the location from the *E.coli* structure (lines 226-8, Extended Data Fig 4). and allow the authors to have more confidence in the effect of the LolF mutations they create (as well as whether the abaucin directly interferes with the acyl chain binding site).

5. The authors utilise *A. baumannii* LolDF expressed in *E. coli* but then also carry out experiments in the related *A. baylyi* strain. The authors should provide a sequence comparison of the *A. baylyi* and *A. baumannii* proteins and if the sequences are different, clarify which protein is being referred to. A lipoprotein (BamE) loaded *A. baylyi* LolDF complex is purified to enable the *in vitro* activity assay. Structural characterisation of this lipoprotein-loaded complex in the presence and absence of abaucin are required to understand how the drug interferes with the lipoprotein transport mechanism. Regarding the *in vitro* transport and ATPase assays, the drug concentration used (20mM) seems very high for compound with micromolar affinity. Was this amount of drug required to see an effect?

6. The Liu study that identified abaucin described LolF mutations, A362T and Y394, that conferred resistance to abaucin in *A. baumannii*. Some comment should be made as to how consistent this is with the proposed binding site in the current study. These mutations increased by 16- and 4-fold respectively. Conversely, mutations in the present study informed by the structure apparently increase the K_d almost 10-fold (e.g. A377D) but barely increase the MIC (from approx. 1 to 1.5 ug/ml), questioning their relevance to the *in vivo* drug binding. It is not clear from the methods whether the LolDF used is complexed with lipoprotein or not and whether it is *A. baumannii* or *A. baylyi* LolDF protein, this should be made clear. Furthermore, does the fitting model take account of the fact that 2 molecules of abaucin are bound per LolDF complex?

7. The authors isolate a derivative of abaucin, AB-1 that is consistently less potent (2-fold) than abaucin but described as more soluble. No details are provided in the methods as to how the solubility comparison was performed and there is no quantitation as to how much more soluble it was, only a photograph of DMSO solubilised compound at an unknown concentration (Figure 5). I note abaucin is sufficiently soluble to be used at 20mM in the *in vitro* assay and ATPase assays (line 854 and 859). Data is also presented demonstrating that neither abaucin nor the AB-1 derivative are hemolytic, but this information would be more appropriate for a supplemental figure. For the characterisation of this derivative to be of sufficient significance for a main figure, then efficacy of the new compound in the *in vivo* model or a comparison demonstrating the relative rates of resistance of *A. baumannii* to the compounds would offer far more insight.

Minor points

Line 77: Mention that at least in some an outer membrane receptor, LolB transfers lipoproteins from LolA into the outer membrane.

Line 79: Clarify that LolDF and LolCDE are the ABC transporter complexes referred to in line 76

Line 88: The deep learning mediate approach that led to the discover of abaucin was not focussed on LolCDE, rather it was aimed to identify small molecules active against *A. baumannii* and the LolDF complex in *A. baumannii* was subsequently discovered as the target.

Line 164: define "OTU"

Line 220: The amino acid at position 375 in LolF is described as F in main text but L in Extended figure 6

Line 232: Misspelling of abaucin.

Line 263: Make clear why LolDFY270A was chosen as a control mutant

Line 844: Correct to "with or without"

Lines 877 and 878: Correct figure references: Fig 4d and Extended data Fig 5d don't exist (presumably Fig 4c and Extended data Fig 5b?)

Figure 33: In the lower panel, in the right-hand lane, DMSO and Abaucin should be marked "+" currently it suggests abaucin is present without DMSO.

Reviewer #2

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3

(Remarks to the Author)

The manuscript by Pang and colleagues is describing the mode of action of the newly identified inhibitor abaucin against the pathogenic *Acinetobacter baumannii*. Previous work identified abaucin as a potent inhibitor against the lipoprotein trafficking transporter LolCDE. Here, the authors characterised the drug in a murine infection model, structurally, by mutagenesis and identified derivatives with better solubility. The murine infection model used clinical isolates and the drug as previously described was able to provide clearance of the infection. The cryo-EM structures identified the binding pose which shows two molecules binding symmetrically and trapping the transporter in an outward-open conformation. The binding site is verified by mutagenesis and binding data. The abaucin prevents or competes with the lipoprotein and prevents the transporter from undergoing a transport cycle. The authors have also attempted to improve the molecule by some modifications and show that loss of trimethylfluorine by a fluoride is capable to retain its activity while improving the relative solubility and toxicity.

Overall this is an excellent study that has been performed to very high standards. It characterises the in-depth activity of abaucin in *in vivo* and *in vitro* and it is likely to have an impact on drug development against *A. baumannii*. I have some comments below that should improve the manuscript and its clarity.

The inclusion of abaucin in the ATPase assay shows only a 50% reduction compared to the uninhibited reactions but these data contradict the loading experiment in Fig 3e. I am not sure the ATPase data are meaningful in their current state as the system is not very relevant along the transport cycle without all the components. The authors should measure the ATPase

activity in the presence of LolA and BamE, and the reduction in ATPase activity is likely to be more significant and correlate with the loading experiment. The current assay only indicates that abaucin can reduce the ATPase activity by interfering with the cycle but the transporter can still go through the whole cycle as it still retains significant activity. The time dependant reduction in ATPase activity is not as significant as the authors portray. It will be more meaningful if they also perform statistical analysis between the 0, 30 and 60min experiments.

The BLI kinetic binding data suggest that ATP is not a requirement for abaucin binding as the experiments were performed in the absence of ATP. Does ATP binding alter the affinity for abaucin?

It is unclear from the manuscript why the loading experiments were performed with *Acinetobacter baylyi*. If it is due to technical issues, the authors should clearly state. They should also report the MIC value of abaucin for *A. baylyi* too.

Line 286-289: the authors should also report theoretical $\log P$ values for the modified compound? Table S2 will also benefit from such values too.

Why was AB-1 not tested in a mouse model, especially if its toxicity is lower than the parent compound. A comprehensive approach would be to look at other toxicity markers between the two drugs.

Can the authors expand the discussion on the analogues and speculate why they have significantly altered MIC behaviour, considering that the trifluoromethyl phenyl group of the abaucin makes only very weak van der Waals interactions with the transporter? Is it likely to be solubility/uptake limitation rather than loss of affinity? (not necessary for the revision, but do the authors have K_d values for the other analogues?). The authors should report/measure the K_d for AB-1 and compare it to the parent compound.

Extended Data Fig. 9: what evidence do the authors have that LolA cannot bind in the presence of abaucin? Is it due to the conformation of the hook?

Extended Data Fig. 7: two cyan triangles in the LolF after position 375 point to a gap region. Please check and correct.

Line 248-249: remove the word non-closable as it is redundant with the next sentence (outward-open conformation).

Are there published genomic data for the clinical isolates in Fig 1? Provide accession codes. Correct Fig 1 and 5b to state clinical isolates not 'clinical isolation'

Lines 358-360: the current sentence implies that AI was used in this study. Rephrase it.

Reviewer #4

(Remarks to the Author)

The manuscript "Molecular basis of precision antimicrobials: conformational hijacking of LolDF by Abaucin in *A. baumannii*" examines the antibacterial effects of an *Acinetobacter*-specific drug, abaucin, in treating clinical strains of *A. baumannii* and inhibiting the essential LolDF protein complex.

A major strength of the manuscript is the elucidation of two different abaucin-inhibited structures of LolDF. This novel structure offers insight into the inhibitory activity of abaucin. The authors present several characterization assays to validate the observed structures. Some of these offer clear insights (e.g. analysis of some resistant variants), but others are confounded. The in vitro analysis of abaucin inhibition and the resulting model for abaucin inhibition of LolDF is also inconsistent with the cryoEM evidence and requires further explanation.

In addition to the cryoEM structural analysis, the manuscript also presents an analysis of abaucin effectiveness against clinically isolated strains of *A. baumannii* that are carbapenem-resistant and demonstrates abaucin efficacy in a pulmonary infection model. These insights are very incremental, expending the results from the abaucin discovery study to a different infection route and to show action against varying clinical strains that all have LolDF. Showing abaucin has no activity against *E. coli* or *K. pneumoniae* or microbiota species is not novel.

A final element of the manuscript presents a chemical effort to test abaucin analogs. Here, the authors offer major conclusions that an abaucin derivative ("AB-1") has better solubility, equivalent antibacterial activity, and less toxicity against human cells. The claim for improved solubility (in DMSO) is weak with the limited evidence presented (Fig. 5a) and seems to have minor impact. The claim for equivalent antibacterial activity is also weak, with AB-1 consistently showing weaker activity than abaucin (MICs in Fig. 5b). The claim for reduced toxicity is not well supported since accounting for ~2-fold increase in MIC for AB-1 compared to abaucin, AB-1 exhibits more HEK cell line toxicity at 1x MIC concentrations than the original abaucin compound.

Additional major comments:

Abaucin mechanism-of-action. CryoEM structures show that abaucin "occupies the substrate-binding cavity and blocks ATP-

dependent cavity". With analysis to lipoprotein liganded LolCDE structure, the authors suggest that abaucin occupies the same cavity that a lipoprotein would occupy in LolDF and prevent its conformational cycling. This is a plausible way for abaucin to inhibit LolDF activity. However, the presented in vitro assay contradicts those observations. Examining purified BamE lipoprotein liganded LolDF complex, the data show that abaucin added to purified BamE-LolDF complex blocks BamE release to the LolA chaperone. How is abaucin able to do this when BamE is already occupying the binding pocket where abaucin needs to bind? The in vitro assay results do not seem to reflect the cryoEM structure very well. The proposed model (extend Fig. 9) does not explain the observed abaucin activity. ATPase inhibition data are clear and it is sensible that abaucin prevents LolDF cycling. But, apparently, abaucin can prevent the transition from state C to state D (ext. Fig. 9) after being liganded. How is the cryoEM evidence consistent with the in vitro data?

Abaucin affinity measurement for LolF variants that are inactive (lethal in vivo) cannot be reliably interpreted and should be removed. The LolF mutation might severely impact complex folding or assembly, and this hasn't been controlled. Analysis of V62D, M63D, L275D, A278D, L285D, F375D, Y378A is therefore not valid. Two of these variants are used to suggest that the door-bar loop is central and sufficient to abaucin engagement. This conclusion is really only supported by A377D and should be reconsidered accordingly.

Several resistant mutations are used to validate the *A. baumannii* LolDF-abaucin cryoEM structures. These mutations are made in the *A. baylyi* lolF gene. The MIC shifts for what are claimed "resistant" mutants are very modest (Fig 4b), at most a 2-fold increased shift in MIC. It doesn't seem that these mutations offer a compelling level of abaucin resistance. It's a bit hard to directly interpret these in vivo data relative to binding data because resistance and binding data are performed using LolF of different *Acinetobacter* species. This requires some explanation or warning for interpretation.

As described above, AB-1 improved solubility is modest and not presented quantitatively. Is abaucin solubility a limitation? At least in animal infection models, it doesn't seem to be a problem.

The AB-1 analog is a very modest alteration to abaucin's chemical structure (Fig 5a), and AB-1 has universally less potency against *A. baumannii* strains tested (Fig 5b, generally a 2-fold MIC increase). The effects of the two compounds on HEK toxicity should therefore not be made on an equal concentration basis. Looking at the lowest MIC scenario of abaucin and AB-1 (1 ug/ml and 2 ug/ml, respectively), the HEK cell line toxicity data is actually worse for AB-1 (35% cell survival at 1x MIC of 2 ug/ml) compared to abaucin (75% cell survival at 1x MIC of 1 ug/ml). In sum, it is not obvious that AB-1 is in any way an improvement over the starting abaucin structure.

AB-1 has a very high MIC against the lab *A. baumannii* strain as ATCC 17978 strain (16 ug/ml). The authors did not test abaucin against this strain (that I can tell). Previous abaucin MICs against ATCC 17978 were reported as ~2 ug/ml. Please measure AB-1 relative potency against this lab strain and comment on the relative efficacy of AB-1.

Minor comments

Please check minor typos throughout. Eg.. "abaucine"

L173-174. The statement is wrong. Abaucin was not originally identified through deep-learning-guided modeling of the *E. coli* LolCDE transporter.

L827: "LolE operon" should be replaced with "bamE gene".

Reviewer #5

(Remarks to the Author)

In their manuscript, Dr. Pan et al. first validated the efficacy and selectivity of abaucin, an antibiotic discovered through deep-learning guided studies, against clinically carbapenem-resistant *A. baumannii* (CRAB) isolates both in vitro and in a neutropenic mouse lung infection model. The authors then use structural biology analysis and demonstrated that abaucin exerts its antibacterial effect by selectively inhibiting the *A. baumannii* lipoprotein transporter LolDF, an essential component of the outer membrane biogenesis pathway. They further modified the scaffold and generated an analogue of abaucin, AB-1, and evaluated its solubility, human cell toxicity, and anti-CRAB activities. The authors suggest that the Lol complex represents an attractive drug target for therapeutic intervention against multidrug-resistant infections.

Acinetobacter baumannii is a multidrug-resistant (MDR) pathogen of significant clinical importance. It causes healthcare-associated infections such as pneumonia and bacteremia. Due to its ability to rapidly acquire resistance to almost all available antibiotics, including carbapenems, *A. baumannii* is listed by WHO and public health agencies in many countries as one of the top MDR pathogens for research and drug development. In this regard, this manuscript addresses an important infectious disease, antimicrobial resistance, and novel antibiotic development area.

Although the in vitro and in vivo evaluation of abaucin and its new analogue, AB-1, has demonstrated their efficacy and safety, the manuscript may be further improved if the authors consider the following comments.

Major comments:

1. Although a non-treatment (vehicle) control and/or an uninfected control (control) were included in most of the experiments, inclusion of additional controls is needed and will likely increase the overall robustness of the study.

a) Ln 154: It is shown that there was no effect of in vivo abaucin treatment on the gut microbiome, an interesting and important study. However, a control group treated with a broad-spectrum antibiotic to demonstrate its effect on the gut microbiome will be important to validate the treatment regimen and technical analysis.

b) Similarly, for the in vivo abaucin efficacy experiment, inclusion of a carbapenem antibiotic in the study to demonstrate its

ineffectiveness will be valuable.

2. The efficacy of AB-1 is not evaluated in the mouse model.

3. Statistical analysis methods are not described in detail.

4. Ln 290: It is nice that the authors have performed some preliminary safety evaluation (cell cytotoxicity and hemolysis) of their newly synthesized AB-1. Please consider including additional cytotoxicity analysis of more advanced assays, which will be important.

Minor comments:

1. More characterization data on the mouse model of intratracheal infection with HX-R7 strain will be needed, such as tissue and blood bacterial burdens, infection course, and bacterial dose titration etc.. These data can be included in the Suppl Data section.

2. Ln 164: Please spell out "OUT";

3. Ln 687: the abaucin administration schedule (every 12 h) is slightly different from that described in Fig. 2a (2, 12, and every 12 hours thereafter);

4. Ln 698: Please provide more details on how the lung injury was scored.

5. Fig. 1: For comparison purposes, please include data for reference *A. baumannii* strains and ampicillin MIC data for gut commensal bacteria and other resistant pathogens.

6. Fig. 2: Please consider including Control Group cytokine data for Fig. 2e and 2f;

7. Fig. 2: The treatment time points and 2nd CTX dose in the figure legend are different from those described in the Materials & Methods.

8. Fig. S1b: Please consider moving the BAL bacterial data into Fig. 1.

9. Fig. 5b: Please include the MIC data of other reference *A. baumannii* strains.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have revised the manuscript which has addressed my major concerns. The removal of the AB-1 data, which was unconvincing, and the addition of the LolDF transporter simultaneously bound to lipoprotein have significantly improved the clarity and impact of the study.

One point remaining is that the abaucin affinity data fitted with a 1: 1 global binding model (main manuscript, Line 969) but this does not seem appropriate given that two molecules of drug are bound per transporter. In fact, in response figure 3.7, the legend states a 1:2 model was used for the same data. Could the authors clarify the model used, please?

A second minor point is that the formatting of the table in Figure 1 could be modified to make the data clearer i.e. insert a break in the line between the "Gut commensal bacteria" and "Multidrug resistance pathogen" data to make it clear that these are separate data. If the line with a single "-" is included just to balance the table, it should be removed. The heading of the left-hand column should be "Clinical isolate" rather than "Clinical isolation."

Those points aside, I believe the manuscript is now suitable for publication in Nature Communications.

Reviewer #2

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3

(Remarks to the Author)

The authors have addressed all my comments with additional data and clarifications. The only concern is the inclusion of the abaucin-lipoprotein complex structure. Inspecting the supplied maps, it appears that the density probably belongs to the LolDF complex and in particular residues 375-381 (the loop is out of density) in one side, and residues 367-381 on the other side (the model is not well built in the density). The lipoprotein density itself is extremely weak and it could also correspond to detergent molecules. The authors have also not identified the type of lipoprotein so it is unclear what the dipeptide represents. The structure is unlikely to represent an inhibitor-lipoprotein complex and I strongly suggest that it is omitted from the manuscript as it is not providing any new insights at its current state.

Reviewer #4

(Remarks to the Author)

My concerns with the prior submission have been adequately addressed. New structures have helped to better define how abaucin interferes in the LolDF transport cycle.

My only comment is that the authors should now also include a discussion of their structures and findings in relation to the recent Nat Comms LoIDF-abaucin publication (PMID 42091888). I think both studies are valuable and some comparative discussion would be beneficial here.

Reviewer #5

(Remarks to the Author)

In their revised manuscript, the authors have made substantial efforts in addressing my initial concerns, including additional experiments and new data. Those modifications have adequately answered my suggestions. However, if the authors can provide the inoculation dose used in their Suppl Fig. 1b, that will be great.

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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Pang et al present a manuscript on the interaction of a previously discovered small molecule, abaucin that inhibits the action of the *Acinetobacter baumannii* lipoprotein transporter LolDF. The authors present data on its effectiveness against Carbapenem resistant *Acinetobacter baumannii* (CRAB) clinical isolates and demonstrate the effectiveness of abaucin in a murine pneumonia model. They go on to solve the structure of *A. baumannii* LolDF complexed with abaucin, validate the binding with by in vivo analysis of mutants in the related species. *A. baylyi*. Finally, they synthesise and characterize a derivative of abaucin. Bacterial lipoprotein synthesis and transport is a key target for new antibiotics and understanding the mechanism of an inhibitor is important for the further development of small molecule inhibitors of *A. baumannii*. The efficacy and specificity of the drug had been addressed in a previous study, the novelty here is in the structural determination of the *A. baumannii* LolDF protein and biophysical/biochemical assessment of drug interaction with the purified transporter. However, these characterisations lack key data that are required to support the conclusions; these issues are detailed below.

Major points

[Comment 1]

The authors utilise murine infection models to demonstrate in vivo efficacy and demonstrate a specificity of abaucin that spares bacterial species making up the microbiome. Similar studies were carried out by Liu et al who originally described abaucin and these studies should be directly acknowledged in these sections. In addition, Figure 1 is missing the data for the *Klebsiella* and *E.coli* strains referred to in the main text (line 116) and Figure 1 legend.

[Response 1]

We thank the reviewer for this helpful comment. We have now explicitly acknowledged the seminal study by Liu et al. in the revised manuscript, including at the point of abaucin's initial identification (line 75) and in the sections describing its microbiome-sparing selectivity (line 108) and in vivo efficacy (line 114 and 143).

To clarify the scope of our work, we have revised the text to emphasize that our MIC profiling, murine pneumonia model, and faecal microbiota analyses are intended to extend and validate the original characterisation of abaucin using independently collected clinical isolates and a distinct *in vivo* infection model, rather than to present these properties as entirely novel observations.

Regarding Fig. 1, we apologize for the lack of clarity. The data for *Klebsiella pneumoniae* and *E. coli* strains are included in the right-hand side of the lower panel. We have revised both the main text and figure legend to explicitly describe these strains as carbapenem-resistant *Klebsiella pneumoniae* and ESBL-producing *E. coli* to ensure consistency and improving clarity.

Clinical isolation	Species	β -lactamase genes	MIC (μ g/mL)	
			Abaucin	Ampicillin
HX-S9	<i>A. baumannii</i>	<i>blaADC-25,blaOXA-51</i>	1-2	8-16
HX-S19	<i>A. baumannii</i>	<i>blaADC-25,blaOXA-23,blaOXA-66,blaTEM-1D</i>	8-16	>128
HX-S31	<i>A. baumannii</i>	<i>blaADC-25,blaOXA-66,blaTEM-1D</i>	8-16	>128
HX-R7	<i>A. baumannii</i>	<i>blaADC-25,blaOXA-23,blaOXA-66</i>	8-16	>128
HX-R9	<i>A. baumannii</i>	<i>blaADC-25,blaOXA-23,blaOXA-66,blaTEM-1D</i>	8-16	>128
HX-R28	<i>A. baumannii</i>	<i>blaADC-25,blaCARB-5,blaOXA-23,blaOXA-91</i>	8-16	>128
Gut commensal bacteria		Multidrug resistance pathogen	MIC (μ g/mL) Abaucin	MIC (μ g/mL) Abaucin
<i>B. vulgatus</i>	>128			>128
<i>B. thetaiotaomicron</i>	>128	-		-
<i>B. fragilis</i>	>128	CRKP		>128

Fig. 1: MICs of abaucin against clinically isolated Gram-negative pathogens and gut commensal bacteria. MICs were determined for carbapenem-susceptible (HX-S9, HX-S19, HX-S31) and carbapenem-resistant (HX-R7, HX-R9, HX-R28) clinical isolates of *A. baumannii*, extended-spectrum β -lactamase (ESBL)-producing *E. coli* (HX-1-7), and carbapenem-resistant *Klebsiella pneumoniae* (CRKP, HX-1-13), as well as representative gut commensal isolates (*Bacteroides vulgatus*, HX-25-48-A1, *Bacteroides thetaiotaomicron*, HX-25-52-D4), *Bacteroides fragilis*, HX-25-56-I2). All strains were isolated from West China Hospital, Sichuan University. MIC measurements were consistent across three independent experiments initiated from single colonies.

[Comment 2]

2. The most significant part of the paper is the structural determination of *A. baumannii* LolDF in complex with abaucin. As the authors note, there is no published structural information for this class of Lol transporter. However, there is little description of how this structure is similar or different from *E. coli* LolCDE. This section should be expanded to clarify this

[Response 2]

We thank the reviewer for this important comment. We agree that the original submission did not sufficiently clarify the structural comparison between *A. baumannii* LolDF and *E. coli* LolCDE. This section has now been revised to provide a clearer and more systematic comparison between the two transporters (Line 181-208).

The revised text describes the conserved structural features shared by LolDF and LolCDE, including the type VII ABC transporter architecture, the transmembrane core, the Sabre and Porter

periplasmic subdomains, and the cytoplasmic association with LolD. It also now defines the main distinguishing features of LolDF more clearly, including its symmetric LolF organisation and the broader outward-open central cavity flanked by paired door-bar elements, whereas LolCDE exhibits an intrinsically asymmetric and functionally differentiated cavity. These additions place the abaucin-bound LolDF structure in a clearer comparative context and better define the structural features of this noncanonical transporter.

[Comment 3]

3. Following on from the previous point, the authors state (line 196) that no lipoprotein density was obtained in the presence of abaucin, but they do not provide a structure of the transporter in the absence of abaucin. This is vital data which is required to understand the mechanism and provide more confidence that the density ascribed to be abaucin is indeed missing from the abaucin structure. Structures of *E.coli* LolCDE, including those previously published by the authors, show co-purified lipoproteins (either endogenous lipoproteins or co-overexpressed). An abaucin-free structure would therefore reasonably be expected to contain lipoprotein and comparison of this with the current abaucin-bound structure are required to draw conclusions about the drug mechanism. If the complex does not contain lipoprotein, then structures of the *A. baylyi* LolDF-lipoprotein complex (see point 5) in the presence and absence of abaucin should be solved.

[Response 3]

We thank the reviewer for this important comment and agree that comparison with a lipoprotein-bound state is critical to support the proposed mechanism.

To address this, we have determined an additional cryo-EM structure of a lipoprotein-loaded *A. baumannii* LolDF complex in the presence of abaucin (Fig. 5c). Specifically, we co-expressed the *A. baumannii* LolDF and lipoprotein BamE in *A. baylyi* and incubated with abaucin. In this structure, the central cavity contains extra densities for two abaucin molecules and the lipoprotein N-terminus (Fig. 5c).

This new structure exhibits a conformation resembling the initially determined narrow-cavity abaucin-LolDF structure, showing a outward-open central cavity, a typical feature of the substrate-bound state of type VII ABC transporters. This suggests that abaucin binding does not prevent lipoprotein binding, but stabilizes the transporter in a substrate-accessible open conformation. Consistent with this, our in vitro transport assay shows that abaucin does not reduce the amount of BamE associated with LolDF but impairs transfer to LolA. The ATPase of the LolDF complex in the presence of abaucin and components of transport reaction was also diminished.

These findings support a model in which abaucin inhibits a post-binding step in lipoprotein transport.

Although an abaucin-free structure of *A.baumannii* LolDF was not obtained, the newly determined substrate-associated complex directly addresses the review's concern by demonstrating lipoprotein occupancy in the presence of abaucin and enabling mechanistic interpretation of its inhibitory effect. We have revised the manuscript accordingly to (i) include this new structure, (ii) clarify the interpretation of density in the abaucin-bound state, and (iii) avoid overinterpretation of the absence of lipoprotein density in the initial structure. These revisions are described in Page 14, result section from lines 278 in the revised manuscript.

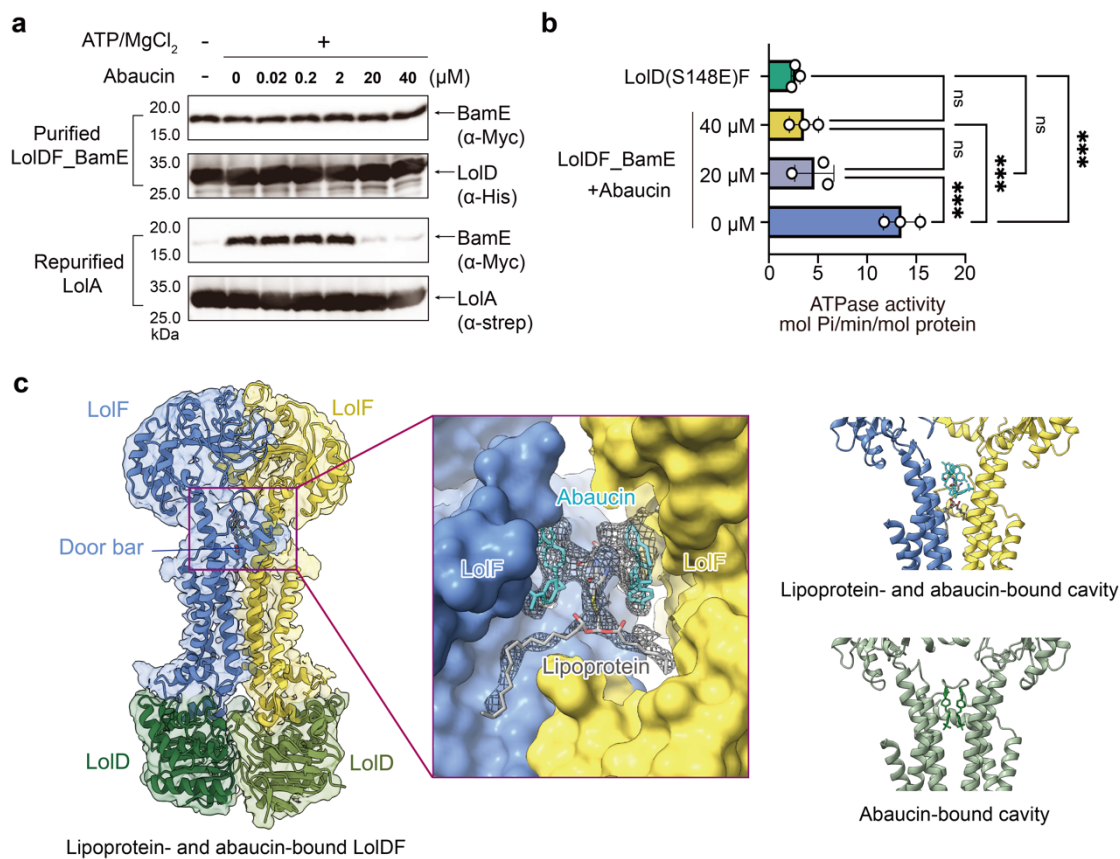


Fig. 5: Abaucin inhibits lipoprotein transfer while sparing lipoprotein extraction. **a**, *In vitro* lipoprotein transport assay in the presence of ATP, MgCl₂, and abaucin. **b**, ATPase activity of detergent-solubilised LolDF-BamE in the presence of LolA and abaucin (***p* < 0.001). Abaucin reduced LolDF-associated ATPase activity to levels comparable to those of the ATPase-impaired LolD(S148E)F control. Data in (a) and (b) are representative of *n* = 3 independent experiments; data in (b) are means ± s.d. Statistical source data for (b) are provided in Source Data. **c**, Cryo-EM structure of the lipoprotein-associated abaucin-bound *A. baumannii* LolDF complex. LolF protomers are shown in blue and yellow, and LolD protomers in green and olive green. The central inset shows density within the transmembrane cavity fitted with two abaucin molecules and the N-terminus of a lipoprotein substrate, including two acyl chains and a short peptide segment. The right panel compares the open cavity conformations of the lipoprotein-associated, abaucin-bound LolDF complex and the abaucin-bound LolDF structures in the absence of substrate.

[Comment 4]

4. *A. baumannii* LolDF is incubated with abaucin in the presence and absence of ATP (line 181), but both structures presented have ATP bound. Was abaucin binding observed in the absence of ATP? The authors do not further discuss the relevance of the two different abaucin bound states. Are these proposed to be sequential states in the inhibitor binding?

[Response 4]

We thank the reviewer for pointing out this inconsistency. The original description of the sample preparation was inaccurate and has now been corrected. The text from line 174 has been revised to clarify that the structures presented in the original submission were obtained from *A. baumannii* LolDF incubated with abaucin in the presence of ATP. To address whether ATP is required for ligand binding, we refer to the additional structure described in Response 3, namely the lipoprotein-associated abaucin-bound *A. baumannii* LolDF, which was obtained in the absence of ATP (Fig. 5c, above). In this ATP-free complex, abaucin adopts a binding mode highly similar to those observed in the ATP-bound structure, indicating that ATP is not required for abaucin binding. The dataset has been incorporated into the revised manuscript to clarify this point.

We have also revised the manuscript to better define the relationship between the two abaucin-bound conformations. We do not interpret the wide and narrow states as definitive sequential stages of inhibitor binding. Rather, they are described as related conformations within an outward-open ensemble, differing primarily in the degree of cavity compaction. In LolCDE, ATP binding is coupled to NBD dimerization and concerted movements of the TMDs and PDs towards the central axis, leading to closure of the substrate-binding cavity and substrate extrusion. By contrast, in both abaucin-bound LolDF structures, the NBDs remain undimerized despite the presence of ATP and magnesium ions. This indicates that the observed narrowing of the transmembrane cavity is not driven by the canonical ATP-dependent allosteric cycle. Instead, it is more consistent with a local conformational response associated with abaucin binding. These revisions are described in Page 11, Lines 190 to 224 of the revised manuscript with highlighted changes.

[Comment 5]

5. *E.coli* LolCDE transports triacylated lipoproteins whereas *A. baumannii* is presumed capable of also transporting diacylated lipoproteins since the enzyme transferring the third acyl chain to lipoproteins is not essential in *Acinetobacter* species. The acyl chain binding sites in the *E.coli* and

A. baumannii proteins are not necessarily identical. A structure of the transporter in complex with lipoprotein would also verify the binding site of the acyl chains in this transporter rather than inferring the location from the *E. coli* structure (lines 226-8, Extended Data Fig 4). and allow the authors to have more confidence in the effect of the LolF mutations they create (as well as whether the abaucin directly interferes with the acyl chain binding site).

[Response 5]

We agree that the substrate-binding site of *A. baumannii* LolDF should not be inferred directly from *E. coli* LolCDE, particularly given the likely differences in substrate handling between the two systems. Accordingly, we have removed the corresponding statement from the revised manuscript (line 209).

As shown in the lipoprotein-associated, abaucin-bound LolDF structure described above, the additional substrate density adopts a characteristic “straddle” configuration resembling the N-amine-linked acyl chains observed in lipoprotein-bound *E. coli* LolCDE structures (Fig. 5c, above). Notably, the two abaucin molecules remain positioned on either side beneath the door bar loops, suggesting that abaucin does not directly compete with the substrate binding.

However, because the local resolution in this region does not permit detailed analysis of the lipid-drug interface, we use this structure primarily as supportive evidence for substrate co-occupancy and a post-binding mode of inhibition, rather than for detailed residue-level interpretation of substrate recognition or acyl-chain interactions. We have revised the manuscript accordingly.

[Comment 6]

6. The authors utilise *A. baumannii* LolDF expressed in *E. coli* but then also carry out experiments in the related *A. baylyi* strain. The authors should provide a sequence comparison of the *A. baylyi* and *A. baumannii* proteins and if the sequences are different, clarify which protein is being referred to. A lipoprotein (BamE) loaded *A. baylyi* LolDF complex is purified to enable the in vitro activity assay. Structural characterisation of this lipoprotein-loaded complex in the presence and absence of abaucin are required to understand how the drug interferes with the lipoprotein transport mechanism. Regarding the in vitro transport and ATPase assays, the drug concentration used (20mM) seems very high for compound with micromolar affinity. Was this amount of drug required to see an effect?

[Response 6]

We thank the reviewer for these helpful comments and for highlighting points that required clarification. We address each issue below.

(1) Use *A. baumannii* and *A. baylyi* LolDF

We agree that the use of proteins from different species for structural and functional assays may have caused confusion. To address this, we have repeated the transport assay using purified *A. baumannii* LolDF (Fig. 5a, above), consistent with the protein used for the structural study. For these experiments, *A. baumannii* LolDF was expressed in *A. baylyi* (ADP1), a non-pathogenic and genetically tractable close relative of *A. baumannii*, rather than in *E. coli*. This change was made because protein expressed in *E. coli* exhibited low substrate occupancy, likely reflecting a difference in substrate handling between the systems. In contrast, the complex expressed from *A. baylyi* showed good substrate occupancy (Supplementary Fig. 8a-c) and remained stable during cryo-EM sample preparation. Moreover, LolDF is highly conserved between the *A. baumannii* and *A. baylyi* (~88% sequence identity, Supplementary Fig. 7a), and *A. baumannii* LolDF functionally complements the lolDF-silenced *A. baylyi* (Supplementary Fig. 7b and 6b), supporting functional conservation between the two species. Therefore, the *A. baumannii* LolDF-BamE complex expressed in *A. baylyi* was used for the in vitro transport assays. We have replaced the transport assay data accordingly and revised the manuscript to clarify the expression system and species to avoid ambiguity.

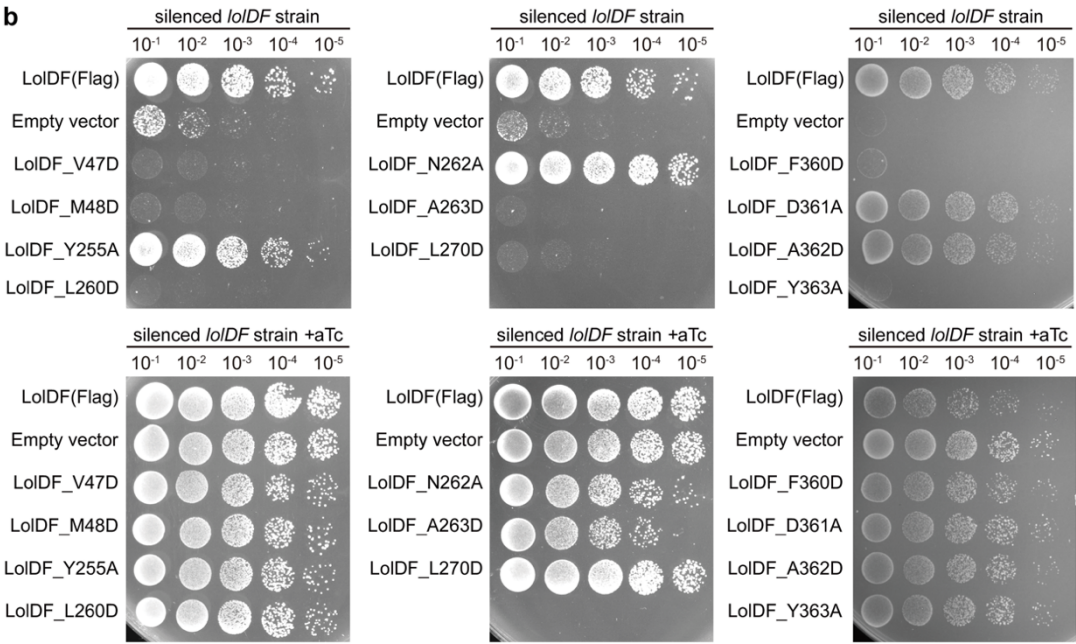
(2) Structural characterisation of the lipoprotein-loaded complex

As described in Responses 3 and 5, we have now determined a cryo-EM structure of a lipoprotein-loaded LolDF complex in the presence of abaucin (Fig. 5c). In this structure, both lipoprotein and abaucin are observed within the central cavity, demonstrating that abaucin does not prevent substrate binding but instead co-occupies the cavity. Together with the transport assay results, these data support a mechanism in which abaucin inhibits a post-binding step in lipoprotein transport.

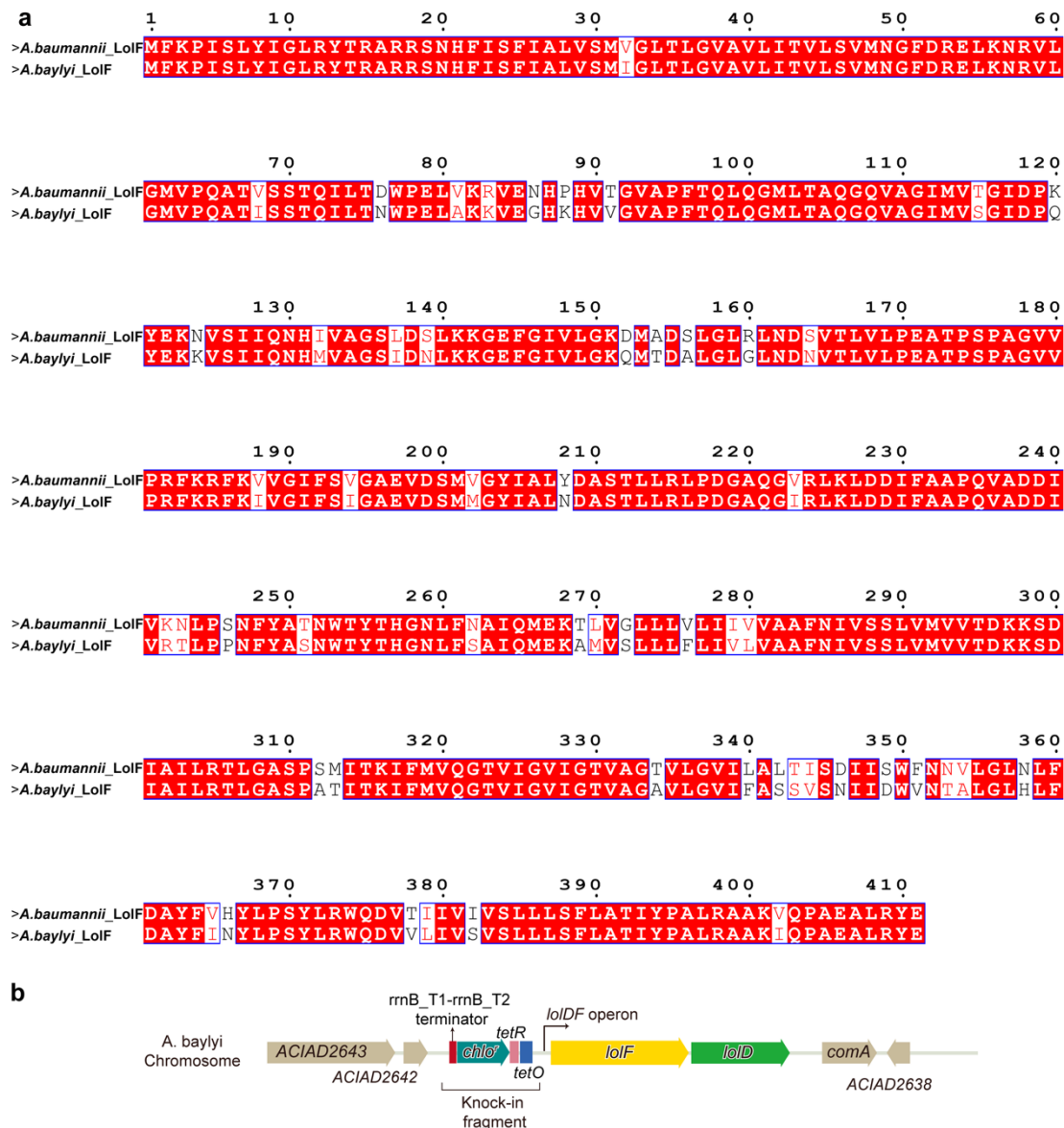
(3) Abaucin concentration used in functional assays

We apologize for the error in the original manuscript. The reported concentration of 20 mM was incorrect; all experiments were performed at 20 μ M abaucin. This has been corrected. To further address the reviewer's concern, we have performed additional dose-response transport and ATPase assays using BamE-loaded *A. baumannii* LolDF. These data show that abaucin inhibits LolDF-dependent transport in a concentration-dependent manner, with significant inhibition observed at 20 μ M and 40 μ M. Consistently, ATPase activity is reduced under these conditions to

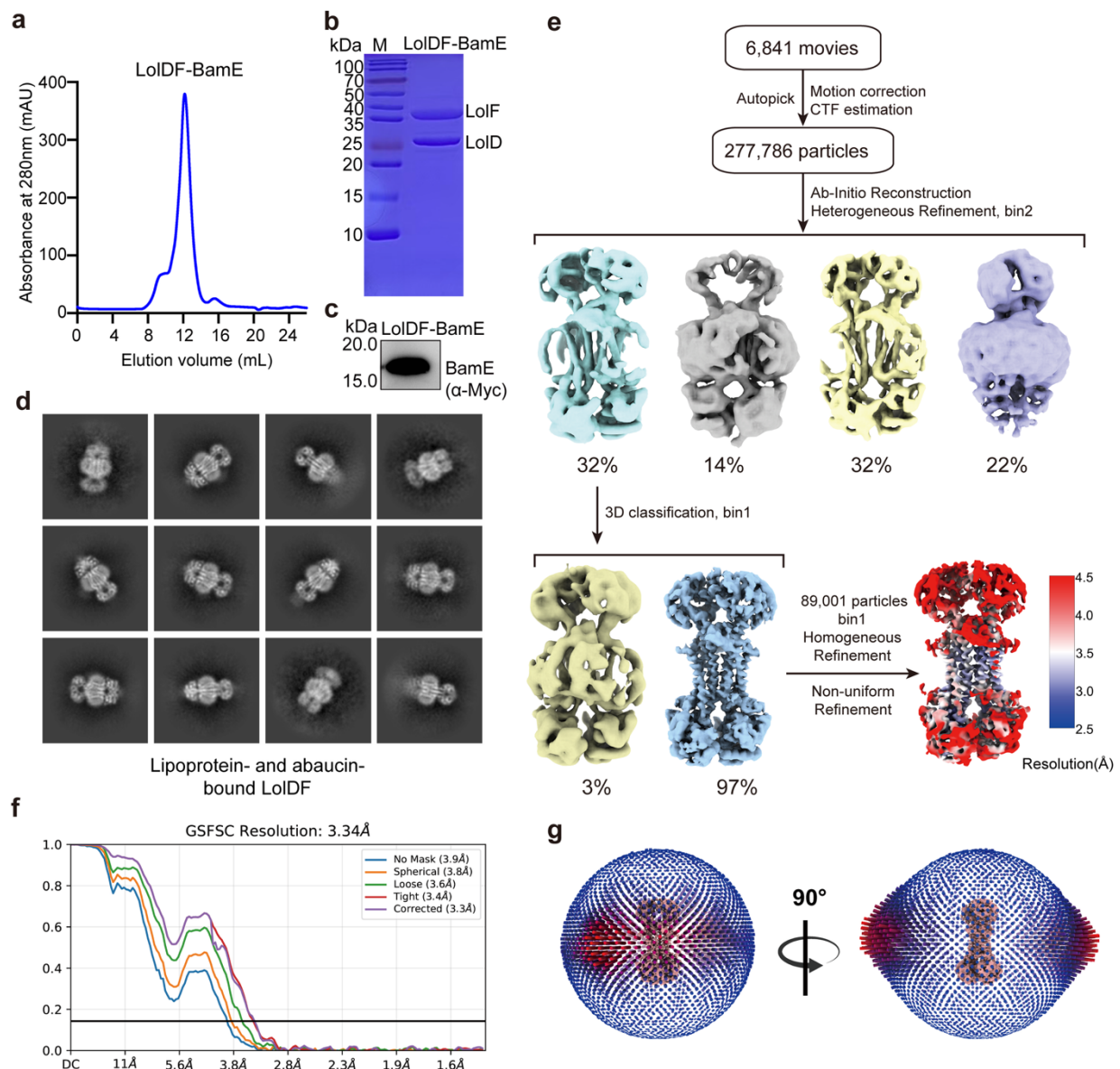
a level comparable to that of the catalytic mutant LolD(S148E)F (Fig. 5a, b). The revised manuscript now includes these data and clarifies the experimental conditions (Page 13, Result section from lines 344).



Supplementary Fig. 6 Recovery of the *lolDF*-silenced strain and expression levels of LolDF mutants. (b) Cell viability of TetR-regulated *lolDF*-silenced *A. baylyi* strain with or without anhydrotetracycline (aTc). Data in (b) are representative of $n = 3$ independent experiments.



Supplementary Fig. 7 Sequence alignment of *A. baumannii* and *A. baylyi* LolF. (a) Sequence alignment of LolF from *A. baumannii* LolF and *A. baylyi*, showing 88% sequence identity. **(b)** Schematic of the engineered *A. baylyi* lolDF operon, in which a TetR-regulated repression module containing an rrnB T1-rrnB T2 terminator, chloramphenicol-resistance cassette and *tetO* operator was inserted upstream of *lolF*.



Supplementary Fig. 8 Cryo-EM structure determination of the abaucin-bound LolDF-lipoprotein complex. (a) Gel-filtration profile for the purified BamE-associated *A. baumannii* LolDF expressed in *A. baylyi*. (b) SDS-PAGE analysis of the purified BamE-associated *A. baumannii* LolDF. (c) Immunoblotting analysis for the presence of BamE associated in the complex. (d) Representative 2D classification of abaucin-bound LolDF with lipoprotein. (e) Cryo-EM data collection, processing, 3D classification and 3D refinement for abaucin-bound LolDF with lipoprotein. (f) Gold-standard Fourier shell correlation (FSC) curves of the final cryo-EM map for abaucin-bound LolDF with lipoprotein. (g) Corresponding angular distribution map of the particles.

[Comment 7]

7. The Liu study that identified abaucin described LolF mutations, A362T and Y394, that conferred resistance to abaucin in *A. baumannii*. Some comment should be made as to how consistent this is with the proposed binding site in the current study. These mutations increased by 16- and 4-fold

respectively. Conversely, mutations in the present study informed by the structure apparently increase the K_d almost 10-fold (e.g. A377D) but barely increase the MIC (from approx. 1 to 1.5 $\mu\text{g/ml}$), questioning their relevance to the in vivo drug binding. It is not clear from the methods whether the LolDF used is complexed with lipoprotein or not and whether it is *A. baumannii* or *A. baylyi* LolDF protein, this should be made clear. Furthermore, does the fitting model take account of the fact that 2 molecules of abaucin

[Response]

We thank the reviewer for these constructive comments and address each point below.

As shown in the sequence alignment below (Response Figure 1.7), residue A362 reported by Liu *et al.* corresponds to A377 in the original numbering used for model building in this study. This discrepancy arose because the initial residue numbering began 15 positions upstream of the authentic initiator methionine, owing to an annotation carry-over during construct design. To eliminate potential confusion and maintain consistency with both the Liu *et al.* study and the NCBI reference sequence (GenBank accession CP053098.1), we have now renumbered the entire sequence accordingly. All occurrences of A377 have been corrected to A362 in the revised manuscript. Importantly, A362 maps to the door-bar loop in our cryo-EM structure and lies within the abaucin-binding cavity, consistent with the resistance mutations reported by Liu *et al.* This agreement supports the assignment of the inhibitor-binding site (revised manuscript line 257).

The reviewer noted that the A362D substitution in our study resulted in a ~10-fold increase in K_D , whereas the A362T mutation reported by Liu *et al.* confers a 16-fold increase in resistance. These results are directionally consistent: weakened binding in the purified LolDF assay and increased resistance in cells both identify A362 as an important determinant of abaucin recognition.

We clarify that both MIC and affinity measurement were conducted using *A. baumannii* LolDF expressed in *A. baylyi*. This experimental design was necessitated by the genetic intractability and biosafety restrictions associated with pathogenic *A. baumannii*, and follows established practice of using non-pathogenic surrogate hosts. However, we agree that cellular susceptibility is inherently multifactorial. Factors such as membrane permeability, expression level, genetic manipulation, and pathway buffering can attenuate the phenotypic impact of altered target engagement. In light of these considerations, and to avoid overinterpretation, we have removed the MIC data from the revised manuscript. The consistency of key binding-site residues (aa377 and aa362) identified in both our study and that of Liu *et al.*, and the results of our mutant binding affinity and morphological assays support the structural assignment and reinforce the validity of our model.

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LoIF NCBI 1 MFKPISLYIGLRYTRARRSNHFISFIALVSMVGLTLGVAVLITVLSVMNGFDRELKNRVL 60
LoIF 15 MFKPISLYIGLRYTRARRSNHFISFIALVSMVGLTLGVAVLITVLSVMNGFDRELKNRVL 75
*****

GMVPQATVSSTQILTDWPELVKRVENHPHVTGVAPFTQLQGMLTAQQQVAGIMVTGIDPK 120
GMVPQATVSSTQILTDWPELVKRVENHPHVTGVAPFTQLQGMLTAQQQVAGIMVTGIDPK 135
*****

YEKNVSIIQNHIVAGSLDSLKKGEFGIVLGKDMADSLGLRLNDSVTLVLPEATPSPAGVV 180
YEKNVSIIQNHIVAGSLDSLKKGEFGIVLGKDMADSLGLRLNDSVTLVLPEATPSPAGVV 195
*****

PRFKRFKVVGIFSVGAEVDSMVG YIALYDASTLLRLPDGAQGVRLKLD DIFAAPQVADDI 240
PRFKRFKVVGIFSVGAEVDSMVG YIALYDASTLLRLPDGAQGVRLKLD DIFAAPQVADDI 255
*****

VKNLPSNFYATNWTYTHGNLFNAIQMEKTLVGLLLVLIIVVAAFNI VSSLVMVVTDKKSD 300
VKNLPSNFYATNWTYTHGNLFNAIQMEKTLVGLLLVLIIVVAAFNI VSSLVMVVTDKKSD 315
*****

IAILRTLGA SPMITKIFMVQGT VIGVIGTVAGTVLGVILALTISDIISWFNNVLGLNLF 360
IAILRTLGA SPMITKIFMVQGT VIGVIGTVAGTVLGVILALTISDIISWFNNVLGLNLF 375
*****
377
DAYFVHYLPSYLRWQDVTIIVIVSLLLSFLATIYPALRAAKVQPAEALRYE 411
DAYFVHYLPSYLRWQDVTIIVIVSLLLSFLATIYPALRAAKVQPAEALRYE 426
*****

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Response Figure 1.7: Alignment of LoIF in this study with the reference sequence. Sequence alignment between the LoIF used in the original manuscript and the NCBI reference sequence shows a uniform 15-residue offset. Thus, residue A377 in the original manuscript corresponds to A362, the position reported by Liu *et al.* The boxed alanine indicates the equivalent residue in the two numbering schemes.

[Comment 8]

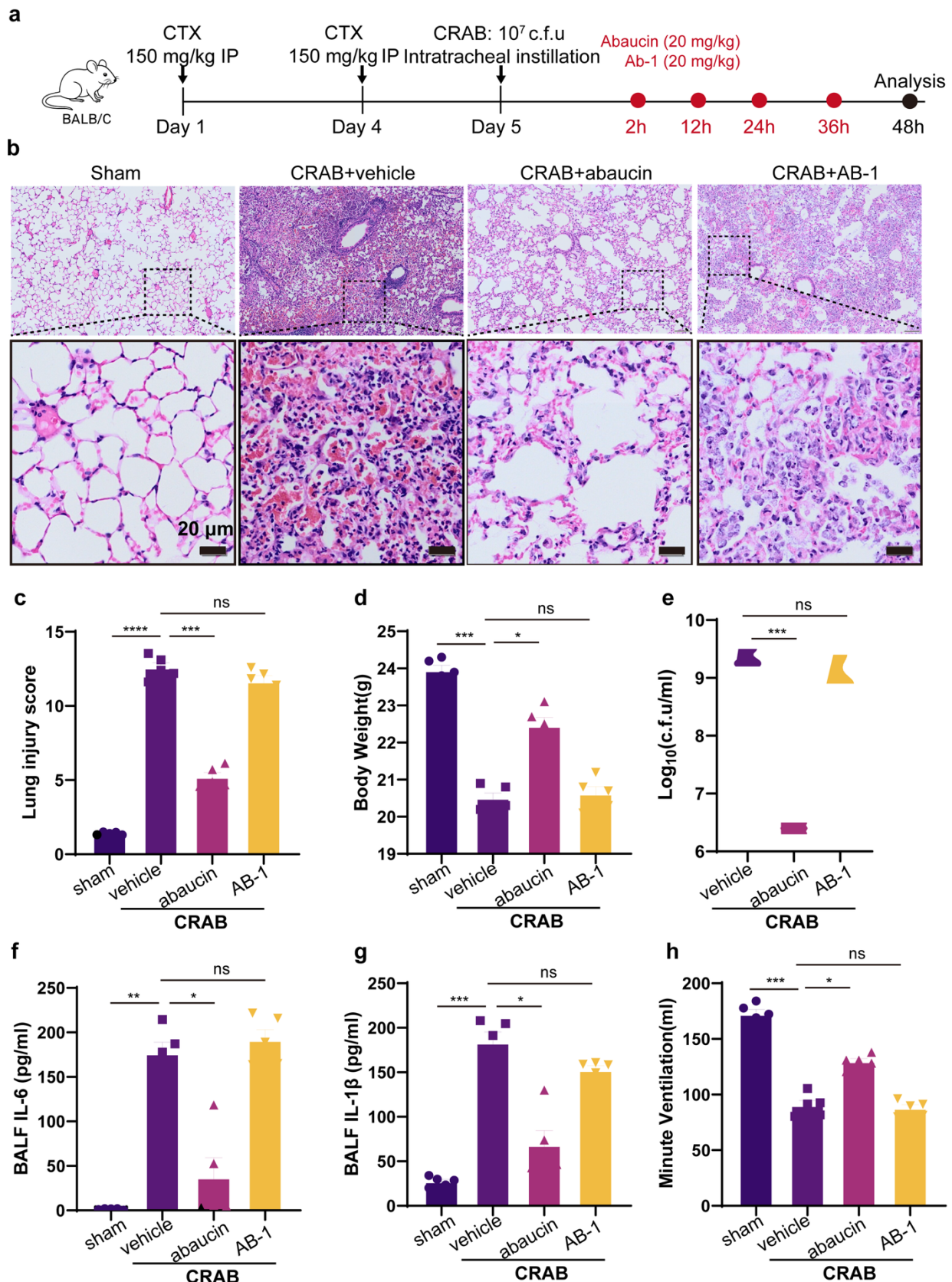
7. The authors isolate a derivative of abaucin, AB-1 that is consistently less potent (2-fold) than abaucin but described as more soluble. No details are provided in the methods as to how the solubility comparison was performed and there is no quantitation as to how much more soluble it was, only a photograph of DMSO solubilised compound at an unknown concentration (Figure 5). I note abaucin is sufficiently soluble to be used at 20mM in the in vitro assay and ATPase assays (line 854 and 859). Data is also presented demonstrating that neither abaucin nor the AB-1 derivative are hemolytic, but this information would be more appropriate for a supplemental figure. For the characterisation of this derivative to be of sufficient significance for a main figure, then

efficacy of the new compound in the *in vivo* model or a comparison demonstrating the relative rates of resistance of *A. baumannii* to the compounds would offer far more insight.

[Response]

We thank the reviewer for this thoughtful comment. We agree that the characterisation of AB-1 in the original submission was not sufficiently complete, particularly given the lack of quantitative solubility analysis and the limited significance of the preliminary haemolysis data on their own.

To address the reviewer's broader concern, we assessed AB-1 in the murine CRAB pneumonia model. Under the conditions tested, AB-1 showed limited *in vivo* efficacy and was consistently less effective than abaucin (Response Figure 1.8). In light of this, and to maintain a clearer focus on the central findings of the study, we have removed the AB-1 section from the revised manuscript. This includes the associated figure and discussion. We believe this revision improves the overall clarity and impact of the manuscript by focusing on the mechanistic and *in vivo* characterization of abaucin supported by robust data.



Response Figure 1.8: **a**, Schematic of treatment groups and dosing regimen for the murine pulmonary infection model. BALB/c mice were assigned to the indicated groups: untreated Control (sham), sham-infected vehicle controls, and infected animals treated with abaucin (20 mg kg⁻¹) and AB-1 (20 mg kg⁻¹). Compounds were administered via intratracheal instillation. Values

listed denote the timing of sample collection or analysis post-infection (hours). **b**, Representative haematoxylin and eosin (H&E)-stained lung sections from sham, vehicle-treated, abaucin-treated, and AB-1-treated mice (scale bar, 20 μ m). **c**, Lung injury scores derived from histopathological assessment of lung sections in (c) ($n = 5$, *** $p < 0.001$, **** $p < 0.0001$). **d**, Body weight changes in sham, vehicle-treated, abaucin-treated and AB-1-treated mice over the course of infection ($n = 5$, * $p < 0.05$; *** $p < 0.001$). **e**, Bacterial burden in BALF from vehicle-treated, abaucin-treated, and AB-1-treated mice, determined by colony-forming unit (c.f.u.) counts after serial dilution ($n = 3$, **** $p < 0.0001$). **f-g**, Concentrations of IL-6 and IL-1 β in BALF from sham, vehicle-treated, abaucin-treated, and AB-1-treated mice ($n = 5$, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). **h**, Lung function parameters, including minute ventilation (MV; h), in sham, vehicle-treated, abaucin-treated, and AB-1-treated mice with CRAB-induced pneumonia ($n = 5$, * $p < 0.05$; *** $p < 0.001$). Data are shown as mean $\pm$ s.e.m, Statistical significance was determined as described in Methods; ns, not significant.

Minor points

Line 77: Mention that at least in some an outer membrane receptor, LolB transfers lipoproteins from LolA into the outer membrane.

Thank the reviewer for this helpful suggestion. The sentence has been revised as follows: After maturation on the inner membrane⁴⁰, lipoproteins are extracted by the ATP-binding cassette (ABC) transporter complex and transferred to the periplasmic chaperone LolA. In organisms encoding the outer-membrane receptor LolB, LolA subsequently delivers these lipoproteins to LolB for insertion into the OM⁴¹⁻⁴⁴.

Line 79: Clarify that LolDF and LolCDE are the ABC transporter complexes referred to in line 76

The text has been revised as follows: Although the overall pathway is conserved, the ABC transport machinery differs among species: *A. baumannii* employs the LolDF complex, whereas *Escherichia coli* (*E. coli*) uses the LolCDE system^{45,46}.

Line 88: The deep learning mediate approach that led to the discover of abaucin was not focussed on LolCDE, rather it was aimed to identify small molecules active against *A. baumannii* and the LolDF complex in *A. baumannii* was subsequently discovered as the target.

We thank the reviewer for this important comment. The sentence has been revised to clarify that abaucin was identified through deep-learning-guided screening for compounds active against *A. baumannii*, rather than through a target-based search. The revised text now reads: "A recent machine-learning-guided screen identified abaucin, an antibiotic that selectively targets *A. baumannii* by perturbing lipoprotein trafficking, providing a unique opportunity to develop a pathogen-selective therapeutic".

Line 164: define “OTU”

We thank the reviewer for pointing this out. Rather than defining “OTU” at this point, we have removed the term from the revised text because the corresponding microbiota panel reports taxonomically aggregated relative abundances rather than OTU-level composition. To avoid ambiguity, the revised manuscript now describes this result as a change in faecal microbial composition.

Line 220: The amino acid at position 375 in LolF is described as F in main text but L in Extended figure 6

We thank the reviewer for identifying this error. Extended Data Fig. 6 has been corrected accordingly. The residue at position 375 in LolF is phenylalanine (F), rather than leucine (L).

Line 232: Misspelling of abaucin.

We thank the reviewer for identifying this typographical error. The text has been corrected accordingly.

Line 263: Make clear why LolDFY270A was chosen as a control mutant

We thank the reviewer for this comment. LolDF^{Y270A} was used as a negative-control mutant because Tyr270 is located outside the abaucin-binding interface and is not predicted to contact the ligand directly. We agree that the rationale for its inclusion was not sufficiently clear and have removed this point in the revised text.

Line 844: Correct to “with or without”

We thank the reviewer for this comment. The text has been corrected to “with or without” .

Lines 877 and 878: Correct figure references: Fig 4d and Extended data Fig 5d don’t exist (presumably Fig 4c and Extended data Fig 5b?)

We thank the reviewer for pointing out these incorrect figure citations. The references have now been corrected to Extended Data Fig. 7b.

Figure 33: In the lower panel, in the right-hand lane, DMSO and Abaucin should be marked “+” currently it suggests abaucin is present without DMSO.

We thank the reviewer for pointing out this labelling error. The figure has been replaced with new dose-dependent assay, now in Fig. 5a.

Reviewer #2 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Thank you very much for taking the time and effort to review our manuscript. We greatly appreciate your insightful comments, which have helped us improve the quality of our work. We have carefully addressed all the points raised and have revised the manuscript accordingly. We hope that the revised manuscript resolves any remaining concerns.

Reviewer #3 (Remarks to the Author):

The manuscript by Pang and colleagues is describing the mode of action of the newly identified inhibitor abaucin against the pathogenic *Acinetobacter baumannii*. Previous work identified abaucin as a potent inhibitor against the lipoprotein trafficking transporter LolCDE. Here, the authors characterised the drug in a murine infection model, structurally, by mutagenesis and identified derivatives with better solubility. The murine infection model used clinical isolates and the drug as previously described was able to provide clearance of the infection. The cryo-EM structures identified the binding pose which shows two molecules binding symmetrically and trapping the transporter in an outward-open conformation. The binding site is verified by mutagenesis and binding data. The abaucin prevents or competes with the lipoprotein and prevents the transporter from undergoing a transport cycle. The authors have also attempted to improve the molecule by some modifications and show that loss of trimethylfluorine by a fluoride is capable to retain its activity while improving the relative solubility and toxicity.

[Comment 1]

Overall this is an excellent study that has been performed to very high standards. It characterises the in-depth activity of abaucin in in vivo and in vitro and it is likely to have an impact on drug development against *A. baumannii*. I have some comments below that should improve the manuscript and its clarity.

The inclusion of abaucin in the ATPase assay shows only a 50% reduction compared to the uninhibited reactions but these data contradict the loading experiment in Fig 3e. I am not sure the ATPase data are meaningful in their current state as the system is not very relevant along the transport cycle without all the components. The authors should measure the ATPase activity in the presence of LolA and BamE, and the reduction in ATPase activity is likely to be more significant and correlate with the loading experiment. The current assay only indicates that abaucin can reduce the ATPase activity by interfering with the cycle but the transporter can still go through the whole cycle as it still retains significant activity.

[Response]

We thank the reviewer for this insightful comment and agree that the initial ATPase assay, performed in the absence of additional transport components, did not fully capture the physiological transport cycle. To address this, we have re-evaluated the effect of abaucin under more functionally relevant conditions by including both the substrate BamE and the periplasmic chaperone LolA. In this context, we first performed in vitro transport assays across a range of

abaucin concentrations and observed complete inhibition of lipoprotein transfer at 20 and 40 μM (Fig. 5a).

We then measured ATPase activity of the LolDF complex under the same conditions (i.e., in the presence of BamE and LolA). Under these transport-relevant conditions, ATPase activity was markedly reduced in the presence of abaucin (20 and 40 μM), approaching the baseline level observed for the ATP hydrolysis-deficient mutant LolD(S148E) (Fig. 5b). These results reconcile the apparent discrepancy noted by the reviewer and indicate that abaucin more effectively suppresses ATPase activity when the transporter is engaged in an active transport cycle.

In addition, we have determined a new cryo-EM structure of the lipoprotein-associated abaucin-bound LolDF complex (Fig. 5c). This structure reveals that abaucin and lipoprotein co-occupy the central cavity, providing direct structural evidence that substrate binding is not prevented.

Together, the functional assays and structural data support a model in which abaucin acts as a conformational trap, inhibiting a post-binding step in lipoprotein transport rather than competing with substrate loading. The revised manuscript now incorporates these additional data and clarifies the interpretation of ATPase activity in the context of the full transport cycle (Page 14, Result section from lines 278 in the revised manuscript).

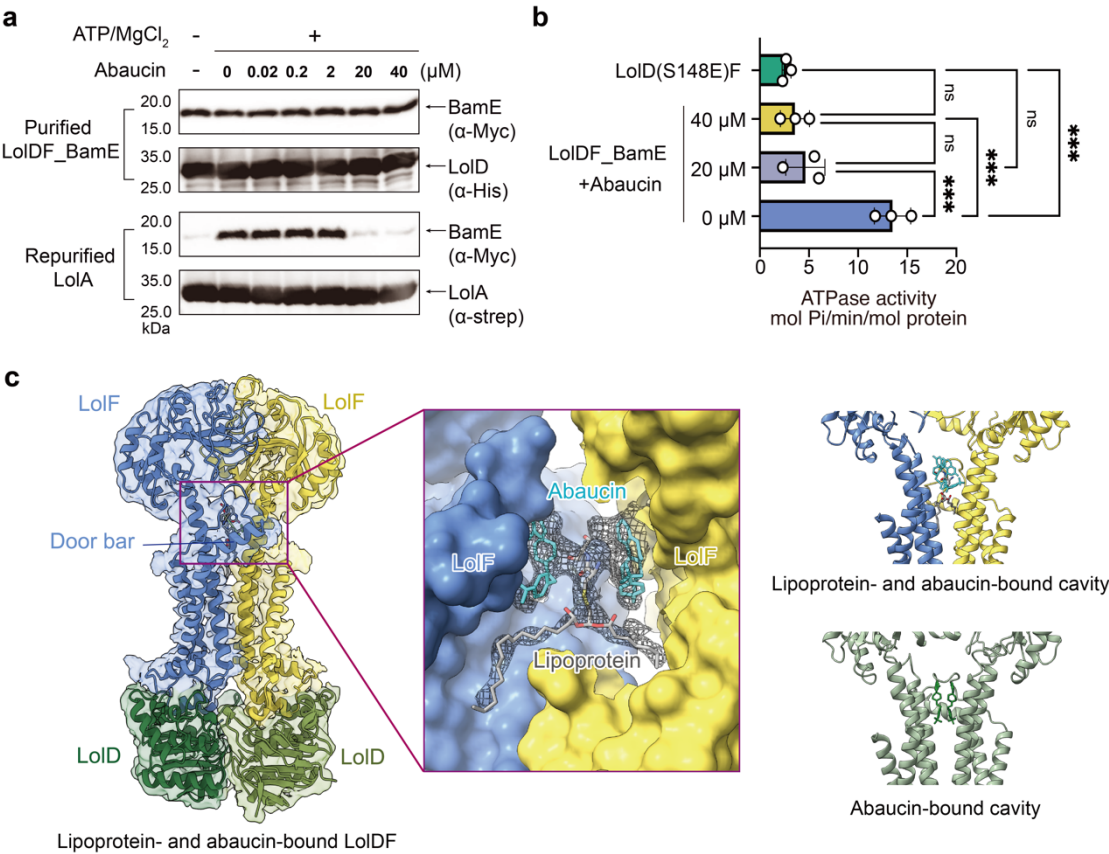


Fig. 5: Abaucin inhibits lipoprotein transfer while sparing lipoprotein extraction. **a**, *In vitro* lipoprotein transport assay in the presence of ATP, MgCl₂, and abaucin. **b**, ATPase activity of detergent-solubilised LolDF-BamE in the presence of LolA and abaucin (***p* < 0.001). Abaucin reduced LolDF-associated ATPase activity to levels comparable to those of the ATPase-impaired LolD(S148E)F control. Data in (a) and (b) are representative of *n* = 3 independent experiments; data in (b) are means ± s.d. Statistical source data for (b) are provided in Source Data. **c**, Cryo-EM structure of the lipoprotein-associated abaucin-bound *A. baumannii* LolDF complex. LolF protomers are shown in blue and yellow, and LolD protomers in green and olive green. The central inset shows density within the transmembrane cavity fitted with two abaucin molecules and the N-terminus of a lipoprotein substrate, including two acyl chains and a short peptide segment. The right panel compares the open cavity conformations of the lipoprotein-associated, abaucin-bound LolDF complex and the abaucin-bound LolDF structures in the absence of substrate.

[Comment 2]

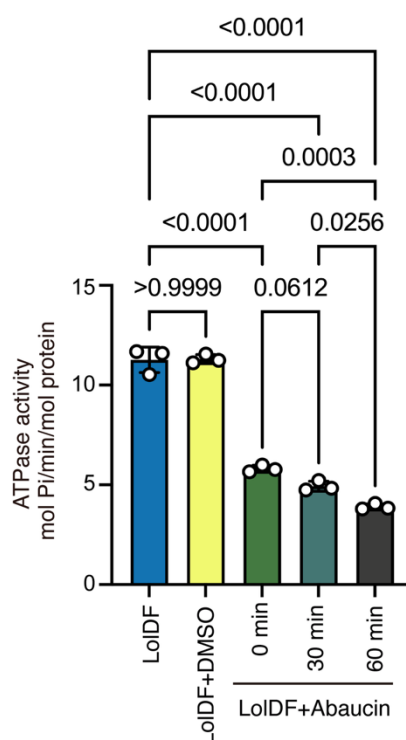
The time dependant reduction in ATPase activity is not as significant as the authors portray. It will be more meaningful if they also perform statistical analysis between the 0, 30 and 60min experiments.

[Response]

We thank the reviewer for this helpful suggestion. We have now performed statistical comparisons between the 0, 30, and 60 min time points (Response figure 3.2).

The reduction in ATPase activity between 0 and 60 min is statistically significant (*P* = 0.0003), indicating a time-dependent inhibitory effect. The change between 0 and 30 min is more modest and does not reach statistical significance (*P* = 0.0612), whereas a significant reduction is observed between 30 and 60 min (*P* = 0.0256). These results suggest that the inhibitory effect of abaucin develops progressively over time, consistent with the kinetics of transporter inhibition observed in our system.

To improve clarity and emphasize physiologically relevant conditions, we have replaced the original figure with updated data and statistical annotations in the revised manuscript (Fig. 5d).



Response Figure 3.2: ATPase activity of the LolDF complex in the presence and absence of inhibitor. ATP hydrolysis was measured for purified LolDF alone, LolDF incubated with DMSO (vehicle control), and LolDF treated with abaucin for 0, 30, and 60 minutes. Data are presented as mean $\pm$ s.d. from three independent experiments. Statistical significance was determined by one-way ANOVA with post-hoc correction; exact P values are indicated above the respective comparisons. ns, not significant ($P > 0.05$).

[Comment 3]

The BLI kinetic binding data suggest that ATP is not a requirement for abaucin binding as the experiments were performed in the absence of ATP. Does ATP binding alter the affinity for abaucin?

[Response]

We thank the reviewer for this insightful suggestion. Our BLI measurements were performed in the absence of ATP, and therefore directly demonstrate that abaucin binding does not require ATP. Consistent with this, we have determined cryo-EM structures of LolDF-abaucin complexes in both the presence (*E. coli*-expressed protein) and absence (*A. baylyi*-expressed protein) of ATP. In both cases, abaucin occupies the central cavity in a highly similar binding mode, indicating that the presence of ATP does not substantially alter abaucin binding mode.

Notably, in the ATP-bound structures, the NBDs remain undimerized and the transporter adopts a cavity-open conformation, suggesting that abaucin stabilizes/locks a non-productive state of the transporter. Given that ATP- and abaucin- binding appear to favor distinct conformational states of LolDF, BLI measurements in the simultaneous presence of both ligands would be difficult

to interpret, as they would reflect a mixture of conformational populations rather than a single well-defined binding equilibrium.

[Comment 4]

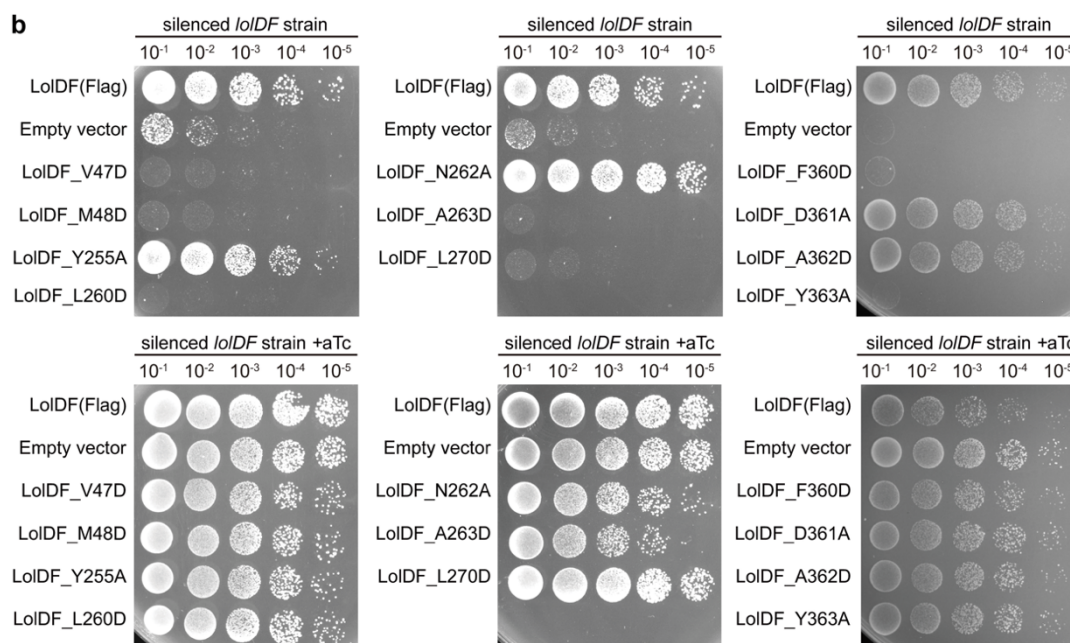
It is unclear from the manuscript why the loading experiments were performed with *Acinetobacter baylyi*. If it is due to technical issues, the authors should clearly state. They should also report the MIC value of abaucin for *A. baylyi* too.

[Response]

We agree that the use of proteins from different species for structural and functional assays may have caused confusion. To address this, we have repeated the loading and transport assays using *A. baumannii* LolDF (Fig. 5a, above), consistent with the structural study.

In these experiments, *A. baumannii* LolDF was expressed in *A. baylyi* ADP1, a non-pathogenic and genetically tractable close relative of *A. baumannii*, rather than in *E. coli*. This change was made because the protein expressed in *E. coli* exhibited low substrate occupancy, likely reflecting differences in substrate-handling between species. LolDF is highly conserved between *A. baumannii* and *A. baylyi* (~88% sequence identity), and *A. baumannii* LolDF functionally complements the lolDF-silenced *A. baylyi* (Supplementary Fig. 6b), supporting functional conservation. Accordingly, the *A. baumannii* LolDF-BamE complex expressed in *A. baylyi* was used for subsequent in vitro binding and transport assays. We have replaced the corresponding datasets and revised the manuscript to clearly specify the expression system and species used, therefore avoiding ambiguity.

For the MIC assay, we expressed WT or mutant *A. baumannii* LolDF in a lolDF-silenced *A. baylyi* background. However, we realized that differences arising from genetic manipulation, non-native expression level, and species-specific membrane properties could influence MIC value and confound interpretation. We therefore removed the MIC data obtained in *A. baylyi* from the revised manuscript to avoid overinterpretation.



Supplementary Fig. 6 Recovery of the *lolDF*-silenced strain and expression levels of LolDF mutants. (b) Cell viability of TetR-regulated *lolDF*-silenced *A. baylyi* strain with or without anhydrotetracycline (aTc). Data in (b) are representative of $n = 3$ independent experiments.

[Comments 5]

Line 286-289: the authors should also report theoretical lopP values for the modified compound? Table S2 will also benefit from such values too.

[Response 5]

We thank the reviewer for the suggestion. As several reviewers raised concerns regarding the limitations of the modified compound, we have removed the related content from the revised manuscript.

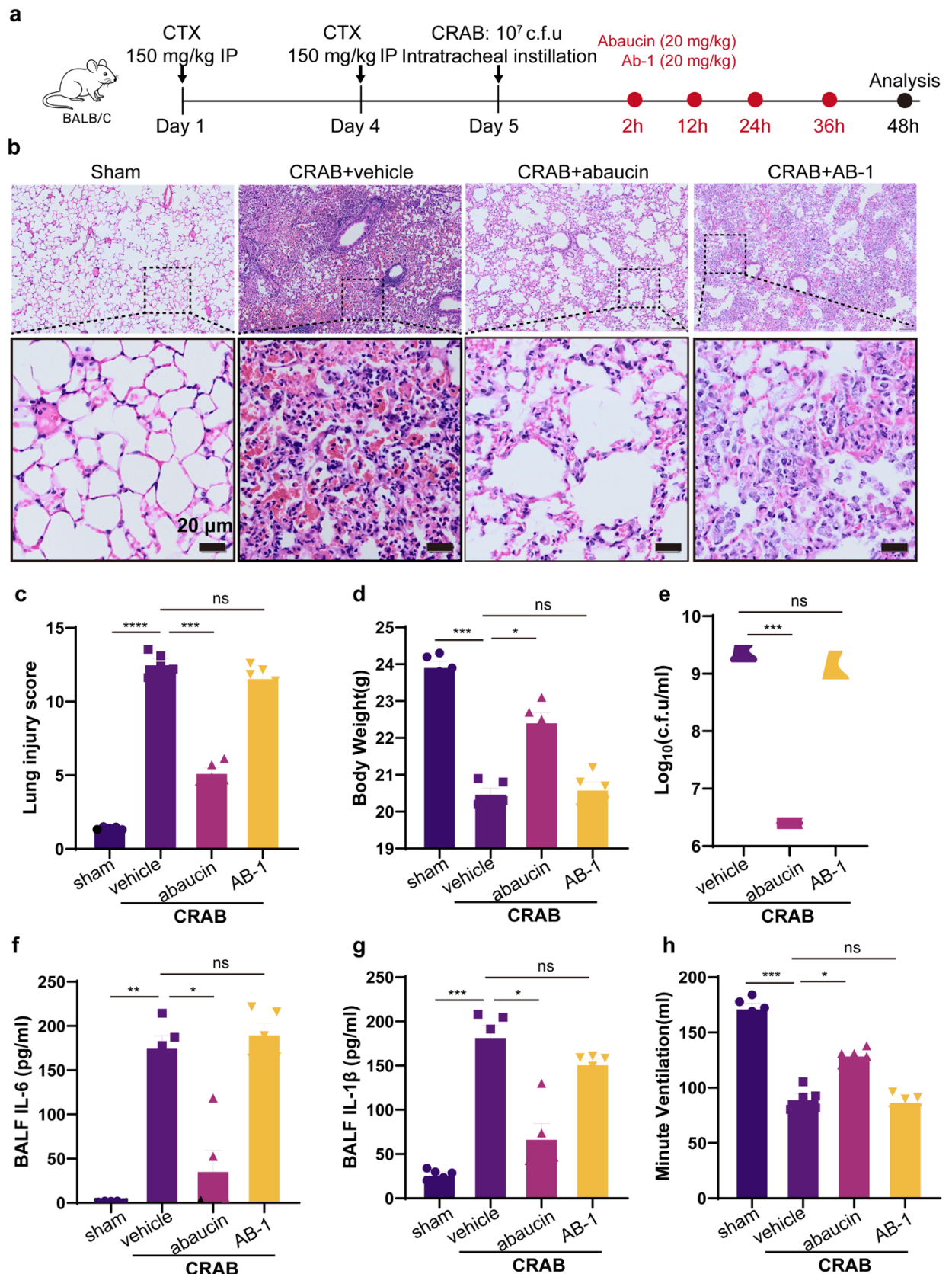
[Comment 6]

Why was AB-1 not tested in a mouse model, especially if its toxicity is lower than the parent compound. A comprehensive approach would be to look at other toxicity markers between the two drugs.

[Response]

In our previous manuscript, AB-1 exhibited slightly lower cytotoxicity than abaucin and appeared to offer solubility advantages. Following the reviewers' suggestions, we extended our evaluation of AB-1 to a mouse pneumonia model to enable a more comprehensive *in vivo* comparison. The resulting therapeutic efficacy data, which are presented in the Response Figure 3.6, show that AB-1 does not achieve superior to the parent compound abaucin.

Given that the central objective of this study is to define the binding mode of abaucin within LolDF and to elucidate the structural basis of transporter inhibition, we concluded that the inclusion of AB-1 does not substantively advance the primary mechanistic narrative. To maintain focus and avoid diluting the key structural and functional findings, we have therefore removed all AB-1-related data from the revised manuscript.



Response Figure 3.6: **a**, Schematic of treatment groups and dosing regimen for the murine pulmonary infection model. BALB/c mice were assigned to the indicated groups: untreated Control (sham), sham-infected vehicle controls, and infected animals treated with abaucin (20 mg kg⁻¹), and AB-1 (20 mg kg⁻¹). Compounds were administered via intratracheal instillation. Values

listed denote the timing of sample collection or analysis post-infection (hours). **b**, Representative haematoxylin and eosin (H&E)-stained lung sections from sham, vehicle-treated, abaucin-treated and AB-1-treated mice (scale bar, 20 μ m). **c**, Lung injury scores derived from histopathological assessment of lung sections in (c) ($n = 5$, *** $p < 0.001$, **** $p < 0.0001$). **d**, Body weight changes in sham, vehicle-treated, abaucin-treated, and AB-1-treated mice over the course of infection ($n = 5$, * $p < 0.05$; *** $p < 0.001$). **e**, Bacterial burden in BALF from vehicle-treated, abaucin-treated, and AB-1-treated mice, determined by colony-forming unit (c.f.u.) counts after serial dilution ($n = 3$, **** $p < 0.0001$). **f-g**, Concentrations of IL-6 and IL-1 β in BALF from sham, vehicle-treated, abaucin-treated, and AB-1-treated mice ($n = 5$, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). **h**, Lung function parameters, including minute ventilation (MV; h), in sham, vehicle-treated, abaucin-treated, and AB-1-treated mice with CRAB-induced pneumonia ($n = 5$, * $p < 0.05$; *** $p < 0.001$). Data are shown as mean $\pm$ s.e.m, Statistical significance was determined as described in Methods; ns, not significant.

[Comment 7]

Can the authors expand the discussion on the analogues and speculate why they have significantly altered MIC behaviour, considering that the trifluoromethyl phenyl group of the abaucin makes only very weak van der Waals interactions with the transporter? Is it likely to be solubility/uptake limitation rather than loss of affinity? (not necessary for the revision, but do the authors have K_d values for the other analogues?). The authors should report/measure the K_d for AB-1 and compare it to the parent compound.

[Response]

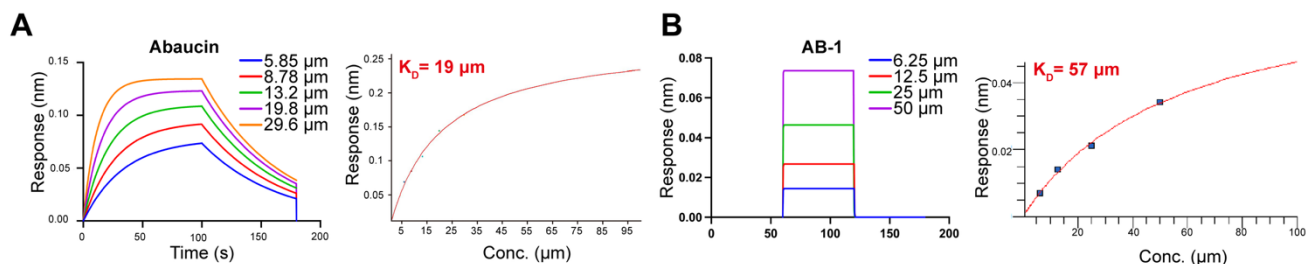
We thank the reviewer for this thoughtful inquiry. We have analyzed the fluorine-substituted analogue AB-1 from the perspectives of target affinity, solubility, and cellular permeability.

1. Comparison of binding Affinity (K_D): We measured the binding affinity of AB-1 to LolDF using Bio-Layer Interferometry (BLI). Under identical conditions (5% DMSO), the K_D for AB-1 was determined to be approximately 57 μ M, compared to 19 μ M for the parent compound, abaucin (Response Figure 3.7). This modest (~ 3 -fold) reduction in target affinity does not fully account for the much more pronounced loss of antibacterial activity observed in MIC assays.

2. Solubility: We agree with the reviewer's hypothesis that solubility and uptake are key variables. AB-1 exhibits significantly better aqueous solubility than abaucin (remaining clear at concentrations where abaucin appears turbid in 4% DMSO, ruling out solubility limitations as the cause for its reduced potency).

3. Cellular Uptake: The trifluoromethyl ($-CF_3$) group on abaucin is a critical pharmacophore that enhances lipophilicity and membrane permeability. Replacing it with a single, smaller fluorine atom in AB-1 likely impairs its ability to penetrate the complex Gram-negative outer membrane or reduces its accumulation within the periplasm. Therefore, the altered MIC behavior of AB-1 is most plausibly attributed to impaired cellular uptake rather than a catastrophic loss of target affinity or poor solubility.

To maintain a concise narrative and ensure the focus remains on the "conformation-hijacking" mechanism of abaucin, which is the most significant contribution of this study, we have elected to remove the AB-1 analogue data from the revised manuscript. We believe this streamlines the text without compromising the rigor or the broader conclusions of our mechanistic model.



Response Figure 3.7: Comparison of abaucin and AB-1 binding affinity measured by biolayer interferometry. **A**, Representative sensorgrams showing the association and dissociation of abaucin at indicated concentrations (5.85-29.6 μM) with immobilized *A. baumannii* LolDF. Steady-state analysis yielded equilibrium dissociation constants $K_D = 19 \mu\text{M}$. **B**, Representative sensorgrams showing the association and dissociation of AB-1 at indicated concentrations (6.25-50 μM) with immobilized *A. baumannii* LolDF. Steady-state analysis yielded equilibrium dissociation constants $K_D = 57 \mu\text{M}$. Data were fit using a [1:2 steady-state / heterogeneous ligand] binding model with global fitting of association and dissociation phases.

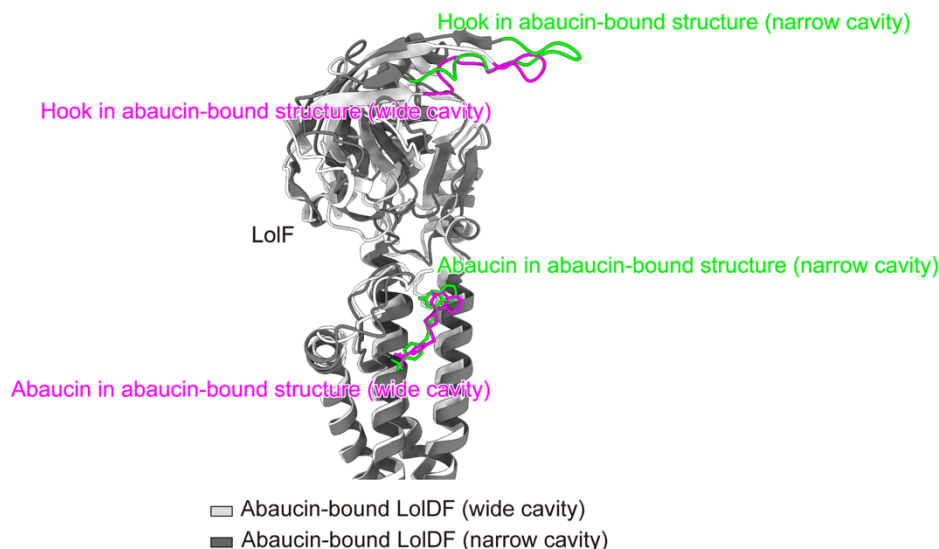
[Comment 8]

Extended Data Fig. 9: what evidence do the authors have that LolA cannot bind in the presence of abaucin? Is it due to the conformation of the hook?

[Response]

Thank you for the question regarding Extended Data Fig. 9. We would like to clarify that this figure represents a structural model depicting our proposed mechanism, rather than direct experimental evidence of LolA binding. In our abaucin-bound LolDF structures, abaucin occupies the transmembrane cavity, which is spatially distant from the periplasmic hook implicated in LolA recruitment (shown in the Response Figure 3.8). We therefore do not infer that abaucin directly blocks LolA binding through steric occlusion at the hook. Instead, based on the newly determined lipoprotein-bound LolDF-abaucin structure and the transport-coupled ATPase activity assays, we propose that the abaucin stabilizes a non-productive conformation of the transporter. In this state, lipoprotein transfer to LolA is impaired, while LolA binding is not necessarily directly prevented.

We have revised the manuscript to clarify that this model reflects an indirect inhibition mechanism and to avoid overinterpretation of LolA binding in the presence of abaucin.



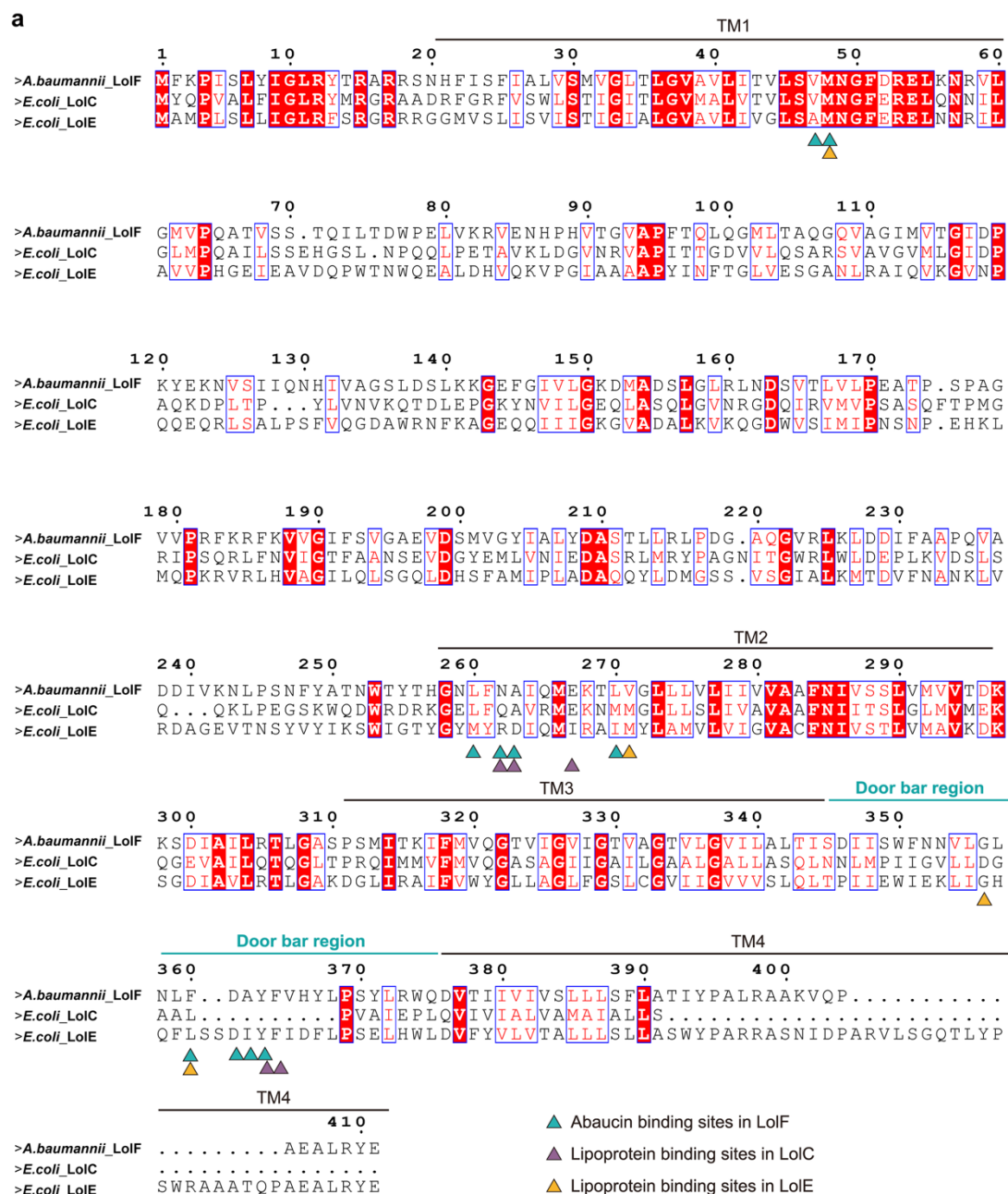
Response Figure 3.8: Structural comparison of abaucin-bound LolDF in wide- and narrow-cavity conformations. Structural superimposition of the wide-cavity (light gray) and narrow-cavity (dim gray) states reveals the "hook" region lies above the central cavity, whereas the bound abaucin molecules occupy a nearly identical central position.

[Comment 9]

Extended Data Fig. 7: two cyan triangles in the LolF after position 375 point to a gap region. Please check and correct.

[Response]

We thank the reviewer for noting this issue. Upon re-examination of Extended Data Fig. 7, we confirm that the two cyan triangles following residue 375 in LolF were incorrectly positioned and pointed to an unintended gap region. This has now been corrected in the revised Supplementary Fig. 4 to accurately reflect the structural information. In addition, the LolDF sequence in the revised manuscript has been renumbered according to the NCBI annotation. The previous numbering included with ribosome-binding site sequence, which caused the discrepancy.



Supplementary Fig. 4 Sequence alignment of *A. baumannii* LolF and *E. coli* LolC and LolE.
(a) Sequence alignment highlighting residues involved in abaucin binding in LolF and those mediating lipoprotein binding in LolC or LolE.

[Comment 10]

Line248-249: remove the word non-closable as it is redundant with the next sentence (outward-open conformation).

[Response]

Thank you for this suggestion. We have revised the sentence at Lines 248-249 by removing the word “non-closable”. The revised sentence now reads: “...trapping the transporter in an outward-open conformation.”

[Comment 11]

Are there published genomic data for the clinical isolates in Fig 1? Provide accession codes.
Correct Fig 1 and 5b to state clinical isolates not 'clinical isolation'

[Response]

We thank the reviewer for the helpful suggestions.

The clinical isolates used in Fig. 1 (Fig. 5 has been removed) were obtained from the West China Hospital Clinical Isolate Bank. While genome sequence data are publicly available for these specific strains in NCBI (SAMN34037527: HX_S9; SAMN34037535: HX_S19; SAMN34037546: HX_S31; SAMN34037486: HX_R7; SAMN34037488: HX_R9; SAMN34037507: HX_R28), and this information has been added into the Method section. We have corrected the figure legends as requested.

[Comment 12]

Lines 358-360: the current sentence implies that AI was used in this study. Rephrase it.

[Response]

We thank the reviewer for this clarification. We agree that the original sentence could be misinterpreted as implying the use of AI-based methods in this study. The intent was to provide broader context on emerging approaches in drug discovery rather than to describe methods used here. As no AI-based approaches were employed in this work, we have removed the sentence to avoid any ambiguity.

Reviewer #4 (Remarks to the Author):

The manuscript “Molecular basis of precision antimicrobials: conformational hijacking of LolDF by Abaucin in *A. baumannii*” examines the antibacterial effects of an *Acinetobacter*-specific drug, abaucin, in treating clinical strains of *A. baumannii* and inhibiting the essential LolDF protein complex.

[Comment 1]

A major strength of the manuscript is the elucidation of two different abaucin-inhibited structures of LolDF. This novel structure offers insight into the inhibitory activity of abaucin. The authors present several characterization assays to validate the observed structures. Some of these offer clear insights (e.g. analysis of some resistant variants), but others are confounded. The *in vitro* analysis of abaucin inhibition and the resulting model for abaucin inhibition of LolDF is also inconsistent with the cryoEM evidence and requires further explanation.

In addition to the cryoEM structural analysis, the manuscript also presents an analysis of abaucin effectiveness against clinically isolated strains of *A. baumannii* that are carbapenem-resistant and demonstrates abaucin efficacy in a pulmonary infection model. These insights are very incremental, expending the results from the abaucin discovery study to a different infection route and to show action against varying clinical strains that all have LolDF. Showing abaucin has no activity against *E. coli* or *K. pneumoniae* or microbiota species is not novel.

A final element of the manuscript presents a chemical effort to test abaucin analogs. Here, the authors offer major conclusions that an abaucin derivative (“AB-1”) has better solubility, equivalent antibacterial activity, and less toxicity against human cells. The claim for improved solubility (in DMSO) is weak with the limited evidence presented (Fig. 5a) and seems to have minor impact. The claim for equivalent antibacterial activity is also weak, with AB-1 consistently showing weaker activity than abaucin (MICs in Fig. 5b). The claim for reduced toxicity is not well supported since accounting for ~2-fold increase in MIC for AB-1 compared to abaucin, AB-1 exhibits more HEK cell line toxicity at 1x MIC concentrations than the original abaucin compound.

[Response]

We thank the reviewer for the rigorous assessment and the constructive criticism, which have been instrumental in improving the clarity and focus of our manuscript. In response to the points raised, we have performed additional structural studies and revised the text as detailed below.

1. Resolving the inhibitory mechanism with direct structural evidence

We appreciate the reviewer’s identification of the apparent discrepancy between our initial structural models and the proposed inhibitory mechanism. To provide definitive clarity, we

determined a new cryo-EM structure of the abaucin-bound, lipoprotein-associated *A. baumannii* LolDF complex.

Co-occupancy: At 3.34 Å resolution, the structure reveals a mixed density within the central cavity: one well-resolved density is assigned to abaucin, while adjacent densities are consistent with two lipoprotein acyl chains and a partial peptide segment (revised Fig. 5c).

Non-competitive inhibition: This ternary complex provides unambiguous evidence that abaucin and the substrate can co-occupy the expanded cavity. It proves that abaucin does not competitively exclude substrate binding but instead traps the transporter in a state that prevents the subsequent translocation stroke.

Functional validation: This "conformation-hijacking" model is further supported by our revised *in vitro* transport and ATPase assays, which confirm that inhibition occurs at a step post lipoprotein binding (revised Fig. 5a, b).

We have updated the Results and Discussion sections to integrate these new structural data, which we believe fully resolve the previous mechanistic inconsistency.

2. As complementary validation of Liu et al.

Animal experiments serve to complement and further substantiate the findings of Liu *et al.* in the context of the structural and mechanistic work presented here. In the revised manuscript, these data are now framed strictly as confirmatory evidence supporting the physiological relevance of abaucin against carbapenem-resistant *A. baumannii* (CRAB).

3. Removal of the AB-1 analog characterization

We fully accept the reviewer's critique regarding the AB-1 analog. Our re-evaluation confirms that the initial claims regarding its improved solubility and reduced toxicity were not sufficiently robust, particularly concerning the normalization of cytotoxicity by MIC values.

To ensure the highest scientific rigour and to avoid misleading comparisons, we have completely removed all AB-1-related data, including figures and associated discussion (formerly Fig. 5).

The revised manuscript now focuses exclusively on the molecular mechanism of the parent compound, abaucin, which remains the primary contribution of this study.

By incorporating the updated complex structure and removing the secondary analog work, we believe the revised manuscript is now more focused, rigorous, and clearly demonstrates the "conformational trap" mechanism that defines abaucin's activity.

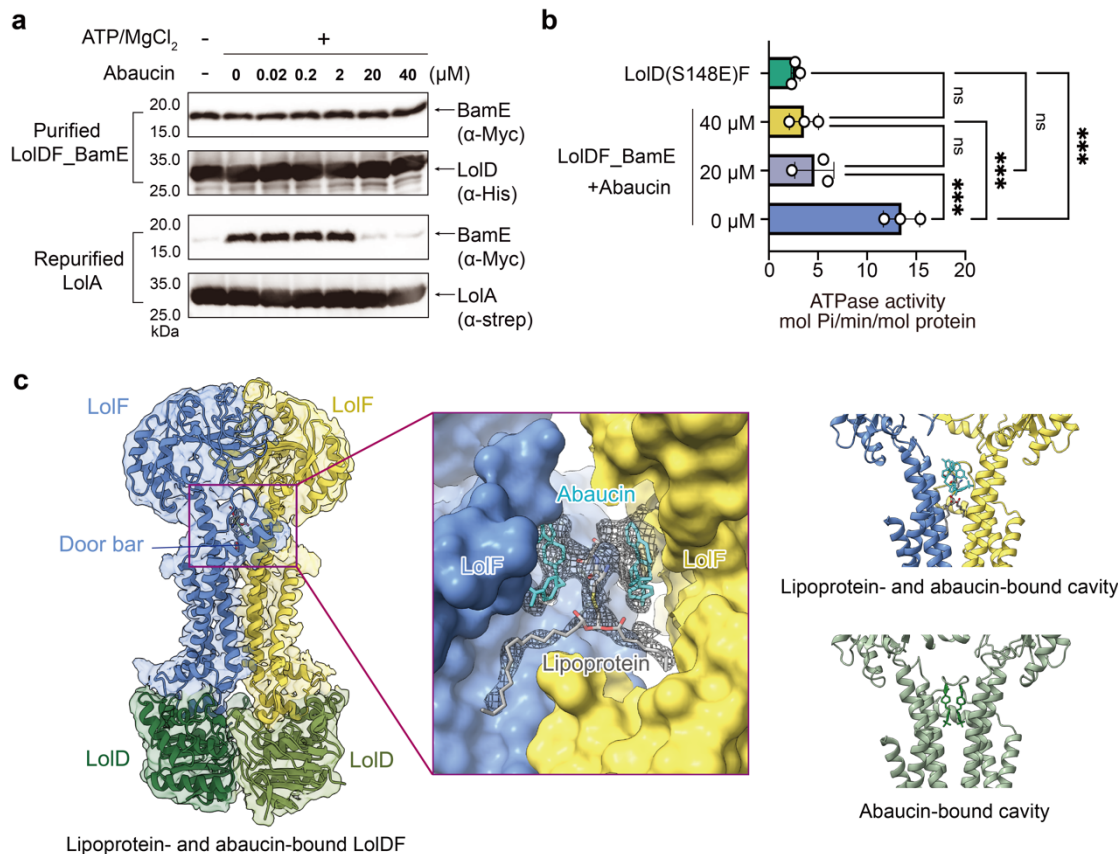


Fig. 5: Abaucin inhibits lipoprotein transfer while sparing lipoprotein extraction. **a**, *In vitro* lipoprotein transport assay in the presence of ATP, MgCl₂, and abaucin. **b**, ATPase activity of detergent-solubilised LolDF-BamE in the presence of LolA and abaucin (***p* < 0.001). Abaucin reduced LolDF-associated ATPase activity to levels comparable to those of the ATPase-impaired LolD(S148E)F control. Data in (a) and (b) are representative of *n* = 3 independent experiments; data in (b) are means ± s.d. Statistical source data for (b) are provided in Source Data. **c**, Cryo-EM structure of the lipoprotein-associated abaucin-bound *A. baumannii* LolDF complex. LolF protomers are shown in blue and yellow, and LolD protomers in green and olive green. The central inset shows density within the transmembrane cavity fitted with two abaucin molecules and the N-terminus of a lipoprotein substrate, including two acyl chains and a short peptide segment. The right panel compares the open cavity conformations of the lipoprotein-associated, abaucin-bound LolDF complex and the abaucin-bound LolDF structures in the absence of substrate.

Additional major comments:

[Comment 2]

Abaucin mechanism-of-action. CryoEM structures show that abaucin “occupies the substrate-binding cavity and blocks ATP-dependent cavity”. With analysis to lipoprotein liganded LolCDE structure, the authors suggest that abaucin occupies the same cavity that a lipoprotein would occupy in LolDF and prevent its conformational cycling. This is a plausible way for abaucin to inhibit LolDF activity. However, the presented *in vitro* assay contradicts those observations. Examining purified BamE lipoprotein liganded LolDF complex, the data show that abaucin added to purified BamE-LolDF complex blocks BamE release to the LolA chaperone. How is abaucin able to do this when BamE is already occupying the binding pocket where abaucin needs to bind?

The in vitro assay results do not seem to reflect the cryoEM structure very well. The proposed model (extend Fig. 9) does not explain the observed abaucin activity. ATPase inhibition data are clear and it is sensible that abaucin prevents LolDF cycling. But, apparently, abaucin can prevent the transition from state C to state D (ext. Fig. 9) after being liganded. How is the cryoEM evidence consistent with the in vitro data?

[Response]

We thank the reviewer for this critical and insightful observation regarding the apparent discrepancy between our initial structural model and the in vitro functional data. This comment has prompted us to perform additional structural characterization that fundamentally clarifies abaucin's mechanism of action.

1. Structural evidence for co-occupancy:

The reviewer's central question, how abaucin binds when the pocket is already occupied by BamE, is directly addressed by our new 3.34 Å cryo-EM structure of abaucin-bound, lipoprotein-associated *A. baumannii* LolDF complex (revised Fig. 5c, above). The structure reveals that the central cavity of *A. baumannii* LolDF is sufficiently expanded to simultaneously accommodate both the inhibitor and the substrate. While abaucin occupies the central cavity, it engages specific "door-bar" motifs that do not fully overlap with the docking site of the lipoprotein acyl chains. This provides unambiguous evidence that abaucin does not function through simple competitive exclusion.

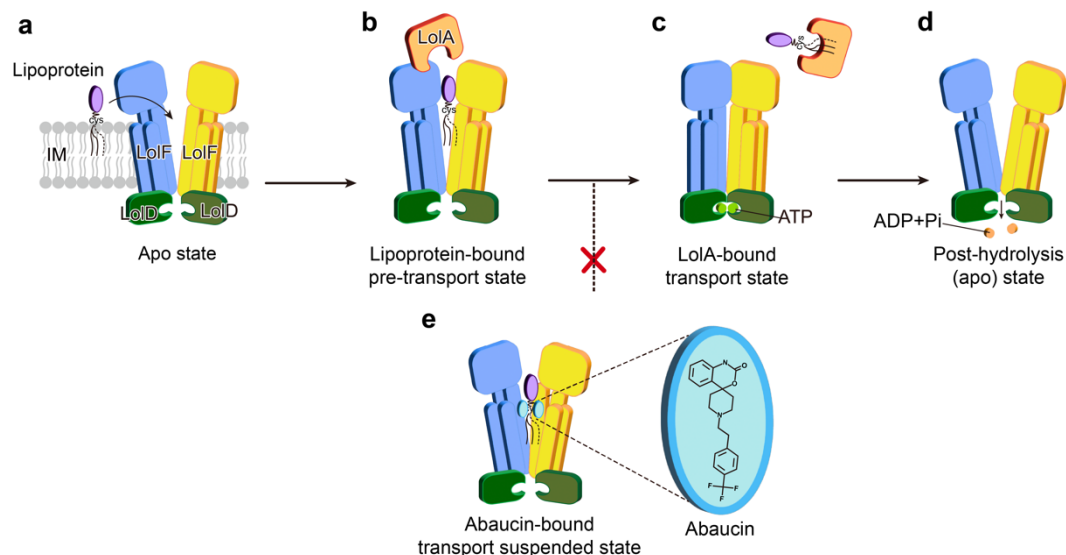
2. Resolving the *in vitro* contradiction:

This structural finding perfectly aligns with our in vitro results showing that abaucin blocks BamE release even when added to a pre-formed LolDF-BamE complex. Abaucin enters the cavity and "hijacks" the complex after the substrate has bound. By locking the transporter in a static, open conformation, abaucin prevents the essential translocation step required to move the substrate from the LolDF cavity to the LolA chaperone.

3. Clarification of the transition from state b to state c (Supplementary Fig. 10)

The reviewer correctly points out that abaucin prevents the transition from State b (liganded) to State c (released). Our model in Supplementary Fig. 10 now reflects this. At state b, abaucin binding locks the transporter in the substrate loaded state, instead of proceeding to State c, the presence of abaucin converts the liganded complex into a catalytically inactive "locked" state. The ATPase inhibition data further support this; abaucin allows the initial "priming" of the system (substrate binding) but strictly abolishes the energy-consuming steps required for substrate extraction and release.

By determining the newly updated structure, we have reconciled the cryo-EM evidence with the biochemical data. We have updated the Results and Discussion sections to emphasize that abaucin is a post-binding conformational trap rather than a competitive binding inhibitor. This discovery significantly strengthens the proposed "conformation-hijacking" model.



Supplementary Fig. 10 Schematic of the LolDF-mediated lipoprotein export cycle in the presence or absence of abaucin. (a to d) Model of lipoprotein trafficking mediated by LolDF. In the apo state (a), unloaded LolDF extracts lipoproteins from the inner membrane (IM). The lipoprotein-bound LolDF (b), associates with the periplasmic chaperone LolA. Binding of ATP/MgCl₂ to the nucleotide-binding domains (NBDs) induces complex closure (c), driving lipoprotein transfer to LolA. Subsequent ATP hydrolysis resets the transporter to the apo state (d), Abaucin binding locks the transporter in a lipoprotein-accessible conformation, thereby preventing ATP-induced complex closure required for lipoprotein export to LolA.

[Comment 3]

Abaucin affinity measurement for LolF variants that are inactive (lethal in vivo) cannot be reliably interpreted and should be removed. The LolF mutation might severely impact complex folding or assembly, and this hasn't been controlled. Analysis of V62D, M63D, L275D, A278D, L285D, F375D, Y378A is therefore not valid. Two of these variants are used to suggest that the door-bar loop is central and sufficient to abaucin engagement. This conclusion is really only supported by A377D and should be reconsidered accordingly.

[Response]

We thank the reviewer for the evaluation of our mutational analysis. In response, we have removed lethal from the affinity assay (now in Fig. 4a). The numbering of the residues has been revised according to NCBI annotation. The initial numbering included 15 amino acids of the upstream ribosome-binding site sequence.

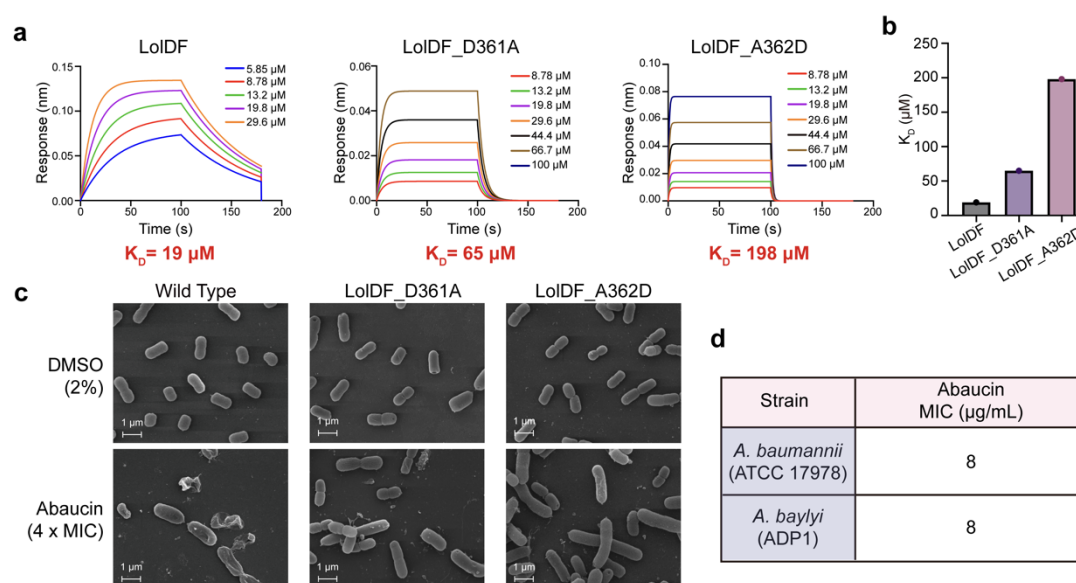


Fig. 4: Characterization of abaucin binding to LolDF. **a**, Bio-layer interferometry (BLI) sensorgrams showing abaucin affinity to wild-type LolDF or LolDF mutants carrying substitutions in the abaucin-binding sites. **b**, Averaged K_D value from the BLI assay. **c**, Scanning electron microscopy (SEM) images of wild-type *A. baylyi* and the corresponding LolDF mutant strains with or without abaucin treatment. **d**, MICs were determined for wild-type *A. baumannii* (ATCC 17978) and *A. baylyi* (ADP1). Data in (a-c) are representative of $n = 3$ independent experiments.

[Comment 4]

Several resistant mutations are used to validate the *A. baumannii* LolDF-abaucin cryoEM structures. These mutations are made in the *A. baylyi* lolF gene. The MIC shifts for what are claimed “resistant” mutants are very modest (Fig 4b), at most a 2-fold increased shift in MIC. It doesn’t seem that these mutations offer a compelling level of abaucin resistance. It’s a bit hard to directly interpret these in vivo data relative to binding data because resistance and binding data are performed using LolF of different *Acinetobacter* species. This requires some explanation or warning for interpretation.

[Response]

We thank the reviewer for this important comment. In the MIC assay, we expressed WT or mutant *A. baumannii* LolDF in a lolDF-silenced *A. baylyi* background. This design was necessitated by the genetic intractability and biosafety restrictions associated with pathogenic *A. baumannii* and follows the established practice of using non-pathogenic surrogate hosts.

However, we realized that differences arising from genetic manipulation, non-native expression level, and species-specific membrane properties could influence MIC value and confound interpretation. We therefore removed the MIC data obtained in *A. baylyi* from the revised manuscript to avoid overinterpretation.

[Comment 5]

As described above, AB-1 improved solubility is modest and not presented quantitatively. Is abaucin solubility a limitation? At least in animal infection models, it doesn't seem to be a problem.

[Response]

We thank the reviewer for this comment. The solubility comparison between abaucin and AB-1 was performed in 4% DMSO. In our animal infection models, abaucin was formulated with PEG to ensure adequate solubility for in vivo administration, and under these conditions solubility did not appear to be a limiting factor for efficacy.

We fully agree that the modest solubility improvement of AB-1, without quantitative assessment, does not establish a meaningful advantage over the parent compound. Given that the primary focus of this study is to elucidate the inhibitory mechanism of abaucin against LolDF and to demonstrate its therapeutic efficacy based on this mechanism, we have removed the AB-1-related data from the manuscript. We appreciate the reviewer's understanding.

[Comment 6]

The AB-1 analog is a very modest alteration to abaucin's chemical structure (Fig 5a), and AB-1 has universally less potency against *A. baumannii* strains tested (Fig 5b, generally a 2-fold MIC increase). The effects of the two compounds on HEK toxicity should therefore not be made on an equal concentration basis. Looking at the lowest MIC scenario of abaucin and AB-1 (1 ug/ml and 2 ug/ml, respectively), the HEK cell line toxicity data is actually worse for AB-1 (35% cell survival at 1x MIC of 2 ug/ml) compared to abaucin (75% cell survival at 1x MIC of 1 ug/ml). In sum, it is not obvious that AB-1 is in any way an improvement over the starting abaucin structure.

[Response]

We thank the reviewer for the rigorous and insightful analysis of the AB-1 cytotoxicity data. The reviewer correctly points out that comparing compounds at equal absolute concentrations is inappropriate when their MICs differ. Therefore, we have removed all AB-1-related data from the manuscript to maintain focus on the mechanistic characterization of the parent compound. We appreciate the reviewer's critical evaluation, which has significantly improved the clarity and rigor of our study.

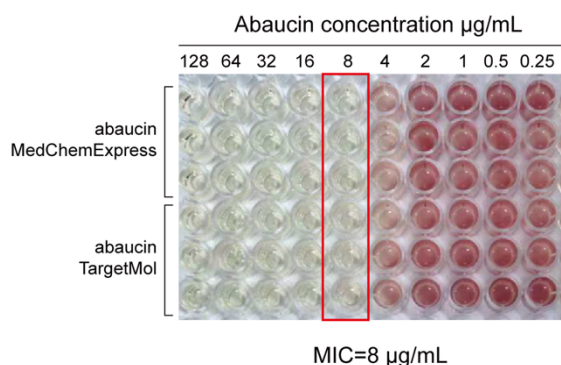
[Comment 7]

AB-1 has a very high MIC against the lab *A. baumannii* strain as ATCC 17978 strain (16 ug/ml). The authors did not test abaucin against this strain (that I can tell). Previous abaucin MICs against

ATCC 17978 were reported as ~2 µg/ml. Please measure AB-1 relative potency against this lab strain and comment on the relative efficacy of AB-1.

[Response]

We thank the reviewer for the careful comparative analysis. We have now measured the MIC of abaucin against the laboratory *A. baumannii* ATCC 17978 strain under the same conditions used for AB-1. Under our assay conditions, abaucin purchased from MedChemExpress (cat: HY-18611A) or TargetMol (cat: T12773) showed an MIC of 8 µg ml⁻¹ against *A. baumannii* ATCC 17978. Following the reviewer's suggestion, we also measured the MIC of AB-1 against the same strain and found it to be 16 µg ml⁻¹. Thus, AB-1 was approximately two-fold less potent than abaucin against ATCC 17978 in our hands fig. However, given that the primary focus of this study is the mechanistic characterization of abaucin, we have removed all AB-1-related data from the manuscript.



Response Figure: Comparative MIC determination of abaucin from two commercial sources. Abaucin obtained from MedChemExpress and TargetMol was tested in a twofold dilution series ranging from 0.25 to 128 µg mL⁻¹ against the indicated *A. baumannii* ATCC17978 strain. Both preparations exhibited identical activity, yielding a MIC of 8 µg mL⁻¹.

Minor comments

Please check minor typos throughout. Eg.. “abaucine”

We thank the reviewer for pointing this out. Minor typographical errors, including “abaucine”, have been corrected throughout the manuscript.

L173-174. The statement is wrong. Abaucin was not originally identified through deep-learning-guided modeling of the *E. coli* LolCDE transporter.

We thank the reviewer for this important comment. The revised text now reads: "[Abaucin was originally identified by Liu et al. in a machine-learning-guided screen for inhibitors of *A. baumannii* growth and was proposed to disrupt lipoprotein trafficking⁵⁵](#)".

L827: “LolE operon” should be replaced with “bamE gene”.

We thank the reviewer for pointing out this error. “LolE operon” has been corrected to “*bamE* gene”.

Reviewer #5 (Remarks to the Author):

In their manuscript, Dr. Pan et al. first validated the efficacy and selectivity of abaucin, an antibiotic discovered through deep-learning guided studies, against clinically carbapenem-resistant *A. baumannii* (CRAB) isolates both in vitro and in a neutropenic mouse lung infection model. The authors then use structural biology analysis and demonstrated that abaucin exerts its antibacterial effect by selectively inhibiting the *A. baumannii* lipoprotein transporter LolDF, an essential component of the outer membrane biogenesis pathway. They further modified the scaffold and generated an analogue of abaucin, AB-1, and evaluated its solubility, human cell toxicity, and anti-CRAB activities. The authors suggest that the Lol complex represents an attractive drug target for therapeutic intervention against multidrug-resistant infections.

Acinetobacter baumannii is a multidrug-resistant (MDR) pathogen of significant clinical importance. It causes healthcare-associated infections such as pneumonia and bacteremia. Due to its ability to rapidly acquire resistance to almost all available antibiotics, including carbapenems, *A. baumannii* is listed by WHO and public health agencies in many countries as one of the top MDR pathogens for research and drug development. In this regard, this manuscript addresses an important infectious disease, antimicrobial resistance, and novel antibiotic development area. Although the in vitro and in vivo evaluation of abaucin and its new analogue, AB-1, has demonstrated their efficacy and safety, the manuscript may be further improved if the authors consider the following comments.

We would like to express our sincere gratitude for your insightful and constructive comments on our manuscript. Your recognition of the clinical importance of targeting the LolDF complex and the efficacy of abaucin is highly encouraging. We have carefully addressed each of your concerns and performed additional experiments to strengthen the study.

Major comments:

[Comment 1]

1. Although a non-treatment (vehicle) control and/or an uninfected control (control) were included in most of the experiments, inclusion of additional controls is needed and will likely increase the overall robustness of the study.

a) Ln 154: It is shown that there was no effect of in vivo abaucin treatment on the gut microbiome, an interesting and important study. However, a control group treated with a broad-spectrum

antibiotic to demonstrate its effect on the gut microbiome will be important to validate the treatment regimen and technical analysis.

b) Similarly, for the in vivo abaucin efficacy experiment, inclusion of a carbapenem antibiotic in the study to demonstrate its ineffectiveness will be valuable.

[Response]

We thank the reviewer for this important suggestion. In the revised manuscript, we have added meropenem- and piperacillin-treated groups as broad-spectrum β -lactam antibiotic controls.

Regarding point (a), we have added meropenem- and piperacillin-treated groups to the gut microbiome analysis. CRAB infection with vehicle treatment caused a marked shift in faecal microbial composition relative to sham controls. Abaucin did not further perturb the microbiota, as both the community composition and Shannon diversity remained broadly comparable to those of vehicle-treated mice. In contrast, meropenem and piperacillin produced distinct microbiota profiles with altered Shannon diversity (please see revised Supplementary Fig. 1d, e).

Regarding point (b), we have included meropenem- and piperacillin-treated groups in the neutropenic mouse CRAB lung infection model (please see revised Fig. 2). Meropenem reduced the bacterial burden of the CRAB HX-R7 strain and ameliorated lung injury, inflammatory cytokine production and respiratory impairment to a degree broadly comparable to abaucin. Piperacillin showed limited protection under the same dosing regimen, as bacterial burden, lung pathology, inflammatory responses and ventilation impairment remained closer to those observed in vehicle-treated infected mice. We have therefore revised the manuscript to present meropenem as an active broad-spectrum comparator rather than as an ineffective carbapenem control. These data further support the in vivo antibacterial efficacy of abaucin while preserving the key distinction that abaucin produced minimal additional gut microbiome perturbation in the corresponding microbiome analysis.

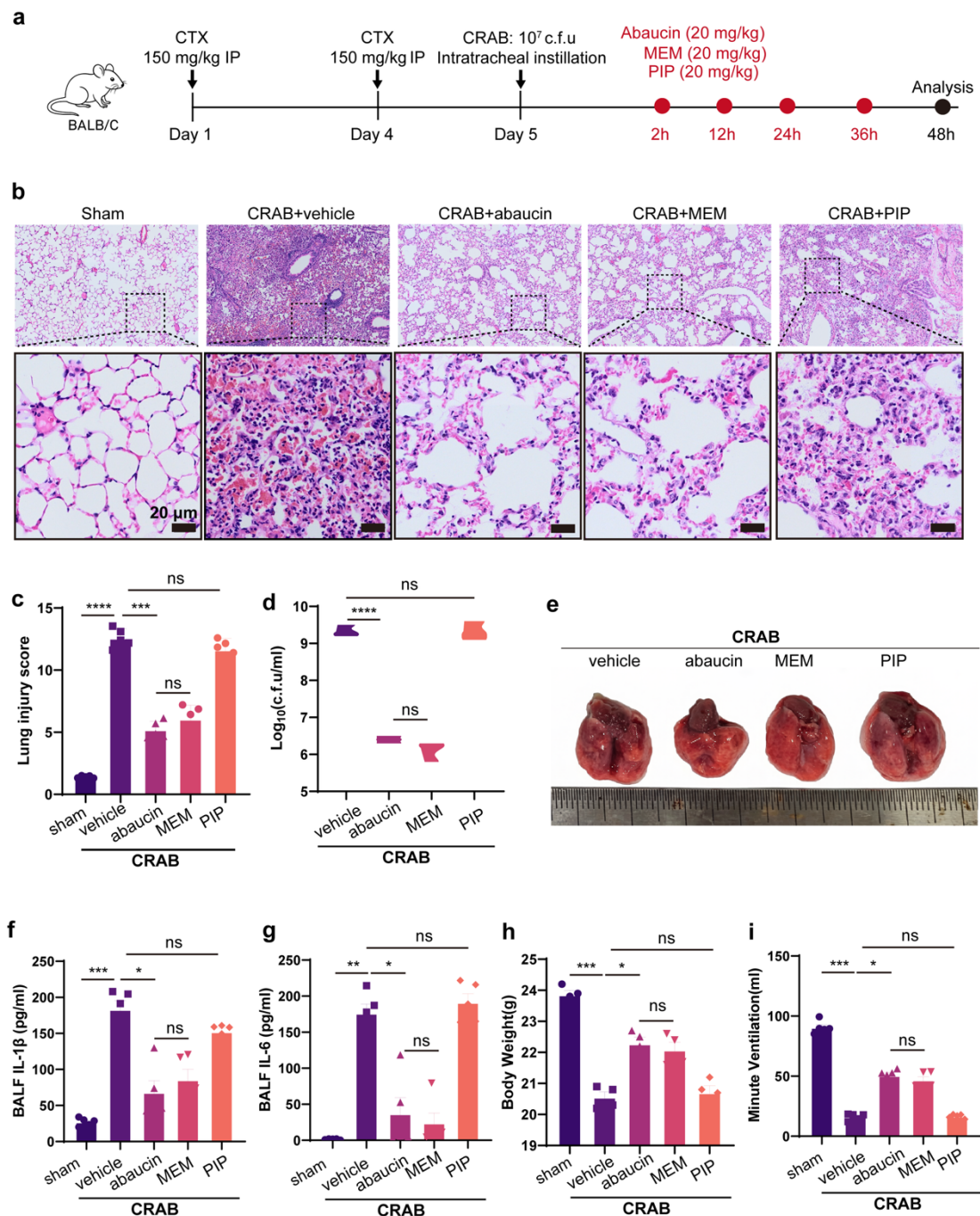
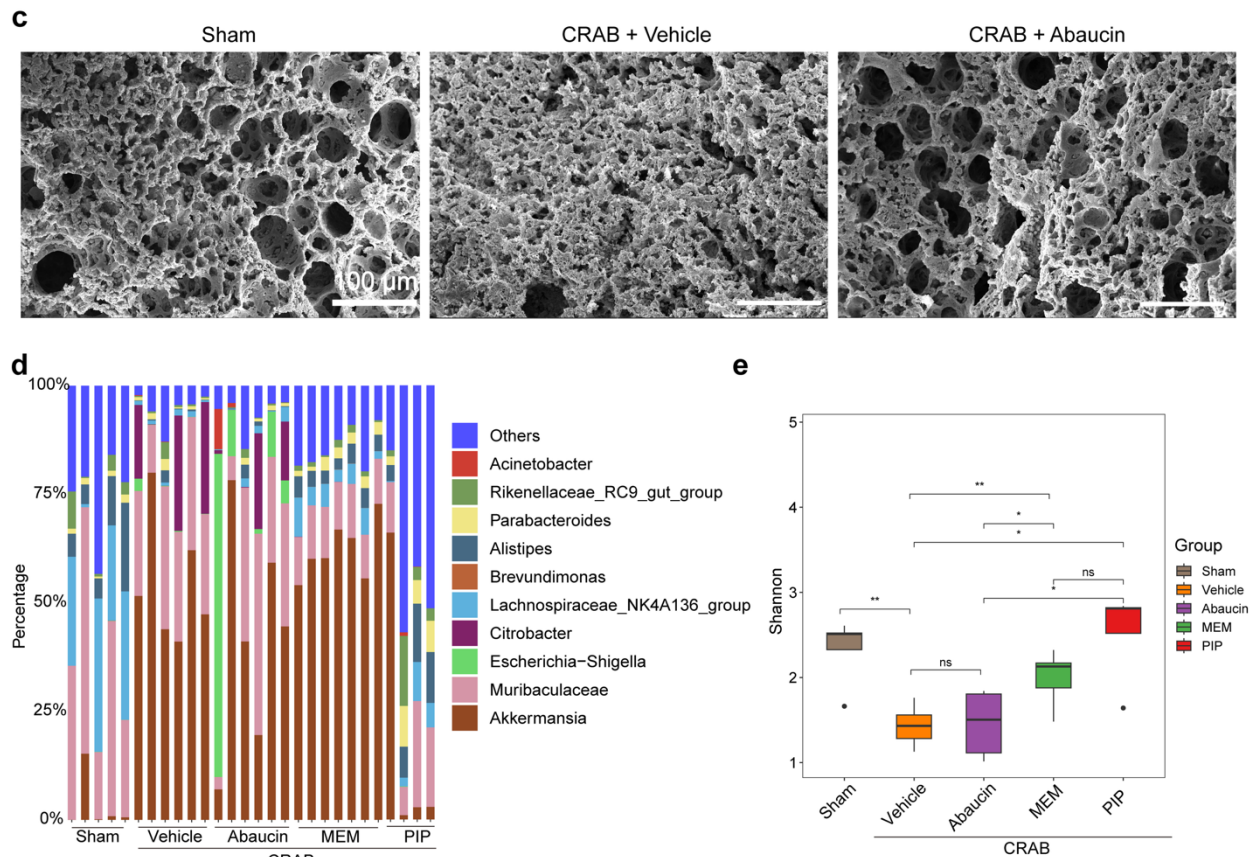


Fig. 2: Abaucin treatment ameliorates CRAB-induced pneumonia in neutropenic mice. **a**, Schematic of the experimental design. Neutropenia was induced by two doses of cyclophosphamide (CTX) administered 96 h and 24 h before infection. At the time of infection, CRAB (HX-R7) was inoculated by intratracheal instillation (1×10^7 c.f.u.). Vehicle, abaucin, meropenem, or piperacillin (20mg/kg) was administered in four doses at 2, 12, 24, and 36 h post infection, and mice were euthanized at 48 h post infection. IP, intraperitoneal injection. **b**, Representative haematoxylin and eosin (H&E)-stained lung sections from sham, vehicle-treated, abaucin-treated mice, meropenem and piperacillin-treated mice (scale bar, 20 μ m). **c**, Lung injury scores derived from histopathological assessment of lung sections shown in (b) ($n = 5$, *** $p < 0.001$, **** $p < 0.0001$). **d**, Bacterial burden in BALF from vehicle-treated, abaucin-treated, meropenem-treated, and piperacillin-treated mice, determined by colony-forming unit (c.f.u.) counts after serial dilution ($n = 3$, **** $p < 0.0001$). **e**, Lung tissue from vehicle-treated, abaucin-

treated, meropenem-treated, and piperacillin-treated mice in the CRAB-induced pneumonia model. **f,g**, Concentrations of IL-1 β (f) and IL-6 (g) in BALF from sham, vehicle-treated, abaucin-treated mice, meropenem-treated, and piperacillin-treated mice ($n = 5$, $*p < 0.05$; $**p < 0.01$; $***p < 0.001$). **h**, Body weight changes in sham, vehicle-treated, abaucin-treated, meropenem-treated, and piperacillin-treated mice over the course of infection ($n = 5$, $*p < 0.05$; $***p < 0.001$). **i**, Minute ventilation (MV; **h**) in sham, vehicle-treated, abaucin-treated, meropenem-treated, and piperacillin-treated mice with CRAB-induced pneumonia ($n = 5$, $*p < 0.05$; $***p < 0.001$). Data are shown as mean $\pm$ s.d. Statistical significance was determined as described in Methods; ns, not significant.



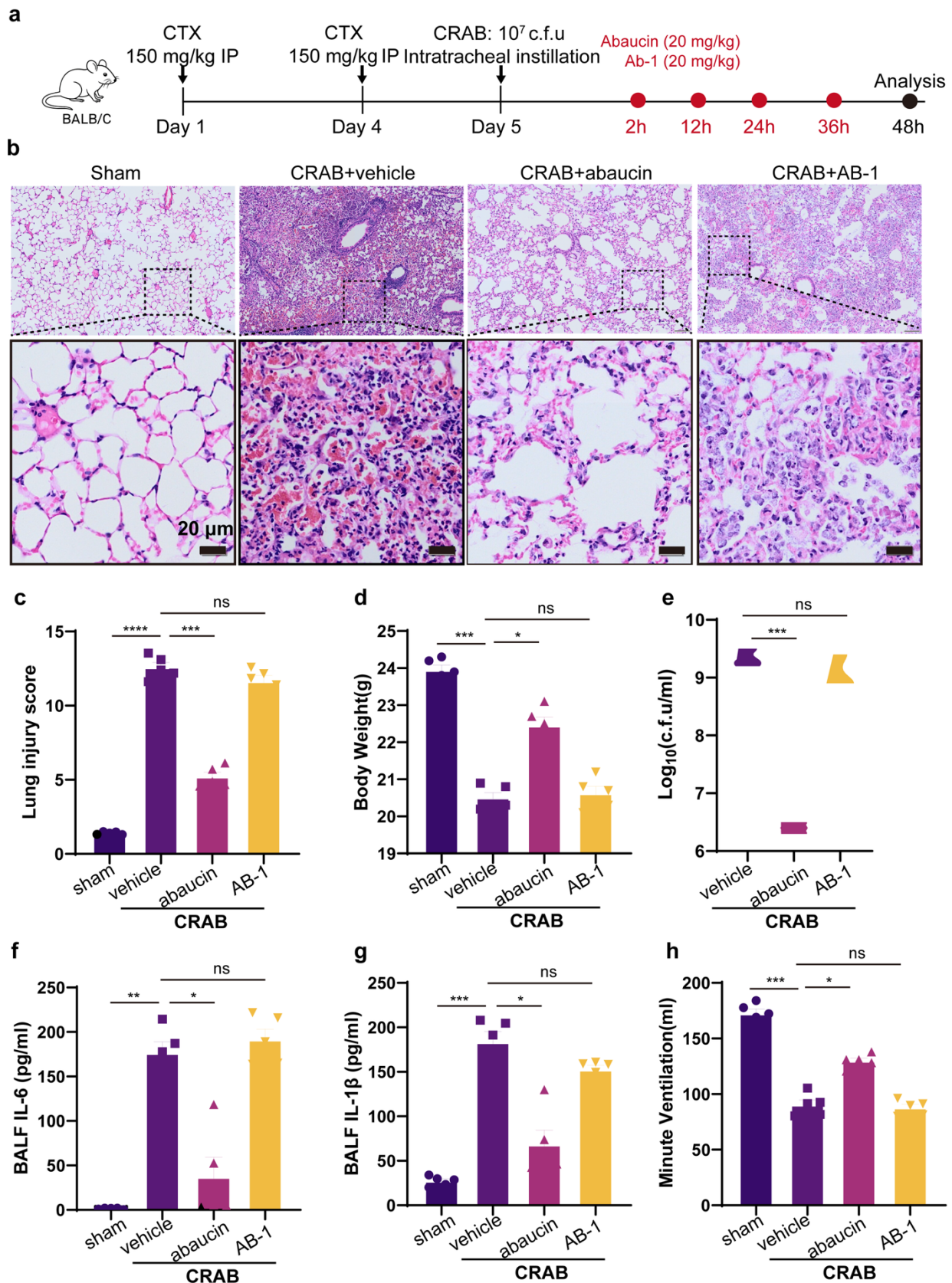
Supplementary Fig. 1c-d Effects of abaucin on the gut microbiota in mice. (c) Representative SEM images of lungs from sham, vehicle-treated and abaucin-treated mice in the CRAB-induced pneumonia model (scale bar, 200 μm ; $n = 5$ mice per group). (d) Taxonomic relative abundance at the family level of faecal samples from sham ($n = 5$), vehicle-treated ($n = 6$), abaucin-treated ($n = 6$), meropenem-treated ($n = 7$) and piperacillin-treated ($n = 4$) mice. Each bar represents one mouse; colours denote the indicated bacterial taxa, with low-abundance taxa grouped as “Others”. CRAB, carbapenem-resistant *A. baumannii*; MEM, meropenem; PIP, piperacillin. (e) Box plots of α -diversity metric (Shannon index) of the faecal microbiota in sham, vehicle-treated, abaucin-treated, meropenem-treated and piperacillin-treated mice (ns, not significant; $*p < 0.05$; $**p < 0.01$). Boxes show the interquartile range, centre lines indicate the median, whiskers indicate the data range and points represent individual mice. Statistical significance was determined as described in Methods.

[Comment 2]

2. The efficacy of AB-1 is not evaluated in the mouse model.

[Response]

We thank the reviewer for this comment. AB-1 was evaluated in the murine CRAB pneumonia model (Response Fig. 5.2). Under the dosing regimen tested, however, AB-1 showed limited in vivo efficacy and was clearly less effective than abaucin, with no material improvement in the principal therapeutic readouts. We therefore do not consider the current AB-1 data sufficiently strong to support inclusion in the revised study. We have removed the AB-1 section from the revised manuscript. As this derivative still requires further optimisation and, in its current form, adds limited value to the conclusions, we believe that its exclusion improves the focus and rigour of the manuscript.



Response Figure 5.2: a, Schematic of treatment groups and dosing regimen for the murine pulmonary infection model. BALB/c mice were assigned to the indicated groups: untreated Control (sham), sham-infected vehicle controls, and infected animals treated with abaucin (20 mg

kg⁻¹), and AB-1 (20 mg kg⁻¹). Compounds were administered via intratracheal instillation. Values listed denote the timing of sample collection or analysis post-infection (hours). **b**, Representative haematoxylin and eosin (H&E)-stained lung sections from sham, vehicle-treated, abaucin-treated and AB-1-treated mice (scale bar, 20 µm). **c**, Lung injury scores derived from histopathological assessment of lung sections in (c) (n = 5, ***p < 0.001, ****p < 0.0001). **d**, Body weight changes in sham, vehicle-treated, abaucin-treated, and AB-1-treated mice over the course of infection (n = 5, *p < 0.05; ***p < 0.001). **e**, Bacterial burden in BALF from vehicle-treated, abaucin-treated, and AB-1-treated mice, determined by colony-forming unit (c.f.u.) counts after serial dilution (n = 3, ****p < 0.0001). **f-g**, Concentrations of IL-6 and IL-1β in BALF from sham, vehicle-treated, abaucin-treated, and AB-1-treated mice (n = 5, *p < 0.05; **p < 0.01; ***p < 0.001). **h**, Lung function parameters, including minute ventilation (MV; h), in sham, vehicle-treated, abaucin-treated, and AB-1-treated mice with CRAB-induced pneumonia (n = 5, *p < 0.05; ***p < 0.001). Data are shown as mean ± s.e.m, Statistical significance was determined as described in Methods; ns, not significant.

[Comment 3]

3. Statistical analysis methods are not described in detail.

[Response]

We have expanded the "Statistical Analysis" section in Materials and Methods:

“All calculations or analyses were performed using Prism 8 software package (GraphPad) or R. Data. All results shown were obtained by at least three biologically independent experiments and the values are presented as means ± standard error of the mean (SEM). The Shapiro-Wilk test was applied to assess the normality of the data. The standard Student’s t-test was applied when there were two groups with a normal distribution, while one-way ANOVA with Bonferroni post hoc test was applied when there were more than two groups with normal distribution. Otherwise, the Mann-Whitney U test was used for two groups of nonparametric analysis. For more than two groups of nonparametric analysis, we applied the Kruskal-Wallis test followed by Dunn’s post hoc test to correct multiple comparisons.”

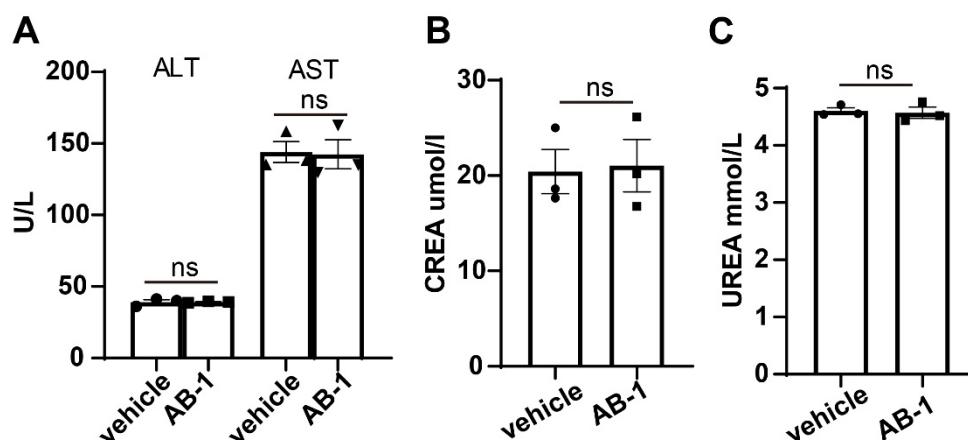
[Comment 4]

4. Ln 290: It is nice that the authors have performed some preliminary safety evaluation (cell cytotoxicity and hemolysis) of their newly synthesized AB-1. Please consider including additional cytotoxicity analysis of more advanced assays, which will be important.

[Response]

We thank the reviewer for this suggestion. We perform additional preliminary in vivo safety studies of AB-1, including serum ALT, AST, creatinine and urea measurements (Response Figure 5.4). Under the tested conditions, these parameters were not detectably altered relative to the vehicle, indicating no evident acute hepatorenal toxicity. However, because AB-1 showed limited

in vivo efficacy, we have therefore removed the entire AB-1 section from the revision. Accordingly, the additional AB-1 toxicity data are also not included. We believe this improves the clarity, focus and rigour of the study.



Response Figure 5.4: Serum biochemical analysis of liver and kidney function in vehicle- and AB-1-treated mice. **A**, Serum alanine aminotransferase (ALT, U/L) and aspartate transaminase (AST, U/L) levels in vehicle- and AB-1-treated mice. **B**, Serum creatinine (CREA, $\mu\text{mol/L}$) levels in vehicle- and AB-1-treated mice. **C**, Serum urea (UREA, mmol/L) levels in vehicle- and AB-1-treated mice. ($n = 3$, ns, not significant).

Minor comments:

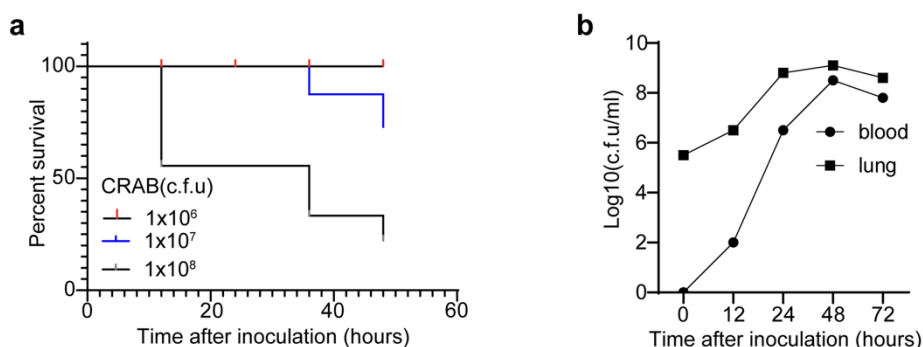
1. More characterization data on the mouse model of intratracheal infection with HX-R7 strain will be needed, such as tissue and blood bacterial burdens, infection course, and bacterial dose titration etc.. These data can be included in the Suppl Data section.

[Response]

We thank the reviewer for this suggestion. We agree that more complete characterisation of the HX-R7 intratracheal infection model improves the robustness of the in vivo study.

Accordingly, we have added model-validation data to Supplementary Fig. 1a,b, including bacterial dose titration and time-course measurements of bacterial burdens in the lung and blood. The dose-titration analysis showed an inoculum-dependent effect on survival and supported the use of 10^7 c.f.u. for the therapeutic infection model. The time-course analysis further showed progressive bacterial accumulation in the lung, followed by dissemination into the blood, thereby defining the kinetics of infection in vivo.

These additions provide a clearer framework for interpreting the treatment experiments and have been incorporated into the Supplementary Data.



Supplementary Fig. 1a, b: Characterization of the murine intratracheal CRAB (HX-R7) infection model. **A**, Survival curves of mice after intratracheal infection with HX-R7 at the indicated inoculation (1×10^6 , 1×10^7 , and 1×10^8 c.f.u.). **B**, Kinetics of bacterial dissemination after intratracheal infection with HX-R7. Bacterial burdens in the lung and blood were determined at the indicated time points and are presented as $\log_{10}$ c.f.u. ml^{-1} .

2. Ln 164: Please spell out “OUT”;

[Response]

We thank the reviewer for this comment. Instead of defining “OTU” in the text, we have removed the term entirely, as the corresponding microbiota panel provides taxonomically aggregated relative abundances rather than OTU-level data. To avoid ambiguity, the revised manuscript now reports this result simply as a change in faecal microbial composition.

3. Ln 687: the abaucin administration schedule (every 12 h) is slightly different from that described in Fig. 2a (2, 12, and every 12 hours thereafter);

[Response]

We thank the reviewer for identifying this discrepancy. We have audited the relevant text and figures and corrected the treatment schedule throughout the manuscript for consistency. The abaucin dosing regimen is now uniformly described as administration at 2 h and 12 h after infection, followed by dosing every 12 h thereafter. The main text and figure legends have been revised accordingly.

4. Ln 698: Please provide more details on how the lung injury was scored.

[Response]

We thank the reviewer for this comment. We have already expanded the description in the revised Materials and Methods. Lung injury was evaluated using a semiquantitative

histopathological scoring system adapted from a published method^{1,2}. Four indices were assessed: alveolar septal thickening, inflammatory cell infiltration, haemorrhage and oedema. Alveolar septal thickening, haemorrhage and oedema were each scored on a 0-4 scale, corresponding to none, mild, moderate, severe and very severe changes. Inflammation was assessed by counting the total number of inflammatory cells per $\times 100$ field. Scoring was performed in a blinded manner on randomly selected fields from each lung section. The manuscript has been revised accordingly.

Reference:

1. Ma, Jingyue *et al.* Elevated GFI1 in Alveolar Macrophages Suppresses ACOD1 Expression and Exacerbates Lipopolysaccharide-Induced Lung Injury in Obesity. *Advanced science* 13, 2413546 (2025).
2. Jiang J, *et al.* Targeting NOX4 alleviates sepsis-induced acute lung injury via attenuation of redox-sensitive activation of CaMKII/ERK1/2/MLCK and endothelial cell barrier dysfunction. *Redox biology* 36, 101638, (2020).

5. Fig. 1: For comparison purposes, please include data for reference *A. baumannii* strains and ampicillin MIC data for gut commensal bacteria and other resistant pathogens.

[Response]

We thank the reviewer for these suggestions. The clinical *A. baumannii* isolates in Fig. 1 are from the West China Hospital Clinical Isolate Bank; their NCBI accession numbers (SAMN34037527, SAMN34037535, SAMN34037546, SAMN34037486, SAMN34037488, SAMN34037507) have now have been added to the Methods section. Regarding the ampicillin MIC data for gut commensals, *Bacteroides* species exhibit intrinsic resistance to penicillins. Following CLSI broth microdilution guidelines, we determined the ampicillin MICs for three representative gut commensal strains and confirmed that all three exhibited MIC values exceeding $16 \mu\text{g mL}^{-1}$ (see Response Table). These results are consistent with the well-documented broad-spectrum activity of ampicillin and the natural resistance profile of anaerobic Gram-negative commensals. Nevertheless, to preserve the manuscript's focus on the structural and mechanistic characterization of abaucin, we have elected not to expand Fig. 1 with these supplementary data. Should the reviewer recommend it, we are prepared to include the ampicillin MIC table as Supporting Information.

Gut commensal bacteria	MIC (µg/mL)	
	Abaucin	AMP
<i>B. vulgatus</i>	>128	>16
<i>B. thetaiotaomicron</i>	>128	>16
<i>B. fragilis</i>	>128	>16

6. Fig. 2: Please consider including Control Group cytokine data for Fig. 2e and 2f;

[Response]

We thank the reviewer for this suggestion. We agree that the control group cytokine data should be shown alongside the infected groups to provide a clearer baseline for comparison. We have therefore added the control group data to Fig. 2 (shown above) in the revised manuscript.

7. Fig. 2: The treatment time points and 2nd CTX dose in the figure legend are different from those described in the Materials & Methods.

[Response]

We thank the reviewer for identifying these inconsistencies. We have carefully checked the relevant text, figure legends and schematic timeline, and corrected them throughout the manuscript for consistency. The abaucin treatment schedule is now uniformly described across the Materials and Methods, figure legends and figures, and the second CTX dose has also been corrected to match the experimental protocol. The revised manuscript now presents a consistent treatment and immunosuppression regimen throughout.

8. Fig. S1b: Please consider moving the BAL bacterial data into Fig. 1.

[Response]

We thank the reviewer for this suggestion. We agree that the BALF bacterial burden is an important component of the in vivo efficacy assessment and should be presented alongside the other principal readouts. We have therefore moved these data from Supplementary Fig. 1 to Fig. 2 in the revised manuscript.

9. Fig. 5b: Please include the MIC data of other reference *A. baumannii* strains.

[Response]

We thank the reviewer for this request. Please note that Figure 5 has been removed entirely from the revised manuscript following the removal of the AB-1-related content. In addition, for *A.*

baylyi strain used as the surrogate host for *A. baumannii* in the genetic modification assays, we have measured the MIC of abaucin in the corresponding model strains (revised Fig. 4d).

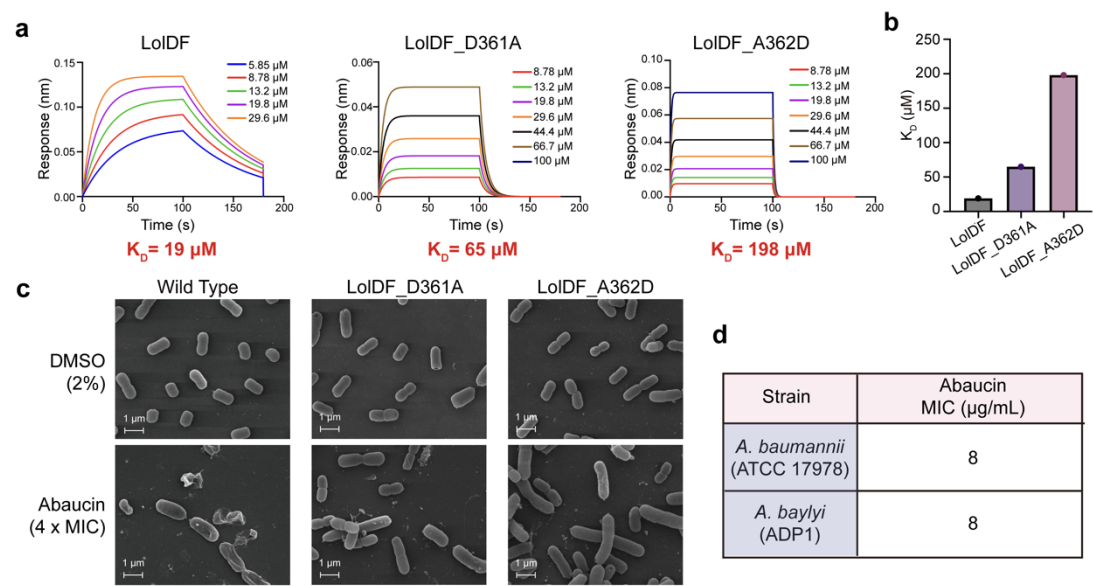


Fig. 4: Characterization of abaucin binding to LolDF. **a**, Bio-layer interferometry (BLI) sensorgrams showing abaucin affinity to wild-type LolDF or LolDF mutants carrying substitutions in the abaucin-binding sites. **b**, Averaged K_D value from the BLI assay. **c**, Scanning electron microscopy (SEM) images of wild-type *A. baylyi* and the corresponding LolDF mutant strains with or without abaucin treatment. **d**, MICs were determined for wild-type *A. baumannii* (ATCC 17978) and *A. baylyi* (ADP1). Data in (a-c) are representative of $n = 3$ independent experiments.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have revised the manuscript which has addressed my major concerns. The removal of the AB-1 data, which was unconvincing, and the addition of the LolDF transporter simultaneously bound to lipoprotein have significantly improved the clarity and impact of the study.

One point remaining is that the abaucin affinity data fitted with a 1: 1 global binding model (main manuscript, Line 969) but this does not seem appropriate given that two molecules of drug are bound per transporter. In fact, in response figure 3.7, the legend states a 1:2 model was used for the same data. Could the authors clarify the model used, please?

A second minor point is that the formatting of the table in Figure 1 could be modified to make the data clearer i.e. insert a break in the line between the “Gut commensal bacteria” and “Multidrug resistance pathogen” data to make it clear that these are separate data. If the line with a single “-“ is included just to balance the table, it should be removed. The heading of the left-hand column should be “Clinical isolate” rather than “Clinical isolation.”

Those points aside, I believe the manuscript is now suitable for publication in Nature Communications.

Response: We thank the reviewer for the positive assessment of our revised manuscript and for recognizing the improvements resulting from the removal of the AB-1 data and the inclusion of the LolDF-lipoprotein complex structure. We are grateful for the reviewer's recommendation for publication.

1. The affinity data were fitted using a 1:2 binding model, consistent with the structural observation that two abaucin molecules bind per LolDF transporter. The description of a "1:1 global binding model" in the manuscript was incorrect and resulted from a typographical oversight during revision. We have corrected the text accordingly to indicate that a 1:2 binding model was used for data analysis.

2. We have revised the formatting of the table in Figure 1 to improve clarity by clearly separating the "Gut commensal bacteria" and "Multidrug-resistant pathogen" sections and removing the unnecessary placeholder row. In addition, the column heading has been corrected from "Clinical isolation" to "Clinical isolate."

Reviewer #2 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Response: We thank the reviewer for taking the time to evaluate our revised manuscript.

Reviewer #3 (Remarks to the Author):

The authors have addressed all my comments with additional data and clarifications. The only concern is the inclusion of the abaucin-lipoprotein complex structure. Inspecting the supplied maps, it appears that the density probably belongs to the LolDF complex and in particular residues 375-381 (the loop is out of density) in one side, and residues 367-381 on the other side (the model is not well built in the density). The lipoprotein density itself is extremely weak and it could also correspond to detergent molecules. The authors have also not identified the type of lipoprotein so it is unclear what the dipeptide represents. The structure is unlikely to represent an inhibitor-lipoprotein complex and I strongly suggest that it is omitted from the manuscript as it is not providing any new insights at its current state.

Response: We thank the reviewer for the careful evaluation of the density and for raising these concerns.

Regarding the assignment of abaucin, we agree that the densities corresponding to the abaucin molecules are located close to, and partially merged with, the loop regions (residues 375-381 and 367-381). We therefore explored the possibility that these densities could be explained by modeling the loops into the map. While portions of the density could indeed be accounted for by extending the loop models, a substantial fraction of the density remained unexplained. In contrast, fitting abaucin molecules into these regions provided a more complete explanation of the observed density, including the surrounding features. Furthermore, these densities closely resemble those observed in the previously reported abaucin-bound LolDF structure in the absence of lipoprotein. Taken together, we believe that assignment of these densities to abaucin is more consistent with the cryo-EM map than attributing them solely to the loop regions.

With respect to the putative lipoprotein density, we acknowledge that the density is relatively weak. For this reason, we did not attempt to interpret detailed interactions between the substrate and the LolDF complex. Rather, the primary objective of this structure was to determine whether the LolDF cavity could simultaneously accommodate both substrate and abaucin. Despite the limited local resolution, the elongated density corresponding to the lipid moiety is clearly visible, and the modeled acyl chains fit this density well.

Importantly, this abaucin- and substrate-bound complex was obtained by co-expressing the lipoprotein BamE, the same construct used in our transport assays. The presence of BamE in the purified sample was independently confirmed by Western blotting using the affinity tag (Supplementary Fig. 8c). We also considered the possibility that the density could arise from detergent molecules. However, the detergent used during purification was DDM, which contains a single dodecyl alkyl chain. This interpretation is difficult to reconcile with the observed density, which is more consistent with elongated diacyl lipid chains spanning the cavity.

Finally, we note that we have been careful not to overinterpret these densities in the manuscript. We do not assign a specific identity to the bound lipoprotein, nor do we draw mechanistic conclusions based on detailed substrate–transporter interactions. Instead, we use this structure to support the conclusion that the abaucin-bound LolDF cavity remains sufficiently accessible to accommodate a lipoprotein substrate, providing structural evidence for a substrate-accessible outward-open state.

Reviewer #4 (Remarks to the Author):

My concerns with the prior submission have been adequately addressed. New structures have helped to better define how abaucin interferes in the LolDF transport cycle.

My only comment is that the authors should now also include a discussion of their structures and findings in relation to the recent Nat Comms LolDF-abaucin publication (PMID 42091888). I think both studies are valuable and some comparative discussion would be beneficial here.

Response: We thank the reviewer for this helpful suggestion. We have revised the Discussion to incorporate a comparison with the recently published LolDF–abaucin study (PMID: 42091888). Specifically, we now state that: *“Consistent with our observations, a recent study reported LolDF structures containing either two or four abaucin molecules, which adopt a similar conformational state⁵⁸.”* The similarity between the conformational states observed in the two studies provides independent structural support for the mechanism by which abaucin stabilizes LolDF in a non-productive conformation.

We note that a more detailed structural comparison was not possible because the corresponding atomic coordinates had not yet been deposited in the Protein Data Bank at the time of our revision. Consequently, our discussion is limited to the structural features and conclusions described in the published article. We believe that inclusion of this comparison appropriately places our findings within the context of the emerging literature while avoiding overinterpretation in the absence of the deposited structural models.

Reviewer #5 (Remarks to the Author):

In their revised manuscript, the authors have made substantial efforts in addressing my initial concerns, including additional experiments and new data. Those modifications have adequately answered my suggestions. However, if the authors can provide the inoculation dose used in their Suppl Fig. 1b, that will be great.

Response: Thank you for this helpful comment. We have revised the legend of Supplementary Fig. 1b with the inoculation dose used. The revised legend reads: “Kinetics of bacterial dissemination after intratracheal infection with HX-R7 at an inoculum of 1×10^7 CFU. Bacterial burdens in the lung and blood were determined at the indicated time points and are presented as $\log_{10}$ CFU mL⁻¹.”