

Molecular prosthetics for CFTR designed for anion selectivity outperform amphotericin B in cystic fibrosis airway epithelia

Corresponding Author: Professor Martin Burke

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)

Thank you for asking me to review this manuscript.

I found it to be carefully written and compiled – it is easy to follow and engage with.

In summary, the researchers have expanded on previous findings that amphotericin B, an anti-fungal drug, forms unselective ion channels when applied to airway epithelial cells and can restore airway surface liquid pH and antibacterial functions in CF cells and pigs (Muraglia et al. Nature 2019). However, at higher concentrations the channels lose their capacity to do this.

In this manuscript the researchers have chemically altered amphotericin B, generating synthetic derivatives, so that the channels formed favour anion permeability and thus increased efficacy.

A logical set of experiments are described demonstrating in relevant cellular models that these derivatives do generate

- Improved relative permeability to anions
- They restore airway surface liquid pH at both high and low concentrations to a greater extent than amphotericin B
- A further step whereby cholesterol binding is minimised and this reduces toxicity of the compounds in CF airway epithelial cells (without this modification there would be an obvious flaw in the therapeutic potential of these compounds if toxic to cells – amphotericin B itself though used clinically does have marked toxicity in humans)

The development of novel approaches to ameliorate the pathophysiology of CF in the microenvironment of the apical surface of airway epithelial cells is welcome. Importantly, 'non-CFTR' based approaches are attractive because they are relevant to i) people who do not have a highly-effective CFTR modulator option available to them (mainly due to genetics and class I variants but also due to inequality in access globally due to cost) and ii) the potential to augment benefits from CFTR modulators as an additional therapy.

I cannot find a major flaw in the experimental approaches, results presented and conclusions drawn.

However, areas that could be expanded include

- Limitations of the approaches to date and next logical steps, e.g. work in animal models versus cell models
- Potential clinical translatability of the compounds, barriers to this and steps necessary to achieve this, how might the compounds be given, e.g. orally, IV, nebulised???

Reviewer #2

(Remarks to the Author)

The paper "Molecular prosthetics for CFTR designed for anion selectivity outperform amphotericin B in cultured cystic fibrosis airway epithelia" by Marin-Toledo et al. developed two novel synthetic derivatives of amphotericin B (AmB), AmB-AA and C2'epiAmB-AA, as a new molecular treatment for abnormal airway surface liquid (ASL) in cystic fibrosis (CF) airway. The new derivatives of AmB evaluated the ASL pH in CF airway epithelial cell culture at both low and high concentrations; also, derivative C2'epiAmB-AA further reduced the toxicity to the cell culture by reducing the cholesterol binding, which

could affect the mammalian lipid bilayers. The new derivatives of AmB could be the optional new molecular therapy for CF patients after further testing. The methodology is sound; however, additional data and clarification would strengthen the conclusions.

1. For the Ussing chamber current clamp experiments done in Figure 1 (probably in Figure 4d as well), AmB and the derivatives were added both sides of the epithelial cell cultures; however, in the ASL pH measurements, $H^{14}CO_3^-$ transport, and toxicity tests, AmB and the derivatives seem to be added only in the apical side of the culture. Would the Ussing chamber results be affected by the presence of AmB and the derivatives in the basolateral side? Many labs had demonstrated the crosstalk between the apical and basolateral membranes happening in the epithelial cells (ex. DOI: 10.1152/ajpcell.1994.267.1.C146), as a result, treating the basolateral membrane with AmB and the derivatives could complicate the conclusion from the Ussing chamber results.
2. ENaC channels are well expressed in airway epithelial cell culture. In your previous publications (DOI: 10.1038/s41586-019-1018-5; DOI: 10.1016/j.jcf.2020.11.018), amiloride was used to minimize the ENaC activity in Ussing chamber experiments. Here, in this paper, there is no mention of using amiloride in the Ussing chamber experiments. Would the PCl/PNa measurements here be affected by ENaC activity in the cell culture?
3. AmB and its derivatives applied in the airway seem not to target a specific cell. What would happen to the cells without the CFTR channel after AmB and its derivatives application? Would there be any unexpected consequences for their functions? Further investigation would be beneficial, and the authors could discuss this in detail.
4. Investigating the effects of AmB-AA and C2'epiAmB-AA on ASL layer height and mucociliary clearance process would further establish the potential of this new molecular therapy for CF.
5. In Figure 1c, the x-axis seems to be wrong. In Figure 1d, voltage measurements from the Ussing chamber experiment only showed 6 minutes. The traces shown in Figure 1b to 1e should be on a similar time scale.

Reviewer #3

(Remarks to the Author)

This manuscript describes the design and validation of chemical modifications aimed at ameliorating the efficacy and toxicity of amphotericin B (AmB) to use as a prosthetic anion channel in the treatment of cystic fibrosis.

This is an interesting paper on an important topic. However, although molecular simulation methods were apparently used to construct an atomistic model of the Am-AA "sponge" formed with cholesterol, that part of the work is not conveyed in adequate or convincing manner. Specifically:

(1) The methodology is not adequately described. The simulated annealing mentioned in the Results (Line) is not mentioned in the Methods. How were the systems prepared? How did the three repeats differ from one another? Enough details should be included for reproducibility by competent researchers. "Previously reported data" (line 734) are not referenced. Please confirm that "a grid density of less than $1 \text{ \AA}^{-3}$ " (line 751) was used in the Ewald calculations.

(2) What were the purpose and the outcome of the simulations? Where are the results shown, analyzed, and discussed? The only place (outside of Methods) where the simulations are mentioned is in the following paragraph, Lines 189-195:

"Building on an extensive series of recently reported biophysical and structural studies with AmB25,57, XPLOr-NIH simulated annealing and molecular dynamics yielded a high-resolution structure of the AmB-AA sponge which showed the presence of a large void volume capable of accommodating the presence of a C16-ethylamine chain (Fig. 4d). These computational models further supported the conclusion that AmB-AA similarly kills mammalian cells via a sterol sponge-mediated cholesterol binding-dependent mechanism." [My emphasis]

Figure 3d (mis-referenced in the text in line 193 as "Fig. 4d") shows what may be a snapshot from the simulations (but is not specified). The caption of Fig. 3d reads: "(d) Model of AmB-AA bound to cholesterol." Unless I missed something, that is the only data shown from the simulations in the manuscript.

What are we looking at? How was this particular structure obtained and how was it chosen for display? How representative is it? If the point was to show "the presence of a large void volume capable of accommodating the presence of a C16-ethylamine chain" as stated in the Results, how was that aspect of the model established and validated?

In conclusion, the simulations should be adequately motivated, described, analyzed, and discussed to justify their inclusion in the manuscript.

Reviewer #4

(Remarks to the Author)

This is an interesting and well-constructed Ms which is a follow on from the authors 2019 Nature paper, which provided strong evidence that amphotericin B (AmB) restored host defences in CF airway, both in vitro and in vivo. In this new paper the authors have performed some nice chemical optimisation of AmB to improve its anion selectivity and reduce cholesterol binding capacity (potential toxicity factor). The new AmB derivatives demonstrate small, but significantly larger, changes in the ASL pH as originally reported for AmB, using polarised CF cell lines and fully differentiated primary CF airway cells. However, this paper lacks sufficient in vitro 'functional' evidence using fully differentiated primary human CF cultures that the new derivatives substantially improve and restore airway defence mechanisms, compared to the parent compound. Furthermore, no in vivo (animal) evidence is provided to substantiate the in vitro findings.

Specific major concerns essential to be addressed to support the conclusions.

1. While the optimised AmB derivatives were able to increase HCO₃⁻ transport and ASL pH, this could be for reasons other than via direct permeation through the AmB derivatives themselves. A more direct way to test this is to measure the anion permeability of the derivatives after reconstitution into artificial lipid bilayers and performing current measurements under different conditions.

2. The ASL pH results for AmB-AA depicted in Figs 2b and 4g don't show any dependence on dose (between 2 and 100uM). Why didn't they test lower doses? This is important in the context of future clinical relevance.

3. It would be extremely informative to compare the ASL pH/HCO₃ transport results obtained from cells treated with AmB and derivatives, to what would be observed for these parameters using CF cell lines (CuFi-1 and 4) or primary CF cells (delF/delF) after pretreatment with CFTR modulators. This would provide stronger evidence that the changes observed with AmB derivatives are likely to have 'clinical' benefit. Furthermore, as the authors suggest that the AmB derivatives work in a CFTR-independent manner, they should test CF null cells which don't produce any CFTR protein to substantiate this conclusion.

4. The Ms would be far more convincing if other 'functional' effects, relevant to CF airway disease, were also performed, such as ASL 'hydration', ASL viscosity, bacterial killing.

5. The Ms doesn't address the fact that these new derivatives have not been approved for clinical use, unlike AmB.

Minor concerns that should be addressed.

1. The number of different CF donors used in the experiments that tested the effect of AmB and derivatives on ASL pH using primary CF airway cells (Fig 4h) is not stated.

2. Methods. No details are provided about CuFi-4 cells.

3. Extended fig 1. Indicate CuFi-1 cells were used in panel f.

4. Statistics. The authors have only employed parametric tests, but there is no information whether they checked if the results were normally distributed.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Thank you for responding to the previous review comments fully.

Reviewer #2

(Remarks to the Author)

Marin-Toledo and colleagues have presented a well written revised manuscript that provides the necessary clarification for my comments, as well as the methodology as other reviewers pointed out. The new data from the cell culture experiments and the discussion section provide significant improvement, addressing most of my concerns.

Reviewer #4

(Remarks to the Author)

This revised version has been significantly improved and the authors have satisfactorily answered all my comments. I have no further comments or suggestions.

This is an important piece of work that could eventually provide significant benefit for all people with CF, and especially those who are not eligible, or who cannot afford, current highly effective CFTR modulators.

Reviewer #5

(Remarks to the Author)

Review focusing on MD simulations

Manuscript: Molecular prosthetics for CFTR designed for anion selectivity outperform amphotericin B in cultured cystic fibrosis airway epithelia

I have been asked to comment on the concerns of Reviewer #3 and to assess the authors' responses and revisions. Please

see my comments below.

Unfortunately, there remain several serious issues related to the molecular dynamics (MD) simulations and their interpretation.

“How were the systems prepared? How did the three repeats differ from one another?”

These points remain unclear and should be addressed explicitly. In particular, the manuscript does not describe the initial structures used for the simulations (see detailed comments below).

Additionally, it is not evident how independent replicates were generated. Did you create them by reassigning velocities (e.g., using different random seeds) after energy minimization? As currently described, it appears that the same NPT-equilibrated structure was used to initiate multiple production runs without modifying the velocity distribution. If so, this would not constitute statistically independent repeats and is not an appropriate approach for replica generation.

The responses to questions regarding the purpose and outcome of the simulations are still not adequately addressed. The revised text does not clearly articulate what specific hypotheses the simulations were intended to test, nor what conclusions can be robustly drawn from them. For example, in Extended Figure 6, the use of RMSF to infer or support similar interaction patterns is not appropriate. RMSF reflects local flexibility and does not provide direct information about interaction networks or binding modes. Drawing conclusions about similarity of interactions based on RMSF alone is therefore not well justified and should be reconsidered or supported with more suitable analyses (e.g., contact maps, interaction frequencies, or distance-based metrics).

Main text

The authors state, as one of the objectives of the MD simulations, that they aim to understand how “the C41-ethylamine side chain would ... change the mechanism by which these compounds interact with lipid bilayers.” However, no simulations including a lipid bilayer are presented. This represents a clear mismatch between the stated objective and the actual simulations performed.

Figure 3d

The description “NMR informed model of AmB AA bound to cholesterol” is insufficiently documented. It is not clear how this model was generated, whether it results from MD simulations, NMR refinement, or a hybrid approach. This should be explicitly described in the Methods.

Additionally, the panel would benefit from clearer visual guidance. In particular, the smaller inset (panel d) is not well connected to the rest of the figure; adding an arrow or similar visual cue would help the reader.

Methods

The section title “System Preparation and Molecular Dynamics Simulations” could be improved for clarity. For example: “Molecular Dynamics Simulations”, or “System Preparation and Parameters for Molecular Dynamics Simulations.”

The statement “The system was prepared using the lowest energy AmB–cholesterol lattice (Fig. 1a) from previously reported and deposited data⁵⁸.” is problematic for several reasons:

- Fig. 1a does not clearly show an AmB–cholesterol lattice.
- It is not evident from the cited reference how the initial structure was obtained.

For reproducibility, it is essential that the authors provide full details of the initial structure. Deposition of the starting coordinates, final structures, and parameter files (e.g., via Zenodo or a similar repository) is strongly recommended and, in practice, expected, required by Nat Comm.

The following sentence is difficult to parse and should be rewritten:

“To maintain the structure of the experimentally resolved sponge, carbon atoms in the macrolide region of all AmB molecules were constrained using gmx genrestr with a force constant of 8000 kJ/mol/nm² and all sterol molecules left unconstrained (???) AmB parameters were obtained from CHARMM General Force Field and sterols parameters were obtained from the CHARMM36 force field.”

Beyond the wording issues, there are important methodological concerns:

A force constant of 8000 kJ/mol/nm² is unusually high, even for early equilibration, and requires justification. It is unclear whether these constraints were released during equilibration or remained during production simulations. Cholesterol (or

sterols in general) was left unconstrained, which is inconsistent given that it is a key structural component of the system. These points raise concerns about whether the system was allowed to equilibrate realistically.

The sentence "For equilibration and production runs, the C-rescale barostat and V-rescale thermostat were employed along with the leapfrog integrator to maintain constant pressure and temperature."

is somewhat unclear. As written, it may suggest that all listed components, including the leapfrog integrator, contribute to maintaining constant pressure and temperature, which is not accurate.

In the "Table of simulations," the simulation length would be clearer if reported as 3×250 ns rather than simply 250 ns.

Extended Figure 6

The statement "Computed RMSF values for each atom in the cholesterol molecule indicate similar intermolecular interaction patterns across all AmB derivative sponge models." is not sufficiently precise. RMSF reflects atomic fluctuations (flexibility) and does not directly measure intermolecular interactions. The interpretation should either be justified more carefully or rephrased.

More generally, the occupied/void volume analysis appears to be a relatively coarse metric. Its dependence on voxelization and occupancy thresholds makes it difficult to interpret in a physically meaningful way or to relate directly to molecular interactions. More informative analyses would include:

- radial distribution functions (RDFs) between AmB and cholesterol,
- contact frequency or residence time analyses,
- or other position-resolved interaction metrics.

Finally, there is an inconsistency between the text and the figure: values are described as 100% in the text, while the figure reports values on a 0-1 scale. This should be made consistent.

Overall

The MD section requires substantial clarification and, in some cases, additional analysis. At present, the description of the simulations, the lack of reproducibility details, and the interpretation of the results do not meet the expected standards.

Version 2:

Reviewer comments:

Reviewer #5

(Remarks to the Author)

The revised work is much more transparent and reproducible, and the addition of RDF/contact analyses is a clear improvement. I appreciate the substantial improvements made in this revision. In particular, the authors now clearly describe the origin of the starting structures, the system construction, the use of randomized initial velocities for the three replicas, the simulation parameters, and the deposition of coordinates and input files. These changes adequately address my previous concerns regarding reproducibility and the generation of replicate trajectories. The revision of the RMSF interpretation and the addition of RDF and contact-frequency analyses are also welcome improvements. However, several important issues remain.

1. Scope of the simulations and interpretation of the restraint-based model

The revised Methods now clearly state that positional restraints of 8000 kJ / mol / nm² were applied to the macrolactone heavy atoms throughout minimization, equilibration, and production. The authors also explicitly state that this protocol probes local relaxation of unrestrained groups within an experimentally resolved scaffold, rather than the de novo thermodynamic stability of the sponge architecture. This is an important clarification. Such restrained simulations can reasonably address whether the C41 modification and cholesterol molecules can be accommodated locally within the NMR-derived sponge scaffold.

However, the conclusions in the Results and Extended Figure 6 still exceed what this protocol can demonstrate. Because the macrolactone scaffold is deliberately maintained close to the NMR-derived geometry, these simulations cannot establish spontaneous sponge assembly, thermodynamic stability of the sponge, preservation of sponge architecture in an unrestrained system, cholesterol extraction from a membrane, or equivalence of the complete toxicity mechanism between AmB and AmB-AA. Accordingly, statements such as "MD yielded a high-resolution sponge structure," "large void volumes were not disrupted," or that the simulations support an identical cholesterol-extraction-dependent mechanism should be softened.

The data support a more limited and defensible conclusion: the C41 ethylenediamine modification appears compatible with the local cholesterol environment of the experimentally derived and restrained sponge scaffold. This would be consistent with the proposed sterol-sponge mechanism, but it does not independently demonstrate sponge stability or cholesterol extraction.

I recommend revising the central conclusion accordingly, both in the Results and in the legend/title of Extended Figure 6. Terms such as "maintaining structural stability" should be avoided unless stability is assessed using a substantially less restrained or unrestrained model.

2. The new interaction analysis does not directly test the full C41 ethylenediamine modification

The reported RDF is calculated between the C2'-hydroxyl oxygen of AmB and cholesterol O3. This seems to be useful for examining the effect of C2' epimerization, but it does not directly test the newly introduced C41 ethylenediamine group. Similarly, the description of the C41 contact analysis is somewhat unclear. The rebuttal states that the C41 region was represented by the C41 carbon, whereas the revised Methods refer more broadly to a C41 carboxylate/carboxamide interaction class. In either case, it is not clear that the analysis includes the full modified side chain, particularly the linker atoms and terminal protonated amine(s). This distinction is important because the ethylenediamine group is precisely the chemical modification whose compatibility with cholesterol packing is being claimed.

Please clarify the exact atom selections used for the C41 interaction analysis. I recommend adding analyses that directly involve the complete modified group, for example: contact frequencies between cholesterol and all atoms of the C41 carboxamide/ethylenediamine moiety, distance distributions or RDFs involving the terminal amine nitrogen(s) and cholesterol hydroxyl atoms, hydrogen-bond occupancy and geometry, and, where relevant, water-mediated interactions or solvent exposure of the new charged group. These analyses should be possible using the existing trajectories.

3. Convergence has not been demonstrated

The MD checklist claims that the RMSF values calculated over the final 80% of each trajectory "represent equilibrium dynamics." The revised Methods similarly state that the final 80% was used for RMSF, contact-frequency, and RDF analyses, with the initial 20% excluded as equilibration. However, equilibration or convergence was not demonstrated – why do 20% was excluded? The fact that "presented RMSF values were calculated for the last 80% (200 ns) of the trajectory and represent equilibrium dynamics" (checklist document) does not mean that the simulations converged.

4. The checklist statement regarding independence from initial configuration should be corrected

The use of different random initial velocities is an acceptable and standard approach for generating dynamical replicas from one starting structure. However, it does not establish independence from the initial spatial configuration, because all systems begin from the same templated NMR-derived geometry. The checklist should therefore not state that independence from initial configuration has been demonstrated. It would be more accurate to state that three trajectories were initiated from the same structural model using independently randomized velocity distributions. This is a reporting issue, no additional starting conformations are required.

5. The occupied/void-volume analysis remains insufficiently defined and should be de-emphasized

The authors have appropriately added RDF and contact analyses as more direct descriptors of local AmB-cholesterol interactions. These should remain the principal analyses. By contrast, the occupied/void-volume analysis remains difficult to interpret. The Methods describe an occupancy map at "0.5 Å³ resolution," which is not clear: does this refer to grid spacing, voxel volume, or another parameter? The coordinate alignment procedure, molecular selections, cavity definition, occupancy criterion, and averaging scheme should be stated explicitly.

Moreover, because the macrolactone scaffold remains strongly restrained, similar void volumes are not independent evidence that the sponge is structurally stable. The analysis should therefore be presented only as a descriptive supplementary metric, with replicate-level uncertainty shown.

Finally, the figure/text should consistently distinguish between fractional occupancy values from 0 to 1 and occupancy percentages from 0 to 100%.

6. Minor but important - should be corrected or clarified:

1. The protonation states and net charges of AmB, AmB-AA, C2'epiAmB, and C2'epiAmB-AA should be explicitly stated. This is particularly important because the biological and mechanistic rationale relies on altered charge at C41.

2. The reference (88) cited for the C-rescale barostat appears not to correspond to the stochastic cell-rescaling method. Please cite the appropriate original method or GROMACS implementation reference.

3. The cited CHARMM36 reference (84) appears to be a protein-force-field paper. Please cite the appropriate CHARMM lipid/cholesterol parametrization source and specify the topology version used.

4. Please remove very long hyphens (—), humans do not use it.

Version 3:

Reviewer comments:

Reviewer #5

(Remarks to the Author)

Thank you for the careful revision and for substantially improving the manuscript.

There remain a few relatively small technical issues that should be corrected before acceptance.

1. RDF analysis terminology. There is a technical wording error in the RDF Methods. The authors describe the RDFs as being calculated between "AmB hydrogen-bond donor atoms" and cholesterol, but then include the C41 carbon among these atoms. The C41 carbon is not a hydrogen-bond donor.

2. Number of molecules used for the volume analysis. The manuscript should state unambiguously whether the occupied/void-volume analysis is performed on the central 27 AmB molecules or on all 125 molecules in the 5 × 5 × 5

assembly.

3. Incorrect Extended Figure 6 panel reference. The Results state that void-volume changes are shown in Extended Fig. 6d. However, panel d contains the contact-frequency analysis, whereas the void-volume analysis is shown in panel g.

4. Water count in Table 1. Table 1 labels values such as 99,786 as “Water molecules count,” while the total atom count for the corresponding system is 135,776. The first number is likely “Water atoms”.

5. Overstatement in Figure 3. The title remains “Toxicity of AmB-AA is mechanistically similar to AmB,” and panel c is described as a representative snapshot from an “equilibrium all-atom MD simulation of the AmB-AA sterol sponge.” Given that the macrolactone scaffold is strongly restrained throughout the simulations, this wording remains stronger than what the simulations themselves establish. I suggest using more restrained wording, for example describing panel c as a representative snapshot from a restrained all-atom MD production trajectory based on the experimentally derived sponge scaffold. Likewise, the Figure 3 title could be softened to indicate that the observations are “consistent with” a mechanistically similar cholesterol-dependent toxicity mechanism rather than demonstrating mechanistic equivalence.

MD checklist

1. Item 1b: equilibration and production periods. The response should explicitly state the complete simulation and analysis scheme rather than mentioning only the 1 ns NPT equilibration. For clarity, it should indicate that each system underwent 1 ns NPT equilibration followed by 250 ns of production MD per replicate, with the final 200 ns used for RMSF, contact-frequency, and RDF analyses, and the final 50 ns used for the hydrogen-bond occupancy analysis. The reason for using a different analysis window for the hydrogen-bond calculation should also be stated.

2. Item 2a: connection to experiment. Rather than simply answering “Yes,” the checklist should briefly state how the simulations are connected to experiment. For example, the simulations begin from an experimentally derived NMR sponge scaffold, and the calculated interaction behavior is interpreted in relation to cholesterol-binding and toxicity measurements.

3. Item 3b: suitability and limitations of the model. The checklist should explicitly point to the Methods passage explaining what the restrained model can and cannot address. In particular, it should note that the simulations probe local relaxation and accommodation of the C41 modification and cholesterol within the experimentally derived scaffold, rather than de novo sponge formation, unrestrained thermodynamic stability, or cholesterol extraction from a membrane.

4. Item 4a: system setup table. This item should not simply state “Yes, Methods section” in its current form. The table itself does not include the 11-nm simulation-box dimension, and the water-molecule counts appear to be mislabeled, as noted above.

5. Item 4b: software versions. Actual versions of VMD and MDAnalysis should also be provided.

6. Item 4d: deposited input and coordinate files. The checklist should state that these materials are deposited on Zenodo, rather than simply saying “supplementary files.” The manuscript specifies that starting and final coordinates, topology files, parameter files, CGenFF outputs, and MDP files for minimization, equilibration, and production are available there.

7. Item 4e: custom parameters. The response should more precisely state that compound-specific “CGenFF parameters were generated for the AmB derivatives and deposited on Zenodo”, rather than referring generally to the compounds as not being parameterized in the standard CHARMM force field.

Overall, the manuscript has been substantially improved, and these remaining points are primarily technical and reporting issues. Addressing them should improve the internal consistency, reproducibility, and precision of the MD section and checklist.

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Molecular prosthetics for CFTR designed for anion selectivity outperform amphotericin B in cultured cystic fibrosis airway epithelia – Response Letter

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We are encouraged that the reviewers like the work and provided constructive feedback to strengthen the manuscript. Below, we provide a detailed, point-by-point response outlining how we have addressed each of their comments.

Reviewer #1 comments:

“Thank you for asking me to review this manuscript.

I found it to be carefully written and compiled – it is easy to follow and engage with.

In summary, the researchers have expanded on previous findings that amphotericin B, an anti-fungal drug, forms unselective ion channels when applied to airway epithelial cells and can restore airway surface liquid pH and antibacterial functions in CF cells and pigs (Muraglia et al. Nature 2019). However, at higher concentrations the channels lose their capacity to do this.

In this manuscript the researchers have chemically altered amphotericin B, generating synthetic derivatives, so that the channels formed favour anion permeability and thus increased efficacy.

A logical set of experiments are described demonstrating in relevant cellular models that these derivatives do generate

- Improved relative permeability to anions*
- They restore airway surface liquid pH at both high and low concentrations to a greater extent than amphotericin B*
- A further step whereby cholesterol binding is minimised and this reduces toxicity of the compounds in CF airway epithelial cells (without this modification there would be an obvious flaw in the therapeutic potential of these compounds if toxic to cells – amphotericin B itself though used clinically does have marked toxicity in humans)*

The development of novel approaches to ameliorate the pathophysiology of CF in the microenvironment of the apical surface of airway epithelial cells is welcome. Importantly, ‘non-CFTR’ based approaches are attractive because they are relevant to i) people who do not have a highly-effective CFTR modulator option available to them (mainly due to genetics and class I variants but also due to inequality in access globally due to cost) and ii) the potential to augment benefits from CFTR modulators as an additional therapy.

I cannot find a major flaw in the experimental approaches, results presented and conclusions drawn.

1. However, areas that could be expanded include: Limitations of the approaches to date and next logical steps, e.g. work in animal models versus cell models.

Response: Thank you for the constructive feedback. The following section has been added to outline the limitations of our studies and next logical steps.

“While the results of these studies are encouraging, several limitations remain. Additional studies in primary airway epithelial cultures from people with CF are warranted to evaluate the effects of the AmB derivatives on ASL viscosity and antibacterial activity. The degree to which the AmB derivatives reported herein favor anion permeability is modest relative to CFTR, and future work will seek to further increase such anion preference. Further investigation of cell-type-specific effects and potential functional consequences in non-CFTR-deficient cells also warrant additional study. We also note that, unlike AmB, the AmB derivatives reported in this manuscript have not been approved for clinical use, and that clinical trials will be required to determine whether such derivatives can improve lung function in people with CF.”

2. Potential clinical translatability of the compounds, barriers to this and steps necessary to achieve this, how might the compounds be given, e.g. orally, IV, nebulised???”

Response: Thank you for the suggestions. Based on your comments, the following has been added to the discussion section to explain the potential clinical translatability of the compounds and their administration:

“AmB has been used clinically since the late 1950s as an antifungal agent and therefore has a well-characterized toxicity and pharmacokinetic profile^{15,68–70}. This clinical precedent enabled the development of the inhaled formulation ABCI-003, which we recently demonstrated is well tolerated in both healthy volunteers and people with CF, achieving lung tissue concentrations of >80 μ M without serious adverse events^{57,71}. These findings support the feasibility of developing next-generation AmB derivative-based molecular prosthetics in formulations suitable for inhalation and provide confidence in the clinical translatability of this therapeutic strategy.”

Reviewer #2 comments:

The paper “Molecular prosthetics for CFTR designed for anion selectivity outperform amphotericin B in cultured cystic fibrosis airway epithelia” by Marin-Toledo et al. developed two novel synthetic derivatives of amphotericin B (AmB), AmB-AA and C2’epiAmB-AA, as a new molecular treatment for abnormal airway surface liquid (ASL) in cystic fibrosis (CF) airway. The new derivatives of AmB evaluated the ASL pH in CF airway epithelial cell culture at both low and high concentrations; also, derivative C2’epiAmB-AA further reduced the toxicity to the cell culture by reducing the cholesterol binding, which could affect the mammalian lipid bilayers. The new derivatives of AmB

could be the optional new molecular therapy for CF patients after further testing. The methodology is sound; however, additional data and clarification would strengthen the conclusions.

1. For the Ussing chamber current clamp experiments done in Figure1 (probably in Figure 4d as well), AmB and the derivatives were added both sides of the epithelial cell cultures; however, in the ASL pH measurements, H₁₄CO₃⁻ transport, and toxicity tests, AmB and the derivatives seem to be added only in the apical side of the culture. Would the Ussing chamber results be affected by the presence of AmB and the derivatives in the basolateral side? Many labs had demonstrated the crosstalk between the apical and basolateral membranes happening in the epithelial cells (ex. DOI: [10.1152/ajpcell.1994.267.1.C146](https://doi.org/10.1152/ajpcell.1994.267.1.C146)), as a result, treating the basolateral membrane with AmB and the derivatives could complicate the conclusion from the Ussing chamber results.

Response: We thank the reviewer for this insight. In the revised manuscript, we have now clearly distinguished between the different cell lines used, which is important for explaining the different protocols used in the epithelia derived from the different cell lines. Our reasoning for why the compounds are uniquely added on both sides of the Fischer Rat Thyroid (FRT) epithelia is explained below.

The purpose of the current clamp experiment was to determine the relative permeability of AmB-based ion channels free of any significant background ion movement caused by the cell's electrogenic transport. To minimize this background noise, this study used Fischer Rat Thyroid (FRT) epithelia. These cells were chosen due to their high transepithelial electrical resistance (TEER) and little to no electrogenic transport (<https://doi.org/10.1152/ajplung.1994.266.4.L405>). This allowed us to be confident that the voltage and conductance changes were due to the addition of the small molecule ion channels. However, because of their low electrogenic transport, this also required the addition of small molecule ion channels on both sides of the membrane to allow transepithelial ion flux.

Alternatively, for the ASL pH and H₁₄CO₃⁻ transport assays, we aimed to directly compare the effectiveness of AmB-based ion channels to replace the lost CFTR channel function found in CuFi-1 or CuFi-4 airway epithelial. In these cells, the necessary ion channels responsible for the movement of ions on the basolateral side are intact, which allows for ion efflux when a channel such as an AmB-based ion channel is introduced to the apical side. Additionally, we have previously demonstrated in our Nature 2019 paper (<https://doi.org/10.1038/s41586-019-1018-5>) that basolateral addition of AmB in these cells were unable to cause a change in the ASL pH, ASL height, or H₁₄CO₃⁻ transport ability.

To clarify these points, we have made the following changes to the text of the manuscript:

“The relative permeability to Cl⁻ and Na⁺ (P_{Cl}/P_{Na}) for ion channels formed by AmB and these two synthetic derivatives were estimated by measuring dilution potentials in

Fischer Rat Thyroid (FRT) cells (Fig. 1b-1f, Extended Fig. 1)^{40,41}. These cells form polarized epithelia with high transepithelial resistance and little to no electrogenic transport, making them ideal for studying the properties of small molecule ion channels⁴⁰.”

We have also added the following comment in the methods section to explain why we added compounds to both sides of these FRT epithelia:

“Due to the high transepithelial electrical resistance and low electrogenic transport of the mature FRT membranes, we required an addition of the small molecule ion channels to both sides of the membrane to allow measurement of transepithelial ion flux.”

2. ENaC channels are well expressed in airway epithelial cell culture. In your previous publications (DOI: 10.1038/s41586-019-1018-5; DOI: 10.1016/j.jcf.2020.11.018), amiloride was used to minimize the ENaC activity in Ussing chamber experiments. Here, in this paper, there is no mention of using amiloride in the Ussing chamber experiments. Would the Pcl/PNa measurements here be affected by ENaC activity in the cell culture?

Response: We thank the reviewer for this comment. Our previously published Ussing chamber measurements were done using CF airway epithelial (CuFi-1 or CuFi-4) to show ion channel activity of AmB-based ion channels. In this manuscript, we aimed to show relative permeability of two different ions, therefore we selected to use Fischer Rat Thyroid (FRT) cells. These cells were chosen due to their high transepithelial resistance (TEER) and little to no electrogenic transport (<https://doi.org/10.1152/ajplung.1994.266.4.L405>). Therefore, ENaC channel activity did not need to be inhibited. We now made sure to clarify this in the text:

“The relative permeability to Cl⁻ and Na⁺ (PCL/PNa) for ion channels formed by AmB and these two synthetic derivatives were estimated by measuring dilution potentials in Fischer Rat Thyroid (FRT) cells (Fig. 1b-1f)^{40,41}. These cells form polarized epithelia with high transepithelial resistance and little to no electrogenic transport, making them ideal for studying the properties of small molecule ion channels⁴⁰.”

3. AmB and its derivatives applied in the airway seem not to target a specific cell. What would happen to the cells without the CFTR channel after AmB and its derivatives application? Would there be any unexpected consequences for their functions? Further investigation would be beneficial, and the authors could discuss this in detail.

We thank the reviewer for raising this important point. Although, in the context of cystic fibrosis, we have not directly investigated the effects of AmB and its derivatives on non-bronchial epithelia, our group previously demonstrated that apical addition of AmB does not alter airway surface liquid pH in non-CF cultured lung epithelia (NuLi) or in non-CF primary human airway epithelia (<https://doi.org/10.1038/s41586-019-1018-5>).

In addition, studies in potassium channel–deficient yeast cells have shown that low concentrations of AmB facilitate potassium influx into the cell, rather than efflux as observed in CF airway epithelia. These findings suggest that ion transport mediated by AmB-based molecular prosthetics is driven by the existing electrochemical gradients associated with ion dyshomeostasis, rather than indiscriminate ion flux.

Consistent with this, early clinical trial data for the inhaled AmB formulation, ABCI-003, showed no serious adverse events following delivery directly to the lungs in healthy volunteers and some people with cystic fibrosis.

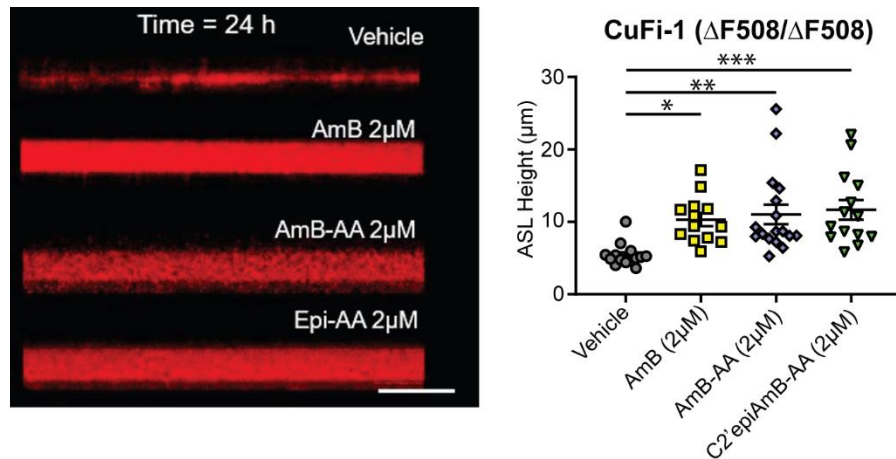
Together, these findings suggest that AmB-based molecular prosthetics do not broadly disrupt epithelial function under non-pathological conditions. Nevertheless, we agree that further investigation of cell-type–specific effects and potential functional consequences in non-CFTR-deficient cells will be important and warrant additional study. We now expand on this point in the discussion section as shown below.

“While the results of these studies are encouraging, several limitations remain. Additional studies in primary airway epithelial cultures from people with CF are warranted to evaluate the effects of the AmB derivatives on ASL viscosity and antibacterial activity. The degree to which the AmB derivatives reported herein favor anion permeability is modest relative to CFTR, and future work will seek to further increase such anion preference. Further investigation of cell-type–specific effects and potential functional consequences in non-CFTR-deficient cells also warrant additional study. We also note that, unlike AmB, the AmB derivatives reported in this manuscript have not been approved for clinical use, and that clinical trials will be required to determine whether such derivatives can improve lung function in people with CF.”

4. Investigating the effects of AmB-AA and C2'epiAmB-AA on ASL layer height and mucociliary clearance process would further establish the potential of this new molecular therapy for CF.

Response: We appreciate the suggestions. We have performed new experiments that probed the effects of 2 μ M of AmB, AmB-AA, or C2'epiAmB-AA on ASL layer height in CuFi-1 airway epithelial. These studies showed that, like AmB (Nature 2019), AmB-AA and C2'epiAmB-AA increase ASL layer height to levels that are comparable to that of AmB. This data is now shown in **Figure 4i**. We have also added the following text:

“ASL hydration is a key airway host defense parameter that is compromised in CF airway epithelia^{62,63}. AmB-based ion channels are able to restore ASL hydration homeostasis in CuFi-1 epithelia to levels similar to non-CF epithelia, as shown by ASL height measurements². Similarly, treatment with 2 μ M of AmB-AA or C2'epiAmB-AA improved the ASL height of CuFi-1 epithelia when compared to the vehicle (**Fig. 4i**).”



“(i) ASL height measurements of CuFi-1 ($\Delta F508/\Delta F508$) pretreated with 2 μM of AmB, AmB-AA, and C2’epiAmB-AA.”

We do not currently have the ability to perform mucociliary clearance studies due to lack of access to cultured primary airway epithelia.

5. In Figure 1c, the x-axis seems to be wrong. In Figure 1d, voltage measurements from the Ussing chamber experiment only showed 6 minutes. The traces shown in Figure 1b to 1e should be on a similar time scale.

Response: The differences in the x-axis in Figure 1d are intentional and a result of the experiment. To accurately calculate the relative permeability of the compounds, the cells needed to be at an equilibrium prior to any addition of compounds or change of solution. However, the time to reach equilibrium can differ. The equilibration time scale prior to compound addition can be affected by the batch of cells used. As for post-compound addition, the different time scales depend on the compound being used, which were expected given the different conductive properties of the compounds.

To clarify this, we have added the following text to the figure 1 caption.

“Graphed representative traces of Ussing chamber current clamp ion-selectivity protocol prior to correcting for junction potentials. Step I) Apical media switch to 70 mM NaCl solution for paracellular ion movement determination. Step II) Addition of DMSO bilaterally. The time needed to equilibrate voltage was varied based on batch of cells and properties of molecular prosthetic added.”

This was also added to the main text to improve clarity.

“Next, following equilibration, the apical [NaCl] was reduced to obtain a dilution potential for the epithelia.”

Reviewer #3 (Remarks to the Author):

This manuscript describes the design and validation of chemical modifications aimed at

ameliorating the efficacy and toxicity of amphotericin B (AmB) to use as a prosthetic anion channel in the treatment of cystic fibrosis.

This is an interesting paper on an important topic. However, although molecular simulation methods were apparently used to construct an atomistic model of the Am-AA “sponge” formed with cholesterol, that part of the work is not conveyed in adequate or convincing manner. Specifically:

(1) The methodology is not adequately described. The simulated annealing mentioned in the Results (Line) is not mentioned in the Methods. How were the systems prepared? How did the three repeats differ from one another? Enough details should be included for reproducibility by competent researchers. “Previously reported data” (line 734) are not referenced. Please confirm that “a grid density of less than 1 Å⁻³” (line 751) was used in the Ewald calculations.

Response: Thank you for this important catch and helpful comments. A detailed description of the methodology has been added and updated to accurately describe the equilibrium simulations that were performed to sample the dynamics of the AmB variants and contrast them with the AmB wild type. The modifications to the methods also address the handling of long-range interactions. The replicas were equilibrated with random seeds for the initial velocities. Please see the newly updated section “Molecular Dynamics Simulations” for the text. And yes, a grid density of less than 1 Å⁻³ was used in the Ewald calculations. This can be verified in the additional supplemental files that are included in the submission.

“System Preparation and Molecular Dynamics Simulations:

Molecular Dynamics Simulations: All molecular simulations were performed using GROMACS 2025^{80,81} and visualized with VMD [<http://www.ks.uiuc.edu/Research/vmd/>]⁸². The system was prepared using the lowest-energy AmB–cholesterol lattice (Fig. 1a) from previously reported and deposited data⁵⁸. The structure for the original AmB-cholesterol lattice was used as a template for modeling the AmB-AA + cholesterol lattices. To maintain the structure of the experimentally resolved sponge, carbon atoms in the macrolide region of all AmB molecules were constrained using gmx genrestr with a force constant of 8000 kJ/mol/nm² and all sterol molecules left unconstrained. AmB parameters were obtained from CHARMM General Force Field⁸³ and sterols parameters were obtained from the CHARMM36 force field⁸⁴. After constructing each AmB-AA system in complex with cholesterol, the sponge was solvated using gmx solvate tool using the TIP3P water model⁸⁵ and neutralized at 150 mM NaCl. Energy minimization was done for 10,000 steps and followed by a 1 ns NPT equilibration run allowing water molecules to relax and system box size to converge. For equilibration and production runs, the C-rescale barostat⁸⁶ and V-rescale thermostat⁸⁷ were employed along with the leapfrog integrator to maintain constant pressure and temperature. The production simulations were carried out using an isothermal–isobaric ensemble at 298 K with a pressure of 1.0 atm. A 1.2 nm cut-off was used for nonbonded interactions with a smoothing function implemented farther than 1 nm. An integration time step of 2 fs was used, and the bond distances of the hydrogen atoms were constrained using the LINCS algorithm⁸⁸. For long-range electrostatic calculations, the particle mesh

Ewald method was used, with a grid density of less than 1 Å⁻³. The production runs were 250 ns long with 3 replicates each.

Table of simulations:

System Description	Atom count	Water molecules count	NaCl conc.	Simulation length (ns)
AmB wt sponge with Cholesterol	135,776	99,786	150 mM	250
epiAmB sponge with Cholesterol	135,866	99,876	150 mM	250
AmB-AA sponge with Cholesterol	136,440	99,075	150 mM	250
EpiAmB-AA sponge with Cholesterol	149,432	112,095	150 mM	250

Volume Calculations: Occupied and void volumes were calculated using the volmap plugin in VMD [<https://www.ks.uiuc.edu/Research/vmd/plugins/volmapgui/>]. Volumes were calculated using an occupancy map at a resolution of 0.5 Å³ and the resulting voxels were counted for occupancy relative to timescale of dynamics. Voxel occupancy was calculated over the course of the entire simulation and averaged. The percentage of simulation time during which a voxel was occupied was determined from the fractional value of a given voxel.

Sterol RMSF calculations: RMSF of each sterol atoms were calculated using the MDAnalysis^{89–91} Python package.”

(2) What were the purpose and the outcome of the simulations? Where are the results shown, analyzed, and discussed? The only place (outside of Methods) where the simulations are mentioned is in the following paragraph, Lines 189-195:

“Building on an extensive series of recently reported biophysical and structural studies with AmB25,57, XPLOR-NIH simulated annealing and molecular dynamics yielded a high-resolution structure of the AmB-AA sponge which showed the presence of a large void volume capable of accommodating the presence of a C16-ethylamine chain (Fig. 4d). These computational models further supported the conclusion that AmB-AA similarly kills mammalian cells via a sterol sponge-mediated cholesterol binding-dependent mechanism.” [My emphasis]

Figure 3d (mis-referenced in the text in line 193 as “Fig. 4d”) shows what may be a snapshot from the simulations (but is not specified). The caption of Fig. 3d reads: “(d) Model of AmB-AA bound to cholesterol.” Unless I missed something, that is the only data shown from the simulations in the manuscript.

What are we looking at? How was this particular structure obtained and how was it chosen for display? How representative is it? If the point was to show “the presence of a large void volume capable of accommodating the presence of a C16-ethylamine chain” as stated in the Results, how was that aspect of the model established and validated?

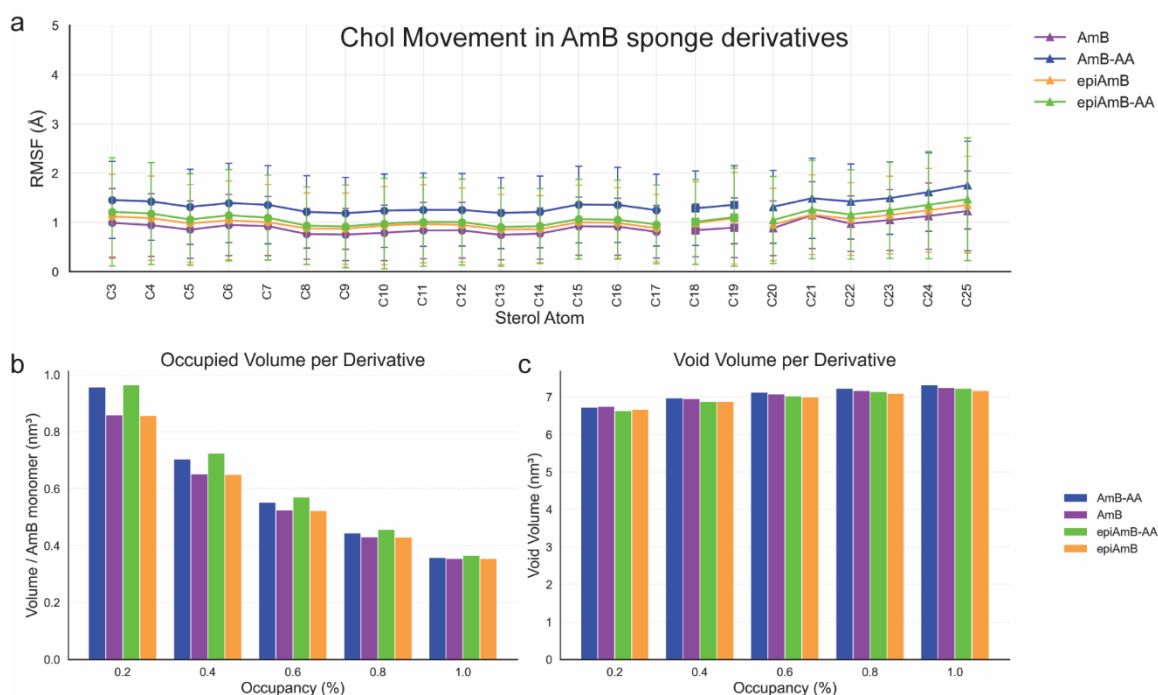
In conclusion, the simulations should be adequately motivated, described, analyzed, and discussed to justify their inclusion in the manuscript.

Response: Thank you for these comments and suggestions. The purpose of the molecular dynamics simulations was to test whether the side chains added at C41 would: a) be sterically incompatible with the structure of the sterol sponge, b) substantially change intramolecular dynamics of AmB derivatives, and/or c) substantially change the intermolecular dynamics in the AmB-sterol sponge, and thus change the mechanism by which these compounds interact with lipid bilayers. All of the results of the MD simulations are consistent with the conclusion that the side chains: a) are readily accommodated by the void volume proximal to the C41 carbon atom in the original sponge structure, b) do not substantially change intramolecular dynamics of AmB derivatives, and c) do not substantially change the intermolecular dynamics in the AmB-sterol sponge, and thus, would not be expected to change the mechanism by which these compounds interact with lipid bilayers. Additional analyses and figures have also been added showing and characterizing the dynamics of the complexed sterol molecules and occupied volumes of the AmB derivatives, all of which further support this conclusion.

To address these points in the revised manuscript, we have added the following text to the manuscript:

“Building on an extensive series of recently reported biophysical and structural studies with AmB^{25,58}, we investigated whether the C41-ethylamine side chain would be incompatible with the structure of the sterol sponge, change intramolecular dynamics of AmB derivatives, and/or change the mechanism by which these compounds interact with lipid bilayers. Molecular dynamics of an AmB-AA sponge complexed with cholesterol based on a prior XPLOR-NIH structure⁵⁸ yielded a high resolution sponge structure showing the presence of a large void volume capable of accommodating the presence of a C41-ethylamine chain (**Fig. 3d and Extended Fig. 6**). Additionally, there are no substantial changes in the intramolecular dynamics for the AmB derivatives and in the AmB-sterol sponge (**Fig. 3d and Extended Fig. 6**). These computational models further supported the conclusion that AmB-AA similarly kills mammalian cells via a sterol sponge-mediated cholesterol extraction-dependent mechanism.”

We have also now added new data showing the dynamics of the complexed sterol molecules and occupied volumes of the AmB derivatives to Extended Figure 6.



“Extended Figure 6. AmB-Cholesterol interactions are preserved across derivatives. (a) Computed RMSF values for each atom in the cholesterol molecule indicate similar intermolecular interaction patterns across all AmB derivative sponge models. **(b)** Occupied volume analysis captures the degree of conformational freedom of each AmB derivative within the sponge structures. Occupancy (%) denotes the fraction of simulation time during which the same voxels remain occupied. A value of 100% corresponds to restrained macrolide atoms. The difference in occupied volume at 0.2 between AmB and AmB-AA arises from the C41 ethylamine side-chain modification. **(c)** Void volume quantifies the change in volume available to accommodate a sterol molecule. Occupancy (%) denotes the fraction of simulation time during which a voxel remains unoccupied. Void volume fluctuations are minimal across the range of occupancy values.”

Following your suggestion, we have also updated the caption of Fig 3d.

“(d) NMR informed model of AmB-AA bound to cholesterol.”

Reviewer #4 (Remarks to the Author):

This is an interesting and well-constructed Ms which is a follow on from the authors 2019 Nature paper, which provided strong evidence that amphotericin B (AmB) restored host defences in CF airway, both in vitro and in vivo. In this new paper the authors have performed some nice chemical optimisation of AmB to improve its anion selectivity and reduce cholesterol binding capacity (potential toxicity factor). The new AmB derivatives demonstrate small, but significantly larger, changes in the ASL pH as originally reported for AmB, using polarised CF cell lines and fully differentiated primary CF airway cells. However, this paper lacks sufficient in vitro ‘functional’ evidence using fully differentiated

primary human CF cultures that the new derivatives substantially improve and restore airway defence mechanisms, compared to the parent compound. Furthermore, no in vivo (animal) evidence is provided to substantiate the in vitro findings.

Specific major concerns essential to be addressed to support the conclusions.

1. While the optimised AmB derivatives were able to increase HCO₃⁻ transport and ASL pH, this could be for reasons other than via direct permeation through the AmB derivatives themselves. A more direct way to test this is to measure the anion permeability of the derivatives after reconstitution into artificial lipid bilayers and performing current measurements under different conditions.

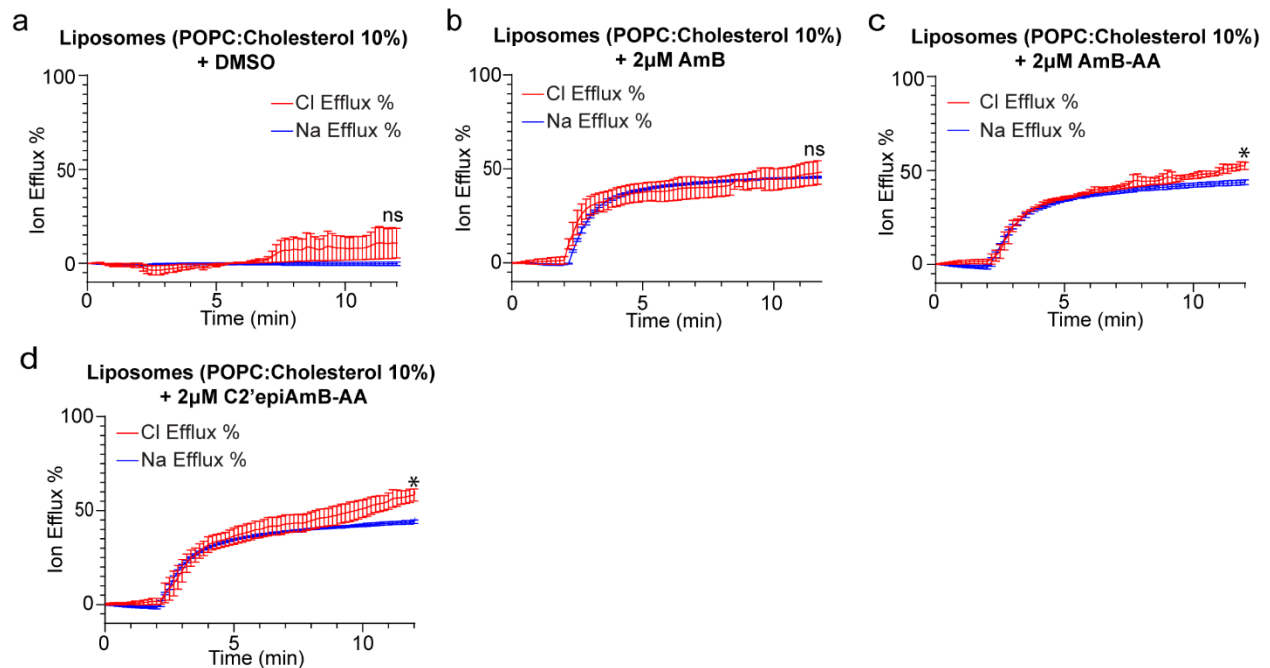
Response: We appreciate the suggestion and agree that directly measuring ion permeability free of other factors such as electrogenic transport is important to show the changes seen in HCO₃⁻ transport and ASL pH using CuFi-1/CuFi-4 airway epithelial. As described below, we have addressed this by performing new experiments in liposomes, and we have also clarified the important data that we previously generated in FRT cells.

We agree that an additional study in a more direct system such as liposomes would help emphasize the effect being caused by the optimized AmB derivatives and seen in FRT cells.

Specifically, the Fischer Rat Thyroid (FRT) epithelial was chosen for our Ussing chamber studies, as they are known for their high transepithelial resistance (TEER) and little to no electrogenic transport (<https://doi.org/10.1152/ajplung.1994.266.4.L405>). We make sure to emphasize this point now on our manuscript as well:

“The relative permeability to Cl⁻ and Na⁺ (P_{Cl}/P_{Na}) for ion channels formed by AmB and these two synthetic derivatives were estimated by measuring dilution potentials in Fischer Rat Thyroid (FRT) cells (Fig. 1b-1f)^{40,41}. These cells form polarized epithelia with high transepithelial resistance and little to no electrogenic transport, making them ideal for studying the properties of small molecule ion channels⁴⁰.”

We also performed new experiments in liposomes as suggested. Specifically, we set up an experiment in 10% cholesterol containing POPC liposomes with two ion-selective electrodes (for sodium and chloride) to measure the efflux% of chloride and sodium caused by the addition of 2μM of AmB, AmB-AA, or C2'epiAmB-AA. Similar experiments and the resulting ion efflux percentages have been previously used to compare the effect of small molecule ion transporters (10.1021/jacs.6b04656) and to estimate their ion selectivity (<https://doi.org/10.1039/d5sc00866b>). This resulted in AmB-AA and C2'epiAmB-AA eliciting a higher chloride efflux % than sodium after 10 minutes of compound addition. This experiment is now shown in **Extended Fig. 8**.



“Extended Figure 8. Sodium and chloride efflux of (a) DMSO, (b) AmB, (c) AmB-AA, and (d) C2’epiAmB-AA (2 μ M) in 10% cholesterol containing liposomes. Unpaired t-test at 12 minutes between Cl Efflux % and Na Efflux %. Statistics utilized the Shapiro-Wilk test to check for normal distribution. ns, not Significant. ** P <0.0001, *** P<0.001, ** P<0.01, * P<0.05.”**

We have added the following text to the manuscript:

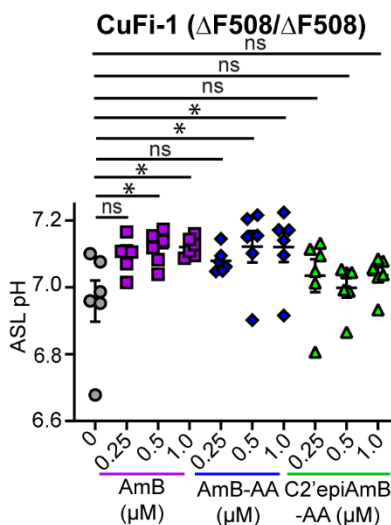
“Consistent with these results, C2’epiAmB-AA channels exhibited greater Cl⁻ efflux relative to Na⁺ efflux in liposomes containing 10% cholesterol when compared to AmB channels (Extended Fig. 8).”

2. The ASL pH results for AmB-AA depicted in Figs 2b and 4g don’t show any dependence on dose (between 2 and 100 μ M). Why didn’t they test lower doses? This is important in the context of future clinical relevance.

Response: We agree that characterizing the activity of AmB-AA at lower concentrations is helpful. We thus ran new experiments in which we compared the activity of AmB, AmBAA and C2’epiAmB-AA at 0.25 μ M, 0.50 μ M, and 1.0 μ M in CuFi-1 (Δ F508/ Δ F508) epithelia. These studies showed that AmB and AmB-AA increased ASL pH at concentrations at or above 0.50 μ M. C2’epiAmB-AA did not increase ASL pH at any of these lower tested concentrations. This data has been added as **Extended Fig.4**. We also added the following text to the manuscript:

“However, we note that, in contrast to AmB and AmB-AA, C2’epiAmB-AA did not increase ASL pH at concentrations below 2 μ M (Extended Fig. 4). We reason that this

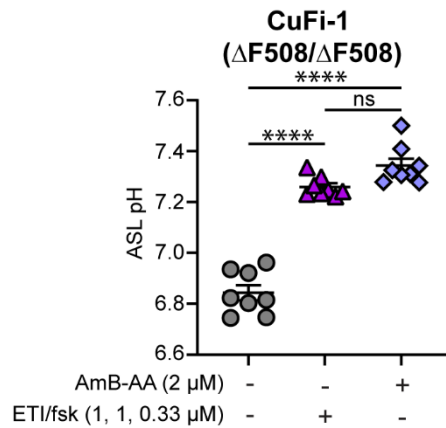
could be due to the decreased cholesterol binding, which was shown to improve ion channel formation in liposomes (Extended Fig. 7)."



“Extended Figure 4. ASL pH measurements following addition of 0.25 μM , 0.50 μM , and 1 μM of AmB, AmB-AA, and C2’epiAmB-AA to CuFi-1 ($\Delta\text{F508}/\Delta\text{F508}$) epithelia. One-Way ANOVA with multiple comparisons. Statistics utilized the Shapiro-Wilk test to check for normal distribution. ns, not Significant. **** P < 0.0001, *** P < 0.001, ** P < 0.01, * P < 0.05.”

3. It would be extremely informative to compare the ASL pH/HC03 transport results obtained from cells treated with AmB and derivatives, to what would be observed for these parameters using CF cell lines (CuFi-1 and 4) or primary CF cells (*delF/delF*) after pretreatment with CFTR modulators. This would provide stronger evidence that the changes observed with AmB derivatives are likely to have ‘clinical’ benefit. Furthermore, as the authors suggest that the AmB derivatives work in a CFTR-independent manner, they should test CF null cells which don’t produce any CFTR protein to substantiate this conclusion.

Response: Thank you for these insightful comments and suggestions. In response, we have performed new experiments in CuFi-1 cultured airway epithelia comparing the activity of AmB-AA to the current clinically approved CFTR modulators (ETI). The improvements in ASL pH observed for AmB-AA and ETI were very similar.



“Extended Figure 3. AmB-AA results in a similar increase in ASL pH as current CFTR modulators. ASL pH measurements comparing the addition of 2 μM AmB-AA to the addition of 1 μM Elexacaftor, 1 μM Tezacaftor, and 0.33 μM Ivacaftor with 0.33 μM forskolin to CuFi-1 (ΔF508/ΔF508) epithelia. One-Way ANOVA with multiple comparisons. Statistics utilized the Shapiro-Wilk test to check for normal distribution. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.”

This data is now included in **Extended Fig. 3**, and we have now added the following text to the manuscript:

“Next, we compared the efficacy of AmB-AA with the leading therapy available to some people with cystic fibrosis, Elexacaftor/Tezacaftor/Ivacaftor (ETI), and found that AmB-AA produced a similar increase in ASL pH in CuFi-1 epithelia (Extended Fig. 3). Whereas we previously showed that increases in ASL pH by AmB channels were suboptimal relative to ETI in cultured CF epithelia⁵⁶.”

Response to: *Furthermore, as the authors suggest that the AmB derivatives work in a CFTR-independent manner, they should test CF null cells which don't produce any CFTR protein to substantiate this conclusion.*

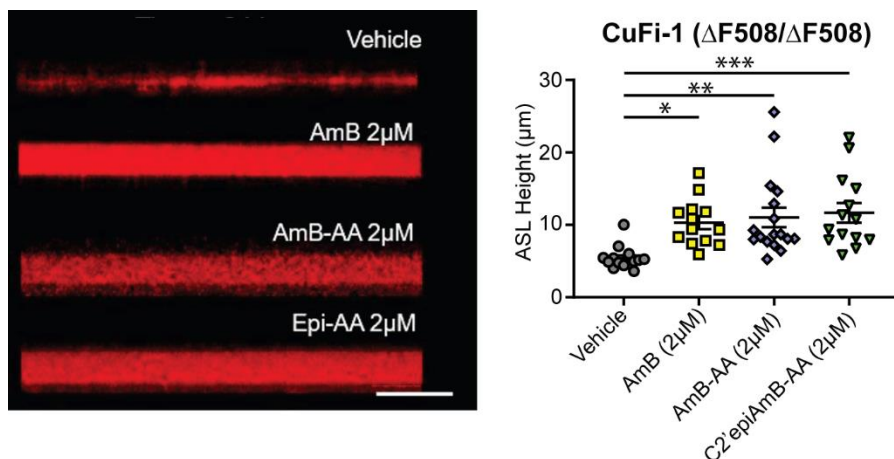
Although we did not test CFTR-null cell lines here, in our previously published paper (*Nature* **2019**, 567,405–408) we demonstrate that AmB increases the ASL pH in the trachea of CFTR $-/-$ pigs. This finding supports the claim that AmB-based channels are CFTR independent.

4. *The Ms would be far more convincing if other ‘functional’ effects, relevant to CF airway disease, were also performed, such as ASL ‘hydration’, ASL viscosity, bacterial killing.*

Response: We appreciate the feedback and agree that other assays that probe for effectiveness of molecular prosthetics in CF models will help reinforce their benefits. Based on this suggestion we conducted additional experiments in an ASL hydration assay on CuFi-1 (ΔF508/ΔF508) epithelia, testing 2 μM of AmB, AmB-AA, or

C2'epiAmB-AA. This study showed that all three molecular prosthetics were effective at restoring ASL height. This data is included in **Figure 4i**, and is referenced in the text as:

“ASL hydration is a key airway host defense parameter that is compromised in CF airway epithelia^{62,63}. AmB-based ion channels are able to restore ASL hydration homeostasis in CuFi-1 epithelia to levels similar to non-CF epithelia, as shown by ASL height measurements². Similarly, treatment with 2 μ M of AmB-AA or C2'epiAmB-AA improved the ASL height of CuFi-1 epithelia when compared to the vehicle (**Fig. 4i**).”



“(i) ASL height measurements of CuFi-1 (Δ F508/ Δ F508) pretreated with 2 μ M of AmB, AmB-AA, and C2'epiAmB-AA.”

As for ASL viscosity and ASL bacterial killing, we previously showed that AmB causes improvements in both of these parameters in cultured primary CF airway epithelia obtained from people with CF that donated their lungs via transplant (<https://doi.org/10.1038/s41586-019-1018-5>).

Due to the success of the CFTR modulators in reducing the need for lung transplants, we are currently unable to source the large number of CF primary airway epithelia needed to run these same experiments with AmB-AA. These assays were optimized for primary CF airway epithelia and not CuFi-1 or CuFi-4 epithelia. We note however that in our previous studies both ASL viscosity and ASL bacterial killing studies have been shown to correlate very well with the ASL pH and ASL hydration results (<https://doi.org/10.1172/JCI83922>; <https://doi.org/10.1038/nature11130>; <https://doi.org/10.1038/s41586-019-1018-5>).

5. The Ms doesn't address the fact that these new derivatives have not been approved for clinical use, unlike AmB.

We have added the following text to the discussion section to address this comment:

“We also note that, unlike AmB, the AmB derivatives reported in this manuscript have not been approved for clinical use, and that clinical trials will be required to determine whether such derivatives can improve lung function in people with CF.”

Minor concerns that should be addressed.

Response: Thank you for all these comments. We have made the corresponding changes to address each point.

1. The number of different CF donors used in the experiments that tested the effect of AmB and derivatives on ASL pH using primary CF airway cells (Fig 4h) is not stated.

In figure 4h, we now state the 2 donors which are (dF508/dF508).

“(h) ASL pH rescue of primary CF airway epithelial (both $\Delta F508/\Delta F508$) pretreated with 2 and 50 μM of AmB, AmB-AA, and Epi-AA.”

2. Methods. No details are provided about CuFi-4 cells.

The handling and growth conditions of CuFi-4 cells have been added to the methods section.

“CuFi-1, CuFi-4, and FRT cells (Welsh Laboratory, University of Iowa) were grown from cryostock on cell culture Treated 75 cm² flasks (Thermo Scientific BioLite). The flasks for CuFi-1 and CuFi-4 cells were previously coated with 4 mL 50 $\mu g/mL$ human placental collagen type IV (Sigma-Aldrich) for a minimum of 1 hours at 37 °C, then rinsed twice with PBS, and dried prior to seeding.”

3. Extended fig 1. Indicate CuFi-1 cells were used in panel f.

Indicated the use of CuFi-1 cells in panel f.

“(f) ASL pH dose response for AmB-NAcC in CuFi-1 ($\Delta F508/\Delta F508$).”

4. Statistics. The authors have only employed parametric tests, but there is no information whether they checked if the results were normally distributed.

The figure captions now state that the statistical tests utilized the Shapiro-Wilk test to check for normal distribution of data.

“Statistics utilized the Shapiro-Wilk test to check for normal distribution.”

Molecular prosthetics for CFTR designed for anion selectivity outperform amphotericin B in cultured cystic fibrosis airway epithelia – Response Letter

Reviewer #1 (Remarks to the Author):

Thank you for responding to the previous review comments fully.

Thank you for your feedback and for helping us strengthen this manuscript.

Reviewer #2 (Remarks to the Author):

Marin-Toledo and colleagues have presented a well written revised manuscript that provides the necessary clarification for my comments, as well as the methodology as other reviewers pointed out. The new data from the cell culture experiments and the discussion section provide significant improvement, addressing most of my concerns.

Thank you for your feedback and for helping us strengthen this manuscript.

Reviewer #4 (Remarks to the Author):

This revised version has been significantly improved and the authors have satisfactorily answered all my comments. I have no further comments or suggestions.

This is an important piece of work that could eventually provide significant benefit for all people with CF, and especially those who are not eligible, or who cannot afford, current highly effective CFTR modulators.

Thank you for your feedback and for helping us strengthen this manuscript.

Reviewer #5 (Remarks to the Author):

Review focusing on MD simulations

Manuscript: Molecular prosthetics for CFTR designed for anion selectivity outperform amphotericin B in cultured cystic fibrosis airway epithelia

I have been asked to comment on the concerns of Reviewer #3 and to assess the authors' responses and revisions. Please see my comments below.

Unfortunately, there remain several serious issues related to the molecular dynamics (MD) simulations and their interpretation.

Response: We appreciate your careful assessment and the opportunity to strengthen the manuscript by addressing the concerns you raised regarding the MD simulations and their interpretation. Below we provide a point-by-point detailed response on how all your concerns were addressed.

1. *How were the systems prepared? How did the three repeats differ from one another? These points remain unclear and should be addressed explicitly. In particular, the manuscript does not describe the initial structures used for the simulations (see detailed comments below).*

Response: The systems were prepared using the Amphotericin-Ergosterol sponge structure resolved in our prior work (*Nature* **623**, 1079–1085 (2023), BMRB Entry 21102). The three repeats were differentiated from one another by using random starting velocities. Please see below for more detailed information on system setup.

For our study, the AmB molecules in BMRB Entry 21102 were replaced with AmB-AA, C2'epiAmB, or C2'epiAmB-AA and the ergosterol molecules were replaced with cholesterol. This follows a similar protocol to (*Nature* **623**, 1079–1085 (2023)) and the Methods section has been revised to include a greater level of detail regarding these calculations, thus enabling reproducibility.

The following information has been added to the main text of the manuscript to clarify how the systems were prepared and the initial structures used for the simulations:

System preparation and parameters: Starting coordinates for each AmB derivative–cholesterol sponge were generated by templating the derivative onto the 5×5×5 AmB lattice of the NMR-resolved native AmB–ergosterol sponge structure²⁵ (BMRB Entry 21102), preserving the experimentally determined intermonomer geometry and the two resolved cholesterol molecules within the central unit cell. Each templated sponge was centered in a cubic box (11 nm side length; ≥2.0 nm solute-to-edge padding), solvated with TIP3P water^{81,82}, and neutralized to 150 mM NaCl (Table 1). AmB parameters were obtained from CHARMM General Force Field⁸³ via their web server [<https://cgenff.com/>]. Cholesterol and other system parameters were obtained from the CHARMM36 force field⁸⁴. To preserve the NMR-resolved scaffold geometry, harmonic positional restraints were applied to the macrolactone heavy atoms of each AmB-derivative using `gmxfgenrestr` with a force constant of 8,000 kJ/mol/nm² (allowing ±0.2 Å fluctuations) while mycosamine atoms, C41 side chain modifications, all hydrogens, and all sterol atoms remained fully unrestrained throughout minimization, equilibration, and production. This protocol probes local relaxation of unrestrained groups — in particular, accommodation of the C41 ethylamine and cholesterol dynamics — within the experimentally resolved scaffold, rather than the de novo thermodynamic stability of the sponge architecture itself.

Simulation Protocol: All simulations were performed using GROMACS^{85,86} 2025. Each solvated, restrained system was energy-minimized for 5,000 steps using the steepest-descent algorithm with a maximum force tolerance of 1,000 kJ mol⁻¹ nm⁻¹. Initial velocities were then assigned from a Maxwell–Boltzmann distribution⁸⁷ at 298 K using `gen_vel = yes` and a pseudorandom seed (`gen_seed = -1`). The system was equilibrated under the NPT ensemble for 1 ns to allow solvent relaxation and convergence of box dimensions. Production simulations were performed in the isothermal–isobaric (NPT) ensemble at 298 K and 1.0 atm. Pressure was maintained using the stochastic cell-rescaling (C-rescale) barostat⁸⁸ with isotropic coupling ($\tau_p = 5.0$ ps; compressibility = 4.5×10^{-5} bar⁻¹), and temperature was maintained using the velocity-rescaling (V-rescale) thermostat ($\tau_t = 0.5$ ps), with the sponge and solvent thermostatted as separate coupling groups. Equations of motion were integrated using the leap-frog algorithm⁸⁹ with a 2-fs time step. Bonds involving hydrogen atoms were constrained using the LINCS algorithm⁹⁰ (LINCS order = 4, iterations = 1). Short-range Lennard-Jones and electrostatic interactions were truncated at 1.2 nm using a force-switch modifier applied from 1.0 nm. Long-range electrostatics were treated with the smooth particle-mesh Ewald (PME)

method^{91,92} with a real-space cutoff of 1.2 nm, a Fourier grid spacing of 0.16 nm (grid density $< 1 \text{ \AA}^{-3}$), fourth-order B-spline interpolation, and a relative tolerance of 10^{-5} . Long-range analytical dispersion corrections were applied to both energy and pressure. Production trajectories were 250 ns in length per replicate; coordinates were written every 10 ps. The production runs were 250 ns long. For each system modeled, 3 replicates were initialized using different random seeds for starting velocities. Visualization and analyses of the trajectories were carried out using VMD⁹³ and the Python package MDAnalysis^{94,95}.

All analyses were performed on the central 3x3x3 lattice of individual AmBs (total 27) and their correspondingly resolved 2 cholesterol (total 54) from the sponges. This was done to ensure that no bulk solvent interactions are introduced as the sponge internal dynamics was characterized.

Table 1 (Revised): System Composition and Simulation Details

Sponge system	Atom count	AmB molecule count	Chol molecules count	Water molecule count	NaCl conc.	Simulation length (ns)
AmB:Chol	135,776	125	250	99,786	150 mM	3 x 250
C2'epiAmB:Chol	135,866	125	250	99,876	150 mM	3 x 250
AmB-AA:Chol	136,440	125	250	99,075	150 mM	3 x 250
C2'epiAmB-AA:Chol	149,432	125	250	112,095	150 mM	3 x 250

To address the concern regarding how replicates were performed, the text (p.18, line 692) was modified to state that replicates were performed with random starting velocities.

“The production runs were 250 ns long. For each system modeled, 3 replicates were initialized using different random seeds for starting velocities. Visualization and analyses of the trajectories were carried out using VMD⁹³ and the Python package MDAnalysis^{94,95}.”

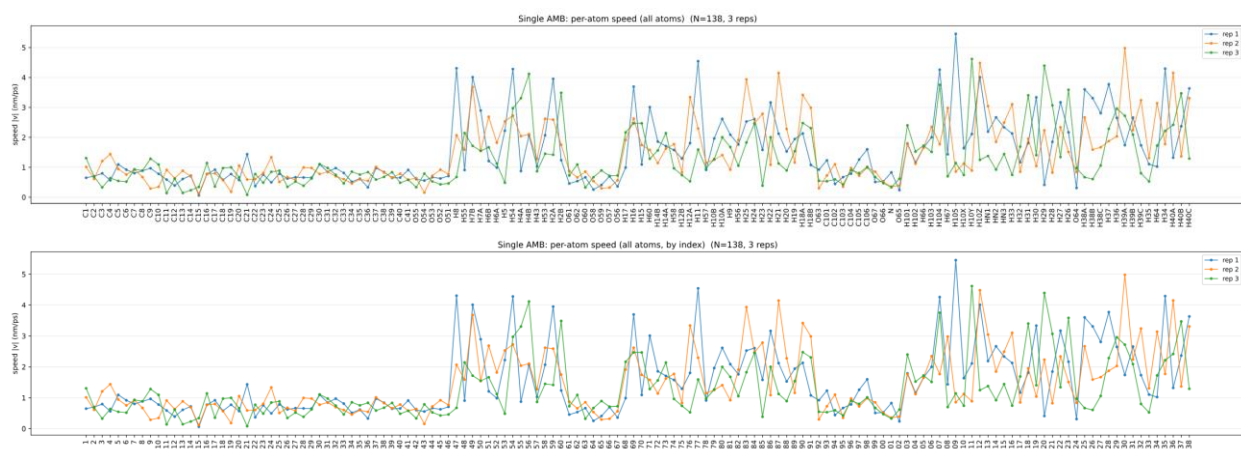
All analysis was performed on the central 3x3x3 lattice of individual AmBs (total 27) and their correspondingly resolved 2 cholesterol from the sponges (total 54). This was done to ensure that no bulk solvent interactions are introduced as the sponge internal dynamics was characterized.”

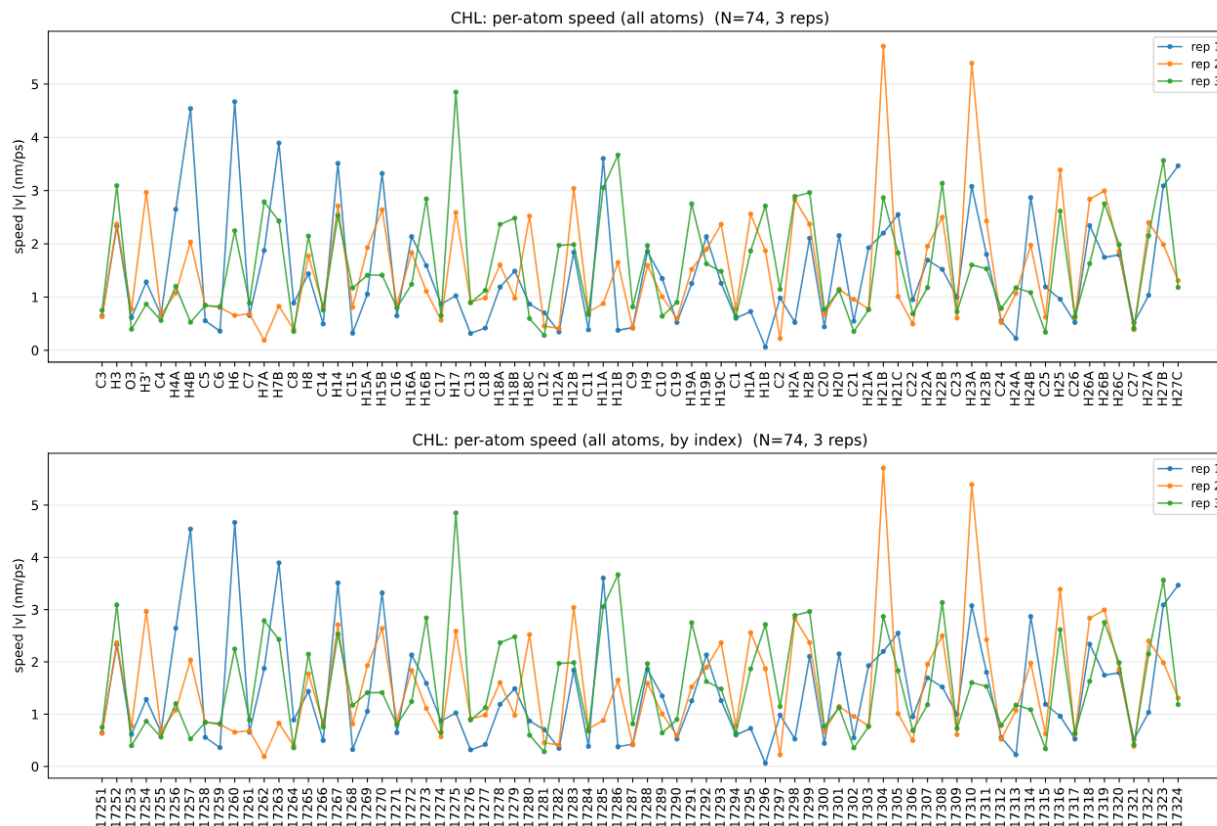
2. Additionally, it is not evident how independent replicates were generated. Did you create them by reassigning velocities (e.g., using different random seeds) after energy minimization? As currently described, it appears that the same NPT-equilibrated structure was used to initiate multiple production runs without modifying the velocity distribution. If so, this would not constitute statistically independent repeats and is not an appropriate approach for replica generation.

Response: The independent replicates were generated by initializing random velocities. To clarify this, the Methods section has been revised to explicitly state that random initial velocities were used per replicate and now reads (p.18, line 672):

"Each solvated, restrained system was energy-minimized for 5,000 steps using the steepest-descent algorithm with a maximum force tolerance of $1,000 \text{ kJ mol}^{-1} \text{ nm}^{-1}$. Initial velocities were then assigned from a Maxwell–Boltzmann distribution⁸⁷ at 298 K using `gen_vel = yes` and a pseudorandom seed (`gen_seed = -1`). The system was equilibrated under the NPT ensemble for 1 ns to allow solvent relaxation and convergence of box dimensions."

To provide direct evidence that this protocol yielded statistically independent initial conditions, we have included a new figure (below), in this response letter, showing per-atom initial speeds for atoms of the same single AmB and same single cholesterol across the three replicates, demonstrating uncorrelated velocity assignment across replicates for the same molecule.





3. *The responses to questions regarding the purpose and outcome of the simulations are still not adequately addressed. The revised text does not clearly articulate what specific hypotheses the simulations were intended to test, nor what conclusions can be robustly drawn from them. For example, in Extended Figure 6, the use of RMSF to infer or support similar interaction patterns is not appropriate. RMSF reflects local flexibility and does not provide direct information about interaction networks or binding modes. Drawing conclusions about similarity of interactions based on RMSF alone is therefore not well justified and should be reconsidered or supported with more suitable analyses (e.g., contact maps, interaction frequencies, or distance-based metrics).*

Response: The purpose of the simulation was to test the specific hypothesis that the modification of the C41 position could be accommodated while preserving the sponge void volumes that previous studies have shown are critical for the proposed cytotoxic AmB-sponge mechanism of action. To clarify this purpose, we revised and reorganized the text so that the rationale and goals of the MD simulations are introduced prior to the biological experiments. The text (p. 5, line 191) now reads:

"An orally inhaled dry powder formulation containing AmB, ABCI-003, was well-tolerated in clinical trials in healthy volunteers⁵⁷, and in people with CF on and off modulators⁵⁷. These results support the use of AmB as a meaningful benchmark for minimum targeted tolerability in cultured airway epithelia. Although AmB-AA shows similar tolerability to AmB in a variety of mammalian cells (**Extended Fig. 5**), toxicity studies in cultured CuFi-1 and

CuFi-4 airway epithelial treated with AmB and AmB-AA demonstrated increased toxicity for the latter (**Fig. 3a, 3b, and Extended Fig. 5d**).

We previously demonstrated that the toxicity of AmB to human cells is primarily driven by the capacity of this natural product to form an extramembranous sterol sponge that binds and extracts cholesterol from mammalian lipid bilayers²⁵. We hypothesized that AmB-AA primarily exerts its cytotoxic activity by the same sterol sponge mechanism. To provide evidence for or against this hypothesis, we performed MD simulations to determine whether the ethylenediamine side chain could be accommodated within the previously determined sterol sponge structure^{25,58}. Notably, these MD simulations, by design, do not include lipid bilayers. However, prior work integrating biophysical experiments in lipid bilayers, SSNMR studies, and MD simulations demonstrated that structural characterization of the sponge assembly itself, independent of explicit lipid bilayer inclusion, can yield meaningful mechanistic insights^{15,22,24,25,58,59}.

Molecular dynamics of an AmB-AA sponge complexed with cholesterol based on a prior NMR-resolved XPLOR-NIH structure²⁵ yielded a high-resolution sponge structure showing the formation of large void volumes that were not disrupted by the presence of a C41-ethylamine chain (**Fig. 3c and Extended Fig. 6**). Additionally, computed AmB-Chol radial distribution functions (RDF) and contact frequencies, along with RMSF values of cholesterol heavy atoms indicate similar stability across all AmB-cholesterol and derivative sponges (**Fig. 3c and Extended Fig. 6**). These computational models suggest that AmB-AA similarly kills mammalian cells via a sterol sponge-mediated cholesterol extraction-dependent mechanism.”

With respect to Extended Fig. 6, upon further reflection, we agree that it is not rigorous to say that RMSF infers or supports similar interaction patterns. Alternatively, it provides evidence regarding the stability of conformational dynamics of the cholesterol molecules within the sponge structure. Accordingly, the corresponding statement in the legend of **Extended Figure 6** has been revised to now read (p. 30, line 1081):

“Root mean square fluctuation (RMSF) values for each cholesterol carbon atom (C1–C25) indicate comparable conformational freedom of cholesterol across all four AmB derivatives.”

We thank the reviewer for suggesting additional analyses to strengthen the interpretation of the simulations. We agree that RMSF alone does not directly assess AmB–cholesterol binding modes. Per your suggestion, we performed additional interaction analyses and added the results to Extended Fig. 6. These specifically include radial distribution functions (RDFs) between AmB and cholesterol reference atoms, and per-atom contact frequencies between cholesterol and defined AmB interaction regions (mycosamine and C41). Full methodological details are provided in the revised methods section.

Also following your suggestion to provide **interaction frequencies** analysis, we performed contact frequency analysis between all carbon atoms in cholesterol and the mycosamine region (identified as the C2'-hydroxyl and C3'-amine) or C41 region (identified as only the C41 carbon) of each AmB derivative. Below we describe the results:

Contact frequencies between cholesterol and the mycosamine and C41 regions are comparable across AmB, AmB-AA, C2'epiAmB, and C2'epiAmB-AA within replicate-to-replicate noise, indicating that none of the chemical modifications excludes cholesterol from any region of the sponge cavity — every interaction region remains geometrically accessible to bound cholesterol across all four derivatives.

Further following your suggestion to run additional **distance-based metric** analysis, we ran Radial Distribution Function (RDF) analysis of the AmB relative to the cholesterol. Below we describe the results:

The RDFs provide higher-resolution information about close-range cholesterol-AmB coordination and reveal a pattern that the binary contact analysis cannot resolve: the AmB C2'-hydroxyl-cholesterol O3 RDF groups by C2' stereochemistry rather than by C41 modification. AmB and AmB-AA show a sharp first-shell peak at ~3 Å, consistent with hydrogen bonding, whereas C2'epiAmB and C2'epiAmB-AA both show this interaction shifted to ~5 Å.

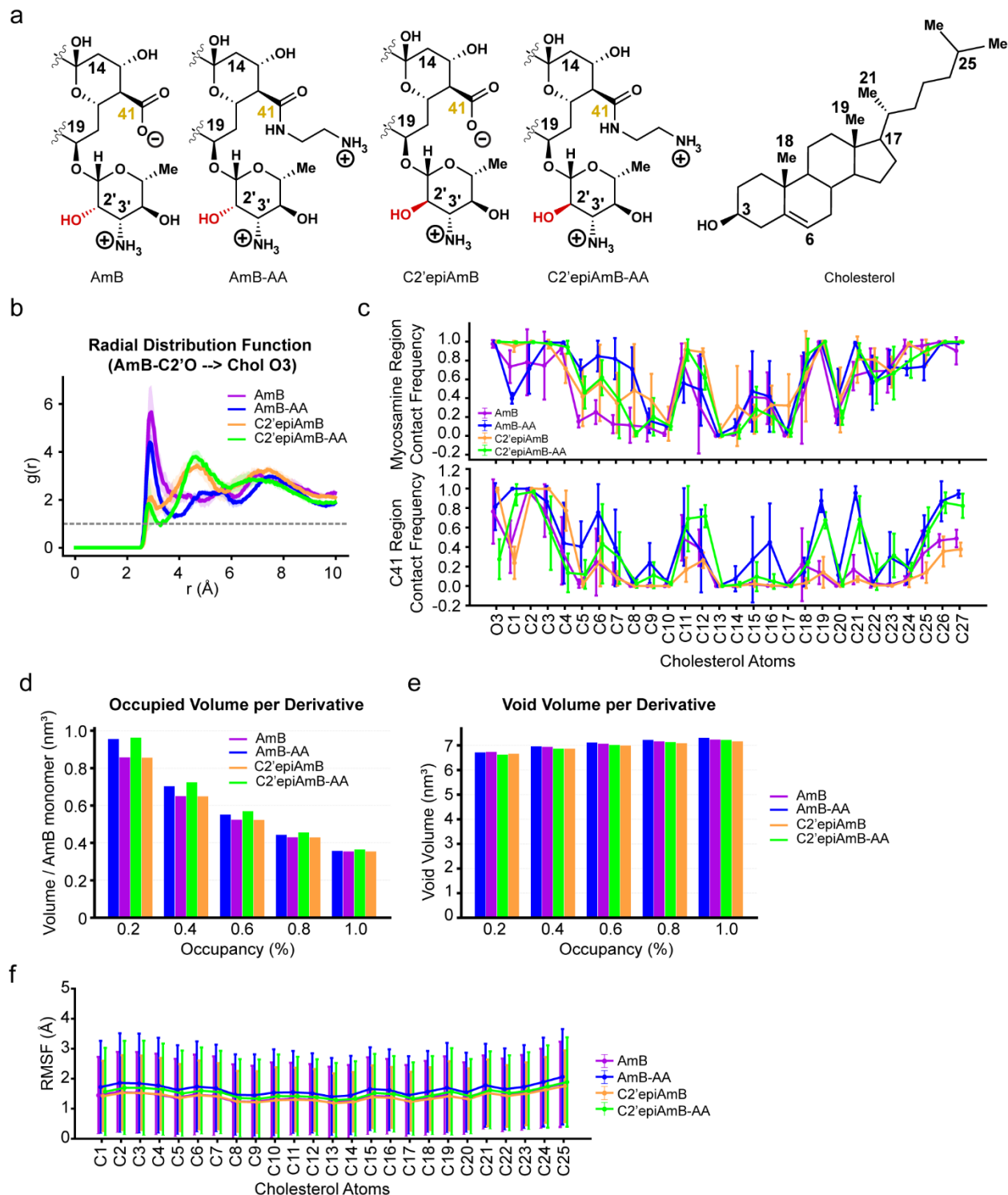
The C41-ethylenediamine modification therefore does not detectably alter close-range cholesterol packing at this contact, consistent with the contact-frequency result and with the preserved cholesterol-binding behavior of AmB-AA observed by UV-Vis (Fig. 3a). In contrast, C2' epimerization reshapes close-range cholesterol ordering at this contact, complementary to the loss of detectable cholesterol binding by C2'epiAmB and C2'epiAmB-AA in the UV-Vis assay (Fig. 4b, Extended Fig. 5f) and reaffirming our previous demonstration that C2' stereochemistry is a primary determinant of mammalian sterol affinity (*Nature* **623**, 1079–1085 (2023)).

Together with the per-atom cholesterol RMSF, the analyses support two conclusions: (i) the C41-ethylenediamine modification does not detectably perturb the sponge-cholesterol binding mode, supporting the conclusion that AmB-AA retains AmB's sterol-sponge-mediated cholesterol-binding mechanism; and (ii) C2' epimerization reshapes close-range cholesterol ordering at the mycosamine-C3'OH contact, providing a structural explanation of the loss of cholesterol binding by C2'epi derivatives.

These analyses are now reflected in the text (p.6, line 212):

“Additionally, computed AmB-Chol radial distribution functions (RDF) and contact frequencies, along with RMSF values of cholesterol heavy atoms indicate similar dynamics across all AmB-cholesterol and derivative sponges (**Fig. 3c and Extended Fig. 6**).”

The analyses are also now updated in Extended Figure 6 (p. 29), shown below:



Extended Figure 6. AmB derivatives interact with cholesterol differently while maintaining structural stability to accommodate cholesterol. (a) Chemical structures of amphotericin B (AmB), AmB-AA, C2'epiAmB, C2'epiAmB-AA and cholesterol. The C2'-hydroxyl of the mycosamine sugar is highlighted in red, while the C41 carboxylate/carboxamide site is highlighted in yellow. **(b)** Radial distribution function, $g(r)$, between the C2'-hydroxyl oxygen of each AmB derivative and the 3-hydroxyl oxygen (O3) of cholesterol (Chol). C2'epiAmB derivatives

demonstrate a loss of close-range contacts (characteristic of hydrogen bonding or approximately 2.8-3.3 Å) between C2'-hydroxyl and the 3-hydroxyl oxygen. Shaded regions denote the standard deviation across replicate simulations. **(c)** Per-atom contact frequency between cholesterol (O3, C1–C27) and the mycosamine region (top; C2'-hydroxyl and C3'-amine) or C41 region (bottom; C41 carbon only) of each AmB derivative. Error bars denote the standard deviation across replicate simulations. **(d)** Occupied volume analysis captures the degree of conformational freedom experienced by C41 modifications and the mycosamine of each AmB derivative within the sponge structures. Occupancy (%) denotes the fraction of simulation time during which the same voxels remain occupied. A value of 1 corresponds to macrolide atoms that are restrained in the MD simulations **(e)** Void volume quantifies the change in volume available to accommodate a sterol molecule. Occupancy (%) denotes the fraction of simulation time during which a voxel remains occupied (occupied volume) or unoccupied (void volume). **(f)** Root mean square fluctuation (RMSF) values for each cholesterol carbon atom (C1–C25) indicate comparable conformational freedom of cholesterol across all four AmB derivatives. Error bars denote the standard deviation across replicate simulations.

Main text

4. *The authors state, as one of the objectives of the MD simulations, that they aim to understand how “the C41-ethylamine side chain would ... change the mechanism by which these compounds interact with lipid bilayers.” However, no simulations including a lipid bilayer are presented. This represents a clear mismatch between the stated objective and the actual simulations performed.*

Response: We appreciate the comment and the opportunity to clarify. In the present study, our objective was not to directly model lipid bilayer interactions, but rather **to determine whether the structure of the sponge would accommodate an ethylenediamine side chain without disrupting its mechanism of action**. As detailed below, an extensive series of prior studies performed by our team already established the interaction of the sterol-sponge with lipid bilayers in the context of its mechanism of cytotoxicity. Thus, we do not think it necessary to repeat such studies in the context of this paper. That said, we agree that the language used in the original manuscript was confusing and could be misinterpreted to suggest that we had intended to do such studies.

Specifically, our manuscript previously stated “Building on an extensive series of recently reported biophysical and structural studies with AmB^{25,58}, we investigated whether the C41-ethylamine side chain would be incompatible with the structure of the sterol sponge, change intramolecular dynamics of AmB derivatives, and/or change the mechanism by which these compounds interact with lipid bilayers, and thereby potentially change the mechanism by which these compounds interact with lipid bilayers.”

We have revised this statement to eliminate the ambiguity, and it now reads (p.6, line 200):

“We hypothesized that AmB-AA primarily exerts its cytotoxic activity by the same sterol sponge mechanism. To provide evidence for or against this hypothesis, we performed MD simulations to determine whether the ethylenediamine side chain could be accommodated within the previously determined sterol sponge structure^{25,58}.”

Additionally, to be maximally transparent regarding the lack of lipids used in these MD simulations, we follow that text with the following statement (p. 6, line 204):

“Notably, these MD simulations, by design, do not include lipid bilayers”

However, given our extensive prior experimental work with lipid bilayers, SSNMR studies, and MD simulations (please see summary below) we believe that we can extrapolate key insights into the mechanism of action of AmB, or similarities between AmB and AmB-AA using MD simulations of the sterol sponge itself without having to explicitly model lipid bilayers. We include this justification in the following revised text (p. 6, line 205):

“However, prior work integrating biophysical experiments in lipid bilayers, SSNMR studies, and MD simulations demonstrated that structural characterization of the sponge assembly itself, independent of explicit lipid bilayer inclusion, can yield meaningful mechanistic insights^{15,22,24,25,58,59.}”

Here we provide a summary of the previous biophysical experiments in lipid bilayers, SSNMR studies, and MD simulations that support this statement.

For decades, the leading textbook model proposed that AmB kills cells primarily by forming ion channels in lipid bilayers (*Antimicrob. Agents Chemother.* **161**, 387–394 (1963); *Arch. Biochem. Biophys.* **102**, 180–188 (1963)). Subsequent computational modeling studies suggested that both the mycosamine ring and the C41-carboxylic acid are required for such ion channel formation (*Biochim. Biophys. Acta.* **511**, 397–407 (1978); *Biochim. Biophys. Acta.* **1108**, 123–127 (1992); *Mol. Pharmacol.* **52**, 560–570 (1997); *Biochim. Biophys. Acta.* **1567**, 63–78 (2002)). Using semisynthesis combined with disc diffusion and broth dilution assays, our group evaluated the antifungal activity of three AmB derivatives: amphoteronolide B (AmdeB) and methylamphoteronolide B (MeAmdeB), both of which lack the mycosamine appendage, and methylamphotericin B (MeAmB), which lacks the carboxylic acid at C41 but still contains the mycosamine ring (*J. Am. Chem. Soc.* **129**, 13804–13805 (2007)). These studies demonstrated that the mycosamine ring is critical for antifungal potency, but the C41-carboxylic acid is not required. These results suggested that the C41 carboxylic acid is not required for ion channel formation and/or ion channel formation is not required for antifungal activity (**Figure 1A**).

We next evaluated these derivatives in a series of lipid bilayer-based biophysical assays (*Proc. Natl. Acad. Sci. U. S. A.* **108**, 6733–6738 (2011)). These included potassium efflux in 10% ergosterol-containing phosphatidylcholine large unilamellar vesicles (LUVs), lipid bilayer binding assays in sterol-free LUVs, and isothermal titration calorimetry studies using sterol-free or ergosterol-containing LUVs. AmdeB and MeAmdeB showed no antifungal activity, no channel activity, and no binding to ergosterol, whereas AmB and MeAmB both showed strong antifungal activity, good channel activity, and strong binding to ergosterol (**Figure 1B**). These findings established that the mycosamine appendage, but not the C41 carboxylic acid, is essential for antifungal activity, ion channel activity, and ergosterol binding. Based on these findings, and noting that another polyene macrolide antifungal, called natamycin, similarly binds ergosterol but

does not form ion channels, we hypothesized that AmB might primarily kill fungi by simply binding ergosterol (**Figure 1C**).

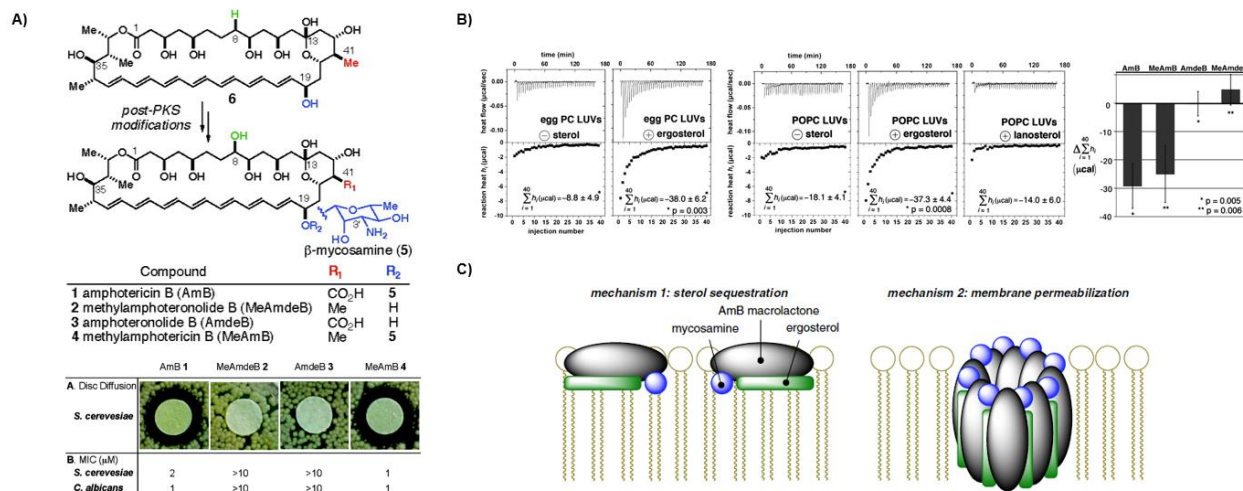


Figure 1. Disc diffusion and MIC studies in yeast performed by Palacios, D.S., ..., Burke, M.D., *JACS* 129, 13804–13805 (2007). Sterol binding experiments in lipid bilayers and the hypothesis that sterol sequestration may be a primary driver of the antifungal activity of AmB studied by Palacios, D.S., ..., Burke, M.D., *PNAS* 108, 6733–6738 (2011).

At this point it remained clear, however, if ergosterol binding was important for ion channel formation which in turn was responsible for antifungal activity, or if ergosterol binding alone is the primary mechanism of fungicidal activity. To distinguish between these possibilities, we sought a derivative of AmB that retains the capacity to bind ergosterol but lacks the capacity to form ion channels (*Proc. Natl. Acad. Sci. U. S. A.* **109**, 2234–2239 (2012)). Guided by leading computational models for the AmB-based ion channel that suggested a critical role for the C35 hydroxyl group in forming ion channels (*Mol. Pharmacol.* **52**, 560–570 (1997); *Biochim. Biophys. Acta* **1567**, 63–78 (2002)) and our own work demonstrating the critical nature of the mycosamine appendage for binding ergosterol, we hypothesized that synthetically deleting the C35-OH group but preserving the mycosamine ring would eliminate the ability of AmB to form ion channels in lipid bilayers while still retaining sterol binding (**Figure 2A**). C35deOAmB was thus synthesized using our bloc chemistry approach (*Science* **347**, 1221–1226 (2015); *Nature* **604**, 92–97 (2022)) (**Figure 2B**). Isothermal titration calorimetry (ITC) experiments showed that C35deOAmB retained ergosterol binding in LUVs (**Figure 2C**). Potassium efflux experiments in LUVs showed that C35deOAmB does not form ion channels (**Figure 2D**). And MIC studies in yeast showed that C35deOAmB retained good fungicidal activity in yeast (**Figure 2E**). **These results demonstrated that AmB kills yeast primarily by simply binding ergosterol.**

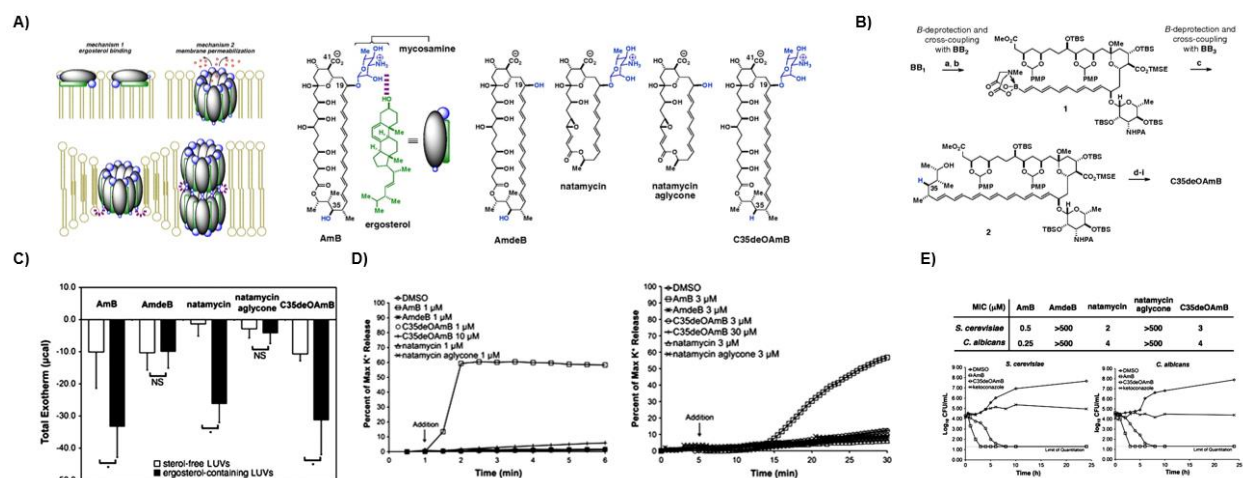


Figure 2. Lipid bilayer-based ITC and potassium efflux experiments combined with MIC studies with C35deOAmB performed by Gray, K. C., ..., Burke, M.D., PNAS 109, 2234–2239 (2012) demonstrating that AmB primarily kills fungi by binding sterols.

Following this discovery, we considered three possible models for the biophysical interactions of AmB (Figure 3) with lipid bilayers that lead to ergosterol binding: a) insertion into lipid bilayers, b) surface adsorption, and c) a new sterol sponge model (Nat. Chem. Biol. 10, 400–406 (2014)).

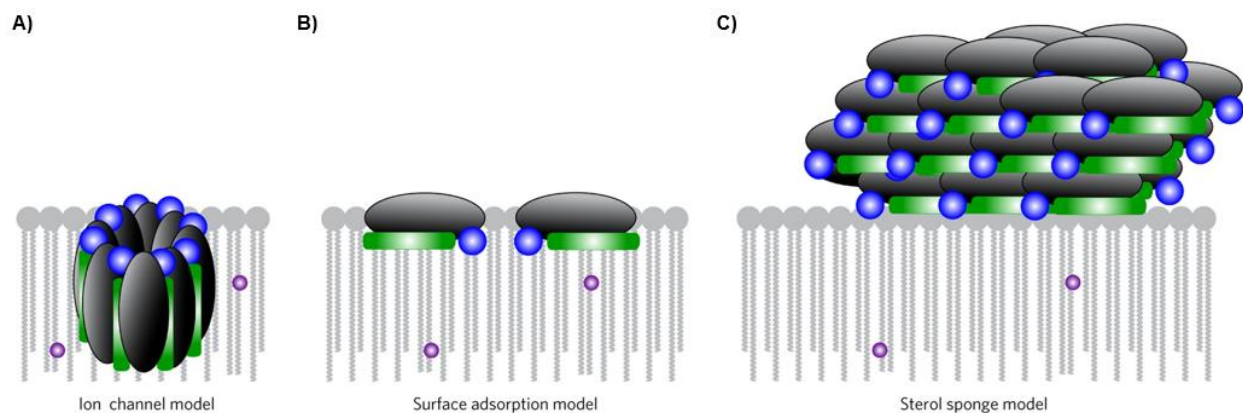


Figure 3. Image from Anderson, T. M., ..., Burke, M.D., Nat. Chem. Biol. 10, 400–406 (2014). (A) Classic ion channel model of AmB. (B) Surface adsorption model. (C) Sterol Sponge Model.

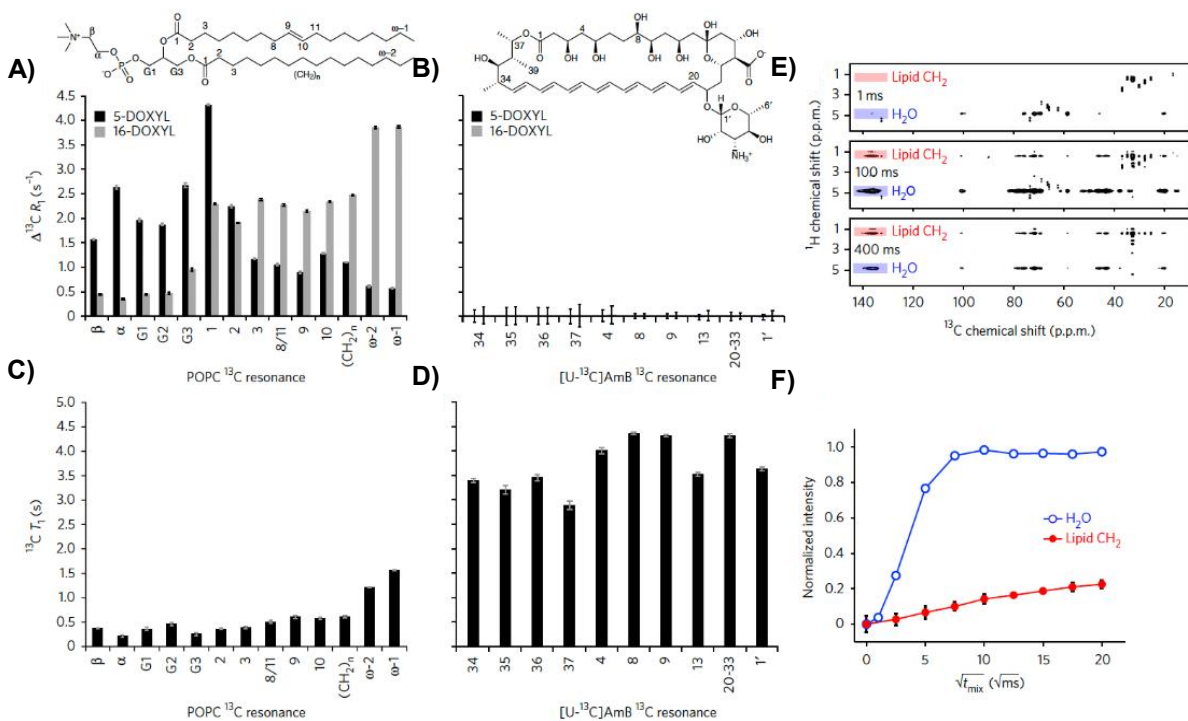


Figure 4. SSNMR experiments in lipid bilayers using paramagnetic relaxation enhancement with ^{13}C -labeled AmB and ^{13}C -labeled ergosterol. Work by Anderson, T. M., ..., Burke, M.D., *Nat. Chem. Biol.* **10**, 400–406 (2014).

To distinguish between these three possibilities, we performed an extensive series of solid-state NMR experiments in the presence of lipid bilayers, including paramagnetic relaxation enhancement (PRE) experiments using ^{13}C -labeled AmB, ^{13}C -labeled ergosterol, and lipid-appended DOXYL spin labels (Figure 4). These studies collectively demonstrated that AmB assembles into a large **extramembranous aggregate** that rapidly extracts ergosterol from lipid bilayers. In fact, most of the AmB molecules in this extramembranous sterol sponge are located $> 20 \text{ \AA}$ away from the lipid bilayers. Transmission electron microscopy (TEM) imaging in the presence of ergosterol-containing LUVs directly validated the presence of a **large extramembranous AmB sponge aggregate associated with ergosterol-containing lipid bilayers** (Figure 5).

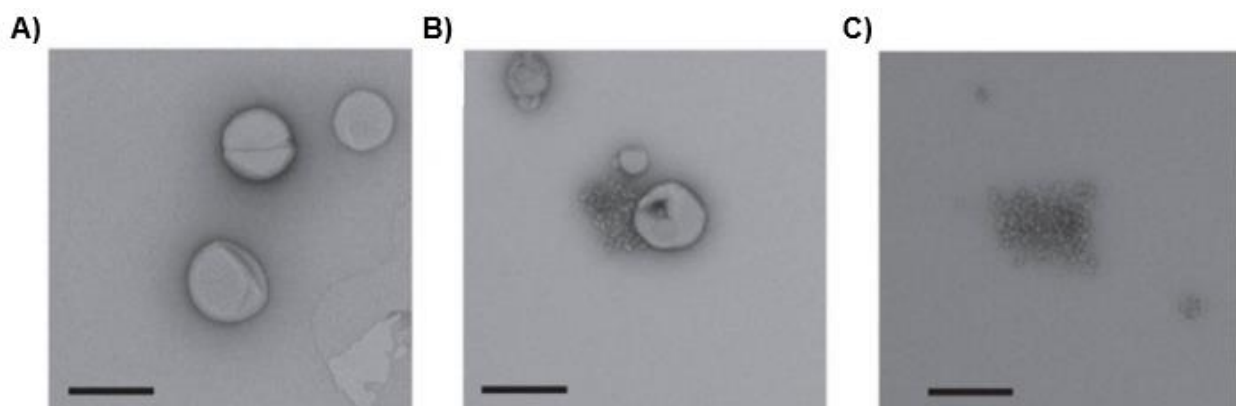


Figure 5. TEM images obtained by Anderson, T. M., ..., Burke, M.D., *Nat. Chem. Biol.* **10**, 400–406 (2014). Showing (A) POPC/Erg 10:1 liposomes (B) POPC/Erg 10:1 liposomes with 1 equiv. (relative to Erg) of added AmB (C) AmB only. Scale bars, 200 nm.

Using additional biophysical studies directly characterizing the content of lipid bilayers in both LUVs and in living fungal cells, we demonstrated that these large extramembranous aggregates physically extract ergosterol from lipid bilayers, and that this physical extraction of ergosterol by the extramembranous sterol sponge is the primary mechanism by which AmB kills fungal cells (**Figure 6**).

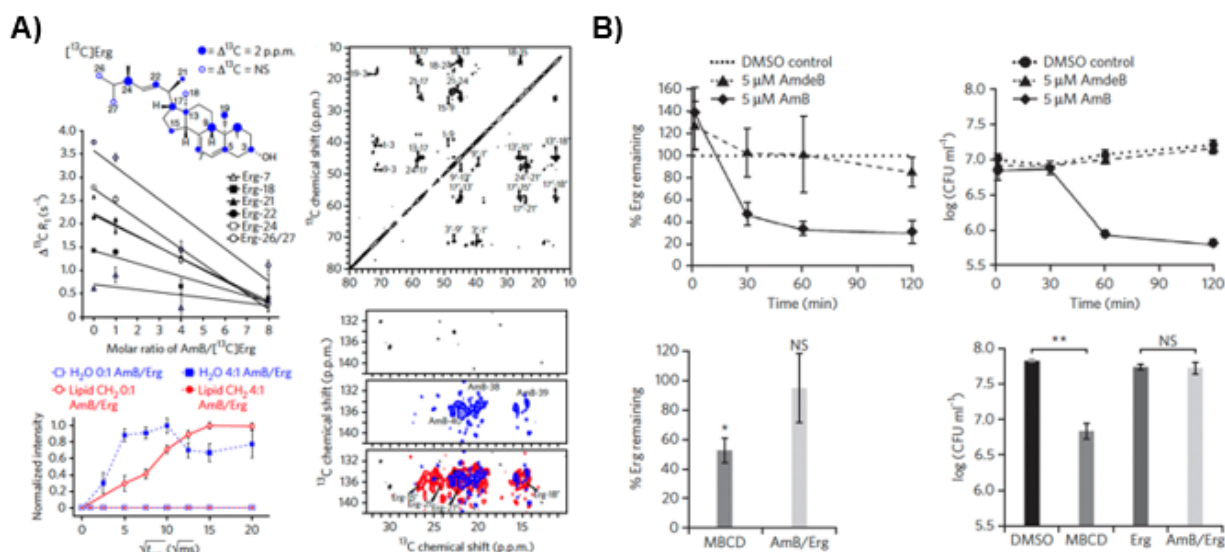


Figure 6. Work by Anderson, T. M., ..., Burke, M.D., *Nat. Chem. Biol.* **10**, 400–406 (2014). (A) PRE effects in SSNMR show that AmB extracts Erg from lipid bilayers. (B) Ergosterol extraction from yeast cell membranes by AmB kills yeast cells.

Having established that AmB primarily kills fungi via the sterol sponge mechanism, and most importantly for the purposes of this response letter, **having demonstrated that this AmB-based sterol sponge resides outside the lipid bilayer**, we carried out extensive biophysical and computational modeling studies to characterize the AmB-based sterol sponge in the absence of lipid bilayers. **As described below, these studies of the AmB-based sterol sponge in the absence of lipid bilayers led to substantial**

additional insights into the fundamental mechanism and biophysical underpinnings via which AmB kills both fungal and human cells, which ultimately enabled the development of a renal sparing derivative of AmB, dubbed AM-2-19 and is now called Turletricin, that has proven to be safe in Phase I human clinical trials (Open Forum Infectious Diseases, 13, ofaf695.1409 (2026); <https://doi.org/10.1093/ofid/ofaf695.1409>) and is now being tested in Phase 2 clinical trials for treating people with life-threatening fungal infections (<https://clinicaltrials.gov/study/NCT07215273>).

Specifically, we first combined high-resolution SSNMR with density functional theory calculations, XPLOR-NIH simulated annealing, and molecular dynamics calculations in lipid-free systems (*Nat Struct Mol Biol* **28**, 972–981 (2021)). These studies demonstrated that the AmB-sponge aggregate is composed of asymmetric head-to-tail homodimers (Figure 7A). Furthermore, large intermolecular void volumes comparable to the size of sterol molecules were revealed (Figure 7B).

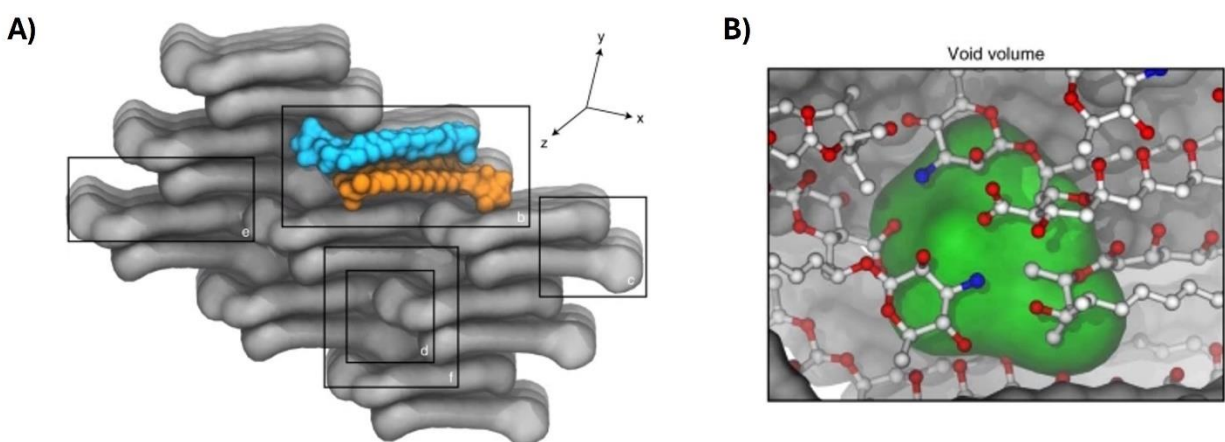


Figure 7. (A) Lowest distance restraint energy (6.8 kcal) XPLOR-NIH structure of the AmB sponge ($3 \times 3 \times 3$) lattice which consists of asymmetric head-to-tail homodimers done by Lewandowska, A., ..., Pogorelov, T.V., Burke, M.D., Rienstra, C. M. *Nat Struct Mol Biol* **28**, 972–981 (2021). (B) Representative void volume in the AmB lattice.

We then utilized frontier SSNMR studies and MD simulations to guide the discovery of a renal-sparing polyene antifungal AM-2-19, which is now called Turletricin (*Nature* **623**, 1079–1085 (2023)). Maji et al. first showed that the sterol sponge mechanism also accounts for the toxicity of AmB to primary human renal cells. We then used extensive series of SSNMR studies in the absence of lipid bilayers to **determine high resolution structures of both ergosterol- and cholesterol-bound AmB sponges in the absence of lipid bilayers (Figure 8A)**. These high-resolution structures illuminated a key interaction between the mycosamine C2'-OH group and both ergosterol or cholesterol, and a second key interaction between the polyene of AmB and the diene of ergosterol that was unique to this fungal sterol (Figure 8B). These insights suggested that eliminating the interactions with the C2'-OH (which is common to both sterols) while preserving the interaction with the polyene (which is unique to ergosterol), would selectively destabilize the binding to cholesterol. This, in turn, should reduce or eliminate mammalian renal toxicity while preserving antifungal activity. Accordingly, we synthesized

a derivative of AmB in which the stereogenic center at C2' was epimerized. With this derivative, we performed extensive additional biophysical studies and biological studies which demonstrated that this derivative retained ergosterol binding but lost detectable cholesterol binding, which in turn eliminated toxicity to primary human renal cells and mouse kidneys in vivo (Figure 8C-H).

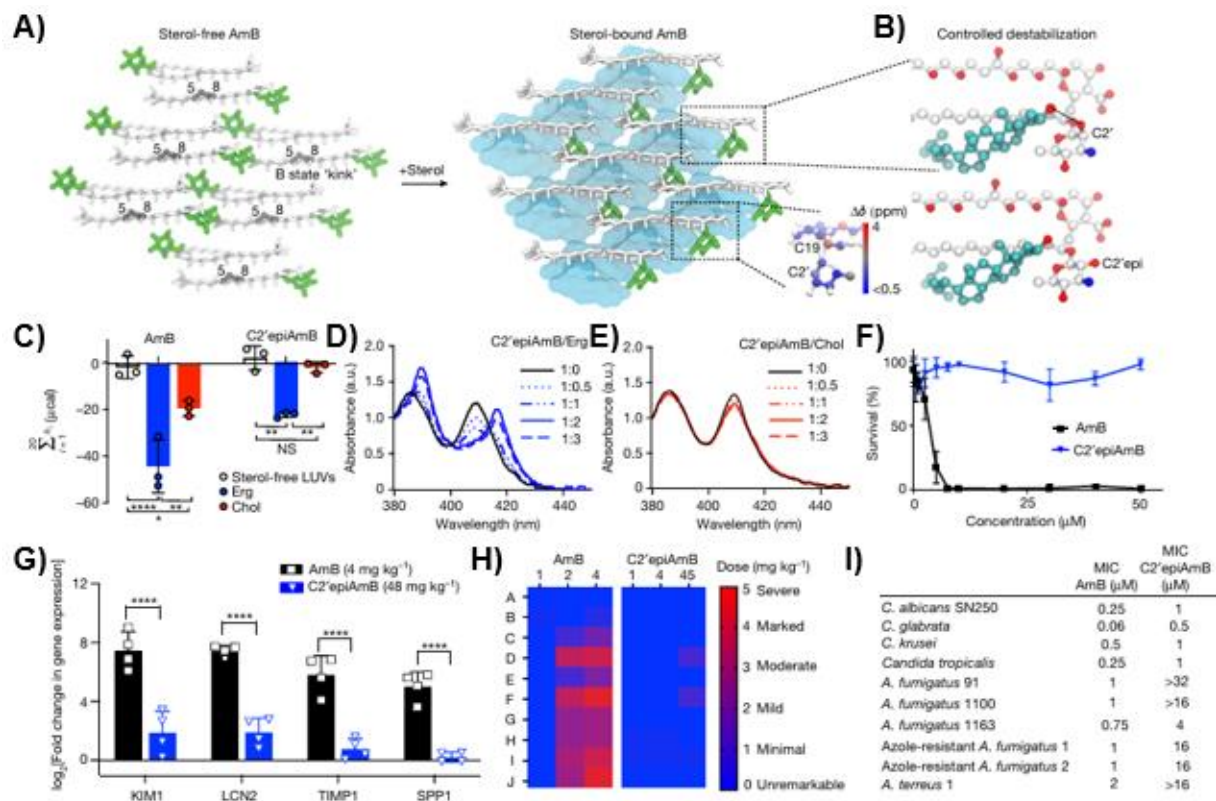


Figure 8. Work by Maji, A., ..., Pogorelov, T.V., ..., Rienstra, C.M., Burke, M.D., Nature 623, 1079–1085 (2023). Comparison between apo-AmB and sterol-bound AmB sponge structure highlights change in mycosamine orientation. Epimerization of the C2'OH resulted in a controlled destabilization of AmB-sterol sponge structure that enabled Erg selectivity.

Additional studies yielded a more advanced derivative, AM-2-19, that is equipotent to AmB, non-toxic to the kidneys in mice (and subsequently in rats, dogs, and people in Phase 1 clinical trials) and is highly effective in a wide range in vivo models for invasive fungal infections (Figure 9 and 10). This compound is now called Turletricin and is being evaluated for the treatment of life-threatening fungal infections in a Phase 2 clinical trial.

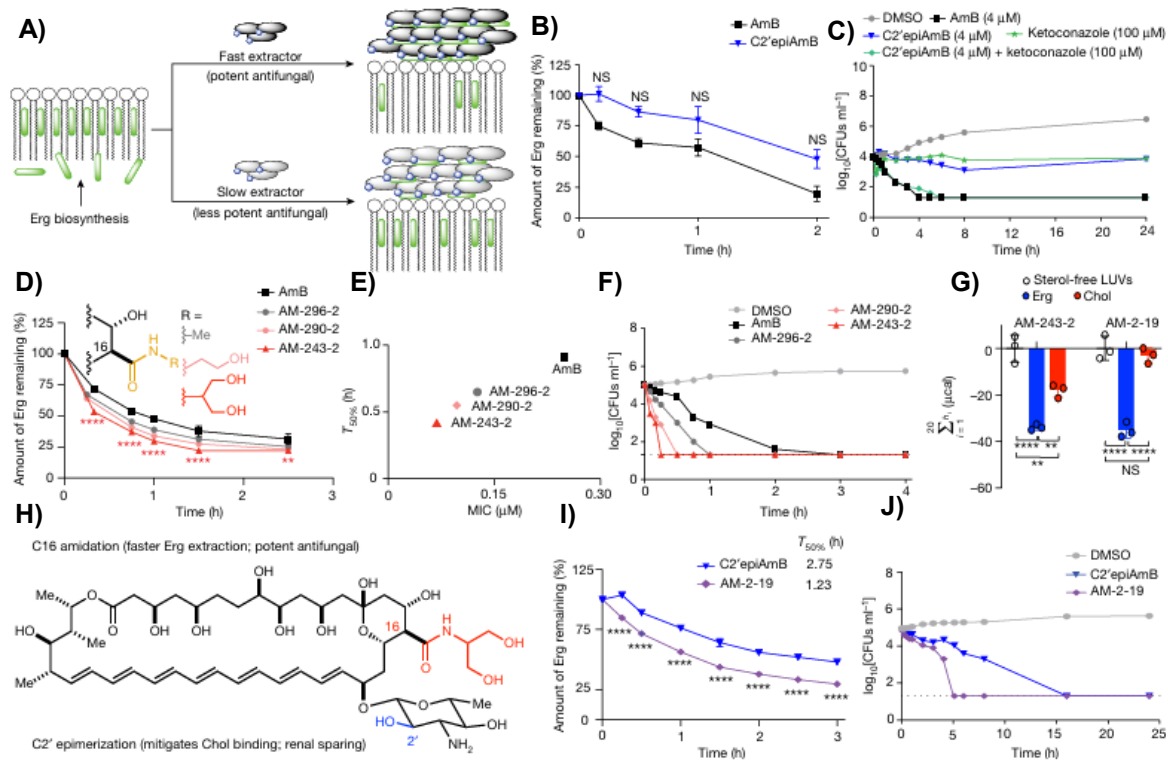


Figure 9. Work by Maji, A., ..., Pogorelov, T.V., ..., Rienstra, C.M., Burke, M.D., Nature 623, 1079–1085 (2023). AM 2-19 combines C2'epimerization with C16 amidation to mitigate cholesterol binding and promote faster ergosterol extraction, respectively.

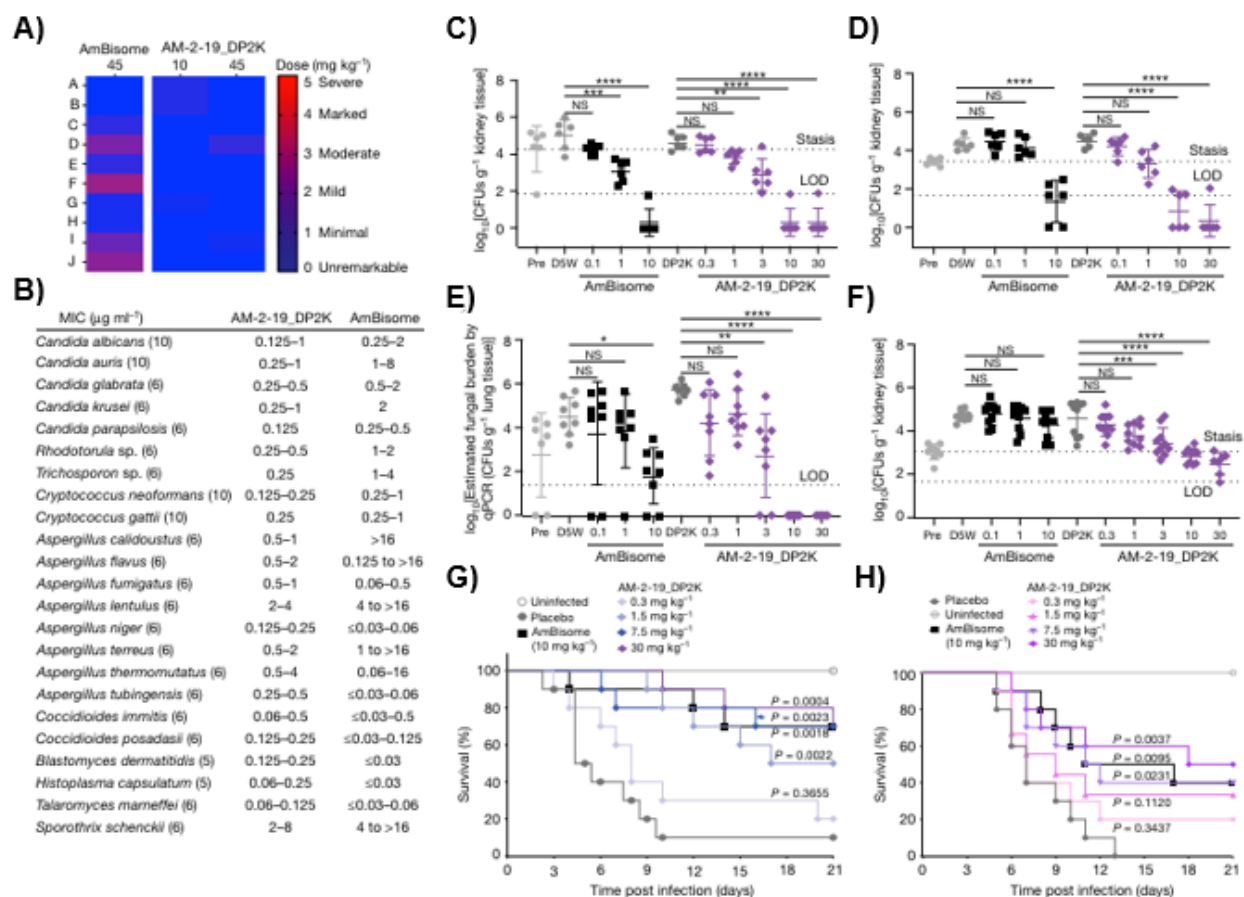


Figure 10. Work by Maji, A., ..., Pogorelov, T.V., ..., Rienstra, C.M., Burke, M.D., Nature 623, 1079–1085 (2023). A micellar formulation of AM-2-19 shows in vivo tolerability and efficacy against candidiasis and aspergillosis mice models.

Most importantly for the purposes of this response letter, critical scientific insights into the mechanism by which the extramembranous sterol sponge kills both fungal and human cells, which in turn led to these real-world advances, **were substantially enabled by computational modeling studies performed in the absence of lipid bilayers**. We respectfully submit that the computational modeling studies of the sterol sponge putatively formed by AmB-AA similarly performed in the absence of lipid bilayers and reported in the current manuscript, add value to the manuscript and can be rigorously interpreted to support the specific conclusion that “the ethylenediamine side chain could be accommodated within the previously determined sterol sponge structure”

5. The description “NMR informed model of AmB AA bound to cholesterol” is insufficiently documented. It is not clear how this model was generated, whether it results from MD simulations, NMR refinement, or a hybrid approach. This should be explicitly described in the Methods.

Response: We agree that this language was confusing. We did not do NMR studies on the AmB-AA sponge; rather, we built our models on the previous NMR studies that we

performed with Amphotericin B and sterols (Nature **623**, 1079–1085 (2023), please see detailed summary above). Specifically, our starting point was a structure of the AmB-sponge bound to ergosterol that was extensively characterized in our previous work by SSNMR in concert with XPLOR-NIH, and MD simulations.

We have modified the Figure 3c legend (p. 8, line 269) for clarity to accurately read:

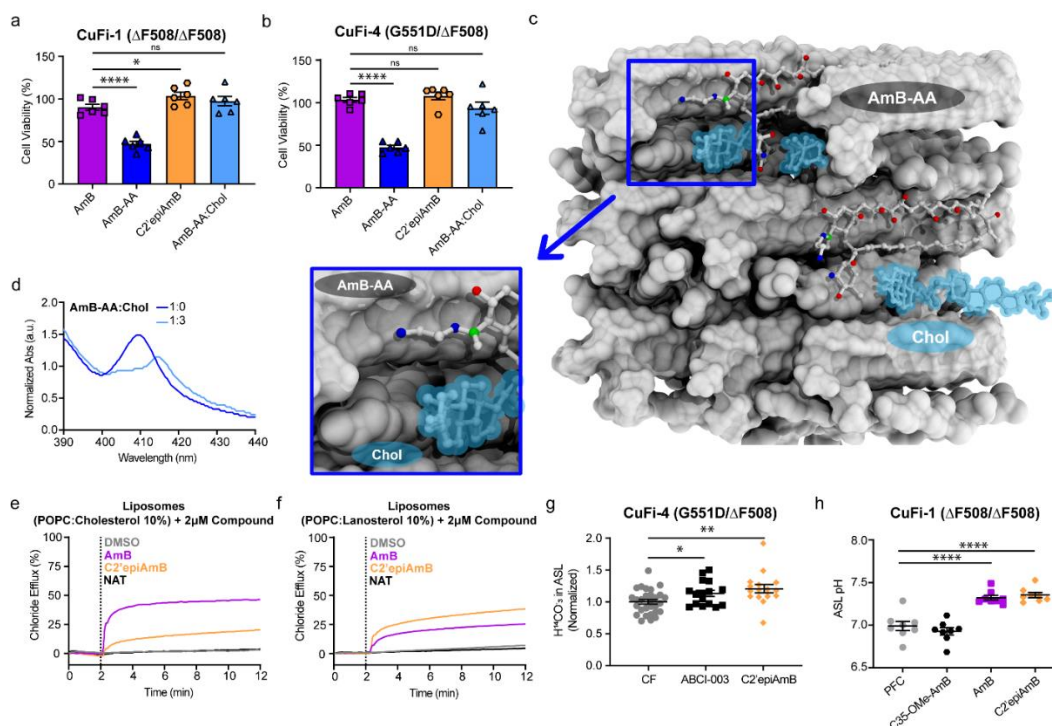
“MD model of AmB-AA bound to cholesterol. Representative snapshot from an equilibrium all-atom MD simulation of the AmB-AA sterol sponge in complex with cholesterol. The starting point for this MD simulation was our previous NMR study²⁵.”

Additionally, we have modified the Methods to explicitly state this (p. 17, line 654) and addressed this in more detail in reply #1:

“Starting coordinates for each AmB derivative–cholesterol sponge were generated by templating the derivative onto the 5×5×5 AmB lattice of the NMR-resolved native AmB–cholesterol sponge structure (BMRB Entry 21102),”

6. Additionally, the panel would benefit from clearer visual guidance. In particular, the smaller inset (panel d) is not well connected to the rest of the figure; adding an arrow or similar visual cue would help the reader.

Response: We agree that some visual guidance would help the reader with Figure 3, previously panel d (now panel c). After modifying the text and moving the panels we have revised the figure so that it now includes visual cues for clarity (below).



Methods

7. The section title “System Preparation and Molecular Dynamics Simulations” could be improved for clarity. For example:

“Molecular Dynamics Simulations”, or

“System Preparation and Parameters for Molecular Dynamics Simulations.”

Response: Thank you, we agree and have modified the section title (p. 17, line 652) to:

“Molecular dynamics simulations:”

To add structure to the section, we also introduced subsections: System preparation and parameters, Simulation Protocol, List of simulations (Table), Volume calculations, etc.

8. *The statement “The system was prepared using the lowest energy AmB–cholesterol lattice (Fig. 1a) from previously reported and deposited data⁵⁸.” is problematic for several reasons:*

- Fig. 1a does not clearly show an AmB–cholesterol lattice.*
- It is not evident from the cited reference how the initial structure was obtained.*

Response: Thank you for catching this oversight. The citation has been correctly updated to the correct reference, which shows the AmB-ergosterol lattice.

The reference was updated to (p. 21, line 825):

25. Maji, A. et al. Tuning sterol extraction kinetics yields a renal-sparing polyene antifungal. *Nature* 623, 1079–1085 (2023).

9. *For reproducibility, it is essential that the authors provide full details of the initial structure. Deposition of the starting coordinates, final structures, and parameter files (e.g., via Zenodo or a similar repository) is strongly recommended and, in practice, expected, required by Nat Comm.*

Response: We agree and have uploaded all the starting coordinates, final structures, and parameter files to Zenodo (DOI: <https://doi.org/10.5281/zenodo.20588529>).

In addition, new main text section Data Availability (p. 20, line 759) includes a section that now reads:

Data Availability

MD Simulation Data: Starting and final coordinates (GRO file format), GROMACS topology (.top/.itp), parameter files (.prm), CGenFF outputs, .mdp files for minimization, NPT equilibration and production are deposited on Zenodo (DOI: <https://doi.org/10.5281/zenodo.20588529>).

10. *The following sentence is difficult to parse and should be rewritten:*

“To maintain the structure of the experimentally resolved sponge, carbon atoms in the macrolide region of all AmB molecules were constrained using gmx genrestr with a force constant of 8000 kJ/mol/nm² and all sterol molecules left unconstrained (???) AmB parameters were obtained from CHARMM General Force Field and sterols parameters were obtained from the CHARMM36 force field.”

Response: Thank you for your suggestion. We have rewritten the Methods section for clarity and details. This section has been updated (p.17, line 663) to now read:

“To preserve the NMR-resolved scaffold geometry, harmonic positional restraints were applied to the macrolactone heavy atoms of each AmB-derivative using `gmx genrestr` with a force constant of 8,000 kJ/mol/nm² (allowing ± 0.2 Å fluctuations) while mycosamine atoms, C41 side chain modifications, all hydrogens, and all sterol atoms remained fully unrestrained throughout minimization, equilibration, and production.”

Beyond the wording issues, there are important methodological concerns:

11. A force constant of 8000 kJ/mol/nm² is unusually high, even for early equilibration, and requires justification. It is unclear whether these constraints were released during equilibration or remained during production simulations. Cholesterol (or sterols in general) was left unconstrained, which is inconsistent given that it is a key structural component of the system.

These points raise concerns about whether the system was allowed to equilibrate realistically.

Response: We used a force constant of 8,000 kJ/mol/nm² to maintain the NMR-resolved structure with the restraints applied to the heavy atoms of the AmB macrolides. While 8,000 kJ/mol/nm² may seem high, our goal was to preserve the experimentally-resolved sponge geometry of Nature **623**, 1079–1085 (2023) throughout production, while leaving all chemically relevant regions free. Under equipartition, this force constant still permits per-atom ± 0.2 Å fluctuations at 298 K, well within the NMR coordinate uncertainty, so the restrained macrolide backbone behaves as a dynamic scaffold reproducing the deposited structure rather than a frozen one. Restraints were maintained throughout minimization, equilibration, and production, and we have stated this explicitly in the revised Methods.

These constraints remained during the entire production simulation across all replicates to maintain the sponge NMR-resolved geometry and leave the chemically important regions (C41 modifications and the mycosamine) unrestrained (Extended Figure 6b-c).

The complementary decision to leave cholesterol fully unrestrained reflects the question the simulations were designed to address whether the experimentally-defined sponge can accommodate the C41-ethylamine modification and whether bound-cholesterol dynamics differ across derivatives. Restraining cholesterol would have eliminated the observable. The mycosamine, the C41 side chain, all hydrogens, and all sterol atoms were therefore unrestrained throughout — i.e., every chemical group whose behavior we report on was free to sample its native ensemble around the fixed scaffold. To add in this version all analyses were performed on the 3x3x3 core of the sponge to reduce influence of bulk water. Please note that the core offers substantial amount of data covering 27 AmB and 54 Chol molecules.

To make this clear the text now reads (p.17, line 663):

“To preserve the NMR-resolved scaffold geometry, harmonic positional restraints were applied to the macrolactone heavy atoms of each AmB-derivative using `gmx genrestr` with a force constant of 8,000 kJ/mol/nm² (allowing ± 0.2 Å fluctuations) while mycosamine atoms, C41 side chain modifications, all hydrogens, and all sterol atoms remained fully unrestrained throughout minimization, equilibration, and production.”

12. *The sentence “For equilibration and production runs, the C-rescale barostat and V-rescale thermostat were employed along with the leapfrog integrator to maintain constant pressure and temperature.” is somewhat unclear. As written, it may suggest that all listed components, including the leapfrog integrator, contribute to maintaining constant pressure and temperature, which is not accurate.*

Response: Thank you for your suggestion. We agree, the text has been re-written to improve clarity, and it now reads (p.18, line 678):

“Production simulations were performed in the isothermal–isobaric (NPT) ensemble at 298 K and 1.0 atm. Pressure was maintained using the stochastic cell-rescaling (C-rescale) barostat⁸⁸ with isotropic coupling ($\tau_p = 5.0$ ps; compressibility = 4.5×10^{-5} bar⁻¹), and temperature was maintained using the velocity-rescaling (V-rescale) thermostat²¹ ($\tau_t = 0.5$ ps), with the sponge and solvent thermostatted as separate coupling groups. Equations of motion were integrated using the leap-frog algorithm⁸⁹ with a 2-fs time step.”

13. *In the “Table of simulations,” the simulation length would be clearer if reported as 3 × 250 ns rather than simply 250 ns.*

Response: Thank you for the feedback. The table has been updated from simply 250 ns to “3 x 250 ns” and additionally the text was updated to make it clear that 3 replicates were done and the total number of AmB and Chol molecules were added, and it now reads (p.18, line 692):

“The production runs were 250 ns long. For each system modeled, 3 replicates were initialized using different random seeds for starting velocities.”

14. *The statement “Computed RMSF values for each atom in the cholesterol molecule indicate similar intermolecular interaction patterns across all AmB derivative sponge models.” is not sufficiently precise. RMSF reflects atomic fluctuations (flexibility) and does not directly measure intermolecular interactions. The interpretation should either be justified more carefully or rephrased.*

Response: Thank you for pointing this out. We agree this statement is not sufficiently precise. We modified the figure legend in Extended Figure 6 now reads (p.30, line 1081):

“(f) Root mean square fluctuation (RMSF) values for each cholesterol carbon atom (C1–C25) indicate comparable conformational freedom of cholesterol across all four AmB derivatives. Error bars denote the standard deviation across replicate simulations.”

15. *More generally, the occupied/void volume analysis appears to be a relatively coarse metric. Its dependence on voxelization and occupancy thresholds makes it difficult to interpret in a physically meaningful way or to relate directly to molecular interactions.*

More informative analyses would include:

- radial distribution functions (RDFs) between AmB and cholesterol,
- contact frequency or residence time analyses,
- or other position-resolved interaction metrics.

Response: We thank the reviewer for these constructive suggestions which have substantially strengthened the molecular interpretation of our simulations.

The results are described in greater detail in response #3. In summary, the suggested additional analyses of MD (RDFs and contact frequencies) support two conclusions:

(1) the C41-ethylenediamine modification does not detectably perturb the sponge-cholesterol binding mode, supporting the conclusion that AmB-AA retains AmB's sterol sponge-mediated cholesterol-binding mechanism;

(2) C2' epimerization reshapes close-range cholesterol ordering at the mycosamine-C3'OH contact, providing a structural explanation of the loss of cholesterol binding by C2'epi derivatives. The occupied/void analysis is retained as a complementary global descriptor of sponge porosity, and the revised text now presents the RDF and contact-based metrics as the principal interaction analyses (p. 19, line 734).

The methods section has also been updated to accurately describe the analysis done (p. 19, line 704):

Volume calculations: Occupied and void volumes were calculated using the `volmap` plugin of VMD [<https://www.ks.uiuc.edu/Research/vmd/plugins/volmapgui/>]. Volumes were calculated using an occupancy map at a resolution of 0.5 Å³ and the resulting voxels were counted for occupancy relative to timescale of dynamics. Voxel occupancy was calculated over the course of the entire simulation and averaged. The percentage of simulation time during which a voxel was occupied was determined from the fractional value of a given voxel.

Cholesterol root mean square fluctuation (RMSF) calculations: RMSF values were computed for each of the core 54 cholesterol heavy atom using the MDAnalysis⁹⁴⁻⁹⁶ Python package. Atomic positions in the first frame, corresponding to the initial NMR-derived structure, were used as the reference. For each atom, the RMSF was calculated as the time-averaged root mean square deviation of its position from the reference over the analyzed trajectory frames. Calculations were performed over the final 80% of each trajectory, consistent with the contact frequency analysis. The reported statistics are based on total of 600 ns MD data 3 trajectory replicas of 27 AmBs and 54 cholesterol, each.

Contact Frequencies: Contact frequencies between AmB interaction regions and individual cholesterol atoms were computed using MDAnalysis^{94,95}. AmB heavy atoms were partitioned into functional interaction classes (mycosamine and C41 carboxylate/carboxamide), and cholesterol heavy atoms were grouped by atom name. For each frame, pairwise distances between all AmB atoms in a given interaction class and all cholesterol atoms were computed using `capped_distance` with periodic boundary corrections. A binary contact was registered for a given interaction class–Chol atom pair if any interatomic distance fell within a 4.0 Å cutoff. The contact frequency was then calculated as the fraction of analyzed frames in which at least one such contact was observed. Analysis was performed over the final 80% of each trajectory to ensure equilibrated sampling. The reported statistics are based on total of 600 nsMD data over 3 trajectory replicas of 27 AmBs and 54 cholesterol each.

Radial Distribution Functions: Radial distribution functions (RDFs) between AmB hydrogen-bond donor atoms and cholesterol heavy atoms were computed using the InterRDF module of MDAnalysis^{94,95}. For each AmB derivative, donor atoms comprising the mycosamine C2 hydroxyl were selected as the reference group, while cholesterol O3 atoms were used as target groups to probe contacts at the sterol C3-hydroxyl. Pairwise distances were binned over 0–10 Å using 100 bins with periodic boundary corrections, sampling every 100ps across the last 80% of each trajectory (frames 20–100%) to exclude initial equilibration. RDFs were computed independently for each replicate and subsequently averaged, with the standard deviation across replicates reported as the spread. The reported statistics are based on total of 600 ns MD data 3 trajectory replicas of 27 AmBs and 54 cholesterol each.

16. Finally, there is an inconsistency between the text and the figure: values are described as 100% in the text, while the figure reports values on a 0-1 scale. This should be made consistent.

Response: Thank you for catching this. The text has been updated to better reflect the figure and now reads p.30, line 1078 (and above):

“A value of 1 corresponds to macrolide atoms that are restrained in the MD simulations”

Overall

The MD section requires substantial clarification and, in some cases, additional analysis. At present, the description of the simulations, the lack of reproducibility details, and the interpretation of the results do not meet the expected standards.

Response: We appreciate your thorough evaluation of the MD section. In response, we have substantially revised the manuscript to clearly define the hypothesis being tested by the MD simulations: **the ethylenediamine side chain appended at the AmB-C41 position can be accommodated within the void volume of the previously determined sterol sponge structure.** Accommodation of this substituent is consistent with the hypothesis that AmB-AA retains the same sterol sponge mediated cholesterol binding mechanism that has been extensively established for AmB and, therefore, shares a similar mechanistic basis for cytotoxicity. To further evaluate this hypothesis, **we performed the additional analyses you suggested**, including radial distribution function, per-atom contact frequency, and RMSF analyses. Collectively, all of these studies are consistent with AmB-AA retaining AmB's sterol sponge-mediated cholesterol binding mechanism. **We also substantially expanded the methodological details necessary for reproducibility and interpretation**, and we believe they significantly strengthen the rigor and transparency of the MD component of the study.

We again thank all the reviewers for their thoughtful feedback. With these revisions, we believe the manuscript has been significantly strengthened and hope that it is now suitable for publication.

Molecular prosthetics for CFTR designed for anion selectivity outperform amphotericin B in cultured cystic fibrosis airway epithelia – Response Letter

Reviewer #5 (Remarks to the Author):

The revised work is much more transparent and reproducible, and the addition of RDF/contact analyses is a clear improvement. I appreciate the substantial improvements made in this revision. In particular, the authors now clearly describe the origin of the starting structures, the system construction, the use of randomized initial velocities for the three replicas, the simulation parameters, and the deposition of coordinates and input files. These changes adequately address my previous concerns regarding reproducibility and the generation of replicate trajectories. The revision of the RMSF interpretation and the addition of RDF and contact-frequency analyses are also welcome improvements. However, several important issues remain.

Response: Thank you for your initial feedback which helped strengthen our manuscript. We appreciate the opportunity to address your remaining concerns. Below we provide a detailed point-by-point response to each of them.

1. Scope of the simulations and interpretation of the restraint-based model
The revised Methods now clearly state that positional restraints of 8000 kJ / mol / nm² were applied to the macrolactone heavy atoms throughout minimization, equilibration, and production. The authors also explicitly state that this protocol probes local relaxation of unrestrained groups within an experimentally resolved scaffold, rather than the de novo thermodynamic stability of the sponge architecture. This is an important clarification. Such restrained simulations can reasonably address whether the C41 modification and cholesterol molecules can be accommodated locally within the NMR-derived sponge scaffold.

However, the conclusions in the Results and Extended Figure 6 still exceed what this protocol can demonstrate. Because the macrolactone scaffold is deliberately maintained close to the NMR-derived geometry, these simulations cannot establish spontaneous sponge assembly, thermodynamic stability of the sponge, preservation of sponge architecture in an unrestrained system, cholesterol extraction from a membrane, or equivalence of the complete toxicity mechanism between AmB and AmB-AA. Accordingly, statements such as “MD yielded a high-resolution sponge structure,” “large void volumes were not disrupted,” or that the simulations support an identical cholesterol-extraction-dependent mechanism should be softened.

The data support a more limited and defensible conclusion: the C41 ethylenediamine modification appears compatible with the local cholesterol environment of the experimentally derived and restrained sponge scaffold. This would be consistent with the proposed sterol-sponge mechanism, but it does not independently demonstrate sponge stability or cholesterol extraction.

I recommend revising the central conclusion accordingly, both in the Results and in the legend/title of Extended Figure 6. Terms such as “maintaining structural stability” should be avoided unless stability is assessed using a substantially less restrained or unrestrained model.

Response: We have accordingly softened the conclusions about the sponge formation and cholesterol extraction mechanism using the suggested language.

Specifically, based on these comments, we revised the results section to introduce the MD simulations with the following (p. 6, line 209):

“Molecular dynamics of the bis-positively charged AmB-AA or C2’epiAmB-AA sponge complexed with cholesterol was based on a prior NMR-resolved XPLOD-NIH structure²⁵ (**Fig. 3c and Extended Fig. 6a**). These structures were used to compare the interactions of the bis-positively charged AmB derivatives with cholesterol.”

Additionally, the conclusions from the MD simulations were softened and now state the following:

“Additionally, void volume changes across all AmB derivatives sponges were computed as a descriptive supplementary metric (**Extended Fig. 6d**). These analyses show that the C41 ethylenediamine modification appears compatible with the local cholesterol environment of the experimentally derived and restrained sponge scaffold. This is consistent with the proposed sterol-sponge mechanism, but it does not independently demonstrate sponge stability or cholesterol extraction.”

We have also revised the title of Extended Figure 6 to now be (p. 30):

“Extended Figure 6. AmB derivatives compatible with the local cholesterol environment of the experimentally derived sponge scaffold show consistency with sterol-sponge mechanism.”

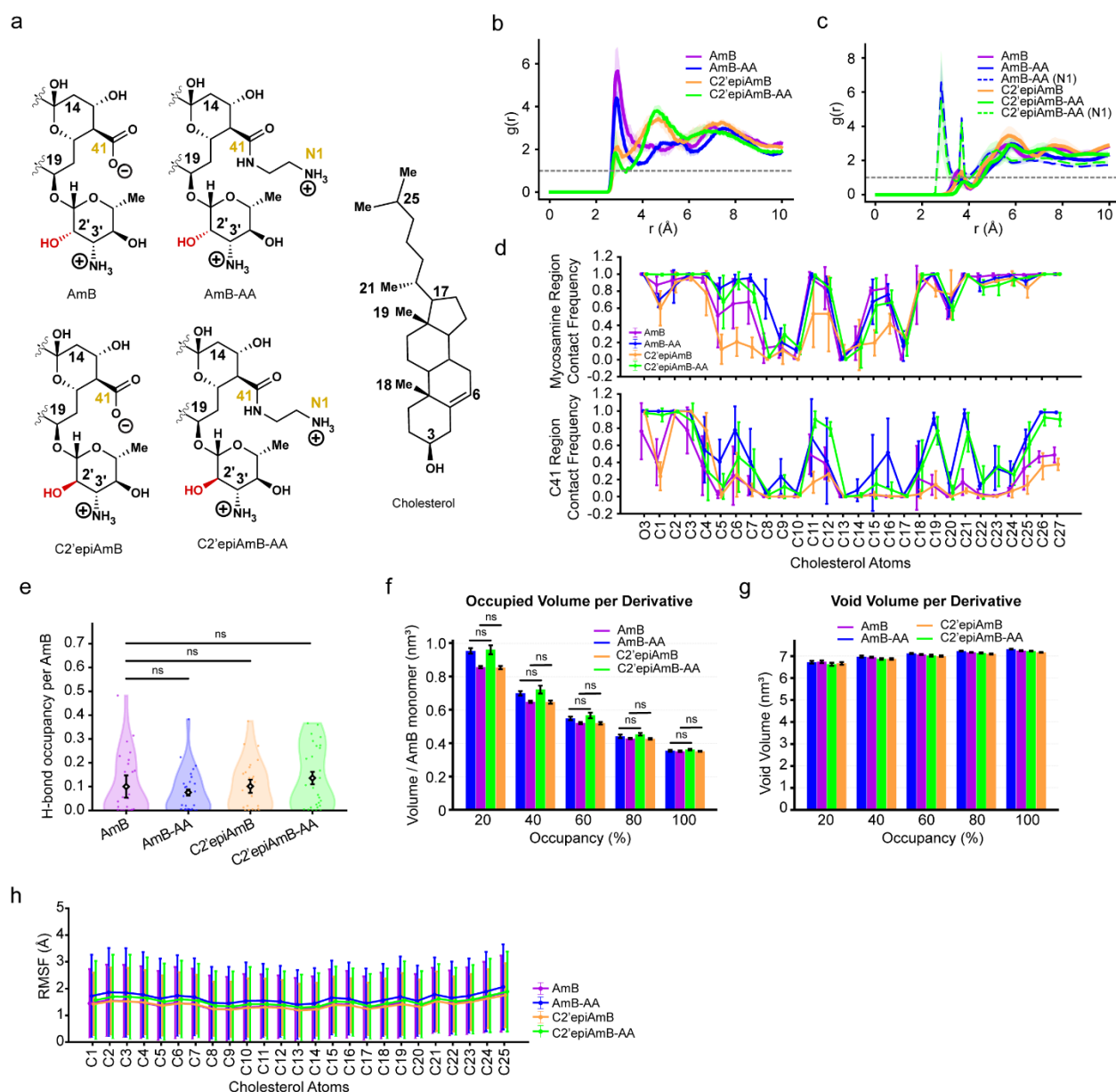
2. The new interaction analysis does not directly test the full C41 ethylenediamine modification

The reported RDF is calculated between the C2'-hydroxyl oxygen of AmB and cholesterol O3. This seems to be useful for examining the effect of C2' epimerization, but it does not directly test the newly introduced C41 ethylenediamine group. Similarly, the description of the C41 contact analysis is somewhat unclear. The rebuttal states that the C41 region

was represented by the C41 carbon, whereas the revised Methods refer more broadly to a C41 carboxylate/carboxamide interaction class. In either case, it is not clear that the analysis includes the full modified side chain, particularly the linker atoms and terminal protonated amine(s). This distinction is important because the ethylenediamine group is precisely the chemical modification whose compatibility with cholesterol packing is being claimed.

Please clarify the exact atom selections used for the C41 interaction analysis. I recommend adding analyses that directly involve the complete modified group, for example: contact frequencies between cholesterol and all atoms of the C41 carboxamide/ethylenediamine moiety, distance distributions or RDFs involving the terminal amine nitrogen(s) and cholesterol hydroxyl atoms, hydrogen-bond occupancy and geometry, and, where relevant, water-mediated interactions or solvent exposure of the new charged group. These analyses should be possible using the existing trajectories.

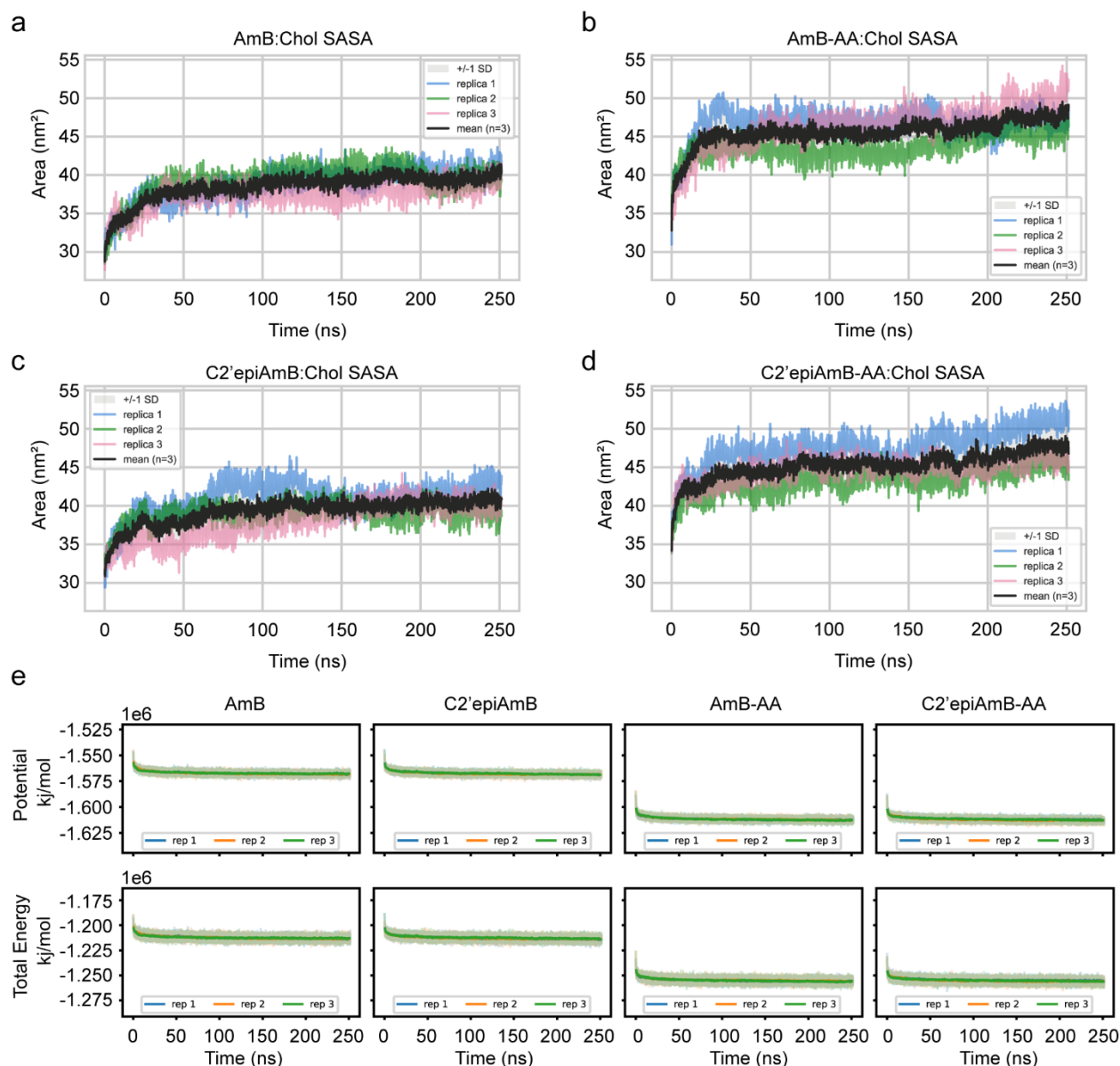
Response: The exact atom used for the original C41 contact interaction analysis with cholesterol was the C41 carbon. We agree with your suggestion that analysis which directly involves the complete modified group rather than just the C41 carbon would strengthen this analysis, and we have performed the additional studies you suggested. Specifically, we have run a new contact frequencies analysis to replace the old analysis (**Extended Figure 6d**), an additional Radial Distribution Functions (RDFs) analysis (**Extended Figure 6c**), and Hydrogen-bond occupancy and geometry analysis (**Extended Figure 6e**) that focused on the entire C41 region and, where possible, focused on the charged amine for the carboxamide/ethylenediamine moieties. The results are summarized in the updated **Extended Figure 6 (p. 30)**, along with its new figure legend, and reflected in the manuscript text shown below:



"Extended Figure 6. AmB derivatives compatible with the local cholesterol environment of the experimentally derived sponge scaffold shows consistency with sterol-sponge mechanism. (a) Chemical structures of amphotericin B (AmB), AmB-AA, C2'epiAmB, C2'epiAmB-AA and cholesterol. The C2'-hydroxyl of the mycosamine sugar is highlighted in red, while the C41 carboxylate/carboxamide site is highlighted in yellow. **(b)** Radial distribution function, $g(r)$, between the C2'-hydroxyl oxygen of each AmB derivative and the 3-hydroxyl oxygen (O3) of cholesterol (Chol). C2'epiAmB derivatives demonstrate a loss of close-range contacts (characteristic of hydrogen bonding) between C2'-hydroxyl on AmB mycosamines and the 3-hydroxyl oxygen on cholesterol. Shaded regions denote the standard deviation across replicate simulations. **(c)** Radial distribution function, $g(r)$, between the AmB C41 region (C41 or

N1) and cholesterol 3-hydroxyl oxygen (O3). The reference atom is C41 for all derivatives, and the terminal N1 amine of the ethylenediamine side chain for AmB-AA and C2'epiAmB-AA. Both ethylenediamine-modified derivatives display close-range N1-O3 contacts. Shaded regions denote the standard deviation across replicate simulations. **(d)** Per-atom contact frequency between cholesterol (O3, C1–C27) and the mycosamine region (top; C2'-hydroxyl and C3'-amine) or C41 region (bottom; C41 carbon and all heavy atoms extending to terminal N1 amine) of each AmB derivative. Error bars denote the standard deviation across replicate simulations. **(e)** AmB hydrogen-bond occupancy with cholesterol, defined as the fraction of frames in which a given AmB forms at least one hydrogen bond with a cholesterol. Statistical significance was assessed using the Welch's t-test (ns, $P > 0.05$) between AmB derivatives. **(f)** Occupied volume analysis captures the degree of conformational freedom experienced by C41 modifications and the mycosamine of each AmB derivative within the sponge structures. Occupancy (%) denotes the fraction of simulation time during which the same voxels remain occupied. A value of 100 corresponds to macrolide atoms that are restrained in the MD simulations. Standard deviation bars show replicate level uncertainty. AmB and C2'epiAmB are directly comparable with the same number of atoms while AmB-AA and C2'epiAmB-AA are directly comparable. Statistical significance was assessed using the Welch's t-test (ns, $P > 0.05$) between AmB derivatives. **(g)** Void volume quantifies the change in volume available to accommodate a sterol molecule. Occupancy (%) denotes the fraction of simulation time during which a voxel remains occupied (occupied volume) or unoccupied (void volume). No statistical significance was found in void volumes between AmB derivatives. Statistical significance was assessed using the Welch's t-test (ns, $P > 0.05$) between AmB derivatives. **(h)** Root mean square fluctuation (RMSF) values for each cholesterol carbon atom (C1–C25) indicate comparable conformational freedom of cholesterol across all four AmB derivatives. Error bars denote the standard deviation across replicate simulations."

Also following your suggestion, we additionally computed changes to solvent accessible area of the sponge upon the ethylenediamine modification of the C41 region, showing that the AmB-AA sponges experience a 17% increase in surface area compared to AmB sponges while retaining the same interaction abilities as AmB. This increase can be attributed to the addition of the ethylenediamine, which increases the solvent accessible surface area of the entire AmB monomer. The results can be seen below as part of **Extended Figure 7a to Extended Figure 7d (p. 32)**.



“Extended Figure 7. Solvent accessible surface area calculations. (a-d) Computed SASA values for the internal core of each AmB:Chol derivative sponge, containing 27 AmD molecules, shows solvent equilibration with C41 and mycosamine regions occur in the first 20% of the simulation. There is a ~17% increase in solvent accessible surface area of AmB-AA and C2'epiAmB-AA sponges at equilibrium. This can be attributed to the ethylenediamine modification at the C41 carbon. **(e)** Internal and potential energies are converged before 20% of simulation time.”

These analyses are also included in the main text, which now reads (p. 6, line 217):

“Solvent accessible surface area was calculated to compare changes in solvent exposure as a direct result of the ethylenediamine modification (**Extended Fig. 7a-d**). Additionally, void volume changes across all AmB derivatives sponges were computed as a descriptive

supplementary metric (**Extended Fig. 6d**). These analyses show that the C41 ethylenediamine modification appears compatible with the local cholesterol environment of the experimentally derived and restrained sponge scaffold. This is consistent with the proposed sterol-sponge mechanism, but it does not independently demonstrate sponge stability or cholesterol extraction.”

Additionally, the methods section has been updated to now include all the new analysis. It now reads (p. 19, line 737):

“Contact Frequencies: Contact frequencies between AmB interaction regions and individual cholesterol atoms were computed using MDAnalysis^{95,96}. The mycosamine and C41 regions of AmB were selected as the interaction classes. The mycosamine region includes the C2'-hydroxyl and C3'-amine and the C41 region includes the C41 carbon and all heavy atoms extending to terminal N1 amine. For each frame, pairwise distances between all AmB atoms in a given interaction class and all cholesterol atoms were computed using `capped_distance` with periodic boundary corrections. A binary contact was registered for a given interaction class-Chol atom pair if any interatomic distance fell within a 4.0 Å cutoff. The contact frequency was then calculated as the fraction of analyzed frames in which at least one such contact was observed. Analysis was performed over the final 80% of each trajectory to ensure equilibrated sampling. The reported statistics are based on total of 600 ns MD data over 3 trajectory replicas of 27 AmBs and 54 cholesterol each.

Radial Distribution Functions: Radial distribution functions (RDFs) between AmB hydrogen-bond donor atoms and cholesterol heavy atoms were computed using the InterRDF module of MDAnalysis^{95,96}. For each AmB derivative (where appropriate), donor atoms comprising the mycosamine C2'-hydroxyl, C41 carbon, and the terminal N1 amine were selected as the reference group, while cholesterol O3 atoms were used as target groups to probe contacts at the sterol 3-hydroxyl. Pairwise distances were binned over 0–10 Å using 100 bins with periodic boundary corrections, sampling every 100ps across the last 80% of each trajectory (frames 20–100%) to exclude initial equilibration. RDFs were computed independently for each replicate and subsequently averaged, with the standard deviation across replicates reported as the spread. The reported statistics are based on total of 600 ns MD data 3 trajectory replicas of 27 AmBs and 54 cholesterol each.

Hydrogen Bond Occupancy Analysis: Hydrogen bonds between the central 27 AmB derivatives (3×3×3 core) and cholesterol were identified using the `HydrogenBondAnalysis` module in MDAnalysis^{95,96}, applying default geometric criteria of a donor-acceptor distance (≤ 3.5 Å) and a donor-acceptor angle ($\geq 150^\circ$). Donor and acceptor atom sets were specified per derivative comprising the mycosamine amine, the C2'-hydroxyl, the terminal N1 amines (AmB-AA and C2'epiAmB-AA), and the 3-hydroxyl oxygen on cholesterol. Analysis was performed on the final 50 ns of each trajectory per replicate, and per-AmB occupancy was defined as the fraction of analyzed frames in

which a given AmB formed at least one hydrogen bond to any cholesterol. Statistical significance was defined using Welch's t-test.

Solvent Accessible Surface Area (SASA) Analysis: SASA were computed using the `gmx sasa` tool in GROMACS, which implements the double-cubic lattice method. The sponge phase, comprising all AmB and cholesterol molecules, was defined as the surface group so that neighboring AmB and cholesterol molecules occlude one another, and the reported area was restricted to the central 27 AmB derivatives (3×3×3 core) as an output group. This yields the exposed surface of the core molecules within the intact sponge rather than isolated molecules. A solvent probe radius of 1.4 Å was applied. Prior to analysis, each trajectory was subsampled to one frame per 100 ps. SASA was computed independently for each replicate and subsequently averaged, with the standard deviation across replicates reported as the spread. The reported statistics are based on a total of 750 ns MD data over all three replicates of 27 AmBs and 54 cholesterol each."

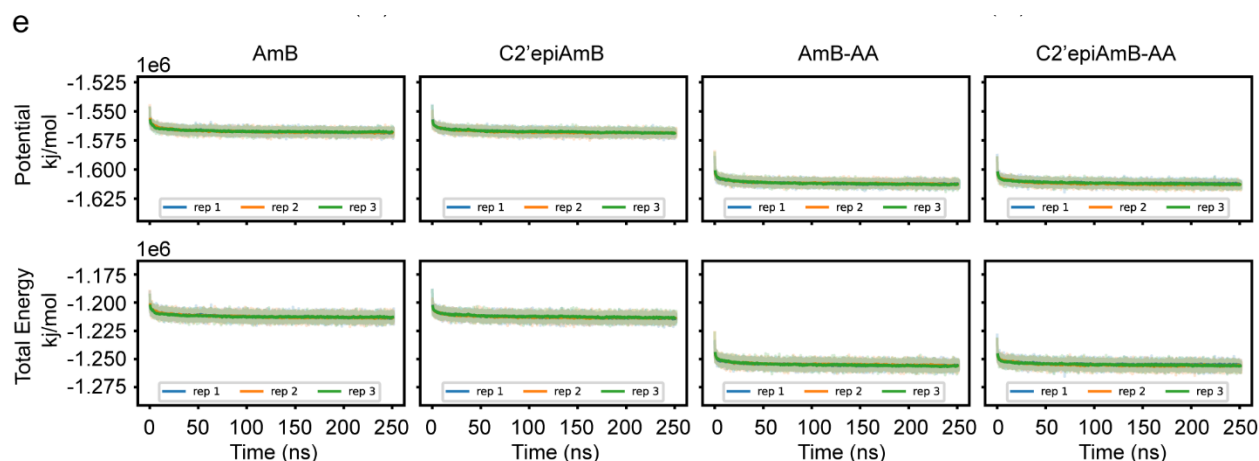
The description of these analyses was clarified in the text (p. 6, line 212):

"Computed AmB-Chol hydrogen bonding occupancies, radial distribution functions (RDF) between cholesterol O3 and either the C2'OH, C41, or N1 of AmB derivatives, contact frequencies between all cholesterol atoms and the entire C41 region of AmB derivatives, and RMSF values of cholesterol heavy atoms collectively indicate similar dynamics across all AmB-cholesterol and derivative sponges (**Fig. 3c and Extended Fig. 6**)."

3. Convergence has not been demonstrated

The MD checklist claims that the RMSF values calculated over the final 80% of each trajectory "represent equilibrium dynamics." The revised Methods similarly state that the final 80% was used for RMSF, contact-frequency, and RDF analyses, with the initial 20% excluded as equilibration. However, equilibration or convergence was not demonstrated – why do 20% was excluded? The fact that "presented RMSF values were calculated for the last 80% (200 ns) of the trajectory and represent equilibrium dynamics" (checklist document) does not mean that the simulations converged.

Response: We have now included as part of **Extended Figure 7e** the potential and total energy diagrams showing the convergence of the energy criteria for all 3 copies of trajectories across all systems. This shows a stable value maintained throughout the remainder of the simulation starting at the 50 ns mark.



The following text was added (Methods section, p. 18, line 708):

“All analyses were performed on the central 3x3x3 lattice of individual AmBs (total 27) and their correspondingly resolved 2 cholesterol (total 54) from the sponges. This was done to ensure that no bulk solvent interactions are introduced as the sponge internal dynamics was characterized. Convergence of the simulations was checked by computing system energy and SASA using gmxdump energy and gmxdump sasa, respectively (Extended Fig. 7).”

4. The checklist statement regarding independence from initial configuration should be corrected

The use of different random initial velocities is an acceptable and standard approach for generating dynamical replicas from one starting structure. However, it does not establish independence from the initial spatial configuration, because all systems begin from the same templated NMR-derived geometry. The checklist should therefore not state that independence from initial configuration has been demonstrated. It would be more accurate to state that three trajectories were initiated from the same structural model using independently randomized velocity distributions. This is a reporting issue, no additional starting conformations are required.

Response: The MD simulation checklist has been updated in 1D to state the following:

“No, three trajectories were initiated from the same NMR derived structural model using independently randomized velocity distributions.”

5. The occupied/void-volume analysis remains insufficiently defined and should be de-emphasized

The authors have appropriately added RDF and contact analyses as more direct

descriptors of local AmB-cholesterol interactions. These should remain the principal analyses. By contrast, the occupied/void-volume analysis remains difficult to interpret. The Methods describe an occupancy map at “0.5 Å³ resolution,” which is not clear: does this refer to grid spacing, voxel volume, or another parameter? The coordinate alignment procedure, molecular selections, cavity definition, occupancy criterion, and averaging scheme should be stated explicitly.

Moreover, because the macrolactone scaffold remains strongly restrained, similar void volumes are not independent evidence that the sponge is structurally stable. The analysis should therefore be presented only as a descriptive supplementary metric, with replicate-level uncertainty shown.

Finally, the figure/text should consistently distinguish between fractional occupancy values from 0 to 1 and occupancy percentages from 0 to 100%.

Response: The text has been accordingly modified to de-emphasize the importance of the void volume calculations as shown in the following (p. 6, line 209):

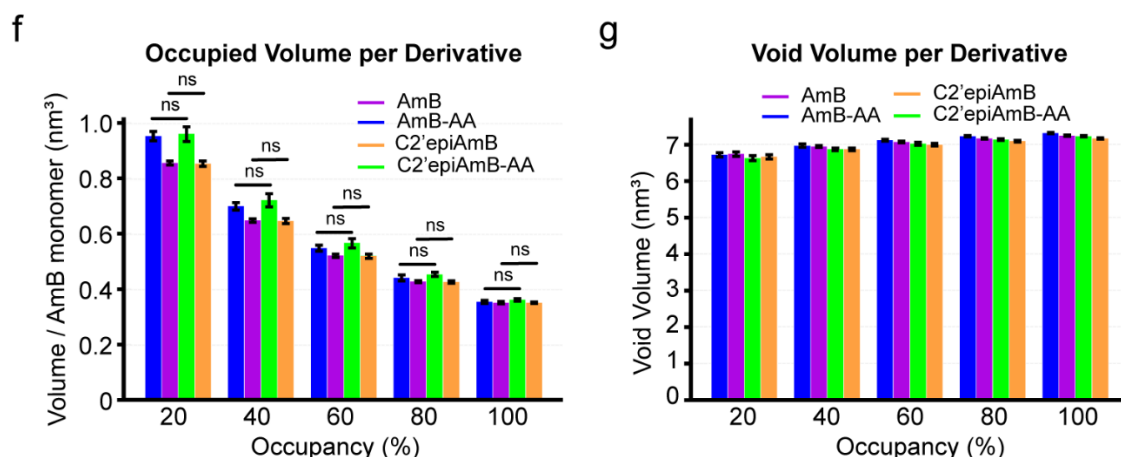
“Molecular dynamics of the bis-positively charged AmB-AA or C2’epiAmB-AA sponge complexed with cholesterol was based on a prior NMR-resolved XPLOr-NIH structure²⁵ (**Fig. 3c and Extended Fig. 6a**). These structures were used to compare the interactions of the bis-positively charged AmB derivatives with cholesterol. Computed AmB-Chol hydrogen bonding occupancies, radial distribution functions (RDF) between cholesterol O3 and either the C2’OH, C41, or N1 of AmB derivatives, contact frequencies between all cholesterol atoms and the entire C41 region of AmB derivatives, and RMSF values of cholesterol heavy atoms collectively indicate similar dynamics across all AmB-cholesterol and derivative sponges (**Fig. 3c and Extended Fig. 6**). Solvent accessible surface area was calculated to compare changes in solvent exposure as a direct result of the ethylenediamine modification (**Extended Fig. 7a-d**). Additionally, void volume changes across all AmB derivatives sponges were computed as a descriptive supplementary metric (**Extended Fig. 6d**). These analyses show that the C41 ethylenediamine modification appears compatible with the local cholesterol environment of the experimentally derived and restrained sponge scaffold. This is consistent with the proposed sterol-sponge mechanism, but it does not independently demonstrate sponge stability or cholesterol extraction.”

The “0.5 Å resolution” refers to the length of each side of the 3-dimensional voxel within which occupancy is determined. This was updated to “0.125 Å³” to clearly reference the voxel volume. The void volume calculation method section has been modified for improved clarity on the molecular selections, cavity definition, occupancy criterion, and averaging scheme. No coordinate alignment procedure was necessary since macrolactone was restrained. The modified methods section now reads (p. 19, line 716):

“Volume calculations: Occupied and void volumes were calculated using the volmap plugin of VMD [<https://www.ks.uiuc.edu/Research/vmd/plugins/volmapgui/>]. For each

frame, atoms of all AmB monomers were mapped onto a Cartesian grid spanning the simulation box with a uniform grid spacing, where an individual voxel volume is $0.125 \text{ \AA}^3$. Occupied volume is defined by whether an AmB monomer occupied a voxel in a given frame. Conversely, unoccupied (void) is defined by a voxel that does not contain an AmB monomer. Occupied volumes were averaged over the last 80% of trajectory length then divided by the number of AmB monomers (125) to provide an average occupied volume per monomer. Averaged over all frames, if the fractional occupancy is 0, there are no AmB monomers occupying the voxel volume, and if occupancy is 1 then all AmB monomers occupy the voxel volume.”

Lastly, the figure and text has been updated to consistently show fractional occupancy percentages from 0 to 100% and with error bars as shown in Extended Figure 6f and Extended Figure 6g below.



6. Minor but important - should be corrected or clarified:

1. The protonation states and net charges of AmB, AmB-AA, C2'epiAmB, and C2'epiAmB-AA should be explicitly stated. This is particularly important because the biological and mechanistic rationale relies on altered charge at C41.

The text has been modified to explicitly state the bis-positively charged nature of AmB-AA and C2'epiAmB-AA as well as refer to the structures drawn in Extended Fig. 6a as shown below (p. 6, line 209):

“Molecular dynamics of the bis-positively charged AmB-AA or C2'epiAmB-AA sponge complexed with cholesterol was based on a prior NMR-resolved XPLOR-NIH structure²⁵ (Fig. 3c and Extended Fig. 6). These structures were used to compare the interactions of the bis-positively charged AmB derivatives with cholesterol.”

2. The reference (88) cited for the C-rescale barostat appears not to correspond to the stochastic cell-rescaling method. Please cite the appropriate original method or GROMACS implementation reference.

The reference has been updated to the following.

Bernetti, M. and Bussi G., “Pressure control using stochastic cell rescaling”, *J. Chem. Phys.*, 153, 114107 (2020)

3. The cited CHARMM36 reference (84) appears to be a protein-force-field paper. Please cite the appropriate CHARMM lipid/cholesterol parametrization source and specify the topology version used.

The methods section has been updated to reflect the appropriate CHARMM lipid/cholesterol parametrization source and specify the topology version used (p. 17, line 668):

“AmB parameters were obtained from CHARMM General Force Field⁸³ via their web server [<https://cgenff.com/>]. Cholesterol and other system parameters were obtained from the CHARMM36 force field^{84,85}. The toppar_c36_jul22/stream/lipid/toppar_all36_lipid_cholesterol.str file, included in the July 2022 version of the CHARMM36 forcefield, was the source for cholesterol topology and parameters.”

The reference was updated to include the following:

Lim, J. B., Rogaski, B. & Klauda, J. B. Update of the cholesterol force field parameters in CHARMM. *J. Phys. Chem. B* **116**, 203–210 (2012).

4. Please remove very long hyphens (—), humans do not use it.

Response: The em dashes were removed.

Reviewer #5 (Remarks to the Author)

Thank you for the careful revision and for substantially improving the manuscript. There remain a few relatively small technical issues that should be corrected before acceptance.

1. RDF analysis terminology: There is a technical wording error in the RDF Methods. The authors describe the RDFs as being calculated between “AmB hydrogen-bond donor atoms” and cholesterol, but then include the C41 carbon among these atoms. The C41 carbon is not a hydrogen-bond donor.

Response: We have clarified the wording error for this methods section. It now reads:

“Radial distribution functions (RDFs) between AmB atoms and cholesterol heavy atoms were computed using the InterRDF module of MDAnalysis^{96,97}. For each AmB derivative (where appropriate), the mycosamine C2 hydroxyl, C41 carbon, and terminal N1 amine were selected as the reference group, while cholesterol O3 atoms were used as the target group, yielding the distance-resolved density of the sterol C3-hydroxyl oxygen around each reference atom relative to a uniform bulk distribution. The position and magnitude of the first peak in each RDF thus report the characteristic separation and relative enrichment of the sterol hydroxyl in the immediate coordination shell of the corresponding reference atom.”

2. Number of molecules used for the volume analysis. The manuscript should state unambiguously whether the occupied/void-volume analysis is performed on the central 27 AmB molecules or on all 125 molecules in the $5 \times 5 \times 5$ assembly.

Response: We have clarified the unambiguous sections of the methods section for clarity. It now reads:

The simulations protocol section specifies that “Volume calculations were performed on the entire $5 \times 5 \times 5$ lattice of individual AmBs. All remaining analyses were performed on the central $3 \times 3 \times 3$ lattice of individual AmBs (total 27) and their correspondingly resolved 2 cholesterol (total 54) from the sponges.”

3. Incorrect Extended Figure 6 panel reference. The Results state that void-volume changes are shown in Extended Fig. 6d. However, panel d contains the contact-frequency analysis, whereas the void-volume analysis is shown in panel g.

Response: This figure reference has been updated in the results section to say Extended Figure 6g as shown below:

“Additionally, void volume changes across all AmB derivatives sponges were computed as a descriptive supplementary metric (**Extended Fig. 6g**).”

4. Water count in Table 1. Table 1 labels values such as 99,786 as “Water molecules count,” while the total atom count for the corresponding system is 135,776. The first number is likely “Water atoms”.

Response: Table 1 has been updated to say, “Water atoms count” rather than “Water molecules count”.

5. Overstatement in Figure 3. The title remains “Toxicity of AmB-AA is mechanistically similar to AmB,” and panel c is described as a representative snapshot from an “equilibrium all-atom MD simulation of the AmB-AA sterol sponge.” Given that the macrolactone scaffold is strongly restrained throughout the simulations, this wording remains stronger than what the simulations themselves establish. I suggest using more restrained wording, for example describing panel c as a representative snapshot from a restrained all-atom MD production trajectory based on the experimentally derived sponge scaffold. Likewise, the Figure 3 title could be softened to indicate that the observations are “consistent with” a mechanistically similar cholesterol-dependent toxicity mechanism rather than demonstrating mechanistic equivalence.

Response: The Figure 3c panel is now described in the figure legend as a: “Representative snapshot from a restrained all-atom MD production trajectory based on the experimentally derived sponge scaffold.”

Also, the title of Figure 3 has now been changed to: “Toxicity of AmB-AA is consistent with the cholesterol-dependent toxicity mechanism of AmB.”

MD checklist

1. Item 1b: equilibration and production periods. The response should explicitly state the complete simulation and analysis scheme rather than mentioning only the 1 ns NPT equilibration. For clarity, it should indicate that each system underwent 1 ns NPT equilibration followed by 250 ns of production MD per replicate, with the final 200 ns used for RMSF, contact-frequency, and RDF analyses, and the final 50 ns used for the hydrogen-bond occupancy analysis. The reason for using a different analysis window for the hydrogen-bond calculation should also be stated.

Response: The response to item 1b of the MD simulation checklist now states:

“Yes, prior production, simulations underwent energy minimization followed by 1 ns of NPT equilibration followed by 250 ns of production MD per replicate, with the final 200 ns used for RMSF, contact-frequency, and RDF analyses, and the final 50 ns used for the hydrogen-bond occupancy analysis. Based on previous studies [<https://doi.org/10.1038/s41598-025-19843-5>] and the known timescales of hydrogen bond occupancy [<https://doi.org/10.1021/acs.langmuir.6c02051>], we performed hydrogen bond occupancy analysis on the final 50 ns of trajectory data.”

2. Item 2a: connection to experiment. Rather than simply answering “Yes,” the checklist should briefly state how the simulations are connected to experiment. For example, the simulations begin from an experimentally derived NMR sponge scaffold, and the calculated interaction behavior is interpreted in relation to cholesterol-binding and toxicity measurements.

Response: The response to item 2a of the MD simulation checklist now states:

“Yes. The simulations begin from an experimentally derived NMR sponge scaffold, and the calculated interaction behavior is interpreted in relation to cholesterol-binding and toxicity measurements.”

3. Item 3b: suitability and limitations of the model. The checklist should explicitly point to the Methods passage explaining what the restrained model can and cannot address. In particular, it should note that the simulations probe local relaxation and accommodation of the C41 modification and cholesterol within the experimentally derived scaffold, rather than de novo sponge formation, unrestrained thermodynamic stability, or cholesterol extraction from a membrane.

Response: The response to item 3b of the MD simulation checklist now states:

“Yes, Methods section. The simulations probe local relaxation and accommodation of the C41 modification and cholesterol within the experimentally derived scaffold, rather than de novo sponge formation, unrestrained thermodynamic stability, or cholesterol extraction from a membrane.”

4. Item 4a: system setup table. This item should not simply state “Yes, Methods section” in its current form. The table itself does not include the 11-nm simulation-box dimension, and the water-molecule counts appear to be mislabeled, as noted above.

Response: Table 1 in the methods section is now correctly labeled as shown below.

Table 1: System Composition and Simulation Details

Sponge system	Atom count	AmB molecule count	Chol molecule count	Water atoms count	NaCl conc. (mM)	Simulation length (ns)	Simulation dimensions (nm)
AmB:Chol	135,776	125	250	99,786	150	3x250	11x11x11
C2'epiAmB:Chol	135,866	125	250	99,876	150	3x250	11x11x11
AmB-AA:Chol	136,440	125	250	99,075	150	3x250	11x11x11
C2'epiAmB-AA:Chol	149,432	125	250	112,095	150	3x250	11x11x11

5. Item 4b: software versions. Actual versions of VMD and MDAnalysis should also be provided.

Response: The methods section has been modified to state:

“Visualization and analyses of the trajectories were carried out using VMD⁹⁵ 1.9.4 and the Python package MDAnalysis^{96,97} 2.8.0.”

6. Item 4d: deposited input and coordinate files. The checklist should state that these materials are deposited on Zenodo, rather than simply saying “supplementary files.” The manuscript specifies that starting and final coordinates, topology files, parameter files, CGenFF outputs, and MDP files for minimization, equilibration, and production are available there.

Response: The response to item 4d of the MD simulation checklist now states:

“Yes. These materials are deposited on Zenodo and available in the supplementary files. The manuscript specifies that starting and final coordinates, topology files, parameter files, CGenFF outputs, and MDP files for minimization, equilibration, and production are available there.”

7. Item 4e: custom parameters. The response should more precisely state that compound-specific “CGenFF parameters were generated for the AmB derivatives and deposited on Zenodo”, rather than referring generally to the compounds as not being parameterized in the standard CHARMM force field.

Response: The response to item 4e of the MD simulation checklist now states:

“CGENFF parameters were generated for the AmB derivatives and deposited on Zenodo.”

Overall, the manuscript has been substantially improved, and these remaining points

are primarily technical and reporting issues. Addressing them should improve the internal consistency, reproducibility, and precision of the MD section and checklist.