Peer Review File

Manuscript Title: A synthetic antibiotic class overcoming bacterial multidrug resistance

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referee #1 (Remarks to the Author):

The prevalence of multidrug resistant bacteria is of increasing concern and discovery of novel compounds to extend our current arsenal is of utmost importance. Here Mitcheltree and coworkers undertake a synthetic approach to identify novel lincosamide-related compounds, such as Iboxamycin, which display excellent activity against ESKAPE pathogens including strains harboring Erm and Cfr rRNA methyltransferase resistance determinants. In vivo studies indicate that iboxamycin is effective in a mouse model in treating both Gram-positive and -negative infections. The authors also complement these findings with stunning mechanism-of-action data, highlighting the surprising and unprecedented mechanism by which iboxamycin overcomes Erm resistance by displacing the methylated A2058 nucleotide within the drug binding site.
Collectively, this is a tour-de-force for antibiotic discovery. The manuscript is clearly written and beautifully presented. In addition to discovery of a compound that could be very important medically, the data also reveal as yet unexplored ways in which compounds can be designed to overcome certain resistance determinants in the future.

Since my expertise is in biochemistry and structural biology, I cannot assess the technical aspects of the synthesis or in vivo studies, but can state categorically that the structures are of excellent quality and the results are appropriately presented and interpreted.

There are only two points that could be addressed that I believe would improve the manuscript.
1. The authors nicely illustrate how Iboxamycin overcomes Erm methylation of A2058 by inducing a 2A shift in the methylated nucleotide, however, the the Cfr methylation is somehow not so explicitly addressed. Maybe I missed this somewhere, but is a similar mechanism being utilized here too? Given the importance, the MoA should also be made clearer in the manuscript and illustrated graphically.
2. It is surprising to me that the authors completely ignore the third resistance mechanism that acts against lincosamides i.e. target protection. From the literature, it appears that this mechanism is gaining interest and traction within the reservoir of resistant microbes, and now even being identified in clinical isolates. It would seem therefore for completeness, to test the activity of IBX against strains also bearing or expressing such ABCF proteins that are readily available. It would not surprise me if IBX is also very effective against this mechanism given that it appears to work by displacement of the drug. I provide just a few recent papers that include most of the relevant references to direct the authors to the relevant findings and organisms for their analysis: PMID: 34117249; PMID: 32587401; PMID: 29415157; PMID: 30597160

Minor point:
The authors state that the compound has no effect on mitochondrial function. Presumably since it is not taken up into the organelle? Presumably IBX nevertheless be expected to bind also to mitochondrial ribosomes, but not to human cytoplasmic ribosomes? Maybe this could be commented on in the manuscript?

Referee #2 (Remarks to the Author):

Summary

This paper reports the component-based synthesis of iboxamycin, where a rigid oxepanoproline scaffold was linked to the aminooctose residue of the antibiotic, clindamycin. The authors demonstrate that iboxamycin is a potent antibiotic displaying activity against critical Gram-positive and Gram-negative pathogens, as well as efficacy in murine models of bacterial infection. Notably, the authors show the ability of iboxamycin to overcome MLSB resistance. The authors confirm that iboxamycin targets the bacterial ribosome and has a low potential to select for resistance. Via toeprinting experiments, the authors demonstrate that iboxamycin associates more strongly with the ribosome than clindamycin. Finally, X-ray crystallographic studies found iboxamycin bound in the canonical binding pocket within the large ribosomal subunit. They also show using Erm-modified 70S ribosomes with the classic A2058 methylation that iboxamycin also bound methylated ribosome in a very similar manner to wildtype but with enhanced target engagement due to new hydrophobic interactions, thus providing sufficient high affinity for iboxamycin to overcome resistance. In all, the study shows the potential of component-based chemical synthesis to identify powerful antibiotics.

Major Comments

This is a well-written and well-organized manuscript. Description of the evolution of OPP-1 to OPP-3 is well done. Overall, OPP-3 demonstrates compelling antibiotic attributes, both in vitro and in vivo. Mechanistic investigations were thorough. In all, the manuscript features a novel antibiotic with a distinctive mode of action that speaks to the evolving role of chemical synthesis in antibacterial drug discovery.

Minor Comments

1. This reviewer found, Figure 2 and Supplementary Table 1 confusing. It is unclear what is in one and not the other? I suggest changing it to a more succinct (and more aesthetically pleasing) demonstration of susceptibility for the main Figure 2 with all details placed in Supplementary.

2. Figure 2 a,b does not include MLSB (i-erm) resistance. Does ibroxymycin overcome the latter? This would have to be shown after bacterial cells have been pre-incubated subtherapeutic levels of inducer to allow proper expression of the erm gene.

3. There seems to be a correlation for poor activity of ibroxamyin with resistance to linezolid, that is not mentioned in the study. Supplementary Table 1, for example, shows S. aureus strains HAV#219 and 220 as resistant to ibroxamyin, and also to linezolid. The same goes for all strains of S. epidermis which are resistant to ibroxamycin (except #279), and similarly resistant to linezolid. Can you comment on these strains? Where linezolid resistance is rather infrequent, target site mutations do arise. Is there a correlation here for resistance to ibroxamycin due to possible mutations in the PTC? Are these strains sequenced that can help further inform mechanisms of cross-resistance?

4. Pseudomonas aeruginosa remains uniquely resistant to iboxamycin; the authors should comment on this and provide a possible reason, given its critical status as a priority pathogen. It is equally worth testing more than one strain. Nonetheless, I did notice the test of HAV#039 (porin/efflux) which is interesting. Again, this is worth noting in the main text, given P. aeruginosa’s importance.

5. The authors mention that traditional physicochemical predictors of Gram-negative activity such as rotatable-bond count, molecular weight, relative polar surface area, or lipophilicity do not explain the enhanced activity against Gram-negative bacteria. The authors do not consider the more up-to-date citations by Hergenrother’s group (PMIDs: 28489819, 33228356) that positively charged, nitrogen-containing functional groups improve accumulation into Gram-negative bacteria.

Indeed, it is possible that enhanced activity could be due to the cationic aminoacyl residue substituent of iboxamycin.

Referee #3 (Remarks to the Author):

The recent manuscript by Myers et al. describes their recent discovery of iboxamycin, a totally synthetic analog of lincomycin, an FDA approved drug, and describes its mechanism of action and efficacy in vivo. Overall, this work is a synthetic tour de force and it is somewhat sad that the entire synthetic sequence is reduced to one part of one figure. Fortunately, the most up to date synthesis of the molecule was recently published in JACS allowing for the synthetic chemists to learn more about this route. I want to point out that even though an updated and streamlined synthesis of iboxamycin was reported that this does NOT detract from the impact of this work. The synthesis shown here differs from the previous method and also has some highlights of novelty including the first aldol reach to produce stereo-enriched proline rings. However, chemistry aside, what really differentiates this paper from others and raises it to the level of Nature is the identification of the mechanism of action and the activity of the molecule. These two aspects are incredibly significant and I cannot stress enough how impactful this work is. I fully expect these lead compounds to be entered into clinical trials as the preliminary data is very impressive. Additionally, having a molecule that works against a common mode of antibiotic resistance is incredibly important for the clinician's arsenal. The crystallographic work validates the target engagement, mouse studies (as requested by previous reviews) demonstrates clinical relevance, and resistance selection assays provide the critical data needed to progress these compounds. Overall, this work clearly demonstrates that the molecule that their group designed and constructed, and one impossible to produce if not for synthetic organic chemistry, is primed for potential commercialization. In the opinion of this reviewer the work is at the bar of importance, impact, and significance compared to other published papers in the journal. The authors should be commended for their fantastic work.

-Bill Wuest

Author Rebuttals to Initial Comments:

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Response to Reviewer #1

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Remarks to the Authors:

The prevalence of multidrug resistant bacteria is of increasing concern and discovery of novel compounds to extend our current arsenal is of utmost importance. Here Mitcheltree and coworkers undertake a synthetic approach to identify novel lincosamide-related compounds, such as Iboxamycin, which display excellent activity against ESKAPE pathogens including strains harboring Erm and Cfr rRNA methyltransferase resistance determinants. In vivo studies indicate that iboxamycin is effective in a mouse model in treating both Gram-positive and -negative infections. The authors also complement these findings with stunning mechanism-of-action data, highlighting the surprising and unprecedented mechanism by which iboxamycin overcomes Erm resistance by displacing the methylated A2058 nucleotide within the drug binding site.

Collectively, this is a tour-de-force for antibiotic discovery. The manuscript is clearly written and beautifully presented. In addition to discovery of a compound that could be very important medically, the data also reveal as yet unexplored ways in which compounds can be designed to overcome certain resistance determinants in the future.

Since my expertise is in biochemistry and structural biology, I cannot assess the technical aspects of the synthesis or in vivo studies, but can state categorically that the structures are of excellent quality and the results are appropriately presented and interpreted.

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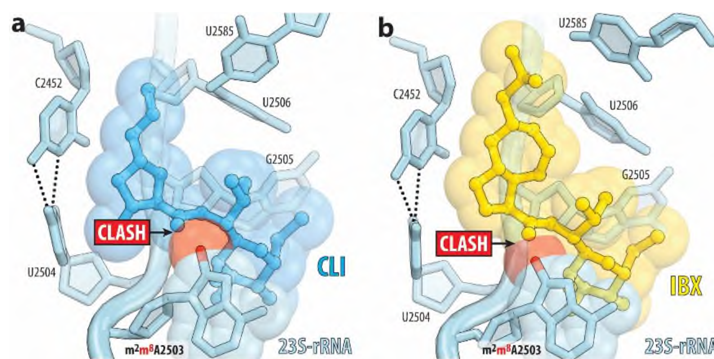
1. *The authors nicely illustrate how Iboxamycin overcomes Erm methylation of A2058 by inducing a 2A shift in the methylated nucleotide, however, the the Cfr methylation is somehow not so explicitly addressed. Maybe I missed this somewhere, but is a similar mechanism being utilized here too? Given the importance, the MoA should also be made clearer in the manuscript and illustrated graphically.*

Response: Indeed, unlike $PhLOPS_A$ antibiotics including lincomycin and clindamycin, iboxamycin is active against bacterial pathogens expressing the *cfr* resistance gene (**Figure 2a,b**). This activity was unexpected, as the Cfr enzyme introduces a methyl group to C8 of the 23S rRNA residue A2503, altering the shape of the PTC pocket and blocking the action of many antibiotics that bind to the ribosomal A site, including clindamycin (**Response Figure 1a**). *In silico* modeling of m^8A2503 in our structure of ribosome-bound iboxamycin shows a clash between the methyl group installed by Cfr and the antibiotic (**Response Figure 1b**). As the reviewer remarks, the sensitivity of *cfr*-positive strains to iboxamycin suggests that when bound to the Cfr-modified ribosome, iboxamycin likely displaces m^8A2503 in a manner analogous to what we observe with m^6_2A2058. In the absence of the corresponding structure, however, we are reluctant to include such a discussion in the body of the manuscript, as the precise mechanism of action against Cfr-modified ribosomes currently remains speculative.



Response Figure 1 | Structural basis for Cfr-mediated resistance to lincosamides. *In silico* molecular modeling of the C8-methylation of 23S rRNA nucleotide A2503 (red sphere) in the existing structures of ribosome-bound clindamycin (**a**, blue, PDB entry 4V7V) and iboxamycin (**b**, yellow) reveals a steric clash with both antibiotics. While this methylation imparts resistance to clindamycin, *in vitro* susceptibility testing against *cfr*-harboring strains shows that iboxamycin overcomes this resistance mechanism.

2. *It is surprising to me that the authors completely ignore the third resistance mechanism that acts against lincosamides i.e. target protection. From the literature, it appears that this mechanism is gaining interest and traction within the reservoir of resistant microbes, and now even being identified in clinical isolates. It would seem therefore for completeness, to test the activity of IBX against strains also bearing or expressing such ABCF proteins*

that are readily available. It would not surprise me if IBX is also very effective against this mechanism given that it appears to work by displacement of the drug. I provide just a few recent papers that include most of the relevant references to direct the authors to the relevant findings and organisms for their analysis: PMID: 34117249; PMID: 32587401; PMID: 29415157; PMID: 30597160

Response: This is an excellent suggestion, and we thank the reviewer for the comment. We have found that iboxamycin is active against a strain of *S. aureus* expressing the MsrA ABCF ATPase, a target-protection protein that confers resistance to macrolide and streptogramin group-B antibiotics (but not to lincosamides),[1] and we have included these data in a revised version of **Figure 2**. More strikingly, we find that iboxamycin overcomes LsaA-mediated lincosamide-, pleuromutilin-, and group-A streptogramin cross-resistance, as evidenced by its activity against the *lsaA*-positive strain of *E. faecium* ATCC 29212. This is notable because the resistance of *E. faecium* to lincosamide antibiotics has been attributed directly to *lsaA*, a gene intrinsic to the species,[2] which in turn illuminates the basis for iboxamycin's breakthrough activity in this enterococcal pathogen. Consequently, we have annotated the revised **Figure 2** to reflect ATCC 29212's *lsaA*-positive genotype and have included corresponding discussion attributing iboxamycin's activity in *E. faecalis* to its overcoming ABCF resistance. Additionally, in the Introduction section of our revised manuscript, we include a discussion of the target-protection resistance mechanism referencing the papers that the reviewer kindly brought back to our attention.

Minor point:

3. *The authors state that the compound has no effect on mitochondrial function. Presumably since it is not taken up into the organelle? Presumably IBX nevertheless be expected to bind also to mitochondrial ribosomes, but not to human cytoplasmic ribosomes? Maybe this could be commented on in the manuscript?*

Response: It is our understanding that iboxamycin, like clindamycin, does not inhibit the function of mammalian ribosomes (both cytosolic and mitochondrial) because these ribosomes both contain rRNA sequences that correspond to the bacterial 23S rRNA mutation A2058G (*E. coli* numbering).[3] This mutation is known to impart MLS_B resistance[4] and was one of the two mutations identified among iboxamycin-resistant mutants selected by growing *E. coli* SQ110DTC on iboxamycin-containing media (**Extended Data Table 4**). Because A2058G confers iboxamycin resistance, we conclude that iboxamycin selectively binds to wild-type bacterial ribosomes due to differences in primary structure when compared to mammalian ribosomes. The revised version of our manuscript contains an additional passage addressing this point.

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[1] L.K.R. Sharkey, T.A. Edwards & A.J. O'Neill. ABC-F Proteins Mediate Antibiotic Resistance through Ribosomal Protection. *mBio* **7**, e01975-15 (2016).
[2] K.V. Singh, G.M. Weinstock & B.E. Murray. An *Enterococcus faecalis* ABC Homologue (Lsa) Is Required for the Resistance of This Species to Clindamycin and Quinupristin-Dalfopristin. *Antimicrob. Agents Chemother.* **46**, 1845–1850 (2002).
[3] E.C. Böttger, B. Springer, T. Prammananan, Y. Kidan & P. Sander. Structural basis for selectivity and toxicity of ribosomal antibiotics. *EMBO Reports* **2**, 318–323 (2001).
[4] B. Vester & S. Douthwaite. Macrolide Resistance Conferred by Base Substitutions in 23S rRNA. *Antimicrob. Agents Chemother.* **45**, 1–12 (2001).

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Response to Reviewer #2

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Remarks to the Authors:

This paper reports the component-based synthesis of iboxamycin, where a rigid oxepanoproline scaffold was linked to the aminooctose residue of the antibiotic, clindamycin. The authors demonstrate that iboxamycin is a potent antibiotic displaying activity against critical Gram-positive and Gram-negative pathogens, as well as efficacy in murine models of bacterial infection. Notably, the authors show the ability of iboxamycin to overcome MLSB resistance. The authors confirm that iboxamycin targets the bacterial ribosome and has a low potential to select for resistance. Via toeprinting experiments, the authors demonstrate that iboxamycin associates more strongly with the ribosome than clindamycin. Finally, X-ray crystallographic studies found iboxamycin bound in the canonical binding pocket within the large ribosomal subunit. They also show using Erm-modified 70S ribosomes with the classic A2058 methylation that iboxamycin also bound methylated ribosome in a very similar manner to wildtype but with enhanced target engagement due to new hydrophobic interactions, thus providing sufficient high affinity for iboxamycin to overcome resistance. In all, the study shows the potential of component-based chemical synthesis to identify powerful antibiotics.

Major comments:

This is a well-written and well-organized manuscript. Description of the evolution of OPP-1 to OPP-3 is well done. Overall, OPP-3 demonstrates compelling antibiotic attributes, both in vitro and in vivo. Mechanistic investigations were thorough. In all, the manuscript features a novel antibiotic with a distinctive mode of action that speaks to the evolving role of chemical synthesis in antibacterial drug discovery.

Response: We thank the reviewer for their complimentary assessment.

Minor comments:

1. *This reviewer found, Figure 2 and Supplementary Table 1 confusing. It is unclear what is in one and not the other? I suggest changing it to a more succinct (and more aesthetically pleasing) demonstration of susceptibility for the main Figure 2 with all details placed in Supplementary.*

Response: We are grateful to the reviewer for this feedback and agree that the correspondence between data presented in **Figure 2** and **Supplementary Table 1** is not self-evident. In order to make clear which strains are highlighted in **Figure 2**, we have annotated **Supplementary Table 1** with an additional column specifying which rows appear in the former and have introduced color-coding in **Supplementary Table 1** matching that of **Figure 2**. In its present form, we believe the layout of **Figure 2** to be as succinct as possible, as the figure is intended to illustrate the considerable breadth of iboxamycin's spectrum of activity, as well as its coverage of a diversity of resistance phenotypes observed in clinical samples.

2. *Figure 2 a,b does not include MLSB (i-erm) resistance. Does ibroxymycin overcome the latter? This would have to be shown after bacterial cells have been pre-incubated subtherapeutic levels of inducer to allow proper expression of the erm gene.*

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Response: In **Supplementary Table 1**, we in fact report the MICs of iboxamycin and clindamycin in a strain of *S. aureus* with inducible Erm resistance (ATCC BAA-977), both with and without sub-MIC (32 μg·mL^{-1}) erythromycin pre-treatment. As would be anticipated based on its activity in c-*erm* strains, iboxamycin is active under both conditions, whereas clindamycin inhibits growth only when *erm* expression has not been induced. Furthermore, because lincosamides in general are not expected to induce expression of i-*erm*,[5] we have elected to highlight iboxamycin's activity against c-*erm*, rather than i-*erm*, in **Figure 2**.

3. *There seems to be a correlation for poor activity of ibroxamyin with resistance to linezolid, that is not mentioned in the study. Supplementary Table 1, for example, shows S. aureus strains HAV#219 and 220 as resistant to ibroxamyin, and also to linezolid. The same goes for all strains of S. epidermis which are resistant to ibroxamycin (except #279), and similarly resistant to linezolid. Can you comment on these strains? Where linezolid resistance is rather infrequent, target site mutations do arise. Is there a correlation here for resistance to ibroxamycin due to possible mutations in the PTC? Are these strains sequenced that can help further inform mechanisms of cross-resistance?*

Response: We thank the reviewer for the astute observation that certain strains of *S. aureus* and *S. epidermidis* show resistance toward iboxamycin that correlates with resistance to linezolid. While we agree that this observation is noteworthy and merits further attention, we believe that a detailed investigation into the specific mechanisms that grant both linezolid and iboxamycin resistance in these strains falls outside the scope of this manuscript. As the reviewer hypothesizes, the observed iboxamycin resistance may well arise from the same mutations at the PTC binding site that grant linezolid resistance. However, it is also known that 23S rRNA mutations distal to the PTC binding site also impart linezolid resistance,[6] and it is not immediately obvious whether such mutations necessarily result in iboxamycin resistance as well (presently, only 23S rRNA mutations A2058G and A2059G are known to impart iboxamycin resistance). The deconvolution of these possibilities, both known and unknown, will likely require a considerable research effort that extends beyond the bounds of work necessary to support the conclusions we defend in our present study.

4. *Pseudomonas aeruginosa remains uniquely resistant to iboxamycin; the authors should comment on this and provide a possible reason, given its critical status as a priority pathogen. It is equally worth testing more than one strain. Nonetheless, I did notice the test of HAV#039 (porin/efflux) which is interesting. Again, this is worth noting in the main text, given P. aeruginosa's importance.*

Response: In response to the referee's suggestion, we include in our revised manuscript a brief discussion of iboxamycin's activity against *Pseudomonas aeruginosa*, which is known to possess "more tools for defying the activity of antimicrobial agents than virtually any other microorganism."[7] These tools include exceedingly restrictive porins that transport solutes up to 100 times slower than those found in *E. coli*, for instance, as well as ten or more genomically encoded multidrug efflux pumps.

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[5] B. Weisblum & V. Demohn. Erythromycin-inducible resistance in Staphylococcus aureus: survey of antibiotic classes involved. *J. Bacteriol.* **98**, 447–452 (1969).
[6] K.S. Long & B.Vester. Resistance to Linezolid Caused by Modifications at Its Binding Site on the Ribosome. *Antimicrob. Agents Chemother.* **56**, 603–612 (2012).
[7] L.B. Rice. Challenges in Identifying New Antimicrobial Agents Effective for Treating Infections with *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. *Clinical Infectious Diseases* **43**, S100–S105 (2006).

While we find that genetic deletion of three major efflux pump systems (Δ*mexR-mexA-mexB-oprM*, Δ*mexX-mexY*, Δ*mexD*; HAV#039) partially restores the activity of iboxamycin, at this time we cannot rule out the possibility that iboxamycin functions as a substrate for other pumps as well, and contend that a more comprehensive account of iboxamycin's weak activity in the species lies beyond the scope of the present work. We are nonetheless encouraged that members of the oxepanoprolinamide class display measurable activity in this uniquely challenging pathogen and expect that further structural modification, coupled to mechanistic studies of *P. aeruginosa* permeation and efflux will afford antibiotics with improved activity against this remaining member of the ESKAPE group.

5. *The authors mention that traditional physicochemical predictors of Gram-negative activity such as rotatable-bond count, molecular weight, relative polar surface area, or lipophilicity do not explain the enhanced activity against Gram-negative bacteria. The authors do not consider the more up-to-date citations by Hergenrother's group (PMIDs: 28489819, 33228356) that positively charged, nitrogen-containing functional groups improve accumulation into Gram-negative bacteria. Indeed, it is possible that enhanced activity could be due to the cationic aminoacyl residue substituent of iboxamycin.*

Response: We are grateful for the reviewer's comment, and have included both a brief discussion of these predictive rules in our revised manuscript, as well as additional information in the **Supplementary Methods** aimed at addressing this point. While iboxamycin does contain a cationic residue, this alone cannot explain its broad-spectrum activity, as clindamycin also bears a basic aminoacyl substituent. Hergenrother and coworkers' findings suggest that secondary amines such as iboxamycin tend to accumulate more in *E. coli* when compared to tertiary amines such as clindamycin.[8] However, we found that *N*-methylation of iboxamycin does not significantly impact the Gram-negative activity of the resulting antibiotic, as N-Me-IBX is roughly equipotent with IBX in both wild-type and permeable (*lptD* mutant) strains of *E. coli* (**Supplementary Methods Figure S1**, reproduced below as **Response Figure 2**). In the course of our research, we introduced primary amines at various positions within the lincosamide scaffold with the aim of improving Gram-negative accumulation while preserving affinity for the ribosome; none afforded an improvement in Gram-negative activity.[9] Hergenrother and coworkers explicitly identify clindamycin's rotatable-bond count and PMI1/MW (the ratio of the first diagonal element of the diagonalized moment of inertia tensor and molecular weight) as molecular descriptors that lie outside the range of Gram-negative accumulators.[8] We note that relative to clindamycin, iboxamycin features no change in rotatable-bond count, and in fact displays a higher PMI1/MW metric associated with poorer compound accumulation. Consequently, we maintain that neither Hergenrother's rules for compound accumulation, nor the other metrics historically advanced for Gram-negative activity engineering are sufficient to explain iboxamycin's broad-spectrum activity. Nonetheless, we acknowledge that continued refinement of the oxepanoprolinamide scaffold with these rules and metrics in mind provides a prudent roadmap by which to achieve yet greater activity in Gram-negative species, including *P. aeruginosa*.

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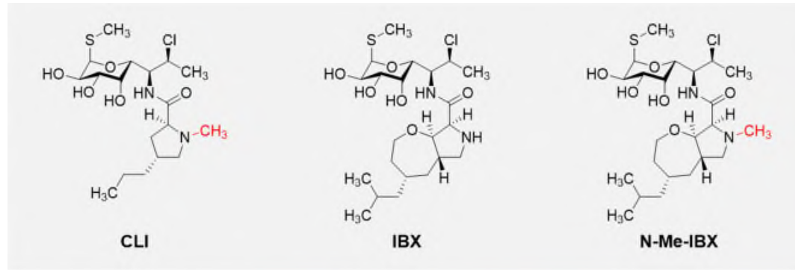
[8] M.F. Richter, B.S. Drown, A.P. Riley, A. Garcia, T. Shirai, R.L. Svec & P.J. Hergenrother. Predictive compound accumulation rules yield a broad-spectrum antibiotic. *Nature* **545**, 299–304 (2017).
[9] M.J. Mitcheltree. A Platform for the Discovery of New Lincosamide Antibiotics. PhD Dissertation, Harvard University (2018). Available online at: http://nrs.harvard.edu/urn-3:HUL.InstRepos:40050042



	Species	Strain	Description	CLI	IBX	N-Me-IBX
Gram +	*S. aureus*	HAV001	ATCC 29213	0.125	0.06	0.125
Gram +	*S. aureus*	HAV031	c-*ermA*	>128	4	4
Gram +	*S. pneumoniae*	HAV003	ATCC 49619	0.06	0.015	0.06
Gram +	*S. pyogenes*	HAV004	ATCC 19615	0.06	0.03	0.06
Gram +	*E. faecalis*	HAV005	ATCC 29212	16	0.06	0.25
Gram +	*E. faecium*	HAV251	Clinical	>128	1	2
Gram –	*E. coli*	HAV006	ATCC 25922	>128	8	16
Gram –	*E. coli*	HAV038	Δ*tolC*; efflux-impaired	4	0.5	2
Gram –	*E. coli*	HAV040	*lptD* mutant; permeable	2	1	2
Gram –	*A. baumannii*	HAV010	ATCC 19606	>128	4	16
Gram –	*K. oxytoca*	HAV450	Clinical	>128	8	16
			Rotatable bonds	7	7	
			PMI1/MW	5.4	6.0	

Response Figure 2 | Effect of N-methylation on the Gram-negative activity of iboxamycin (IBX). The Gram-negative activity of iboxamycin cannot be attributed to differing N-substitution, rotatable bond count, or reduced PMI1/MW relative to clindamycin.

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Response to Reviewer #3

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The recent manuscript by Myers et al. describes their recent discovery of iboxamycin, a totally synthetic analog of lincomycin, an FDA approved drug, and describes its mechanism of action and efficacy in vivo. Overall, this work is a synthetic tour de force and it is somewhat sad that the entire synthetic sequence is reduced to one part of one figure. Fortunately, the most up to date synthesis of the molecule was recently published in JACS allowing for the synthetic chemists to learn more about this route. I want to point out that even though an updated and streamlined synthesis of iboxamycin was reported that this does NOT detract from the impact of this work. The synthesis shown here differs from the previous method and also has some highlights of novelty including the first aldol reach to produce stereo-enriched proline rings. However, chemistry aside, what really differentiates this paper from others and raises it to the level of Nature is the identification of the mechanism of action and the activity of the molecule. These two aspects are incredibly significant and I cannot stress enough how impactful this work is. I fully expect these lead compounds to be entered into clinical trials as the preliminary data is very impressive. Additionally, having a molecule that works against a common mode of antibiotic resistance is incredibly important for the clinician's arsenal. The crystallographic work validates the target engagement, mouse studies (as requested by previous reviews) demonstrates clinical relevance, and resistance selection assays provide the critical data needed to progress these compounds. Overall, this work clearly demonstrates that the molecule that their group designed and constructed, and one impossible to produce if not for synthetic organic chemistry, is primed for potential commercialization. In the opinion of this reviewer the work is at the bar of importance, impact, and significance compared to other published papers in the journal. The authors should be commended for their fantastic work.-Bill Wuest

Response: We thank Prof. Wuest for his kind appreciation of our work.

Reviewer Reports on the First Revision:

Referee #1 (Remarks to the Author):

The authors have added additional data addressing the ability of IBX to overcome ABCF-mediated target protection mechanisms, which I think strengthens the paper. I like the response figure 1 and would suggest that the authors include it in the supplementary material. I realise that it is speculation to suggest that IBX forces the methylated A2503 out of the way analogous to A2058 but I am surprised that the authors don't want to mention this in the discussion as a hypothesis that can be tested in the future. The argument is quite compelling.
Otherwise I congratulate the authors on an exciting paper!

Referee #2 (Remarks to the Author):

The authors have responded in earnest and adequately to all critique/comments.

Author Rebuttals to First Revision:

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Response to Reviewer #1

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Otherwise I congratulate the authors on an exciting paper!

Response: While we remain reluctant to present speculative hypotheses regarding the precise mechanism for oxepanoprolinamides' activity in *cfr*-expressing strains within the main text of the manuscript, we agree that interested readers may benefit from the additions that the reviewer suggests. Consequently, our Supplementary Methods document now includes a section dedicated to illustrating the structural basis for Cfr-mediated lincosamide resistance based on *in silico* modeling of A2503 C8 methylation in static structures of antibiotic–ribosome complexes.

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Response to Reviewer #2

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Remarks to the Authors:

The authors have responded in earnest and adequately to all critique/comments.

Response: We are pleased to learn the reviewer found our responses satisfactory, and offer thanks once again for their insightful questions.

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