JAC Antimicrob Resist
https://doi.org/10.1093/jacamr/dlac061

JAC-Antimicrobial Resistance

Synthetic oxepanoprolinamide iboxamycin is active against *Listeria monocytogenes* despite the intrinsic resistance mediated by VgaL/Lmo0919 ABCF ATPase

Tetiana Brodiazhenko[1]†, Kathryn Jane Turnbull[2]†, Kelvin J. Y. Wu[3], Hiraku Takada[4,5], Ben I. C. Tresco[3], Tanel Tenson[1], Andrew G. Myers[3] and Vasili Hauryliuk[1,4]*

[1]University of Tartu, Institute of Technology, 50411 Tartu, Estonia; [2]Department of Clinical Microbiology, Rigshospitalet, 2200 Copenhagen, Denmark; [3]Department of Chemistry and Chemical Biology, Harvard University, Cambridge, MA, USA; [4]Department of Experimental Medicine, University of Lund, 221 84 Lund, Sweden; [5]Faculty of Life Sciences, Kyoto Sangyo University, Kamigamo, Motoyama, Kita-ku, Kyoto 603-8555, Japan

*Corresponding author. E-mail: vasili.hauryliuk@med.lu.se
†Equal contribution.
@hauryliuk

Received 6 April 2022; accepted 17 May 2022

Background: Listeriosis is a food-borne disease caused by the Gram-positive Bacillota (Firmicute) bacterium *Listeria monocytogenes*. Clinical *L. monocytogenes* isolates are often resistant to clinically used lincosamide clindamycin, thus excluding clindamycin as a viable treatment option.

Objectives: We have established newly developed lincosamide iboxamycin as a potential novel antilisterial agent.

Methods: We determined MICs of the lincosamides lincomycin, clindamycin and iboxamycin for *L. monocytogenes*, *Enterococcus faecalis* and *Bacillus subtilis* strains expressing synergetic antibiotic resistance determinants: ABCF ATPases that directly displace antibiotics from the ribosome and Cfr, a 23S rRNA methyltransferase that compromises antibiotic binding. For *L. monocytogenes* strains, either expressing VgaL/Lmo0919 or lacking the resistance factor, we performed time-kill kinetics and post-antibiotic effect assays.

Results: We show that the synthetic lincosamide iboxamycin is highly active against *L. monocytogenes* and can overcome the intrinsic lincosamide resistance mediated by VgaL/Lmo0919 ABCF ATPase. While iboxamycin is not bactericidal against *L. monocytogenes*, it displays a pronounced post-antibiotic effect, which is a valuable pharmacokinetic feature. We demonstrate that VmlR ABCF of *B. subtilis* grants significant (33-fold increase in MIC) protection from iboxamycin, while LsaA ABCF of *E. faecalis* grants an 8-fold protective effect. Furthermore, the VmlR-mediated iboxamycin resistance is cooperative with that mediated by the Cfr, resulting in up to a 512-fold increase in MIC.

Conclusions: While iboxamycin is a promising new antilisterial agent, our findings suggest that emergence and spread of ABCF ARE variants capable of defeating next-generation lincosamides in the clinic is possible and should be closely monitored.

Introduction

Lincosamides constitute an important class of antibiotics used both in veterinary and human medicine.[1] These compounds inhibit protein synthesis by binding to and compromising the enzymatic activity of the peptidyl transferase centre (PTC) of the ribosome,[2–5] resulting in bacteriostasis.[6] Representatives of this antibiotic class share a common architecture and are typically comprised of a 4′-substituted L-proline residue connected via an amide bond to a unique *S*-glycosidic amino sugar moiety (Figure 1a and b). The first lincosamide to be discovered, lincomycin (Figure 1a), is a natural product produced by *Streptomyces lincolnensis* subsp. *lincolnensis* and is active against streptococcal, pneumococcal and staphylococcal infections.[7] Its semi-synthetic derivative, clindamycin (Figure 1b), can be produced via a one-step stereoinvertive deoxychlorination of lincomycin.[8] Clindamycin is more potent than lincomycin and is currently the lincosamide of choice for human medicine.[9] Like lincomycin,

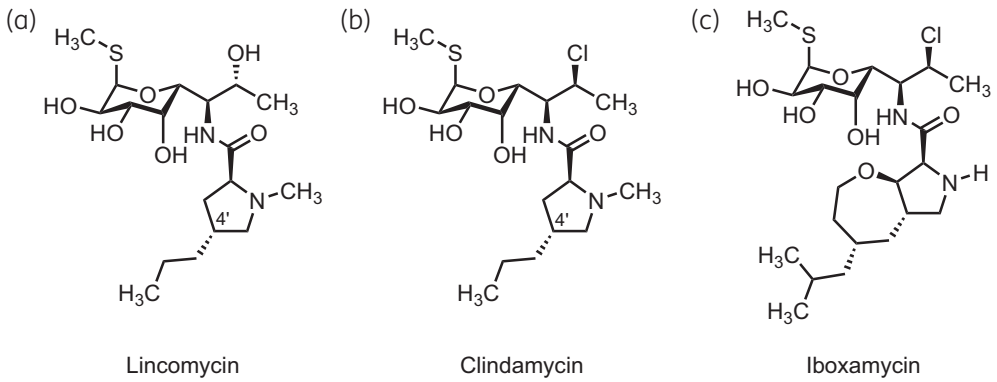

Figure 1. Chemical structures of lincosamide antibiotics lincomycin (a), clindamycin (b) and iboxamycin (c).

clindamycin is mostly active against Gram-positive but not Gram-negative bacteria, which restricts the spectrum of its applications.[10] A *cis*-4-ethyl-L-pipecolic acid amide of clindamycin, pirlimycin, has a similar spectrum of antibacterial activity[11,12] and is approved for veterinary applications in the United States and European Union. Finally, a recently developed semisynthetic derivative of lincomycin ('compound A') was shown to be able to overcome clindamycin resistance in *Staphylococcus aureus* mediated by ribosomal RNA (rRNA) methylation by ErmA and ErmB antibiotic resistance determinants.[13]

Iboxamycin (Figure 1c) is a newly developed lincosamide with an exceptionally broad spectrum of antibacterial activity.[14] Featuring a fully synthetic, bicyclic oxepanoprolinamide aminoacyl fragment, iboxamycin improves upon previous lincosamides in its activity against both Gram-positive and Gram-negative pathogens.[14] Iboxamycin was found to be more potent than clindamycin against Gram-positive pathogens and overcomes lincosamide resistance mediated by rRNA modification by Erm and Cfr 23S rRNA methyltransferases, both of which are highly clinically important and widespread antibiotic resistance determinants.[15–18] While the presence of Cfr renders clinical isolates of *S. aureus* and *Staphylococcus epidermidis* virtually non-susceptible to clindamycin (MIC >128 mg/L), the resistance to iboxamycin conferred by Cfr (MIC of 2–8 mg/L compared with 0.06 mg/L for *cfr*−strains) is not sufficient to render the drug fully inactive in the context of infection treatment.[14] Importantly, iboxamycin is also highly active against *Enterococcus faecalis* (MIC 0.06 mg/L as compared with 16 mg/L for clindamycin)—a species that is intrinsically resistant to 'classical' lincosamides as it encodes the LsaA antibiotic resistance (ARE) factor in its chromosomal genome,[19] a member of the ABCF ATPase protein family that includes multiple resistance factors.[20–22] LsaA provides resistance to pleuromutilin, lincosamide and streptogramin A (PLS_A) antibiotics by displacing the drug from the ribosome,[23] acting similarly to other ARE ABCFs.[24–27] As evident from the 96- to 256-fold higher susceptibility to clindamycin and lincomycin in a Δ*lsaA E. faecalis* strain as compared with *E. faecalis* ectopically expressing LsaA,[23] LsaA is a potent lincosamide resistance determinant. The high susceptibility of *E. faecalis* to iboxamycin suggests that this compound has the potential to overcome resistance mediated by other ARE ABCFs as well.

Listeriosis is a dangerous food-borne bacterial disease caused by the Gram-positive Bacillota (formerly Firmicute) bacterium *Listeria monocytogenes*, which infects people through contaminated meat, fish and dairy products.[28,29] While it is a relatively rare infection that mainly affects people with weakened immune systems, or who are pregnant,[30] the majority of listeriosis cases require hospitalization and mortality rates can be as high as 20%–30% even with antibiotic treatment.[31,32] Antibiotic treatment options for *L. monocytogenes* infections include cell wall synthesis disruptors ampicillin and vancomycin, folic acid synthesis inhibitors sulfamethoxazole and trimethoprim, and protein synthesis inhibitors, such as gentamicin and azithromycin.[33] *L. monocytogenes* strains reported in recent years are often resistant to clindamycin, with the resistant fraction ranging from 29% to 76%, depending on the collection,[34–37] thus excluding clindamycin as a viable option for treatment of *L. monocytogenes* infections. Importantly, just as *E. faecalis* encodes the ABCF ATPase LsaA, *L. monocytogenes* encodes the ARE ABCF PLS_A resistance factor VgaL/Lmo0919 in its core genome.[38] As with LsaA, VgaL operates on the ribosome,[23] and loss of VgaL results in increased susceptibility to lincosamides, with the Δ*lmo0919 L. monocytogenes* strain being 8- to 16-fold more susceptible to lincomycin as compared with the isogenic wild type (WT).[23] Finally, a model Bacillota, *Bacillus subtilis*, also encodes an ARE ABCF PLS_A resistance factor—VmlR.[27,39]

In this report, using lincomycin and clindamycin as reference compounds, we (i) characterized the efficacy of iboxamycin against *L. monocytogenes*; (ii) probed its ability to specifically counter resistance mediated by ARE ABCF Lmo0919 in *L. monocytogenes*, LsaA in *E. faecalis* and VmlR in *B. subtilis*; (iii) characterized its bactericidal/bacteriostatic mechanism of action; and, finally, (iv) assessed the strength of its post-antibiotic effect (PAE).

Materials and methods

Synthesis of iboxamycin

Iboxamycin was prepared according to the method reported by Mason and colleagues.[40]

Strains and media

WT *L. monocytogenes* 10403S was provided by Daniel A. Portnoy, WT *L. monocytogenes* EGD-e was provided by Jörgen Johansson,

construction of *L. monocytogenes* EDG-e Δ*lmo0919* was described earlier,[23]

E. faecalis Δ*lsaA* (*lsa*::Kan) strain TX5332[19] was provided by Barbara E. Murray, *E. faecalis* Δ*lsaA* pCIE$_{spec}$ and *E. faecalis* Δ*lsaA* pCIE$_{spec}$ LsaA were described earlier.[23] WT 168 *trpC B. subtilis* (laboratory stock) was used. *B. subtilis* strains *trpC* Δ*vmlR* (VHB5) and Δ*vmlR thrC*::P$_{hy\text{-}spank}$-*vmlR* (VHB44) were described earlier.[27] To construct *B. subtilis thrC*::P$_{hy\text{-}spank}$-*cfr* (VHB138) and Δ*vmlR thrC*::P$_{hy\text{-}spank}$-*cfr* (VHB139), a PCR product encoding *Staphylococcus sciuri cfr* gene optimized to *Escherichia coli* codon usage[41] was PCR-amplified from the pBRCfr plasmid using primers VHT25 (5′-CGGATAACAATTAAGCTTAGTCGACTTAAGGAGGTGTGTCTCATGAACTTTAACAACAAAACCAAATAC-3′) and VHT26 (5′-GTTTCCACCGAATTAGCTTGCATGCTCACTGGGAGTTCTGATAGTTACCATACA-3′). The second PCR fragment encoding a kanamycin-resistance marker, a polylinker downstream of the P$_{hy\text{-}spank}$ promoter and the *lac* repressor ORF—all inserted in the middle of the *thrC* gene—was PCR-amplified from pHT009 plasmid using primers pHT002_F (5′-GTCGACTAAGCTTAATTGTTATCCGCTCACAATTACACACATTATGCC-3′) and pHT002_R (5′-GCATGCAAGCTAATTCGGTGGAAACGAGGTCATC-3′). The two fragments were ligated using the NEBuilder HiFi DNA Assembly master mix (New England BioLabs, Ipswich, MA, USA) yielding the pHT009-*cfr* plasmid (VHp439), which was used to transform either WT 168 *trpC2* or Δ*vmlR* (VHB5) strain. Selection for kanamycin resistance yielded the desired VHB138 and VHB139 strain.

Growth assays, MIC, cidality and PAE assays with *L. monocytogenes* were performed in MH-F broth, *E. faecalis* MIC assays were performed in BHI broth and *B. subtilis* MIC assays were performed in LB broth. The media were prepared as per EUCAST guidelines (https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Disk_test_documents/2020_manuals/Media_preparation_v_6.0_EUCAST_AST.pdf) and contained 95% Mueller–Hinton broth (MHB) media (Sigma, Lot# BCCB5572), 5% lysed horse blood (defibrinated 50% stock, Hatunalab AB cat. no. 139) and 20 mg/mL β-NAD (Sigma, Lot# SLCD5502). Prior to use the 50% horse blood stock was freeze thawed five times and clarified via centrifugation twice for 30 min at 18 000 rpm at 4°C and then filtrated using 0.2 μm membrane filter, aliquotted and stored at −20°C. Solid agar plates were prepared from BHI broth media (VMR, Lot# G0113W) supplemented with 1% (final concentration) agar.

Liquid growth assays

L. monocytogenes was pre-grown on BHI agar plates at 37°C for 48 h. Individual fresh colonies were used to inoculate 2 mL of MH-F broth in 15 mL round bottom tubes, which were then incubated overnight at 37°C with shaking at 180 rpm. The overnight cultures were diluted then with MH-F broth to final OD$_{600}$ of 0.005 and incubated for 8 h in a water bath shaker (Eppendorf™ Inova™ 3100 High-Temperature) at 37°C with shaking at 160 rpm. bacterial growth was monitored by OD$_{600}$ measurements every 30 min.

Antibiotic susceptibility testing

The MIC antibiotic susceptibility testing was performed according to EUCAST guidelines (http://www.eucast.org/ast_of_bacteria/mic_determination), as described earlier.[23]

L. monocytogenes strains were grown in MH-F broth inoculated with 5×10^5 cfu/mL (OD$_{600}$ of approximately 0.0015) with increasing concentrations of antibiotics. After 24–48 h of incubation at 37°C without shaking, the presence or absence of bacterial growth was scored by eye.

E. faecalis strains were grown in BHI media supplemented with 2 mg/mL kanamycin (to prevent *lsa* revertants), 0.1 mg/mL spectinomycin (to maintain the pCIE$_{spec}$ plasmid), 100 ng/mL of cCF10 peptide (to induce expression of LsaA protein) as well as increasing concentrations of antibiotics, was inoculated with 5×10^5 cfu/mL (OD$_{600}$ of approximately 0.0005) of *E. faecalis* Δ*lsaA* (*lsa::Kan*) strain TX5332 transformed either with empty pCIE$_{spec}$ plasmid, or with pCIE$_{spec}$ encoding LsaA. After 16–20 h at 37°C without shaking, the presence or absence of bacterial growth was scored by eye.

B. subtilis strains were grown in LB medium supplemented with increasing concentrations of antibiotics. The cultures were inoculated with 5×10^5 cfu/mL (OD$_{600}$ of approximately 0.0005), and after 16–20 h at 37°C without shaking the presence or absence of bacterial growth was scored by eye.

Time-kill kinetics assay

The protocol was based on CLSI[42] and Svetlov *et al.*[43] Exponential *L. monocytogenes* cultures in MH-F broth (OD$_{600} \approx 0.3$) were diluted to 10^5 cfu/mL (OD$_{600} = 0.001$) in 10 mL of MH-F broth either supplemented with appropriate antibiotic at 4-fold MIC concentration or without antibiotics (positive growth control), and the resultant cultures were incubated at 37°C without shaking. Aliquots (1 mL) were taken at incremental incubation times (0, 2, 4, 6, 8 and 10 h), spun down at 4000 rpm for 5 min at room temperature and cell pellets were gently washed twice with 900 μL of 1 × PBS. Cell pellets were resuspended in 100 μL of 1 × PBS, 10-fold serial dilutions were prepared in 96-well plates (10^{-1}–10^{-8}), and 10 μL resultant 10-fold seral dilutions were spotted on BHI agar plates. Colony forming units were scored after 24–48 h incubation at 37°C.

PAE assay

Exponential cultures of *L. monocytogenes* strains in MH-F blood broth media (OD$_{600} \approx 0.3$) were diluted to 10^5 cfu/mL (≈OD$_{600}$ of 0.001) in 5 mL of MH-F media either supplemented with appropriate antibiotic at 4-fold MIC concentration or without antibiotics (positive growth control) and incubated at 37°C without shaking for 2 h. After the 2 h pretreatment, antibiotics were removed by 1:100 dilution of 100 μL into 10 mL of fresh prewarmed MH-F blood broth media. At incremental timepoints (0, 2, 4, 6, 8 and 10 h), 1 mL of the 100 × diluted cell culture was harvested, centrifuged for 5 min at 4000 rpm, 900 μL of the medium was removed, and the pellets were resuspended in the remaining 100 μL. The volume was adjusted to 1 mL with 1 × PBS. Control cultures without antibiotics were handled similarly. Cell solutions were then serially diluted 10-fold to 10^{-8}, and 10 μL was spotted on BHI agar plates. Plates for individual timepoints were incubated at room temperature until the last set of plates were spotted (10 h timepoint), and then incubated at 37°C. The plates were scored after 24 and 48 h incubation at 37°C and imaged using ImageQuant LAS 4000 (GE Healthcare). The last timepoint (24 h) was processed separately analogously to 0–10 h timepoints (see above).

Results

L. monocytogenes is highly susceptible to iboxamycin despite VgaL/Lmo0919 ABCF resistance factor

To test the lincosamide susceptibility of *L. monocytogenes* we used two widely used WT strains, both belonging to serovar 1/2a: EGD-e[44] and 10403S, a streptomycin-resistant variant of 10403.[45] The two WTs are genomically distinct, e.g. the virulence master-regulator PrfA is overexpressed in EGD-e and the prophage content differs between the two strains.[46] In addition to the two WTs, we also tested an *L. monocytogenes* EDG-e derivative that was genomically modified to abrogate the expression of VgaL/Lmo0919 PLS$_A$ resistance factor (EDG-e Δ*lmo0919*).[23]

Both WT *L. monocytogenes* strains are much more susceptible to iboxamycin (MIC of 0.125–0.5 mg/L) as compared with clindamycin (MIC of 1 mg/L) and lincomycin (MIC of 2–8 mg/L) (Table 1). In agreement with the higher susceptibility of Δ*lmo0919* EDG-e to lincomycin,[23] this strain is 2–8-fold more susceptible to

iboxamycin than the corresponding WT. This indicates that while VgaL does confer some protection from iboxamycin, the high potency of the synthetic antibiotic would likely allow the drug to overcome resistance in clinical settings. A likely explanation is that increased affinity of the synthetic drug for the ribosome renders antibiotic displacement by ABCF ATPases inefficient.

Importantly, expression of Lmo0919 is not constitutive: it is elicited by antibiotic-induced ribosomal stalling on the regulatory short open reading frame upstream of the *lmo0919* gene.[38] Therefore, the difference in iboxamycin susceptibility between WT and Δ*lmo0919* EDG-e strains reflects both the ability of Lmo0919 to protect the ribosome from the antibiotic as well as the efficiency of iboxamycin-mediated induction of Lmo0919. To deconvolute these two effects, in the following experiments we used engineered *E. faecalis* and *B. subtilis* strains that allow for ectopic inducible expression of ARE ABCFs: *E. faecalis* LsaA and *B. subtilis* VmlR, respectively.

E. faecalis ABCF LsaA grants a moderate protective effect against iboxamycin

To test the ability of other ABCF PLS_A resistance factors to confer resistance to iboxamycin, we compared a pair of *E. faecalis* strains: one lacking the chromosomally encoded LsaA (Δ*lsaA* $pCIE_{spec}$) and the other allowing cCF10-peptide-inducible expression of LsaA (Δ*lsaA* $pCIE_{spec}$ LsaA).[23] Using this experimental set up, we could specifically assess the ability of LsaA to protect the strain from lincosamides. While expression of LsaA dramatically increases resistance to clindamycin and lincomycin (96- to 256-fold, respectively), it results in a mere 8-fold protective effect against iboxamycin (MIC of 0.0625 and 0.5 mg/L, respectively) (Table 1), demonstrating that iboxamycin can also largely overcome LsaA-mediated resistance. Our current results are in agreement with our earlier MIC measurements for WT *E. faecalis lsaA*+ OG1RF strain (lincomycin 32 mg/L, clindamycin 16–32 mg/L)[23] and WT *E. faecalis* ATCC 29212 *lsaA*+ strain (clindamycin 16 mg/L and iboxamycin 0.6 mg/L).[14]

B. subtilis ABCF VmlR acts cooperatively with rRNA methyltransferase Cfr to grant significant protection against iboxamycin

Next we tested a set of *B. subtilis* strains: WT 168 *B. subtilis*, Δ*vmlR* (VHB5) as well as a Δ*vmlR* strain in which VmlR is expressed under the control of IPTG-inducible $P_{hy\text{-}spank}$ promoter (VHB44)[47] (Table 1). Disruption of *vmlR* results in a 33-fold increase in iboxamycin susceptibility (MIC of 2 and 0.06 mg/L, respectively), and resistance is restored upon ectopic expression of VmlR (MIC of 4 mg/L, 2-fold increase over the WT levels). The iboxamycin susceptibility of Δ*lmo0919 L. monocytogenes* EDG-e and Δ*vmlR B. subtilis* is near-identical, indicating that the 16-/4-fold difference in iboxamycin susceptibility between WT *L. monocytogenes* and *B. subtilis* is due to the different efficiency of resistance granted by Lmo0919 and VmlR, respectively.

Importantly, VmlR loss results in the same *relative* increase in susceptibility to all lincosamides tested—iboxamycin, clindamycin and lincomycin; 32–33-fold—regardless of the potency of the lincosamide (Table 1). This suggests that if the affinity of iboxamycin to the target were to be decreased by, for instance, rRNA modification, direct target protection by the ABCF could cooperatively lead to high levels of resistance. To probe this hypothesis, we have characterized the lincosamide susceptibility of *B. subtilis* strains that express Cfr 23S rRNA methyltransferase under the control of IPTG-inducible $P_{hy\text{-}spank}$ promotor, either in the presence or absence of the chromosomally encoded VmlR. Ectopic expression of Cfr in *vmlR*+ *B. subtilis* effected a cooperative resistance to iboxamycin, resulting in MICs of 16–32 mg/L as opposed to 2 mg/L when either of these resistance determinants are expressed individually (Table 1). As expected, Cfr also granted high levels of lincomycin and clindamycin resistance when ectopically expressed in both WT and Δ*vmlR* strains (MIC ranging from 320 to excess of 640 mg/L).

Taken together, our *B. subtilis* MIC results demonstrate that despite the cooperative action of the two resistance determinants, iboxamycin is a much more potent antibiotic against *cfr*+ *abcf*+ strains as compared with lincomycin and clindamycin.

Table 1. Broth microdilution MIC testing of lincosamide antibiotics against *L. monocytogenes*, *E. faecalis* and *B. subtilis* strains

	Antibiotic MIC, mg/L		
Species/strain	lincomycin	clindamycin	iboxamycin
L. monocytogenes 10403S	4–8	2	0.125–0.25
L. monocytogenes EDG-e	8	1–2	0.125–0.5
L. monocytogenes EDG-e Δ*lmo0919*	0.25–1	0.125–0.5	0.0625
E. faecalis Δ*lsaA* $pCIE_{spec}$	0.125	0.125	0.0625
E. faecalis Δ*lsaA* $pCIE_{spec}$ LsaA	16–32	16	0.5
B. subtilis WT 168	80	4	2
B. subtilis Δ*vmlR*	2.5	0.125	0.06
B. subtilis Δ*vmlR thrC*::$P_{hy\text{-}spank}$-*vmlR* (IPTG: 1 mM)	160	8	4
B. subtilis thrC::$P_{hy\text{-}spank}$-*cfr* (IPTG: 1 mM)	>640	640	16–32
B. subtilis Δ*vmlR thrC*::$P_{hy\text{-}spank}$-*cfr* (IPTG: 1 mM)	>640	320	2

In the case of *L. monocytogenes* strains, MIC testing was carried out in MH-F broth and growth inhibition was scored after 48 h incubation at 37°C. *E. faecalis* MIC testing was carried out in BHI broth supplemented with 2 mg/mL kanamycin (to prevent *lsa* revertants), 0.1 mg/mL spectinomycin (to maintain the $pCIE_{spec}$ plasmid) and 100 ng/mL of cCF10 peptide (to induce expression of LsaA protein). *B. subtilis* MIC testing was carried out in either LB medium or LB supplemented with 1 mM IPTG to induce expression of either VmlR or Cfr protein, and growth inhibition was scored after 16–20 h at 37°C. The MIC experiments were performed as three (*L. monocytogenes* strains) or two (*B. subtilis* and *E. faecalis* strains) biological replicates.

Iboxamycin is bacteriostatic against L. monocytogenes and displays a strong PAE

Macrolide antibiotics that tightly bind the ribosome and dissociate slowly are bactericidal, while macrolides that dissociate rapidly are bacteriostatic.[43] As with lincomycin and clindamycin, iboxamycin was shown to be bacteriostatic against a panel of bacterial species.[14] However, since effects on *L. monocytogenes*

were not assessed in the original report—and the species is highly susceptible to iboxamycin—we tested for potential bactericidal effects of iboxamycin against this pathogen. The three *L. monocytogenes* strains that we used for the MIC measurements—WT 10403 and EGD-e as well as ABCF-deficient EDG-e Δ*lmo0919*—were treated with 4 × MIC concentration of either iboxamycin, clindamycin and lincomycin for increasing periods of time (from 2 to 24 h), washed, and then plated on BHI agar plates that contained no antibiotic. The bacterial growth expressed in cfu was scored after either 24 or 48 h incubation of plates at 37°C. When the colony counting was performed after 24 h, we observed potentially bactericidal behaviour of iboxamycin, with almost a two $\log_{10}$ drop in cfu after the 10 h treatment with the antibiotic (Figure 2a–c). Importantly, no similar cfu decrease was observed for either clindamycin or lincomycin (Figure 2a–c). However, this apparent cfu drop effect of iboxamycin disappeared after 48 h of incubation (Figure 2d–f), suggesting slow regrowth rather than cidality, indicative of the so-called PAE.[48,49]

PAE is characterized by the time after antibiotic removal where no growth of the treated bacteria is observed. This prolonged action of iboxamycin has been previously noted for *S. aureus* and *Enterococcus faecium*.[14] Therefore, we next performed PAE experiments in *L. monocytogenes*, demonstrating that, indeed, iboxamycin displays pronounced PAE, suppressing the growth of the WT 10403S and WT EGD-e for 6 and 8 h, respectively (Figure 3b and c). Clindamycin demonstrates a weaker PAE against EGD-e (2 h) and similar PAE against 10403S. No clear PAE is detectible for lincomycin. Compared with the isogenic WT, EDG-e Δ*lmo0919* displays similar PAE in the case of clindamycin, and, possibly, somewhat more pronounced PAE in the case of iboxamycin.

Discussion

In this report we have evaluated the efficiency of the oxepanoprolinamide iboxamycin against *L. monocytogenes*. The antibiotic can largely overcome the intrinsic PLS_A resistance of this species that is mediated by the ribosome-associated ATPase VgaL/Lmo0919, and can similarly counteract the intrinsic resistance mediated by ARE ABCF LsaA in *E. faecalis*. ARE ABCF PLS_A resistance factors are broadly distributed among bacterial pathogens,[20,22,50,51] and therefore the ability of iboxamycin to largely counteract the ABCF-mediated resistance is a valuable feature

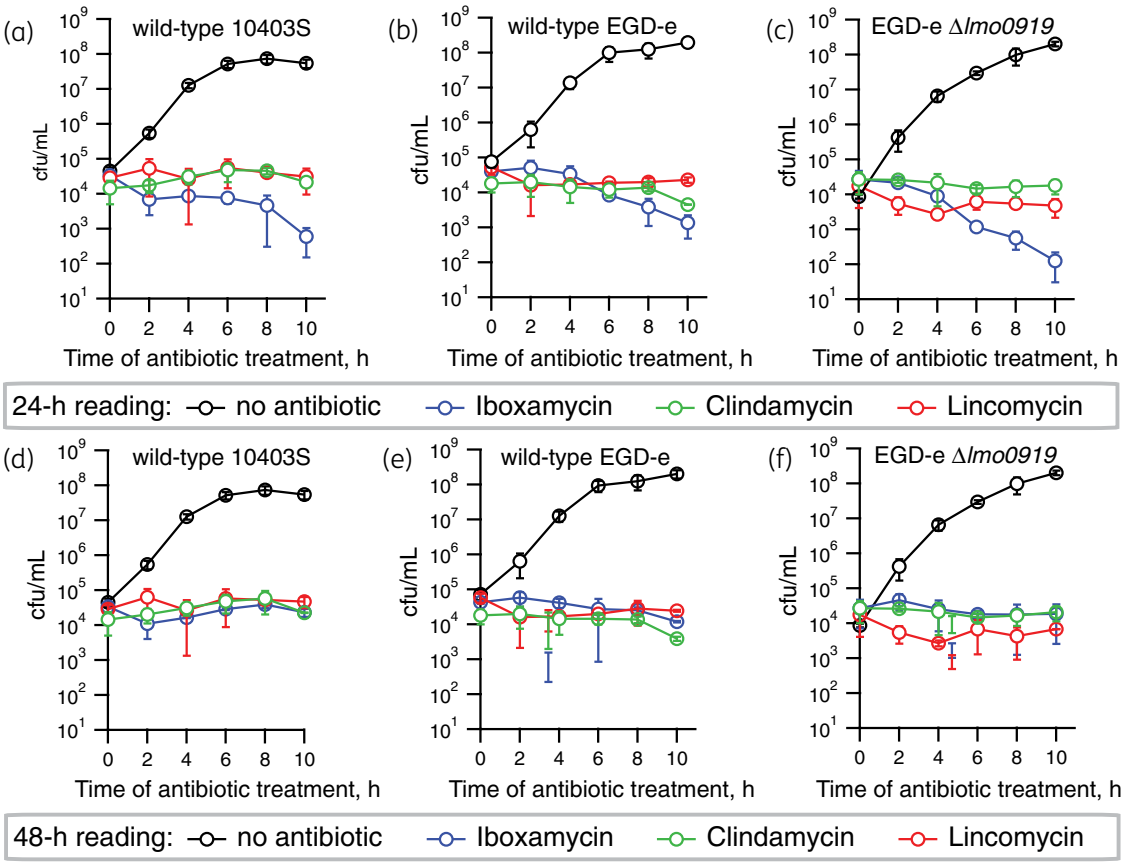

Figure 2. Iboxamycin is bacteriostatic against *L. monocytogenes*. Exponentially growing *L. monocytogenes* type strains; 10403S (a and d), EDG-e (b and e) or VgaA-deficient EDG-e Δ*lmo0919* (c and e) were treated with 4 × MIC of either iboxamycin, clindamycin or lincomycin or no antibiotic as control. Cells were harvested at given timepoints and washed before plating. After 24 (a–c) or 48 h (d–f) of incubation, colonies were counted to determine cfu/mL. All experiments were carried out in MH-F broth at 37°C without shaking, data points are from three biological replicates and standard deviation is indicated with error bars.

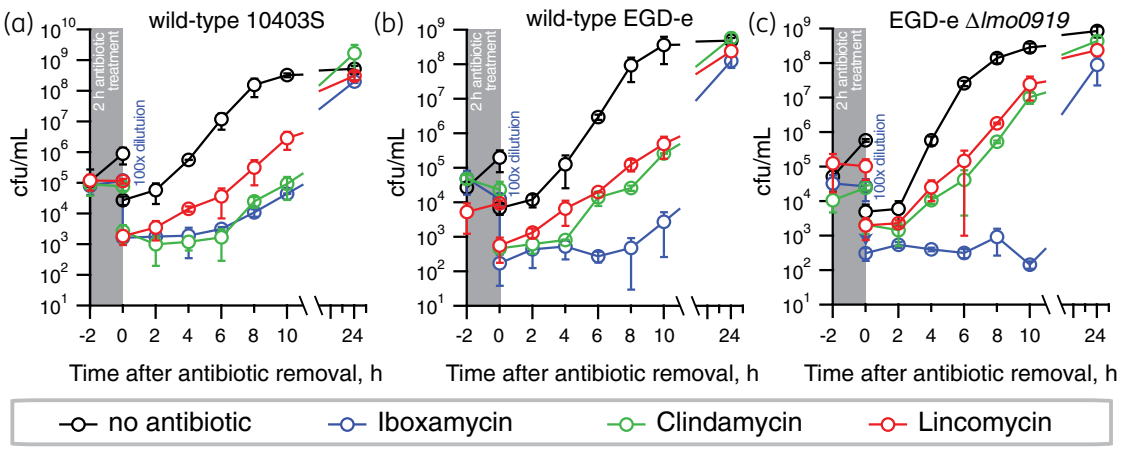

Figure 3. Iboxamycin displays strong PAE against *L. monocytogenes*. To determine the time taken for antibiotic treated *L. monocytogenes* strains to resume growth after a 2 h antibiotic treatment, exponentially growing type strains; 10403S (a), EDG-e (b) or VgaA-deficient EDG-e Δ*lmo0919* (c) were treated with 4x MIC of either iboxamycin, clindamycin, lincomycin, or no antibiotic as control, for 2 h. Cells were then diluted by 100-fold to remove the antibiotic, and samples taken every 2 h subsequently for viability counting. All experiments were carried out in MH-F broth at 37°C with shaking at 180 rpm, data points are from three biological replicates and standard deviation is indicated with error bars.

of the new antibiotic. However, given that *B. subtilis* VmlR does confer significant levels of iboxamycin resistance (33-fold increase in MIC) and is cooperative with the Cfr rRNA methyltransferase resistance determinant, emergence and spread of ABCF ARE variants capable of defeating next-generation lincosamides in the clinic is possible and should be closely monitored.

Furthermore, we demonstrate that iboxamycin displays a strong PAE against *L. monocytogenes*, compromising bacterial regrowth for many hours post-antibiotic removal. In clinical settings the longer PAE would allow the design of dosing regiments with larger dosing intervals, resulting in fewer daily administrations of the drug.[52,53] The PAE is considerably stronger than that of clindamycin while lincomycin displays no PAE. It is possible that the strength of the PAE reflects how tightly the antibiotic binds to the target, the ribosome—and how slowly it dissociates from it. The pronounced PAE suggests that development of even more tight-binding lincosamides could produce effectively bactericidal drugs in the context of infection. Further biochemical studies are necessary to substantiate this hypothesis. Experiments in *L. monocytogenes* infection models are necessary to further assess the potential of iboxamycin as a novel drug for the treatment of listeriosis.

Acknowledgements

We are grateful to Daniel A. Portnoy for sharing WT *L. monocytogenes* 10403S, Jörgen Johansson for sharing WT *L. monocytogenes* EGD-e, Barbara E. Murray for sharing *E. faecalis* Δ*lsaA* (*lsa*::Kan) strain TX5332[19] and Birte Vester for sharing the *S. sciuri cfr*-encoding plasmid.[41]

Funding

This work was supported by the funds from the European Regional Development Fund through the Centre of Excellence for Molecular Cell Technology (V.H., T.T.); the Estonian Research Council (grant PRG335 to V.H., T.T.); Swedish Research council (project grants 2017-03783 and 2021-01146, grant 2018-00956 within the RIBOTARGET consortium under the framework of JPIAMR); and the Ragnar Söderberg foundation (V.H.). K.J.Y.W. was supported by a National Science Scholarship (PhD) by the Agency for Science, Technology and Research, Singapore.

Transparency declarations

A.G.M. is an inventor in a provisional patent application submitted by the President and Fellows of Harvard College covering oxepanoprolinamide antibiotics described in this work. A.G.M. has filed the following international patent applications: WO/2019/032936 'Lincosamide Antibiotics and Uses Thereof' and WO/2019/032956 'Lincosamide Antibiotics and Uses Thereof'. All other authors: none to declare.

References

1 Schwarz S, Shen J, Kadlec K *et al.* Lincosamides, streptogramins, phenicols, and pleuromutilins: mode of action and mechanisms of resistance. *Cold Spring Harb Perspect Med* 2016; **6**: a027037.

2 Matzov D, Eyal Z, Benhamou RI *et al.* Structural insights of lincosamides targeting the ribosome of *Staphylococcus aureus*. *Nucleic Acids Res* 2017; **45**: 10284–92.

3 Tu D, Blaha G, Moore PB *et al.* Structures of MLS_BK antibiotics bound to mutated large ribosomal subunits provide a structural explanation for resistance. *Cell* 2005; **121**: 257–70.

4 Dunkle JA, Xiong L, Mankin AS *et al.* Structures of the *Escherichia coli* ribosome with antibiotics bound near the peptidyl transferase center explain spectra of drug action. *Proc Natl Acad Sci U S A* 2010; **107**: 17152–7.

5 Schlunzen F, Zarivach R, Harms J *et al.* Structural basis for the interaction of antibiotics with the peptidyl transferase centre in eubacteria. *Nature* 2001; **413**: 814–21.

6 Spížek J, Řezanka T. Lincosamides: Chemical structure, biosynthesis, mechanism of action, resistance, and applications. *Biochem Pharmacol* 2017; **133**: 20–8.

7 Macleod AJ, Ross HB, Ozere RL *et al.* Lincomycin: a new antibiotic active against staphylococci and other Gram-positive cocci: clinical and laboratory studies. *Can Med Assoc J* 1964; **91**: 1056–60.

8 Birkenmeyer RD, Lincomycin KF. XI. Synthesis and structure of clindamycin. A potent antibacterial agent. *J Med Chem* 1970; **13**: 616–9.

9 Phillips I. Past and current use of clindamycin and lincomycin. *J Antimicrob Chemother* 1981; **7** Suppl A: 11–8.

10 Smieja M. Current indications for the use of clindamycin: a critical review. *Can J Infect Dis* 1998; **9**: 22–8.

11 Ahonkhai VI, Cherubin CE, Shulman MA *et al*. *In vitro* activity of U-57930E, a new clindamycin analog, against aerobic Gram-positive bacteria. *Antimicrob Agents Chemother* 1982; **21**: 902–5.

12 Birkenmeyer RD, Kroll SJ, Lewis C *et al*. Synthesis and antimicrobial activity of clindamycin analogues: pirlimycin, a potent antibacterial agent. *J Med Chem* 1984; **27**: 216–23.

13 Hirai Y, Maebashi K, Yamada K *et al*. Characterization of compound A, a novel lincomycin derivative active against methicillin-resistant *Staphylococcus aureus*. *J Antibiot (Tokyo)* 2021; **74**: 124–32.

14 Mitcheltree MJ, Pisipati A, Syroegin EA *et al*. A synthetic antibiotic class overcoming bacterial multidrug resistance. *Nature* 2021; **599**: 507–12.

15 Long KS, Poehlsgaard J, Kehrenberg C *et al*. The Cfr rRNA methyltransferase confers resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A antibiotics. *Antimicrob Agents Chemother* 2006; **50**: 2500–5.

16 Schwarz S, Werckenthin C, Kehrenberg C. Identification of a plasmid-borne chloramphenicol-florfenicol resistance gene in *Staphylococcus sciuri*. *Antimicrob Agents Chemother* 2000; **44**: 2530–3.

17 Uchiyama H, Weisblum B. N-Methyl transferase of *Streptomyces erythraeus* that confers resistance to the macrolide-lincosamide-streptogramin B antibiotics: amino acid sequence and its homology to cognate R-factor enzymes from pathogenic bacilli and cocci. *Gene* 1985; **38**: 103–10.

18 Maravic G. Macrolide resistance based on the Erm-mediated rRNA methylation. *Curr Drug Targets Infect Disord* 2004; **4**: 193–202.

19 Singh KV, Weinstock GM, Murray BE. An *Enterococcus faecalis* ABC homologue (Lsa) is required for the resistance of this species to clindamycin and quinupristin-dalfopristin. *Antimicrob Agents Chemother* 2002; **46**: 1845–50.

20 Wilson DN, Hauryliuk V, Atkinson GC *et al*. Target protection as a key antibiotic resistance mechanism. *Nat Rev Microbiol* 2020; **18**: 637–48.

21 Murina V, Kasari M, Takada H *et al*. ABCF ATPases involved in protein synthesis, ribosome assembly and antibiotic resistance: structural and functional diversification across the tree of life. *J Mol Biol* 2019; **431**: 3568–90.

22 Ero R, Kumar V, Su W *et al*. Ribosome protection by ABC-F proteins-molecular mechanism and potential drug design. *Protein Sci* 2019; **28**: 684–93.

23 Crowe-McAuliffe C, Murina V, Turnbull KJ *et al*. Structural basis of ABCF-mediated resistance to pleuromutilin, lincosamide, and streptogramin A antibiotics in Gram-positive pathogens. *Nat Commun* 2021; **12**: 3577.

24 Murina V, Kasari M, Hauryliuk V *et al*. Antibiotic resistance ABCF proteins reset the peptidyl transferase centre of the ribosome to counter translational arrest. *Nucleic Acids Res* 2018; **46**: 3753–63.

25 Sharkey LK, Edwards TA, O'Neill AJ. ABC-F proteins mediate antibiotic resistance through ribosomal protection. *mBio* 2016; **7**: e01975.

26 Su W, Kumar V, Ding Y *et al*. Ribosome protection by antibiotic resistance ATP-binding cassette protein. *Proc Natl Acad Sci U S A* 2018; **115**: 5157–62.

27 Crowe-McAuliffe C, Graf M, Huter P *et al*. Structural basis for antibiotic resistance mediated by the *Bacillus subtilis* ABCF ATPase VmlR. *Proc Natl Acad Sci U S A* 2018; **115**: 8978–83.

28 Radoshevich L, Cossart P. *Listeria monocytogenes*: towards a complete picture of its physiology and pathogenesis. *Nat Rev Microbiol* 2018; **16**: 32–46.

29 Schlech WF 3rd, Lavigne PM, Bortolussi RA *et al*. Epidemic listeriosis–evidence for transmission by food. *N Engl J Med* 1983; **308**: 203–6.

30 Southwick FS, Purich DL. Intracellular pathogenesis of listeriosis. *N Engl J Med* 1996; **334**: 770–6.

31 de Noordhout CM, Devleesschauwer B, Angulo FJ *et al*. The global burden of listeriosis: a systematic review and meta-analysis. *Lancet Infect Dis* 2014; **14**: 1073–82.

32 Mylonakis E, Hohmann EL, Calderwood SB. Central nervous system infection with *Listeria monocytogenes*. 33 years' experience at a general hospital and review of 776 episodes from the literature. *Medicine (Baltimore)* 1998; **77**: 313–36.

33 Temple ME, Nahata MC. Treatment of listeriosis. *Ann Pharmacother* 2000; **34**: 656–61.

34 Caruso M, Fraccalvieri R, Pasquali F *et al*. Antimicrobial susceptibility and multilocus sequence typing of *Listeria monocytogenes* isolated over 11 years from food, humans, and the environment in Italy. *Foodborne Pathog Dis* 2020; **17**: 284–94.

35 Andriyanov PA, Zhurilov PA, Liskova EA *et al*. Antimicrobial resistance of *Listeria monocytogenes* strains isolated from humans, animals, and food products in Russia in 1950-1980, 2000-2005, and 2018-2021. *Antibiotics (Basel)* 2021; **10**: 1206.

36 Rugna G, Carra E, Bergamini F *et al*. Distribution, virulence, genotypic characteristics and antibiotic resistance of *Listeria monocytogenes* isolated over one-year monitoring from two pig slaughterhouses and processing plants and their fresh hams. *Int J Food Microbiol* 2021; **336**: 108912.

37 Tirziu E, Herman V, Nichita I *et al*. Diversity and antibiotic resistance profiles of *Listeria monocytogenes* serogroups in different food products from the Transylvania Region of Central Romania. *J Food Prot* 2022; **85**: 54–59.

38 Dar D, Shamir M, Mellin JR *et al*. Term-seq reveals abundant ribo-regulation of antibiotics resistance in bacteria. *Science* 2016; **352**: aad9822.

39 Ohki R, Tateno K, Takizawa T *et al*. Transcriptional termination control of a novel ABC transporter gene involved in antibiotic resistance in *Bacillus subtilis*. *J Bacteriol* 2005; **187**: 5946–54.

40 Mason JD, Terwilliger DW, Pote AR *et al*. Practical Gram-scale synthesis of iboxamycin, a potent antibiotic candidate. *J Am Chem Soc* 2021; **143**: 11019–25.

41 Ntokou E, Hansen LH, Kongsted J *et al*. Biochemical and computational analysis of the substrate specificities of Cfr and RlmN methyltransferases. *PLoS One* 2015; **10**: e0145655.

42 CLSI. *Performance Standards for Antimicrobial Susceptibility Testing—Twenty-Sixth Edition: M100*. 2016.

43 Svetlov MS, Vazquez-Laslop N, Mankin AS. Kinetics of drug-ribosome interactions defines the cidality of macrolide antibiotics. *Proc Natl Acad Sci U S A* 2017; **114**: 13673–8.

44 Glaser P, Frangeul L, Buchrieser C *et al*. Comparative genomics of *Listeria* species. *Science* 2001; **294**: 849–52.

45 Edman DC, Pollock MB, Hall ER. *Listeria monocytogenes* L forms. I. Induction maintenance, and biological characteristics. *J Bacteriol* 1968; **96**: 352–7.

46 Bécavin C, Bouchier C, Lechat P *et al*. Comparison of widely used *Listeria monocytogenes* strains EGD, 10403S, and EGD-e highlights genomic variations underlying differences in pathogenicity. *mBio* 2014; **5**: e00969-14.

47 Britton RA, Eichenberger P, Gonzalez-Pastor JE *et al*. Genome-wide analysis of the stationary-phase sigma factor (sigma-H) regulon of *Bacillus subtilis*. *J Bacteriol* 2002; **184**: 4881–90.

48 Walkup GK, You Z, Ross PL *et al*. Translating slow-binding inhibition kinetics into cellular and *in vivo* effects. *Nat Chem Biol* 2015; **11**: 416–23.

49 Bundtzen RW, Gerber AU, Cohn DL *et al*. Postantibiotic suppression of bacterial growth. *Rev Infect Dis* 1981; **3**: 28–37.

50 Mohamad M, Nicholson D, Saha CK *et al*. Sal-type ABC-F proteins: intrinsic and common mediators of pleuromutilin resistance by target protection in staphylococci. *Nucleic Acids Res* 2022; **50**: 2128–42.

51 Sharkey LKR, O'Neill AJ. Antibiotic resistance ABC-F proteins: bringing target protection into the limelight. *ACS Infect Dis* 2018; **4**: 239–46.

52 ter Braak EW, de Vries PJ, Bouter KP *et al*. Once-daily dosing regimen for aminoglycoside plus β-lactam combination therapy of serious bacterial infections: comparative trial with netilmicin plus ceftriaxone. *Am J Med* 1990; **89**: 58–66.

53 Gilbert DN. Once-daily aminoglycoside therapy. *Antimicrob Agents Chemother* 1991; **35**: 399–405.