A synthetic antibiotic class overcoming bacterial multidrug resistance

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The dearth of new medicines effective against antibiotic-resistant bacteria presents a growing global public health concern. For more than five decades, the search for new antibiotics has relied heavily on the chemical modification of natural products (semisynthesis), a method ill-equipped to combat rapidly evolving resistance threats. Semisynthetic modifications are typically of limited scope within polyfunctional antibiotics, usually increase molecular weight, and seldom permit modifications of the underlying scaffold. When properly designed, fully synthetic routes can easily address these shortcomings. Here we report the structure-guided design and component-based synthesis of a rigid o xoepanoproline scaffold which, when linked to the aminooctose residue of clindamycin, produces an antibiotic of exceptional potency and spectrum of activity, which we name iboxamycin. Iboxamycin is effective against ES KA PE pathogens including strains expressing Erm and Cfr ribosomal RNA methyltransferase enzymes, products of genes that confer resistance to all clinically relevant antibiotics targeting the large ribosomal subunit, namely macrolides, lincosamides, phenicols, oxazolidinones, pleuromutilins and streptogramins. X-ray crystallographic studies of iboxamycin in complex with the native bacterial ribosome, as well as with the Erm-methylated ribosome, uncover the structural basis for this enhanced activity, including a displacement of the m15A2058 nucleotide upon antibiotic binding. Iboxamycin is orally bioavailable, safe and effective in treating both Gram-positive and Gram-negative bacterial infections in mice, attesting to the capacity for chemical synthesis to provide new antibiotics in an era of increasing resistance.

The rise and dissemination of bacteria resistant to most or all known antibiotics raises concerns of an impending global infectious-disease crisis. Any plan to address the problem depends on the discovery of new antibiotics effective against modern bacterial pathogens, but opinions diverge on which strategies can best achieve these discoveries. For decades, natural products have served as both conceptual and material starting points for antibiotics discovery, but making specific chemical modifications to structurally complex natural products (that is, semisynthesis) is inherently challenging, and the pace of drug discovery by this route has slowed markedly. In an alternative approach, the development of fully synthetic platforms to construct tetracycline, macrolide, group A streptogramin and arylomycin antibiotics has enabled deep-seated structural modifications that are not achievable by semisynthesis. These technologies enable chemists to envision and reduce to practice an almost limitless array of design hypotheses. Here we use component-based chemical synthesis to achieve a substantive re-scaffolding of the lincosamide antibiotics, giving rise to a broad-spectrum agent that is effective against a wide range of multidrug-resistant bacterial pathogens.
Clindamycin-resistant bacteria are globally distributed. Acquired resistance to clindamycin most commonly occurs through N\(^{-}\)dimethylation of 23S ribosomal RNA (rRNA) residue A2058 (nucleotide numbering in this Article refers to Escherichia coli) by erythromycin resistance rRNA methyltransferases\(^{17}\). These enzymes, which are encoded by \(erm\) genes, give rise to macrolide, lincosamide and streptogramin B (MLS\(_{B}\)) cross-resistance; expression of these genes may be constitutive (\(c\)-\(erm\)), or inducible upon exposure to macrolide antibiotics\(^{26,27}\) (\(i\)-\(erm\)). The prevalence of \(erm\) genes among clinical streptococcal and staphylococcal isolates has led the US Centers for Disease Control and Prevention (CDC) to list MLS\(_{B}\)-resistant pathogens among its most pressing threats in their 2019 report\(^{1}\). A distinct rRNA methyltransferase, \(Cfr\) (encoded by the horizontally transferrable gene \(cfr\))\(^{20}\) methylates C8 of the 23S RNA residue A2503 and confers resistance to phenicol, lincosamide, oxazolidinone, pleuromutilin and streptogramin A\(^{18,22}\) (PHLOPS\(_{A}\)) antibiotics as well as 16-membered macrolides\(^{31}\). Moreover, recent studies have elucidated a third major resistance mechanism affecting the lincosamides, whereby target-protection proteins (for example, LsaA) bind to antibiotic-inhibited ribosomes, evict the drug and in so doing restore protein synthesis\(^{24,25}\). The prevalence of \(erm\), \(cfr\) and target-protection resistance genotypes in staphylococcal, streptococcal and enterococcal clinical isolates, together with the risk of \textit{Clostridium difficile} colitis with the use of clindamycin challenge the continued utility of this antibiotic in patients.

### Discovery of oxepanoprolinamides

To enable full structural exploration of the lincosamides, we developed modular synthetic routes to both the aminooctose and aminoacyl residues, then linked these components by amide-bond formation to produce more than 500 antibacterial candidates\(^{26-29}\). Thus a wide array of structural changes were examined; we observed and report in this Article that bicyclic scaffold modifications of the aminooctose hemisphere in combination with the aminooctose residue of clindamycin were especially advantageous with respect to antibacterial activity, notably among clindamycin-resistant strains. In brief, the bicyclic scaffold of oxepanoprolinamide 1 (OPP-1) was conceived to rigidify both the \(\gamma\)-\(endo\) proline ring pucker and the extended \(4\text{\textquoteright}-\text{\textprime}\)-propyl chain of clindamycin as observed when bound to the ribosome\(^{30}\) (Extended Data Fig. 1a). These explorations were inspired and made feasible by a stereoselective aldol reaction developed for a key fragment coupling in the synthesis of macrolide antibiotics\(^{7}\). Thus, enolization of (\(R,R\))-4, a chiral glycine equivalent\(^{31}\), followed by addition of the bis-electrophile 5 led to sequential \(\text{syn}\)-aldol addition, then spontaneous intramolecular \(N\)-alkylation, furnishing the \(\beta\)-hydroxy proline derivative 6 in a single operation (64% yield, 2.3 g scale) (Fig. 1c). A three-step sequence involving cleavage of the chiral auxiliary (with \(>99%\) recovery of pseudoephedrine), \(N\)-Boc protection, and esterification provided the suitably protected intermediate 7 in 85% overall yield. Construction of the oxepene ring was then achieved through palladium-catalysed O-allylation followed by ring-closing metathesis using the first-generation Hoveyda–Grubbs catalyst\(^{32}\). The target OPP-1 was assembled following an established sequence of saponification, amide coupling to the aminooctose component (7-chloro-1-methylthio-lincosamine, 7-Cl-MTL)\(^{33}\) and \(N\)-Boc removal\(^{31}\).

Microbiological testing of OPP-1 showed it to be comparable in activity to clindamycin (Extended Data Fig. 2a). Substitution of position 7\(^{\prime}\) of the oxepene ring was prioritized next, as molecular modelling suggested that substituents there would project towards a hydrophobic cleft formed by 23S rRNA residues A2451 and C2452 (Extended Data Fig. 1a). Wacker oxidation of intermediate 8 using Grubbs’ protocol\(^{34}\) proceeded with complete regioselectivity to provide the 7-keto product 10 with a yield of 74% (Fig. 1c); this selectivity is thought to arise from the \(\omega\)-withdrawing effect of the oxygen atom embedded within the 7-membered ring. Deoxydifferufination of intermediate 8 with \(N,N\)-diethylenamino-sulfur trifluoride (DAST) followed by the same three-step sequence described earlier provided oxepanoprolinamide OPP-2, which exhibited substantially improved activity against multidrug-resistant Gram-positive strains (\textit{Streptococcus pneumoniae}\(\textit{Streptococcus pneumoniae}\))...
Fig. 2a) This sequence provided a viable discovery route to OPP-\(c\)-ermA; Streptococcus pyogenes \(c\)-ermB\), as well as against Klebsiella pneumoniae (Extended Data Fig. 2a), lending support to our design strategy. Alkyl (and aryl) substitution of position 7 was made possible by non-regioselective enolization of \(35\) and its less active 7′-β stereoisomer \(\text{ermA} \rightarrow \text{ermB}\). For example, IBX overcame resistance and is effective in vivoFig. 2b) in vitro and in vivo antibacterial activity of the broad-spectrum antibiotic IBX. a–c, MICs in \(\mu\)g ml\(^{-1}\) of IBX in standard and multidrug-resistant bacterial strains. a, IBX displays a broad antibacterial spectrum of activity, and overcomes \(\text{erm}, \text{cfr}\) and \(\text{IsaA}\) resistance. \(\text{B. fragilis}\), \(\text{Bacteroides fragilis}\); \(\text{H. influenzae}\), \(\text{Haemophilus influenzae}\); \(\text{K. oxytoca}\), \(\text{Klebsiella oxytoca}\); \(\text{S. epidermidis}\), \(\text{Staphylococcus epidermidis}\); \(\text{S. haemolyticus}\), \(\text{Staphylococcus haemolyticus}\). Comparisons of IBX with standard antibiotics against clinical Gram-positive (b) and Gram-negative (c) isolates illustrate the differentiated activity of the oxepanoprolinamide class. CLI, clindamycin; CTR, ceftriaxone; LEVO, levofloxacin; AZM, azithromycin; LNZ, linezolid; VAN, vancomycin; GEN, gentamicin. d, Efficacy of IBX in a neutropenic mouse thigh infection model; colours correspond to strains highlighted in a–c. Mice received vehicle or IBX intraperitoneally, and bacterial counts were determined 12 h after treatment. Data are mean ± s.d.; \(n = 8\) thighs from 4 mice examined over 2 experiments; two-tailed unpaired Welch’s t-test. CFU, colony-forming units. e, Survival of mice receiving IBX or vehicle following systemic infection with \(\text{S. pyogenes}\). 3GC-R, third-generation cephalosporin-resistant; CRAB, carbapenem-resistant \(\text{A. baumannii}\); CRE, carbapenem-resistant Enterobacteriaceae; ESBL, extended-spectrum beta-lactamase; FQR, fluoroquinolone-resistant; LNZ/R, linezolid-resistant; MDR, multidrug-resistant; MEC-R, methicillin-resistant; MRSA, methicillin-resistant \(\text{S. aureus}\); VAN-R, vancomycin-resistant; VRE, vancomycin-resistant Enterococci.
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biotics (MIC = 8 μg ml⁻¹; Fig 2c). Clinical strains of

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displayed activity against methicillin-resistant

phenotype37, with MICs at least 1,000× lower than that of clindamycin

ermB

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To assess the efficacy of oxepanoprolinamide antibiotics, we evaluated IBX in animal models of infection using both Gram-positive and Gram-negative bacteria. Cell culture safety profiling experiments showed that IBX is non-haemolytic and non-toxic towards mammalian cells (concentration causing 50% cell growth inhibition (GL50) > 50 μM, Extended Data Fig. 3a, d–f), and has no effect on membrane integrity or mitochondrial function (Extended Data Fig. 3b, c). In mice, IBX achieved greater exposure than clindamycin, exhibiting an intravenous mean residence time (MRT) of 1.2 h and 24% oral bioavailability (Extended Data Table 1). Next, we studied the efficacy of IBX in a murine neutropenic thigh infection model using standard strains of S. pyogenes and A. baumannii, a clinical strain of methicillin-resistant S. aureus, and a clinical strain of E. coli displaying aminoglycoside, tetracycline, fluoroquinolone and third-generation cephalosporin multi-resistance. In each experiment, when compared to vehicle, IBX achieved a statistically significant reduction in bacterial burden 12 h following treatment (Fig 2d, Extended Data Fig. 4a–d). Finally, in a model of systemic infection, we studied the ability of IBX to rescue mice from a lethal challenge of intraperitoneally administered S. pyogenes ATCC 19606. IBX was well-tolerated at all dose levels in this experiment, and resulted in the rescue of infected mice, with all mice treated with 3 or 10 mg kg⁻¹ IBX surviving (Fig 2e, Extended Data Fig. 4e). Together, these results illustrate the potential of the oxepanoprolinamide class to provide orally available, broad-spectrum antibiotics effective against a range of resistance mechanisms.

IBX targets wild-type and resistant ribosomes

The remarkable antibacterial profile of IBX prompted us to study its mechanism of action, including the structural basis for its activity against Erm-methylated MLS₅,₉-resistant ribosomes. Broth culture profiling revealed that, like clindamycin, IBX is bacteriostatic but exhibits prolonged effects on bacterial growth following even brief exposure; concentration-dependent cidality was observed for certain highly susceptible strains (Extended Data Fig. 5). Spontaneous resistance was slow to develop in standard and clinical bacterial strains (approximate frequency of resistance ≤ 10⁻⁹ at 4× MIC; Extended Data Table 2). By contrast, IBX-resistant mutants did emerge in E. coli SQ110DTC, a strain specifically engineered for selecting mutations in rRNA that render cells resistant to ribosome-targeting inhibitors50. This strain lacks six out of seven rRNA alleles and the major multidrug efflux pump TolC. When SQ110DTC cells were plated on agar containing IBX, resistant clones with MICs in the range of 2–4 μg ml⁻¹ appeared with a frequency of approximately 10⁻⁴. Of the 14 randomly selected clones we sequenced, all carried the single-nucleotide mutations A2058G or A2059G within the 23S rRNA (Extended Data Table 3), corresponding to base changes in the canonical lincosamide binding site known

of outer-membrane penetration and transporter-mediated efflux to Gram-negative activity4. Whereas clindamycin exhibited activity only against E. coli strains with loss-of-function mutations to major efflux transporter (tolC) or outer-membrane assembly (lptD) genes, we found that, as a class, oxepanoprolinamides were significantly less susceptible to these mechanisms of intrinsic antibiotic resistance (Extended Data Fig. 2b). Pseudomonas aeruginosa, while measurably inhibited by IBX (MIC = 128 μg ml⁻¹; Fig 2a), was comparatively resistant towards the antibiotic, reflecting the unique challenges facing the discovery of antibiotics effective against this pathogen51. It is notable that the expanded spectrum of activity of IBX relative to clindamycin is not readily explained by traditional physicochemical predictors of Gram-negative activity such as rotatable-bond count, molecular weight, relative polar surface area or lipophilicity. Nor does it reinforce recently advanced rules for Gram-negative accumulation (see Supplementary Methods). Nevertheless, we believe that enhanced Gram-negative potency may be realized through optimization of the former metrics within the oxepanoprolinamide class.

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The binding of IBX to the functional *T. thermophilus* ribosome complex containing mRNA and tRNAs corresponds closely to the binding of clindamycin to the tRNA-free ribosome of *E. coli* or the large ribosomal subunit of the archaean *Haloarcula marismortui* (Extended Data Fig. 7c). With clindamycin and lincomycin, a network of hydrogen bonds anchors the aminooxocete moiety of IBX to NBP tetrolides A2058, A2059, and A2503 (Fig. 3b). Similarly, the cationic aminocly residue of IBX occupies a hydrophilic pocket formed by the PTC residues G2061 and U2504 (Fig. 3b), displacing a divalent magnesium ion otherwise observed in the drug-free ribosome (Extended Data 7c). Consistent with our design hypothesis (Extended Data Fig. 1b), the oxepane ring of IBX overlays closely with the n-propyl-group atoms of clindamycin and presents the 7'-isobutyl group for interaction with the A-site clef formed by 23S RNA residues A2451 and C2452 (Fig. 3c). This cleft, which normally accommodates the side-chains of incoming amino acids, has a key role in the positioning of aminoclaycet 3' ends of A-site tRNAs within the PTC during translation (14). Electron density corresponding to the CCA terminus of the A-site tRNA is poorly resolved in our structure, suggesting that IBX blocks proper coordination of the aminoclaycet 3' end of A-site tRNAs within the PTC (Extended Data Fig. 7d). Notably, the 7'-isobutyl substituent of IBX extends deep enough within the A-site cleft to overlap not only with incoming amino acids (as the n-propyl group of clindamycin does, albeit to a lesser extent) but also with the P-site amino acid (Fig. 3d, Extended Data Fig. 7e). As tight coordination and proper placement of the P-site substrate are particularly important at the outset of protein synthesis, these observations, together with the toe-printing results described above, lead us to propose that compared to lincomycin and clindamycin, IBX more readily interferes with translation initiation.

The activity of IBX against bacteria harboung erm resistance genes prompted us to determine the crystal structure of IBX bound to the m2A2058-containing ribosome. Erm-mediated methylation of A2058 confers resistance to clindamycin by blocking the association of its 7'-CT-MTL residue with the NPE (11), and because IBX bears the same aminooxocete residue, its ability to overcome MLSR resistance was unexpected. Erm-modified 70S ribosomes with A2058 dimethylation levels of approximately 60% were isolated from *T. thermophilius* Co-crystallization of these ribosomes with IBX provided crystals diffracting to 2.6 Å resolution (Supplementary Table 2). Of note, the corresponding electron density map revealed that IBX binds to the methylated ribosome in a manner almost identical to its positioning within the wild-type ribosome, whereas m2A2058 undergoes a movement of about 2 Å relative to its canonical position (Extended Data 1a). By contrast, present at the same concentration (50 μM), IBX firmly locked the ribosome at the start codons, allowing virtually no escape, suggesting that IBX associates more strongly with the ribosome than does clindamycin.

To illuminate the structural basis for this improved activity, we determined the structure of IBX bound to the bacterial ribosome using X-ray crystallography. 70S Ribosomes from the Gram-negative bacterium *Thermus thermophilus* were co-crystallized with IBX, mRNA, non-hydrolyzable aminocly tRNA analogue Met-tRNA(thi), (located within the P site), and acylated tRNA(thi) (within the A and E sites). The crystals we obtained diffracted to 2.30 Å resolution (Supplementary Table 2). The unbiased Fobs−Fcalc difference Fourier map revealed positive electron density peaks resembling characteristic chemical features of IBX (Extended Data Fig. 7a, b), and confirmed that the antibiotic binds in the canonical binding pocket within the large ribosomal subunit (Fig. 3a), spanning the peptidyl transferase center (PTC) and extending into the nascent peptide exit tunnel (NPET) (Supplementary Video 1).

Among clindamycin-resistant bacteria (13). Notably, mammalian cytosolic and mitochondrial ribosomes carry G2058 residues (13), explaining the non-toxicity of IBX in human cell culture experiments. These results demonstrated that, like clindamycin, IBX selectively targets the bacterial ribosomes and thus has a low potential to select for resistance owing to the redundancy of rRNA genes in the genomes of most bacterial pathogens (14).

To understand the effect of IBX on ribosomal function, we performed primer extension inhibition analysis (‘toeprinting’), an in vitro technique that detects the position of drug-arrested ribosomes on mRNA (15). Consistent with previous reports (13), clindamycin arrested translation at the start codons of the model open reading frames (ORFs), yet allowed a fraction of ribosomes to translate ORFs up to the first stop codon, a point at which ribosomes are arrested owing to the lack of isoleucyl- tRNA in the in vitro translation system (Extended Data Fig. 6). By contrast, present at the same concentration (50 μM), IBX firmly locked the ribosome at the start codons, allowing virtually no escape, suggesting that IBX associates more strongly with the ribosome than does clindamycin.

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Conclusion
Using a platform for antibiotics discovery rooted in component-based synthesis and structure-guided design, we identified the emergent oxazoloprolinamide antibiotic class. Departing radically from their lincosamide progenitors, the oxazoloprolinamides overcome Ern-, Cfr- and ABCF-mediated multidrug resistance and display activity against high-priority Gram-positive and Gram-negative pathogens, including multidrug-resistant strains of *S. aureus, E. faecalis, E. coli, K. pneumoniae* and *A. baumannii*. Methodologically, our findings attest to the continued role of empiricism in antibiotics discovery, as the activity of oxazoloprolinamides对抗 posttranscriptionally methylated ribosomes could not have been predicted on the basis of existing antibiotic–ribosome co-crystal structures. Continued refinement of the scaffold, including modifications to the aminocoustic residue whose unexpected capacity to displace m7A2OSβ we illustrate here, is likely to expand the oxazoloprolinamides’ spectrum of activity further. These findings, together with the safety, pharmacokinetic profile and efficacy of IBX in mouse models of Gram-positive and Gram-negative bacterial infection reaffirm the capacity for chemical synthesis to replenish our antibiotics armamentarium, delivering broad-spectrum agents capable of overcoming increasingly widespread resistance mechanisms.

Online content
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**Methods**

**In vitro susceptibility**

MICs were determined by the broth microdilution method or the agar dilution method following the Clinical and Laboratory Standards Institute (CLSI) guidelines. Standard bacterial strains were obtained from American Type Culture Collection (ATCC). Other bacterial strains were obtained from hospitals and commercial sources, as noted in Supplementary Table 1. Before the start of the MIC experiment, standard and test compound stock solutions were prepared in dimethyl sulfoxide (DMSO, Aldrich D2650) at a stock concentration of 5,120 μg ml⁻¹. The compound concentration range typically employed for each experiment was 128–0.06 μg ml⁻¹ (2.5% final DMSO concentration). Clindamycin was used as a comparator in all experiments and each experiment was performed in triplicate. Specialized procedures were employed for streptococci, anaerobic species and N. gonorrhoeae (see Supplementary Methods for details). All other bacterial strains were sub-cultured on blood agar plates (tryptic soy agar with 5% sheep blood, Hardy Diagnostics) and incubated overnight at 35 °C. Organisms were suspended in cation-adjusted Mueller–Hinton broth (CaMHB, BD 212322) and optical density was adjusted to 0.5 McFarland standard. Suspensions were further diluted to obtain a final inoculum of 5 × 10⁵ CFU ml⁻¹ for broth microdilution experiments. The minimum concentration of compound required to inhibit visible bacterial growth after 24 h of incubation was recorded as the MIC.

**Time-kill studies**

Time-kill studies were performed using four different concentrations of standard and test compounds (1, 2, 4 and 10×MIC). Experiments were performed in duplicate following CLSI guidelines. An inoculum was prepared in CaMHB containing 0.5–5 × 10⁵ CFU ml⁻¹ of test organism. Cultures were incubated at 37 °C in a shaker incubator at 110 rpm; a flask containing bacteria left unexposed to antibiotic was used as untreated control. At time points 0, 1, 3, 6 and 24 h following administration of antibiotic, bacterial counts were determined from each flask by serial dilution and plating on brain heart infusion agar (BD, Sparks, MD). Plates were incubated at 35 °C in incubators for 18–24 h to determine bacterial growth. Compounds exhibiting ≥3 log₁₀ CFU ml⁻¹ reduction compared to initial counts are considered bactericidal. Compounds exhibiting ≥2 log₁₀ CFU reduction, or which maintain counts similar to the initial bacterial counts, are considered bacteriostatic.

**Post-antibiotic and post-antibiotic sub-MIC effect**

Measurements of post-antibiotic effect (PAE) and post-antibiotic sub-MIC effect (PA-SME) durations were performed according to the methods described by Odenholt-Tornqvist and colleagues. Bacterial strains were cultured overnight on blood agar plates. Optical density was adjusted to 0.5 McFarland standard in CaMHB and suspensions were diluted further into 50-ml sterile Erlenmeyer flasks containing 20 ml CaMHB to achieve a bacterial load of 0.5–1 × 10⁶ CFU ml⁻¹. Cultures were then exposed to test and standard antibiotic at 1, 2, 4 and 10×MIC concentrations for 1 h at 37 °C in a shaker incubator at 110 rpm (0.5% final DMSO concentration). A flask containing the unexposed bacterial strain was left as untreated control. Post-exposure, 100 μl of bacterial sample was immediately diluted further in 19.9 ml of sterile CaMHB (1:2,000 final dilution). For PA-SME studies, post-exposure and dilution, each flask (except those in the control group) was supplemented with 0.25 and 0.5×MIC per ml of specific antibiotic (0.5% final DMSO concentration). Flasks were incubated at 37 °C in a shaker incubator (110 rpm). Samples (100 μl from each flask per time point) were collected at 0, 1, 3, 6 and 24 h from each flask and were serially diluted. From each dilution, 100 μl and 10 μl from each dilution were plated in duplicate on tryptic soy agar plates. Plates were incubated overnight at 35 °C to determine the bacterial counts (limit of detection = 20 CFU per plate or 2 × 10³ CFU ml⁻¹). These counts were plotted against time with linear interpolation between time points to obtain the growth kinetic curves. PAE and PA-SME were determined from these curves using the formulae:

\[ \text{PAE} = T - C \]

where \( T \) is the time required for bacteria previously exposed to antibiotic to multiply 1 log₁₀ above counts immediately following dilution, and \( C \) is the corresponding time required for untreated culture to do the same; and

\[ \text{PA-SME} = T_{pa} - C \]

where \( T_{pa} \) is the time taken for bacteria previously exposed to antibiotic and then re-exposed to sub-MIC concentrations to increase 1 log₁₀ above counts immediately following dilution.

**Mammalian cell experiments**

All cell lines were purchased from ATCC and used at a passage number of no greater than 8; cell lines were not authenticated or tested for mycoplasma contamination. All compounds were dissolved in sterile DMSO to a stock concentration of 20 μM and aliquoted prior to freezing at −20 °C. Aliquots were limited to a maximum of three freeze–thaw cycles. A549 (human pulmonary carcinoma; male) cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum. K562 (human chronic myelogenous leukemia; female) cells were cultured in Iscove’s Modification of DMEM (IMDM) media supplemented with 10% fetal bovine serum. HCT116 (human colorectal carcinoma; male) cells were cultured in McCoy’s 5a (Iwakata and Grace Modification) media supplemented with 10% fetal bovine serum. HepG2 (human hepatocellular carcinoma; male) cells were cultured in Eagle’s Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum. All cell cultures were maintained in a 5% CO₂ incubator at 35 °C.

Growth inhibition was studied using the Promega CellTiter-Blue dye cell viability assay pursuant to the manufacturer’s protocol. In brief, cells were harvested, diluted, and mixed with the desired concentration of test compound (or DMSO, used to define 0% growth inhibition). This suspension was then added to a 96-well plate (5 × 10⁴ cells ml⁻¹, 100 μl per well, 5 replicates per test compound), and the plates were incubated for 72 h at 37 °C in a 5% CO₂ incubator. Next, 20 μl of CellTiter-Blue reagent was added to each well and the plates were incubated for 4 h. Plates were read on a SpectraMax i3 plate reader. GI₅₀ values were determined by nonlinear regression using GraphPad Prism and represent the mean of at least three independent experiments.

Mitochondrial toxicity assays were conducted using the Promega Mitochondrial ToxGlo assay, according to the manufacturer’s protocol.

**Animal studies**

Animal experiments were performed at the Biology Research Infrastructure Laboratory of Harvard University following Institutional Animal Care and Use Committee (IACUC)-approved protocols. Animals were maintained in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Pathogen-free, 5- to 6-week-old, female CD-1 mice weighing 22–26 g were obtained from Charles River Laboratories, Inc. Animals were acclimated for a minimum of three days prior to start of the studies. Mice were caged as a group of four per cage, were housed at 21–23 °C with humidity ranging from 30–70%, were exposed to 12-h light and dark cycles, and were supplied with food and water ad libitum.

**Mouse thigh infection studies**

Mice were rendered neutropenic by administering cyclophosphamide intraperitoneally, 150 mg kg⁻¹ 4 days prior, and 100 mg kg⁻¹ 1 day prior to infection. Bacterial strains were cultured overnight on agar plates at 35 °C in 5% CO₂. Bacterial inoculum was prepared in sterile brain
heart infusion broth (BHIB) and OD was adjusted to 0.1 at 600 nm; the inoculum was further diluted to achieve a bacterial load of 0.5–1 × 10^9 CFU ml⁻¹. Mice were infected by intramuscular injection of 0.1 ml of inoculum into each thigh (0.2 ml of inoculum was used per mouse). Mice were randomly divided into 4 treatment groups: IBX, comparator antibiotic, vehicle-treated control and untreated baseline. At 2 h post-infection (t = 0), the untreated mice (n = 2) were euthanized to obtain pre-treatment bacterial counts, while the others received a single dose of IBX (6 mg ml⁻¹ in 10% Capsitol; 250 µl, 60 mg kg⁻¹), comparator antibiotic (clindamycin phosphate, ciprofloxacin, or azithromycin, 6 mg ml⁻¹ in sterile saline, 250 µl, 60 mg kg⁻¹), or sterile vehicle (10% Capsitol, 250 µl) by intraperitoneal injection. Intramuscular bacterial counts were determined after euthanizing mice from each group (n = 2) by CO₂ inhalation. Thighs (4 per time point) were aseptically removed and homogenized in sterile saline, and homogenates were serially diluted and plated (each sample was plated in duplicate). Bacterial counts (log₈ CFU per thigh) were determined after incubating the plates overnight at 35°C in 5% CO₂. Each experiment was repeated twice, and mean values were calculated (n = 16 counts across 4 biologically independent mice per time point). Because the outcome of the experiment (bacterial counts) was determined by serial dilution, a quantitative technique, blinding was not used. The results of these experiments are displayed in Extended Data Fig. 4.

Mouse systemic infection study

*S. pyogenes* ATCC 19615 was cultured overnight on blood agar plates in 5% CO₂ at 35°C before being suspended in BHIB. The optical density of the bacterial suspension was adjusted to 0.1 at 600 nm using sterile BHIB and was then further diluted 1:1 in 10% hog gastric mucin (HGM, type III) to prepare the infecting inoculum in 5% HGM. Mice were infected by intraperitoneal injection of 250 µl infecting inoculum, representing 1–2 × 10^8 CFU per mouse. Mice were randomly assigned to treatment groups; at time points 1.5, 17 and 29 h post-infection, mice were administered intravenous infusions of IBX (1, 3 or 10 mg kg⁻¹, 250 µl in 10% Capsitol; 8 mice per dose level), clindamycin phosphate (1.3 or 10 mg kg⁻¹, 250 µl in normal saline; 8 mice per dose level), or vehicle as negative control (10% Capsitol, 250 µl; 10 mice). Mice were monitored for survival for seven days following infection. Because the outcome of the experiment (survival) is objective, blinding was not used.

Frequency of resistance

Triplicates of agar plates containing IBX at concentrations corresponding to 4×MIC, 8×MIC, and 16×MIC were prepared. Bacterial cultures (*S. aureus* ATCC 29213, *S. aureus* ATCC 700699, *E. faecalis* ATCC 29212, *E. faecium* HAV-251, and *K. pneumoniae* ATCC 10031) were prepared by overnight culture, and counts were determined by serial dilution and plating. Bacterial inocula (250 µl, containing approximately 1 × 10^8, 1 × 10^7, 1 × 10^6, or 1 × 10^5 CFU) were spread onto the media plates. The plates were incubated at 37°C for 72 h and were monitored for the appearance of colonies. The colony appearing after plating 10^6 CFU of *S. aureus* ATCC 29213 on IBX-containing agar (4×MIC) was examined further to determine the fold change in antibiotic susceptibility; for details, see Supplementary Methods.

Selection of IBX-resistant mutants in SQI10DTC

Approximately 10^8 CFU (1.2 ml of the exponentially growing cell culture with optical density of A₆₀₀ = 1.2), were plated on LB agar plates containing either 0.25 µg ml⁻¹ or 1 µg ml⁻¹ of IBX (10× or 30×MIC, respectively). Approximately 20 colonies appeared on both plates after incubation at 37°C for 24 h. The segment of the 23S rRNA gene corresponding to domains V and VI of the 23S rRNA was PCR-amplified from 14 randomly selected colonies using the primers 2020R (CCCCGAGCTACGTTAAAGATTTGAATTCG) and L2904 (AAGGGTACGCTTCTAGGACGCC). PCR products were sequenced using the primer L2667 (GGTCTCTCGTACTAGGGACG).

Toepirnking analysis

The *ermB* DNA template for toeprinting was generated by a 4-primer cross-over PCR using primers T7, NTV, ermB3-F and ermB3-R. The *ermDL* template was generated in the same way using primers RLR-fwd, RLR-rev, T7-SD-fwd and T7-SD-rev. In vitro translation in the PURExpress system (New England Biolabs) and toeprinting analysis were carried out as described previously. The antibiotics (mupirocin, clindamycin, erythromycin or IBX) were present in the reactions at a final concentration of 50 µg ml⁻¹.

Primer sequences used for toeprinting are as follows: T7 (TAATAC GACTATAGGG); NTV (GGTTATAAGTCCTGTTAATAC); ermB3-R (TAATACGACTATAGGGCTTGAATTTGGAGAGAAAAATATGTTCTTCAAGTTGAAATGAGACTTCGTATTTTCCC); ermB3-F (GGTATAAGGTTAAGCTCACGGG); T7-SD-fwd (TAATACGACTATAGGGCTTGAATTTGGAGAGAAAAATATGTTCTTCAAGTTGAAATGAGACTTCGTATTTTCCC); T7-SD-rev (TAATACGACTATAGGGCTTGAATTTGGAGAGAAAAATATGTTCTTCAAGTTGAAATGAGACTTCGTATTTTCCC); T7-SD-fwd (TAATACGACTATAGGGCTTGAATTTGGAGAGAAAAATATGTTCTTCAAGTTGAAATGAGACTTCGTATTTTCCC); T7-SD-rev (TAATACGACTATAGGGCTTGAATTTGGAGAGAAAAATATGTTCTTCAAGTTGAAATGAGACTTCGTATTTTCCC).

Crystalization of IBX in complex with wild-type and Erm-modified *T. thermophilus* 70S ribosomes

Wild-type 70S ribosomes from *T. thermophilus* (strain HB8) containing unmodified residue A2058 of the 23S rRNA were prepared as described previously. Purification of the Erm-modified 70S ribosomes from *T. thermophilus* (strain HB27 expressing Erm-like enzyme from *B. thermophilus*) containing 6-dimethylated A2058 residue in the 23S rRNA was accomplished as optimized previously for the wild-type 70S ribosomes from *T. thermophilus*. Decacylated tRNA and non-hydrolyzable aminoacylated fMet-NH₂-tRNA₆₅₄ were prepared as described previously. Complexes of the A2058-dimethylated or unmethylated *T. thermophilus* 70S ribosomes with mRNA, decacylated A-site tRNA₆₅₄, and P-site hydrolysis-resistant aminoacylated fMet-NH₂-tRNA₆₅₄ were formed as described previously. For *T. thermophilus* 70S ribosome complexes with IBX, the antibiotic was included in the crystallization mixture (250 µM each) and then later added to the stabilization buffers (250 µM each). Collection and processing of the X-ray diffraction data, model building, and structure refinement were performed as described in our previous reports. Diffraction data were collected at beamlines 24ID-C and 24ID-D at the Advanced Photon Source (Argonne National Laboratory). A complete dataset for each complex was collected using 0.979 Å irradiation at 100 K from multiple regions of the same crystal, using 0.3-degree oscillations. Raw data were integrated and scaled using XDS software (5 Feb 2021). Molecular replacement was performed using Phaser from the CCP4 program suite (version 7.0). The search model was generated from the previously published structures of *T. thermophilus* 70S ribosome with bound mRNA and aminoacylated tRNAs (PDB entry 6XHW). Initial molecular replacement solutions were refined by rigid-body refinement with the ribosome split into multiple domains, followed by positional and individual B-factor refinement using PHENIX software (version 1.17). Non-crystallographic symmetry restraints were applied to four parts of the 30S ribosomal subunit (head, body, spur, and helix 44), and four parts of the 50S subunit (body, L1-stalk, L10-stalk, and C-terminus of the L9 protein). Structural models were built in Coot (version 0.8.2). Structural models and restraints for IBX were generated using PRODRG online software (http://prodrg2.dynsb.org). The statistics of data collection and refinement are compiled in Supplementary Table 2. All figures showing atomic models were rendered using PyMol software.

Statistical analyses

Statistical analysis was performed using GraphPad Prism. Mouse thigh infection study data (12-h time points) were compared using Welch's t-test.


**Reporting summary**
Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

**Data availability**
Coordinates and structure factors are deposited in the RCSB Protein Data Bank with accession codes 7RQ8 for the *T. thermophilus* 70S ribosome in complex with IBX, mRNA, deacylated A-site tRNA^Phe^, aminoacylated P-site fMet-NH-tRNA^Met^, and decacylated E-site tRNA^Met^; and 7RQ9 for the mRNA* T. thermophilus* 70S ribosome in complex with IBX, mRNA, deacylated A-site tRNA^Phe^, aminoacylated P-site fMet-NH-tRNA^Met^, single-crystal X-ray crystallographic data for compound 6 are deposited at the Cambridge Crystallographic Data Centre under deposition number 2072277. All previously published structures that were used in this work for model building and structural comparisons were retrieved from the RCSB Protein Data Bank under accession codes 6XHW^4^, 4V7V^3^, and 1YJN^5^.

Source data are provided with this paper.

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Extended Data Fig. 1 | Structure-based design of 7′-substituted oxepanoprolinamides. a, Superposition of the X-ray crystal structure of ribosome-bound lincosamide antibiotic clindamycin (2, blue, PDB entry 4V7V) with the energy-minimized structure of OPP-1 (green). Note that in this configuration, the C7′-atom of OPP-1 contacts the lipophilic surface of the A-site cleft. b, The same structure, overlaid with the X-ray crystal structure of iboxamycin (IBX, yellow) bound to the bacterial ribosome.
Extended Data Fig. 2 | MICs (μg ml⁻¹) of antibiotics containing a bicyclic aminoacyl residue. 

**a**, Effects of 7′ substitution, ring size, and saturation on antibacterial activity. 

**b**, Effects of 7′-alkyl substituent chain length on antibacterial activity, including against MLS₆-resistant S. aureus and strains of E. coli engineered to lack key efflux or outer-membrane assembly machinery. c-ermA/B, constitutively expressed erythromycin ribosome methylase A/B gene.

### Table A

| Species         | Strain Description | MIC (μg ml⁻¹) | CLI | OPP-1 | OPP-2 | OPP-4 | OPP-5 | OPP-6 | OPP-3 | epi-OPP-3 |
|-----------------|--------------------|---------------|-----|-------|-------|-------|-------|-------|-------|-----------|
| S. aureus       | ATCC 29213         | 1.25          | 1.25| 0.25  | 0.125 | 0.5   | 0.06  | 0.06  | >128  | 4         |
| S. aureus       | MMX 3035; c-ermA    | >128          | NT  | NT    | NT    | NT    | NT    | NT    | NT    | NT        |
| S. pneumoniae   | ATCC 49619         | 0.06          | 0.06| 0.06  | ≤0.06 | ≤0.06 | ≤0.03 | 0.06  | 0.03  | ≤0.06     |
| S. pneumoniae   | MMX 3028; c-ermB    | 256           | 64  | 64    | NT    | NT    | NT    | 8     | 0.25  | 0.5       |
| S. pyogenes     | ATCC 19615         | 0.06          | 0.03| 0.03  | ≤0.03 | ≤0.03 | ≤0.03 | 0.03  | 0.03  | ≤0.06     |
| S. pyogenes     | MMX 946; c-ermB     | >256          | 16  | 4     | NT    | NT    | 32    | 0.25  | 0.5    |           |
| E. faecalis     | ATCC 29212         | 16            | 16  | 4     | >32   | 32    | 0.125 | 0.06  | ≤0.06 |           |
| K. pneumoniae   | ATCC 10031         | 8             | >64 | 16    | 8     | >32   | NT    | 0.25  | NT    |           |
| E. coli         | ATCC 29222         | >128          | >64 | >64   | >64   | 32    | 32    | 32    | 8     | 16        |
| A. baumannii    | ATCC 19606         | >128          | >32 | >32   | >32   | >32   | NT    | 4     | NT    |           |
| H. influenzae   | ATCC 49247         | 8             | 1   | 4     | 8     | 32    | 8     | 1     | 2     |           |

### Table B

| Specie          | Strain Description | MIC (μg ml⁻¹) | CLI | OPP-3 | OPP-7 | OPP-8 | OPP-9 |
|-----------------|--------------------|---------------|-----|-------|-------|-------|-------|
| S. aureus       | ATCC 29213         | 0.125         | 0.06| 0.06  | 0.125 | 1     |
| S. aureus       | MMX 3035; c-ermA    | >128          | 4   | 2     | 8     | 64    |
| E. faecalis     | ATCC 29212         | 16            | 0.06| 0.06  | 0.25  | 16    |
| E. faecalis     | Clinical; VRE      | >128          | 1   | 0.5   | 8     | 64    |
| A. baumannii    | ATCC 19606         | >128          | 4   | 8     | 32    | >128  |
| E. coli         | ATCC 29222         | >128          | 8   | 4     | 16    | 64    |
| E. coli         | ΔtoC; efflux-impaired | 4         | 0.5 | 0.125 | 0.5   | 8     |
| E. coli         | iptD mutant; permeable | 2         | 1   | 1     | 2     | 8     |
Extended Data Fig. 3 | Effects of iboxamycin on mammalian cells.
a. Normalized hemolysis (mean ± s.d.) of human erythrocytes by IBX (n = 3 replicates) and clindamycin (n = 5 replicates) relative to Triton X-100 (n = 45 replicates) measured over one independent experiment.
b, c. Mitochondrial ToxGlo data showing effects of IBX on HepG2 cellular membrane integrity and ATP production relative to vehicle-treated control (antimycin serves as a positive control for mitotoxicity). Data are mean ± s.d.; n = 3 technical replicates from one independent experiment.
d–f. Comparison of effects of IBX, clindamycin, doxycycline, and azithromycin on cell viability (CellTiter-Blue). Data are the mean ± s.d. of n = 3 independent experiments performed in technical quintuplicate. Where applicable, GI₅₀ values (μM) are reported beside the dose-response curves.
Extended Data Fig. 4 | Efficacy of iboxamycin (IBX) in mouse models of infection. Bacterial counts were quantified in the thighs of neutropenic mice infected with *S. pyogenes* ATCC 19615. (a), *S. aureus* MRSA HAV017 (b), *A. baumannii* ATCC 19616 (c) or *E. coli* MDR HAV504 (d) treated with IBX, vehicle, or comparator antibiotic at the listed time points post-treatment. Data are mean ± s.d.; n = 8 thighs from 4 mice examined over 2 experiments, with the exception the sub-set of part c where mice received IBX via intravenous administration (n = 4 thighs from 2 mice examined over a single experiment). 

e, Mouse survival in an *S. pyogenes* systemic infection model. Abbreviations: AZM, azithromycin; CLI, clindamycin; CPFX, ciprofloxacin; IP, intraperitoneal administration; IV, intravenous administration...
Extended Data Fig. 5 | Time-kill kinetics, post-antibiotic effect, and post-antibiotic sub-MIC effect data of iboxamycin (IBX) against susceptible strains. a, Arrayed growth curves for three susceptible strains showing concentration effects on growth inhibition (time-kill), growth kinetics following exposure to antibiotic at 4×MIC (PAE), and growth kinetics under sub-MIC concentrations following exposure to antibiotic at 4×MIC (PA-SME). Points represent mean values from n = 2 biologically independent experiments. b, Tabulated PAE and PA-SME durations (determined as the difference in time required for bacterial counts to rise 10× between experimental and untreated control arms). Abbreviations: CLI, clindamycin; LNZ, linezolid.
Extended Data Fig. 6 | Iboxamycin (IBX) efficiently arrests translation at the start codon. Toeprinting analysis showing sites of IBX-induced translation arrest of ErmBL and ErmDL leader peptides. Because all reactions contained mupirocin, an inhibitor of Ile-tRNA synthetase, the ribosomes that escape inhibition by ribosome-targeting antibiotics are trapped at the codon preceding Ile (black arrowheads). Red arrowheads mark translation arrest at the start codon, while cyan arrowheads denote known erythromycin-induced arrest sites D10 (ermBL) and L7 (ermDL). Each gel is representative of two independent experiments, for source data see Supplementary Figure 1. ERY, erythromycin; CLI, clindamycin.
Extended Data Fig. 7 | High-resolution electron density maps of iboxamycin (IBX) bound to the bacterial ribosome. **a, b**, Unbiased $F_o - F_c$ and $2F_o - F_c$ electron density maps of IBX in complex with the *T. thermophilus* 70S ribosome (green and blue mesh, respectively). The refined model of IBX is displayed in its electron density before (a) and after (b) the refinement contoured at 3.0σ and 1.5σ, respectively. **c**, Superposition of ribosome-bound IBX (yellow) with prior structures of clindamycin bound to the tRNA-free 70S ribosome from eubacterium *E. coli* (blue, PDB entry 4V7V46) or to the 50S ribosomal subunit from archaeon *H. marismortui* harboring the 23S rRNA mutation G2099A (green, PDB entry 1YJN48). All structures were aligned based on domain V of the 23S rRNA. **d**, $2F_o - F_c$ electron density map (blue mesh) corresponding to ribosome-bound IBX (yellow), deacylated A-site tRNA$^{fMet}$ (green) and aminoacylated initiator P-site fMet-NH-tRNA$^{Met}$ (dark blue). The refined models of tRNAs are displayed in their respective electron-density maps contoured at 1.0σ. In **d**, the entire bodies of the A- and P-site tRNAs are viewed from the back of the 50S subunit, as indicated by the inset. Ribosome subunits are omitted for clarity. Note that IBX binding to the ribosome prevents accommodation of the aminoacyl-bearing CCA-end of the A-site tRNA. **e**, Close-up view of the P-site tRNA CCA-end bearing a formyl-methionyl (cyan) residue. **f**, Detailed arrangement of the hydrogen bonds formed between the aminooctose component of IBX with 23S rRNA residues A2058 and G2505 (light blue).
### Extended Data Table 1 | Pharmacokinetics of clindamycin and iboxamycin

| Parameters                     | Clindamycin          | Iboxamycin         |
|-------------------------------|----------------------|--------------------|
|                               | Species | i.v. | p.o. | i.v. | p.o. |
| Dose (mg·kg⁻¹)                | m       | 60   | 60   | 60   | 60   |
| AUC (µg·h·mL⁻¹)               |         |      |      | 6.8 ± 1.3 | 1.6 ± 0.4 |
| C_max (µg·mL⁻¹)               | 2.9 ± 0.7 | 0.52 ± 0.10 | 7.9 ± 3.0 | 1.6 ± 0.7 |
| MRT (h)                       | 0.9 ± 0.3 | -    | 1.2 ± 0.2 | -    |
| CL (L·kg⁻¹·h⁻¹)               | 29 ± 7   | -    | 8.9 ± 1.8 | -    |
| Vss (L·kg⁻¹)                  | 25 ± 1   | -    | 12.5 ± 0.4 | -    |
| Bioavailability               | -       | 24% ± 7% | - | 24% ± 7% |
| Clint,mic (µg·min⁻¹·mg⁻¹)     | m       | NT   | 21   |
|                               | h       | 55   | 34   |
| Plasma protein binding        | m       | 72%  | 95%  |
|                               | h       | 87%  | 92%  |

Parameters were calculated by a non-compartmental model (n=3 mice per study arm), and are reported as their mean ± s.d., where applicable. i.v., intravenous; p.o., per os; AUC, area under the curve; MRT, mean retention time; CL, clearance; Vss, volume of distribution at steady state; Clint,mic, liver microsome intrinsic clearance; h, human; m, mouse.
## Extended Data Table 2 | Frequency of resistance to iboxamycin in susceptible bacterial strains

| Species      | Strain                  | Bacterial Count (CFU) | Colonies Growing on iboxamycin-containing media |
|--------------|-------------------------|-----------------------|-------------------------------------------------|
|              |                         |                       | 4 × MIC | 8 × MIC | 16 × MIC |
| S. aureus    | ATCC 29213              | $1 \times 10^7$       | none    | none    | none     |
|              |                         | $1 \times 10^8$       | none    | none    | none     |
|              |                         | $1 \times 10^9$       | 4       | none    | none     |
|              |                         | $1 \times 10^{10}$    | 2       | none    | none     |
| S. aureus    | ATCC 700699             | $1 \times 10^7$       | none    | none    | none     |
|              | cErmA, VISA             | $1 \times 10^8$       | none    | none    | none     |
|              |                         | $1 \times 10^9$       | none    | none    | none     |
|              |                         | $1 \times 10^{10}$    | none    | none    | none     |
| E. faecalis  | ATCC 29212              | $1 \times 10^9$       | 5       | none    | none     |
|              |                         | $1 \times 10^{10}$    | 8       | none    | none     |
| E. faecium   | HAV 251                 | $1 \times 10^9$       | none    | none    | none     |
|              | Clinical, VRE           | $1 \times 10^{10}$    | none    | none    | none     |
| K. pneumonia | ATCC 10031              | $1 \times 10^9$       | 1       | none    | none     |
|              |                         | $1 \times 10^{10}$    | 5       | 3       | none     |
Extended Data Table 3 | Mutations selected in *E. coli* strain SQ110DTC conferring resistance to iboxamycin

| Clone     | Iboxamycin concentration on selection plate (µg·mL⁻¹) | Mutation | MIC (µg·mL⁻¹) |
|-----------|-------------------------------------------------------|----------|---------------|
|           |                                                       |          | Iboxamycin    | Clindamycin   |
| SQ110DTC  |                                                       | WT       | 0.03          | 16            |
| OPP-3R1   | 1                                                     | A2058G   | 4             | >128          |
| OPP-3R13  | 1                                                     | A2059G   | 2             | >128          |
| OPP-3R14  | 1                                                     | A2058G   | ND            | ND            |
| OPP-3R2   | 0.25                                                  | A2059G   | 2             | >128          |
| OPP-3R3   | 0.25                                                  | A2059G   | ND            | ND            |
| OPP-3R4   | 0.25                                                  | A2058G   | ND            | ND            |
| OPP-3R5   | 0.25                                                  | A2059G   | ND            | ND            |
| OPP-3R6   | 0.25                                                  | A2059G   | ND            | ND            |
| OPP-3R7   | 0.25                                                  | A2059G   | ND            | ND            |
| OPP-3R8   | 0.25                                                  | A2059G   | ND            | ND            |
| OPP-3R9   | 0.25                                                  | A2058G   | 4             | >128          |
| OPP-3R10  | 0.25                                                  | A2058G   | ND            | ND            |
| OPP-3R11  | 0.25                                                  | A2058G   | ND            | ND            |
| OPP-3R12  | 0.25                                                  | A2059G   | ND            | ND            |
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Data collection

X-ray diffraction data was collected at beamlines 24ID-C and 24ID-E at the Advanced Photon Source (Argonne National Laboratory) using NEXUS Remote Access v6.2.0.

Data analysis

Raw X-ray crystallographic data were integrated and scaled using XDS software (Feb 5, 2021). Molecular replacement was performed using PHASER from the CCP4 program suite (version 7.0). All structures were refined using PHENIX software (version 1.17). Structural models were built in Coot (version 0.8.2). Atomic models of iboxamycin were generated from its known chemical structure using PRODRG free online software (http://prodrg1.dyndns.org). All figures showing atomic models were generated using PyMol software (version 1.8). GraphPad Prism 8 was used to plot graphs and to perform statistical analyses.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Coordinates and structure factors are available in the RCSB Protein Data Bank with accession codes 7RQ8 for the T. thermophilus 70S ribosome in complex with iboxamycin, mRNA, deacylated A-site tRNAPhe, aminocylated P-site fMet-NH-tRNAiMet, and deacylated E-site tRNAPhe; and 7RQ9 for the A2058-dimethylated T. thermophilus 70S ribosome in complex with iboxamycin, mRNA, deacylated A-site tRNAPhe, aminocylated P-site fMet-NH-tRNAiMet. Single-crystal X-ray
crystallographic data for compound 6 are deposited at the Cambridge Crystallographic Data Centre under deposition number 2072277. Source data from mouse thigh-infection experiments are provided with this paper. Publicly available crystallographic datasets describing the structure of the bacterial ribosome were used in this study, and are available from the RCSB Protein Data Bank under accession codes 6XHW, 4V7V, and 1YJN.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- [ ] Life sciences
- [ ] Behavioural & social sciences
- [ ] Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

**Sample size**

For mouse thigh-infection experiments, sample size (4 thighs per time point) was chosen in order to provide 80% power to detect significant (p=0.05) difference between treated and untreated animals, anticipating bacterial counts of ~8±1 Log10CFU in untreated thighs and ~6±1 Log10CFU in treated thighs. Sample size in the mouse systemic-infection experiment (8 mice per treatment arm) was chosen in order to provide 90% power to detect significant (p=0.05) difference in ED50 between clindamycin and iboxamycin, anticipating values of 8.6±2.8 mg/kg and 3.5 mg/kg, respectively.

**Data exclusions**

No data was excluded.

**Replication**

MICs were determined in biological duplicate. Time-kill, PAE, and PA-SME studies were performed in biological duplicate, and bacterial counts within these studies were measured in technical duplicate. Mouse thigh-infection experiments were performed in biological duplicate, and bacterial counts from each thigh were measured in technical duplicate. Hemolysis and mitotoxicity assays (ToxGlo) were performed in technical triplicate. Mammalian cell viability assays (CellTiter-Blue) were performed in biological triplicate and technical quintuplicate. The mRNA toe-printing gel depicted is representative of one of two independent experiments. All attempts at replication were successful.

**Randomization**

Mice were randomly assigned to treatment groups receiving iboxamycin, comparator (clindamycin, ciprofloxacin, or azithromycin), or vehicle. Iboxamycin-resistant colonies of E. coli SQ110DTC were randomly selected for gene sequencing. MIC, time-kill, PAE, PA-SME, mammalian cytotoxicity, and frequency-of-resistance experiments were intrinsically randomized, as the experimental groups constituted equally sized aliquots of a common cellular suspension. Likewise, no extrinsic randomization was necessary for mRNA toeprinting, which used a homogeneous cell-free transcription-translation reagent system. For cross-validation during crystallographic model building and refinement, an R-free set was used.

**Blinding**

Blinding was not used in MIC determination because the outcome (turbidity) is not deemed subjective outside the margin of error (one binary dilution) typical of this experiment. Blinding was not used in time-kill, PAE, PA-SME, or mouse thigh-infection studies because the outcome (bacterial counts) was determined by serial dilution, a quantitative technique. Blinding was not used in mammalian cytotoxicity experiments because the outcomes were determined using microplate photometry, a quantitative technique. Blinding was not used in the mouse systemic-infection study because the outcome (survival) is objective. Blinding was not used in frequency-of-resistance experiments because the outcome (number of colonies) is objective. Blinding was not used in mRNA toeprinting experiments because the outcome (differences in translation-arrest sites and inhibition-escape) are evident upon inspection of unprocessed gel images, and no attempt to quantify these differences was made. Blinding was not used in X-ray crystallographic experiments.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
|---------------------------------|---------|
| n/a                             | n/a     |
| [ ] Antibodies                  | [ ] ChiP-seq |
| [ ] Eukaryotic cell lines       | [ ] Flow cytometry |
| [ ] Palaeontology and archaeology | [ ] MRI-based neuroimaging |
| [ ] Animals and other organisms |         |
| [ ] Human research participants |         |
| [ ] Clinical data               |         |
| [ ] Dual use research of concern|         |
## Eukaryotic cell lines

Policy information about cell lines

| Cell line source(s)                                                                 | All cell lines were purchased from ATCC and used at a passage number no greater than 8.
| HepG2 (ATCC HB-8065): Human male, epithelial, hepatocellular carcinoma              |
| HCT116 (ATCC CCL-247): Human male, epithelial, colorectal carcinoma                 |
| K562 (ATCC CCL-243): Human female, lymphoblast, chronic myelogenous leukemia        |
| AS49 (ATCC CCL-185): Human male, epithelial, lung carcinoma                         |

Authentication

None of the cell lines were authenticated.

Mycoplasma contamination

Cell lines were not tested for mycoplasma contamination.

Commonly misidentified lines

None were used.

## Animals and other organisms

Policy information about studies involving animals. ARRIVE guidelines recommended for reporting animal research

| Laboratory animals                                                                 | Five- to six-week-old female CD-1 mice (weighing 22-26 grams) were used in animal experiments. |
| Wild animals                                                                       | The study did not involve wild animals. |
| Field-collected samples                                                             | The study did not involve samples collected from the field. |

Ethics oversight

Animal experiments were reviewed and approved by the IACUC of Harvard University.

Note that full information on the approval of the study protocol must also be provided in the manuscript.