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Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see our Editorial Policies and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed	
☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒	A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒	The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☒	☐	A description of all covariates tested
☐	☒	A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒	A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒	For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	X-ray diffraction data was collected at beamlines 24ID-C and 24ID-E at the Advanced Photon Source (Argonne National Laboratory) using NE-CAT 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, aminoacylated 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, aminoacylated 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

n/a	Involved in the study
☒	☐ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

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 A549 (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 (See ICLAC register)	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.