A long-distance rRNA base pair impacts the ability of macrolide antibiotics to kill bacteria

Maxim S. Svetlov, Sophie Cohen, Nada Alsuhebany, Nora Vázquez-Laslop, and Alexander S. Mankin

*Center for Biomolecular Sciences, Department of Pharmaceutical Sciences, University of Illinois at Chicago, Chicago, IL 60607

Edited by F. Ulrich Hartl, Max Planck Institute of Biochemistry, Martinsried, Germany, and approved December 17, 2019 (received for review November 4, 2019)

While most of the ribosome-targeting antibiotics are bacteriostatic, some members of the macrolide class demonstrate considerable bactericidal activity. We previously showed that an extended alkyl-aryl side chain is the key structural element determining the macrolides’ slow dissociation from the ribosome and likely accounts for the antibiotics’ cidality. In the nontranslating Escherichia coli ribosome, the extended side chain of macrolides interacts with 23S ribosomal RNA (rRNA) nucleotides A752 and U2609, that were proposed to form a base pair. However, the existence of this base pair in the translating ribosome, its possible functional role, and its impact on the binding and cidality of the antibiotic remain unknown. By engineering E. coli cells carrying individual and compensatory mutations at the 752 and 2609 rRNA positions, we show that integrity of the base pair helps to modulate the ribosomal response to regulatory nascent peptides, determines the slow dissociation rate of the extended macrolides from the ribosome, and increases their bactericidal effect. Our findings demonstrate that the ability of antibiotics to kill bacterial cells relies not only on the chemical nature of the inhibitor, but also on structural features of the target.

Results and Discussion

Disruption of the A752-U2609 Base Pair Affects the Ribosomal Response to Regulatory Nascent Peptides. We engineered two E. coli strains to carry the single mutations A752G or U2609C that would partially or completely disrupt the putative base pair, and a third strain where mutations A752G and U2609C were introduced simultaneously to restore the base-pairing potential. Because in the engineered strains the rRNA is exclusively expressed from a plasmid (21), the cells contain pure populations of mutant ribosomes.

Significance

The bactericidal activity of macrolide antibiotics correlates with the presence of an extended alkyl-aryl side chain, which accounts for their slow departure rate from the ribosome. Here, we found that the base pair between 23S ribosomal RNA (rRNA) nucleotides 752 and 2609 located in the macrolide binding site is important for the ribosome functionality and for establishing the unique interactions with the extended side chain of macrolide antibiotics. Disruption of the 752-2609 base pair accelerates the departure of extended macrolides from the ribosome and reduces their cidality. Our results demonstrate that not only the chemical features of the antibiotic, but also the structure of the target site contribute to the ability of the inhibitor to kill bacteria.

Author contributions: M.S.S., N.V.-L., and A.S.M. designed research; M.S.S., S.C., and N.A. performed research; M.S.S., N.V.-L., and A.S.M. analyzed data; and M.S.S., N.V.-L., and A.S.M. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

This open access article is distributed under Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND).

To whom correspondence may be addressed. Email: shura@uic.edu.

First published January 13, 2020.
The doubling time of all three mutants in rich media was practically indistinguishable from that of the WT cells (τ ~ 32 min), and the in vitro activity of the isolated wt and mutant ribosomes was comparable (Fig. 2A). Thus, neither the identity of the 23S rRNA residues 752 or 2609, nor their potential to form a base pair, is critical for cell growth or for general ribosomal functions.

Since nucleotides A752 and U2609 are located in the NPET, where they likely interact with growing proteins, we wondered whether they could play a role in the ribosomal response to regulatory nascent peptides (22). Indeed, disruption of the A752-U2609 base pair decreases the efficiency of translational arrest mediated by SecM (Fig. 2B, lanes 3 and 4), a nascent peptide involved in sensing secretion stress (23). Remarkably, SecM-mediated stalling was reduced in single mutants but was restored in the A752G/U2609C double mutant (Fig. 2B, lane 5). These findings parallel the previous observation that the integrity of the 752-2609 base pair is important for ribosome stalling during translation of the regulatory leader peptide TnaC of the tryptophanase operon (24). Altogether, our data argue that the A752-U2609 base pair does form in the translating ribosome, facilitating the response to nascent peptides.

### The Ribosomes with an Intact or Disrupted 752-2609 Base Pair Bind Macrolides with Comparable Affinities

Because crystallographic studies have indicated that the alkyl-aryl side chain of macrolides can interact with the paired A752/U2609 nucleotides (11, 17) (Fig. 1), it was suggested that disruption of this base pair would decrease the affinity of the drug for the ribosome (11). However, equilibrium binding of SOL to the ribosome was minimally affected by the mutations (Fig. 3A). Hence, varying the identities of the 23S rRNA residues 752 and 2609 or disrupting their base-pairing

### Table 1. Kinetic parameters of dissociation of ERY and SOL from WT or mutant E. coli ribosomes

| Mutation(s)          | ERY*  | SOL†          |
|----------------------|-------|---------------|
|                      | k, min⁻¹ | k_{trap} min⁻¹ | k_{slow} min⁻¹ | Fraction of fast population, % |
| WT(A752/U2609)       | 0.32 ± 0.020 | 0.063 ± 0.018 | 0.0036 ± 0.0008 | 42.7 ± 6.3 |
| U2609C               | 0.15 ± 0.015 | 0.120 ± 0.019 | 0.0085 ± 0.0002 | 68.1 ± 5.4 |
| A752G                | 0.13 ± 0.010 | 0.089 ± 0.020 | 0.0101 ± 0.0002 | 53.1 ± 8.2 |
| A752G/U2609C         | 0.36 ± 0.023 | 0.063 ± 0.045 | 0.0050 ± 0.0001 | 26.9 ± 11.2 |

*The single rate constants for ERY were estimated from the dissociation curves shown in Fig. 3C.
†The fast and slow rate dissociation constants for SOL were obtained by fitting the data of the curves shown in Fig. 3B to double-exponential functions.

### Fig. 2. Role of the 752-2609 base pair in ribosomal activities.

(A) In vitro translation of the sf-GFP protein by WT and mutant ribosomes monitored by fluorescence. Error bars represent SD of three independent experiments. (B) Toeprinting analysis of the SecM-mediated programmed ribosome stalling during translation of the secM gene by WT and mutant ribosomes. Bands representing SecM-arrested ribosomes (Pro codon in the A site) are marked by a red arrowhead. Reactions in lanes 6–9 contained indolmycin, an inhibitor of tryptophanyl-tRNA synthetase, which leads to trapping ribosomes at the Trp codon (gray arrowhead) prior to the secM programmed arrest site. The comparable intensity of the trap-site bands shows that the general translation of secM is not affected by the ribosomal mutations. (C) The bar graph showing relative intensity of the secM arrest bands in samples 2–5. Error bars represent SEM of two independent experiments.
capacity does not impact the macrolides overall affinity for the ribosome.

**The Integrity of the A752-U2609 Base Pair Slows the Departure of the Extended Macrolides from the Ribosome.** To further characterize the interactions between macrolides and ribosomes with or without the A752G/U2609C base pair, we measured the rate of antibiotic dissociation. Consistent with our previous data (10), SOL slowly dissociates from WT ribosomes with biphasic kinetics, likely reflecting the existence of fast and slowly dissociating ribosome–drug complexes (Fig. 3B and Table 1). Similar kinetic was observed for the mutant ribosomes with the restored A752G/U2609C base pair (Fig. 3B and Table 1). In contrast, the single A752G or U2609C mutations accelerated the SOL off rate in both the fast-dissociating and slow-dissociating populations. In addition, the fast-dissociating population became predominant, leading to an overall significantly expedited departure of SOL from the ribosome (Fig. 3B and Table 1). Unlike their effect on dissociation kinetics of SOL, these mutations had only a minor influence on the monophasic dissociation kinetics of ERY (Fig. 3C and Table 1), a drug that lacks the alkyl-aryl side chain (Fig. 1). These data suggest that the effect of the 752-2609 base pair on the rate of drug dissociation from the ribosome is specifically mediated by the interaction established with the macrolide side chain.

**Disruption of the 752-2609 Base Pair Alleviates Macrolide Cidal Property.** We asked whether disrupting the ability of 752-2609 nucleotides to base pair would also interfere with the cidal property of the extended macrolides. First, we determined the minimal inhibitory concentration (MIC) of SOL and found that neither the individual A752G or U2609C, nor the compensatory A752G/U2609C mutations had any pronounced effect on the sensitivity to the drug (MIC-SOL = 0.25–0.5 μg/mL). Of note, the previously reported 4- to 8-fold resistance of the U2609C mutant to the extended macrolides (15) was observed only in strains with the intact *tolC* gene, whereas it was not manifested in the *ΔtolC* cells used in the present experiments.

Having established that the rRNA mutations do not affect MIC-SOL in our strains, we examined the survival of the WT and mutant cells after their exposure to SOL. When exposed to high concentrations of SOL, only ~0.01% of the cells with the 752-2609 base pair could resume growth whereas, in contrast, ~10 times more cells survived the equivalent antibiotic treatment when the 752-2609 base pair was weakened or disrupted by the single mutations (Fig. 4D). The same trend was observed when cells were exposed for varying time intervals to a fourfold MIC.
of SOL (Fig. 4B). This result shows that the integrity of the 752-2609 base pair defines not only the dynamics of the interaction between the drug and the ribosome but also the bactericidal activity of the drug against E. coli. The mutations had only minimal effect on cidality of the side chain-lacking ERY (Fig. 4C).

Although in ~95% of analyzed bacterial genomes the identities of the 23S rRNA residues 752 and 2609 (E. coli numbering) support the base pair formation, the pairing ability is not universal (25). Nevertheless, macrolides with extended side chain can exhibit cidality even against some bacteria lacking the base pair (26). This agrees well with our data showing that although the disruption of the 752-2609 base pair reduced SOL cidality, this antibiotic still killed the mutant cells more readily than ERY, which lacks the alkyl-aryl side chain (Fig. 4C). Conceivably, when the 752-2609 base pair cannot be formed, the macrolide’s extended side chain may maintain stacking interaction with one of the unpaired bases or, alternatively, may reorient and interact with other nearby rRNA residues (20, 27). It is also possible that even in the species where pairing of the 23S rRNA residues 752 and 2609 can occur, the base pair may form only transiently in response to additional cues, e.g., the presence of a nascent protein with a specific amino acid sequence in the NPET or the binding with a specific small molecules, including the extended-chain macrolides (6). The dynamic nature of this base pair could account for the biphasic kinetics of SOL dissociation from the E. coli ribosomes observed in our experiments (Fig. 3B), as well as biphasic binding mode of extended macrolides reported previously (28), where the off and on rates of the antibiotic could depend on the formation or disruption of the pairing of the 752 and 2609 residues.

Although our experiments have been carried out only with SOL, we expect that similar effects would be observed with other extended macrolide antibiotics, e.g., telithromycin, whose alkylaryl side chain establishes equivalent interactions with the A752-U2609 base pair (11, 12). Altogether, our findings suggest that optimizing the interactions with the A752-U2609 base pair by modifying the side chain of macrolides or other drugs binding in a similar ribosomal location could be a strategy to improve the antibiotics cidal activity.

Materials and Methods

Construction of Mutant Strains. Single A752G or U2609C or double A752/G U2609C mutations were introduced by site-directed mutagenesis into 23S rRNA gene of the pAM552 plasmid (29) using the QuikChange Lightning Multi Site-directed mutagenesis Kit (Agilent Technologies). Plasmids were transformed into the Δo70C E. coli SO171 strain lacking chromosomal RNA alleles (21, 30), and transformants were cured off the resident pCsCAB plasmid encoding WT rRNA (31). The presence of the mutation and the purity of the ribosome population carrying the mutant rRNA was verified by sequencing and by primer extension on the total cellular rRNA.

Ribosome Preparation and Binding Studies. WT and mutant ribosomes were purified according to ref. 32. Equilibrium and kinetic binding studies were performed as described in ref. 10.

In Vitro Translation and Toeprinting. Translation and toeprinting reactions were carried out in the Aribosib PURExpress system (New England Biolabs) supplemented with isolated WT or mutant ribosomes as described in ref. 29. Plasmid pv71-efGFP (33) was used as a template for translation of EGFP reporter protein. Expression of EGFP was continuously monitored by fluorescence (485–520 nm) in a microplate reader (Tecan). Toeprinting analysis was carried out as described in ref. 34 using a DNA template prepared by PCR encoding the last 29 codons of the secM gene.

MIC Determination and Cidality Testing. MIC was determined in 96-well plates by serial dilution of antibiotic and incubating plates overnight at 37°C without shaking. The optical density of the standard overnight bacterial cultures was A000 = 0.001. For the analyses of bactericidal action, overnight cultures were diluted 1:500 and grown at 37°C to A000 = 0.2. Various concentrations of SOL or ERY were added and culture dilutions were plated after 4 h incubation. For the time-kil measurements, cells were incubated with 4× MICSOL (2 μg/mL for WT, A752G, and A752G/U2609C mutants, 1 μg/mL for U2609C mutant). Aliquots were withdrawn, and culture dilutions were plated. Colonies were counted following 48- to 72-h incubation at 37°C. For comparison of SOL and ERY cidality, cells were incubated for 4 h with 7× MIC concentrations of the drugs: SOL (3.5 μg/mL for WT, A752G, and A752G/U2609C mutants, 1.75 μg/mL for U2609C mutant); ERY (7 μg/mL for WT, A752G, and A752G/U2609C mutants, 3.5 μg/mL for U2609C mutant).

Data Availability. The engineered strains are available upon request.

ACKNOWLEDGMENTS. We thank Dorota Klepacki, Tanja Florin, and Prabha Fernandes for help and advice; and Yury Polikanov for helpful discussions and help with figure preparation. This work was supported by Grant R21 AI137584 from the National Institutes of Health.

1. D. R. Osmon, Antimicrobial prophylaxis in adults. Mayo Clin. Proc. 75, 98–109 (2000).
2. S. W. Sinner, A. R. Tunkel, Antimicrobial agents in the treatment of bacterial meningitis. Infect. Dis. North Am. 18, 581–602 (2004).
3. E. W. Hooik, 3rd, R. B. Roberts, M. A. Sande, Antimicrobial therapy of experimental enterococcal endocarditis. Antimicrob. Agents Chemother. 8, 564–570 (1975).
4. M. A. Kohnan, D. J. Dwyer, J. Wierzbowsk, G. Cottarel, J. J. Collins, Mitranslation of membrane proteins and two-component system activation triggered antibiotic-mediated cell death. Cell 135, 679–690 (2008).
5. P. Fernandes, E. Martens, D. Bertrand, D. Pereira, The solithromycin journey-It is all in the chemistry. Bioorg. Med. Chem. 24, 6420–6428 (2016).
6. N. Vazquez-Laslop, A. S. Mankin, How macrolide antibiotics work. Trends Microbiol. 34, 668–684 (2018).
7. G. H. Hawks, Antibiotic therapy of staphylococcal infections. Can. Med. Assoc. J. 93, 848–853 (1965).
8. L. J. Norica, A. M. Silva, S. F. Hayashi, Studies on time-kil kinetics of different classes of antibiotics against veterinary pathogenic bacteria including Pasteurella, Actinobacillus and Escherichia coli. J. Antibiot. (Tokyo) 52, 52–60 (1999).
9. L. N. Woosley, M. Castanheira, R. N. Jones, CEM-101 activity against Gram-positive organisms. Antimicrob. Agents Chemother. 54, 2182–2187 (2010).
10. M. S. Svetlov, N. Vazquez-Laslop, A. S. Mankin, Kinetics of drug-ribosome interactions defines the modality of macrolide antibiotics. Proc. Natl. Acad. Sci. U.S.A. 114, 13673–13678 (2017).
26. I. Gustafsson, L. Engstrand, O. Cars, In vitro pharmacodynamic studies of activities of ketolides HMR 3647 (Telithromycin) and HMR 3004 against extracellular or intracellular Helicobacter pylori. *Antimicrob. Agents Chemother.* **45**, 353–355 (2001).

27. D. Tu, G. Blaha, P. B. Moore, T. A. Steitz, Structures of MLSBK antibiotics bound to mutated large ribosomal subunits provide a structural explanation for resistance. *Cell* **121**, 257–270 (2005).

28. O. N. Kostopoulou, A. D. Petropoulos, G. P. Dinos, T. Choli-Papadopoulou, D. L. Kalpaits, Investigating the entire course of telithromycin binding to Escherichia coli ribosomes. *Nucleic Acids Res.* **40**, 5078–5087 (2012).

29. C. Orelle et al., Protein synthesis by ribosomes with tethered subunits. *Nature* **524**, 119–124 (2015).

30. K. Kannan, N. Vázquez-Laslop, A. S. Mankin, Selective protein synthesis by ribosomes with a drug-obstructed exit tunnel. *Cell* **151**, 508–520 (2012).

31. D. Zaporojets, S. French, C. L. Squires, Products transcribed from rearranged rrn genes of Escherichia coli can assemble to form functional ribosomes. *J. Bacteriol.* **185**, 6921–6927 (2003).

32. H. Ohashi, Y. Shimizu, B. W. Ying, T. Ueda, Efficient protein selection based on ribosome display system with purified components. *Biochem. Biophys. Res. Commun.* **352**, 270–276 (2007).

33. B. C. Bundy, J. R. Swartz, Site-specific incorporation of p-propargyloxyphenylalanine in a cell-free environment for direct protein-protein click conjugation. *Bioconjug. Chem.* **21**, 255–263 (2010).

34. C. Orelle et al., Identifying the targets of aminoacyl-tRNA synthetase inhibitors by primer extension inhibition. *Nucleic Acids Res.* **41**, e144 (2013).