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Context-Specific Action of Ribosomal Antibiotics

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Abstract

The ribosome is a major antibiotic target. Many types of inhibitors can stop cells from growing by binding at functional centers of the ribosome and interfering with its ability to synthesize proteins. These antibiotics were usually viewed as general protein synthesis inhibitors, which indiscriminately stop translation at every codon of every mRNA, preventing the ribosome from making any protein. However, at each step of the translation cycle, the ribosome interacts with multiple ligands (mRNAs, tRNA substrates, translation factors, etc.), and as a result, the properties of the translation complex vary from codon to codon and from gene to gene. Therefore, rather than being indiscriminate inhibitors, many ribosomal antibiotics impact protein synthesis in a context-specific manner. This review presents a snapshot of the growing body of evidence that some, and possibly most, ribosome-targeting antibiotics manifest site specificity of action, which is modulated by the nature of the nascent protein, the mRNA, or the tRNAs.

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INTRODUCTION

Antibiotics, drugs that inhibit the growth of bacteria, including pathogenic ones, are the most successful medicines found by humans and have saved millions of lives. Unfortunately, the broad use of antibiotics, both legitimate and frivolous, has resulted in the spread of strains resistant to many antibiotics. Therefore, finding new natural and synthetic antibacterials, or modifying strategies to use known compounds, is one of the most urgent tasks of the modern health sciences.

The majority of currently known antibiotics have been found serendipitously, by testing secondary metabolites produced by soil or marine microorganisms or by screening immense libraries of synthetic compounds. Optimization of prototype antibiotics has generally not been driven by knowledge of the mechanism of action but has been accomplished by synthesizing a large number of derivatives and testing them in various assays. Although these approaches have been occasionally successful in yielding excellent antibiotics currently used in the clinic, the paucity of new drugs in the pipeline calls for unorthodox and innovative approaches to revitalize the discovery process. We strongly believe that exploitation of the knowledge of how antibiotics work should become a guiding principle for drug discovery.

Our current knowledge of the mechanisms of antibiotic action is in fact very limited. In many instances the commonly accepted views on how an antibiotic achieves its inhibitory effect on its target and cell growth are based on research carried out decades ago, as early as the 1960s and 1970s, using the fairly limited arsenal of methods available at the time. Furthermore, the findings of those studies were interpreted within the framework of the contemporary understanding of

the biology and chemistry of the cell and its components. Breakthrough discoveries in the areas of gene expression, cell physiology, and structural biology since then have not necessarily been incorporated into models of the mechanisms of action of many antibiotic families.

The majority of antibiotics inhibit growth and proliferation of bacteria by targeting one of the essential enzymes. Most of such enzymes catalyze fairly simple biochemical reactions, where two substrates combine to form a new molecule, an individual compound is split into two products, or a molecule is transformed into its isoform. Inhibition of such enzymes generally means that the sole reaction they catalyze becomes too slow to sustain cell growth. However, the straightforward rules of enzyme inhibition do not necessarily apply to one of the main antibiotic targets in the bacterial cell—the ribosome—because it handles a multiplicity of substrates and functional partners during the complex process of protein synthesis.

A great variety of clinically used and investigational antibiotics achieve their therapeutic effects by interfering with the functions of the ribosome, which is the central component of the gene expression apparatus and indispensable for sustaining life. In a fast-growing cell, the majority of ribosomes are involved in translation; therefore, antibiotics encounter ribosomes executing varying steps of protein synthesis (**Figure 1**). By analogy with the conventional enzymes, it has been largely assumed that ribosomal antibiotics arrest translation equally efficiently at every codon of a gene. However, one should keep in mind that at each codon the translation complex is different, and antibiotics encounter ribosomes engaged with different mRNAs and translation factors, associated with diverse combinations of tRNA substrates and carrying distinct nascent peptides. Thus, during progression through the mRNA's coding sequences, the ribosome catalyzes peptide bond formation between 400 possible combinations of substrates (since any of the major 20 amino acids can participate in the reaction as donor or acceptor) and is transiently occupied with an even greater number of tRNA combinations. Thus, even without considering the contribution of the ribosome-associated nascent proteins and mRNAs, the variety of ribosomal complexes potentially available in the cell to interact with an antibiotic is enormous.

It is well established that the nature of ligands can significantly affect the properties of ribosomes. tRNAs can bind with distinct kinetics (18, 27) and accuracies (16, 78) and can be translocated with different efficiencies and rates (23, 28, 44). In addition, the efficiency of peptide bond formation is critically influenced by the nature of the donor and acceptor amino acids (39, 106). Because these factors modify the properties of the ribosome as a decoder and catalyst, they should also have a profound impact on the action of the antibiotics that interfere with ribosomal functions. It is actually hard to imagine that inhibitors of protein synthesis, whose action would be independent of the nature of the substrates associated with the ribosome, could even exist.

In this review, we discuss the accumulating evidence that several classes of ribosomal antibiotics inhibit translation in a ligand- or context-specific way. We present several well-documented examples of major classes of ribosome-targeting antibiotics whose activities are modulated by mRNA or nascent protein contexts and discuss the implications of codon- and gene-specific action of antibiotics for combating resistance and developing more efficient treatments. We also describe the current experimental tools that are suitable for investigating site-specific effects of antibiotics on translation.

NEW GENOMIC AND BIOCHEMICAL TECHNIQUES PROVIDE TOOLS FOR REVEALING CONTEXT SPECIFICITY OF ANTIBIOTIC ACTION

Why has the concept that the action of ribosomal antibiotics could be ligand or context specific not been explored in early studies? A simple explanation is that the techniques available at the dawn of the antibiotics era were inadequate for either detecting or examining the gene-specific

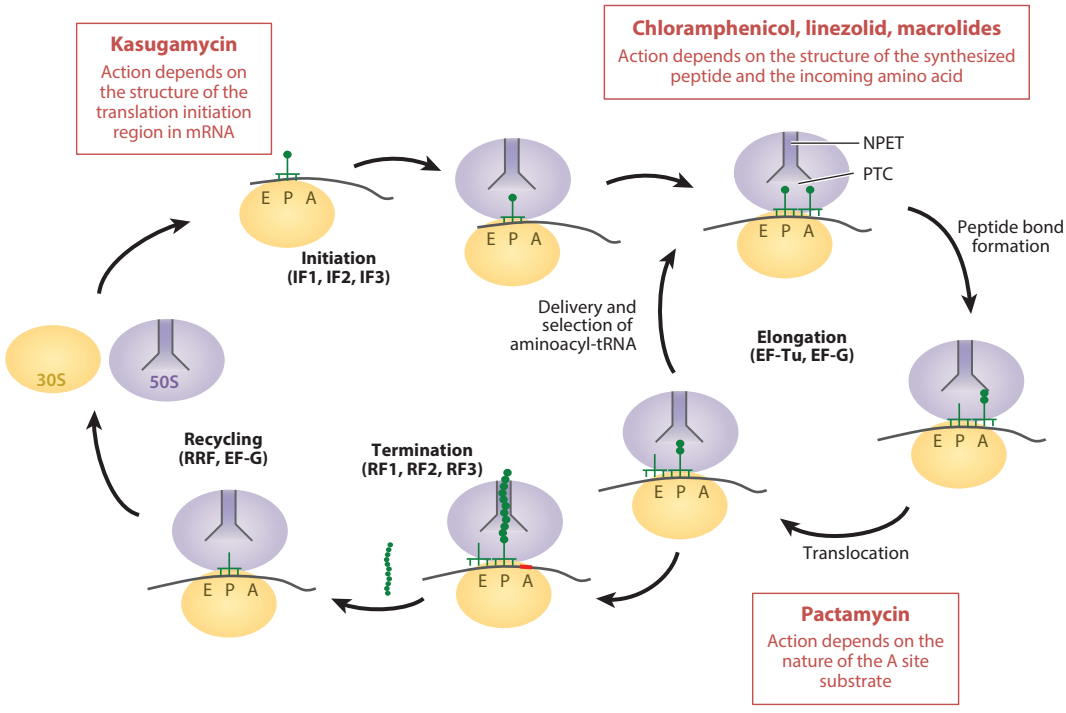


Figure 1

An overview of protein synthesis and the steps inhibited by antibiotics with context-specific mode of action. At the initiation step, the ribosome, assisted by initiation factors (IF1, IF2, IF3), locates the start codon in mRNA and binds the initiator tRNA in the peptidyl (P) site. Kasugamycin selectively interferes with initiation of translation of some mRNAs depending on the structure of their ribosome binding site. During translation elongation, EF-Tu-delivered aminoacyl-tRNAs are selected and then accommodated in the aminoacyl (A) site. As the peptide bond is formed in the peptidyl transferase center (PTC), the growing nascent protein is transferred to the A site tRNA. The antibiotic-mediated direct (chloramphenicol and linezolid) or allosteric (macrolides) inhibition of peptide bond formation depends on the structure of the nascent protein. Subsequent translocation of the ribosome is promoted by the elongation factor EF-G. In the course of translocation, the A site–bound peptidyl-tRNA moves into the P site and the deacylated tRNA is relocated into the exit (E) site. Inhibition of translocation by pactamycin may depend on the nature of the A site substrate. During translation elongation, the growing nascent protein is threaded through the nascent peptide exit tunnel (NPET). When the ribosome encounters a stop codon (*red*), it enters the termination phase, during which the completed protein is released with the help of termination factors (RF1 or RF2, and RF3). Finally, at the recycling phase, the combined action of ribosome recycling factor (RRF) and EF-G splits the ribosome into its subunits.

and moreover codon-specific action of ribosomal inhibitors. Studying inhibition of bulk protein synthesis in vivo or inhibition of translation of a single reporter gene in vitro does not shed light on whether the effect of the drug is the same on all of the cellular proteins. Furthermore, biochemical studies, commonly involving surrogates of tRNA substrates, tell little about the possible influence of various natural ribosomal ligands on drug action.

The major advances in our understanding of how ribosomal antibiotics work have been brought about by several recently developed, powerful in vivo and in vitro techniques. Arguably, the most notable among them are ribosome profiling (Ribo-seq) and primer-extension inhibition (toeprinting) analysis.

Ribo-seq shows the distribution of ribosomes along translated mRNAs in the cell (36, 59) (**Figure 2*a***). It is based on isolation and deep sequencing of the ribosome-protected mRNA fragments and mapping such ribosome footprints to the genome. The Ribo-seq data reveal the average

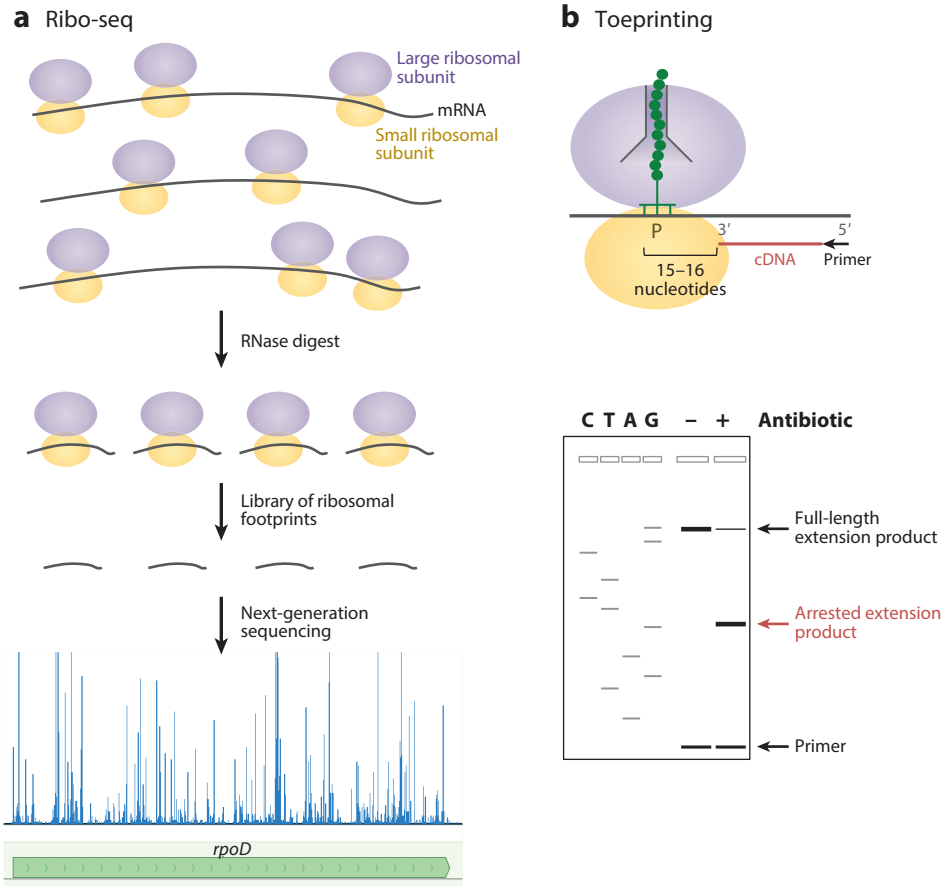


Figure 2

Methodologies for studying the context-specific action of ribosomal antibiotics. (*a*) Ribo-seq, also known as ribosome profiling, involves isolation, deep sequencing, and mapping to the genome of ribosomal footprints, the mRNA segments occupied by ribosomes. Analysis of antibiotic-induced changes in ribosomal footprint pattern at the codon or gene level reveals that the action of several antibiotic families is context specific. (*b*) In vitro toeprinting analysis allows for mapping the position of an arrested ribosome on mRNA with codon precision. (*Top*) Extension of a DNA primer (*black arrow*) by reverse transcriptase (not shown) is interrupted by the presence of a stalled ribosome. The first nucleotide of the codon occupying the peptidyl (P) site and the 3′-terminal end of the truncated cDNA are separated by 15–16 nucleotides. (*Bottom*) The cDNA products, along with sequencing reactions, are resolved in a sequencing gel. The position of the stalled ribosome is inferred from the migration of the toeprint band (*red arrow*). Antibiotic-dependent appearance of toeprint bands reveals the specific codons where translation is arrested.

ribosome occupancy of every codon of the gene. Comparison of individual codon occupancy between the antibiotic-treated cells and the untreated controls uncovers the mRNA sites where the drugs exert the most pronounced inhibition of translation: The occupancy of such codons will be increased in antibiotic-treated cells. Conversely, the codons where the ribosome could successfully evade the inhibitory action of the drug will show decreased occupancy.

In vitro toeprinting analysis allows locating the ribosome within an mRNA with codon precision (33). The assay is based on annealing a DNA primer to the in vitro–translated mRNA template and extending it with reverse transcriptase. The reverse transcriptase stops when it encounters a

ribosome paused on the mRNA, and mapping the 3′ end of the synthesized complementary DNA allows the precise determination of the codon at which the ribosome has stalled (**Figure 2*b***). The application of toeprinting for studying ribosome progression along mRNA has been made possible by the development of the so-called PURE system, where each of the proteins required for translation, tRNAs, and ribosomes are individually purified and then combined together (84). The toeprinting technique has been successfully applied to studies of intrinsic and drug-induced translation arrest (56, 100).

PTC: peptidyl transferase center

CHL: chloramphenicol

LZD: linezolid

Several other technologies can also advance the understanding of antibiotic specificity. The single-molecule fluorescence resonance energy transfer (smFRET) technique allows us to follow the progression of a single ribosome through a number of mRNA codons by monitoring the intersubunit rotation and substrate binding (14, 79). Analysis of smFRET traces makes it possible to observe antibiotic-induced changes in the kinetics of the ribosome traversing through mRNA, as well as the effect of the drugs on the lifetimes of specific functional states of the ribosome at individual codons. Finally, the progress in increasing the resolution of crystallographic and cryo–electron microscopy (cryo-EM) reconstructions has been instrumental in revealing the interactions of antibiotics with ribosomal functional complexes (4, 5, 72, 73, 75). Innovative techniques have made it possible to gain insights into the specificity of action of several classes of ribosomal inhibitors that we discuss in the following sections.

ACTION OF PEPTIDYL TRANSFERASE–TARGETING ANTIBIOTICS DEPENDS ON THE NATURE OF THE DONOR AND ACCEPTOR SUBSTRATES

Formation of peptide bonds and peptide release, which take place in the peptidyl transferase center (PTC), are the only reactions directly catalyzed by the ribosome that involve rearrangement of covalent bonds. Although ribosomal RNA is involved in the proton relay required for peptidyl transfer (26, 74), the ribosome acts primarily as an entropic catalyst, and the major acceleration of the reaction comes from the proper placement of the donor (peptidyl-tRNA) and acceptor (aminoacyl-tRNA) substrates (69, 86, 108).

The PTC is a prevalent target for protein synthesis inhibitors (70, 104). Most PTC drugs sterically hinder accommodation of the substrates in the active site. One of the oldest known PTC-targeting antibiotics is chloramphenicol (CHL), a natural compound produced by several *Streptomyces* species (96) (**Figure 3*a***). CHL has always been considered a classic PTC inhibitor (55, 67, 103), a view compatible with structural studies that show its binding in the aminoacyl (A) site, occupying the space required for the placement of the acceptor amino acid (12, 24, 81) (**Figure 3*b***). Because the proper placement of the acceptor substrate is critical for peptide bond formation, CHL was thought to efficiently block formation of any and every peptide bond.

Although the model of CHL action as a universal inhibitor of peptide bond formation has been broadly accepted and was supported by the ability of the drug to inhibit peptidyl transfer in some model assays, it could not easily account for a number of experimental results. For example, in vivo synthesis of specific polypeptides was seemingly less sensitive to the presence of CHL than translation of other proteins (reviewed in 67). Furthermore, appreciable in vitro inhibition of peptide bond formation between some substrates required strikingly high ($\geq$1 mM) concentrations of CHL (13, 31, 66).

A generally similar controversy has been accumulating about the mode of action of another PTC-targeting drug, linezolid (LZD), a synthetic oxazolidinone antibiotic introduced in the clinic 50 years after CHL (52) (**Figure 3*a***). LZD binds to a ribosomal site that closely matches that of CHL (9, 37, 46, 105) (**Figure 3*b***). Therefore, similar to CHL, LZD was thought to act as a

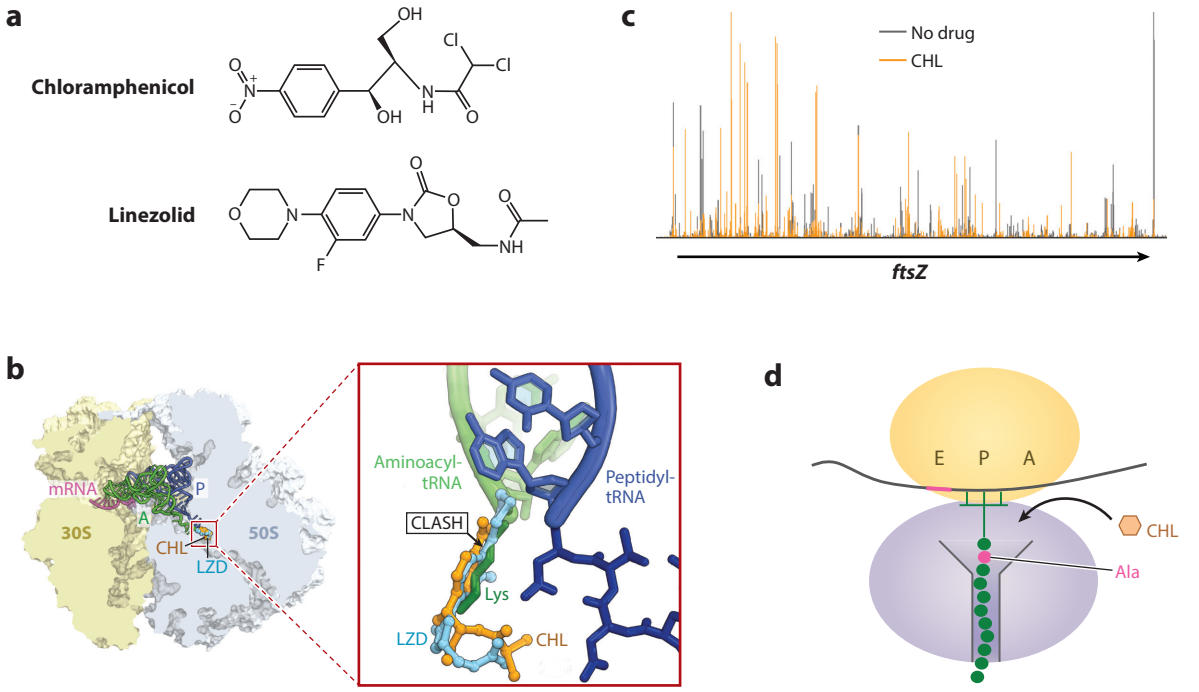


Figure 3

The context-specific action of peptidyl transferase center inhibitors chloramphenicol (CHL) and linezolid (LZD). (*a*) The chemical structures of CHL and LZD. (*b*) The binding site of CHL and LZD overlaps with that of the aminoacyl moiety of the aminoacyl (A) site tRNA. (*c*) Ribo-seq analysis shows that CHL and LZD cause redistribution of ribosomes on mRNA. Changes in the pattern of ribosomal footprints in the *ftsZ* gene illustrate the general trend observed in other genes (51). In the presence of either one of the inhibitors, ribosomes are preferentially arrested at the codons that immediately follow Ala codons. (*d*) The preferred target of CHL (or LZD) is a ribosome carrying a nascent peptide whose penultimate residue is an Ala. The ribosomal A site, peptidyl (P) site, and exit (E) site are indicated.

universal inhibitor of peptidyl transfer because it distorts the placement of the acceptor amino acid in the PTC. However, in model assays utilizing initiator fMet-tRNA$_i^{Met}$ as a donor substrate, LZD either completely failed to inhibit peptide bond formation (43) or produced only modest inhibition even at high concentrations (65). Yet, it could readily inhibit in vitro synthesis of reporter proteins or the overall protein synthesis in bacterial cells (85).

Many of the discrepancies regarding the action of CHL and LZD have been resolved by understanding that neither CHL nor LZD is a universal inhibitor of peptide bond formation. Instead, these PTC-binding drugs interfere with translation in a context-specific manner, that is, by preferentially arresting ribosomes at specific mRNA sites. The key data that revealed context specificity of action of these drugs came from Ribo-seq experiments, in which *Escherichia coli* cells were exposed to high concentrations of CHL or LZD for a short period of time sufficient to completely inhibit protein synthesis, and the distribution of ribosomes along mRNAs was analyzed (51, 57). If, as the classical model would have predicted, CHL and LZD were to inhibit equally well formation of any peptide bond, the drugs would have simply "frozen" the ribosomes on the mRNA codons where the encounter with the antibiotic had occurred. In this case, the distribution of the ribosome footprints along mRNA would not have changed relative to untreated control cells. However, analysis of the Ribo-seq data revealed something very different: Exposure to CHL

ERY: erythromycin
AZI: azithromycin

or LZD caused reallocation of the ribosomes along mRNAs (**Figure 3*c***). It became evident that upon encountering the antibiotic, the majority of the ribosomes still polymerize a few peptide bonds and progress through several codons before being arrested at specific sites.

What distinguishes the sites of preferential action of CHL and LZD? Is it the nature of the amino acids participating in peptide bond formation, the structure of tRNAs, or a unique folding of the mRNA? Bioinformatics analysis of the Ribo-seq data showed that the primary factor that defined the sites of the most pronounced inhibition of translation by CHL or LZD was none of those factors, but the nature of the nascent peptide. Specifically, CHL most efficiently arrested ribosomes when the penultimate amino acid of the growing protein was an Ala (**Figure 3*d***) or, somewhat less frequently, a Thr or a Ser (51). Because of that, the ribosomes were most readily arrested by CHL at the codons that followed Ala, Thr, or Ser codons. The trend was even more pronounced with LZD, where the majority of the arrest sites occurred at the codons that were preceded specifically by an Ala codon. While the penultimate residue of the nascent peptide played the major role in defining the sites of CHL and LZD action, the identities of the amino acids at the peptidyl (P) and A sites also influenced CHL and LZD specificity. In particular, Gly in the P or A sites of the PTC prevented LZD and CHL from arresting translation (51).

For a number of sites, the trend revealed by Ribo-seq could be reproduced in cell-free translation assays. Toeprinting analysis confirmed that CHL and LZD most efficiently arrest translation when the ribosome carries a nascent chain with an Ala in the penultimate position, and mutagenizing this residue to other amino acids decreased antibiotic-mediated arrest (51). In agreement with the Ribo-seq data, Gly residues in the P or A sites of the PTC counteract the in vitro action of either of the inhibitors.

Notably, toeprinting experiments also showed that neither CHL nor LZD could efficiently inhibit formation of the first peptide bond. In view of the aforementioned findings, this is not really surprising: The initiating ribosome does not carry a nascent peptide with a penultimate Ala. Corroborating this observation, metagene analysis of the Ribo-seq data shows that in cells treated with CHL or LZD, the ribosomes do not stall at the initiator codons of the genes but redistribute to the downstream codons, where they are eventually arrested (51). In retrospect, because most of the classic in vitro assays of peptide bond formation employed fMet-tRNA$_i^{Met}$ as a donor substrate, it is not surprising that very high concentrations of CHL or LZD were required to achieve even marginal inhibition of the transpeptidation reaction in these assays (43, 55).

The mechanistic underpinnings of site specificity of action of the PTC-targeting CHL and LZD remain to be elucidated. One plausible scenario is that the presence of Ala, Thr, or Ser in the penultimate position of the nascent protein favors drug binding due to a direct interaction of the A site–bound antibiotic with the short side chain of these amino acids. Other amino acids at the same position may directly or allosterically hinder antibiotic binding or, in the case of glycine, not provide the contacts that would increase the drug's affinity for the PTC. The inability of CHL or LZD to efficiently inhibit peptide bond formation involving Gly as an acceptor could be explained by coaccommodation of the antibiotic and an amino acid with the simplest possible side chain (a hydrogen atom) in the PTC A site.

MACROLIDE ANTIBIOTICS ARREST THE RIBOSOME AT SITES WITH SPECIFIC SEQUENCE MOTIFS

Macrolides are among the most clinically successful ribosome-targeting drugs. The prototype macrolide, erythromycin (ERY) (**Figure 4*a***), as well as drugs of the second generation [such as azithromycin (AZI) or clarithromycin] have been broadly used for decades for the treatment of infections caused by gram-positive pathogens (2, 22). The medical use of macrolides of the third

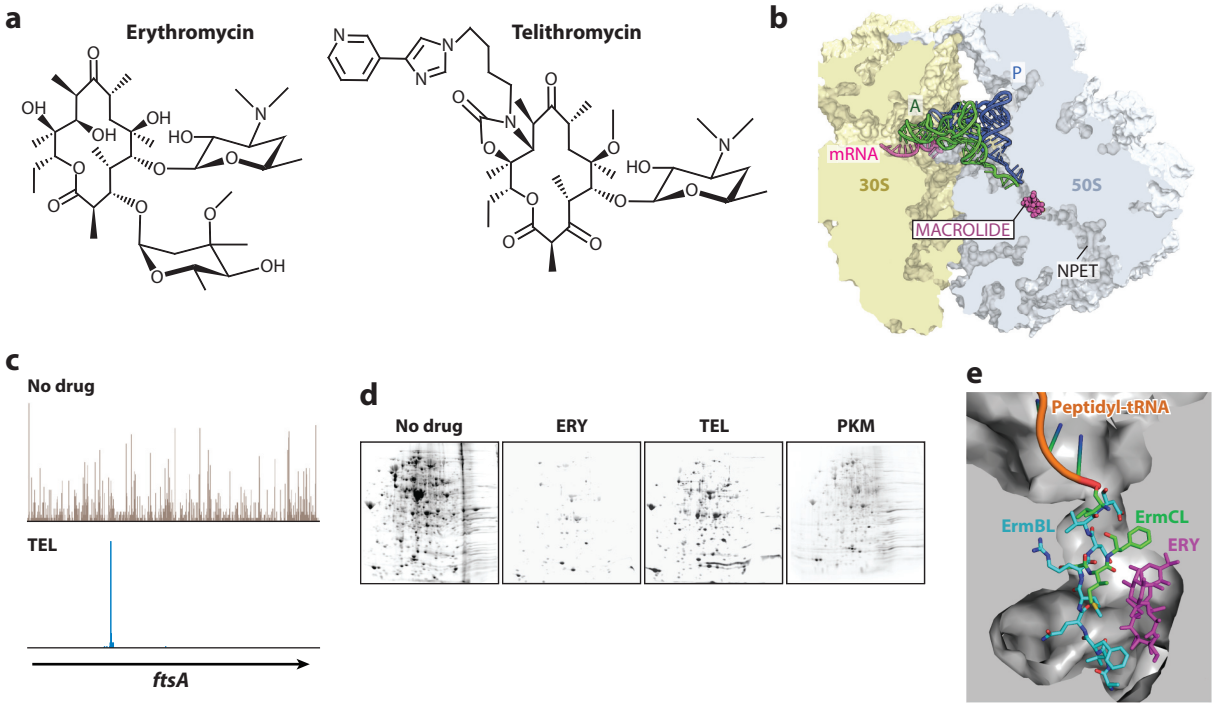


Figure 4

Macrolide antibiotics halt translation of proteins carrying specific sequence motifs. (*a*) The chemical structures of the prototype macrolide erythromycin (ERY) and the ketolide telithromycin (TEL). (*b*) Macrolides and ketolides bind at the nascent peptide exit tunnel (NPET) and partially obstruct the passage of the newly made protein. Aminoacyl (A) site and peptidyl (P) site tRNAs are also shown. (*c*) Ribo-seq data demonstrate that ERY (as well as other macrolides) causes translation arrest at discrete and specific sites along mRNAs. The pattern shown for the *ftsA* gene illustrates the pattern observed with many other genes (41). The peak of ribosome density in the TEL-treated sample corresponds to ribosomes stalled at the 121st (Val) codon of the *ftsA* gene, which constitutes the middle codon of the Arg-X-Arg arrest motif. (*d*) Two-dimensional electrophoresis analysis demonstrating that de novo protein synthesis (determined by active incorporation of radiolabeled [^{35}S]-Met into polypeptides) occurs in *Escherichia coli* cells despite their exposure to saturating concentrations of ERY, TEL, or the natural ketolide pikromycin (PKM). (*e*) The ErmBL and ErmCL nascent peptides can be threaded through the NPET occupied by an ERY molecule (4, 6). Note that nascent protein chains with distinct amino acid sequences take different paths in the tunnel partially obstructed by the antibiotic.

generation, the ketolides [e.g., telithromycin (TEL)] (**Figure 4*a***), is thus far limited due to toxicity issues, but their significantly increased efficacy compared to that of previous macrolides attracts important efforts for developing them into clinically useful antibacterials (reviewed in 11, 29). A recent, highly innovative chemical platform allows combinatorial synthesis of a great variety of new macrolide compounds from their basic building blocks (83) and increases the plausibility of finding new clinically efficient compounds of this class.

Macrolides bind at a short distance from the PTC in the nascent peptide exit tunnel (NPET) (12, 24, 81, 94), the conduit through which the newly made protein leaves the ribosome (**Figure 4*b***). Because macrolides are bulky and the NPET is rather narrow, it has been thought that these drugs obstruct the NPET so significantly that translation of every protein should be aborted when the nascent chain becomes 4–10 amino acids long and its N terminus reaches the macrolide binding site (91). Strangely, however, macrolides are unable to completely inhibit bulk protein synthesis, as estimated by incorporation of radiolabeled [^{35}S]-Met into polypeptides. Even

TEL: telithromycin

NPET: nascent peptide exit tunnel

when *E. coli* cells are exposed to an ERY concentration exceeding the minimal inhibitory concentration (MIC) by a factor of 100, translation continues for several hours at around 7% of the level of the untreated control (42). The incomplete inhibition effect is even more striking with ketolides. Residual translation remains at approximately 20% in cells treated with 100-fold MIC of TEL (42), whereas at equivalently high concentrations, the natural ketolide pikromycin inhibits protein synthesis by only 60% (1). These findings corroborate the results of in vitro experiments that showed that synthesis of some reporters (e.g., poly-Lys) is highly sensitive to macrolide inhibition, but production of other polypeptides (poly-Phe or green fluorescent protein) is nearly indifferent to the presence of specific macrolides (58, 63, 89). The conventional view of macrolides as dull plugs of the NPET was also hard to reconcile with the ability of short peptides with defined sequences to displace macrolide molecules from their binding site in the NPET (49, 92, 93).

Ribo-seq experiments carried out in gram-positive *Staphylococcus aureus* and gram-negative *E. coli* cells treated with macrolides offered an explanation for many of these paradoxes (20, 41). It turned out that macrolides arrest the ribosomes only at a limited number of discrete mRNA sites defined by the presence of specific sequence motifs (**Figure 4*c***).

One of the most prevalent macrolide-arrest motifs revealed by Ribo-seq is Arg/Lys–X–Arg/Lys (where X represents any amino acid). The macrolide-bound ribosome halts translation at the second codon of the motif, when it needs to catalyze the transfer of the nascent protein carrying Arg or Lys in its penultimate position to the acceptor residue of the incoming Lys- or Arg-tRNA. Polymerizing this sequence, which was called the +X+ motif because of the positive charge of Arg and Lys, represents a problem for the ribosome bound to any of the tested macrolides (20, 41, 88). Other sequences could also become troublesome, but only when specific macrolides are bound to the ribosome. For example, ERY and AZI, but less so TEL, inhibit peptide bond formation between Pro and either positively or negatively charged amino acids and also impede the transfer of a nascent chain ending with Asp to Lys-tRNA (20, 41). In addition, some stretches of hydrophobic amino acids, such as the IFVI sequence of the ErmCL peptide encoded in the regulatory open reading frame (ORF) of the inducible macrolide resistance gene *ermC*, cause translation arrest of the ribosome complexed with cladinose-containing macrolides, but not with ketolides (35, 97, 100). Importantly, not every occurrence of the main stalling motif +X+ is necessarily associated with macrolide-induced translation arrest: In some instances, a more extended context, likely involving more distal segments of the nascent chain, could modulate the inhibitory action of the macrolide antibiotic (S. Sothiselvam, A.S. Mankin & N. Vázquez-Laslop, unpublished observations).

Although CHL and LZD act in a context-specific manner, they nevertheless inhibit synthesis of all cellular proteins because virtually every protein in the bacterial cell contains Ala, Thr, and/or Ser, which define the sites of preferential action of these drugs. When it comes to macrolide antibiotics, the situation is different, because the action of these inhibitors calls for the presence of specific amino acid sequences that occur only sparingly in the structures of the proteins. If a protein lacks the sequence that is problematic for the macrolide-bound ribosome, then it can be synthesized even in cells exposed to the macrolide. Furthermore, because the nature and prevalence of arrest motifs depend on the structure of the antibiotic, different macrolides allow for continued translation of different subsets of polypeptides. Thus, electrophoresis analysis shows that cells exposed to TEL synthesize a significant number of proteins (42) (**Figure 4*d***). However, because not only the +X+ but also other motifs are problematic for the ERY-bound ribosome, fewer proteins escape inhibition by ERY (42) (**Figure 4*d***). Conversely, many more proteins are synthesized in the cells treated with the natural ketolide pikromycin, which arrests ribosomes less efficiently even at the commonly difficult +X+ motif (1) (**Figure 4*d***). These observations demonstrate an important point: A protein synthesis inhibitor does not need to completely abolish translation to

be an active antibiotic. As long as the expression of at least one essential protein is interrupted, the bacteria will not be able to grow and proliferate.

Although we still lack a detailed understanding of the molecular mechanisms that define the context selectivity of macrolide action, recent studies have provided several useful insights (4, 6, 7, 77, 88, 99, 100). In the case of the +X+ motif, the extensive direct interaction of the drug in the NPET with the nascent protein segment preceding the arrest motif is apparently not required for halting translation. Thus, synthesis of the MRLR peptide is arrested at the third codon of its gene, when the tripeptide MRL barely reaches the antibiotic molecule in the NPET (87). To account for this result, it was proposed that macrolides allosterically affect the properties of the PTC, interfering with its ability to catalyze peptide bond formation between certain combinations of donor and acceptor substrates (87). In agreement with this model, biochemical and structural studies show rearrangement of key PTC nucleotides in response to the binding of macrolides in the NPET (4, 6, 7, 32, 87). Direct drug-nascent chain interactions may play a more important role with other arrest motifs. Thus, the stalling sequence IFVI of ErmCL failed to arrest the ERY-bound ribosome when present at the N terminus of a protein, whereas it actively halts translation if preceded by five more amino acids (87, 100).

KSG: kasugamycin

Cryo-EM studies showed that the path of the growing protein chain in the macrolide-obstructed tunnel depends on the nascent peptide sequence and the structure of the NPET-bound antibiotic (4, 6, 7) (**Figure 4*e***). In a general scenario, macrolide-induced site-specific translation arrest requires the integration of the signals coming from the structurally restrained nascent chain in the drug-obstructed NPET and the antibiotic molecule (4, 6, 99).

We are only starting to understand why certain substrates cannot properly react in the PTC when macrolides are present. Biochemical studies have shown that not only the positive charge of the key amino acids of the +X+ motif but also the length of their side chains plays an important role in making this sequence particularly problematic (88). It is possible that the presence of the antibiotic bound in the vicinity of the PTC makes it energetically favorable for the extended positively charged side chain of the penultimate Arg or Lys of the nascent peptide to protrude in the direction of the PTC A site and obstruct the accommodation of the similarly charged and equally bulky acceptor amino acid. Comparable scenarios may explain other examples of macrolide-induced translation arrest because smFRET studies and cryo-EM reconstructions show that an ERY-stalled ribosome is reluctant to accept the incoming aminoacyl-tRNA (4, 7, 38).

THE INITIATION INHIBITOR KASUGAMYCIN INTERFERES WITH TRANSLATION OF A SPECIFIC CLASS OF mRNAs

Kasugamycin (KSG) is an inhibitor of translation initiation (60, 71) whose binding site in the 30S subunit overlaps with the last two nucleotides of the exit (E) site codon and encroaches upon the first nucleotide of the P site codon (72, 80, 82) (**Figure 5*a*,*b***). The antibiotic distorts the mRNA path in the ribosome, preventing recognition of the start codon by the initiator fMet-tRNA$_i^{Met}$. On the basis of this proposed mechanism, one would predict that initiation of synthesis of any protein should be disrupted by KSG. However, experimental results show that KSG action depends on the nature and structure of the mRNA.

Early studies showed that KSG differentially inhibits synthesis of cytoplasmic and envelope proteins in *E. coli*, and even expression of individual envelope polypeptides was inhibited to different extents (34). Treatment of *E. coli* cells infected by bacteriophage MS2 or f2 with KSG inhibited synthesis of the phage maturation proteins much more than the coat proteins (45, 61), possibly reflecting idiosyncratic structural features of the ribosome binding sites (RBSs) of their mRNAs

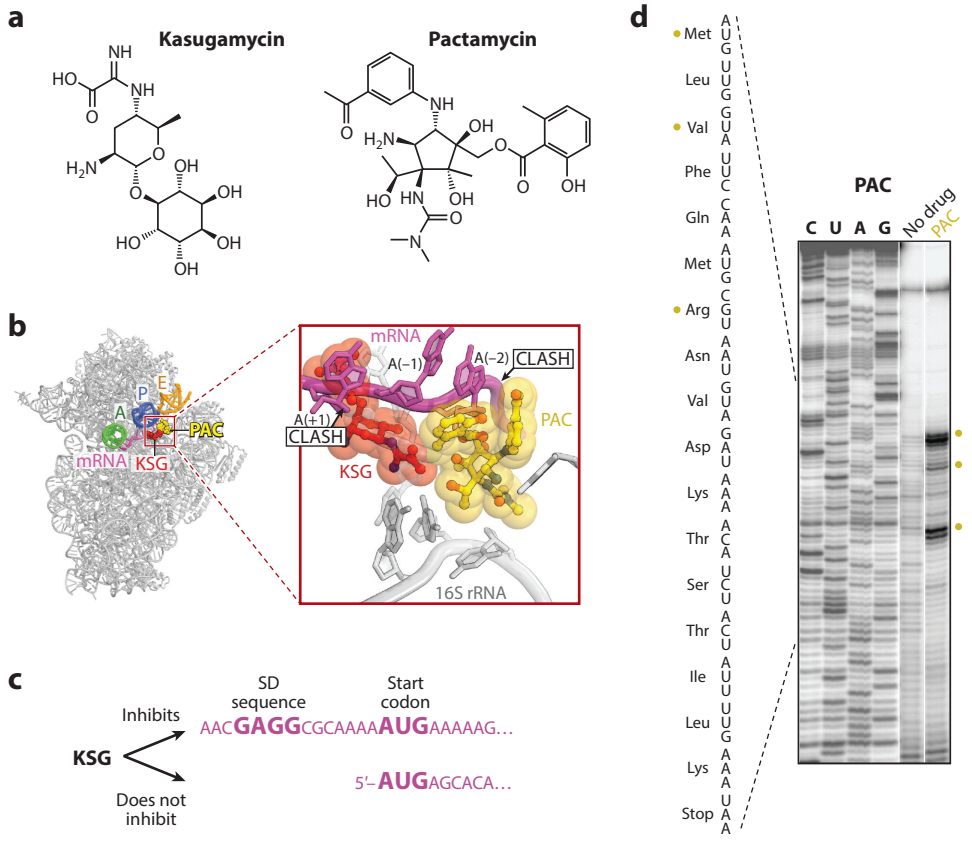


Figure 5

Kasugamycin (KSG) action depends on the structure of mRNA, whereas the activity of pactamycin (PAC) relies on the nature of the aminoacyl (A) site tRNA. (*a*) Chemical structures of KSG and PAC. (*b*) KSG and PAC bind to the small ribosomal subunit and alter the path of the mRNA at the exit (E) site. The first nucleotide of the P site codon is designated (+1). The anticodon stem-loops at the A, peptidyl (P), and E sites are also shown on the left. (*c*) KSG efficiently inhibits translation of leadered mRNAs containing a Shine-Dalgarno (SD) sequence, whereas translation of leaderless transcripts continues in the presence of the antibiotic. (*d*) Toeprinting analysis gel illustrating that PAC inhibits in vitro translation at specific codons of *ermBL* mRNA (62).

(80). In support of this notion, KSG could readily inhibit translation of the reporter *lacZ* gene, whose initiation region had the sequence **AGGA**AACACCC**AUG** [where the Shine-Dalgarno (SD) sequence and start codon are boldface], but not that of the *gst* gene, whose initiation region, **AGGA**AACACUU **AUG**, differed by only two nucleotides (underlined) (82). Extrapolating these observations, one would expect that expression of genes with different RBS sequences could show significantly dissimilar response to KSG treatment. Ribo-seq analysis would be a suitable technique for illuminating at a genome-wide level the RBS variants preferentially targeted by KSG.

A vivid demonstration of the mRNA specificity of KSG action is the inability of the drug to inhibit translation of leaderless mRNAs (15, 53) (**Figure 5*c***). Some *E. coli* phages and transposons carry genes that start immediately with the initiation codon and thus lack the SD sequence. In contrast to the canonical translation initiation, which is driven by the 30S subunit, translation of

leaderless mRNAs is initiated by the 70S ribosome (8, 54, 95). In vivo and in vitro studies showed that neither binding of the ribosome to the start codon of the leaderless mRNAs nor initiation of translation is significantly affected by KSG (15, 53).

In contrast to *E. coli*, whose core genome contains none or at the most very few leaderless genes (101), the genomes of some bacterial species encode a significant number of leaderless mRNAs. It remains to be investigated whether such bacteria are particularly resistant to KSG and whether their treatment with the drug leads to a differential expression of leadered and leaderless mRNAs.

The discrimination by KSG between leadered and leaderless mRNAs in *E. coli* could be further influenced by the accumulation of what are proposed to be specialized ribosomes in the drug-treated cells. Upon exposure to KSG, a fraction of the *E. coli* ribosomes undergo partial disassembly, during which the 30S subunits lose a subset of ribosomal proteins (40). The resulting 61S ribosomal particles can translate leaderless mRNAs, but not mRNAs with an SD sequence. The accumulation of the 61S particles was reported to require the simultaneous presence of KSG and leaderless mRNA (40). It appears, therefore, that the detachment of the 30S subunit proteins occurs when the drug-bound ribosome attempts to initiate translation from the leaderless mRNA start codon, but the mechanistic aspects of the process remain entirely unclear.

PAC: pactamycin

INHIBITION OF TRANSLOCATION BY PACTAMYCIN MAY DEPEND ON THE NATURE OF THE A SITE tRNA

Pactamycin (PAC) inhibits protein synthesis in bacteria, archaea, and eukaryotes by binding to the small ribosomal subunit (10, 25, 50, 107). Similar to KSG, PAC binds at the 30S subunit E site, in the mRNA channel, and likely diverts the mRNA E site codon from its regular placement (10) (**Figure 5*a*,*b***). Although PAC was originally viewed as a specific inhibitor of initiation (30), subsequent studies suggested that the drug interferes with translocation (21). Strangely, however, this activity strongly depends on the nature of the pretranslocation complex, specifically, on the type of the A site substrate. The drug could readily inhibit translocation of the ribosome carrying $tRNA_i^{Met}$ in the P site and the peptidyl-tRNA mimic, N_α-Ac-Lys-$tRNA^{Lys}$, in the A site. However, in a similar complex with *N*-Ac-Phe-$tRNA^{Phe}$ in the A site, no inhibition of translocation was observed even at elevated concentrations of PAC (21). None of the currently available structural data explain why the nature of the mRNA codon, the tRNA body, and/or the nascent chain esterifying the A site tRNA would influence the action of PAC. Nevertheless, the general site specificity of PAC action has been confirmed by toeprinting (62). In the presence of PAC, in vitro translation of several model genes was arrested at preferential sites (**Figure 5*d***), which varied among mRNA templates. The effect of PAC at the genome-wide level and the molecular mechanisms responsible for site specificity of the drug action are yet to be investigated.

AN EXPANDING NUMBER OF RIBOSOME-TARGETING ANTIBACTERIALS WITH CONTEXT-SPECIFIC ACTION

Although, as we have discussed, context-specific action has been investigated for only a few classes of antibiotics, this phenomenon potentially applies to several (possibly many) more classes of ribosome-targeting drugs. Studies employing in vitro toeprinting analysis suggest that several of the long-known ribosomal inhibitors (such as orthosomycins or streptogramin B) and newer antibiotics (e.g., odilorhabdins) arrest translation specifically at distinct mRNA sites (62, 64). Ongoing ribosome-profiling experiments confirm that several of these antibiotics preferentially inhibit cellular translation also at specific mRNA codons (T. Florin, D. Klepacki, K. Mangano, A.S. Mankin & N. Vázquez-Laslop, unpublished results).

A SMALL-MOLECULE INHIBITOR OF THE HUMAN RIBOSOME ABOLISHES PRODUCTION ONLY OF SPECIFIC PROTEINS

Taken to the extreme, the idea of context specificity of antibiotic action opens the possibility of finding ribosome inhibitors that, while allowing translation of most of the cellular polypeptides, could prevent production of a handful of others, or potentially only one particular protein. A spectacular validation of these seemingly far-fetched aspirations came not from the studies of antibacterials, but from an attempt to identify inhibitors of a particular human protein. Proprotein convertase subtilisin/kexin type 9 (PCSK9) regulates the level of low-density lipoprotein cholesterol (LDL-C) in the blood. Lowering PCSK9 levels results in reduction of the LDL-C concentration and decreases the incidence of cardiovascular disease (3, 17). High-throughput screening identified a compound capable of lowering PCSK9 secretion. Subsequent cell culture studies demonstrated that reduced secretion of PSCK9 resulted from selective inhibition of its translation (68). Further optimization yielded compound PF-06446846 (**Figure 6*a***), an even stronger inhibitor of PCSK9 expression (47). Toeprinting and mutational analysis showed that the amino acid sequence of the nascent protein specified by the first 33 codons of the PCSK9 gene is required for PF-06446846 to be able to reduce protein production. Treatment of cultured human cells with PF-06446846 led to specific arrest of translation at codon 34 of the PCSK9 gene, as revealed by Ribo-seq (**Figure 6*b***). However, the Ribo-seq data showed that the compound affected translation of another 22 proteins by arresting translation at specific codons of the corresponding genes, and cell-free experiments confirmed that the expression of a number of these polypeptides was indeed sensitive to PF-06446846.

PF-06446846 directly binds to the ribosome (47). Its mechanism of action is reminiscent of that of macrolide antibiotics, and the dependence of translation stalling on the nature of the nascent peptide is consistent with the binding of the inhibitor in the NPET of the human ribosome. The mechanistic details of action of PF-06446846 are unclear. Except for the prevalence of Leu-rich stretches, the sequences of the nascent peptides in the NPET of the arrested ribosome do not show any obvious similarity (**Figure 6*c***). Furthermore, translation of many other human proteins containing comparable Leu-rich motifs is unaffected by PF-06446846, indicating that either special folding of the nascent chain in the NPET or unrecognized features of the sequence context define the genes that could be inhibited by the compound.

The unwanted side effects precluded further development of PF-06446846 into a clinical drug. Nevertheless, its discovery demonstrates the feasibility of searching for (and finding!) ribosome inhibitors whose action is highly context specific.

WHY DOES CONTEXT SPECIFICITY OF RIBOSOMAL ANTIBIOTICS MATTER?

One might ask how the understanding of context specificity or gene specificity of antibiotic action helps to develop better drugs or expand our knowledge of cell biology. We offer here just three of many reasons.

Context Specificity Helps to Understand and Fight Antibiotic Resistance

Many resistance genes are activated only when the cell is exposed to the antibiotic. Expression of such genes relies on a sensing mechanism, which recognizes the presence of the inducing antibiotic, and on a response circuit that triggers gene expression when the inducer is detected. If the antibiotic is not recognized as an inducer, the resistance gene stays silent and cells remain

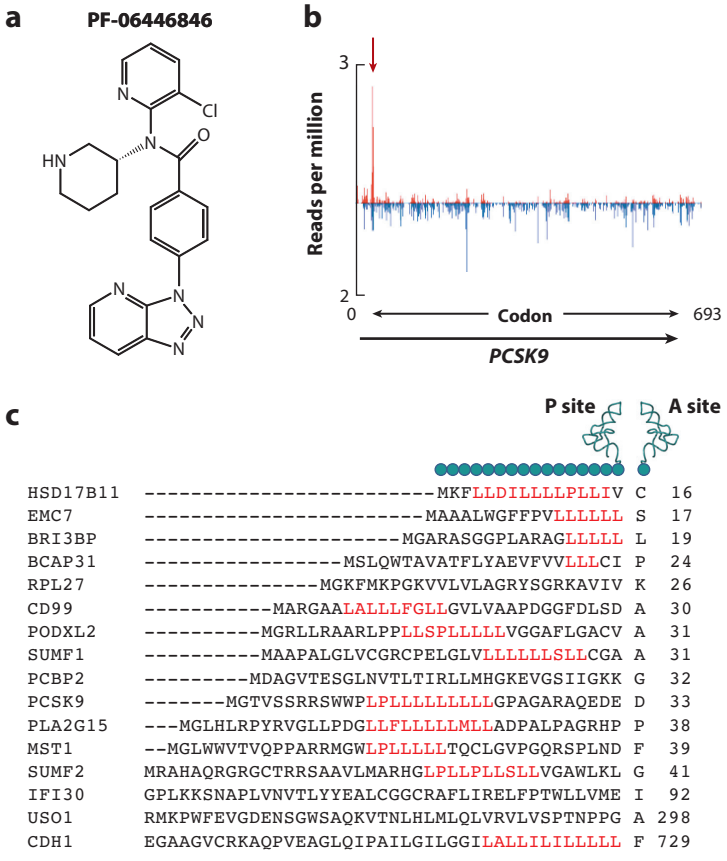
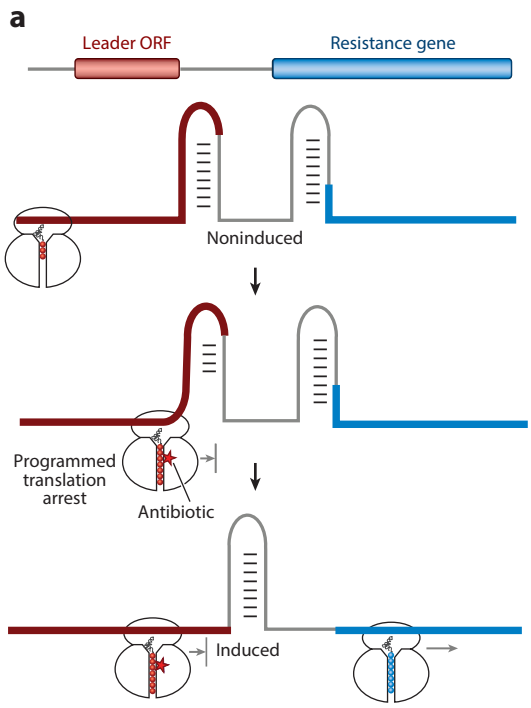


Figure 6

A highly specific small-molecule inhibitor of the human ribosome whose activity depends on the sequence of the nascent protein. (*a*) The chemical structure of compound PF-06446846. (*b*) Ribosome profiling shows that PF-06446846 specifically arrests translation at codon 34 of the human PCSK9 gene (47). (*c*) The amino acid sequences of the nascent peptides at the sites of translation arrest induced by PF-06446846 and the nature of the incoming aminoacyl (A) site amino acid (47). Several of the inhibited peptides contain Leu-rich sequences (*red*), hinting that specific amino acid motifs may be important for PF-06446846-mediated translation arrest. The codon number within the corresponding gene at which major arrest occurs is indicated to the right of the sequence. Panel *b* adapted from Reference 47 (CC BY license).

susceptible to the drug. Understanding the mechanism of activation of such genes could lead to the development of antibiotics that would be undetectable by the sensing system and would not activate the resistance mechanism.

Regulation of inducible genes conferring resistance to the ribosomal inhibitors often exploits programmed translation arrest within the regulatory leader ORFs (reviewed in 48, 90, 98, 102) (**Figure 7*a***). In particular, this mechanism controls expression of macrolides and CHL resistance genes. In the absence of antibiotic, the leader ORF is constitutively translated while the resistance cistron remains silent because its RBS is sequestered in mRNA secondary structure (**Figure 7*a***). When the inducing antibiotic is present, the ribosome stalls at a specific codon of the leader ORF. This triggers a conformational change in the mRNA that releases the previously occluded RBS and results in activation of the resistance gene.



b

Leader ORF	Amino acid sequences of the encoded peptides
msrSAL	MTASMRLK
msrCL	MTASMKLRFELLNN…
ermDL	MHFIRLRFLVLNK…
ermQL	MNGGIASIRLRR
erm39L	MASMSVTYIRLRI
ermXL	MLISGTAFLRLRTNRT…
ermUL	MTPSFPPYSHINDGKIQRALARLR…
ermDL(2)	MIKRNAFGFRRYDRLRNRIL…
pikR2L	MQFCHSQARYMRLRIS

Figure 7

Activation of antibiotic-resistance genes relies on the context-specific action of ribosome-targeting antibiotics. (*a*) Programmed translation arrest at the leader open reading frame (ORF) mediates the change in mRNA conformation necessary to activate expression of the resistance gene. (*b*) Many of the regulatory peptides responsible for activating macrolide-resistance genes contain the sequence motif +X+ (*red*), a prevalent arrest motif for macrolide antibiotics.

Before the context specificity of the action of macrolides or CHL was uncovered, it was unclear how antibiotic-induced programmed translation arrest operates: Why in the presence of the inducing antibiotic can the ribosome translate through several codons of the leader ORF and then stop at one specific codon? The rules of context specificity of antibiotics explain how the system works. The peptides encoded in the leader ORFs carry the sequence that is poorly translated by the ribosome bound with the inducing drug. Thus, many leader ORFs of macrolide resistance genes carry the +X+ motif (**Figure 7*b***) or other motifs known to be problematic for the macrolide-bound ribosome (20, 76, 87, 90). Similarly, programmed translation arrest in inducible CHL resistance genes exploits the specificity of CHL action: The site of the programmed ribosome stalling is defined by the appearance of the first Ala codon (the leader ORF of the *cmlA* gene) or the first Thr codon (the leader ORF of the *cat86A* gene) (51).

An understanding of the principles of context specificity not only allows prediction of which antibiotics would remain invisible to the sensory system and fail to activate resistance but also could direct future efforts toward developing noninducing antibiotics.

Protein-Selective Antibiotic Action Could Lead to Species-Specific Drugs

Context specificity of macrolides, and perhaps several other classes of ribosomal antibiotics, is manifested as inhibition of a defined subset of cellular proteins (1, 42, 89) (**Figure 4*d***). However,

the sequences of homologous proteins could vary significantly in different bacterial phyla. Therefore, the spectra of polypeptides inhibited by the same drug in diverse bacteria could be dissimilar. This could be one of the reasons why the efficacy of antibiotics against different bacterial species can differ significantly.

The context specificity of antibiotics depends on the structure of the compound and thus is tunable by altering the drug's chemical makeup (1, 32, 89, 97). Optimizing the antibiotic structure for inhibiting specific motifs, and thus preventing translation of a defined subset of cellular proteins, could be a way to target specific pathogenic bacteria while sparing the beneficial species of the human microbiome. With the expansion of our knowledge of context specificity, this concept could become a guiding principle for the rational optimization of ribosome-targeting therapies.

Ribosome-Targeting Antibiotics as Translation Modulators

The growing understanding of the context specificity of ribosomal antibiotics suggests that we should view them not simply as inhibitors of protein synthesis but rather as modulators of translation (19, 32). Therefore, antibiotics and other ribosome-targeting small molecules could be used not simply to halt translation but to remodel the cellular proteome. The case of the specific inhibitor of PCSK9 synthesis in human cells discussed above is one manifestation of this principle. Once the context specificity of drug action is better understood, the efforts to identify ribosome-targeting compounds capable of interfering with the synthesis of only the unwanted proteins would have the proper knowledge-based foundation. Hopefully, we are on the way to making new medicines by clearly understanding how they work rather than by expanding our ability to blindly screen millions of random compounds in search of fortuitous hits.

CONCLUDING REMARKS

The concept of context specificity of antibiotic action is fairly new. Like many advances in science it is propelled by novel techniques that allow us to take a fresh look at drugs that have been studied for decades as well as at emerging ribosome inhibitors. So far, only a few attempts have been made to take advantage of Ribo-seq, toeprinting, smFRET, and other codon- or gene-specific techniques to expand our understanding of the mechanisms of antibiotic action. We are confident, however, that we will soon learn of new examples of ribosome-binding small molecules that act as modulators rather than general inhibitors of translation.

So far, we have only scratched the surface of the phenomenon of context specificity of ribosomal drugs. We still have a very limited understanding of why particular motifs are problematic for the antibiotic-bound ribosome and how changes in the structures of drugs affect the specificity of their action. Answers to these questions not only will expand our arsenal of approaches for knowledge-based design of better drugs but also will likely illuminate the yet unknown important aspects of ribosome function.

SUMMARY POINTS

1. Several ribosomal antibiotics inhibit translation in a context-specific manner.
2. The action of chloramphenicol and linezolid depends on the nature of the penultimate amino acid of the nascent protein.

3. Specific amino acid sequences are difficult to synthesize for the macrolide-bound ribosome.
4. Macrolides inhibit synthesis of a subset of bacterial proteins; the spectrum of the inhibited proteins depends on the antibiotic structure.
5. Kasugamycin differentially inhibits initiation of translation of mRNAs depending on the presence and the structure of the nucleotide sequence preceding the start codon.
6. The inhibition of translocation by pactamycin may depend on the nature of the A site substrate.
7. A small-molecule inhibitor of the human ribosome selectively arrests translation of a small subset of proteins.

FUTURE ISSUES

1. Applying mRNA- and codon-specific technologies, such as Ribo-seq and toeprinting, to a broad range of ribosomal antibiotics in order to identify inhibitors whose action is context specific.
2. Unraveling the molecular mechanisms of context specificity.
3. Using the knowledge of context-specific action of antibiotics as a tool to learn about properties of the ribosome that depend on the nature of its associated ligands and substrates.
4. Exploiting context specificity of antibiotic action to develop new and better medicines.

DISCLOSURE STATEMENT

Over the years, research in the Mankin/Vázquez-Laslop laboratory has been supported in part by grants from the pharmaceutical industry, including companies developing some of the antibiotics mentioned in this article. We believe this has not affected the objectivity of this review.

ACKNOWLEDGMENTS

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33. Introduction of toeprinting analysis, one of the methodologies that can reveal context specificity of ribosomal inhibitors.

36. Introduction of Ribo-seq, an approach able to reveal context-specific action of antibiotics in vivo.

40. Report that kasugamycin induces formation of specialized ribosomes that preferentially translate a subset of proteins.

41. Along with Reference 20, first evidence of the context specificity of macrolide antibiotics action in vivo.

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