Macrolide Antibiotics: Binding Site, Mechanism of Action, Resistance

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Abstract: Macrolides are among the most clinically important antibiotics. However, many aspects of macrolide action and resistance remain obscure. In this review we summarize the current knowledge, as well as unsolved questions, regarding the principles of macrolide binding to the large ribosomal subunit and the mechanism of drug action. Two mechanisms of macrolide resistance, inducible expression of Erm methyltransferase and peptide-mediated resistance, appear to depend on specific interactions between the ribosome-bound macrolide molecule and the nascent peptide. The similarity between these mechanisms and their relation to the general mode of macrolide action is discussed and the discrepancies between currently available data are highlighted.

Key words: macrolides, erythromycin, ketolides, azithromycin, clarithromycin, tylosin, carbomycin, spiramycin, ribosome, resistance.

INTRODUCTION

Macrolides belong to one of the most commonly used families of clinically important antibiotics used to treat infections caused by Gram-positive bacteria such as Staphylococcus aureus, Streptococcus pneumoniae and Streptococcus pyogenes. Chemically, macrolides are represented by a 14-, 15- or 16-membered lactone ring carrying one or more sugar moieties and additional substitutions linked to various atoms of the lactone ring, Fig (1).

Erythromycin A, a 14-membered ring drug, was the first clinically used macrolide. Drug delivery problems resulting from acid instability, prompted the design of newer macrolides. Increased acid stability as well as an increase in the range of antimicrobial activity characterized the second generation, which includes 14-membered ring drugs, such as clarithromycin and roxithromycin as well as the 15-membered ring azithromycin. In addition, the 16-membered ring macrolides such as tylosin, carbomycin A and spiramycin and others also exhibited significant antimicrobial activity, and were originally thought to be the answer to the growing occurrence of erythromycin resistant infections. Unfortunately, all of these drugs became prone to the selection of resistant strains. The newest generation of macrolides, the ketolides, whose clinical use is in its early stage, are characterized by improved activity against some of the resistant strains.

It has been well documented that macrolides bind to the large ribosomal subunit in the vicinity of the peptidyl transferase center and cause cell growth arrest due to inhibition of protein synthesis [1,2]. However, in spite of more than 50 years of research, the mode of macrolide inhibition of ribosome activity is understood only in the most general terms.

The extensive use of these antibiotics has led inevitably to the spread of resistant strains. Expression of some of the resistance determinants is inducible by macrolides. Of particular interest are Erm methyltransferases, which specifically methylate a unique nucleotide within the macrolide binding site. The mechanism of Erm induction depends on ribosome stall within the translated regulatory open reading frame preceding the Erm cistron, and is apparently closely related to the general mode of macrolide action on protein synthesis. However, the details of the mechanism of Erm induction are not known.

In addition, a novel mechanism of macrolide resistance has recently been described which is mediated by the expression of specific short peptides in the cell. Although it is not entirely clear how expression of short peptides can render cells resistant to macrolides, the underlying principles maybe related to the mechanisms of inducible resistance and the mode of drug-induced inhibition of translation.

In this brief review we try to summarize the current understanding of the mechanism of macrolide action, and the molecular mechanisms of inducible erm expression and peptide-mediated macrolide resistance. Several reviews dealing with these subjects appeared in the past and we refer the reader interested in specific details to those publications [1-4]. Instead, we will try to focus on the apparent discrepancies and less clearly defined details in the existing models. We strongly believe that understanding these discrepancies exist and finding ways to resolve them will...
help to direct further research focused on the molecular mechanisms of macrolide action and drug resistance.

**THE MACROLIDE BINDING SITE**

The general location of the macrolide binding site on the large ribosomal subunit has been initially mapped using a combination of biochemical and genetic methods [2,5-9]. Nonetheless, the details of the molecular interactions of the various classes of macrolides with the ribosome have just started to emerge with the release of several crystallographic structures of the archaeal and bacterial large ribosomal subunits and their complexes with antibiotics [10-13].

The X-ray structures confirm conclusions drawn from biochemical data, which indicated that RNA constitutes the primary component of the macrolide binding site, Fig. (2A). A number of nucleotide residues in domain V of 23S rRNA interact with the macrolide molecule. Important contacts, which contribute markedly to the strength of interaction of the macrolide molecule with the ribosome, are formed between the C5 mono- or disaccharide side chains of 14-15- and 16-membered ring macrolides and rRNA. The desosamine sugar of erythromycin and other related 14-membered ring macrolides form hydrogen bond interactions with the nitrogen bases of the nucleotide residues A2058, A2059 (here and throughout the entire paper we will use *E. coli* nucleotide numbering to facilitate the discussion). The
base pair 2611-2057, more specifically, the nucleotide occupying position 2057, may also be involved in hydrogen bonding with the C5 desosamine of 14-membered ring macrolides (as seen in the bacterial, \textit{Deinococcus radiodurans} structures) \cite{12}, or it may establish hydrophobic interactions with the lactone ring (as seen in the complexes of 15- and 16-membered ring macrolides with the archaeal 50S ribosomal subunit) \cite{13}. In addition, the desosamine sugar can potentially interact with the backbone phosphate oxygen of G2505. The mycaminose O2 of the mycaminose-mycarose disaccharide of 16-membered ring macrolides also forms a hydrogen bond with adenine at the position 2058 revealing this nucleotide as one of the main binding determinants of all macrolides. The C5 mycaminose-mycarose disaccharides of tylosin, carbomycin A and spiramycin form a number of additional interactions, mainly of a hydrophobic nature. The interactions of the C5 sugar residues with positions 2057-2059 explain why mutations at these nucleotides or dimethylation of A2058 can render macrolide resistance \cite{12}.

Interaction of the lactone ring with the ribosome may account for more than 25% of the free binding energy of the drug \cite{14}. Hydrophobic interactions appear to contribute significantly to this interaction. In particular, the lactone ring interacts hydrophobically with a crevice formed by rRNA bases 2057-2059 \cite{14}. The crystallographic structures of complexes of the 16-membered ring macrolides, tylosin, carbomycin A and spiramycin, with the large ribosomal subunit of \textit{Halocarcula marismortui} \cite{13} suggest that the acetaldehyde group at C6 position of the lactone ring forms a reversible covalent bond with the N6 of A2062, which may contribute to the binding energy of these drugs. This interaction is not possible for the 14-membered ring macrolides of the erythromycin group or the 15-member ring azithromycin, which carry either a hydroxyl or an ester at the C6 position of the lactone ring. This explains why 2062 mutation confers resistance specifically to 16-member ring, but not 14- or 15-membered ring macrolides \cite{15}.

Ketolides are the most recently introduced generation of macrolides. They are characterized by the replacement of the C3 cladinose sugar with a keto group. In addition, most clinically relevant ketolides also contain extended alkyl-aryl side chain appendages as well as an 11, 12-carbamate cycle. Ketolides exhibit increased binding to the ribosome compared to macrolides of previous generations \cite{16,17}. In contrast to expectations, the recently solved structure of a complex of a ketolide, ABT-773, with the \textit{D. radiodurans} ribosome (Schlünzen and Yonath, personal communication), did not provide clear clues into the molecular determinants of increased ketolide affinity. The 11-OH and 12-OH lactone hydroxyls of erythromycin and related drugs could form hydrogen bonds with O4 of U2609. The carbamate cycle that replaces these hydroxyls in ABT-773 (and other ketolides) is seen interacting with O4 of U2609 in \textit{D. radiodurans} 50S subunits. Biochemical data show that this interaction is more important for ketolides than for the drugs of the erythromycin group because the mutation of U2609 to C confers resistance specifically to ketolides but not other macrolides \cite{14}. However, crystal structures did not provide a clear explanation of the significance of this mutation.

Biochemical and genetic data revealed interaction of various macrolides, including erythromycin, ketolides and tylosin, with the loop of helix 35 in domain II of 23S rRNA \cite{7,9}. Mutations or changes in posttranscriptional modification in this rRNA region confer weak resistance to macrolides \cite{7,18}. In addition, a bound macrolide either enhanced or decreased accessibility of nucleotides in the helix 35 loop to chemical modification in the RNA probing experiments \cite{5,7,9,14}. Protection or enhancement of accessibility of A752 to dimethyl-sulfate modification appeared to correlate with the chemical structure of the alkyl-ary side chain of ketolides, suggesting that the side chain might contact the loop of helix 35 \cite{16}, (Xiong and Mankin, unpublished). Although crystallographic structures show that the mucinoside side chain of 16-member ring tylosin can apparently reach the loop of helix 35 \cite{14}, no interactions of 14-membered ring macrolides of the erythromycin type, ABT-773 or azithromycin with this rRNA region were revealed \cite{12}, (Schlünzen and Yonath, unpublished). The quinolyllalyl side chain of ABT-773 forms hydrogen bonds with C1782 in domain IV of 23S rRNA and U790 in domain II. However, the latter interaction it is not close enough to the loop of helix 35 to explain the protection of A752 by ABT-773 observed in the RNA footprinting experiments \cite{14}. Thus, it remains unclear whether the ketolide side chain and/or the loop of helix 35 in the crystal structure are positioned exactly as in the “live and breathing” ribosome. Altogether, crystallographic studies did not show any strong interactions of the ketolide side chain with the ribosome, leaving the 11, 12-carbamate and its interaction with U2609 as potentially the major factor enhancing ketolide binding.

A macrolide molecule is coordinated in its binding site by multiple hydrophobic and hydrogen bonds (and possibly, a covalent bond in case of some 16-membered ring macrolides) between its functional groups and 23S rRNA. These interactions with RNA account for most of the free energy of drug binding. In addition, some macrolides can reach ribosomal proteins L4 and/or L22. The mucinoside sugar of tylosin interacts with L22 and the furosamine residue of spiramycin interacts with L4 \cite{14}. The proximity of these proteins to the macrolide binding site explains why mutations in L22 and L4 protein genes can render cells resistant to macrolides \cite{19-22}.

When analyzing crystallographic structures of the ribosome-macrolide complexes, it is important to keep in mind that although the general features of the drug-ribosome interactions observed with the 50S subunit of a halophilic archaeon (\textit{H. marismortui}) and a bacterium (\textit{D. radiodurans}) are in general agreement, some details vary. For example, the precise position and conformation of the lactone ring of several macrolides seen in structures solved by the Yonath group \cite{12} differ from those seen by the Steitz laboratory \cite{14}. At this point it is unclear whether the discrepancies are due to uncertainty in the crystallographic structures, differences in the ribosomes used, or the difference in crystallization procedures (co-crystallization versus crystal soaking). It is important to note, that in the 23S rRNA of \textit{H. marismortui} several positions involved in macrolide binding are different from those in the bacterial 23S rRNA.
These include such important positions as 2058 (A in bacteria, G in archaea) and 2609 (U in bacteria, C in H. marismortui). The nucleotide sequence differences might potentially affect the affinity of the macrolide molecule to the ribosome and precise molecular interactions in the binding site. Indeed, the mutation of G2058 to A in the closely related archaeon, Halobacterium halobium, significantly increases sensitivity of the mutant to erythromycin and other macrolides (Mankin, Xiong, Tait-Kamradt, unpublished). However, even the drug complexes with the bacterial ribosome of D. radiodurans should be considered cautiously: having only one bacterial structure is not sufficient to distinguish between the general and species-specific determinants of macrolide binding. For example, two molecules of azithromycin were found bound to the D. radiodurans ribosome in the co-crystallization experiments. One of the azithromycin molecules occupied the “conventional” site while the other is seen bound immediately next to it, making direct contact with the proteins L4 and L22 as well as domain II of 23S rRNA. The significance of this second site with regard to azithromycin inhibitory action remains unclear. It could possibly be a species-specific feature because the amino acid Gly60 in protein L4 that is contacted by the drug is unique in D. radiodurans compared to other bacteria (Franceschi, personal communication).

LOCATION OF THE MACROLIDE BINDING SITE IN THE RIBOSOME AND THE MECHANISM OF ACTION

The precise mechanism of protein synthesis inhibition by macrolides depends on the specific chemical structure of the drug molecule. This affects its interaction with the ribosome as well as the mode of the inhibitory action. Four modes of inhibition of protein synthesis have been ascribed to macrolides: 1) Inhibition of the progression of the nascent peptide chain during early rounds of translation [23,24]; 2) Promotion of peptidyl tRNA dissociation from the ribosome [25]; 3) Inhibition of peptide bond formation [23]; and 4) Interference with 50S subunit assembly [26]. All of these mechanisms have some correlation with the location of the macrolide binding site on the ribosome.

The macrolide binding site is located on the large ribosomal subunit inside the nascent peptide exit tunnel near the peptidyl transferase center, Fig. (2B and 2C). Its proximity to the peptidyl transferase center explains the inhibitory effect of some macrolides on peptide bond formation. The sugar residues attached at the C5 position of the lactone ring protrude towards the peptidyl transferase center. The long disaccharide mycaminose-mycarose side chains of the 16-membered ring drugs tylosin, spiramycin and carbomycin A stretch far enough towards the active site of the peptidyl transferase to directly interfere with the catalysis of peptide bond formation [13,27]. The shorter desosamine monosaccharide residues of the 14-membered ring macrolides do not reach the peptidyl transferase, which explains the lack of inhibitory effects of these drugs on the reaction of transpeptidation [24,28,29]. In contrast, some mild stimulation of peptidyl transfer by erythromycin is observed in the puromycin reaction [23].

The main mechanism of inhibition of protein synthesis by macrolides is related to their binding in the nascent peptide exit tunnel. The exit tunnel is formed primarily by 23S rRNA. It starts at the peptidyl transferase center and spans the entire body of the subunit finally opening at its “back” [10,30-32]. Although originally viewed as an inert conduit for nascent peptides of any sequence synthesized by the ribosome, the tunnel is now considered an active and dynamic functional entity [33]. Several studies showed that interactions between the ribosome and the nascent peptide that take place inside the exit tunnel affect the progression of protein synthesis as well as the reactions catalyzed by the ribosomal peptidyl transferase [34,35], (see [36] for review). The tunnel is relatively wide (15 Å average). However, it contains a constriction, (ca. 10 Å wide) formed by proteins L4 and L22, which is located a short distance from the peptidyl transferase center. Macrolides bind close to this constriction and pose a molecular road block for the growing polypeptide chain [12,13,33]. In the presence of bound 14- and 15-membered ring macrolides, polymerization of the first several amino acids continues unperturbed and inhibition of polypeptide growth occurs only after the nascent peptide becomes large enough to reach the bound macrolide near the tunnel constriction. The inhibition of peptide progression eventually results in the dissociation of peptidyl tRNA from the ribosome. The latter apparently takes place during an attempted act of translocation during which the contacts of tRNA with the ribosome must be “loosened”.

This mechanistic model explains biochemical observations regarding macrolide activity and is generally consistent with the macrolide location in the ribosome [24,25,37]. However, the main question that remains unresolved is the length of the nascent peptide that the macrolide-bound ribosome can synthesize. In poly (A)-dependent cell-free translation systems, macrolides were reported to cause accumulation of di-lysine but inhibited synthesis of longer peptides. In poly (U)-dependent protein synthesis, accumulation of Phe2 and Phe3 was observed in the presence of erythromycin, while formation of longer peptides was inhibited [28,29]. Also noteworthy is the observation that the exact point of erythromycin inhibition of elongation appears to be dependent on the amino acid sequence of the nascent peptide. While the drug does not affect transfer of Ac-Gly-Gly to puromycin, the transfer of Ac-Pro-Gly is efficiently inhibited [23]. The effect of erythromycin on elongation of very short peptides appears to contradict more recent observations using an in vitro translation system driven by synthetic mRNAs, in which erythromycin caused the accumulation of peptidyl-tRNAs carrying significantly longer - 6 to 8 amino acid residue-long peptides (Tenson, personal communication). Furthermore, the ketolide telithromycin allowed the ribosome to synthesize nascent peptides that were even longer – 9 to 12 amino acids long. Modeling studies suggest that 6 to 8 amino acids can potentially fit between the peptidyl transferase active site and the macrolide roadblock [12]. If so, then how can erythromycin affect elongation of a dipeptide? And how can a 12-amino-acid-long nascent peptide synthesized in the presence of ketolides fit? Is it folded in a specific way within the ribosome? Another question that
Fig. (2). Molecular interactions and location of the macrolide binding site in the large ribosomal subunit. A. Nucleotide environment of a macrolide (erythromycin) in the 50S ribosomal subunit of the bacterial (*Deinococcus radiodurans*) ribosome [12]. The erythromycin molecule is shown red. Nucleotide residues are numbered as in *E. coli* 23S rRNA. B. Position of erythromycin in the nascent peptide exit tunnel, front view. 23S rRNA is shown light purple, 5S rRNA is green, ribosomal proteins are dark purple and erythromycin red. The view is from the interface of the large ribosomal subunit down the peptidyl transferase center. C. Position of erythromycin in the nascent peptide exit tunnel, side view. tRNA and ribosomal proteins are shown light gray and dark gray, respectively. The P-site-bound tRNA is shown blue (modeled from [61]), modeled nascent peptide is gold and erythromycin molecule is red. View is from the side of the L11 stalk. (Fig. (2) was prepared by Dr. Joerg Harms (Max-Planck Research Unit for Ribosomal Structure) using RIBBONS [62]).
remains unclear whether the length of the nascent peptide produced in the presence of erythromycin or related macrolides depends on the peptide amino acid composition and/or sequence. One would expect that the growth of a nascent peptide composed of bulky amino acids would be arrested earlier than that of a peptide composed of smaller residues. Furthermore, since the macrolide molecule and the nascent peptide appear to be competing for the same space in the exit tunnel, it is possible that certain peptides might exhibit affinity for the components of the tunnel wall that interact with a macrolide molecule, and could competitively remove macrolide from its binding site. In this scenario, synthesis of different proteins can be inhibited to a different extent by macrolides and such specificity may depend both on the structure of the drug and the structure of the nascent peptide and their interactions with the ribosome.

Interference with ribosome assembly may contribute further to the overall inhibitory activity of macrolides [38,39]. In the presence of macrolides, precursors of 50S ribosomal subunits accumulate. These precursors do not contain a full protein complement of the 50S subunit and sediment in a sucrose gradient as 30S particles [40]. The 23S rRNA in 50S precursor particles is more accessible for chemical modification than in the fully assembled 50S subunits (Xiong, Champney and Mankin, unpublished). Although the location of the macrolide binding site in the assembly intermediates remains to be determined; it is conceivable that binding of the macrolide to the subunit precursor (in its “conventional” site or a different site) may prevent specific conformational rearrangements and/or protein binding required for the completion of assembly [41]. A correlation between the inhibition of protein synthesis and inhibition of ribosome assembly suggests that not only drug binding to the precursor, but also either general reduction of protein synthesis or inhibition of production of a specific protein(s) (for example, chaperons which may contribute to ribosome biogenesis [42,43]) is required for inhibiting assembly of the 50S subunit by macrolides. The importance of assembly inhibition for the overall inhibitory action of macrolides remains to be determined. Since inhibition of the assembly takes place within the background of the arrested protein synthesis, it is unclear whether reduced production of new ribosomes amplifies the cell’s “suffering” when protein synthesis is already being inhibited.

**MACROLIDE RESISTANCE DUE TO THE TARGET SITE MODIFICATION**

High efficacy and safety of macrolides as well as their use as an alternate therapy for penicillin-intolerant patients made them popular drugs. The wide use of these antibiotics led inevitably to the spread of the resistance strains. The two most common mechanisms of resistance are excretion of the drug from the cell and modification of the drug target site [2]. The latter mechanism comes in the form of a site-specific posttranscriptional modification of 23S rRNA or mutations in 23S rRNA or ribosomal proteins.

Mutations in protein L4 directly or allosterically affect macrolide binding and cause resistance by preventing drug binding to the ribosome [21,22]. Mutations in another ribosomal protein, L22, do not significantly perturb drug affinity, but appear to act through an indirect mechanism. These mutants exhibit a wider opening of the tunnel so that the nascent peptide can apparently “slip by” the macrolide molecule bound in the tunnel (and/or possibly displace the drug) [21,33]. Mutations in ribosomal protein genes are a frequent cause of drug resistance because a single mutational event is sufficient to render cells resistant to a macrolide. In contrast, resistance due to rRNA mutations is less common [44]. Because of the multiplicity of rRNA genes in the majority of bacteria, the beneficiary (for the pathogen) effect of the mutation in one rRNA gene copy is usually masked by the abundance of wild type rRNA transcribed from unmutated gene copies. Therefore, this mechanism of resistance is more often found in the organisms with a low copy number of rRNA cistrons (for example, *Helicobacter pylori* that has two rRNA operons) [see (2,3) for review].

The most frequently found mechanism of macrolide binding site modification is dimethylation of a single 23S rRNA nucleotide, A2058, by Erm-type methyltransferases. The *erm* genes are found in macrolide producers from which they were apparently disseminated to the clinical pathogens through horizontal gene transfer [45]. Dimethylation of A2058, which is located within the macrolide binding site, drastically decreases drug affinity due to steric hindrance thus, rendering bacteria resistant to high concentrations of macrolide antibiotics [12,16,46]. A2058 is also intimately involved in the binding of lincosamides and streptogramin B [12]. Hence, Erm-based methylation (termed MLSB resistance) renders the cell resistant to at least three unrelated classes of antibiotics. Importantly, the fully assembled ribosome is not a substrate for *erm* methylation because A2058 is buried deep inside the large ribosomal subunit and apparently is not accessible to Erm methyltransferase [47]. Methylation of A2058 can take place only during ribosome assembly, which leaves a very narrow time window for the *erm* enzyme to methylate its rRNA target.

Expression of *erm* genes can either be constitutive or inducible. Inducible *erm*-based resistance, where production of Erm methyltransferase is activated only when cells are exposed to the drug, is found in some antibiotic producers as well as in many clinical pathogens. The frequent occurrence of inducibly expressed *erm* [48,49] suggests that A2058 dimethylation is apparently “unhealthy” for unperturbed protein synthesis. Bacteria appear to avoid unnecessary methylation of this nucleotide unless it becomes essential for survival in the presence of the drug.

The molecular mechanisms of Erm induction are related to the general mode of macrolide action, but they are largely enigmatic. The inducible *erm* cassette consists of two functional parts – the *erm* gene whose expression is normally repressed due to sequestration the *erm* ribosome binding site in the mRNA secondary structure and a constitutively translated leader peptide cistron that precedes *erm* [50,51], Fig. (3A). In the presence of erythromycin, the ribosome is believed to stall around the 8th –9th codon of the leader peptide open reading frame (ORF). The stall of the ribosome triggers a conformational rearrangement in mRNA resulting in the opening of the *erm* ribosome binding site...
and thus, activation of \textit{erm} translation, Fig. (3B) [52,53]. Since stall on the leader ORF occurs only in the presence of the drug, the ribosome translating the leader peptide must bind a macrolide molecule. On the other hand, the ribosome that will translate the \textit{erm} cistron, should be drug free at least during early steps of \textit{erm} translation in order to be able to synthesize the entire polypeptide. This implies that efficiency of \textit{erm} induction should depend critically on macrolide concentration. At very low drug concentrations, too few ribosomes will carry the antibiotic and thus stalling on the leader ORF will not occur. On the other hand, if the drug concentration abruptly becomes very high, translation of the Erm cistron maybe inhibited and expression of the resistance may be delayed. Moreover, only newly assembled ribosomes can carry methylated 23S rRNA, as assembled ribosomes are not \textit{erm} methylase targets (see above). Thus, the ability of a macrolide to induce \textit{erm} resistance may depend on the kinetic interplay of drug binding and dissociation from the ribosome and the rate of translation. It is conceivable that macrolides with a high off-rate and a moderate-affinity binding will induce \textit{erm} expression at a broader range of drug concentrations, while the tightly binding macrolides with a slow off-rate will be less-efficient inducers. Indeed, the ketolides, which bind much tighter to the ribosome than macrolides appear to be poor inducers of \textit{erm} [16,17,54].

\textbf{Fig. (3). Inducible Erm resistance.} The secondary structure of the leader region of the bi-cistronic mRNA coding for the leader peptide and Erm methyltransferase in the non-induced (A) and induced (B) configuration [51]. The sequence of the short peptide encoded in the leader open reading frame and its Shine-Dalgarno region (S.D.\textsubscript{L}) are shown. Shine-Dalgarno region (S.D.\textsubscript{E}) of the Erm cistron and the location of Met initiator codon are indicated. In the presence of an inducing macrolide antibiotic, the ribosome stalls around 8th-9th codon of the leader cistron. Stalling causes rearrangement of the mRNA secondary structure which renders the translation initiation region of the Erm cistron accessible.
The critical and the least understood component of the mechanism of $erm$ induction is the dependence of ribosome stall on both the sequence of the leader nascent peptide and the chemical nature of the inducing macrolide. This suggests that a specific interaction between the peptide and the bound macrolide molecule is required for ribosome stall [51]. Since the latest experiments show that macrolides (erythromycin) normally inhibit ribosome progression when the nascent peptide is between 2 and 8 amino acids long (Tenson, personal communication), it appears that the ribosome should stall at approximately the correct place in the leader peptide ORF to elicit induction, irrespective of the leader peptide sequence. However, this is not true. The identity of leader peptide amino acids 5-9 (S-I-F-V-I) is critical for induction [55]. Since these C-terminus-proximal residues, but not the N-terminus of the nascent peptide appear to determine the efficiency of ribosome stall, the nascent peptide in the stalled complex might be folded in a specific way to allow direct contact between the critical amino acids and the drug (Fig. (5A)). The immediate functional consequences of such interactions are not clear. One possibility is that stall occurs because dissociation of peptidyl-tRNA is inhibited due to a specific drug-nascent peptide interaction. However, other mechanisms are possible.

**PEPTIDE-MEDIATED MACROLIDE RESISTANCE.**

The potential structure-specific interaction between the nascent peptide and the macrolide on the ribosome, which may be the key component of the $erm$ induction mechanism, is reminiscent of interactions that were proposed to take place in a completely different mechanism of macrolide resistance. In this mechanism, resistance to macrolides is mediated by expression of specific peptides, 4-6 amino acid-long [56,57] (see [4] for review). Biochemical evidence indicates that the peptides act in cis, rendering the ribosome on which they were translated refractory to inhibition by macrolides. Screening of random 5-codon-long mini-gene libraries showed that only mini-genes coding for peptides with a specific amino acid sequence could confer resistance, Fig. (4). Thus, peptides conferring the highest resistance to erythromycin (E-peptides) are characterized by the consensus sequence M-(L)-L/I-(F)-V where the third position is almost always occupied by Leu or Ile, while the second and fourth positions show strong preference for hydrophobic amino acids, more frequently Leu/Ile and Phe, respectively [57]. Although cells expressing E-peptides also exhibit some resistance to ketolides, the peptides conferring the highest ketolide resistance (K-peptides) conform to a different consensus: M-K/R-(F/L/V)-X-X, where X indicate positions which do not show an obvious amino acid preference [58]. Peptides with yet different consensus sequences render cells resistant to the 14-membered ring drugs oleandomycin and troleandomycin, 15-membered ring azithromycin, 16-membered ring josamycin and other macrolides [4] and (Gaynor, Mankin and Tenson, unpublished). The strong correlation between the sequence of the resistance peptide and the structure of the drug suggests that a direct interaction between the drug and the peptide takes place on the ribosome. The current model proposes that the newly synthesized resistance peptide interacts with the ribosome-

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**E-peptides**

| ATG CTT TTA CGT ATC TAA | M L L R I |
| ATG ACA TTA AAA GTC TAA | M T L K V |
| ATG ATT CTA AAG TTA TAA | M I L K L |
| ATG ATG CTA AAA TGG TAA | M M L K L |
| ATG CTG CTT AGC GTA TAA | M L L T V |
| ATG CTG TTA TGT GTA TAA | M L L L V |
| ATG CTG CTA TTG GTA TAA | M L L L V |
| ATG CTT TTA TGT TAA | M R L F V |
| ATG TTA TTT TGG TAA | M L L W V |
| ATG GTA ATT TGG GTA TAA | M V I L V |
| ATG GTA ATT TTG GTA TAA | M V I L V |
| ATG GTA ATC ACA GTA TAA | M L I T V |
| ATG GCT TTA AAA TAC TAA | M A L Y T |
| ATG GTA CAA ACA GTA TAA | M V Q T V |
| ATG GTA AAC ACA ATC TAA | M V Y T I |

**K-peptides**

| ATG AAA TTA AAA CTC TAA | M K L K L |
| ATG AAA CTG AAG CTC TAA | M K L K L |
| ATG AAA ATG AAA GTT TAA | M K M K V |
| ATG AAA ATG AAA CTC TAA | M K M K L |
| ATG CGC TTT TTT GTC TAA | M R F F V |
| ATG CGG TTT TTT GTC TAA | M R F F V |
| ATG AGG CTT TTT GCT TAA | M R F F A |
| ATG CTT CTT GTG GTA TAA | M R L F V |
| ATG CTT GTT TTT GTA TAA | M R L F V |
| ATG AAA TTC TTT GTA TAA | M K F F V |
| ATG CTA GTA TAC CGA TAA | M R V Y R |
| ATG AGG CTT TTT ATT TAA | M R R F I |
| ATG CTT CTT GGG TAA | M L R W W |

Fig. (4). Short peptides conferring resistance to erythromycin (E-peptides) or ketolide ABT-773 (K-peptides) selected from a random five-codon-long mini-gene library [57]. The nucleotide sequences of the mini-genes and the amino acid sequences of the encoded peptides are shown.
bound macrolide molecule and evicts the drug molecule from its binding site on the ribosome therefore allowing the ribosome to get re-engaged, at least temporarily, in translation of cellular proteins [58]. Since the size of the peptide appears to be important [56], the act of translation termination (release of the nascent peptide from peptidyl-tRNA) and/or subsequent events may contribute to the drug displacement [59].

The parallel between the mechanism of *erm* induction and peptide-mediated macrolide resistance is obvious. In both cases, the mode of ribosome function appears to depend directly on interaction between the short nascent peptide and the drug molecule bound in the exit tunnel. Strikingly, the consensus sequence of the pentapeptides that render cells resistant to erythromycin, M-(L)-L/I-(F)-V, has a clear resemblance to the C-terminal amino acids of the *erm* leader peptide in the stalled complex, (... I-F-V). It is tempting to think that this is not a mere fortuitous coincidence and that the two mechanisms exploit similar interactions between the drug and the nascent peptide on the ribosome, Fig. (5). However, the consequences of such interactions may be different – ribosome stalling during *erm* induction and drug dissociation (probably accompanied by the release of the ribosome from the mini-gene transcript) in the case of E-peptide expression. How either of these events may occur remains unclear.

**FUTURE DIRECTIONS**

We mentioned some main achievements and problems in understanding the mechanism of action of macrolide antibiotics and mechanisms of macrolide resistance. However, it is clear that comprehensive knowledge of the principles of drug interaction with the ribosome and the molecular mechanisms of drug resistance are essential prerequisites for the development of better drugs. The atomic structures of the ribosome and the ribosome-drug complexes will definitely aid the rational design of new macrolide derivatives, which might exhibit tighter binding to the target. It is unclear, however, whether it is possible to design a molecular platform that would strengthen the interaction of the lactone ring and its side chains with the ribosome while avoiding steric clash with the A2058 dimethyl moiety of an *erm*-methylated ribosome.

The mechanism of macrolide action is only starting to emerge. Again, the precise knowledge of the mode of macrolide binding to the ribosome will be instrumental in understanding how various macrolides affect different aspects of protein synthesis. The length of the nascent peptide synthesized in the presence of macrolides, and the relation between inhibition of elongation and the nascent peptide sequence need to be investigated. Kinetics and mechanisms of peptidyl-tRNA dissociation induced by macrolides also require more detailed biochemical experimentation. The

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**Fig. (5). Putative similarity of the mechanisms of *Erm* induction and peptide-mediated macrolide resistance.** A. Schematic representation of the ribosome stalled on the *Erm* leader cistron. The ribosome is shown with the 8th (Val codon) in the P-site and the 9th (Ile) codon in the A-site. Erythromycin (black octagonal) is shown bound near the constriction of the exit tunnel. The amino acid residues whose identities are important for ribosome stall are shown gray. The model reflects our proposal that the nascent peptide may fold back in order to allow direct contact between the critical amino acid residues and the macrolide. B. The pre-termination complex formed by the ribosome which has finished translation of a resistance peptide. The sequence of one of the E-peptides (see Fig. (4)) is shown. According to the “bottle-brush” model [58], the release of the E-peptide will promote dissociation of the bound macrolide (in this case, erythromycin) from the ribosome. The amino acid residues whose identities are important to confer erythromycin resistance are shown in gray.
release of the ribosome from mRNA, resulting from peptidyl-tRNA dissociation, should potentially allow this ribosome to re-engage in protein synthesis. One would wonder, therefore, if blocking the exit tunnel farther away from the peptidyl transferase center would produce a more efficient inhibition of protein synthesis. The longer nascent peptide stuck in the exit tunnel may prevent peptidyl-tRNA from dissociating and thus, could irreversibly incapacitate the large ribosomal subunit.

Though the general inhibition of protein synthesis by macrolides is obviously an important factor in cell growth arrest, it remains to be elucidated whether production of all proteins is inhibited to the same extent or if translation of some cistrons is more sensitive to macrolide inhibition than the others. The importance of the inhibition of ribosome assembly is yet another parameter which must be studied in more detail.

The mechanism of ribosome stall during induction of 

erm resistance and its relation to peptide-mediated resistance is unclear and thus, will require attention. Modeling or direct crystallographic studies of short nascent peptides on the ribosome may provide a useful structural background to approach these problems. It is obvious that detailed knowledge of the correlation between the structures of inducing macrolides and the sequences of the nascent peptides mediating ribosome stall will be essential.

Although no clinical cases of peptide-mediated macrolide resistance have been reported to date, this type of resistance can be easily selected in the laboratory setting [56, 60], and thus may potentially contribute to the overall resistance of a clinical isolate to macrolide antibiotics. Bacterial genomes contain multiple short open reading frames, some encoding peptide sequences, which can potentially confer macrolide resistance. Expression of such ORFs may easily avoid detection. Thus a more focused investigation is required to evaluate the clinical importance of this resistance mechanism.

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