Ketolide Resistance Conferred by Short Peptides*

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Shaila Tripathi, Patricia S. Kloss, and Alexander S. Mankin‡

From the Center for Pharmaceutical Biotechnology, University of Illinois, Chicago, Illinois 60607

Clones expressing pentapeptides conferring resistance to a ketolide antibiotic, HMR3004, were selected from a random pentapeptide mini-gene library. The pentapeptide MRFFV conferred the highest level of resistance and was encoded in three different mini-genes. Comparison of amino acid sequences of pentapeptides conferring resistance to a ketolide with those conferring resistance to erythromycin reveals a correspondence between the peptide sequence and the chemical structure of macrolide antibiotic, indicating possible interaction between the peptide and the drug on the ribosome. Based on these observations, a “bottle brush” model of action of macrolide resistance peptides is proposed, in which newly translated peptide interacts with the macrolide molecule on the ribosome and actively displaces it from its binding site. Temporal “cleaning” of the ribosome from the bound antibiotic may be sufficient to allow continuation of protein synthesis even despite the presence of the drug in the medium.

Erythromycin and other macrolides are important antibacterial antibiotics. The primary mode of action of macrolides is inhibition of protein synthesis, although they can also interfere with ribosome assembly (1–3). The best studied macrolide, erythromycin, binds to the large ribosomal subunit in the vicinity of the peptidyl transferase center, where it forms tight contacts with rRNA (4–6) and maybe ribosomal proteins (7, 8). Although the molecular mechanisms of action of erythromycin remain obscure, it is clear that the antibiotic blocks the elongation step of translation during early rounds of protein synthesis. The drug has a high affinity for ribosomes with nascent peptides shorter than 2–5 amino acids, but does not bind to ribosomes that carry long nascent peptide chains (9). It was suggested that erythromycin sterically hinders growth of the nascent peptide chain (2) and may promote dissociation of peptidyl-tRNA (10, 11).

The clinical use of macrolides is significantly hampered by the growing number of resistant strains. Different mechanisms of resistance have been described (reviewed in Ref. 6). We have recently described a novel mechanism of erythromycin resistance, which is based on interaction of specific short peptides with the ribosome. The discovery of this resistance mechanism came from an observation that overexpression of a short segment of Escherichia coli 23 S rRNA (positions 1235–1268) rendered cells resistant to erythromycin (12, 13). Mutational and biochemical analyses demonstrated that resistance is caused by translation of a pentapeptide open reading frame (ORF) encoded in E. coli 23 S rRNA and is mediated by interaction of the newly translated pentapeptide with the ribosome. The rRNA-encoded pentapeptide is not normally expressed because the Shine-Dalgarno region of the peptide ORF is sequestered in the 23 S rRNA secondary structure. However, the peptide expression can be activated by site-specific fragmentation of rRNA or by rRNA mutations that increase accessibility of the Shine-Dalgarno region of E-peptide ORF (14). In order to get insights into peptide structural features that are essential for erythromycin resistance, other erythromycin resistance peptides were selected from in vivo expressed random peptide libraries (15). It was found that only short peptides, ranging in size from 3 to 6 amino acids, with specific amino acid sequence can confer erythromycin resistance. Analysis of more than 70 pentapeptides that can confer resistance to erythromycin (E-peptides) revealed a consensus sequence, MXLXV, which could be recognized in the majority of E-peptides and was especially pronounced in the most active E-peptides that could confer very high levels of erythromycin resistance (15).

In vitro studies suggested that the ribosome is the most plausible target of action of E-peptides (12). Interestingly, we observed that chemically synthesized peptides added exogenously to the cell-free translation mixture did not render ribosomes resistant to erythromycin. In contrast, ribosomes that could translate E-peptide mRNA exhibited increased resistance to erythromycin in a cell-free translation system (15). Based on this observation, a model of cis-acting E-peptide was proposed where a newly synthesized E-peptide remains tightly bound to the ribosome and prevents binding of erythromycin into its functional site. Although this model accounted for most of the experimental data, it did not satisfactorily explain why synthetic peptides were not functional in vitro. Furthermore, in order to explain erythromycin resistance of cells expressing E-peptide, we had to invoke a remote possibility that the ribosome-bound E-peptide could be displaced by a newly initiated growing nascent peptide chain.

According to this model, E-peptide did not have to interact directly with the drug, and the only requirement for peptide activity was its tight binding to the ribosome in the vicinity of the erythromycin binding site. To test whether there is an interaction between the drug and peptide on the ribosome, we selected peptides conferring resistance to a chemically different macrolide with a mode of action similar to that of erythromycin. We report here isolation of pentapeptides conferring resistance to a new class of macrolide antibiotics, ketolides. The results of this study show a correspondence between peptide structure and the chemical nature of macrolides to which peptide confers resistance, suggesting direct interaction between the resistance peptide and macrolide antibiotic on the ribosome. 

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‡ To whom correspondence should be addressed: Center for Pharmaceutical Biotechnology, m/c 870, University of Illinois, 900 S. Ashland Ave., Chicago, IL 60607. Tel.: 312-413-1406; Fax: 312-413-9303; E-mail: shura@uic.edu.

1 The abbreviations used are: ORF, open reading frame; E-peptide, pentapeptide conferring erythromycin resistance; K-peptide, pentapeptide conferring ketolide resistance; IPTG, isopropyl-1-thio-β-D-galactopyranoside.
Peptides Conferring Ketolide Resistance

**EXPERIMENTAL PROCEDURES**

Reagents, Strains, and Plasmids—Ketolide (HMR3004) was from Roussel Uclaf, France. Erythromycin was purchased from Sigma. *E. coli* JM109 strain (16) (endA1, recA1, gyrA96, thi, hsdR17 (rK-, mK-), supE44, Δlac-proAB), F' traD36, proAB, lacIqZDM15) was used for propagation of the random pentapeptide library. Construction of the library in the pPOT1AE vector has been described previously (15), and the physical map of the library plasmid is shown in Fig. 1.

Selection of Ketolide Resistance Peptides—The mini-gene library (15) was transformed into competent *E. coli* JM109 cells and plated onto agar plates containing 100 \( \mu \text{g/m} \) ampicillin, 60 \( \mu \text{m} \) ketolide, and 2 \( \text{mM} \) IPTG. Plates were incubated overnight at 37 °C. Twenty-five individual colonies that appeared on the plate were grown in liquid cultures. Plasmids were isolated from 3-ml cultures and used to transform fresh *E. coli* cells. Transformants were plated onto ampicillin plates, and individual colonies of transformed cells were streaked onto plates containing 100 \( \mu \text{g/m} \) ampicillin and 60 \( \mu \text{m} \) ketolide with or without addition of 2 \( \text{mM} \) IPTG. All the selected clones exhibited IPTG-dependent ketolide-resistant phenotype co-transferable with the plasmids. The level of ketolide resistance of the isolated clones was tested in liquid cultures as described previously (15).

Mini-genes in the selected plasmids were sequenced using CCW sequencing primer d(GCATCGGAAGCTGTTG) (Fig. 1).

**RESULTS**

Construction of a random five-codon mini-gene library was described previously (15). In this library, mini-genes, containing the ATG initiator codon, followed by four random codons and the TAA terminator codon, were cloned into the pPOT1AE vector where they are expressed from an IPTG-inducible lac promoter (Fig. 1). This library has been used previously to select for clones that express peptides rendering cells resistant to erythromycin (E-peptides) (15). In the present study, we asked the question whether the amino acid sequence of the selected resistance peptides depends on the chemical structure of macrolide antibiotic used in selection.

Ketolides represent a new generation of 14-member-ring macrolide antibiotics exhibiting increased activity toward erythromycin-resistant ribosomes (17, 18). The l-cladinose sugar moiety at position 3 of the erythromycin macrolide ring is replaced by a keto group in ketolides (Fig. 2). The general mechanism of action of ketolides is probably similar to that of erythromycin, and both macrolides have overlapping binding sites on the ribosome.\(^2\) We used one of the ketolide derivatives, HMR3004, to select clones expressing ketolide resistance peptides.

The minimal inhibitory concentration of HMR3004 for *E. coli* JM109 was 10 \( \mu \text{M} \). Clones expressing ketolide resistance peptides (K-peptides) were selected by plating the library onto an agar plate containing 2 \( \text{mM} \) IPTG and 60 \( \mu \text{M} \) ketolide. Several clones appeared on the plate after 24 h of incubation, and 25 clones were analyzed. Resistance phenotypes were co-transferable with plasmids isolated from the clones. Furthermore, dependence of ketolide resistance on the presence of IPTG clearly indicated that it was mediated by expression of plasmid-encoded peptide mini-genes (Fig. 3). Sequencing of plasmids from 25 isolated ketolide-resistant clones revealed 6 different peptide mini-genes (Table I), each found in several independent isolates. Remarkably, three mini-genes (clones K3, K9, and K17), which had different nucleotide sequences, encoded the same pentapeptide, MRFFV. The minimal inhibitory concentration of HMR3004 for clones expressing different K-peptides ranged from 60 to 100 \( \mu \text{M} \) and thus, significantly exceeded the drug concentration required to inhibit growth of the control *E. coli* cells (Table II).

In previous studies, we found that the amino acid sequence of E-peptides conferring erythromycin resistance conformed to the consensus MXLXV, where the second and the fourth positions were significantly less conserved than the third and the fifth (the first position was occupied by methionine by default) (15). Interestingly, the sequence of the K-peptide MRFFV (see Table I) closely resembled that of the E-peptides MRLFV, which conferred resistance to a very high erythromycin concentration (up to 1 mg/ml) (15). We tested whether peptides selected with one antibiotic could confer resistance to the other (K-peptides to erythromycin and, conversely, E-peptides to ketolide). The clone K3 expressing the MRFFV K-peptide and the clone expressing the E-peptide MRLFV were streaked onto agar plates containing ampicillin, IPTG, and either ketolide or erythromycin (Fig. 4). Cells expressing E-peptide were resistant to low concentrations of ketolide, while K-peptide could confer resistance to low concentrations of erythromycin (data not shown). Remarkably, however, only E-peptide rendered cells resistant to high erythromycin concentration and, conversely, only K-peptide conferred high level of ketolide resistance. Thus, different peptides exhibit a clear specificity toward different types of macrolide antibiotics.

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\(^2\) L. Xiong, S. Shah, P. Mauvais, and A. S. Mankin, unpublished data.

\(^3\) S. Douthwaite, personal communication.
The amino acid sequences of the selected K-peptides and nucleotide sequences of corresponding mini-genes

| Clone | No. of isolates | Mini-gene sequence | Peptide sequence |
|-------|-----------------|--------------------|-----------------|
| K1    | 7               | ATG TTA TAT AAA CCT TAA | M L Y K P      |
| K2    | 3               | ATG AAG GAT ACT TCA TAA | M K D T S      |
| K15   | 5               | ATG TCG TGG AAA ATA TAA | M S W K I      |
| K3    | 3               | ATG CGC TTT TTT GTC TAA | M R F F V      |
| K9    | 4               | ATG CGG TTC TTT GTC TAA | M R F F V      |
| K17   | 3               | ATG CGG TTC TTT GTC TAA | M R F F V      |

Minimal inhibitory concentration of ketolide HMR3004 for E. coli

| Clone | Peptide sequence | MIC* (μM) |
|-------|-----------------|-----------|
| pPOT1AE | None            | 10        |
| K1     | M L Y K P       | 80        |
| K2     | M K D T S       | 60        |
| K3     | M R F F V       | 100       |
| K15    | M S W K I       | 80        |

* MIC, minimal inhibitory concentration.

**DISCUSSION**

**Ketolide Resistance Peptides**—Unraveling the mechanism of peptide-mediated macrolide resistance may provide important clues for understanding the mode of action of macrolide antibiotics. It may also provide insights into interactions between the ribosome and the nascent peptide. In the current study, we investigated whether resistance peptides act simply by competing with the antibiotic for the ribosomal binding site, or whether macrolide resistance conferred by short peptides is mediated by direct interaction between the peptide and the drug on the ribosome. To address this question, peptides conferring resistance to a ketolide, a macrolide antibiotic different from erythromycin, were selected and their amino acid sequences were compared with those of E-peptides.

Out of 25 analyzed ketolide-resistant clones, 10 expressed K-peptide MRFFV. This peptide is apparently one of the “best” peptides conferring resistance to the ketolide HMR3004 used in the selection. Cells expressing this peptide exhibited the highest level of ketolide resistance compared with other selected clones (Table II). Furthermore, this same peptide was encoded in three different mini-genes (Table I), demonstrating strong selection for a specific amino acid sequence. Since one of the best E-peptides had the sequence MRLFV, which differed from the K-peptide MRFFV only in the third amino acid position, one can suppose that, within this context, it is the third amino acid (leucine, in the E-peptide or phenylalanine, in K-peptide) that allows the peptide to discriminate between the two macrolides.

The Mechanism of Peptide-mediated Macrolide Resistance—We have previously demonstrated the cis-mode of E-peptide action so that only the ribosome that translated an E-peptide became resistant to erythromycin, while exogenously added E-peptide did not affect inhibition of cell-free translation by erythromycin (12). Based on this observation, a model of action of E-peptides was proposed in which the newly trans-
lated E-peptide remained bound to the ribosome in the vicinity of the peptidyl transferase center thus blocking the erythromycin-binding site (15). Within this model, the peptide did not interact directly with the erythromycin molecule and resistance depended only on the relative affinities of the E-peptide and the drug to the ribosome. Our new results, however, show that the macrolide and newly synthesized peptide do interact with each other; such interaction is reflected in correlation between the chemical structure of macrolide and the amino acid sequence of resistance peptides. To reconcile the model with the new experimental data, we suggest that the newly synthesized peptide does not just passively occupy the drug binding site, but instead, actively displaces the macrolide from the ribosome (Fig. 5). During this process, the peptide directly interacts with the drug. In the previous model, which was based on an idea of tight association of E-peptides with the ribosome, we had to explain why E-peptide bound in the nascent peptide channel does not inhibit protein synthesis. Therefore, we had to speculate that after initiation of a new polypeptide synthesis, the growing nascent peptide chain has either to displace the E-peptide from its binding site or go around it. This problem is easily solved within the new model which does not require association of the resistance peptide with the ribosome. Instead, the peptide acts as a “bottle brush” that “cleans” the ribosome from the bound antibiotic. After antibiotic is removed, the ribosome can either initiate synthesis of a new polypeptide or bind another molecule of the drug. In the former case, when the nascent peptide becomes longer than 5 amino acids, the ribosome will become “resistant” to macrolides until completion of polypeptide translation because macrolides cannot bind to ribosomes with long nascent peptide chains (19, 20). Thus, translation of macrolide resistance peptides opens for ribosomes a window of opportunity to translated cellular proteins. Notably, the new model is fully compatible with the cis-mode of the peptide action, because only the ribosome that translated the resistance peptide mini-gene would become transiently competent for translation of other genes, which, in an experiment, would be observed as antibiotic resistance.

Macrolide Resistance Peptides and Translation—The correspondence between the peptide amino acid sequence and chemical nature of the macrolide strongly suggests a possibility of interaction between the resistance peptide and the antibiotic. However, free peptide apparently does not interact with the drug, since even 1000-fold excess of the synthetic E-peptide added to the cell-free translation system did not reduce inhibitory action of erythromycin (12). Therefore, the peptide-drug interaction occurs most probably only on the ribosome. Previously, we have shown that only short peptides (3–6 amino acids long) could confer resistance to erythromycin (15). The size of active peptides is compatible with direct interaction between resistance peptide and the drug on the ribosome. Erythromycin hinders growth of nascent polypeptide when it reaches the size of 2–5 amino acids. Therefore, when the ribosome decodes the stop codon of an E- or K-peptide mini-gene, the newly synthesized pentapeptide should be in contact with the ribosome-bound antibiotic. Such interaction may reduce affinity of the drug to its binding site on the ribosome, and it can be ejected together with the completed peptide.

The known mechanisms of antibiotic resistance are usually divided into three main groups: 1) mechanisms affecting accumulation of the drug in the cell, 2) mechanisms involving modification of the drug target, and, finally, 3) mechanisms based on modification of the drug. The proposed mechanism of action of macrolide resistance peptides does not fall within any

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**Fig. 5. A model of action of macrolide resistance peptides.**

I, binding of a macrolide (hatched) to the large ribosomal subunit hinders growth of the nascent peptide chain in the early rounds of protein synthesis. II, translation of a macrolide resistance peptide “cleans” the ribosome from antibiotic. If an antibiotic-free ribosome initiates synthesis of a cellular protein and polymerizes the first 2–5 amino acids, it will become “immune” to an antibiotic until the completion of polypeptide synthesis because macrolides cannot bind to ribosomes containing long nascent peptides.
of the three categories, but rather represents a hybrid of the latter two groups, since “modification of the drug” (its ability to bind to the ribosome) occurs directly within the target (the ribosome). Multiple examples of peptide sequences capable of conferring resistance to different macrolide antibiotics suggest that similar mechanisms of antibiotic resistance may potentially operate in nature and may account for macrolide resistance of some clinical pathogens.

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