IMPLICATION OF COHESIVE BINDING OF A MACROLIDE ANTIBIOTIC, 
ROKITAMYCIN, TO RIBOSOMES FROM Staphylococcus aureus

Kikutarou Endou, Mayumi Matsuoka, Hirofumi Taniguchi, and Yoshinori Nakajima*

Division of Microbiology, Hokkaido Institute of Pharmaceutical Sciences, 7-1 Katsuraoka-cho, Otaru, Hokkaido 047-02, Japan

(Received for publication July 13, 1992)

In a previous paper we reported that rokitamycin (RKM) which killed some types of RKM-susceptible staphylococci bound cohesively to ribosomes obtained from such bacteria whereas other macrolides such as erythromycin and josamycin, which are generally known to be bacteriostatic, bound to these ribosomes only reversibly. From this observation, we speculated that such cohesive binding of RKM to certain ribosomes probably resulted in cell killing (ENDOU, K. et al., FEMS Microbiology Letters, 72: 93-96, 1990). However, this speculation was based only on circumstantial evidence and we did not show directly that reversible binding of RKM to ribosomes from other strains would bring about bacteriostasis only. A clinically isolated strain, Staphylococcus aureus S704, was found to be susceptible to RKM, mycinamicin and tylosin as well as lincosamide and streptogramin type B antibiotics but not to other macrolides (erythromycin, josamycin, rosamicin, etc.). RKM showed bacteriostatic, but not bactericidal activity, on the strain. Determinant(s) responsible for the bacteriostatic phenotype was transferred into strain NCTC8325 using bacteriophage 80L2; the obtained transductant was referred to as strain 8325MMT7. The drug bound reversibly, not cohesively, to the ribosomes from both strains S704 and 8325MMT7, confirming our earlier hypothesis that rokitamycin can cause bacteriostasis or cell death depending upon whether it binds reversibly or cohesively to the ribosomes of a given strain.

Close to the respective MIC values, rokitamycin (RKM),1,2) a 3”-O-propionyl derivative of leucomycin A5, has bactericidal action against some, but not all, strains of Staphylococcus aureus, whereas both erythromycin (EM) and josamycin (JM; leucomycin A3) always exhibit bacteriostatic effects on susceptible strains.3~5) The MICs of RKM, however, were usually somewhat higher than those of EM.3) This observation was repeatedly confirmed (as 50% inhibitory doses of macrolides) by us.6) In addition to such lower inhibitory activities of RKM against the growth of S. aureus, the affinity of RKM for ribosomes obtained from a susceptible strain, NCTC8325, was lower than that of EM or JM (apparent dissociation constants were 3.4 × 10⁻⁸ M for RKM, 1.6 × 10⁻⁸ M for EM, and 2.7 × 10⁻⁸ M for JM), which also failed to explain the killing activity of RKM against strains such as NCTC8325. Cohesive binding of the drug to ribosomes from NCTC8325 was found by chance. According to this finding, we speculated that the cohesive binding of RKM could lead to cell death.6)

Meanwhile, it was found, from a clinical source, that the drug caused bacteriostatic inhibition of strain S704. This strain, shows high constitutive resistance to EM, oleandomycin, spiramycin and JM, but is susceptible to tylosin (TS) and RKM as well as to lincosamides and mikamycin B, the latter belonging to the streptogramin type B group of antibiotics.7,8) The purpose of this investigation was to find out why RKM is bacteriostatic towards strain S704 and bactericidal towards the other RKM-susceptible strain, NCTC8325.


Materials and Methods

Bacteria and Phage Strains
S. aureus S704—a clinical isolate which is phenotypically and constitutively resistant to some macrolide antibiotics excluding RKM, mycinamicin (MCM) and TS, but susceptible to lincosamide and streptogramin type B antibiotics (a phenotype hereafter referred to as partial macrolide resistance (PM-resistant))—was used; S. aureus NCTC8325 as a susceptible strain to all of the employed antibiotics, and also as a recipient for transduction of the PM-resistance determinant(s) from strain S704; S. aureus 8325MMT7—a transductant selected for the PM-resistance phenotype by two separate steps of transduction. S. aureus MS15009 (pI258) was kindly donated by Dr. INOUE, Department of Microbiology, School of Medicine, Kitasato University, Sagamihara, 228, Japan: the strain carries a plasmid encoding constitutive resistance to macrolide, lincosamide, and streptogramin type B antibiotics (MLS-resistance).

Bacteriophage strains 80 and 80L2 (a clear, large-plaque-forming mutant derived from phage 80 grown on strain NCTC8325) were utilized for the transduction.

Transduction
This was carried out according to the method of IORDĂNESCU. The transductants were selected on brain heart infusion (BHI, BBL) agar supplemented with 20 μg of EM per ml. Because direct transfer of PM-resistance determinant(s) between the donor strain S704 and recipient strain NCTC8325 was difficult, two separate steps of transduction were performed: first the PM-resistance determinant(s) from the donor were transduced into a susceptible strain SA113 (restriction-deficient mutant of NCTC8325) using bacteriophage 80 at a frequency of 3.8 × 10⁻¹¹ per SA113 cell, and second the resistance determinant(s) from such a transductant were transferred into strain NCTC8325 using bacteriophage 80L2 at a frequency of 1.0 × 10⁻⁹ per NCTC8325 cell. One of transductants obtained was referred to as 8325MMT7 strain.

Antibiotic-susceptibility Test
An MIC was determined by the two-fold agar dilution method described in a previous paper.

Test for Induction of Resistance
The inducibility of resistance to macrolide antibiotics by EM was assayed by the agar disc diffusion test: soft agar (8 ml) including bacteria (10⁶ cells) was poured onto previously hardened base agar (40 ml) containing peptone, 5 g; yeast extract, 5 g; K₂HPO₄, 1 g; glucose, 2 g; agar, 20 g per liter. The soft agar had the same composition as the base agar except that it contained 5 g of agar per liter. After the soft agar layer was hardened, several paper discs (8 mm in diameter, Toyo Seisakusho Co., Ltd.)—impregnated individually with antibiotics—were separately placed on the agar surface. The plates were then incubated at 37°C for 18 to 24 hours.

Growth Inhibition
An overnight culture of S. aureus in BHI broth was diluted 1:9 with a fresh culture medium H (pH 7.6), i.e., 50 mM NaOH - HEPES buffer containing gelysate peptone, 5 g; beef extract, 3 g; yeast extract, 2 g per liter. After 2-hour incubation at 37°C, the culture was further diluted to an OD of about 0.1 at 610 nm in an L-shaped tube (L-tube) containing medium H plus antibiotic, (total volume of 10 ml) incubated at 37°C for one hour with shaking, and chilled rapidly to 0°C. The chilled cells were harvested by centrifugation (Hitachi, HIMAC Centrifuge CR5B), at 4,000 × g for 10 minutes at 4°C and washed twice with two 5-ml portions of cold 0.9% NaCl solution. The washed cells were suspended in 10 ml of antibiotic-free medium H, and adjusted to an OD of about 0.1 at 610 nm. The cell suspension in an L-tube was further incubated at 37°C with shaking, and the growth of the suspension was determined by monitoring OD at 610 nm.

Viable Cell Count
One ml of an overnight culture of S. aureus NCTC8325 in BHI was added to each of several L-tubes containing 9 ml of a fresh medium H, which were incubated at 37°C for about 2 hours with shaking. When OD₆₁₀ reached a value of 0.7, the cultures were each diluted to a final concentration of about 10⁸ cfu/ml
The diluted cultures were added into newly sterilized L-tubes in a total volume of 10 ml of fresh medium H containing various concentrations of the indicated antibiotics, and incubated at 37°C. A 0.5-ml portion of the culture was removed at 30-minute intervals, and the viable cells (cfu/ml) were determined by a colony counting method.

Preparation of Ribosomes

Ammonium chloride-washed 70S ribosomes of *S. aureus* strains were prepared as previously described. Dissociation of ribosomes into 30S and 50S subunits was brought about by suspending the washed ribosomes in 10 mM Tris-HCl buffer (pH 7.6) containing 0.1 mM Mg(OAc)₂, 50 mM NH₄Cl, and 0.1 mM dithiothreitol (Rib-dissociation buffer).

Displaceable Binding of Rokitamycin to the Ribosomal Particles

Displacement of ¹⁴C-RKM, previously bound to ribosomal particles, by unlabeled RKM was performed as described previously.

Sucrose Density Gradient Analysis of Ribosome-macrolide Complex

Whether ¹⁴C-RKM can bind to 50S ribosomal subunits was determined according to a minor modification of Mao's method: ¹⁴C-RKM (620 pmol), in a total volume of 0.25 ml, was incubated with 20 OD₅₀ units (620 pmol) of ribosomes in Rib-dissociation buffer at 37°C for 30 minutes, following which the reaction mixture was chilled to 0°C with ice. A 0.2-ml aliquot of the mixture was layered on a 10 to 28% sucrose density gradient (30 ml) and centrifuged for 15 hours at 72,000 x g with a Hitachi ultracentrifuge RU-72P equipped with a RPS27 rotor. Gradient fractions (0.5 ml each) were collected from the top and the radioactivity and absorbance of each were determined.

Fig. 1. Effect of some antibiotics on susceptibility in *Staphylococcus aureus* NCTC8325 (A), S704 (B), 8325MMT7 (C), and MS15009 (pL258) (D).

Discs impregnated with the indicated antibiotics were laid on each plate seeded with an indicated strain so as to be arranged them in three rows except that all discs in the second row contained erythromycin (10 µg/disc). The plate was incubated at 37°C for 18 to 20 hours.

Abbreviations and the amount of each antibiotic contained in a paper disc (µg/disc) are as follows:

- OL, oleandomycin, 25; TS, tylosin, 10; MDM, midecamycin, 10; MOM, miokamycin, 10; RSM, rosamicin, 10; LCM, lincomycin, 5; SPM, spiramycin, 25; JM, josamycin, 10; RKM, rokitamycin, 10; MCM, mycinamicin I, 10; MKM-B, mikamycin B, 25; CLDM, clindamycin, 5; except that, in the case of strain MS15009 (pL258), 25 µg/disc were used for TS, MDM, MOM, RSM, JM, RKM, MCM; 50 µg/disc for SPM and MKM-B.
Antibiotics

All antibiotics and radioactive macrolide antibiotics used were the same drugs as described in a previous paper, except that streptomycin sulfate (SM) was purchased from Boehringer Manheim Yamanouchi Co., Japan.

Results

Effects of Antibiotics on the Growth of S. aureus

S. aureus 8325MMT7, a transductant of strain NCTC8325 that was selected for the EM-resistance phenotype, possesses the same resistance characteristics as the donor strain S704 (Fig. 1 and Table 1). This result reveals that the transductant strain 8325MMT7 has determinant(s) mediating PM-resistance. Strain MS15009 (pI258) shows a typical type of resistance to MLS antibiotics except SM (Fig. 1 and Table 1). Though MS15009 strain has a gene which codes for constitutive MLS-resistance, and which is present in the plasmid pI258, foggy, “D-shaped”, leakily growth-inhibited zones were always recognized around the discs of TS, RKM, and MCM (D in Fig. 1). This cause is unknown. The finding, however, suggests that the gene coding for such a phenotype may originate from a certain inducible type of MLS-resistant gene. This suggestion agrees with the results of Weisblum et al.: constitutively MLS-resistant mutants of S. aureus were obtained in vitro from the inducibly MLS-resistant strains.

We made an attempt to enlarge, toward cellular level, an observation described previously that RKM binds cohesively to MLS-sensitive ribosomes such as those from strain NCTC8325.

Even when cells of strain NCTC8325, exposed to RKM or SM for one hour, were washed with drug-free NaCl solution and suspended with drug-free medium H, their growth was greatly inhibited (Fig. 2A). In our test, SM which is well known to have a killing activity for some types of susceptible bacteria was used as a bactericidal control. On the contrary, the growth of strain NCTC8325 after one-hour exposure to EM or JM was only slightly affected (Fig. 2A). Each antibiotic used caused severe inhibition of growth at a concentration of 1 µg/ml (data not shown).

In similar experiments to those described above, we made an attempt to enlarge, toward cellular level, an observation described previously that RKM binds cohesively to MLS-sensitive ribosomes such as those from strain NCTC8325.

Table 1. MICs of some macrolide antibiotics and streptomycin against S. aureus.

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EM</td>
</tr>
<tr>
<td>NCTC8325</td>
<td>0.39</td>
</tr>
<tr>
<td>8325MMT7</td>
<td>&gt; 800</td>
</tr>
<tr>
<td>S704</td>
<td>&gt; 800</td>
</tr>
<tr>
<td>MS15009</td>
<td>&gt; 800</td>
</tr>
</tbody>
</table>

* EM, Erythromycin; JM, josamycin; RKM, rokitamycin; SM, streptomycin.

Fig. 2. Effect of macrolide antibiotics and streptomycin on the growth of S. aureus.

Strains NCTC8325 (A) and 8325MMT7 (B) were grown until the exponential phase of growth was attained, exposed to the indicated drug during one hour ((A) one and (B) ten µg/ml, respectively), and the cells of each of the strains were collected by centrifugation (4,000 x g, 10 minutes), washed twice with NaCl (0.9%) solution, and suspended in antibiotic-free medium H (OD_{610}: 0.1). Growth in the suspension was turbidimetrically determined.
the growth of strain 8325MMT7 in drug-free media after one-hour exposure to the same panel of drugs was virtually almost uninhibited, except with SM (Fig. 2B). Moreover, growth was hardly inhibited even in the presence of a larger amount (10 μg/ml) of the drugs except RKM and SM (data not shown).

**Fig. 3.** Effect of macrolide antibiotics and streptomycin on the viability of *S. aureus* cells.

△ Erythromycin, □ josamycin, ● rokitamycin, ▲ streptomycin, ○ a control run without antibiotic.

Exponential growing cells of strains NCTC8325 (A) and 8325MMT7 (B) in a medium H containing the indicated antibiotic ((A) one and (B) ten μg/ml, respectively), were shaken at 37°C, and the number of viable cells in samples withdrawn at 30-minute intervals from the cultures containing each of the drugs were determined by a colony-counting method.

**Fig. 4.** Binding of 14C-rokitamycin to ribosomal subunits.

The reaction mixture, in a total volume 0.25 ml, containing 20 OD_{260} units of dissociated ribosomes from either *S. aureus* NCTC8325 (A) or 8325MMT7 (B), together with 620 pmol of 14C-rokitamycin (1.6 x 10^8 Bq/mmol), was incubated at 37°C for 30 minutes. Samples of 0.2 ml were layered over sucrose gradients (10 to 28%) in ribosomal dissociation buffer^{14} and centrifuged at 72,000 x g for 15 hours at 4°C. Solid and dotted lines represent the lines of OD at 260 nm and radioactivity (cpm) of the antibiotic, respectively.
Fig. 3A shows that RKM and SM produce a bactericidal effect for NCTC8325 cells, whereas EM and JM cause bacteriostatic actions at vicinity of MICs of all the drugs used. The exposure of 8325MMT7 cells to RKM, however, resulted in only bacteriostasis, though SM acted lethally on the cells (Fig. 3B). Moreover, EM and JM (even at 10 μg/ml) yielded an increase rather than a decrease in the viability of 8325MMT7 cells, since the strain shows resistance to EM and JM (Table 1).

Cohesive Binding of \(^{14}\)C-Rokitamycin to Ribosomes

We posed the following question: why does RKM show bactericidal activity for NCTC8325 strain, and bacteriostatic activity for 8325MMT7 though both strains are susceptible to the antibiotic (Table 1)? Hence we intended to study the characteristics of RKM binding to ribosomes from both the strains.

Radiolabeled RKM bound to only 50S but not 30S ribosomal subunits from strains NCTC8325, and 8325MMT7 (Fig. 4). The level of such binding was a little lower with particles from strain 8325MMT7, in agreement with the observation that \(^{14}\)C-RKM binding to 70S ribosomes from strain 8325MMT7 was less than with ribosomes the extent of parent strain NCTC8325 (control values in Table 2), and that the RKM bound to ribosomes of strain 8325MMT7 is more easily displaced by cold RKM (Table 2).

The experimental evidence obtained supports our previous hypothesis that the cohesive binding of a macrolide antibiotic such as RKM to ribosomes may be related to an initial event in the killing of strain NCTC8325 cells, since macrolide antibiotics primarily inhibit ribosomal function in protein synthesis.\(^{17,18}\)

### Table 2. Effect of unlabeled rokitamycin (RKM) on release of \(^{14}\)C-RKM from the drug-ribosome complex constructed by using ribosomes obtained from \(S.\) aureus NCTC8325, S704, or 8325MMT7.

<table>
<thead>
<tr>
<th>Amount of (^{14})C-RKM displaced by the cold drug (pmol/155 pmol ribosome)(^{a})</th>
<th>NCTC8325 ribosome</th>
<th>S704 ribosome</th>
<th>8325MMT7 ribosome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control(^{b})</td>
<td>113</td>
<td>65</td>
<td>72</td>
</tr>
<tr>
<td>RKM (A)</td>
<td>0</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>(B)</td>
<td>4</td>
<td>19</td>
<td>34</td>
</tr>
<tr>
<td>(C)</td>
<td>8</td>
<td>25</td>
<td>42</td>
</tr>
</tbody>
</table>

After 30-minute preincubation (a total volume of 0.45 ml) of the indicated ribosomes (5 OD\(_{260}\) units: 155 pmol) at 37°C with the \(^{14}\)C-RKM (150 pmol, S.A.: \(1.6 \times 10^{8}\) Bq/mmol), 150 (A), 375 (B), and 750 (C) pmol of the cold RKM were added into respective reaction mixtures in a total volume of 0.5 ml, the mixtures were further incubated at 37°C for 30 minutes, and radioactivities of RKM remaining on ribosome were determined.\(^{6}\)

\(^{a}\) Each value except those in control is calculated by the following equation: amount of \(^{14}\)C-RKM displaced by cold RKM=\(\) (a value of bound hot RKM in control\(^{b}\)) \(-\) (a value of hot RKM which remained on ribosomes under the condition of experiment A, B, or C).

\(^{b}\) The control represents the amount of \(^{14}\)C-RKM bound to 155 pmol of the indicated ribosomes.

**Discussion**

Macrolide antibiotics show their activities against most of Gram-positive bacteria and some of certain Gram-negative bacteria (\(H.\) \(aemophilis\) spp., \(N.\) \(eisseria\) spp., and some \(B.\) \(aerogenes\) spp.) and \(M.\) \(ycoplasma\) spp. The drugs in general inhibit protein synthesis by binding to the 50S subunit of 70S ribosomes of microorganisms, \(i.e.,\) they block the peptidyl transferase action and/or translocation, resulting in bacteriostatic effects.\(^{19\sim23}\)

Though RKM also inhibits protein synthesis, Morohoshi \textit{et al}.\(^{3}\) have reported that a remarkable thickening of the septum of the cell wall takes place in susceptible \(S.\) \(aureus\) strains such as ATCC6538P and S-10 in the presence of RKM after 6-hour incubation. It is not known whether their observation is directly related to the cohesive binding of RKM to ribosomes from these susceptible strain.\(^{6}\)

Toriya \textit{et al}.\(^{4}\) have demonstrated that RKM binds to 50S ribosomal subunits from \(S.\) \(aureus\), and that the drug produces lethal effects on 20 strains of \(S.\) \(aureus\) isolated from clinical sources. Their result
was confirmed in the strain NCTC8325 (Fig. 4). Growth of other RKM-susceptible strains, such as 8325MMT7, was almost unaffected by prior exposure to, and subsequent removal of, RKM (Fig. 2B). However, the number of viable cells of strain 8325MMT7, remained nearly constant in the presence of the drug (Fig. 3B). In a previous paper, the hypothesis that cohesive binding of RKM to ribosomes might cause lethality in susceptible staphylococci was derived from a difference in the ribosome-binding characteristics of RKM and other macrolides such as EM and JM. In order to confirm the hypothesis, it was necessary to present evidence showing that reversible binding of RKM to ribosomes would result in a bacteriostatic effect. We, therefore, concentrated our effort in finding the occurrence of such binding between RKM and ribosomes obtained from susceptible Staphylococcus aureus. In preliminary experiments, using the method of Hash et al. with three antibiotics (RKM, EM and JM), RKM gave the fastest and strongest inhibition of the incorporation of radioactive valine into protein fractions in growing cells of strain NCTC8325. The inhibition of protein synthesis took place within one hour, whereas neither RNA nor DNA synthesis was inhibited during the same period (data not shown).

Schröder and Nierhaus have estimated an apparent Kd to be $8.4 \times 10^{-7}$ M for the binding of dihydrostreptomycin (DHSM) to 70S ribosomes of Escherichia coli A19, and DHSM is known to have about the same degree of antibacterial activity as SM. There has yet been no evidence of cohesive binding of DHSM to ribosomes. An apparent Kd for the complex of RKM with ribosomes from a susceptible strain NCTC8325 was $3.4 \times 10^{-8}$ M indicating that RKM binds to ribosomes with a greater affinity than DHSM. A preliminary study was made on damages to the cell membrane which is generally known as the site of the bactericidal action of SM, and, unlike with DHSM, no leakage of K+ from NCTC8325 cells was found in the presence of RKM. The lethal action of RKM, therefore, appears to occur in a different mechanism from that of SM.

Leucomycin causes polysome degradation in E. coli, whereas EM does not work in this way. In the presence of EM the formation of polysomes in E. coli continues despite a severe depression in protein synthesis. Since RKM is a semi-synthetic derivative of leucomycin A5, it seems likely that RKM also may break down polysomes in S. aureus and that separated ribosomes may bind RKM cohesively. Hence they will not be sufficiently available for protein synthesis in the bacterial cells. We speculate that RKM-bound ribosomes may then be degraded and that this could account for killing of the cells.

In conclusion, the obtained results appear to supply evidence for a hypothesis that the cohesive binding of RKM to ribosomes is closely related to an initial event leading to cell death, though a molecular mechanism of the next event remains yet to be elucidated. With consideration for cohesiveness of RKM to sensitive ribosomes from S. aureus, we are currently investigating mechanisms of the cohesive interaction between RKM and ribosomes, and of the cell-killing resulting from it.

Acknowledgments

We wish to thank M. Aoki, S. Takahashi, and S. Watanabe for their skilled technical assistance. This work was supported in part by research grant 03771750 from the Ministry of Education.

References


