KINETICS OF MACROLIDE ACTION: THE JOSAMYCIN AND ERYTHROMYCIN CASES

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Running title: The kinetics of macrolide action

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Summary

Members of the macrolide class of antibiotics inhibit peptide elongation on the ribosome by binding close to the peptidyl transferase center and blocking the peptide exit tunnel in the large ribosomal subunit. We have studied the modes of action of the macrolides josamycin, with a 16-membered lactone ring, and erythromycin, with a 14-membered lactone ring, in a cell free mRNA translation system with pure components from *E. coli*. We have found that the average lifetime on the ribosome is three hours for josamycin, while less than two minutes for erythromycin and that the dissociation constants for josamycin and erythromycin binding to the ribosome are 5.5 and 11 nM, respectively. Josamycin slows down formation of the first peptide bond of a nascent peptide in an amino acid dependent way and completely inhibits formation of the second or third peptide bond, depending on peptide sequence. Erythromycin allows formation of longer peptide chains before the onset of inhibition. Both drugs stimulate the rate constants for drop-off of peptidyl-tRNA from the ribosome. In the josamycin case, drop-off is much faster than drug dissociation, while these rate constants are comparable in the erythromycin case. Therefore, at a saturating drug concentration, synthesis of full-length proteins is completely shut down by josamycin but not by erythromycin. It is likely that the bacterio-toxic effects of the drugs are caused by a combination of inhibition of protein elongation, on one hand, and depletion of the intracellular pools of aminoacyl-tRNAs available for protein synthesis by drop-off and incomplete peptidyl-tRNA hydrolase activity, on the other.
Introduction

Macrolides form a large group of antibiotics that target the protein synthesis machinery (1,2). All macrolides contain a 14-, a 15- or a 16-membered lactone ring with sugar residues (2). This study concerns the action of two members of the macrolide family: erythromycin and josamycin. Erythromycin, a frequently used drug in the clinic (2), contains a 14-membered lactone ring, while josamycin contains a 16-membered lactone ring (Fig. 1A).

The molecular mechanism of macrolide action has remained puzzling during decades of scientific studies. All macrolides bind competitively to a site in the vicinity of the peptidyl transferase center (3), but direct inhibition of the peptidyl transferase reaction has been observed only for macrolides with 16-membered lactone rings (4,5). Erythromycin, in contrast, allows synthesis of short peptides before nascent protein elongation is inhibited (5). Macrolides also induce drop-off of peptidyl-tRNAs from translating ribosomes both in vitro (6,7) and in vivo (8). The macrolide induced drop-off events may be toxic for bacterial cells because accumulation of peptidyl-tRNA in the cell depletes pools of free tRNA isoacceptors (9-11).

Recently, high-resolution crystal structures of several macrolides in complex with the large ribosomal subunit were obtained (12-16). Those structures have, together with new biochemical data from a cell free translation system (7), clarified some aspects of macrolide action. The crystal structures show that all macrolides bind to the beginning of the nascent peptide exit tunnel, with their C5 sugars extending towards the peptidyl transferase center (Fig. 1). The biochemical data suggest a common mechanism for
macrolide action, based on the space that is available between the peptidyl transferase center and the ribosome bound macrolide (7).

[Figure 1 here]

At the same time, several questions have remained unanswered: (i) are the rate constants for peptidyl-tRNA drop-off enhanced by macrolides, or are these events an indirect consequence of the inhibition of the peptidyl transferase reaction by the drugs? (ii) at which step in the elongation cycle does peptidyl-tRNA drop-off occur? (iii) do macrolides inhibit translocation of peptidyl-tRNA from A to P site? (iv) can protein synthesis be resumed by the dissociation of a macrolide that has caused ribosomal stalling? (v) are the growth inhibitory effects of macrolides due to direct inhibition of protein elongation on the ribosome or an indirect effect of depletion of tRNA pools due to frequent drop-off events and insufficient intracellular peptidyl-tRNA hydrolase activity?

In this study we have clarified some of these issues for the macrolides erythromycin and josamycin by studying their kinetic properties in a cell free system for protein synthesis with *E. coli* components of high purity (7,17).

**Experimental procedures**

**Chemicals and buffers**

GTP, ATP and [³H]Met were from Amersham Biosciences. Putrescine, spermidine, phosphoenolpyruvate (PEP), myokinase (MK) and non-radioactive amino acids were from Sigma. Pyruvate kinase (PK) was from Boehringer-Mannheim. Erythromycin was from Sigma and josamycin was from ICN Biomedicals.
All experiments were performed in polymix buffer, containing 5 mM magnesium acetate, 5 mM ammonium chloride, 95 mM potassium chloride, 0.5 mM calcium chloride, 8 mM putrescine, 1 mM spermidine, 5 mM potassium phosphate and 1 mM dithioerythritol (DTE) (18).

**mRNA**

The template DNAs for *in vitro* transcription were prepared by annealing the following oligonucleotides at the complementary sequences (underlined) and filling the gaps by PCR.

Forward oligo:  
CTCTCTGGTACCGAAATTAATACGACTCACTATAGGAATTCCGGCCCTTGTTAACAATTAAGGAGG

Reverse oligo for MVSN:  
TTTTTTTTTTTTTTTTTTTTCTGCAGATTTAGTTAGAAACCATAGTATACCTCCTTAA
TTGTTAACAGGGCCC

Reverse oligo for MFSN:  
TTTTTTTTTTTTTTTTTTCTGCAGATTTAGTTAGAAACCATAGTATACCTCCTTAA
TTGTTAACAGGGCCC

The reverse oligo used to prepare the MS mRNA was previously described (7). *In vitro* transcription and purification of mRNAs containing a poly(A) tail were as described in (19).
Protein synthesis with factors of high purity

Components of the translation system were purified as described in (7,20) and references therein.

Composition of the translation reactions

All experiments were performed in polymix buffer at 37°C using equal volumes of pre-initiated ribosomes and pre-formed ternary complexes containing EF-Tu, GTP and aminoacyl-tRNA. The reactions were quenched by adding ten volumes of 15% formic acid to one volume of reaction mixture and the peptides were analyzed as described in (7).

The initiation mixture, containing ribosomes (1.6 µM, ~50% active in peptidyl transfer), \[^{3}\text{H}]\text{fMet-tRNA}^{\text{Met}}\ (2 \mu\text{M}), \text{mRNA} \ 3.2 \mu\text{M}, \text{IF2} \ (0.3 \mu\text{M}), \text{IF1} \ (0.6 \mu\text{M}) \text{ and IF3} \ (0.6 \mu\text{M}), \text{was pre-incubated for 10 minutes at 37°C to allow for formation of initiated ribosome complexes.}

The elongation mixture, containing EF-G (1.6 µM), EF-Tu (24 µM), EF-Ts (0.24 µM) and tRNA\text{bulk} \ (~0.18 \text{mM}), the relevant aminoacyl-tRNA synthetases (AARS) (0.1 Units/\mu\text{l}) (defined in (21)), peptidyl-tRNA hydrolase (1.12 \mu\text{M}) and amino acids (160 \mu\text{M each}), was pre-incubated for 8 minutes at 37°C to allow for formation of ternary complexes.

In addition, both mixtures contained ATP (1 mM), GTP (1 mM), PEP (10 mM), MK (3 \mu\text{g/ml}) \text{ and PK (50 \mu\text{g/ml}).}

Procedures

Kinetic experiments with quench-flow techniques
Pre-initiated ribosomes, formed in the absence or presence of josamycin, were rapidly mixed with the elongation mixture in a quench-flow instrument (KinTek Corp.) and the reaction was quenched with formic acid after varying incubation times. The composition of peptides was determined by RP-HPLC, as described in (7).

Association rate constant for josamycin
Josamycin at different concentrations (2, 3, 4 and 6 µM) was added to pre-initiated ribosomes to start the incubation. One volume of elongation mix was added to one volume of reaction mix at each incubation time, and after ten seconds the reaction was quenched with formic acid. The association rates were estimated from the fraction of tri-peptide forming ribosomes.

Ratio between the association rate constants for erythromycin and josamycin
An experiment, where josamycin and erythromycin competed for ribosome binding, was carried out by adding mixtures of josamycin and erythromycin to pre-initiated ribosomal complexes. The josamycin concentration was 100 µM and the erythromycin concentration was varied between 0.5 and 16 µM. After twenty seconds of incubation, one volume of elongation mix was added to one volume of reaction mix and the reaction was quenched by formic acid after ten seconds. The extent of tri-peptide formation was analyzed as in the previous case.

Macrolide dissociation rate constants from chase experiments
To determine the dissociation rate constants for josamycin and erythromycin, in a first experiment initiated ribosome complexes were formed in the presence of 4 µM josamycin during ten minutes. Then, 300 µM erythromycin was added to start the chase. At varying incubation times, one volume of elongation mixture was added to one volume
of reaction mixture. The reaction was quenched with formic acid after ten seconds and the amount of tri-peptide formed was analyzed by RP-HPLC as in the previous experiments. The fraction of ribosomes competent in tri-peptide formation increased with a rate determined by the first order compounded rate constant $k_{obs1}$.

In a second experiment, the initiated ribosome complexes were formed with 4 µM erythromycin by incubation for ten minutes at 37°C. Then, 250 µM josamycin was added to start the chase. The reaction was stopped and the extent of tri-peptide formation was analyzed as described above. The fraction of ribosomes competent in tri-peptide formation decayed with a rate determined by the compounded rate constant $k_{obs2}$. The estimates of $k_{obs1}$, $k_{obs2}$ were, together with the association rate constants for josamycin ($k_{ja}$) and erythromycin ($k_{Ea}$), used to estimate the dissociation rate constants for josamycin ($k_{jd}$) and erythromycin ($k_{Ed}$), as described below.

**Peptidyl-tRNA drop-off**

Pre-initiated ribosome complexes were mixed with translation factors including peptidyl-tRNA hydrolase. The reaction was quenched with formic acid after varying incubation times, and the amounts of peptides on the ribosome (pellet) and peptides originating from drop-off events (supernatant) were analyzed by RP-HPLC as described in (7).

**Quantitative analysis**

**Translation and drop-off model**

To estimate the compounded rate constants $k_1$-$k_5$ in Fig. 4 from the translation and drop-off experiments we described the model with five ordinary differential equations:
\[
dc_1(t)/dt = -k_1 \cdot c_1(t)
\]
\[
dc_2(t)/dt = k_1 \cdot c_1(t) - (k_2 + k_4) \cdot c_2(t)
\]
\[
dc_3(t)/dt = k_2 \cdot c_2(t) - (k_3 + k_5) \cdot c_3(t)
\]
\[
dc_4(t)/dt = k_3 \cdot c_3(t)
\]
\[
dc_5(t)/dt = k_4 \cdot c_4(t) + k_5 \cdot c_5(t)
\]

\[c_1(0) = 1, \quad c_2(0) = c_3(0) = c_4(0) = c_5(0) = 0\]

\[c_1\] is the concentration of initiation complex normalized to the concentration of active ribosomes, \[c_2\] and \[c_3\] are the normalized concentrations of di-peptide complexes before and after translocation, respectively. \[c_4\] is the normalized concentration of tri-peptide complex and \[c_5\] is the normalized concentration of peptidyl-tRNA that has dissociated from ribosomes in drop-off events. In the translation experiments (Fig. 2) we could not separate \[c_2\] from \[c_3\] and therefore rate constants \[k_2\] and \[k_3\] were combined to \[k_{23}\] when fitting the model parameters to the experimental data:

\[
k_{23} = \frac{k_2 k_3}{k_2 + k_3}
\]

The equation system was solved analytically using Maple 7 (Waterloo Maple Inc.) and the solution was used to fit the parameters (\(k_1\) to \(k_5\)) to the experimental data. The fitting was performed using the Marquardt-algorithm (22) implemented in Origin 7 (OriginLab Corp.).

Rate constants for macrolide binding to and dissociation from active ribosomes

It has been suggested (23,24) that macrolide antibiotics bind to the ribosome in two steps, according to

\[
S + R \underset{k_{-1}}{\xrightarrow{k_1}} C_1 \underset{k_{-2}}{\xrightarrow{k_{12}}} C_2
\]

(1)

\[S\] is free antibiotic, \[R\] is free ribosome, \[C_1\] is an initial weak complex and \[C_2\] is a strong complex formed between the ribosome and the drug. When the total drug concentration \(s_0\) is much larger than the total ribosome concentration \(r_0\), then the kinetics of drug binding
is described by two coupled linear differential equations and the solution is always the sum of two exponentially decaying terms and one constant term. When \( k_{-1} \gg k_{12} \), the first binding step equilibrates much faster than the subsequent strong binding of the drug. Elimination of the fast variable (25) can then be used to simplify the slow binding step according to the scheme (26):

\[
S + R \xrightleftharpoons[k]{K} C_1 \xrightleftharpoons[k_{12}]{k_{21}} C_2
\]  

(2)

Formation of \( C_2 \) can then be approximated by

\[
c_2(t) = c(1 - e^{-k_{obs} t})
\]

(3)

\( c_2(t) \) and \( c \) are the current and equilibrium concentrations of \( C_2 \), respectively. The compounded rate constant \( k_{obs} \) is given by

\[
k_{obs} = k_{12} \cdot \frac{s_0}{s_0 + k} + k_{21}
\]

(4)

The binding of josamycin at different concentrations to the ribosome (Fig. 3A) can be described as a single step reaction and the equilibration rate constant \( k_{obs} \) is perfectly linear in the josamycin concentration (Fig. 3A, insert). This means that in this concentration interval, \( s_0 \ll K \) and \( s_0 k_{12} \gg k_{21} \), meaning that Eq. (4) approximates to

\[
k_{obs} = k_{d} s_0 = \left(k_{12} / K\right) s_0
\]

(5)

A putative pre-equilibrium complex \( C_1 \) for ribosome bound josamycin must have a dissociation constant \( K \) larger than \( 5 \cdot 10^{-5} \) M to be compatible with the observed linear dependence of \( k_{obs} \) on \( s_0 \) (Fig. 3A, insert).

To enhance the precision of the evaluation by accounting for the decrease of concentration of free josamycin, the data points in Fig. 3A were not fitted directly to Eq. (3) but instead to:
Tripeptides (%) = 100 \left\{ 1 - j_0 \left( e^{(r_0-j_0)k_{ja}t} - 1 \right) \right\} \\
- j_0 + r_0 e^{(r_0-j_0)k_{ja}t}

where \( j_0 \) is the total josamycin concentration and \( r_0 \) is the total concentration of active ribosomes.

When the two macrolide antibiotics, josamycin (J) and erythromycin (E), compete for the same binding site on the ribosome, their binding kinetics is described by an extended version of the scheme in Eq. (2), provided that the criteria for elimination of fast variables are fulfilled:

\[
J + R \rightleftharpoons C_{J1} \xrightleftharpoons[k_{jd}]{k_{ja}} C_{J2} \\
E + R \rightleftharpoons C_{E1} \xrightleftharpoons[k_{ed}]{k_{ea}} C_{E2}
\]  

(6)

In experiments at high concentrations \( j_0 \) and \( e_0 \) of J and E, respectively, the probability \( P_J \) that a ribosome becomes strongly bound to J rather than to E is given by

\[
P_J = \frac{k_{ja} \cdot j_0}{k_{ja} \cdot j_0 + k_{ea} \cdot e_0} \quad \text{so that} \quad \frac{1}{P_J} = 1 + \frac{k_{ea} \cdot e_0}{k_{ja} \cdot j_0}
\]  

(7)

The probability \( P_E \) that the ribosome binds strongly to E instead, follows from conservation of probability as

\[
P_E = \frac{k_{ea} \cdot e_0}{k_{ja} \cdot j_0 + k_{ea} \cdot e_0}
\]  

(8)

The compounded rate constants \( k_{ja} \) and \( k_{ea} \) are defined in analogy with \( k_a \) in Eq. (5) as

\[
k_{ja} = \frac{k_j}{K_j} \quad \text{and} \quad k_{ea} = \frac{k_E}{K_E}
\]  

(9)

The effective association rate constant \( k_{ja} \) or \( k_{ea} \) approximates either one of the association rate constants for drug-binding in the pre-equilibrium complex \( C_1 \) multiplied with the probability that it continues to the strong complex \( C_2 \) instead of dissociating. The
approximation is valid in the limit of high dissociation probability \((k_{-1} >> k_{12})\), which brings Eq. (1) to Eq. (2). The experiment in Fig. 3B was designed with josamycin and erythromycin concentrations in large excess over the ribosome concentration. The incubation time (20 seconds) was long enough to allow for formation of a strong binding complex with either one of the drugs, as verified in experiments where the extent of binding was monitored as a function of time. The incubation time was also short enough to make exchange of drugs between their strong binding sites negligible (see Table 1 and below). The slope of the straight line in Fig. 3B was used to obtain the ratio between \(k_{Ed}\) and \(k_{Jd}\) according to Eq. (7).

When ribosomes have been equilibrated with one macrolide and then another macrolide is added, there will be an exchange of macrolides strongly bound to the ribosome, and this can be monitored experimentally. When, in addition, both macrolides are present in large excess over ribosomes as well as over their respective dissociation constants and the affinity of each drug to its strong binding site is large compared to its affinity to the pre-equilibrium site, the exchange kinetics takes a particularly simple form.

To see this, assume that all ribosomes are bound to a macrolide at all times. Assume further that \(k_J >> k_{Jd}\) and \(k_E >> k_{Ed}\) so that the ribosomes are in one of their strong complexes \(C_{J2}\) or \(C_{E2}\). Under those conditions, the full reaction dynamics can be accounted for as transitions between \(C_{J2}\) and \(C_{E2}\) complexes:

\[
C_{J2} \xrightarrow{k_{JE}} C_{E2}
\]

The compounded rate constant \(k_{JE}\) is the dissociation rate constant \(k_{Jd}\) for J leaving complex \(C_{J2}\) multiplied with the probability \(P_E\) that J will be replaced by E. Similarly, the
compounded rate constant \( k_{EJ} \) is the dissociation rate constant \( k_{Ed} \) for E leaving complex \( C_{E2} \) multiplied by the probability \( P_J \) that E is replaced by J. Accordingly

\[
k_{JE} = k_{Jd} \cdot P_E =, \quad k_{EJ} = k_{Ed} \cdot P_J
\]

(10)

The time variation of the concentration \( c_{J2} \) is determined by the differential equation

\[
\frac{dc_{J2}}{dt} = -k_{JE}c_{J2} + k_{EJ}c_{E2}
\]

The concentration \( c_{E2} \) of complex \( C_{E2} \) can be eliminated by the conservation relation

\( c_{J2} + c_{E2} = r_0 \). With the relaxation rate \( k_{obs} = k_{JE} + k_{EJ} \), this gives

\[
\frac{dc_{J2}}{dt} = -k_{obs} \cdot c_{J2} + k_{EJ} \cdot r_0
\]

(11)

Two chase experiments can be used to determine the dissociation rate constants \( k_{Jd} \) and \( k_{Ed} \). In the first, ribosomes are pre-equilibrated with J and then E is added (relaxation rate \( k_{obs1} \), Fig. 3C). In the second experiment, ribosomes are pre-equilibrated with E and then J is added (relaxation rate \( k_{obs2} \), Fig. 3D). The dissociation rate constants follow from the equation system

\[
\begin{align*}
k_{obs1} &= k_{Jd}P_{E1} + k_{Ed}P_{J1} \\
k_{obs2} &= k_{Jd}P_{E2} + k_{Ed}P_{J2}
\end{align*}
\]

(12)

which has the solution

\[
k_{Jd} = \frac{k_{obs1}P_{E2} - k_{obs2}P_{J1}}{P_{J2}P_{E1} - P_{J1}P_{E2}}, \quad k_{Ed} = \frac{k_{obs2}P_{E1} - k_{obs1}P_{E2}}{P_{J2}P_{E1} - P_{J1}P_{E2}}
\]

(13)

The approximations leading to Eq. (13) require that the \( C_2 \) complexes have much higher affinity than the \( C_1 \) complexes and that the ribosomes are fully saturated with a macrolide at all times. We have estimated the pre-equilibriums for josamycin and erythromycin to be at least a factor of 10000 or 100, respectively, weaker than their strong binding states,
so that these criteria are fulfilled in the experiments in Figs 3C and 3D. For the analysis in this section we have assumed for simplicity that the total drug concentrations are always much larger than the ribosome concentration. In some cases, the drug-excess was only three-fold, and here we have accounted for the disappearance of drugs from their free state by ribosome binding in our analysis.

**Results**

Josamycin and erythromycin belong to two different sub-classes of the macrolide family, and have been reported to inhibit protein synthesis in principally different ways (4). This study elucidates their modes of action with kinetic data from a cell free translation system with pure *E. coli* components (7,19).

**Inhibition of di- and tri-peptide formation by josamycin**

It was proposed from the crystal structure of the 50S ribosomal subunit in complex with carbomycin A, a macrolide very similar to josamycin (Fig. 1A), that the isobutyrate residue of the drug occupies the same site as the side chain of the amino acid on the aminoacyl-tRNA in the A site of the large subunit (Fig. 1C) (13). This is in line with results from biochemical experiments, showing that carbomycin A inactivates peptidyl transfer in an artificial system with puromycin as the acceptor and N-acetyl-Phe-tRNA\(^{\text{Phe}}\) as the donor (4,5). In contrast, experiments in a more realistic system with heteropolymeric mRNAs show that both fMet-Val-tRNA\(^{\text{Val}}\) and fMet-Gly-Ile-tRNA\(^{\text{Ile}}\) can be formed on ribosomes in complex with josamycin (7). This suggests that the inhibitory action of josamycin can be affected by the identities of the amino acids on the tRNAs in the A and P sites as well as by the length of the amino acid chain on the
peptidyl-tRNA in the P site. To study this phenomenon further, we prepared two different mRNAs; one encoding fMet-Phe-Ser (MFS) and the other encoding fMet-Val-Ser (MVS). Ribosomes, initiated with either one of these mRNAs in the absence or presence of josamycin, were rapidly mixed in a quench-flow instrument with all components necessary for the formation MVS or MFS tri-peptides. The amounts of di- and tri-peptides at different incubation times were quantified in an HPLC system with on-line radiometry and the results are shown in Fig. 2. The kinetics of the four reactions could in each case be approximated by a scheme containing only one rate constant for di-peptide formation ($k_1$) and one combined rate constant for tri-peptide formation ($k_{23}$), including both the rate constant for translocation ($k_2$) and second peptidyl transfer rate constant ($k_3$) (Fig. 4).

[Figure 2 here]

In the absence of josamycin, $k_1$ was 54 s$^{-1}$ for fMet-Phe formation and 61 s$^{-1}$ for fMet-Val formation. The rate constant $k_{23}$ was 5.7 s$^{-1}$ for fMet-Phe-Ser formation and 2.3 s$^{-1}$ for fMet-Val-Ser formation in the absence of josamycin. In the presence of josamycin, $k_1$ was about 0.06 s$^{-1}$ for fMet-Phe formation and 14 s$^{-1}$ for fMet-Val formation. The rate constant $k_{23}$ was too slow to be detected both for fMet-Phe-Ser and fMet-Val-Ser formation in the presence of josamycin. We also studied the effects of erythromycin on the rate constants for di- and tri-peptide formation in the same two cases, and found no inhibition of any rate constant for either of the two mRNAs (data not shown). In summary, the rate of di-peptide formation was inhibited about one thousand-fold by josamycin in the formation of fMet-Phe, and only about five-fold in the formation of fMet-Val. The rate of tri-peptide formation was reduced to virtually zero by josamycin,
but the rates of both di- and tri-peptide formation were unaffected by erythromycin for both mRNAs. This means that ribosomes that contain josamycin can be separated from those that do not contain a macrolide or contain erythromycin by their ability to form the tri-peptide fMet-Phe-Ser. This kinetic difference between ribosomes containing josamycin and ribosomes that do not, was used to obtain the complete binding kinetics of both josamycin and erythromycin, as will be described next.

**Kinetics of josamycin and erythromycin binding to the ribosome**

Josamycin and erythromycin bind competitively to the ribosome, but they block protein synthesis at different lengths of the nascent peptide (7). A ribosome that contains josamycin cannot form an fMet-Phe-Ser peptide, while a ribosome that contains erythromycin makes this tri-peptide with the same rate and efficiency as a macrolide-free ribosome (Fig. 2). Using this assay we only monitor the ribosomes that are active in peptidyl transfer. The advantage is that our results become independent of whether macrolides bind to the non-active ribosomes or not, as long as we use macrolides in large stoichiometric excess over ribosomes.

To measure the rate constant for josamycin binding to the ribosome, we prepared ribosome complexes that were programmed with the mRNA encoding fMet-Phe-Ser and contained fMet-tRNA_{fMet} in the P site. Then, josamycin was added at different concentrations and the ribosomes ability to form fMet-Phe-Ser was probed at varying incubation times by adding all factors necessary for tri-peptide formation. The elongation reaction was quenched after ten seconds and the labeled fMet was separated from di- and tri-peptides with RP-HPLC (7). The disappearance rate of ribosomes that were active in
tri-peptide formation was estimated for four different josamycin concentrations (Fig. 3A) and the association rate constant ($k_{ja}$) was estimated to be $3.25 \cdot 10^4 \text{ M}^{-1}\text{s}^{-1}$.

To determine the rate constant for association of erythromycin to the same pre-initiated ribosomes ($k_{EA}$), mixtures with high concentration of josamycin and erythromycin in different ratios were added to the initiation complexes. The fraction of josamycin bound ribosomes, unable to rapidly form tri-peptides, was monitored. With this experimental design one can neglect re-dissociation events after the initial binding step, meaning that the fraction of ribosome bound josamycin corresponds to the probability that this drug, and not erythromycin, binds first to a ribosome. When the inverse of this probability was plotted versus the ratio between the erythromycin and josamycin concentrations (Fig. 3B) a straight line was obtained, with a slope (30.8) equal to the ratio between the association rate constants for erythromycin and josamycin. From the association rate constant for josamycin we estimated the rate constant for erythromycin binding to the ribosome to be $30.8 \cdot 3.25 \cdot 10^4 = 1.0 \cdot 10^6 \text{ M}^{-1}\text{s}^{-1}$.

To determine the rate constants for dissociation of josamycin ($k_{jd}$) and erythromycin ($k_{Ed}$) from active ribosomes either josamycin or erythromycin was first added at a saturating concentration to the pre-initiated ribosome complexes and the binding reaction was allowed to equilibrate. The ribosomes, which had been saturated with josamycin, were then chased by a 75-fold excess of erythromycin over josamycin, and the gain in their ability to synthesize fMet-Phe-Ser was probed at varying incubation times. This gain reaction followed first order kinetics with a compounded rate constant $k_{obs1}$ (Fig. 3C). To the ribosomes that had been saturated with erythromycin, josamycin
was added at a 62-fold excess over erythromycin, and the loss in their ability to synthesize fMet-Phe-Ser was monitored at varying incubation times. Also this reaction followed first order kinetics, with a compounded rate constant \( k_{\text{obs}2} \) (Fig. 3D). The compounded rate constants \( k_{\text{obs}1} \) and \( k_{\text{obs}2} \) depend on the rate constants for dissociation of josamycin (\( k_{\text{jd}} \)) and erythromycin (\( k_{\text{ed}} \)) from the ribosome, the association rate constants for both drugs (\( k_{\text{ja}} \) and \( k_{\text{ea}} \)) as well as on their concentrations in the two experiments (Experimental procedures). Note that with an infinitely high concentration of the chasing compound, \( i.e. \) erythromycin in the first experiment and josamycin in the second, the observed rate constants would have been equal to the dissociation rate for the chased compound. From these data we estimated that \( k_{\text{jd}} = 1.8 \times 10^{-4} \text{ s}^{-1} \) and \( k_{\text{ed}} = 1.08 \times 10^{-2} \text{ s}^{-1} \). The equilibrium dissociation constant for josamycin (\( K_{\text{jd}} \)) we estimated to be 5.5 nM and the dissociation constant for erythromycin (\( K_{\text{ed}} \)) to be 10.8 nM.

[Table 1 here]

These experiments revealed that the average time for josamycin to dissociate from the ribosome is about 1.5 hours, while it takes on the average about 1.5 minutes for erythromycin to dissociate.

**Peptidyl-tRNA drop-off**

It is known that macrolide treatment causes peptidyl-tRNA drop-off from ribosomes both \textit{in vitro} (6,7) and \textit{in vivo} (8). It is, however, not known if macrolides enhance the rate constant for peptidyl-tRNA drop-off, or whether this reaction is an indirect effect of macrolide-dependent stalling of ribosomes. It is also not known at which step in the elongation cycle the drop-off reaction can occur. To clarify those issues we designed experiments that allowed selective monitoring of the josamycin-dependent rate of
peptidyl-tRNA drop-off from the A and P sites of the ribosome. In a different type of experiment we also measured the overall rate of erythromycin-induced drop-off of peptidyl-tRNA.

To study the josamycin-induced drop-off reaction, pre-initiated ribosomes containing either mRNA encoding fMet-Val-Ser or mRNA encoding fMet-Phe-Ser, were prepared as before (See the scheme of events in Fig. 4). To the first initiation complex was added ternary complex containing EF-Tu, GTP and Val-tRNAVal for formation fMet-Val and to the second initiation complex was added ternary complex containing EF-Tu, GTP and Phe-tRNA\(^{\text{Phe}}\) for formation of fMet-Phe. The experiments were performed both in the absence and presence of EF-G·GTP. In the absence of EF-G, the di-peptidyl-tRNAs remained in the A site after peptidyl-transfer, and in the presence of EF-G they were translocated to the P site (Fig. 4). When josamycin was present, the drug was either added to the pre-initiated ribosomes or, alternatively, together with the ternary complexes so that peptide bond formation occurred before, rather than after, josamycin binding to the ribosome.

The drop-off reactions were monitored by the emergence of free di-peptides by the action of peptidyl-tRNA hydrolase (Pth) that was present in the incubation mixes at a high concentration. Pth hydrolyses free, but not ribosome bound peptidyl-tRNA (11), and rapidly created a free di-peptide after each drop-off event. Since the free di-peptides remained in the supernatant after formic acid precipitation, they could easily be distinguished from their corresponding di-peptidyl-tRNAs in the pellet. The results of the drop-off experiments are shown in Fig. 5 and are summarized in Table 2.
In the absence of josamycin, the rate constants for drop-off of fMet-Phe-tRNA\textsuperscript{Phe} and fMet-Val-tRNA\textsuperscript{Val} from the A site ($k_4$) were 0.040 s\(^{-1}\) and 0.0080 s\(^{-1}\), respectively. The rate constants for drop-off of the same di-peptidyl-tRNAs from the P site ($k_5$) were about an order of magnitude smaller and were estimated to be 0.0032 s\(^{-1}\) and 0.0011 s\(^{-1}\), respectively. In the presence of josamycin, added to the pre-initiated ribosomes, the rate constants for drop-off from both A and P sites were considerably faster than in the absence of the macrolide. The rate constants for drop-off of fMet-Phe-tRNA\textsuperscript{Phe} and fMet-Val-tRNA\textsuperscript{Val} from the A site ($k_4$) were in this case 0.26 s\(^{-1}\) and 0.086 s\(^{-1}\), respectively. The rate constants for drop-off of the same di-peptidyl-tRNAs from the P site ($k_5$) in the presence of josamycin were 0.0091 s\(^{-1}\) and 0.011 s\(^{-1}\). This means that josamycin accelerated drop-off of fMet-Phe-tRNA\textsuperscript{Phe} six-fold from the A site and three-fold from the P site. The drop-off of fMet-Val-tRNA\textsuperscript{Val} was accelerated about ten-fold from both the A and P sites. Very similar drop-off rate constants were obtained when josamycin became ribosome bound after peptidyl-transfer and, when EF-G was present, after translocation (Table 2). This comparison shows that the drop-off of peptidyl-tRNAs does not occur concomitantly with the peptidyl-transfer or the translocation reaction, but from the A site after peptidyl-transfer or from the P site after translocation.

[Table 2 here]

Measuring erythromycin-induced peptidyl-tRNA drop-off is technically more demanding, since erythromycin allows formation of peptides with lengths in the interval between six and eight amino acids before ribosomes are stalled and drop-off events occur (7). Ribosomes were initiated with fMet-tRNA\textsuperscript{fMet} and an mRNA encoding the N-terminal fragment of the MS2 phage coat protein, as described earlier (7). After addition
of all factors required for protein elongation, the extent of drop-off was monitored as a function of time from the amount of \[^{3}H\]fMet-labeled peptides that by the action of Pth were rapidly cleaved off peptidyl-tRNAs that had dissociated from the ribosome in drop-off events caused by the presence of erythromycin (Fig. 5E). From these experiments, we obtained a drop-off rate constant of $8.7 \cdot 10^{-3} \text{ s}^{-1}$, which is comparable to the rate constants for drop-off of fMet-Val-tRNA\text{Val} and fMet-Phe-tRNA\text{Phe} from the ribosomal P site in the presence of josamycin (Table 2).

**Inhibition of synthesis of long peptides by erythromycin and josamycin**

At a saturating concentration of josamycin or erythromycin, all initiated ribosomes in a bacterial cell will carry either one of these macrolides. We were interested in the question if synthesis of long peptide chains, like full length proteins, is completely shut down under those conditions, or whether residual synthetic activity remains. In the case of josamycin, a di-peptidyl-tRNA (Fig. 2) or a tri-peptidyl-tRNA (7) may form and then leave the ribosome in a drop-off event. The rate constants for drop-off are a hundred times larger (Table 2) than the rate constant for dissociation of josamycin from the ribosome (Table 1). Therefore, the event that josamycin dissociates before the drop-off of the peptidyl-tRNA is expected to be less than around 1 %, which leads to the prediction that josamycin at a saturating concentration will completely shut down the synthesis of long peptides. However, in the case of erythromycin, the situation is different. Here, we measured the rate constant for erythromycin dissociation ($10.8 \cdot 10^{-3} \text{ s}^{-1}$; Table 1) to be in the same range as the drop-off rate constant of peptidyl-tRNA ($8.7 \cdot 10^{-3} \text{ s}^{-1}$). The prediction here is that when erythromycin and a peptidyl-tRNA are simultaneously bound
to a stalled ribosome, the drug will dissociate before drop-off of the peptidyl-tRNA in about 50% of the cases. When this happens, protein elongation will be resumed very rapidly and the ribosome will become refractory to erythromycin (27,28), which will allow the synthesis of long peptides like full length proteins. From this, one can predict that even when the erythromycin concentration is high enough to saturate all initiated ribosomes, about 50% of residual synthesis of long peptides will remain.

These predictions were tested in experiments where the mRNA that encodes the first twelve amino acids of the N-terminal of the MS2 coat protein (7) was translated in the presence of varying concentrations of josamycin or erythromycin. At saturating josamycin concentration, synthesis of the dodeka-peptide was completely inhibited. At saturating erythromycin concentration, in contrast, almost 40% of the ribosomes were able to synthesize the dodeka-peptide (Fig. 6), in line with the hypothesis.

Discussion

There are several reports in the literature on the mechanism of action of macrolide antibiotics. It has been found that 16-membered ring macrolides such as josamycin, but not 14-membered macrolides such as erythromycin, inhibit the peptidyl transferase (PT) reaction itself (4,5). It has also been shown that both these types of macrolides cause peptidyl-tRNA drop-off from the ribosome (8). However, the kinetic relations between PT inhibition and drop-off have never been described, and the present results fill that gap in our knowledge. Josamycin was chosen as one of the model compounds, as it reaches close to the PT center (13) (Fig. 1) and abolishes synthesis of peptides containing more than three amino acids (7). Macrolides that do not reach the PT center allow formation of
longer peptides and erythromycin, which belongs to this sub-class, was chosen as the other model compound. The mechanisms of action of josamycin and erythromycin were studied in a cell free translation system, reconstituted from pure *E. coli* components (7,17).

We have found that josamycin decreases the PT rate constant for formation of fMet-Phe-tRNA<sub>Phe</sub> or fMet-Val-tRNA<sub>Val</sub> one thousand-fold or five-fold, respectively (Fig. 2; Table 2). The rates of formation of the tri-peptides fMet-Phe-Ser and fMet-Val-Ser were virtually zero in the presence of josamycin (Fig. 2), while formation of the tri-peptide fMet-Gly-Ile has previously been observed (7). This demonstrates that the effects of josamycin on peptide synthesis strongly depend on both peptide length and amino acid sequence. Sequence effects have also been reported for erythromycin, that poorly inhibits polyphenylalanine synthesis (29-31), but strongly inhibits incorporation of basic amino acids into nascent peptides (32). Interestingly, expression of erythromycin resistance genes is regulated with the help of the peptide sequence specific inhibitory properties of erythromycin (33,34).

We have also characterised the kinetics of binding of josamycin and erythromycin to active ribosomes, by taking advantage of the observation that formation of the tri-peptide fMet-Phe-Ser is totally inhibited by the presence of josamycin (Fig. 2) but unhindered by the presence of erythromycin (Table 1). The effective rate constant for association of erythromycin to the ribosome is 1.0 μM<sup>-1</sup>s<sup>-1</sup> and about thirty times larger than the effective association rate constant for josamycin. The dissociation rate constant for the complex between erythromycin and the ribosome is about 0.01 s<sup>-1</sup> and about sixty times faster than the corresponding parameter for josamycin. This very large difference in
the dissociation rate constants for the two macrolides is in line with a suggestion, based on structural data, that carbomycin A, which is structurally similar to josamycin (Fig. 1A), forms a covalent bond with ribosomal RNA and that erythromycin does not (13).

Although the two drugs have very similar dissociation constants at about 10 nM, erythromycin associates to and dissociates from the ribosome much more rapidly than does josamycin (Table 1). Slow-binding enzyme inhibitors have been extensively discussed by Morrison and Walsh (35), and it has been suggested that such reactions should occur in two steps (36). In line with this, it was found that a number of macrolides, including erythromycin, bind to ribosomes in two steps: first the antibiotic forms a relatively weak complex that equilibrates rapidly with the free state and then the drug binds to a site with high affinity and slow dissociation rate (23,24,37). In the present work we have not been able to detect such two-step mechanisms, but published erythromycin data obtained by Dinos and Kalpaxis (23) could still allow comparisons. We measure a $K_D$ value for erythromycin binding of 10 nM compared to 36 nM obtained from filter binding techniques or 4.2 nM obtained from bulk solution data (23). They report dissociation rate constants of 0.01 s$^{-1}$ from filter binding and an order of magnitude slower dissociation for the reaction in bulk solution, and we obtain 0.01 s$^{-1}$. We report an effective association rate constant of $1.0 \times 10^6$ M$^{-1}$s$^{-1}$ and they observe $2.5 \times 10^4$ M$^{-1}$s$^{-1}$ from filter binding or $2.5 \times 10^5$ M$^{-1}$s$^{-1}$ from bulk solution experiments. Since our data were obtained from bulk solution, the appropriate comparison is with their bulk solution observations. The conclusion is that we have somewhat faster kinetics for binding and dissociation and somewhat lower affinity of the drug to ribosomes. These differences could all be explained as arising from different experimental conditions and, in particular,
from the fact that our experiments were performed at 37 °C and theirs at 25 °C. Under the same conditions they observe a dissociation constant of 40 nM for a first binding step of the drug, while we fail to detect a two-step mechanism and estimate that such complexes must have a dissociation constant larger than about 1.0 µM. Our conclusion is in line with NMR data on erythromycin binding to ribosomes (38), but not with the conclusions drawn by Dinos and Kalpaxis (23). It is possible that also this difference can be accounted for by different experimental conditions, but a direct comparison between our experimental observations and those of Dinos and Kalpaxis is difficult due to an inconsistency between their experimental observations and the exact version of their kinetic model (23). They have analyzed the data in their Fig. 5 (23) in terms of an approximation based on Eq. (A4) in their appendix, and the same type of simplification has been used in later publications (24,37). The approximation neglects the formation of stable ribosome:erythromycin complexes and it is good up to about half a minute, but at longer incubation times there are large deviations between their approximation and their exact model (our on line supplementary material). In the long time range there are also large deviations between the exact model and the experimental data, while there is perfect correspondence between the approximate model and the experiments (23). However, since the approximation has meaning only when it agrees with the exact model, their experiments lack a consistent interpretation in terms of a kinetic model.

The main difference between josamycin and erythromycin is that the former has much slower binding and dissociation kinetics than the latter. The slow association rate constants for both josamycin and erythromycin implies that they are slow-binding inhibitors according to the definition by Morrison and Walsh (35). The rate of binding of
josamycin to the ribosome increases linearly with drug concentration from 0 to 6 µM (Fig. 3A). From this we infer that, if there is a first binding step for this antibiotic, it must have a dissociation constant larger than 50 µM, meaning that the affinity must be very low. This also means that the forward rate constant of a putative second step must be larger than 1.6 s⁻¹.

We have found that the rate constants for drop-off of peptidyl-tRNAs increase with about an order of magnitude by the presence of josamycin (Table 2), showing that these drop-off reactions are accelerated by the drug and not merely a side effect of inhibited PT reactions. Our kinetic analysis has revealed that the drop-off rate constants of peptidyl-tRNAs in the presence of josamycin (Table 2) are more than one hundred-fold larger than the dissociation rate constant of the drug (Table 1). At the same time, the drop-off rate constant of peptidyl-tRNA in the presence of erythromycin is comparable to the dissociation rate constant of the drug (Table 2). From this, we predicted that synthesis of long peptides can be completely shut down by the presence of a saturating concentration of josamycin, but not by a saturating concentration of erythromycin. These predictions were verified by experiments, where a dodeka-peptide was synthesised at increasing concentrations of either josamycin or erythromycin (Fig. 6). This result is probably not specific for the particular nascent peptide sequence, since similar observations were made for an unrelated peptide (7).

**Acknowledgement**

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References

Figure Legends

Figure 1. Structures of the macrolides in this study.

(A) Chemical structures of the antibiotics discussed in this paper: josamycin, carbomycin A and erythromycin. The crystal structure of carbomycin A (Fig. 1C) has been used to model the binding of josamycin and the green ovals indicate the differences between the macrolides. Carbomycin A and erythromycin are color coded according to their respective chemical structures.

(B) Crystal structure of the *Haloarcula marismortui* 50S subunit with a dipeptidyl-tRNA analogue in the A site (39). Parts of the analogue (caproic acid and biotin residues) are not shown for clarity. The N-terminal phenylalanine is shown in red, the next tyrosine residue in black and A76 of tRNA in magenta. Nucleotides A2058, A2059, A2062 (*E. coli* numbering) of the 23S ribosomal RNA, essential for macrolide binding, are shown in green, A2451, an important component of the peptidyl transferase center (40-42), in red and C2452 in blue. N3 of A2451 of 23S RNA is shown in yellow and the proteins L4 and L22 that form part of the tunnel wall (40-42) are shown in blue and yellow, respectively.

(C) Carbomycin A bound to the *H. marismortui* 50S ribosomal subunit (13). Carbomycin A has been used to illustrate josamycin-ribosome interactions in this study. These compounds have very similar chemical structures and have the same groups (mycaminose, mycarose and isobutyrate residues) approaching the peptidyl-transferase center (Fig. 1A). The lactone ring is shown in red, the mycaminose and mycarose residues in black and the isobutyrate residue, that approaches the peptidyl transferase center, in magenta.
(D) Erythromycin, bound to the *Deinococcus radiodurans* 50S ribosomal subunit (12). The desosamine residue of erythromycin is shown in black and the cladinose moiety (not present in carbomycin A or josamycin) in magenta.

**Figure 2. Inhibition of di- and tri-peptide formation by josamycin.**

Formation and disappearance of fMet-Phe (Panel A) or fMet-Val (Panel B) followed by formation of fMet-Phe-Ser (Panel A) or fMet-Val-Ser (Panel B) in the presence or absence of josamycin. Di-peptides are described by circular discs (empty without and filled with josamycin). Tri-peptides are described by triangles (empty without and filled with josamycin). The unbroken lines were obtained from the model in Fig. 4 by fitting its parameter values (Table 2) to the data in Fig. 2.

**Figure 3. Binding kinetics for josamycin and erythromycin.**

This set of experiments was used to determine binding kinetics for both josamycin and erythromycin. The equations used to fit the data points are in the Experimental procedures section.

(A) Fraction of ribosomes free of josamycin plotted as a function of the time after addition of different concentrations of josamycin. The josamycin free ribosome fraction was determined by the extent of fMet-Phe-Ser formation, which occurs in the absence, but not in the presence, of josamycin. The concentrations were 2 µM (■), 3 µM (●), 4 µM (▲) and 6µM (▼). **Insert:** The estimated rates are proportional to the concentration of josamycin. The slope gives the compounded association rate constant $k_{ja}$. 
(B) The inverse of the fraction of josamycin-bound ribosomes plotted as a function of the ratio between the concentrations of erythromycin and josamycin in an experiment where the two drugs were added simultaneously to drug free ribosomes. The incubation time was 20 seconds and the fraction of josamycin free ribosomes was obtained from the extent of tri-peptide formation at each point.

(C) The fraction of ribosomes, originally bound to josamycin, competent in tri-peptide formation plotted as a function of time after addition of erythromycin in 75-fold excess over josamycin. The reaction is described by a single exponential with the compounded rate constant $k_{\text{obs}}$.

(D) The fraction of ribosomes, originally bound to erythromycin, competent in tri-peptide formation plotted as a function of time after addition of josamycin in 62-fold excess over erythromycin. The reaction is described by a single exponential with the compounded rate constant $k_{\text{obs}}$. The rate constants were used to estimate the rate constants for dissociation of josamycin and erythromycin from the ribosome as described (Experimental procedures).

Figure 4. Scheme for di- and tri-peptide formation with drop-off events.

A cartoon explaining the kinetic model (Experimental procedures) for di- and tri-peptide formation and drop-off events. This model was used to obtain the compounded kinetic parameters presented in Table 2 from the data in Figs. 2 and 5A-D. Parameters: $k_1$ is overall rate of di-peptide formation, $k_2$ is translocation rate and $k_3$ is rate of the second peptidyl transfer, $k_4$ and $k_5$ are rate constants of peptidyl-tRNA drop-off from A or P site, respectively.
Figure 5. Drop-off rates for josamycin and erythromycin.

Amount of dipeptides as a fraction of the amount of active ribosomes plotted as a function of time. The upper plot in each panel shows the amount of di-peptides bound to ribosomes and the lower plot shows di-peptides dissociated from the ribosomes.

(A)-(D) The filled squares represent experiments where josamycin was added before formation of di-peptide, the half filled squares indicate that the di-peptide was formed before binding of josamycin and the empty squares are control experiments without josamycin. The lines are least-square fittings to the model described in Fig. 4 resulting in parameters presented in Table 2. The data points from the decrease of the ribosome bound di-peptidyl tRNA have been fitted together with the increase in the corresponding dissociated di-peptidyl tRNA.

(E) The peptidyl-tRNA drop-off induced by erythromycin during translation of the MS mRNA. These drop-off products contained six or seven amino acids and they were detected using RP-HPLC as previously described (7).

Figure 6. Inhibition of synthesis of a dodeka-peptide by josamycin or erythromycin

The fraction of synthesis of a dodeka-peptide plotted as a function of varying concentrations of josamycin (▲) or erythromycin (●).
Table 1. *Josamycin* and *erythromycin* ribosome binding kinetics.

<table>
<thead>
<tr>
<th></th>
<th><em>Josamycin</em></th>
<th><em>Erythromycin</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_a$ (µM⁻¹ s⁻¹)</td>
<td>0.0325 ± 0.0007</td>
<td>1.00 ± 0.04</td>
</tr>
<tr>
<td>$k_d$ (s⁻¹)</td>
<td>0.18·10⁻³ ± 0.02·10⁻³</td>
<td>10.8·10⁻³ ± 0.7·10⁻³</td>
</tr>
<tr>
<td>$K_D$ (nM)</td>
<td>5.5 ± 0.5</td>
<td>10.8 ± 0.3</td>
</tr>
</tbody>
</table>
Table 2. **Compounded rate constants in the josamycin experiments (Fig. 4).**

<table>
<thead>
<tr>
<th>Rate constant</th>
<th>MFS</th>
<th>josamycin</th>
<th>MVS</th>
<th>josamycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_1$ (s$^{-1}$)</td>
<td>54 ± 5</td>
<td>0.059 ± 0.01</td>
<td>61 ± 6</td>
<td>13.5 ± 0.7</td>
</tr>
<tr>
<td>$k_2$ (s$^{-1}$)</td>
<td>5.7 ± 0.5</td>
<td>n.d.</td>
<td>2.3 ± 0.2</td>
<td>n.d.</td>
</tr>
<tr>
<td><strong>Drop-off rate</strong></td>
<td>---</td>
<td>josamycin</td>
<td>Josamycin* after PT</td>
<td>---</td>
</tr>
<tr>
<td>$k_4$ (s$^{-1}$)</td>
<td>0.040 ± 0.003</td>
<td>0.26 ± 0.09</td>
<td>0.15 ± 0.01</td>
<td>0.0080 ± 0.0007</td>
</tr>
<tr>
<td>$k_5$ (s$^{-1}$)</td>
<td>0.0032 ± 0.0007</td>
<td>0.0091 ± 0.003</td>
<td>0.011 ± 0.007</td>
<td>0.0011 ± 0.0004</td>
</tr>
</tbody>
</table>

* Josamycin was added to a final concentration of 15 µM.
Figure 1.
Figure 3.
Figure 4.
Figure 5.

A. MF-peptide from A-site

B. MF-peptide from P-site

C. MV-peptide from A-site

D. MV-peptide from P-site

E. Erythromycin induced drop-off
Figure 6.

Comparison between an exact evaluation of the model in scheme 3 (red curves) and the approximation (black curves) in Fig. 5A in the paper by Dinos and Kalpaxis (Biochemistry, 2000, vol. 39, 11621-11628). “Eryt” stands for erythromycin.

Dinos and Kalpaxis assume that the concentration of the ribosome:erythromycin complex [C*Er] is negligible (page 11627). Although a good approximation very close to the zero time point, this assumption is not valid for later time points. Their approximation agrees perfectly with their experimental data, but the exact evaluation of their model deviates significantly both from the approximation and the experimental observations.

Using the values of the constants from the filter binding experiments presented in their Table 1 we have recreated their Fig. 5A. We have compared the approximation described in their paper with accurate numerical calculations based on the complete model presented in their Scheme 3.

The black lines are linear, and have the slope $F$. $F$ is the “apparent rate constant of inactivation” and is calculated from their Eq. A6 for different erythromycin concentrations. The red lines are the numerical solutions for different erythromycin concentrations of the system of differential equations that describes the complete model in their scheme 3. The difference between the red (exact) and the black (approximate) lines arises by the neglect of the term [C*Er] in their Eq. A4.

Note: The absolute values on the Y-axis are arbitrary because the logarithm is unit-less. We use the logarithm of the remaining concentration of active ribosomes in molar (starting with $10^{-8}$ M to avoid stoichiometry problem with the macrolide concentrations), while the authors have probably used the logarithm of the activity from the scintillation counter. This difference does not in any way affect the conclusions.
Kinetics of macrolide action: The josamycin and erythromycin cases
Martin Lovmar, Tanel Tenson and Måns Ehrenberg

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