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Erythromycin, Roxithromycin, and Clarithromycin: Use of Slow-Binding Kinetics to Compare Their in Vitro Interaction with a Bacterial Ribosomal Complex Active in Peptide Bond Formation

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ABSTRACT

In a cell-free system derived from *Escherichia coli,* it is shown that clarithromycin and roxithromycin, like their parent compound erythromycin, do not inhibit the puromycin reaction (i.e., the peptide bond formation between puromycin and AcPhe-tRNA bound at the P-site of 70S ribosomes programmed with heteropolymeric mRNA). Nevertheless, all three antibiotics compete for binding on the ribosome with tylosin, a 16-membered ring macrolide that behaves as a slow-binding, slowly reversible inhibitor of peptidyltransferase. The mutually exclusive binding of these macrolides to ribosomes is also corroborated by the fact that they protect overlapping sites in domain V of 23S rRNA from chemical modification by dimethyl sulfate. From this competition effect, detailed kinetic analysis revealed that roxithromycin or clarithromycin (A), like erythromycin, reacts rapidly with AcPhe-tRNA·MF-mRNA·70S ribosomal complex (C) to form the encounter complex CA which is then slowly isomerized to a more tight complex, termed C*A. The value of the overall dissociation constant, K_A^*, encompassing both steps of macrolide interaction with complex C, is 36 nM for erythromycin, 20 nM for roxithromycin, and 8 nM for clarithromycin. Because the off-rate constant of C*A complex does not significantly differ among the three macrolides, the superiority of clarithromycin as an inhibitor of translation in *E. coli* cells and many Gram-positive bacteria may be correlated with its greater rate of association with ribosomes.

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Antibiotics are useful not only as therapeutic agents, but also as important tools for blocking and analyzing functional steps of protein synthesis. Crystal structures of ribosomal subunits in complex with a variety of antibiotics have been published recently (Brodersen et al., 2000; Ogle et al., 2001; Pioletti et al., 2001; Schluenzen et al., 2001; Hansen et al., 2002), enabling us to better understand the structural basis of antibiotic action. Among antibiotics, macrolides have played a key role in the treatment of bacterial infections. They consist of a 12- to 16-membered lactone ring, to which one or more sugar substituents, some of them amino-sugars, are attached (Vazquez, 1979). Erythromycin and its second-generation derivatives roxithromycin, clarithromycin and azithromycin (Fig. 1), are the most widely used macrolide antibiotics. Erythromycin derivatives have gained interest for their potential use in the treatment of gastrointestinal disorders and inflammatory diseases as well as for the synthesis of ketolides used in the treatment of emerging drug-resistant bacterial strains (Kirst, 1998; Ma et al., 2001). Although macrolides are structurally related to each other and bind at similar or overlapping sites on the 23S rRNA (Douthwaite and Champney, 2001), they use dissimilar mechanisms to inhibit protein synthesis (Ballesta and Lazaro, 1990; Spahn and Prescott, 1996). Erythromycin, and presumably all 14-membered macrolides, acts during the early stages of protein synthesis by blocking the nascent polypeptide exit tunnel (Nissen et al., 2000; Schluenzen et al., 2001), a process that may also cause destabilization and premature release of peptidyl-tRNA from the ribosome (Menninger and Otto, 1982). The mode of action of 16-membered macrolides is less characterized, although they have been shown to inhibit peptide bond formation in most reference cell-free systems by directly blocking the PTase activity (Dinos et al., 1993; Porse et al., 1995; Dinos and Kalpaxis, 2000; Poulsen et al., 2000). The extremely low regeneration rate of PTase activity from ribosomes complexed with spiramycin or tylosin implies that the binding of these macrolides to the ribosome is almost irreversible. Recently, the X-ray structures for spiramycin and tylosin bound to the 50S subunit of the archaebacterium *Haloarcula marismortui* were solved to 3 Å resolution (Hansen et al., 2002), showing that both anti-

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ABBREVIATIONS: complex C, the Ac[^{3}H]Phe-tRNA·mRNA·70S ribosome complex that bears Ac[^{3}H]Phe-tRNA bound to the ribosomal P-site; PTase, peptidyltransferase; DMS, dimethyl sulfate; MF-mRNA, heteropolymeric mRNA.

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biotics form a covalent bond between the C6-ethylaldehyde group of the antibiotic and the N6-amino group of A2103 of the *H. marismortui* 23S rRNA (A2062 in *Escherichia coli*). In addition, the disaccharide attached to the C5 position of the antibiotic extends up to the tunnel toward the PTase center, which could be expected to directly inhibit the PTase reaction, in contrast with those macrolides with shorter disaccharide branches at this position. A second and equally important inhibitory activity of both 14- and 16-membered macrolides has been discovered recently. Namely, these drugs exhibit an inhibitory action on 50S ribosomal subunit assembly (Champney et al., 1998; Champney, 2001).

Clarithromycin has been synthesized by methylation of the C6-OH group of erythromycin, whereas roxithromycin has been produced by the insertion of an etheroxime chain at the C9 position (Fig. 1). It has been assumed that their higher inhibitory activity against Gram-negative bacteria, compared with that of erythromycin, is presumably because of their enhanced ability to penetrate the cell envelope of Gram-negative cells (Douthwaite et al., 2000). However, the potential interference of their altered structure with the binding of these antibiotics to ribosome has never been assiduously tested. Reported investigations operate on the assumption that interaction of macrolides with ribosomes is mediated by a one-step mechanism (Pestka, 1974; Di Giambattista et al., 1987; Douthwaite et al., 2000). However, recent studies contradict this seemingly satisfactory model and suggest that macrolides, upon binding to ribosomes, cause a slow rearrangement of the encounter complex to another more tight complex (Dinos et al., 1993, 2001; Bertho et al., 1998a; Dinos and Kalpaxis, 2000). In view of these observations, we believed it should be examined whether clarithromycin and roxithromycin, beyond their enhanced acid stability and intracellular accumulation, also exhibit increased affinity for active ribosomes. The data presented here demonstrate that clarithromycin and roxithromycin, like erythromycin, do not inhibit PTase activity. Nevertheless, both antibiotics compete with tylosin for overlapping binding sites on ribosomes programmed with MF-mRNA and bearing AcPhe-tRNA at the P-site. By this competition, we show that clarithromycin and roxithromycin behave as slow-binding ligands of the ribosome and follow a two-step mechanism, a behavior reminiscent of the erythromycin properties (Dinos and Kalpaxis, 2000). In addition, we demonstrate that clarithromycin forms the tightest complex with ribosomes, a property which could be correlated with clarithromycin's superior potency against Gram-positive bacteria (Zuckerman, 2000).

Materials and Methods

Materials. Puromycin dihydrochloride (disodium salt), tylosin, erythromycin, roxithromycin, GTP, ATP, tRNA from *E. coli* strain W, and dimethyl sulfate (DMS) were purchased from Sigma (St. Louis, MO). Clarithromycin was kindly provided by Dr. Markopoulou (Abbott Laboratories, Hellas A.B.E.E., Greece). L-[2,3,4,5,6-^{3}H]Phenylalanine and [γ-^{32}P]ATP were purchased from Amersham Biosciences Inc. (Piscataway, NJ). Avian myeloblastosis virus reverse transcriptase and T4 polynucleotide kinase were from Roche Diagnostics (Mannheim, Germany). Cellulose nitrate filters (type HA; 24-mm diameter, 0.45-μm pore size) were from Millipore Corporation (Bedford, MA).

Biochemical Preparations. Salt-washed ribosomes (0.5 M NH_4Cl) and partially purified translation factors were obtained from *E. coli* B cells as reported previously (Synetos and Coutsogeorgopoulos, 1987). Complex C (i.e., the Ac[^{3}H]Phe-tRNA·MF-mRNA·70S ribosome complex) was prepared as described previously (Synetos and Coutsogeorgopoulos, 1987), with the modification that, instead of poly(U), heteropolymeric mRNA (MF-mRNA) was used as the template. MF-mRNA, a 46-nucleotide long oligonucleotide with an AUG (Met) codon in the middle, followed by a UUC (Phe) codon, was prepared with run-off transcription as described by Triana et al. (1995). Complex C formed in the presence of translation factors was adsorbed on cellulose nitrate filters and then washed with three 4-ml portions of cold buffer A (100 mM Tris/HCl, pH 7.2, 50 mM KCl, 10 mM $MgCl_2$, and 6 mM β-mercaptoethanol). Approximately 20% of the ribosomes absorbed on the filters were in the form of complex C. More than 95% of this complex was reactive toward puromycin, indicating that almost all of the bound Ac[^{3}H]Phe-tRNA was at the P-site.

Puromycin Reaction. The reaction between complex C and excess puromycin was carried out at 10 mM Mg^{2+} and 100 mM NH_4^+,

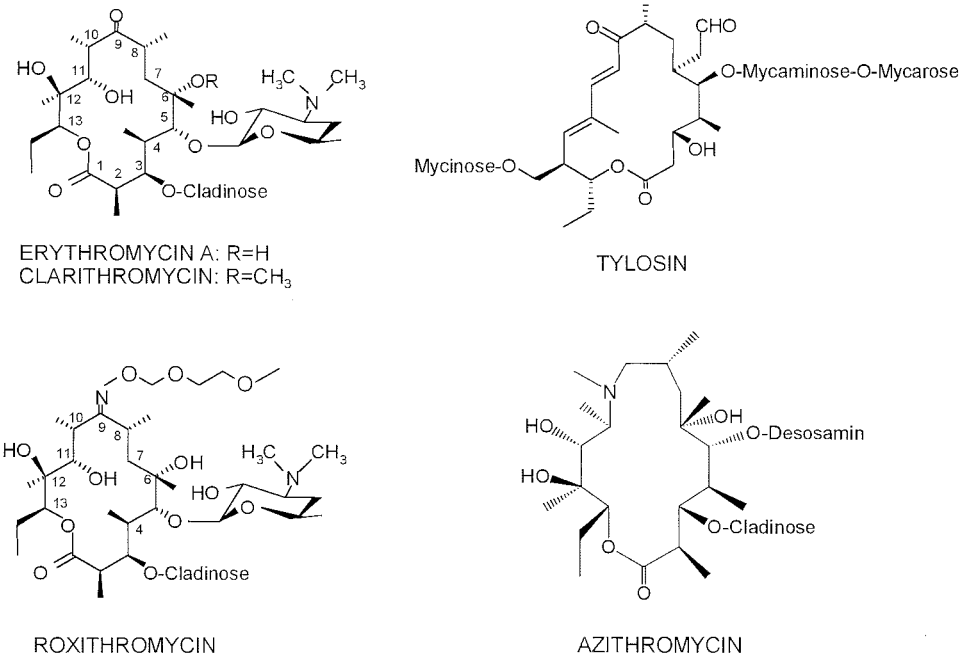

Fig. 1. Chemical structures of erythromycin A, clarithromycin, tylosin, roxithromycin, and azithromycin.

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as described previously (Dinos and Kalpaxis, 2000). Briefly, complex C reacted with an excess of puromycin in the presence or absence of macrolides, and the progress of the reaction was analyzed over a wide range of puromycin and macrolide concentrations. The reaction was terminated by adding an equal volume of 1 M NaOH. The product, AcPhe-puromycin, was extracted in ethyl acetate, and its radioactivity was measured in a liquid scintillation spectrometer. The product was expressed as a percentage of the isolated complex C on the filter. Controls without puromycin were included in each experiment, and the values obtained were subtracted.

Inactivation of Complex C by Tylosin in the Absence or Presence of 14-Membered Ring Macrolides. Complex C, adsorbed on a cellulose nitrate filter, was reacted with specified concentrations of tylosin in 2 ml of buffer B (100 mM Tris-HCl, pH 7.2, 100 mM NH_4Cl, 10 mM Mg^{2+} acetate, 6 mM β-mercaptoethanol). The reaction was allowed to proceed at 25°C for specified time intervals and was stopped by immersing the filter in 15 ml of cold buffer A. The amount of the remaining activity of complex C, after washing the filter with the same buffer to remove traces of nonspecifically bound tylosin, was determined by titration with puromycin (2 mM for 2 min at 25°C). The inactivation of complex C by tylosin was also examined in the presence of erythromycin, clarithromycin, roxithromycin, or azithromycin. In another series of experiments, complex C that was first exposed to each one of the four antibiotics for 10 min (preincubation step) was subsequently reacted with tylosin, and its inactivation was monitored as described above. All data illustrated in the figures denote the mean values obtained from four independent experiments.

Antibiotic Probing and Chemical Modification. Aliquots of 70S ribosomes, 50 pmol per tube, were incubated with (1 μM) or without antibiotic in buffer (20 mM HEPES/KOH, pH 7.8, 100 mM NH_4Cl, and 4 mM β-mercaptoethanol) for 10 min at 25°C. After cooling on ice, chemical modification of ribosomes was carried out by adding 2 μl of DMS (diluted 1:5 in ethanol) and incubating for 10 min at 37°C. The DMS reactions were stopped with 25 μl of stop solution (1 M Tris/HCl, pH 7.5, 1 M β-mercaptoethanol, and 1 mM EDTA), and then ethanol precipitated the reaction. The pellets were resuspended in 50 μl of buffer (10 mM Tris/HCl, pH 7.5, 100 mM NH_4Cl, 5 mM EDTA, and 0.5% SDS) and then extracted with phenol and chloroform. The rRNA was precipitated by ethanol and resuspended in water.

Primer Extension. The modifications in 23S rRNA were monitored by primer extension analysis using reverse transcriptase and 5′-labeled primer. The used primer was complementary to nucleotides 2105 to 2121. The cDNA products of the primer extension reactions were separated by electrophoresis on 6% polyacrylamide sequencing gels. Gels were scanned and analyzed with PhosphorImager (Amersham Biosciences). The positions of the stops in cDNA synthesis were identified by reference to dideoxy sequencing reactions on 23S rRNA, run in parallel (Stern et al., 1988)

Results

The capability of roxithromycin and clarithromycin of acting as PTase inhibitors was tested using the puromycin reaction. This reaction is a model assay system for testing PTase activity and takes place according to kinetic scheme 1.

$$C + S \underset{}{\overset{K_s}{\rightleftharpoons}} CS \xrightarrow{k_3} C' + P$$

Scheme 1. S is the acceptor substrate (puromycin), P is the product Ac[^{3}H]Phe-puromycin, and C′ is a modified species of complex C, stripped of Ac[^{3}H]Phe-tRNA, and thus unable to react for a second cycle with puromycin.

In the presence of excess puromycin, the reaction follows pseudo first-order kinetics and the relationship

$$\ln\frac{C_o}{C_o - P} = k_{obs} \times t \qquad (1)$$

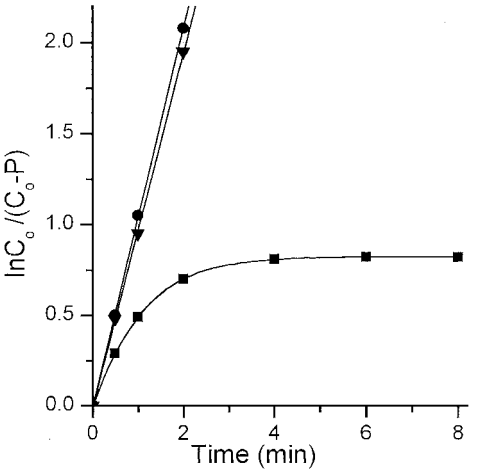
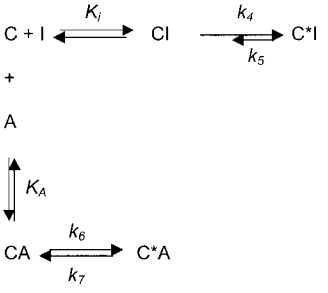


Fig. 2. First-order time plots for AcPhe-puromycin formation in the absence or presence of antibiotics. Complex C adsorbed on a cellulose nitrate filter reacted with 0.4 mM puromycin alone (▼) or in a mixture with 3 μM tylosin (■) and 1 μM clarithromycin or roxithromycin (●).

holds; C_o is the reactive complex C at zero time, and k_{obs} is the apparent rate constant of product formation. In agreement with eq. 1, the progress curve of the puromycin reaction was a straight line. Such a plot obtained at 400 μM puromycin is shown in Fig. 2. As predicted by earlier studies (Dinos and Kalpaxis, 2000), tylosin inhibited the puromycin reaction producing nonlinear progress curves whose initial slope and plateau level varied as a function of the inhibitor concentration. Such a plot, obtained at 400 μM puromycin and 3 μM tylosin, is presented in Fig. 2. In contrast, clarithromycin and roxithromycin failed to inhibit the puromycin reaction (Fig. 2). Nevertheless, both antibiotics competed with tylosin for binding to complex C. This was supported by kinetic experiments in which complex C was mixed with a solution containing both tylosin and each one of the tested macrolides. For instance, when tylosin in mixture with increasing concentrations of clarithromycin was used, a progressive decrease in the apparent rate constant of inactivation, F, occurred (Fig. 3A). High concentrations of clarithromycin provided complex C with complete protection against inactivation by tylosin (Fig. 3A, top line). Furthermore, the slope replot of the inactivation plots (1/F versus clarithromycin concentration) was linear, intercepting the vertical axis at a point above zero (Fig. 3B). This supports the hypothesis that clarithromycin (A) competes with tylosin (I) for binding to complex C, following kinetic scheme 2.



Scheme 2.

Assuming that kinetic scheme 2 is correct, the reciprocal of

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the apparent rate constant of inactivation, F, is given by eq. 2 or 3 (Dinos and Kalpaxis, 2000):

$$\frac{1}{F} = \frac{K_i + [I]}{k_4[I]} + \frac{K_i[A]}{k_4 K_A[I]} \quad (2)$$

$$\frac{1}{F} = \frac{1}{k_4} + \frac{K_i\left(1 + \frac{[A]}{K_A}\right)}{k_4} \times \frac{1}{[I]} \quad (3)$$

Equation 2 is consistent with the linearity of the plot shown in Fig. 3B. The value of the dissociation constant K_A was estimated from the slope of this plot (slope = $K_i/k_4 \cdot K_A[I]$). K_A values measured at several concentrations of tylosin do not vary by more than 8% (Table 1). On the other hand, eq. 3 predicts that at each concentration of clarithromycin, the plot $1/F$ versus the reciprocal of tylosin concentration is a straight line. Such a plot obtained at 0.3 μM clarithromycin and varying tylosin concentrations, is shown in Fig. 4B. The values of K_i and k_4 concerning the tylosin interaction with complex C were determined from the intercepts of the plots shown in Figs. 3B and 4B and were found to be equal to 3 μM and 1.5 min^{-1}, respectively. These are in good agreement with values obtained previously by another type of kinetic analysis (Dinos and Kalpaxis, 2000).

When complex C was preincubated with clarithromycin before the addition of tylosin, a further decrease in the apparent rate constant of inactivation was observed, supporting the notion that the equilibrium between C and A is not established instantaneously but instead through a slow-onset process. Upon preincubation conditions, the estimated dissociation constant, K_A^*, concerns both steps of clarithromycin binding to complex C (scheme 2) and is expressed by the equation

$$K_A^* = K_A\left(\frac{k_7}{k_6 + k_7}\right) \quad (4)$$

Equation 4 allowed us to calculate the isomerization constant k_6/k_7. This value was equal to 24.

To calculate the individual values of k_6 and k_7, complex C prepared in the presence of 1 μM clarithromycin and isolated on cellulose nitrate filter was exposed to 1 μM tylosin for various time intervals, and the inactivation of PTase activity was monitored by the puromycin reaction. As shown in Fig. 5, after an early phase of inactivation in which tylosin reacted mostly with preexisting complex C, the PTase activity was inactivated through the regeneration of complex C from the species C*A, via the rate-limiting step k_7. Therefore, the late slope of the inactivation curve provided the k_7 value. With k_7 and k_6/k_7 known, the k_6 was easily estimated (Table 1).

Roxithromycin exhibited behavior similar to that of clarithromycin, either interacting with complex C or competing with tylosin for binding to ribosomes. The corresponding values of K_A, k_6, and k_7, are presented in Table 1.

The erythromycin and azithromycin interaction with poly(U)-programmed ribosomes has been already analyzed in previous studies (Dinos and Kalpaxis, 2000; Dinos et al., 2001). In the present work, these interactions were reinvestigated by using ribosomes programmed with MF-mRNA. The obtained results revealed that the replacement of poly(U) by MF-mRNA did not cause any change in the mechanism of erythromycin or azithromycin interaction with complex C, nor did it alter the values of kinetic parameters. For the sake of comparison, these values are also included in Table 1.

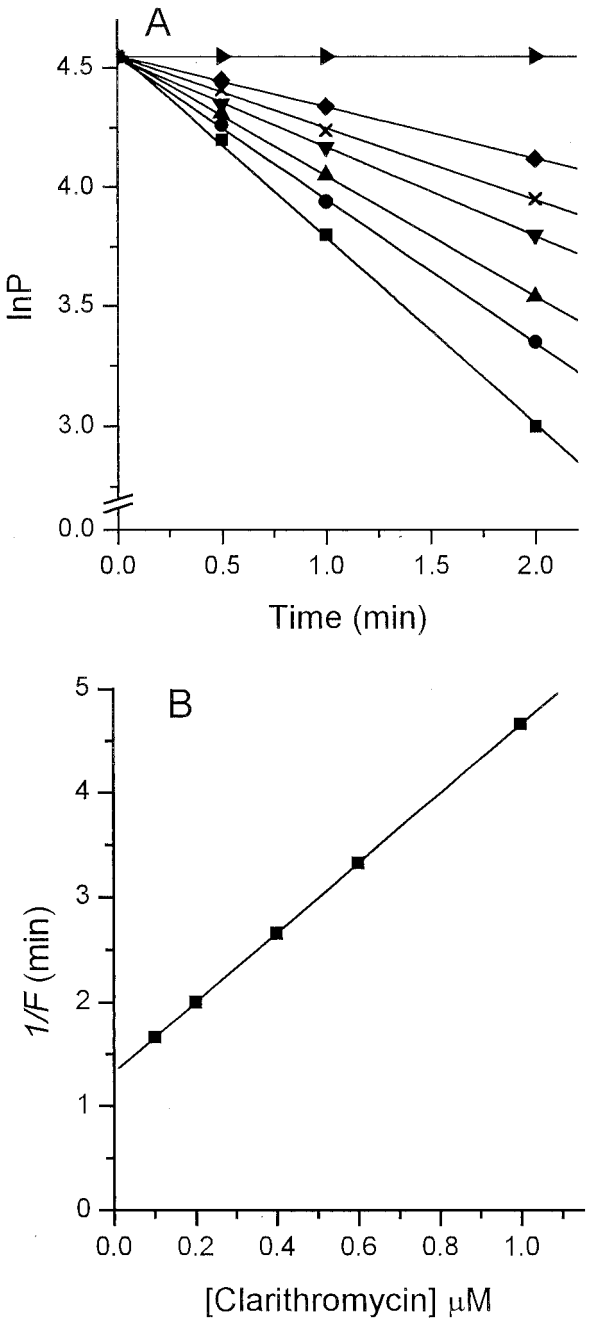

Fig. 3. Inactivation of complex C by tylosin in the absence or presence of increasing concentrations of clarithromycin. A, complex C adsorbed on a cellulose nitrate filter reacted at 25°C with 3 μM tylosin alone (■) or with a solution containing 3 μM tylosin and clarithromycin at the following concentrations: 0.1 μM (●), 0.2 μM (▲), 0.4 μM (▼), 0.6 μM (×),1 μM (♦), and 20 μM (▶), The excess of antibiotics was then removed, and the remaining active complex C was titrated with 2 mM puromycin (2 min at 25°C). B, variation of $1/F$ as a function of the concentration of clarithromycin. The parameter F represents the apparent rate constant of complex C inactivation, and its values were estimated from the slope of the A plots.

The competition of clarithromycin and roxithromycin with tylosin for common binding sites on complex C is consistent with macrolide-mediated protection of 23S rRNA from DMS modification. RNA footprinting in domain V of 23S rRNA

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TABLE 1
Kinetic and equilibrium constants derived from analysis of the interaction between complex C and macrolide antibiotics

Constant	Tylosin	Clarithromycin	Roxithromycin	Erythromycin	Azithromycin
K_i (μM)	3.00 ± 0.03				
k_4 (min^{-1})	1.50 ± 0.09				
k_5 ($min^{-1} \times 10^{-3}$)	2.50 ± 0.20				
k_A (nM)		200 ± 16	400 ± 29	390 ± 16	48 ± 5
K_A^* (nM)		8 ± 0.5	20 ± 1	36.0 ± 1.8	7.1 ± 0.4
k_6 (min^{-1})		1.20 ± 0.13	1.20 ± 0.12	0.60 ± 0.05	0.086 ± 0.011
k_7 (min^{-1})		0.050 ± 0.002	0.06 ± 0.003	0.060 ± 0.003	0.015 ± 0.001
$k_{assoc} \times 10^4$ (M^{-1} s^{-1})		10.4 ± 0.8	5.2 ± 0.4	2.8 ± 0.1	3.5 ± 0.5

revealed that, like tylosin, clarithromycin and roxithromycin protect nucleosides A2058 and A2059 from DMS modification (Fig. 6). In addition, both antibiotics protect G2505 from kethoxal modification (data not shown). Previous studies have demonstrated that tylosin at 100 mM causes additional weak protections at U2506 and A2062 (Poulsen et al., 2000). Because the tylosin concentration used in our experiments was 100-fold lower, the tylosin-mediated protection of A2062 from DMS was fainted (Fig. 6).

Discussion

In the present study, we examined the interaction of the erythromycin derivatives clarithromycin and roxithromycin with a bacterial ribosomal complex active in peptide bond formation. Such functional studies are scarce in the literature because both antibiotics fail to inhibit the puromycin reaction or the poly(U)-directed poly(Phe) synthesis. To bypass this difficulty, our kinetic study was not designed in consideration of the inhibition of peptide bond formation per se but rather of the competition of roxithromycin or clarithromycin with tylosin for binding to the functional ribosomal complex C.

According to our results, the apparent association rate constant of clarithromycin and roxithromycin binding, k_{assoc} [$k_{assoc} = (k_6+k_7)/K_A$], equals 1.0×10^5 $M^{-1}s^{-1}$ and 0.52×10^5 $M^{-1}s^{-1}$, respectively (Table 1). Both values are lower than the upper limit of 10^6 $M^{-1}s^{-1}$ set for the characterization of a drug as a slow-binding ligand (Morrison and Walsh, 1988). Moreover, the value of the k_6/k_7 ratio in both antibiotics is much greater than 1. Therefore, we can classify clarithromycin and roxithromycin in the family of slow-binding, slowly reversible drugs. Corroborative evidence is also derived from the preincubation effect (i.e., the strengthening of competition with tylosin when complex C is preincubated with the drug before the addition of tylosin). The competition increases with increasing concentrations of clarithromycin or roxithromycin (Fig. 3A). This fact, combined with the linearity of the slope replots shown in Fig. 3B and 4B, suggests that both antibiotics interact with complex C in a time-dependent, two-step mechanism. This is consistent with the biphasic pattern of plot shown in Fig. 5; even when complex C is fully saturated with clarithromycin or roxithromycin, an equilibrium between CA and C*A exists that is not affected by the drug concentration. The two-step mechanism has been also suggested by transferred nuclear Overhauser effect spectroscopy studies in vacant ribosomes (Bertho et al., 1998a,b,c). It was concluded from these studies that compounds unable to take part in a two-step interaction with bacterial ribosomes do not exert antibiotic activity.

Assuming that the two-step-mechanism of macrolide inter-

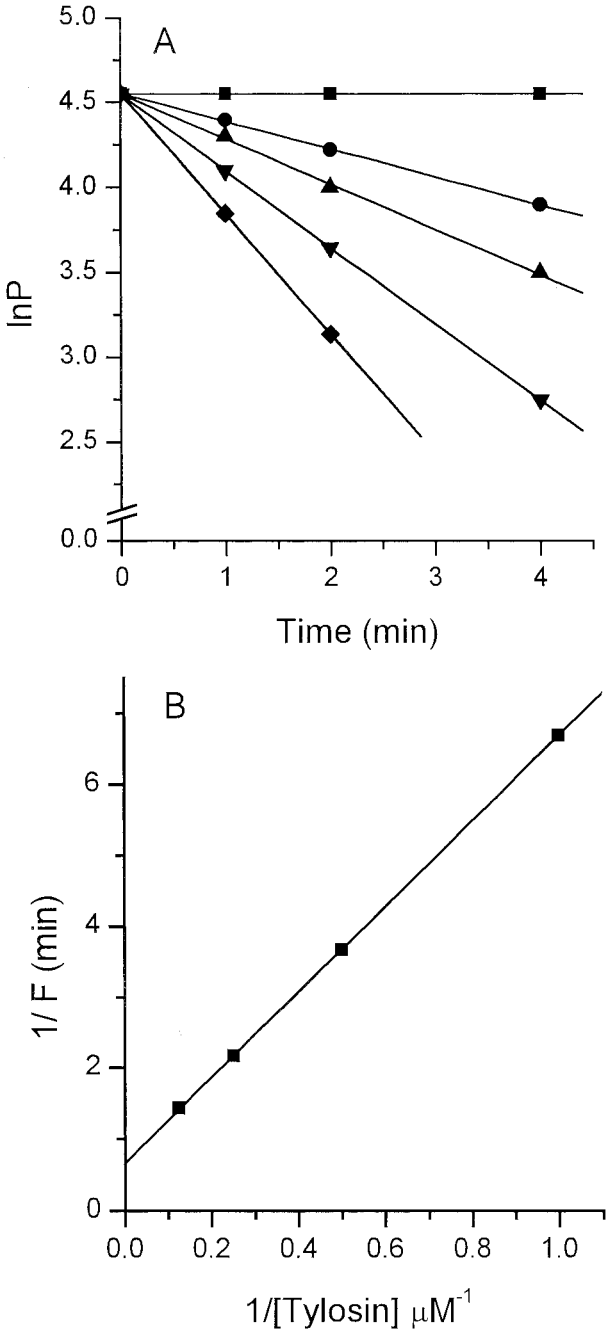

Fig. 4. Variation of F as a function of the concentration of tylosin. A, complex C adsorbed on a cellulose nitrate filter reacted with a solution containing 0.6 μM clarithromycin and tylosin at the following concentrations: none (■), 1 μM (●), 2 μM (▲), 4 μM (▼), and 8 μM (♦). B, variation of $1/F$ as a function of 1/[tylosin]. The parameter F represents the apparent rate constant of complex C inactivation.

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action with ribosomes is correct, it becomes clear that the potency of a macrolide cannot be expressed on the basis of K_A alone. To describe late events of the ribosome/drug interaction, the use of constants additional to K_A are necessary. This criterion can be satisfied by the use of the overall dissociation constant K_A^* which concerns both steps of macrolide interaction with ribosomes. According to K_A^* values (Table 1), we could surmise that the ranking order of drug affinity for complex C is azithromycin ≥ clarithromycin > roxithromycin > erythromycin. This is in agreement with previous studies demonstrating the superiority of azithromycin as an antimicrobial agent against *E. coli* cells (Retsema et al., 1987; Fass, 1993; Zuckerman, 2000; Garza-Ramos et al., 2001; Nilius et al., 2001). Also, a direct correlation is observed between the K_A^* values and the in vitro activity of each drug against susceptible organisms (Hardy et al., 1988; Zuckerman, 2000; Nilius et al., 2001). It should be mentioned that the K_A^* values illustrated in Table 1 are in good proximity to K_{dis} values measured by equilibrium dialysis (Pestka, 1974), fluorescence stop-flow analysis (Moureau et al., 1983), and footprinting protection experiments (Douthwaite and Aagaard 1993; Douthwaite et al., 2000). Small numerical deviations may occur because functional ribosomal complexes, instead of vacant 70S ribosomes or 50S ribosomal subunits, have been used in the present work.

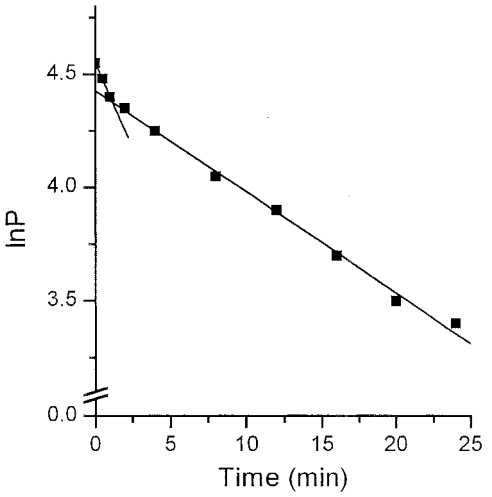

Fig. 5. Determination of the dissociation rate constant k_7. Complex C formed in the presence of 10 μM clarithromycin and adsorbed on a cellulose nitrate filter was exposed to 1 μM tylosin for the time intervals indicated. After removing the excess of the antibiotics, the remaining activity was titrated by 2 mM puromycin (2 min at 25°C). The k_7 value was estimated from the late slope of the plot.

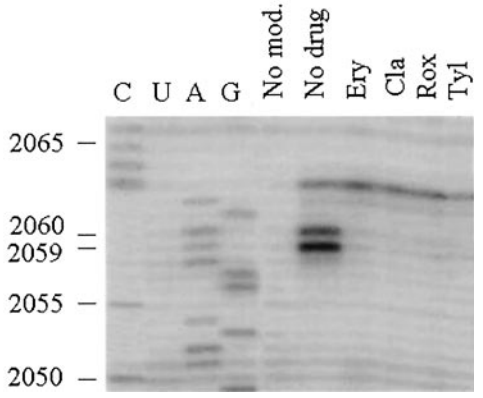

Fig. 6. Alterations in chemical reactivities toward DMS in the central loop region of domain V of 23S rRNA caused by drug binding to 70S ribosomes. Reverse transcription was done with a primer complementary to 23S rRNA nucleotides 2105 to 2121. Positions read from sequencing products (lanes C, U, A, and G) are shown. Reverse transcriptase stops one nucleoside before the modified site. No mod., unmodified 70S ribosomes; no drug, 70S ribosomes modified by DMS in the absence of drug; Ery, Cla, Rox, and Tyl, 70S ribosomes preincubated with 1 μM erythromycin, 1 μM clarithromycin, 1 μM roxithromycin, and 1 μM tylosin, respectively, and then modified by DMS.

According to eq. 4, the high affinity of azithromycin for complex C is caused by the low K_A and k_7 values exhibited by this drug. Clarithromycin compared with roxithromycin and erythromycin displays almost the same k_7 rate constant (Table 1). This implies that the tightness of C*A complex does not essentially differ among these three antibiotics. In contrast, clarithromycin exhibits a higher apparent association rate constant k_{assoc} than roxithromycin or erythromycin (Table 1). Therefore, it is reasonable to conclude that clarithromycin induces more efficiently the formation of complex C*A. This conclusion cannot be easily drawn from the footprinting results. All three antibiotics, in agreement with previous observations (Moazed and Noller, 1987; Hansen et al., 1999; Douthwaite et al., 2000; Poulsen et al., 2000), exhibit identical footprinting patterns in the central loop of 23S rRNA V domain (Fig. 6). Distinctively, tylosin has been found to additionally protect U2506, as a result of the mycarose moiety present in this drug. This difference has been correlated with the ability of 16-membered macrolides to inhibit PTase (Poulsen et al., 2000). On the other hand, high-resolution crystallographic studies using 50S ribosomal subunits from *Deinococcus radiodurans*, complexed with each one of the three antibiotics, have identified seven hydrogen bonds implicated in the drug binding (Schluenzen et al., 2001). All of these hydrogen-bond interactions are mediated by the same reactive groups of the desosamine amino-sugar, and the lactone ring, thus, does not differ among the three drugs. In contrast, recent X-ray data for the binding of 15- and 16-membered ring macrolides to *H. marismortui* 50S subunits did not reveal a similar H-bond network; instead, the interactions were predominantly via Van der Waals forces, particularly through both the lactone ring and sugar extension (Hansen et al., 2002). Accordingly, transferred nuclear Overhauser effect spectroscopy studies have indicated that each one of these antibiotics binds to ribosomes by an identical "surface" involving the C13 to C5 lactone region of the aglycon and both sugar rings, essentially the cladinose one (Bertho et al., 1998b). Taking into account that the erythromycin derivatives are more hydrophobic than the parent compound (Bertho et al., 1998c), their lower K_A^* values may be related to such hydrophobic interactions, complementary to the existing hydrogen bonds. In fact, most of the 14-membered ring macrolides are characterized by a hydrophobicity difference between the two sides of the lactone ring (Ma et al., 2001). It is believed that this bifacial nature of macrolide molecules is very important for many of their biological functions.

In conclusion, azithromycin and clarithromycin, compared with erythromycin, display a substantially lower overall dissociation constant, K_A^*. This property may contribute significantly to their in vivo antimicrobial activity. On the other hand, the relatively smaller difference in K_A^* values between roxithromycin and erythromycin indicates that the higher in

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vivo activity of roxithromycin may be related to a higher drug stability and intracellular accumulation rather than to differences in the interaction with ribosomes.

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