

Insights into the Mechanism of Azithromycin Interaction with an *Escherichia coli* Functional Ribosomal Complex

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ABSTRACT

Azithromycin, a derivative of erythromycin with improved activity against Gram-negative bacteria, exhibits a marginal inhibition effect in a model system derived from *Escherichia coli*, in which a peptide bond is formed between puromycin and AcPhe-tRNA bound at the P-site of poly(U)-programmed ribosomes. This renders the study of azithromycin interaction with Ac^[3H]Phe-tRNA · poly(U) · 70S ribosome complex (complex C) impossible, if we analyze its effect on peptide bond formation. To overcome this problem, we have used an alternative approach to investigate kinetically the azithromycin interaction with complex C and to compare the azithromycin binding properties with those of erythromycin. This approach was based on

the ability of azithromycin to compete with tylosin, a macrolide antibiotic strongly inhibiting the puromycin reaction. Detailed kinetic analysis revealed that the encounter complex CA between complex C and azithromycin (A) undergoes a slow isomerization to a tighter complex C*A, which remains active toward puromycin. The determination of inhibition and isomerization rate constants enabled us to classify azithromycin as a slow-binding ligand of ribosomes. Compared with erythromycin, azithromycin is a better inducer and stabilizer of the C*A complex. This finding may explain the superiority of azithromycin as inhibitor of translation in *E. coli* cells and many other Gram-negative bacteria.

Macrolide antibiotics are powerful inhibitors of protein synthesis in bacteria. They are composed of a large aglycone ring (from 14 to 16 carbon atoms) on which several sugars are attached, some of which are amino sugars containing a diethylamino group (Gale et al., 1981). Macrolides with a 16-membered lactone ring, such as spiramycin and tylosin, bind to the 50S ribosomal subunit and inhibit PTase, possibly interfering with the interaction of peptidyl-tRNA with the ribosomal P-site, a process that may also lead to destabilization and premature release of peptidyl-tRNA (Brisson-Noel et al., 1988). Additional evidence suggests that spiramycin as well as tylosin do not act simply by binding but by inducing through their binding a conformational change on the ribosome, thereby interfering with the substrate attachment at the acceptor site (Dinos et al., 1993; Dinos and Kalpaxis, 2000). It has been recently demonstrated (Champney and Tober, 2000) that spiramycin and tylosin, in addition to their inhibitory effect on translation, prevent the formation of the 50S ribosomal subunit in growing bacterial cells. On the contrary, erythromycin, a 14-membered macrolide (Fig. 1), fails to inhibit peptide bond formation in most of the reference cell-free systems, unless donor substrates of specific characteristics are used (Ballesta and Lazaro, 1990). The differentiated behavior of erythromycin is probably caused by

the small size of the drug molecule, which cannot allow functional groups of erythromycin to extend into the catalytic cavity of PTase (Porse et al., 1995). Despite the inability of erythromycin to inhibit the PTase activity, this drug affects the interaction of peptidyl-tRNA with the P-site and blocks peptide elongation by steric hindrance with the growing polypeptide chain (Menninger, 1985; Chinali et al., 1988; Odom et al., 1991). Thus, mutations that cause resistance to erythromycin have been detected preferentially in ribosomal proteins L4 and L22 (Chittum and Champney, 1994), both of which form part of the surface of the polypeptide exit tunnel (Nissen et al., 2000). Erythromycin, like the large macrolides, also interferes with the formation of the 50S ribosomal subunits (Chittum and Champney, 1995).

Azithromycin, an azalide antimicrobial agent, is a derivative of erythromycin with a 15-membered aglycone ring possessing an additional nitrogen (Fig. 1). This modification increases the basicity of the molecule and improves the drug activity against Gram-negative bacteria. Its minimum inhibitory concentration for 90% of *Escherichia* species strains is 2 µg/ml compared with 32 µg/ml exhibited by erythromycin (Retsema et al., 1987; Zuckerman, 2000). Like erythromycin, this drug prevents bacterial protein-biosynthesis by binding to the large ribosomal subunit and interfering equivalently with the assembly of 50S ribosomal subunit and the growth

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ABBREVIATIONS: Complex C, the Ac^[3H]Phe-tRNA · poly(U) · 70S ribosome complex that bears Ac^[3H]Phe-tRNA bound to the ribosomal P-site; PTase, peptidyltransferase.

of the nascent polypeptide chain (Champney and Burdine, 1998a,b).

Resistance to erythromycin and tylosin, whether inducible or constitutive, is mainly caused by adenine methylation or mutations situated in domains II and V of 23S rRNA, as well as in ribosomal proteins localized near the PTase center (Weisblum, 1995; Spahn and Prescott, 1996; Tait-Kamradt et al., 2000). Azithromycin, like erythromycin and tylosin, does not interact well with methylated ribosomes (Retsema et al., 1987). This explains the observed cross-resistance, and suggests a competition for common or overlapping binding sites on the large ribosomal subunit. Although the potentially useful activity of azithromycin against *Escherichia coli* has been attributed to its faster penetration of the outer membranes (Vaara, 1993), the better ability of azithromycin than erythromycin to compete for [¹⁴C]erythromycin-binding sites might indicate a higher affinity of azithromycin for the susceptible ribosomes (Retsema et al., 1987). However, the affinity of azithromycin for ribosomal complexes active in peptide bond formation has never been determined.

In view of the observations above, it was of interest to examine the interaction of this drug with *E. coli* initiation ribosomal complex and compare its binding properties with those of erythromycin. To bypass the difficulty raised by the fact that azithromycin exhibits a marginal inhibition effect on puromycin reaction, which is usually used as a model reaction for peptide bond formation, an alternative kinetic approach was applied. This was based on the ability of azithromycin to compete with tylosin, a macrolide behaving as a slow-binding, slowly reversible inhibitor of PTase (Dinos and Kalpaxis, 2000).

Experimental Procedures

Materials. Puromycin dihydrochloride, tRNA from *E. coli* strain W, tylosin, and erythromycin were obtained from Sigma (St. Louis, MO). L-[2,3,4,5,6-³H]Phenylalanine was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Cellulose nitrate filters (type HA; 24-mm diameter, 0.45- μ m pore size) were from Millipore Corp. (Bedford, MA). Azithromycin was kindly provided by Dr. C. Theri-

anos of Pfizer Hellas A.E. (Athens, Greece). Stock solutions of antibiotics were prepared by dissolving aliquots of each compound in a small volume of methanol and bringing the solutions to the final volume with 0.1 M Tris-HCl, pH 7.2.

Biochemical Preparations. Ribosomes from *E. coli* B cells, crude Ac[³H]Phe-tRNA charged with 18.5 pmol of [³H]Phe (106,700 cpm total) per A_{260} unit and initiation complex C (i.e., the Ac[³H]Phe-tRNA · poly(U) · 70S ribosome complex), were prepared as described previously (Kalpaxis et al., 1986). The formed complex C was adsorbed on a cellulose nitrate filter and washed with three 4-ml portions of cold buffer A (100 mM Tris-HCl, pH 7.2, 100 mM NH₄Cl, 10 mM magnesium acetate, and 6 mM β -mercaptoethanol).

Puromycin Reaction. The PTase activity of ribosomes was assessed by the puromycin reaction performed at 25°C in the presence of 10 mM Mg²⁺ and 100 mM NH₄⁺. Briefly, complex C adsorbed on a cellulose nitrate filter reacted with excess puromycin in the presence or absence of macrolides, and the progress of the reaction was analyzed over a wide range of macrolide and puromycin concentrations. The product (P), Ac-Phe-puromycin, was expressed as a percentage (x) of the isolated complex C on the filter ($x = 100 \times P / C_0$). It should be mentioned that the value of x was corrected, taking into account the parallel inactivation of complex C during the puromycin reaction and the intervention of other species, except of complex C. Control samples without poly(U) and puromycin were included in each experiment, and the values obtained were subtracted.

Inactivation of Complex C by Tylosin in the Absence or Presence of Azithromycin. Buffer A (2 ml) containing tylosin at specified concentrations and complex C adsorbed on cellulose nitrate filter were added to each of a series of small beakers and allowed to react at 25°C. After the desired reaction time had elapsed, the filter was immersed in 15 ml of cold buffer A and washed by filtration with the same buffer to remove traces of tylosin nonspecifically bound. The remaining active complex C was determined by titration with puromycin (2 mM, 2 min at 25°C). The inactivation of complex C by tylosin was also examined in the presence of various concentrations of azithromycin. In parallel, complex C was preincubated with azithromycin for 10 min, and subsequently reacted with tylosin. The values of the equilibrium and rate constants were determined from the plots of eqs. 1 and 2 by linear regression. All data presented in the figures denote the mean values obtained from four independent experiments.

Results

Inhibition of Peptide Bond Formation by Macrolides. The reaction between complex C and excess puromycin (S), carried out at 25°C in the presence of 10 mM Mg²⁺ and 100 mM NH₄⁺, displays pseudo-first-order kinetics. The anticipated reaction scheme is:



Complex C participates in only one cycle of catalysis since the produced species C' cannot reform reactive ribosomal complex (irreversible inactivation of the enzyme). The relationship

$$\ln \frac{100}{100 - x} = k_{obs}t \quad (1)$$

holds, where k_{obs} is the apparent rate constant of product formation. Equation 1 predicts that the progress curve of the puromycin reaction is a straight line. Such a plot obtained at 200 μ M puromycin is given in Fig. 2 (upper line). From the slope of this plot, a k_{obs} value equal to $0.660 \pm 0.030/\text{min}$ is calculated. In the presence of 20 μ M erythromycin or azithromycin, the slope of the line does not change. However, when

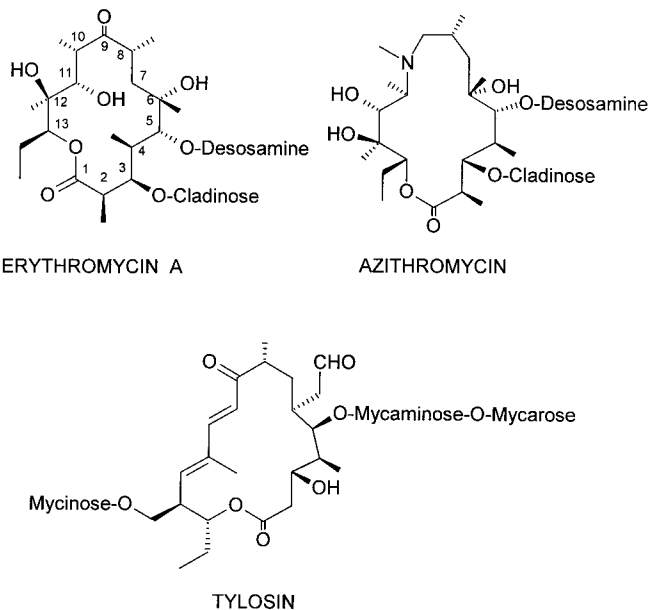
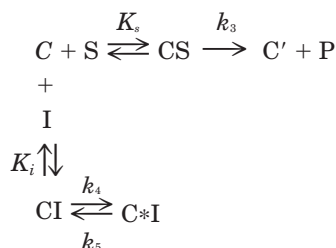


Fig. 1. Chemical structures of erythromycin A, azithromycin, and tylosin.

AcPhe-puromycin synthesis is carried out in the presence of 2 μM tylosin, the rate of product formation is slower, progressively reaching a plateau. Interestingly, a solution containing both 2 μM tylosin and erythromycin or azithromycin at 20 μM fails to inhibit the puromycin reaction (Fig. 2). Detailed kinetic analysis of complex C inactivation by tylosin confirmed previous results (Dinos and Kalpaxis, 2000) suggesting that tylosin (I) reacts rapidly with complex C to form the encounter complex CI, which is subsequently isomerized slowly to a tighter complex C*I, still inactive toward puromycin. These events can be described by kinetic scheme 1:



The K_i , k_4 , and k_5 values, which are in good agreement with the values obtained previously, are presented in Table 1.

Inactivation of Complex C by Tylosin in the Presence of Azithromycin. As shown in Fig. 2, azithromycin fails to inhibit the puromycin reaction with complex C. This makes the study of azithromycin's effect on peptide bond formation impossible. However, azithromycin antagonizes tylosin for binding to complex C (Fig. 2). This is consistent with earlier studies that have demonstrated that azithromycin and other macrolides (including tylosin and erythromycin) share similar or overlapping binding sites on ribosome (Retsema et al., 1987; Porse et al., 1995). Thus, insights into the antagonistic interaction of azithromycin and tylosin with complex C can be gathered by kinetic experiments in which complex C is mixed with a solution containing both tylosin

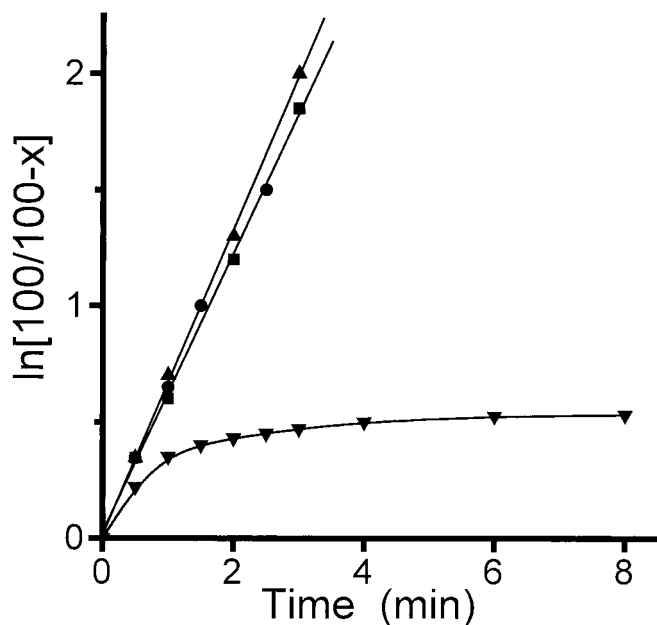
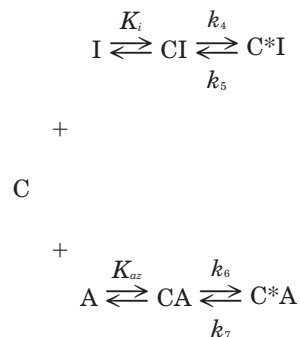


Fig. 2. First-order time plots for AcPhe-puromycin synthesis in the presence or in the absence of macrolides. Complex C reacted with 200 μM puromycin alone (\blacktriangle) or in mixture with 2 μM tylosin (\blacktriangledown), erythromycin or azithromycin at 20 μM (\bullet), or both tylosin at 2 μM and erythromycin or azithromycin at 20 μM (\blacksquare).

and azithromycin, the latter at increasing concentrations. As shown in Fig. 3A, a progressive decrease in the apparent rate constant of complex C inactivation by tylosin occurs as the concentration of azithromycin increases. At high concentrations of azithromycin, the inactivation of complex C is completely reversed (Fig. 3A, upper line). This behavior of azithromycin is reminiscent of the properties of erythromycin (Dinos and Kalpaxis, 2000). The similarity of kinetics suggests that the mechanism of azithromycin may be similar to that of erythromycin. If this is the case, the slope of the plots, like those presented in Fig. 3A, gives the apparent rate constant of inactivation (F), which is related to the azithromycin (A) concentration by the equation:

$$\frac{1}{F} = \frac{K_i + [I]}{k_4[I]} + \frac{K_i[A]}{k_4K_{az}[I]} \quad (2)$$

In eq. 2, K_{az} represents the dissociation constant of complex CA. As predicted by eq. 2, at each concentration of tylosin (I), the plot of $1/F$ versus azithromycin concentration should be a straight line. Figure 3B shows such a plot obtained at 4 μM tylosin with various concentrations of azithromycin, which is linear and satisfies the hypothesized similarity of the two mechanisms. From the slope of this plot, a K_{az} value equal to 48 nM can be estimated. A kinetic scheme that interprets the competition of tylosin reaction by azithromycin can be represented by the kinetic scheme 2:



By preincubating complex C with azithromycin for 10 min before the addition of tylosin, a further decrease in the inactivation constant F is observed, suggesting that at least one of the sequential steps of complex C interaction with azithromycin is slow. Under such conditions, the estimated dissociation constant determines the overall dissociation constant (K_{az}^*) concerning both steps of azithromycin interaction with

TABLE 1

Kinetic parameters of the interaction between ribosomal complex C and the macrolides tylosin, erythromycin and azithromycin

Parameter	Tylosin	Erythromycin	Azithromycin
K_i (nM)	2950 \pm 100		
K_i^* (nM)	4.98 \pm 0.30		
k_4 (min^{-1})	1.36 \pm 0.20		
k_5 ($\text{min}^{-1} \times 10^{-3}$)	2.30 \pm 0.30		
K_{er} (nM)		393 \pm 22	
K_{er}^* (nM)		36 \pm 3	
K_{az} (nM)			48 \pm 5
K_{az}^* (nM)			7.1 \pm 0.4
k_6 (min^{-1})		0.59 \pm 0.09	0.086 \pm 0.011
k_7 (min^{-1})		0.060 \pm 0.008	0.015 \pm 0.001
k_4/K_i ($\text{M}^{-1} \text{s}^{-1} \times 10^4$)	0.77 \pm 0.11		
k_6/K_{er} ($\text{M}^{-1} \text{s}^{-1} \times 10^4$)		2.5 \pm 0.2	
k_6/K_{az} ($\text{M}^{-1} \text{s}^{-1} \times 10^4$)			3.0 \pm 0.3

complex C. According to the slow-onset inhibition theory (Morrison and Walsh, 1985), the isomerization constant k_6/k_7 can be determined by eq. 3:

$$K_{az}^* = K_{az} \left(\frac{k_7}{k_6 + k_7} \right) \quad (3)$$

This value equals 5.72.

To determine the k_7 value, complex C formed in the presence of 0.5 μM azithromycin was adsorbed on a cellulose nitrate filter and, after exposure to 4 μM tylosin for various

time intervals, its activity was titrated with puromycin. The time plot of the reaction was biphasic, displaying an early and a late slope (Fig. 4). We assume that the early phase corresponds to the reaction of tylosin with preexisting active complex C, whereas the late slope represents the reaction of tylosin with complex C regenerated slowly from the complex C*A. Because $k_7 < k_4$, the rate of C*I complex formation is limited by the net flux from C*A to C*I, via the rate limiting step k_7 . From the late slope, a value of k_7 equal to 0.015/min is determined. The value of k_6 estimated from the ratio k_6/k_7 and the value of the apparent association rate constant (k_6/K_{az}) are given in Table 1. For the sake of comparison, the values of the kinetic constants concerning the interaction of erythromycin with complex C are also included.

Discussion

The potency of a macrolide as a pharmaceutical agent depends on its structural resistance to chemical modifications as well as on its ability to penetrate the plasma membrane and to accumulate into the microbial cells. For instance, one of the major disadvantages in the use of erythromycin, compared with azithromycin, is its extreme acid sensitivity leading to degradation in the stomach after oral administration (Mord et al., 2000; Zuckerman, 2000). However, the most important factor contributing to the potency of a macrolide is its ability to interact with the target site(s) on the ribosome (Douthwaite et al., 2000). Kinetic studies of the azithromycin interaction with functional ribosomes are rather scarce in the literature; therefore, we were prompted toward such an investigation.

By monitoring the effect of azithromycin on the inhibition of peptide-bond formation by tylosin, we established that this antibiotic derives its potency through a slow onset of competition with tylosin for common binding sites on ribosomes. The observation that competition is enhanced by increasing concentrations of azithromycin (Fig. 3A) precludes an isomerization of complex C to C* before the antibiotic attachment (Erion and Walsh, 1987). On the other hand, the replot

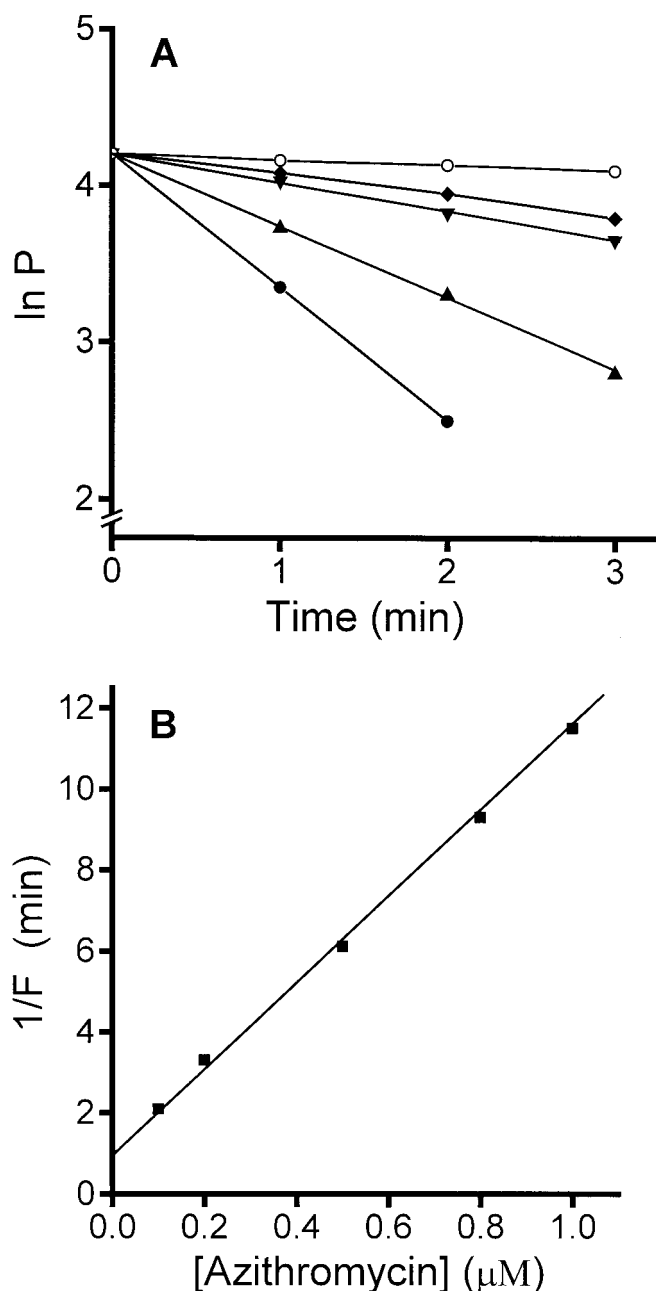


Fig. 3. Effect of azithromycin on the inactivation of ribosomal complex C by tylosin. A, complex C reacted with 4 μM tylosin alone (●) or with a solution containing both 4 μM tylosin and azithromycin at 0.2 μM (▲), 0.5 μM (▼), 0.8 μM (◆), and 1 μM (○). B, variation of $1/F$ as a function of the azithromycin concentration. The parameter F represents the apparent rate constant of complex C inactivation, and its value is estimated from the slope of the A plots.

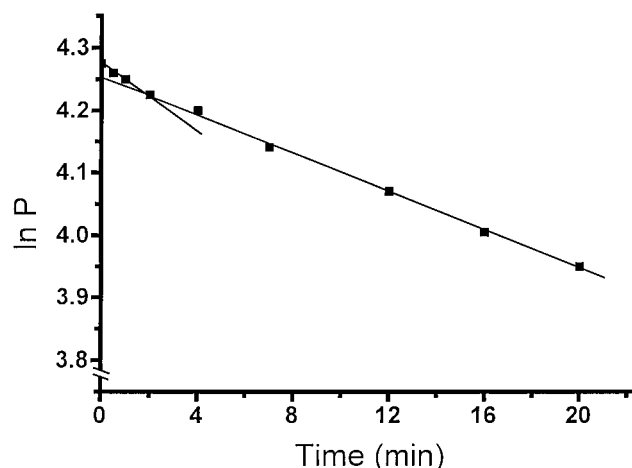


Fig. 4. Determination of the rate constant k_7 . Complex C formed in the presence of 0.5 μM azithromycin, was isolated on cellulose nitrate filter and exposed to 4 μM tylosin for the time intervals indicated. The remaining catalytic activity of complex C was then titrated by 2 mM puromycin (10 min, at 25°C). The k_7 constant represents the rate constant of activity regeneration from the azithromycin complex C*A, and its value is estimated from the late slope of the plot.

of $1/F$ versus azithromycin concentration is linear, intercepting the vertical axis at a point above zero (Fig. 3B). This finding is inconsistent with a binding mechanism of the type $C + A \rightleftharpoons C^*A$. If a single-step mechanism could exist, the inactivation constant F should be independent of the concentration of azithromycin, as time approached 0 (experiments without preincubation). Consequently, our results suggest that azithromycin interacts with complex C in a two-step mechanism, resembling the binding mechanism followed by tylosin or erythromycin (Dinos and Kalpaxis, 2000). Corroborative evidence is also coming from the plot shown in Fig. 4; even when complex C is fully saturated with azithromycin, not all of complex C is in form C^*A . This is consistent with an equilibrium between CA and C^*A that is not affected directly by the drug concentration. The apparent association rate constant (k_6/K_{a2}) of azithromycin binding equals $3.0 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, a value much lower than the upper limit of $10^6 \text{ M}^{-1} \text{ s}^{-1}$ set for the characterization of a drug as a slow-binding ligand (Morrison and Walsh, 1985). In addition, the reverse rate constant k_7 is less than the forward rate constant k_6 ($k_6/k_7 = 5.72$). Both values enable us to classify azithromycin as a slow-binding, slowly reversible drug, interacting with complex C. This conclusion is also supported by the preincubation effect (i.e., the strengthening of azithromycin competition with tylosin when preincubation of complex C with the drug precedes the addition of tylosin). Transferred nuclear Overhauser effect measurements (Bertho et al., 1998a,b), equilibrium dialysis studies (Pestka, 1974), membrane filtration studies (Di Giambattista et al., 1987), and footprinting experiments (Douthwaite and Aagaard, 1993), postulate that such a two-step process may exist for the binding of several macrolides to ribosomes, including erythromycin.

Compared with tylosin and erythromycin, azithromycin exhibits a higher apparent association rate constant (Table 1). This justifies the hypothesis that azithromycin is a better inducer of the C^*A complex formation. From the standpoint of pharmaceutical applications, a fast rate of association with ribosomes is desirable because it may reduce the time required for inhibition at a given drug concentration. Moreover, azithromycin compared with erythromycin, displays a lower k_7 value, which results in a longer-lived C^*A complex. The superiority of azithromycin potency has also been established by binding studies using *Staphylococcus aureus* ribosomes (Retsema et al., 1987). In contrast, results from another study in *S. aureus* cells have showed that erythromycin is much more potent inhibitor of translation than azithromycin (Champney et al., 1998b). However, in the latter study, the relative protein synthesis rate has been measured by ^{35}S -amino acid incorporation in growing cells. Therefore, factors related to the uptake and efflux of azithromycin may have influenced the drug efficiency.

The present work demonstrates that azithromycin, like erythromycin, upon binding to bacterial ribosomes causes a slow rearrangement of the encounter complex to another more tight species. Therefore, the use of constants in addition to K_i is required to evaluate late events of the ribosome-drug interaction. In addition, this work shows that azithromycin, compared with erythromycin, displays a better ability to bind bacterial ribosomes.

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