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 bound at the P-site of poly(U)-programmed ribosomes possible, 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 an diethylenamin 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; Chiniali 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 sub-units (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

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 $^{14}$C]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-$^3$H]Phenylalanine was purchased from Amersham (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$^3$H]Phe-tRNA charged with 18.5 pmol of $^3$H]Phe (106,700 cpm total) per 100 unit and initiation complex C (i.e., the Ac$^3$H]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 NH$_4$Cl, 10 mM magnesium acetate, and 6 mM $\beta$-mercaptoethanol).

**Puromycin Reaction.** The PTase activity of ribosomes was assessed by the puromycin reaction performed at 25°C in the presence of 10 mM Mg$^{2+}$ and 100 mM NH$_4^+$ . 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 ($x = 100 \times P/C_{C}$). 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 value equal to 0.660

\[
 k_{obs} = \frac{100}{100 - x} = k_{obs}t
\]

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 mM puromycin is given in Fig. 2 (upper line). From the slope of this plot, a $k_{obs}$ value equal to 0.660 ± 0.030/min is calculated. In the presence of 20 mM erythromycin or azithromycin, the slope of the line does not change.
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:

\[
\begin{align*}
    & K_s + S \rightleftharpoons CS \rightarrow C' + P \\
    & I \\
    & K_i \uparrow \\
    & CI \rightleftharpoons C*I \\
    & k_i \\
\end{align*}
\]

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 ribosomes (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 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:

\[
\frac{1}{F} = \frac{K_{ai} + [I]}{k_i} + \frac{K_{i}[A]}{k_i K_{ai}[I]}.
\]

In eq. 2, \(K_{ai}\) represents the dissociation constant of complex CA. As predicted by eq. 2, at each concentration of tylosin (I), the slope of 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 \(K_{ai}\) 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:

\[
\begin{align*}
    & K_s + S \rightleftharpoons CS \rightarrow C' + P \\
    & I \\
    & K_i \uparrow \\
    & CI \rightleftharpoons C*I \\
    & k_i \\
    & + \\
    & A \rightleftharpoons CA \rightleftharpoons C*A \\
    & k_i \\
\end{align*}
\]

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 \(K_{ai,*}\) concerning both steps of azithromycin interaction with

**Table 1**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Tylosin</th>
<th>Erythromycin</th>
<th>Azithromycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>(K_s) (nM)</td>
<td>2950 ± 100</td>
<td>4.98 ± 0.30</td>
<td>4.98 ± 0.30</td>
</tr>
<tr>
<td>(k_{ai,*}) (nM)</td>
<td>1.36 ± 0.20</td>
<td>2.30 ± 0.30</td>
<td>2.30 ± 0.30</td>
</tr>
<tr>
<td>(K_{ai,*}) (nM)</td>
<td>383 ± 22</td>
<td>36 ± 3</td>
<td>36 ± 3</td>
</tr>
<tr>
<td>(K_{ai}) (nM)</td>
<td>48 ± 5</td>
<td>7.1 ± 0.4</td>
<td>7.1 ± 0.4</td>
</tr>
<tr>
<td>(k_{ai}) (min⁻¹)</td>
<td>0.59 ± 0.09</td>
<td>0.060 ± 0.008</td>
<td>0.060 ± 0.008</td>
</tr>
<tr>
<td>(k_{ai}^*) (min⁻¹)</td>
<td>0.77 ± 0.11</td>
<td>0.015 ± 0.001</td>
<td>0.015 ± 0.001</td>
</tr>
<tr>
<td>(k_{ai}/K_{ai,*}) (M⁻¹ s⁻¹ × 10⁴)</td>
<td>0.77 ± 0.11</td>
<td>0.015 ± 0.001</td>
<td>0.015 ± 0.001</td>
</tr>
<tr>
<td>(k_{ai}/K_{ai,*}) (M⁻¹ s⁻¹ × 10⁴)</td>
<td>2.5 ± 0.2</td>
<td>3.0 ± 0.3</td>
<td>3.0 ± 0.3</td>
</tr>
</tbody>
</table>
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:

$$K'_{az} = K_{az} \left( \frac{k_7}{k_6 + k_7} \right)$$

(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*1 complex formation is limited by the net flux from C*A to C*1, 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 ($k_{az}/K_{az}$) 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 replota
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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 ⇌ 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 Kallipaxis, 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 (kₐ/Kₐ) of azithromycin binding equals 3.0 × 10⁶ M⁻¹ s⁻¹, a value much lower than the upper limit of 10⁶ M⁻¹ s⁻¹ set for the characterization of a drug as a slow-binding ligand (Morrison and Walsh, 1985). In addition, the reverse rate constant kₛ is less than the forward rate constant kₐ (kₐ/kₛ = 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 Giammatteita 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 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 1) exhibits a higher apparent association rate constant (Table

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