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Binding parameters and microbiological activity of macrolides, lincosamides and streptogramins against *Staphylococcus aureus*

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Parameters of erythromycin binding to *Staphylococcus aureus* were measured in-vitro using an equilibrium method with [^{3}H]erythromycin. The dissociation constant of the complex, erythromycin–*S. aureus* sensitive strain, was K_D = 0·11 μM. The maximal binding, representing the density of binding sites was 14 847 molecules/cell. No binding was detectable on the constitutive resistant strain. Macrolides, streptogramins and lincosamides displaced bound [^{3}H]erythromycin by a competitive process indicating that these compounds share common binding sites on the bacteria, i.e. 50 S ribosomal subunits. A good correlation (r = 0·99) was demonstrated between the corresponding inhibition constants (K_i) and the minimal inhibitory concentration. It is proposed that knowledge of the binding parameters provides a good indication of bacterial susceptibility and may serve as a useful adjunct in developing new compounds.

Macrolides inhibit protein synthesis as a consequence of their ability to bind tightly to the 50 S subunit of bacterial ribosomes. Moreover, it is known that the stoichiometry of erythromycin binding to ribosomal particles of *Staphylococcus aureus* involves one molecule bound to each 50 S subunit (Mao 1967). We have previously shown that the binding parameters of macrolides obtained using whole bacteria actually reflect binding to the active sites, that is the 50 S ribosomal subunit, where these antibiotics exert their action (Barre et al 1986). This paper reports the binding parameters for the interaction of macrolides, lincosamides and streptogramins (MLS) to sensitive and constitutive resistant strains of *S. aureus*. Thus, these antibiotics have been ranked in terms of their affinity for bacteria. At the same time, the minimal inhibitory concentrations of the same antibiotics were determined microbiologically. For some bacterial strains, the evaluation of minimal inhibitory concentrations may be difficult because of special requirements of media and growth conditions. Thus, the determination of binding parameters may be much easier and faster but a labelled antibiotic with a high specific activity is required. For the latter approach to be valid, it is necessary to establish that a relationship exists between these two types of determination of antibiotic activity. For this reason, we have compared the parameters for MLS binding to *S. aureus* with the corresponding minimal inhibitory concentration of the drugs. Thus, such studies would be used alone or in conjunction with microbiological assays for primary screening of active analogues or other compounds which interfere with [^{3}H]erythromycin binding to intact bacteria.

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Materials and methods

[^{3}H]Erythromycin (25 Ci mmol^{-1}) was synthesized by the Laboratory of Structural Organic Chemistry (Université des Sciences et des Techniques du Languedoc, Montpellier). The radiochemical purity, determined by thin layer chromatography on silica gel plates in carbon tetrachloride–ethanol–dimethylformamide (7:2:1), was better than 98%. Labelled and unlabelled compounds were supplied by Abbott Laboratories. Other antibiotics used were josamycin (Spret-Mauchan), pristinamycin (Upjohn), virginiamycin (SKF), rosaramicin (Schering), oleandomycin (Rosaphytopharma), midecamycin (Clin-Midy), a new macrolide, 9-(2-methoxy)ethoxymethoxyimino)erythromycin (RU 28965: roxithromycin) (Roussel Uclaf), lincomycin and clindamycin (Upjohn), and spiramycin (Specia).

Bacteria strains and media. The two *S. aureus* strains tested, i.e. sensitive (209 P CNCM 53156) and constitutive resistant ('Brossy') strains, were obtained from the department of bacteriology of CHU Henri Mondor. Media cultures were prepared with distilled water and brain heart infusion broth (BHI) (2%). All binding experiments were performed in 50 mM Tris-HCl buffer, pH = 7·4 at 37 °C.

Ultrafiltration. All binding experiments were performed by ultrafiltration. The bacteria and bound antibiotic fraction were collected on the filter and washed to eliminate the free fraction.

Equilibrium studies of [^{3}H]erythromycin binding. The binding reaction between an antibiotic, A, and a bacterium, B, is a second order reaction obeying simple mass action principle.

$$[A^*] + [B] \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} [A^* - B]$$

at equilibrium:

$$[A^*] \cdot [B]/[A^* - B] = K_d = 1/K_a$$

where [B] is the concentration of free bacteria, [A*] the concentration of unbound antibiotic, and [A* − B] the

concentration of antibiotic-bacterial complex. k_{+1} and k_{-1} are the binding kinetic rate constants for association and dissociation, respectively. K_d and K_a are the equilibrium dissociation and association constants.

Equilibrium studies allowed us to determine whether the binding of the antibiotic to bacteria is saturable, and, also, the dissociation constant (K_d) of the antibiotic-bacterial complex, the number of the binding sites family and the concentration of binding sites (B_{max}).

The reaction mixture contained 400 μL of *S. aureus* suspension (10^7 cells mL^{-1}) and varying amounts of [^{3}H]erythromycin (10^{-9} to 4×10^{-7} M) in a final volume of 500 μL. The solution was incubated at 37 °C for 50 min then filtered through a Millipore filter (EHWP, 0·5 μm) and washed twice with 10 mL of ice-cold Tris-HCl. Specific binding was defined as the difference between the binding observed in the presence and absence of 10^{-4} M unlabelled erythromycin. The binding of [^{3}H]erythromycin to bacteria was determined by filtering 400 μL of the reaction mixture. After filtration, filters were washed twice with 10 mL of ice-cold Tris-HCl buffer. The radioactivity of the filters, in 5 mL of Picofluor 30, was counted in a Packard 460 CD liquid scintillation counter.

Inhibition of [^{3}H]erythromycin binding by different antibiotics. By displacing the labelled antibiotic binding to the bacteria by unlabelled antibiotic, the dissociation constant of the competitor is obtained. Thereby, a classification of the antibiotics as a function of their affinity for the bacteria is established. A 250 μL sample of *S. aureus* suspension (10^7 cells mL^{-1}), [^{3}H]erythromycin (10^{-7} M) and various amounts (10^{-11} to 10^{-4} M) of other antibiotics in a final volume of 500 μL were incubated at 37 °C for 60 min. The ultrafiltration technique was used as described above.

Determination of minimal inhibitory concentration of the antibiotics. Bacteriological activity of macrolides was appraised by measurement of minimal inhibitory concentrations (MIC). These were determined by the agar dilution method in Mueller-Hinton medium. Concentrations of antibiotic were prepared to a progressive 1·25 geometrical ratio. A bacterial suspension 10^5 to 10^6 bacteria mL^{-1}, was sown onto agar plates which were then incubated at 37 °C for 24 h. MIC was defined as the minimal concentration inhibiting the overall culture of bacteria; however, the presence of one to three colonies was neglected for that determination.

Results

Equilibrium studies of [^{3}H]erythromycin to S. aureus. Total, non-specific and specific binding of [^{3}H]erythromycin to bacteria are shown in Fig. 1. The specific binding was determined by the difference between the total and the non-specific binding of [^{3}H]erythromycin. The specific binding was saturable, as shown by the linear Scatchard plot (Fig. 1 insert), with one class of

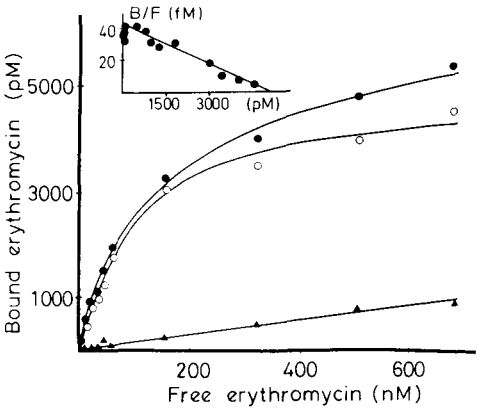

FIG. 1. Equilibrium studies of [^{3}H]erythromycin binding to *S. aureus*. The equilibrium binding study was performed at 37 °C for 60 min using [^{3}H]erythromycin at various concentrations (10^{-9} to 7×10^{-7} M). Total (●——●), specific (○——○) and non-specific (▲——▲) binding were calculated as indicated in the text. The Scatchard plot for specific [^{2}H]erythromycin binding to *S. aureus* is shown in the insert.

binding sites. Binding parameters were calculated by the non-linear method described by Zini et al (1983). For *S. aureus* 209 P, the value of the dissociation constant was 0·11 ± 0·01 μM, whereas the density of binding sites, B_{max}, was 14 847 ± 361 molecules/cell. For the *S. aureus* 'Brossy', constitutive resistant strain, specific binding was not detectable as reported by Saito et al (1968).

Inhibition of [^{3}H]erythromycin binding to S. aureus *by different antibiotics.* Increasing concentrations of the various antibiotics progressively inhibited [^{3}H]erythromycin binding. These inhibitory effects have been studied with two mathematical models: the classical and Hill models which were compared with a Fisher test as described by Molinoff et al (1981). The value of the Hill pseudo-number of all MLS was not significantly different from 1 (Table 1); so the classical model was chosen.

Table 1, Inhibition constants (K_i) and minimal inhibitory concentration (MIC) values for macrolides, lincosamides and streptogramins against *S. aureus*. The inhibition constant was obtained by measuring the binding of [^{3}H]erythromycin (10^{-7} M) under equilibrium conditions in the presence of various concentrations (10^{-10} to 10^{-4} M) of unlabelled antibiotics. The MIC value was determined on Mueller-Hinton media (MH).

Antibiotics	K_i (μM)	N_{Hill}	MIC (μg mL^{-1})
Rosaramicin	0·10 ± 0·01	1·01 ± 0·16	0·16
Pristinamycin	0·11 ± 0·02	1·18 ± 0·32	0·16
Erythromycin	0·11 ± 0·01	0·96 ± 0·08	0·12
Virginiamycin	0·12 ± 0·03	1·19 ± 0·30	0·12
Roxithromycin	0·14 ± 0·01	1·10 ± 0·11	0·16
Clindamycin	0·15 ± 0·02	0·93 ± 0·08	0·12
Lincomycin	0·31 ± 0·05	0·84 ± 0·08	0·39
Midecamycin	0·38 ± 0·04	1·03 ± 0·13	0·63
Josamycin	0·42 ± 0·06	1·04 ± 0·23	0·78
Oleandomycin	0·44 ± 0·04	1·02 ± 0·12	0·78
Spiramycin	0·73 ± 0·01	1·12 ± 0·35	1·25

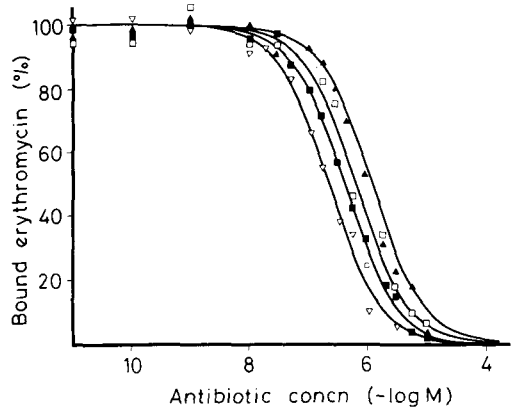

FIG. 2. Inhibition of [^{3}H]erythromycin binding to *S. aureus* by different antibiotics. Binding of [^{3}H]erythromycin (10^{-7} M) to *S. aureus* was measured under equilibrium conditions in the presence of various concentrations (10^{-10} to 10^{-4} M of unlabelled antibiotics. ▲: spiramycin, □ josamycin, ■ lincomycin, ▽ erythromycin.

The IC50 values, defined as the concentration of these unlabelled antibiotics capable of displacing 50% of maximum specific binding of [^{3}H]erythromycin, were used to obtain the inhibition constants (K_i) of these drugs according to the equation developed by Cheng & Prussof (1973):

$$K_i = IC50/(1 + [A^*]/K_g)$$

where [A*] and K_g represent, respectively, [^{3}H]erythromycin concentration and the dissociation constant of the *S. aureus* erythromycin complex (Fig. 2). K_i values were determined for all antibiotics (Table 1). Rosaramicin, erythromycin, roxithromycin, pristinamycin, virginiamycin and clindamycin exhibit the same inhibition constant (K_i = 0·11 μM) while lincomycin, midecamycin, josamicin, oleandomycin and spiramycin exhibit values of $K_i \geq 0{\cdot}31$ μM.

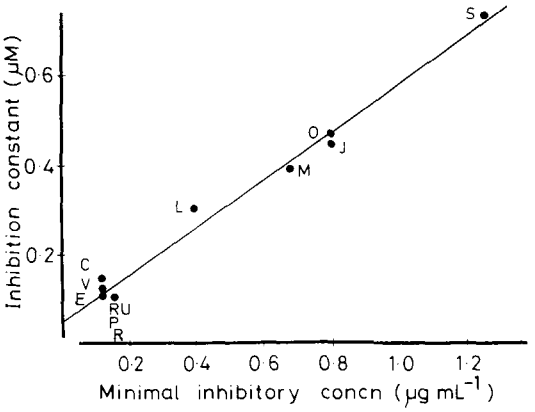

FIG. 3. Correlation between inhibition constant (K_i) and minimal inhibitory concentration (MIC) of macrolides, lincosamides and streptogramins against *S. aureus*. E erythromycin, V virginiamycin, C clindamycin, RU roxithromycin, P pristinamycin, R rosaramycin, L lincomycin, M midecamycin, O oleandomycin, J josamycin, S spiramycin.

MIC determinations. The MIC of antibiotics tested against *S. aureus* 209 P varied as follows: 0·12 μg mL^{-1} for erythromycin, virginiamycin and clindamycin, 0·15 μg mL^{-1} for pristinamycin and roxithromycin, and ≥ 0·39 μg mL^{-1} for lincomycin, midecamycin, josamycin, oleandomycin and spiramycin (Table 1).

Discussion

The dissociation constants, K_d, of the [^{3}H]erythromycin-*S. aureus* complex determined either directly from equilibrium binding of [^{3}H]erythromycin, or indirectly from the inhibition of binding of [^{3}H]erythromycin, are identical at 0·11 μM—the same value as that found by Mao & Putterman (1979). Erythromycin binds to the bacteria at only a single class of binding sites which probably represents the 50 S ribosomal subunit, the concentration of these binding sites is 14 847 ± 361 molecules per cell.

It is important that the constitutive resistant strain exhibits negligible binding of erythromycin. This observation is consistent with the findings of Weisblum et al (1971) and Lai et al (1973) who demonstrated that ribosomes from constitutively resistant cells showed reduced affinity for erythromycin owing to the presence of a methylated base, N^6-dimethyladenine, in 23 S r RNA, that is absent from the r RNA of ribosomes of the sensitive cells.

The inhibitory effects of the various antibiotics on the binding of erythromycin to *S. aureus* clearly show that competitive inhibition occurs. Similar studies have been realized with another labelled ligand, [^{3}H]roxithromycin. Identical results have been obtained for all the MLS with *S. aureus* (unpublished results). Consequently, it is assumed that the MLS share the same binding sites. The determination of the inhibition constants K_i permits the antibiotics to be ranked in order of inhibitory activity (Fig. 3). Thus, rosaramicin, erythromycin, roxithromycin, pristinamycin, virginiamycin and clindamycin exhibit the same affinity while lincomycin, midecamycin, josamycin, oleandomycin and spiramycin have a lower affinity for *S. aureus* 209 P. Of the MLS tested, their minimal inhibitory concentrations have the same ranks order of potency.

A strong correlation ($r = 0{\cdot}989$, $P < 0{\cdot}001$) was observed between the microbiological activities (MIC) and the binding parameters (K_i) for MLS, against *S. aureus* 209 P. This correlation can be explained on the basis that these drugs share the same binding sites which also correspond to their sites of action on the 50 S ribosomal subunit. Pestka et al (1974) have demonstrated a correlation between inhibition constants of erythromycin analogues with *E. coli* and their antibacterial activities against *B. subtilis*. Omura & Nakagawa (1975) have demonstrated correlations between the

IC50 of lincosamides with respect to [^{14}C]erythromycin-ribosomal binding and MICs against *E. coli, B. subtilis* and *S. aureus*. On the other hand, Rakhit & Singh (1974) compared antibacterial activity against *B. subtilis* and *S. pyogenes* with inhibition of protein synthesis, for compounds obtained by chemical transformation of lincosamides; they have found a correlation between these two parameters.

Thus, the effect of erythromycin analogues on [^{3}H]erythromycin binding to whole bacteria provides a sensitive assay for these compounds. Such studies permit a rapid estimation of the general activity of antibiotics. Variables such as metabolic conversions or modifications of the compounds are excluded, but the bacterial permeability is also studied. Such binding studies can be used alone or in parallel with microbiological assays and may serve as a useful adjunct in developing new compounds.

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