Biochemical Pharmacology, Vol. 36, No. 20, pp. 3495–3500, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

BINDING STUDIES OF MACROLIDES, LINCOSAMIDES AND STREPTOGRAMINS TO *STREPTOCOCCUS* G GROUP USING [^{3}H]-ERYTHROMYCIN

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(*Received* 3 *November* 1986; *accepted* 23 *April* 1987)

Abstract—Parameters of [^{3}H]-erythromycin binding to *Streptococcus* are determined *in vivo* using both equilibrium and kinetic methods. This binding is saturable, reversible and independent of energetic systems. Whatever the methods used, the binding parameters are identical as 14 nM for the dissociation constant of the complex erythromycin–*Streptococcus* and a density of binding sites of 11,865 molecules/cell. Other macrolides, streptogramins and lincosamides competitively displaced bound [^{3}H]-erythromycin suggesting that these compounds share common binding sites on the bacteria. In parallel, the MIC values of these antibiotics against *Streptococcus* are determined by agar dilution method in Mueller-Hinton medium with 5% of horse blood in order to compare the binding and microbiological parameters.

A strong correlation ($n = 0.863$) has been found between the corresponding inhibition constants and MIC values. Such binding studies could be used in conjunction with microbiological assays for primary screening of active analogous or other compounds with interfere with [^{3}H]-erythromycin binding to the bacteria.

The affinity labeling studies of macrolides, lincosamides and streptogramins (M.L.S.) to *Streptococcus* were realized to confirm previous results obtained with other strains. These results have enabled us to show that a correlation exists between the dissociation constant (K_D), and the minimal inhibitory concentration (MIC) of the M.L.S. Such studies were previously realized with the *Staphylococcus aureus* [1] and the *Legionella pneumophila* [2] and now with the *Streptococcus*.

The *Streptococcus* was chosen because it presented MIC values for the M.L.S. lower than these of the strains already tested. If the correlation is confirmed, with these other strains, it will have a more general value.

The affinity of [^{3}H]-erythromycin for the *Streptococcus* was determined by both equilibrium and kinetic binding experiments. The affinities of other macrolides, streptogramins and lincosamides were determined as their ability to displace, at equilibrium, the labeled erythromycin bound to the bacteria.

Then, the binding parameters characterizing the affinity of the antibiotics were compared with the microbiological data indicating the susceptibility of the organisms to the same compounds to determine whether a possible relationship exists between the binding and the microbiological parameters. If such a correlation is confirmed, these binding constants would reflect the antibacterial activity of the compounds and would serve as a useful adjunct in developing new compounds.

MATERIALS AND METHODS

Materials. [^{3}H]-erythromycin (25 Ci/mmole) was synthesized by the Laboratory of Structural Organic Chemistry (Université des Sciences et des Techniques du Languedoc, Montpellier, France). The radiochemical purity, determined by thin layer chromatography on silica gel plates in carbon tetrachloride : ethanol : dimethylformamide (7:2:1), was better than 98%. Labeled and unlabeled compounds were supplied by Abbott Laboratories. Other antibiotics used were josamycin (Spret-Mauchan, Paris), pristinamycin (Upjohn, Kalamazoo, MI), virginiamycin (Smith, Kline & French, Philadelphia, PA), rosaramicin (Schering, Bloomfield, NJ), oleanodomycin (Rosaphytopharma, Paris), midecamycin (Clin-Midy, Paris), roxithromycin (Roussel Uclaf, Paris), lincomycin and clindamycin (Upjohn, Paris), and spiramycin (Specia, Paris).

Bacteria strains and media. The *Streptococcus* G group strain was obtained from the Department of Bacteriology (CHU Henri Mondor). Media cultures were prepared with distilled water and Brain Heart Infusion broth (BHI, 10%). In these conditions, bacteria number remains constant for 3 hr. All binding experiments were performed in 50 mM Tris–HCl buffer, pH = 7.4 at 37°. The number of *Streptococcus* per ml was determined by the measure at 340 nm of the suspension optical density. Previous studies have shown a strong correlation between absorbance at 340 nm and bacterial concentration determined by numeration of the suspension (Fig. 1).

Ultrafiltration. The bacteria and bound antibiotic fraction are collected on the filter (Millipore®, EHWP, 0.5 μm) and washed (2 × 10 ml Tris–HCl buffer) to eliminate antibiotic free fraction.

Methods. The binding reaction between an antibiotic A^* and a bacterium B, is a second order

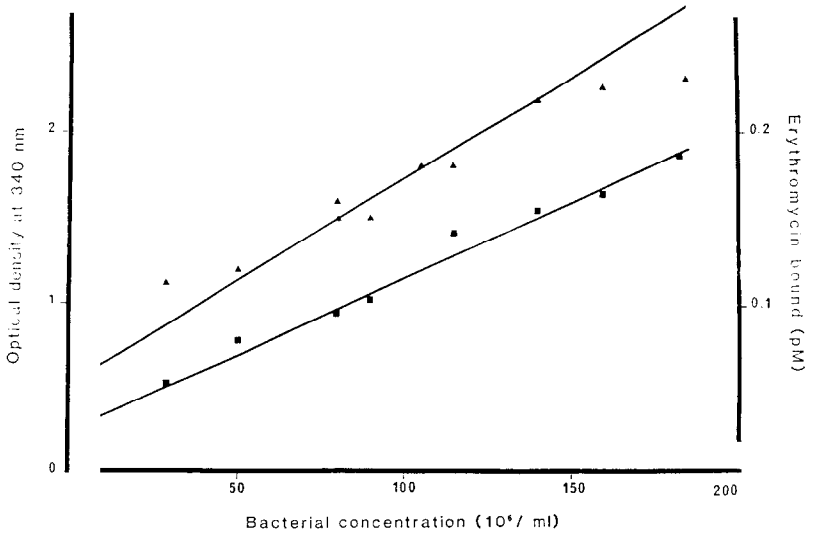

Fig. 1. Correlations between the absorbance (■–■) at 340 nm of a suspension of *Streptococcus* in distilled water with 10% of BHI and the concentration of bound erythromycin to the bacteria (▲–▲) with the number of *Streptococcus* per ml. The number of bacteria was obtained by counting on Mueller–Hinton medium with 5% of horse blood. The specific binding of erythromycin (10^{-8} M), to bacteria is defined as the difference between total and non-specific binding; the latter determined in the presence of 10^{-5} M unlabelled erythromycin under equilibrium conditions.

reaction obeying simple mass action principles.

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

At equilibrium

$$[A^*][B]/[A^* \cdot B] = K_D = 1/K_A$$

where $[B]$ is the concentration of free bacteria. $[A^*]$ the concentration of unbound antibiotic, and $[A^* \cdot B]$ the concentration of antibiotic–bacteria 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. The number of binding sites class and the concentration of binding sites are determined by equilibrium binding studies.

Binding kinetics of [^{3}H]-*erythromycin by* Streptococcus. These studies have been realized always at 37° in shaking bath. First, an association kinetic was performed to define the equilibrium time of the binding reaction and also the association binding kinetic rate k_{+1}. Secondly, the reversibility of the binding reaction was demonstrated by a dissociation kinetic and the binding kinetic rate corresponding k_{-1} was calculated. At last, the effect of energy inhibitors on the association binding kinetic was studied to show if an energetic system is involved in the uptake of erythromycin by *Streptococcus*. The binding kinetics of [^{3}H]-erythromycin to *Streptococcus* were measured by filtering 400 μl of bacterial suspension (10^8 cells/ml) containing [^{3}H]-erythromycin (10^{-8} M) through a filter (Millipore®, EHWP, 0.5 μm) at different times. After, the filter was washed with 2 × 10 ml of Tris–HCl, pH = 7.4 at 4° and was counted in 5 ml of Picofluor 30 (Packard) in a liquid scintillation counter (Packard 460 CD). For the dissociation, at equilibrium reaction time, unlabeled erythromycin (10^{-5} M) was added. Effects of energy inhibitors on the binding kinetic were studied with sodium azide (10^{-2} M) and with potassium cyanide (10^{-3} M).

Equilibrium studies of [^{3}H]-*erythromycin binding.* The reaction mixture contained 400 μl of *Streptococcus* suspension (10^8 cells/ml) and various amounts of [^{3}H]-erythromycin (10^{-9} to 6×10^{-7} M) in a final volume of 500 μl. The mixture was incubated at 37° for the equilibrium reaction time, then filtered. The specific binding of [^{3}H]-erythromycin to *Streptococcus* was determined as the difference between the binding observed in the presence and absence of 10^{-5} M unlabeled erythromycin. Equilibrium studies allowed determination of the dissociation constant K_D of the complex erythromycin–*Streptococcus*, the number of the binding sites class and the concentration of binding sites. Previous studies have shown that maximal specific binding of [^{3}H]-erythromycin to *Streptococcus* (B_{max}) and bacterial concentration present a strong correlation (Fig. 1). So, the bacterial capacity of binding was expressed as the number of [^{3}H]-erythromycin bound moles per bacteria.

Inhibition of [^{3}H]-*erythromycin binding by different antibiotics.* By displacing the labeled antibiotic binding to bacteria by unlabeled antibiotic, the dissociation constant of these competitors is obtained. Thus, a classification of these antibiotics in function of their affinity for the bacteria is established. 250 μl of *Streptococcus* suspension (10^8 cells/ml), [^{3}H]-erythromycin (10^{-8} M) and various concentrations (10^{-11} to 10^{-4} M) of other antibiotics in a final volume of 500 μl were incubated at 37° for 60 min. The ultrafiltration technique was performed as described above.

MIC determination of the antibiotics. Bacteriological activity of macrolides, lincosamides and strep-

togramins was appraised by measurement of minimal inhibitory concentration (MIC). MICs were determined by the agar dilution method in Mueller–Hinton medium with 5% of horse blood. Several concentrations of antibiotic were performed in progression of 1.25 geometrical ratio. A bacterial suspension, 10^5–10^6 cells per ml, was sowed onto agar plates. Then, these plates were incubated at 37° for 24 hr. MIC was defined as the minimal concentration affording complete inhibition of bacteria growth; however, the presence of 1–3 colonies was neglected for that determination.

RESULTS

Association and dissociation binding kinetics

The time course of [3H]-erythromycin uptake by *Streptococcus* is shown in Fig. 2. The equilibrium level was obtained after 60 min. The reversibility of [3H]-erythromycin binding to *Streptococcus* was demonstrated by adding 10^{-5} M unlabeled erythromycin to a bacterial suspension previously incubated for 60 min with [3H]-erythromycin (Fig. 2). From the kinetic binding data, the association (k_{+1}) and dissociation (k_{-1}) rate constants were determined. These constants were calculated according to the equation of Engel *et al.* [3], which were $k_{+1} = 4 \times 1\ 10^6\ \text{min}^{-1} \cdot \text{M}^{-1}$ and $k_{-1} = 0.059\ \text{min}^{-1}$. The ratio of these two parameters yielded the dissociation constant of the erythromycin–*Streptococcus* complex, $K_D = k_{-1}/k_{+1} = 14.4$ nM.

Effect of energy inhibitors on the binding kinetics

Binding kinetics have been performed in presence of NaN_3 (an uncoupler of oxidative phosphorylation from electron transport) and KCN an (inhibitor of electron transport), no modifications of the binding kinetics were observed (Fig. 3). Thus the binding of [3H]-erythromycin to *Streptococcus* was not affected by the presence of energy inhibitors. So this binding did not require oxidative phosphorylation and electron transport.

Equilibrium binding studies

Total, non-specific and specific bindings of [3H]-erythromycin to *Streptococcus* are shown in Fig. 4A. The specific binding was saturable, as shown by the linear Scatchard plot (Fig. 4B), with one class of binding sites. Binding parameters were calculated by the non-linear method described elsewhere [4]. The value of the dissociation constant of the erythromycin–*Streptococcus* complex was 15.7 ± 1.4 nM whereas the density of binding sites, B_{max}, was 11,865 ± 475 molecules per cell.

Effect of macrolides, lincosamides and streptogramins on the [3H]*-erythromycin binding to* Streptococcus

Increasing concentrations of the unlabeled antibiotics progressively inhibited [3H]-erythromycin binding to *Streptococcus* as shown in Fig. 5. The concentration, IC_{50}, at which [3H]-erythromycin binding was inhibited at 50% by these macrolides derivatives are determined. The inhibition constant (K_I) of these drugs are calculated according to the equation developed by Cheng and Prussoff [5]:

$$IC_{50} = K_I/(1 + [A^*]/K_D)$$

where $[A^*]$ and K_D represent respectively the concentration and the dissociation constant of labeled antibiotic.

The inhibition constant of each antibiotic tested are summarized in Table 1. The Hill number (n_H) was calculated and always equal to one, meaning that the binding sites were independent. All the macrolides, streptogramins and lincosamides present a high affinity for the *Streptococcus* with an inhibition constant lower than 2×10^{-7} M.

Determination of MIC

The MICs of macrolides, lincosamides and streptogramins against *Streptococcus* varied as following

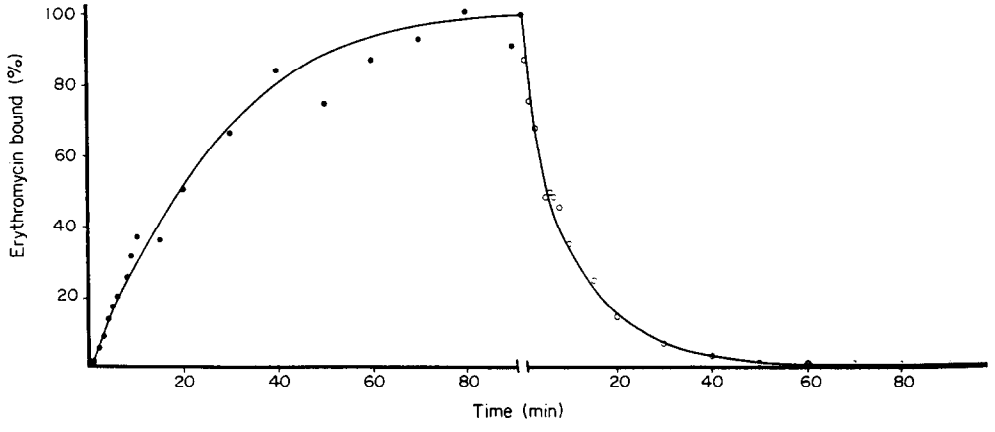

Fig. 2. Association and dissociation kinetics. Association kinetics (●–●) were studied by incubating *Streptococcus* (10^8 cells/ml) and [3H]-erythromycin (10^{-8} M) for up to 90 min at 37°. Non-specific binding, determined in the presence of 10^{-5} M unlabeled erythromycin, was substracted from total binding. After equilibrium was reached, the dissociation kinetics (○–○) was observed after adding sufficient unlabeled erythromycin (10^{-5} M).

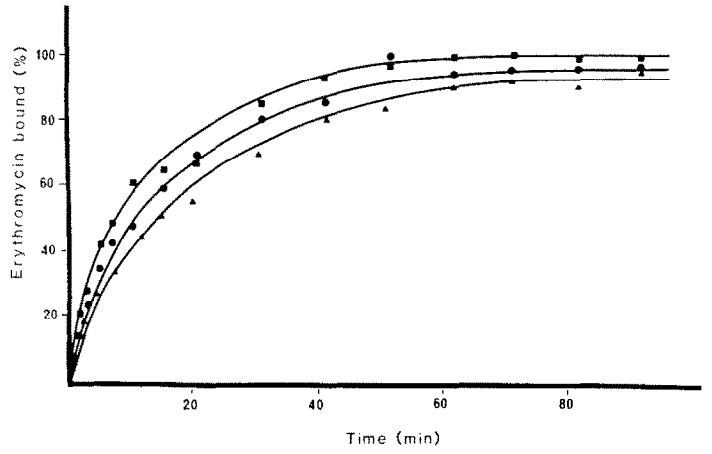

Fig. 3. Kinetic studies in presence of uncoupling agents of oxidative phosphorylation. Association kinetics were studied by incubating *Streptococcus* (10^8 cells/ml) and [^{3}H]-erythromycin (10^{-8} M), in absence (●–●) or in presence of NaN_3 10^{-2} M (■–■) and KCN 10^{-3} M (▲–▲), up to 90 min at 37°. Non-specific binding determined in the presence of 10^{-5} M unlabeled erythromycin, was substracted from total binding.

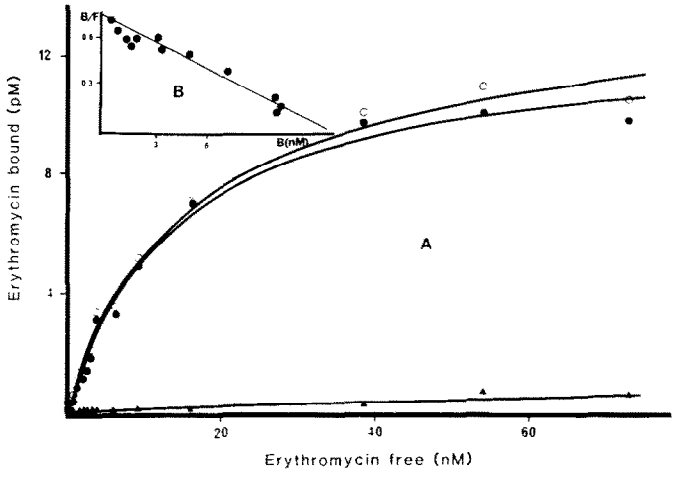

Fig. 4. Equilibrium studies of [^{3}H]-erythromycin binding to *Streptococcus*. Binding was determined after equilibration at 37° for 60 min using [^{3}H]-erythromycin at various concentrations ranging from 10^{-9} M to 6×10^{-7} M with a suspension of *Streptococcus* (10^8 cells/ml). (A) Total (○–○), non-specific (▲–▲) and specific (●–●) binding were obtained as described in the text. (B) Scatchard plot for specific [^{3}H]-erythromycin binding to *Streptococcus*.

0.023 μg/ml for erythromycin, lower than 0.12 μg/ml for virginiamycin, clindamycin, lincomycin, roxithromycin, spiramycin and midecamycin, and higher than 0.19 μg/ml for josamycin, pristinamycin, oleandomycin, rosaramycin (Table 1).

DISCUSSION

The present results show that the [^{3}H]-erythromycin binding to *Streptococcus* is reversible (Fig. 2). Active transport does not appear to be involved in the erythromycin binding to *Streptococcus* (Fig. 3); Mao *et al.* [6] have found same results with [^{14}C]-erythromycin binding to *Staphylococcus*. The association and dissociation kinetics experiments provided rate constants whose ratio (k_{-1}/k_{+1}) yielded a dissociation constant (K_D = 14.4 nM) of the erythromycin–*Streptococcus* complex identical to that determined in the equilibrium binding experiments (K_D = 15.7 nM and K_I = 13.7 nM). The erythromycin dissociation constant is identical for *Streptococcus* and *Escherichia coli* (10 nM) [7] and 10-fold higher for *Staphylococcus aureus* (100 nM) [8]. For the *Escherichia coli* ribosomes, dihydrorosaramicin [9] presents a dissociation constant (200 nM) 20-fold higher than that of the representative 16-membered macrolides, as tetrahydroleucomycin (10 nM) [10]. The erythromycin binding to *Streptococcus* showed only one class of binding sites which represents the 50S ribosomal subunit, as Mao *et al.* [6, 8] and Barré *et al.* [11] have demonstrated it for [^{3}H]-erythromycin binding to *Staphylococcus*. Tejedor *et al.* [12] have showed that

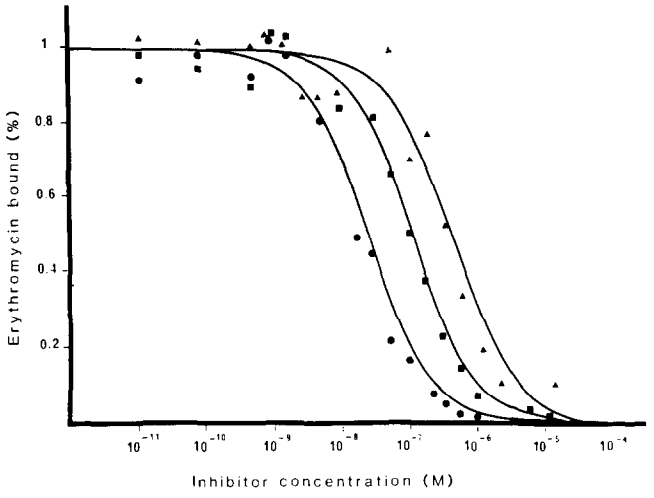

Fig. 5. Inhibition of [^{3}H]-erythromycin binding to *Streptococcus* by antibiotics. Binding of [^{3}H]-erythromycin (10^{-8} M) to *Streptococcus* was measured under equilibrium conditions in the presence of various concentrations (10^{-10}–10^{-4} M) of unlabeled antibiotics: ● erythromycin; ■, roxithromycin; ▲, rosaramicin.

protein L27 is probably a very important part of the macrolide binding site. The *Streptococcus* concentration of [^{3}H]-erythromycin binding sites (B_{max} = 11,865 molecules/cell) is close to the value previously determined [11] for *Staphylococcus* (B_{max} = 14,847 molecules/cell). So, it is clear that these two gram-positive bacteria possess the same number of ribosomal subunits per cell.

The ability of macrolides, lincosamides and streptogramins to interfere with [^{3}H]-erythromycin binding to *Streptococcus* provides an estimate of their ability to interact with the erythromycin ribosomal binding sites. In our previous studies, it has been shown that macrolides, lincosamides and streptogramins displaced the [^{3}H]-erythromycin binding to *Staphylococcus* and to *Legionella*. Inhibitory effects of these antibiotics on [^{3}H]-erythromycin binding to *Streptococcus* clearly show that competitive inhibition occurs between these various drugs. For all antibiotics, the calculated Hill number is close to one indicating that the binding sites are independent. This result shows also that these antibiotics share likely common binding sites to the bacteria. So, the determination of the inhibition constants K_I even for compounds owing low dissociation constants (10^{-4} M) as shown by Pestka *et al.* [13] allows the antibiotics to be ranked in order of inhibitory activity.

The inhibition constants (K_I), characterizing the affinity of the antibiotics for the bacteria have been compared with the microbiological data (MIC) indicating the susceptibility of the organisms to the same antibiotic.

A strong linear correlation ($r = 0.863$, $p < 0.001$) is observed between the antimicrobial activities (MIC) and the binding contants (K_I) of macrolides, lincosamides and streptogramins against *Streptococcus* (Fig. 6). 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 50S ribosomal subunit.

Previously, we have found an identical correlation with these antibiotics against *Staphylococcus* [1] and *Legionella* [2]. Pestka *et al.* [13] have demonstrated a correlation between inhibition constants of erythromycin analogous and their antibacterial activities against *E. coli*. Omura *et al.* [14] have obtained correlations between the IC_{50} 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 and Singh [15] have compared antibacterial activity against *B. subtilis* and *S. pyogenes* with inhibition of protein synthesis, for compounds obtained by chemical transformation of lincosamides, and have found a correlation between these two parameters.

Thus, the correspondence between the ability of these agents to displace [^{3}H]-erythromycin binding

Table 1. Inhibition constants (K_I) and minimal inhibitory concentration (MIC) values for macrolides, lincosamides and streptogramins against *Streptococcus*

Antibiotics	K_I (nM)	MIC (μg/ml)
Erythromycin	13.7 ± 2.3	0.023
Spiramycin	20.0 ± 2.1	0.080
Virginiamycin	32.8 ± 16.1	0.047
Midecamycin	58.4 ± 9.3	0.120
Clindamycin	60.9 ± 5.1	0.047
Roxithromycin	68.7 ± 12.2	0.080
Lincomycin	69.3 ± 5.5	0.063
Josamycin	75.3 ± 11.3	0.190
Pristinamycin	103.1 ± 10.2	0.190
Oleandomycin	109.2 ± 12.2	0.200
Rosaramicin	128.0 ± 32.1	0.250

The inhibition constant was obtained by measuring the binding of [^{3}H]-erythromycin (10^{-8} M) under equilibrium conditions in the presence of various concentrations (10^{-10}–10^{-4} M) of unlabeled antibiotics. The MIC values was determined on Mueller–Hinton medium with 5% of horse blood.

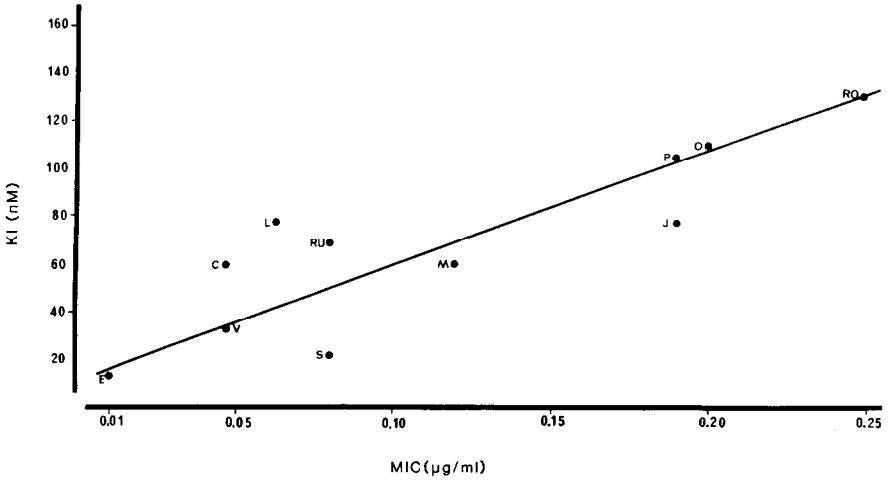

Fig. 6. Correlation between inhibition constant (K_I) and the minimal inhibitory concentration (MIC) of macrolides, lincosamides and streptogramins against *Streptococcus*. E, erythromycin; V, virginiamycin; C, clindamycin; L, lincomycin; RU, roxithromycin; S, spiramycin; M, midecamycin; J, josamycin; P, pristinamycin; O, oleandomycin; RO, rosaramycin.

from the bacteria and their antibacterial activity is quite strong.

The effect of erythromycin analogous on [^{3}H]-erythromycin binding from the whole bacteria provides a sensitive assay for these compounds. Such studies allow a rapid screening of biological activity of drugs. So, it can be used in conjunction with microbiological assays and may serve as a useful adjunct in developing new compounds.

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