ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1974, p. 606-612

Vol. 6, No. 5
Printed in U.S.A.

Effect of Leucomycins and Analogues on Binding [^{14}C]Erythromycin to *Escherichia coli* Ribosomes

SIDNEY PESTKA, AKIRA NAKAGAWA, AND SATOSHI ŌMURA

Roche Institute of Molecular Biology, Nutley, New Jersey 07110, and The Kitasato Institute and Kitasato University, Minato-Ku, Tokyo, Japan

Received for publication 1 July 1974

We examined the effect of leucomycins, leucomycin derivatives, and other 16-membered macrolides (tylosin, niddamycin, spiramycin I, and spiramycin III) on [^{14}C]erythromycin binding to ribosomes. Results of these studies enabled determination of the association and dissociation constants for the binding of each of these compounds to *Escherichia coli* ribosomes. In addition, the binding of the leucomycins and the leucomycin derivatives to ribosomes in general correlated with their antimicrobial activity.

We recently examined the effect of erythromycin and its derivatives on chloramphenicol and erythromycin binding to ribosomes from *Escherichia coli* (10, 11). Their effects on [^{14}C]erythromycin binding to ribosomes permitted the determination of the association and dissociation constants for the binding of these derivatives to ribosomes as well as their interaction coefficients (11). Furthermore, there appeared to be a reasonable correlation with the binding of these analogs to ribosomes and their antibacterial activity (12).

Since a number of leucomycin analogues have been synthesized and the antibacterial activity of various derivatives have been evaluated (3-7), it was of interest to examine the effect of these 16-membered macrolides on both chloramphenicol and erythromycin binding to ribosomes. In addition, since leucomycins interfere with [^{14}C]erythromycin binding to ribosomes, it was possible to determine association and dissociation constants as well as interaction coefficients for each of these derivatives for binding to *E. coli* ribosomes, by the method previously described (1). Therefore, in this paper the effects of leucomycins and similar compounds on [^{14}C]chloramphenicol and [^{14}C]erythromycin binding to ribosomes are reported. Furthermore, these results were correlated with their antibacterial activities.

MATERIALS AND METHODS

Antibiotics. Leucomycin derivatives (Fig. 1) were prepared as previously described (3-6). Tylosin was obtained from Eli Lilly and Co. (Indianapolis, Ind.), spiramycins I and III were from Rhone-Poulenc, and niddamycin was from Farbwerke-Hoechst. The structures of these antibiotics are also shown in Fig. 1. Each compound was dissolved in dimethyl sulfoxide to make a stock solution of 0.01 M. Dilutions were made directly into water. [^{14}C]erythromycin A (45.7 mCi/mmol) was synthesized and ribosomes were prepared as described previously (9).

Determination of [^{14}C]chloramphenicol and [^{14}C]erythromycin binding to ribosomes. Binding of [^{14}C]chloramphenicol and [^{14}C]erythromycin to ribosomes was determined as described in previous reports (8, 9). [^{14}C]chloramphenicol binding to ribosomes was determined in 0.05-ml reaction mixtures (Fig 2). Each reaction mixture for determination of binding of [^{14}C]erythromycin to ribosomes contained, in a volume of 0.50 ml, the following components: 0.004 M $MgCl_2$; 0.1 M KCl; 0.01 M NH_4Cl; 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.2); 5.6 to 7.5 units of absorbancy at 260 nm (A_{260}) of NH_4Cl-washed *E. coli* B or *E. coli* A19 ribosomes; 1.2 μM [^{14}C]erythromycin A unless otherwise specified; and erythromycin A, leucomycin, or other compounds as indicated. Reactions were started by adding ribosomes last to the reaction mixtures. Incubations were performed at 24 C for 30 min. At the end of the incubation, reactions were stopped by diluting the reaction mixture with 3 ml of cold solution A (0.005 M $MgCl_2$, 0.15 M KCl, and 0.01 M Tris-hydrochloride [pH 7.2]). The diluted reaction mixture was filtered through a 25-mm diameter membrane filter (HAWP; Millipore Corp.); the tube and filter were immediately washed an additional three times with 3 ml of cold solution A. The filters were then dried under an infrared lamp and radioactivity was determined with a scintillation spectrometer as previously reported (9).

RESULTS AND DISCUSSION

Effect of leucomycin and derivatives on [^{14}C]chloramphenicol binding to ribosomes. The effect of leucomycin A_3 and various derivatives on [^{14}C]chloramphenicol binding to ribosomes is presented in Fig. 2. The concentrations

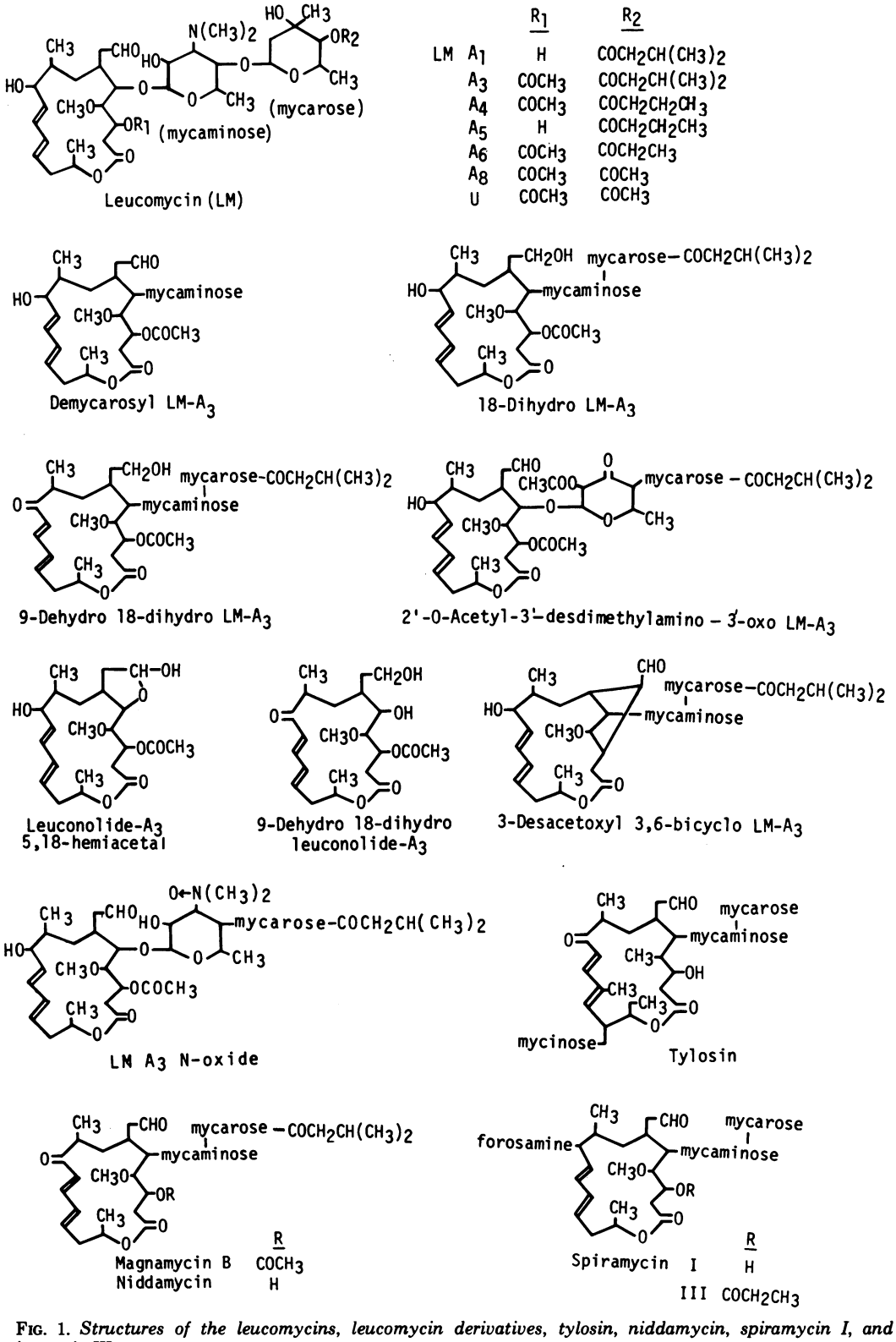

FIG. 1. *Structures of the leucomycins, leucomycin derivatives, tylosin, niddamycin, spiramycin I, and spiramycin III.*

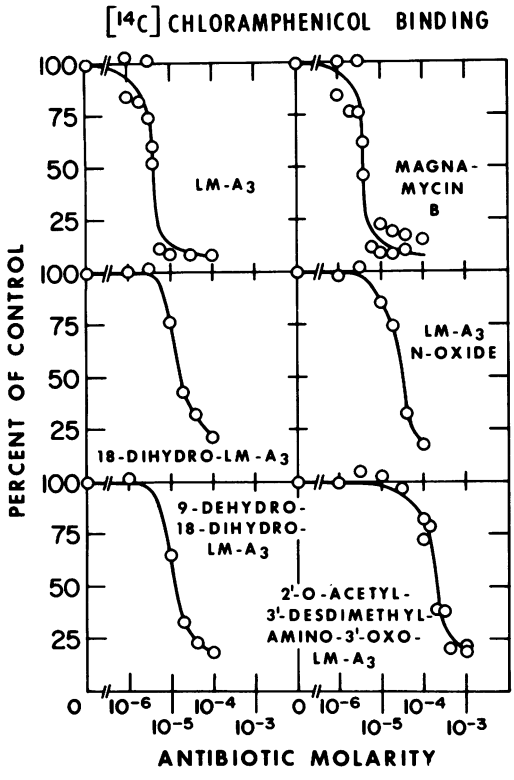

FIG. 2. *Effect of leucomycin, leucomycin derivatives, and related compounds on [14C]chloramphenicol binding to E. coli ribosomes. Each 0.050-ml reaction mixture contained the following components: 0.01 M tris-hydrochloride (pH 7.2); 0.1 M KCl; 0.004 M $MgCl_2$; 0.1 M NH_4Cl; 7.5 A_{260} units of ammonium chloride-washed E. coli B ribosomes; 10 μM [14C]chloramphenicol; and leucomycin, other leucomycin derivatives, or analogs as indicated on the abscissa. Reactions were performed at 24 C for 15 min at which time chloramphenicol binding to ribosomes was determined as previously described (10).*

TABLE 1. *Effect of leucomycin and derivatives on [14C]chloramphenicol binding to E. coli ribosomes*

Antibiotic	Concn for 50% inhibition of binding (μM)	$pK_{50\%}$
Leucomycin A_3	4	5.4
18-Dihydroleucomycin A_3	16	4.8
9-Dehydro-18-dihydro-leucomycin A_3	10	4.9
Leucomycin A_3 *N*-oxide	32	4.5
2'-*O*-Acetyl-3'-desdimethylamino-3'-oxo-leucomycin A_3	200	3.7
Magnamycin B	5	5.4

at which [14C]chloramphenicol binding was inhibited 50% by these leucomycin derivatives are summarized in Table 1. Leucomycin A_3 and magnamycin B were most effective in inhibiting [14C]chloramphenicol binding to ribosomes. Reduction of the aldehyde group of leucomycin A_3 to an alcohol (18-dihydroleucomycin A_3) produced a derivative with about one-fourth the activity of leucomycin A_3 in inhibiting [14C]chloramphenicol binding to ribosomes. Conversion of 18-dihydroleucomycin A_3 to 9-dehydro-18-dihydroleucomycin A_3 produced a compound with slightly increased activity in inhibiting [14C]chloramphenicol binding to ribosomes; it was about 1/2.5 as active as leucomycin A_3. Leucomycin A_3 *N*-oxide had about one-eighth the activity of leucomycin A_3 in inhibiting chloramphenicol binding to ribosomes. The 2'-*O*-acetyl-3'-desdimethylamino-3'-oxo-leucomycin A_3 had relatively little ability to inhibit [14C]chloramphenicol binding to ribo-

TABLE 2. *Concentration of leucomycins, analogues, other 16-membered macrolides and erythromycin which produce 50% inhibition of [14C]erythromycin binding to ribosomes*[a]

Antibiotic	Concn for 50% inhibition (μM)	$pK_{50\%}$	n
Leucomycin A_3	4.2	5.38	1.17
18-Dihydroleucomycin A_3	209	3.68	0.96
9-Dehydro-18-dihydroleucomycin A_3	123	3.91	0.94
Leucomycin A_3 *N*-oxide	30	4.52	1.07
2'-*O*-Acetyl-3'-desdimethylamino-3'-oxo-leucomycin A_3	288	3.54	1.38
Leuconolide A_3 5,18-hemiacetal	309	3.51	1.03
9-Dehydro-18-dihydro-leuconolide A_3	3,160	2.5	1.27
Demycarosyl leucomycin A_3	91	4.04	0.89
3-Desacetoxyl-3,6-bicyclo-leucomycin A_3	447	3.35	0.85
Leucomycin A_1	2.1	5.68	1.12
Leucomycin A_4	2.6	5.58	1.04
Leucomycin A_5	1.8	5.74	1.23
Leucomycin A_6	3.5	5.45	0.87
Leucomycin A_9	14	4.84	0.93
Leucomycin U	21	4.68	1.03
Magnamycin B	4.2	5.38	1.16
Erythromycin A	1.9	5.73	0.89
Tylosin	3.4	5.47	1.01
Niddamycin	4.8	5.32	1.29
Spiramycin I	1.1	5.96	1.07
Spiramycin III	1.8	5.74	1.20

[a] The 50% inhibition and $pK_{50\%}$ values were determined from Fig. 3 and similar data. The $pK_{50\%}$ is the negative log of the molar 50% inhibition value; n represents the interaction coefficients obtained from Hill plots of the data.

somes; it was approximately 1/50 as active as leucomycin A_3. Magnamycin B was approximately equivalent to leucomycin A_3 in inhibiting chloramphenicol binding to ribosomes.

Effects of leucomycins, leucomycin derivatives and other macrolides on [^{14}C]erythromycin binding to ribosomes. The effect of these antibiotics and derivatives on [^{14}C]erythromycin binding to ribosomes is shown in Fig. 3. The data for the concentration of leucomycins and analogues which produce 50% inhibition of [^{14}C]erythromycin binding to ribosomes are summarized in Table 2. These values were determined by Hill plots of the data shown in Fig. 3. The interaction coefficients for the various compounds as determined by Hill plots are also summarized in Table 2. Unlabeled erythromycin with a 14-membered lactone ring was included in these studies as a control. As can be seen, the interaction coefficients for most of the compounds are close to one. The association and dissociation constants as well as the interaction coefficients for these antibiotics (1) are summarized in Table 3. Leucomycin A_6, containing a hydroxyl group on the lactone ring at C3, appeared to bind to ribosomes with the greatest affinity. Leucomycin A_1, A_3, A_4, A_5, A_6, and magnamycin B, erythromycin A, tylosin niddamycin, spiramycin I, and spiramycin III had association constants of 1.2×10^7 M^{-1} or greater. Mao (2), and Wilhelm et al. (13) previously showed that erythromycin analogues and other macrolides inhibited erythromycin binding to ribosomes. Leucomycin A_8 and U had association constants of 6.5×10^6 M^{-1} and 3.8×10^6 M^{-1}, respectively. Reduction of the aldehyde group (18-dihydroleucomycin A_3) reduced the ability of the derivatives to inhibit [^{14}C]erythromycin binding to ribosomes to about 1/20 that of leucomycin A_3. The additional introduction of a keto group at C9 produced 9-dehydro-18-dihydroleucomycin A_3 with about one-eighth the activity of leucomycin A_3 in inhibiting [^{14}C]erythromycin binding. Leucomycin A_3 *N*-oxide also had about one-eighth the activity of leucomycin A_3. Introduction of an acetyl at C2′ and removal of the dimethylamino group yielded 2′-*O*-acetyl-3′-desdimethylamino-3′-oxo-leucomycin A_3 with 1/229 the activity of leucomycin A_3 in inhibiting [^{14}C]-erythromycin binding to ribosomes.

The leuconolide A_3 5,18-hemiacetal with both

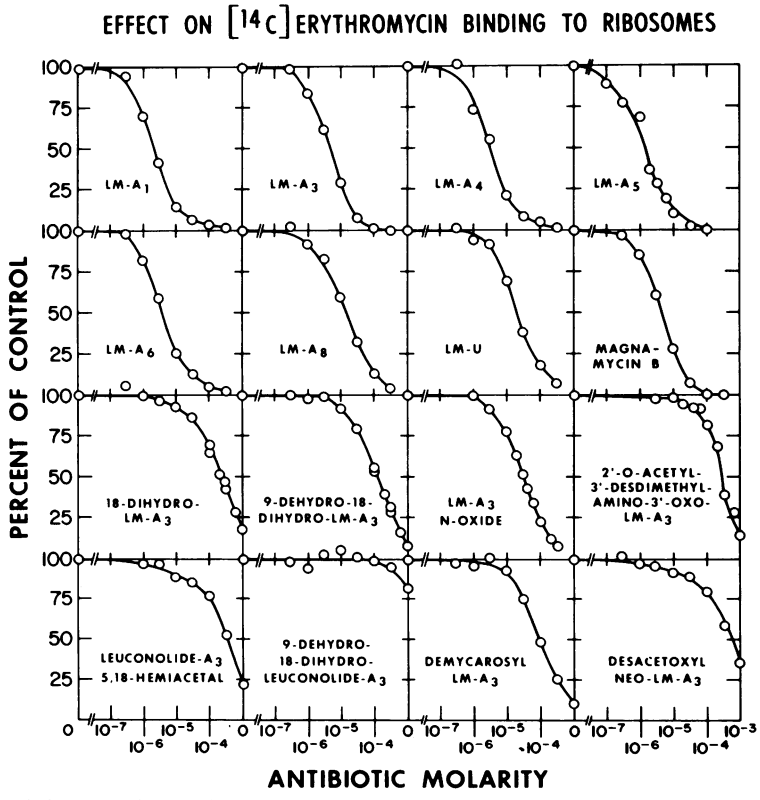

FIG. 3. *Effect of leucomycins and other 16-membered macrolides on [^{14}C]erythromycin binding to ribosomes.*

sugars removed retained substantial activity; it was about 1/25 as active as leucomycin A_3. The 9-dehydro-18-dihydroleuconolide A_3 was essentially inactive; it was 1/1,320 as active as leucomycin A_3 and substantially less active than the leuconolide A_3 5,18-hemiacetal. Removal of mycarose to produce demycarosyl leucomycin A_3 reduced the activity to approximately 1/10 that of leucomycin A_3. 3-Desacetoxyl-3,6-bicyclo-leucomycin A_3 was 1/40 as active as leucomycin A_3.

Antibacterial activity of antibiotics. The antibacterial activities of the various leucomycin analogues are presented in Table 4, together with their concentrations for 50% inhibition of [^{14}C]erythromycin binding to ribosomes as given in Table 2. The compounds were ranked according to their ability to inhibit [^{14}C]erythromycin binding to ribosomes. It can be seen that, in general, their antibacterial activities correlate with their ability to bind to ribosomes (Fig. 4). This is particularly true for all compounds with a concentration for 50% inhibition of erythromycin binding of 21 μM or less. Compounds with a 50% inhibition concentration of greater than 300 μM showed no detectable antibacterial activity up to the maximum concentration tested (200 μg of compound per ml). The 18-dihydroleucomycin A_3 with a 50% inhibition concentration of 209 μM showed no antibacterial activity, despite the findings that the 9-dehydro-18-dihydroleucomycin A_3 and the 2′-*O*-acetyl-3′-desdimethylamino-3′-oxo leucomycin A_3, with 50% inhibition concentrations of 123 and 288 μM, respectively, were active. If cellular modification of these compounds did not occur, it therefore appears that the bacteria were impermeable to 18-dihydroleucomycin A_3, for it would have been expected that the minimum inhibitory concentration (MIC) should be 74 μg of compound per ml based on a 50% inhibitory concentration of 209 μM. Of note is that the interaction coefficients (Tables 2 and 3) for 2′-*O*-acetyl-3′-desdimethylamino-3′-oxo leucomycin A_3 were significantly greater than 1.0. This may possibly indicate some unusual ribosomal interactions.

Furthermore, the leuconolide A_3 5,18-hemiacetal and the 3-desacetoxyl-3,6-bicyclo-leucomycin A_3 were inactive despite the finding that their 50% inhibition concentrations were 309 and 447 μM, respectively. By extrapolation

TABLE 3. *Dissociation and association constants for binding of leucomycins, analogs, other 16-membered macrolides, and erythromycin to E. coli ribosomes at 1 × 10^{-6} M [^{14}C]erythromycin*[a]

Antibiotic	n	pK$_d$	K_d (M)	K_f (M^{-1})
Leucomycin A_3	1.21	7.1	8.3×10^{-8}	1.2×10^{7}
	1.21	7.2	5.7×10^{-8}	1.8×10^{7}
18-Dihydroleucomycin A_3	0.93	5.8	1.4×10^{-6}	7.1×10^{5}
9-Dehydro-18-dihydroleucomycin A_3	0.89	6.2	6.2×10^{-7}	1.6×10^{6}
Leucomycin A_3 *N*-oxide	1.17	6.2	6.4×10^{-7}	1.6×10^{6}
	1.13	5.6	2.6×10^{-6}	3.9×10^{5}
2′-*O*-Acetyl-3′-desdimethylamino-3′-oxo-leucomycin A_3	1.66	4.7	1.9×10^{-5}	5.3×10^{4}
	1.35	5.1	8.7×10^{-6}	1.2×10^{5}
Leuconolide A_3 5,18-hemiacetal	0.91	5.7	2.1×10^{-6}	4.8×10^{5}
9-Dehydro-18-dihydro-leuconolide A_3	1.31	4.0	1.1×10^{-4}	9.4×10^{3}
Demycarosyl leucomycin A_3	0.97	6.1	8.8×10^{-7}	1.1×10^{6}
3-Desacetoxyl-3,6-bicyclo-leucomycin A_3	0.91	5.5	3.3×10^{-6}	3.1×10^{5}
Leucomycin A_1	0.97	7.6	2.4×10^{-8}	4.1×10^{7}
	1.24	7.2	7.0×10^{-8}	1.4×10^{7}
Leucomycin A_4	0.91	7.7	1.9×10^{-8}	5.2×10^{7}
Leucomycin A_5	1.03	7.8	1.5×10^{-8}	6.7×10^{7}
	1.20	7.2	5.6×10^{-8}	1.8×10^{7}
Leucomycin A_6	1.00	7.3	4.6×10^{-8}	2.2×10^{7}
Leucomycin A_8	0.99	6.8	1.5×10^{-7}	6.5×10^{6}
Leucomycin U	1.03	6.6	2.6×10^{-7}	3.8×10^{6}
Magnamycin B	1.11	7.2	6.2×10^{-8}	1.6×10^{7}
Erythromycin A	1.00	7.7	2.1×10^{-8}	4.8×10^{7}
	1.05	7.8	1.6×10^{-8}	6.3×10^{7}
Tylosin	1.39	7.0	9.8×10^{-8}	1.0×10^{7}
Niddamycin	1.10	7.2	6.1×10^{-8}	1.6×10^{7}
Spiramycin I	1.09	7.9	1.3×10^{-8}	7.8×10^{7}
Spiramycin III	1.26	7.3	4.8×10^{-8}	2.1×10^{7}

[a] K_f, K_d, and n represent association constant, dissociation constant, and interaction coefficient determined as previously described (1, 11).

TABLE 4. *Antimicrobial activities of leucomycins and derivatives*

Compounds	MIC (μg/ml)			Conc for 50% inhibition of [^{14}C]erythromycin binding to ribosomes (μM)
	B. subtilis PCI 219	*S. aureus* FDA 209P	*E. coli* NIHJ	
1. Leucomycin A_5	0.2	0.1	12.5	1.8
2. Leucomycin A_1	0.2	0.1	12.5	2.1
3. Leucomycin A_4	0.4	0.2	25	2.6
4. Leucomycin A_6	0.78	0.4	100	3.5
5. Leucomycin A_3	0.2	0.2	25	4.2
6. Magnamycin B	0.4	0.4	25	4.2
7. Leucomycin A_8	1.6	1.6	100	14
8. Leucomycin U	6.25	6.25	200	21
9. Leucomycin A_3 *N*-oxide	50	100	>200	30
10. Demycarosyl leucomycin A_3	50	25	>200	91
11. 9-Dehydro-18-dihydroleucomycin A_3	25	25	>200	123
12. 18-Dihydroleucomycin A_3	200	>200	>200	209
13. 2′-*O*-Acetyl-3′-desdimethylamino-3′-oxo leucomycin A_3	100	100	>200	288
14. Leuconolide A_3 5,18-hemiacetal	>200	>200	>200	309
15. 3-Desacetoxyl-3,6-bicyclo-leucomycin A_3	>200	>200	>200	447
16. 9-Dehydro-18-dihydro-leuconolide A_3	>200	>200	>200	3,160

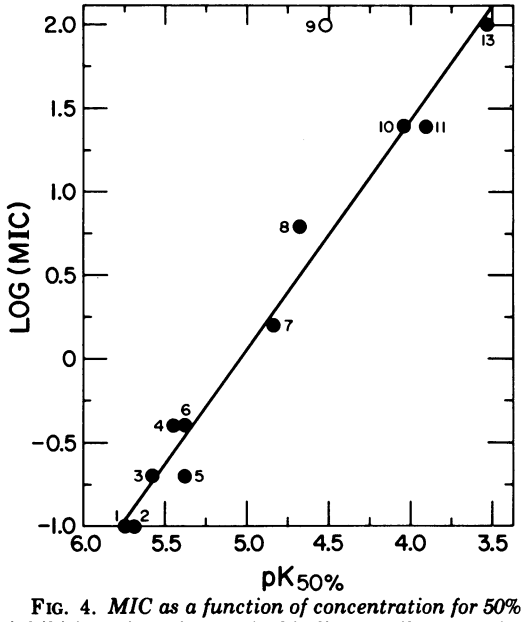

FIG. 4. *MIC as a function of concentration for 50% inhibition of erythromycin binding to ribosomes for leucomycins and leucomycin derivatives. The log of the MIC in micrograms per milliliter for each of the compounds as determined against S. aureus (Table 4) was plotted as a function of the $pK_{50\%}$ (Table 2) for these same compounds. The numbers in the figure refer to the compounds as numbered in Table 4. The line of best fit was determined by the method of least squares, with a computer program. The index of determination for this line of best fit was 0.98, and the standard error of the estimate for Y was 0.159. For determination of this line of best fit, only the solid circles were used. This line is described by the following equation: log (MIC) = 6.861 − 1.362 $pK_{50\%}$ Thus, for any $pK_{50\%}$ value the estimated MIC can be calculated.*

from Fig. 4, it might be predicted they would have MIC values for *Staphylococtus aureus* of 127 and 212 μg of compound per ml, respectively. It is possible that the cells may be less permeable to these compounds as well or that they are metabolically altered by the cells to less active derivatives.

Barring consideration of other variables, it should be possible in many instances to predict reasonable values for the MIC from the $pK_{50\%}$ (Fig. 4). In fact, estimates for the MIC can be made for compounds with little activity. Thus, in Table 5 are presented the estimated MIC values based on the determination of $pK_{50\%}$ Where discrepancies exist (as discussed above), other considerations may be relevant. Thus, leucomycin A_3 *N*-oxide, 18-dihydroleucomycin A_3, and leuconolide A_3 5,18-hemiacetal were less active than would be predicted from the binding data (Fig. 4; Table 5). Perhaps, these compounds were inactivated during the lengthy incubations for determination of MIC values or perhaps the cells are less permeable to these compounds compared with most of the other derivatives. Further study of compounds with discrepancies between actual and calculated MIC values may suggest other factors relevant to antibacterial activity. Thus, such comparisons may permit an understanding of these other parameters relevant to the design of

TABLE 5. *Estimated MIC values against S. aureus for leucomycins and leucomycin derivatives*[a]

Compound	MIC (μg/ml)	
	Actual	Calculated
Leucomycin A_6	0.1	0.11
Leucomycin A_1	0.1	0.13
Leucomycin A_4	0.2	0.18
Leucomycin A_6	0.4	0.27
Leucomycin A_2	0.2	0.34
Magnamycin B	0.4	0.34
Leucomycin A_8	1.6	1.8
Leucomycin U	6.25	3.1
Leucomycin A_3 *N*-oxide	100	5.1
Demycarosyl leucomycin A_3	25	23
9-Dehydro-18-dihydroleucomycin A_3	25	34
18-Dihydroleucomycin A_3	>200	71
2′-*O*-Acetyl-3′-desdimethyl-amino-3′-oxo leucomycin A_3	100	109
Leuconolide A_3 5,18-hemiacetal	>200	120
3-Desacetoxyl-3,6-bicyclo-leucomycin A_3	>200	199
9-Dehydro-18-dihydro-leuconolide A_3	>200	2858

[a] Calculated MIC values were determined with the use of the equation $\log_e(\text{MIC}) = 6.861 - 1.362\ pK_{50\%}$ as noted in the legend to Fig. 4.

antibacterial agents. Additional studies are required to delineate these other factors.

LITERATURE CITED

1. Harris, R., and S. Pestka. 1973. Studies on the formation of transfer ribonucleic acid-ribosome complexes. XXIV. Effects on binding of aminoacyl-oligonucleotides to ribosomes. J. Biol. Chem. **248**:1168–1174.
2. Mao-J.C.-H. 1971. Mode of action of erythromycin, p. 153–175. *In* S. Mitsuhashi (ed.), Drug action and drug resistance in bacteria. University Park Press, Baltimore.
3. Nakagawa, A., K. Suzuki, K. Iwasaki, T. Hata, and S. Ōmura. 1974. Chemistry of leucomycins. XI. Chemical transformation of a basic macrolide to a neutral macrolide. Chem. Pharm. Bull. (Tokyo) **22**:1426–1428.
4. Ōmura, S., M. Katagira, I. Umezawa, K. Komiyama, T. Maekawa, K. Sekikawa, A. Matsumae, and T. Hata. 1968. Structure-biological activities relationships among leucomycins and their derivatives. J. Antibiot. **21**:532–538.
5. Ōmura, S., A Nakagawa, K. Suzuki, T. Hata, A. Jakubowski, and M. Tishler. 1974. Isolation and structure of leuconolide A_3 5,18-hemiacetal and 9-dehydro 18-dihydro leuconolide A_3. J. Antibiot. **27**:147–149.
6. Ōmura, S., A. Nakagawa, K. Suzuki, and T. Hata. 1974. A new bicyclo lactone from leucomycin A_3 by alkali treatment. J. Antibiot. **27**:370–372.
7. Ōmura, S., M. Tishler, A. Nakagawa, Y. Hironaka, and T. Hata. 1972. Relationship of structures and microbiological activities of the 16-membered macrolides. J. Med. Chem. **15**:1011–1015.
8. Pestka, S. 1974. Antibiotics as probes of ribosome structure: binding of chloramphenicol and erythromycin to polyribosomes; effect of other antibiotics. Antimicrob. Ag. Chemother. **5**:255–267.
9. Pestka, S. 1974. Binding of [^{14}C]erythromycin to *Escherichia coli* ribosomes. Antimicrob. Ag. Chemother. **6**:474–478.
10. Pestka, S., and R. A. LeMahieu. 1974. Inhibition of [^{14}C]chloramphenicol binding to *Escherichia coli* ribosomes by erythromycin derivatives. Antimicrob. Ag. Chemother. **6**:39–45.
11. Pestka, S., and R. A. LeMahieu. 1974. Effect of erythromycin analogues on binding of [^{14}C]erythromycin to *Escherichia coli* ribosomes. Antimicrob. Ag. Chemother. **6**:479–488.
12. Pestka, S., R. A. LeMahieu, and P. Miller. 1974. Correlation of effects of erythromycin analogues on intact bacteria and on [^{14}C]erythromycin binding to *Escherichia coli* ribosomes. Antimicrob. Ag. Chemother. **6**:489–491.
13. Wilhelm, J. M., N. L. Oleinick, and J. W. Corcoran. 1968. Interaction of antibiotics with ribosomes: structure-function relationships and a possible common mechanism for the antibacterial action of the macrolides and lincomycin, p. 236–250. Antimicrob. Ag. Chemother. 1967.