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INHIBITION OF PROTEIN SYNTHESIS BY THREE ERYTHROMYCIN-DERIVATIVES

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SUMMARY. Three N-substituted 9(S)-erythromicylamines, which possess a lower minimal inhibitory concentration (MIC) against gram negative bacteria than erythromycin itself, were tested for their inhibitory effect on protein synthesis in a pro- and eukaryotic system, in comparison to erythromycin. The derivatives inhibit poly(A) dependent polylysine synthesis stronger than erythromycin. Competition experiments under conditions of equilibrium dialysis and tests of various ribosomal functions demonstrate that the stronger action of the derivatives is at least partially due to a higher affinity for the 50S subunit and is not due to an altered specificity of inhibition.

INTRODUCTION. The antibiotic erythromycin belongs to the macrolide group and specifically inhibits prokaryotic protein synthesis (1-3). The drug binds to the large ribosomal subunit (4-6). The inhibition effect against bacteria seems to be due to a premature dissociation of the peptidyl tRNA from the ribosome (7, 8), whereas in a cell free system erythromycin prevents the poly(A) directed synthesis of polylysine and causes the accumulation of di-, tri- and tetralysines (9, 10).

Many erythromycin derivatives, including various N-substituted 9(S)-erythromicylamines, have been studied with respect to their antibacterial activity (11) and their ability to inhibit the [^{14}C]erythromycin binding (12, 13). The binding capacity of the derivatives did not parallel in all cases the antimicrobial potency (13, 14). Possible reasons for this discrepancy are 1) an altered permeability due to the modification, 2) intra-

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cellular inactivation, 3) an altered binding or 4) an altered specificity of inhibition. Concerning the last possibility it seems insufficient to test the derivatives with respect to their antimicrobial activity and binding to ribosomes only.

In this paper we use a highly specific assay for the measurement of the action of erythromycin, namely the analysis of the products of poly(Lys) synthesis in presence of the drugs. In addition, the effects of the drugs in various ribosomal test systems were studied. With these techniques the inhibition patterns of three erythromycin derivatives are determined in comparison to the authentic erythromycin.

MATERIALS AND METHODS. Erythromycin was obtained from Abbott Laboratories, North Chicago, Illinois, USA. The erythromycin derivatives were kindly provided by Dr. E. Woitun, Abteilung Chemische Forschung, Dr. Karl Thomae GmbH. Fusidic acid was received from Leo Pharmaceuticals, Ballerup/Denmark. The other antibiotics used in the experiments were obtained from Serva, Heidelberg. The minimal inhibitory concentrations (MIC) of erythromycin and the derivatives were determined by a broth dilution test (15). The nutrient agar consists of 1 % peptone; 0,8 % meat extract; 0,3 % NaCl and 0,2 % Na_2HPO_4 at pH 7,2. The gram negative strains Escherichia coli ATCC 9637 and Klebsiella pneumoniae ATCC 10 031 were obtained from the American Type Culture Collection, and Proteus vulgaris was isolated from clinical materials. The inoculum consisted of 10^5 cells/ml, and the incubation time totalled 18 hours. E. coli 30S and 50S ribosomal subunits were isolated as described (16) and the preparation of the S-150 enzymes from E. coli followed reference 17. 80S ribosomes and S-100 enzymes from yeast were a kind gift from Dr. B. Schulz-Harder, Zentralinstitut für Biochemie und Biophysik, FU Berlin. Elongation factor EF-G was kindly provided by P. Wurmbach, MPI for Molekulare Genetik, Berlin-Dahlem. The test systems used were according to the following references: poly(A) dependent poly(Lys) synthesis (10), the aminoacyl tRNA-ligase test (18), the peptidyl transferase assay (19), the EF-G dependent GTPase test (20) and the analysis of oligolysines synthesized in the poly(A) system (10). The analysis of the nonenzymatic tRNA binding followed reference 21, except that the binding of [^{14}C]Phe-tRNA was measured in presence of 5 or 15 mM Mg^{++}. The test system for the [^{14}C]erythromycin (specific activity: 15 Ci/mole) binding to ribosomes under equilibrium conditions will be described elsewhere (Teraoka and Nierhaus, manuscript in preparation).

RESULTS AND DISCUSSION. Three N-substituted 9(S)-erythromi-

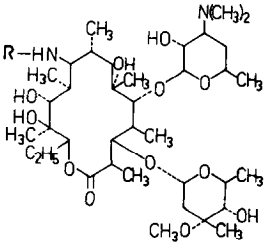
cylamines were synthesized, in which the C_9-amino group is substituted with either a 3-(pyrimidylamino)-propylgroup or a 3-(chinazolylamino)-propylgroup (see Figure 1). In the broth dilution test, these three derivatives show a significant reduction in the MIC values (see Table 1). Therefore the following experiments were carried out to investigate the molecular basis for the action of these derivatives. The derivatives were tested for competition with [^{14}C]erythromycin in binding experiments. As can be seen from Figure 2 the derivatives have the same, or a slightly higher, affinity for the ribosome as compared to erythromycin.

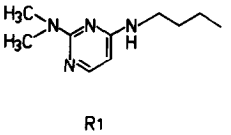
In support of this finding, the three derivatives show a stronger inhibition than erythromycin in a prokaryotic poly(A) directed polylysine system, whereas the polylysine synthesis in an eukaryotic system is affected neither by erythromycin nor by the derivatives (Figures 3A and 3B, respectively).

The lysyl-peptides were analysed by paper chromatography following protein synthesis in the presence and absence of 1 μM of erythromycin or the erythromycin derivatives. As shown in Fig. 4, all erythromycin derivatives induce a marked accumulation of di- and tri-lysines, which is characteristic for the action of erythromycin (22).

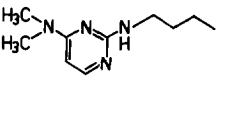
The reason for this strong inhibitory effect of the derivatives in the prokaryotic system could be due to an improved permeability, a multiple step inhibition within the protein synthesis and/or could be caused by a higher affinity for the 50S ribosomal subunit than that of erythromycin. Therefore, the inhibition of various functional steps of the protein synthesis was investigated.

First, the charging of a mixture of *E. coli* tRNA with





R1



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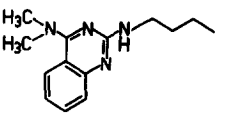

R3

Figure 1:
Structural formula of erythromycin derivatives. The derivatives contain the following substitution E-I, R = R1, E-II, R = R2, E-III, R = R3.

Table 1 Minimal inhibitory concentration of erythromycin and the derivatives against three gram negative bacteria

Strain	Antibiotic µg/ml			
	Erythromycin	E-I	E-II	E-III
Escherichia coli ATCC 9637	32	3	4	5
Klebsiella pneumoniae ATCC 10031	31	8	8	9
Proteus vulgaris	28	14	16	12

[^{14}C]-amino acids was tested in the presence or the absence of erythromycin and its derivatives. As can be seen from Table 2, erythromycin and the derivatives only show an inhibition of the aminoacyl tRNA-ligase when present in a much higher concentration than that used in the polylysine synthesis.

In the next experiment the non-enzymatic binding of tRNA

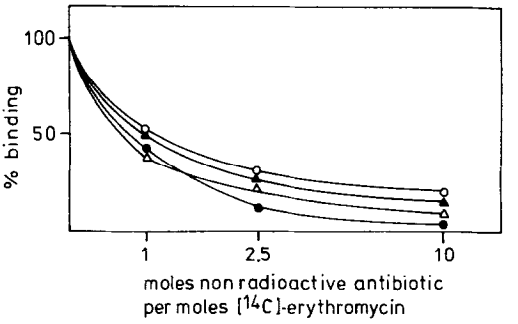

Figure 2:
Competition between non-radioactive antibiotics [o, erythromycin or its derivatives ● , E-I; △ , E-II; ▲ , E-III] and (^{14}C) erythromycin for the binding to 50S subunits. The concentration of (^{14}C)erythromycin was 2 μM in all equilibrium dialysis experiments. 100 % = 826 cpm.

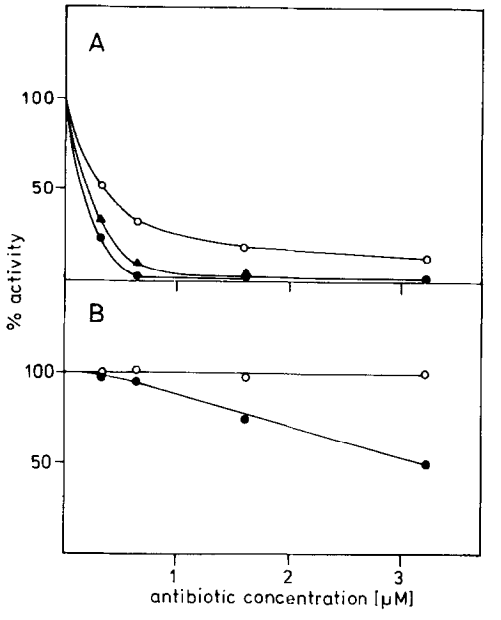

Figure 3:
Inhibition of poly(A) directed polylysine synthesis by erythromycin and its derivatives.
A: prokaryotic system; o, erythromycin; ●, E-I or E-II; ▲, E-III, 100 % = 39,121 cpm.
B: eukaryotic system; o, erythromycin and its derivatives; ●, cycloheximide, 100 % = 7,493 cpm.

to the ribosome was tested. Chlorotetracycline inhibits preferentially tRNA binding to the A-site (23). Controls in the

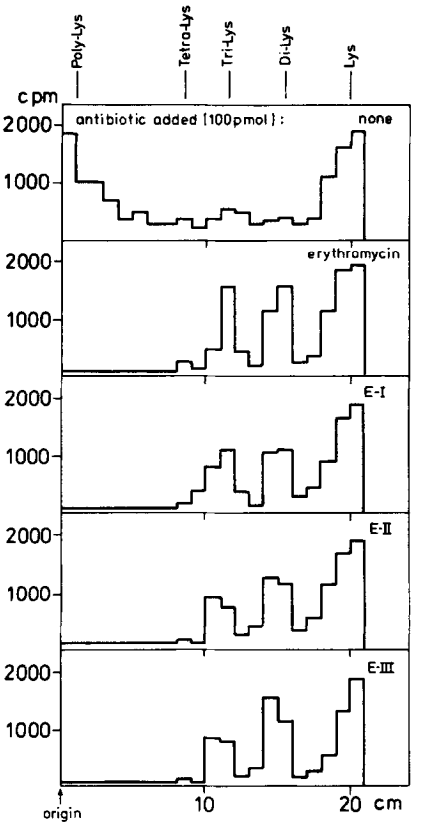

Figure 4:
Paper chromatography of the (^{14}C)lysyl-peptides synthesized in a poly(A) system in presence or absence of erythromycin and its derivatives (100 pmol per assay equivalent to 1 μM).

presence of chlorotetracycline demonstrated a marked inhibition of tRNA binding at 15 mM Mg^{++}, whereas no or low inhibition was detected at 5 mM (data not shown), indicating a predominant tRNA binding to the A-site at 15 mM and to the P-site at 5 mM Mg^{++} (24). Furthermore, controls were performed in the presence of puromycin. The puromycin reaction took place only at 5 mM Mg^{++}.

As can be seen from Table 3, the binding of [^{3}H]Phe-tRNA in the A-site is unaffected by erythromycin and the derivatives, whereas the binding of Ac-[^{3}H]Phe-tRNA into the P-site

Table 2 Effect of erythromycin and its derivatives on aminoacyl-tRNA ligase activity. 100 % = 41122 cpm.

Antibiotic	% Activity in presence of antibiotics		
	5	50	500 (μM)
Erythromycin	99	100	90
E-I	99	96	76
E-II	100	97	74
E-III	99	96	83
none		100	

Table 3 Nonenzymatic poly(U) dependent tRNA binding to 70S ribosomes. A-site: 100 % = 1710 cpm. P-site: 100 % = 1580 cpm.

Antibiotic (5 μM)	Nonenzymatic tRNA binding (%)	
	(^{3}H)Phe-tRNA to A-site (15 mM Mg^{++})	Ac(^{3}H)Phe-tRNA to P-site (5 mM Mg^{++})
none	100	100
Erythromycin	103	124
E-I	102	120
E-II	104	117
E-III	108	128
control (minus poly(U))	9	11

is stimulated about 20 % by erythromycin and the derivatives, at a concentration of 5 μM.

The peptidyl transferase activity is stimulated by erythromycin in agreement with earlier results (25); the derivatives show a similar stimulatory effect (Figure 5A), whereas chlor-

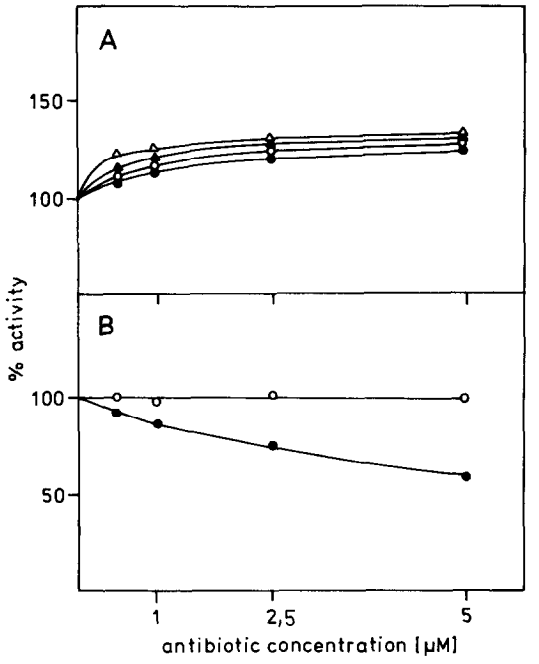

Figure 5:
The effect of erythromycin and its derivatives.
A: Peptidyl transferase activity; 100 % = 14,802 cpm.
o, erythromycin; ●, E-I; △, E-II; ▲, E-III; a control experiment in the presence of 35 μM chloramphenicol gave an inhibition of about 40 % (data not shown).
B: EF-G dependent GTPase activity; 100 % = 50,128 cpm.
o, erythromycin and its derivatives; ●, fusidic acid.

amphenicol, a well-known inhibitor of prokaryotic peptidyl transferase (for review see 26), shows the expected inhibition.

Erythromycin as well as the derivatives do not influence the EF-G dependent GTPase activity (Figure 5B). As a control we included fusidic acid which inhibits the dissociation of EF-G from the ribosome and therefore reduces the EF-G dependent GTPase reaction (Figure 5B, for review see 26).

In conclusion, the stronger inhibitory effect of the synthetic erythromycin derivatives in the poly(A) dependent polylysine synthesis can (at least partially) be explained by a higher affinity of the derivatives for the 50S ribosomal

subunit, and is not due to an altered inhibition specificity as compared to erythromycin.

Since the mode of penetration of erythromycin into bacterial cells is by passive diffusion (27), and since the erythromycin derivatives display a higher affinity than erythromycin for the 50S ribosomal subunit, a stronger concentration gradient is maintained across the cell membrane in the case of the erythromycin derivatives. Furthermore, it cannot be ruled out that the modifications producing the more hydrophilic derivatives facilitate the penetration of the drugs into gram negative bacteria.

Possibly both explanations are the reasons why the erythromycin derivatives display a greater inhibitory effect against the gram negative bacteria tested as compared with erythromycin itself.

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REFERENCES

(1) Brock, T.D. and Brock, M.L. (1959) Biochim.Biophys.Acta 33, 274-275.
(2) Taubman, S.B., Young, F.E. and Corcoran, J.W. (1963) Proc.Natl.Acad.Sci. US 50, 955-962.
(3) Wolfe, A.D. and Hahn, F.E. (1964) Science 143, 1445-1446.
(4) Mao, J., C.-H. (1967) Biochem.Pharmacol. 16, 2441-2443.
(5) Tanaka, K., Teraoka, H., Tamaki, M., Otaka, E. and Osawa, S. (1968) Science 162, 576-578.
(6) Taubman, S.B., Jones, N.R., Young, F.E. and Corcoran, J.W. (1966) Biochim.Biophys.Acta 123, 438-440.
(7) Menninger, J.R. (1976) J.Biol.Chem. 251, 3392-3398.
(8) Otaka, T. and Kaji, A. (1975) Proc.Natl.Acad.Sci. US 72, 2649-2652.
(9) Tanaka, K. and Teraoka, H. (1966) Biochim.Biophys.Acta 114, 204-206.
(10) Tanaka, K. and Teraoka, H. (1968) J.Biochem., Tokyo 64, 635-648.
(11) Massey, E.J., Kitchell, B.S., Martin, L.D. and Gerzon, K. (1974) J.Med.Chem. 17, 105-107.
(12) Pestka, S. and Lemahieu, R.A. (1974) Antimicrob.Ag. Chemother. 6, 479-488.

(13) Vince, R., Weiss, D. and Pestka, S. (1976) Antimicrob. Ag.Chemother. 9, 131-136.
(14) Pestka, S., Lemahieu, R. and Miller, P. (1974) Antimicrob. Ag.Chemother. 6, 489-491.
(15) Ericsson and Sherris 1971 Antibiotic Sensitivity testing. Report of an International Collaborative Study. Acta.Pathol.Microbiol.Scand.Sect. B Suppl. 217.
(16) Schreiner, G. and Nierhaus, K.H. (1973) J.Mol.Biol. 81, 71-82.
(17) Ulbrich, B. and Nierhaus, K.H. (1975) Eur.J.Biochem. 57, 49-54.
(18) Werner, R., Thorpe, L., Reuter, W. and Nierhaus, K.H. (1976) Eur.J.Biochem. 68, 1-3.
(19) Nierhaus, K.H. and Montejo, V. (1973) Proc.Natl.Acad.Sci. US 70, 1931-1935.
(20) Parmeggiani, A., Singer, C. and Gottschalk, E.M. (1971) in "Methods in Enzymology" (Academic Press, New York and London) Vol. XX, part C, pp. 291-302.
(21) Watanabe, S. (1972) J.Mol.Biol. 67, 443-357.
(22) Teraoka, H., Tanaka, K. and Tamaki, M. (1969) Biochim. Biophys.Acta 174, 776-778.
(23) Bodley, J.W. and Zieve (1969) Biochem.Biophys.Res.Commun. 36, 463-468.
(24) Zagorska, L., Dondon, J., Lelong, J.G., Gros, F. and Grunberg-Manago, M. (1971) Biochemie 53, 63-70.
(25) Teraoka, H. and Tanaka, K. (1971) Biochim.Biophys.Acta 232, 509-513.
(26) Vazquez, D. (1974) FEBS Lett. 40, Supplement, S63-S84.
(27) Franklin, T.J.: Industrial Aspects of Biochemistry 30, Part 2, p. 549-577, 9. FEBS-Meeting, Dublin, April 1973.