EFFECT OF THE LEUCOMYCIN-LIKE MACROLIDE ANTIBIOTIC TURIMYCIN ON RIBOSOMAL PEPTIDYLTRANSFERASE FROM *ESCHERICHIA COLI*

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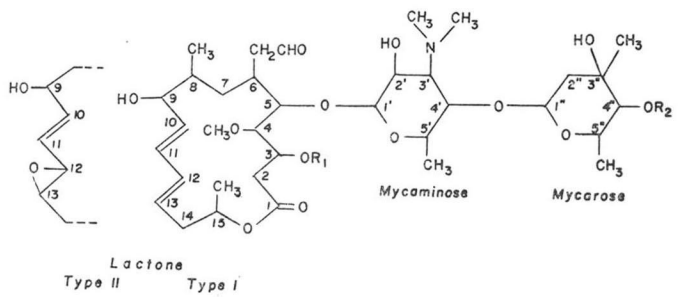
The relationship between the effect of different turimycin components on ribosomal peptidyltransferase of *E. coli*, antimicrobial activity and chemical structure were studied. Inhibition of peptidyltransferase as well as antimicrobial activity increased with the length of the aliphatic side chain in 4″-position of mycarose and decreased with acylation in 3-position of the lactone ring. Inhibition of peptidyltransferase is paralleled by inhibition of acceptor substrate binding.

Turimycin is a new macrolide antibiotic complex produced by fermentation of *Streptomyces hygroscopicus* JA 6599[1]. As a result of structural studies (unpublished results) turimycin components isolated from the complex were found to be identical with known basic macrolide antibiotics of the leucomycin-carbomycin family as shown in Table 1. The chemical structures of turimycin components are characterized by two types of 16-membered lactone rings with different substituents in 3-position, the amino sugar mycaminose and the neutral sugar mycarose which bears different acyl groups in 4″-position (Fig. 1, Table 1). Macrolide antibiotics are known to inhibit protein biosynthesis by interfering with peptidyltransferase[8]. Investigation of relationship between the effect of different turimycin components on ribosomal peptidyltransferase, antimicrobial activity and chemical structure are the subjects of the present paper.

Fig. 1. Chemical structure of turimycins.



Materials and Methods

Ribosomes were prepared from *Escherichia coli* B as described elsewhere[4]. Ac[^{14}C]Leu-pentanucleotide (CACCA-acLeu) was prepared as described earlier[5] and [^{3}H]Phe-pentanucleotide (CACCA-Phe) was prepared according to PESTKA[6]. The transfer of the ac-Leu-residue from CACCA-ac[^{14}C]Leu

fragment to puromycin was measured according to MONRO *et al.*[5]. The puromycin reaction with $[Lys]_n$-tRNA was determined as described previously[7]. For assay of CACCA-acLeu binding to the donor site the method of CELMA *et al.*[8] was used whereas CACCA-Phe binding to the acceptor site was measured according to PESTKA[9]. The incubation mixtures and the reaction conditions are described in the legends.

Turimycins were dissolved in methanol to make a stock solution of 0.001 M. Dilutions were made with water. Relative antimicrobial activity was determined by agar diffusion assay method using *Bacillus subtilis* SG 119 as a test organism. The activity of each compound was compared with the activity of a standard of turimycin complex (1 mg=1,000 units).

Results

1. Effect of Turimycins on the Fragment Reaction with CACCA-acLeu as Donor Substrate

The effect of 11 turimycin components on the acLeu-transfer from CACCA-acLeu to puromycin catalysed by 70S-ribosomes is shown in Fig. 2. According to the substituent in 3-position of the lactone ring turimycin components are classified into turimycin H, A and P group with R_1=H, acetyl and propionyl, respectively (Table 1).

Table 1. Structure and antimicrobial activities of turimycins.

Compound	Abbreviation	Substituents		Identical with (references in[2])	Relative antimicrobial activity units/mg
		R_1	R_2		
Lactone type I					
Turimycin H_5	TM-H_5	H	isovaleryl	Leucomycin A_1	1,673
〃 H_4	TM-H_4	H	*n*- and iso-butyryl	〃 A_5 (R_2=*n*-butyryl)	1,557
〃 H_3	TM-H_3	H	propionyl	〃 A_7	1,171
〃 H_2	TM-H_2	H	acetyl	〃 A_9	414
〃 H_0	TM-H_0	H	H	〃 V	249
Turimycin A_5	TM-A_5	acetyl	isovaleryl	Leucomycin A_3	1,215
〃 A_3	TM-A_3	〃	propionyl	〃 A_6	649
〃 A_2	TM-A_2	〃	acetyl	〃 A_8	174
Turimycin P_5	TM-P_5	propionyl	isovaleryl	YL-704 A_1	1,233
〃 P_3	TM-P_3	〃	propionyl	SF-837 A; YL-704 B_1	704
				Espinomycin A_1	247
〃 P_2	TM-P_2	〃	acetyl	YL-704 C_2	
Lactone type II					
Turimycin EA_3	TM-EA_3	acetyl	propionyl	Maridomycin IV	504
Turimycin EP_5	TM-EP_5	propionyl	isovaleryl	Maridomycin I; YL-704 C	1,432
〃 EP_3	TM-EP_3	〃	propionyl	Maridomycin III; YL-704 C_1	859
〃 EP_2	TM-EP_2	〃	acetyl	Maridomycin V	326

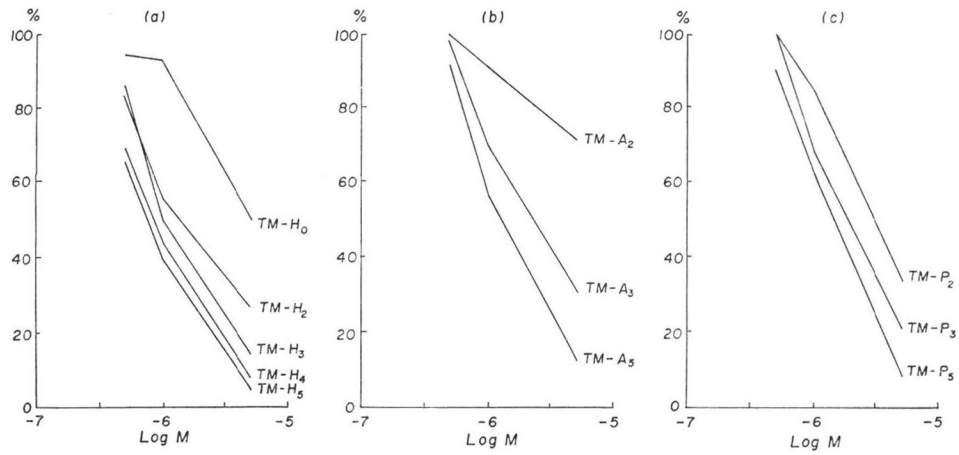
The transfer reaction is inhibited by all turimycins tested. Turimycin components with the same substituent R_1 but different side chains in 4″-position of mycarose (R_2) differ in their activity. Within every group with the same substituent R_1 inhibitory activity increases with the length of the side chain R_2. The most pronounced effects were obtained with components which contain isovaleric acid as R_2. These results are in good agreement with antibacterial activity of these compounds (Table 1). The greatest differences between components which differ in R_2 were observed in the turimycin A group (Fig. 2 b).

Fig. 2. Effect of turimycins on the fragment reaction of acLeu-pentanucleotide with puromycin.

Reaction mixtures contained (before adding methanol) 50 mM Tris–HCl (pH 7.4), 400 mM KCl, 20 mM magnesium acetate, ribosomes (150 μg), ac[^{14}C]Leu-pentanucleotide (2000 cpm) and 500 μM puromycin in 100 μl. The reaction was started with 50 μl methanol. Incubation at 0°C was terminated after 30 minutes by adding 100 μl 200 mM sodium acetate (pH 5.5) saturated with $MgSO_4$. The acLeupuromycin formed was extracted with ethyl acetate (1.5 ml) and the radioactivity was measured on planchets in a flow counter.

LogM=concentrations of turimycins; %=acLeu-puromycin formation as % of control without inhibitor (about 1000 cpm transferred).

(a) R_1=H, (b) R_1=acetyl, (c) R_1=propionyl. Abbreviations used are shown in Table 1.



In contrast to acylation in 4″-position of mycarose, acylation of 3-position of the lactone ring resulted in a decrease of biological activity (Table 1). It is seen from Fig. 2 that acylation of 3-hydroxyl group decreases activity in fragment reaction, too. Antibiotics with hydrogen as R_1 have in every case the greatest effect, when substances with the same side chain R_2 are compared.

Our experiments have shown that turimycin components of the lactone type II inhibited fragment reaction to the same or nearly the same extent as the corresponding turimycins of the lactone type I (data are not shown).

2. Effect of the Turimycins A_2, A_3 and A_5 on the Puromycin Reaction with $[Lys]_n$-tRNA as Donor Substrate

The transfer of lysine peptides to puromycin with intact molecules of $[Lys]_n$-tRNA and poly A as messenger RNA is inhibited by turimycins, too (Table 2). The turimycin components A_2, A_3 and A_5 inhibit transfer reaction in

Table 2. The effect of the turimycins A_5, A_3 and A_2 on the transfer of lysine peptides from $(Lys)_n$-tRNA to puromycin.

	Conc. (μM)	Released activity	
		cpm	%
Control	—	500	100
TM–A_5	0.5	303	61
	0.7	138	28
	0.9	32	6
TM–A_3	0.5	272	54
	0.7	146	29
	0.9	75	15
TM–A_2	0.5	289	58
	0.7	234	47
	0.9	154	31

The reaction mixture contained in 100 μl: 100 mM Tris-acetate (pH 7.2), 100 mM ammonium acetate, 10 mM magnesium acetate, [^{14}C]$(Lys)_n$–tRNA (5 μg, 1625 cpm), ribosomes (260 μg) and poly A (10 μg). After preincubation for 20 minutes at 35°C puromycin (100 μM) was added and the incubation was continued for further 20 minutes at 35°C. Material insoluble in 5% trichloroacetic acid was filtered through Millipore filters and measured in toluene scintillation fluid.

the same order but to a greater extent as the fragment reaction with CACCA-acLeu. Antibiotic concentrations are two to tenfold lower than those needed for comparable inhibition of fragment reaction. Also in the transfer reaction effectiveness increases with the length of the side chain R_2 in mycarose, but the differences are less pronounced as in the fragment reaction.

3. Effect of the Turimycins A_2, A_3 and A_5 on the Substrate Binding to the Donor and Acceptor Site

The effect of the turimycins A_2, A_3 and A_5 on the binding of CACCA-acLeu to the donor site and the binding of CACCA-Phe to the acceptor site of the 70S ribosomes is shown in Table 3. Binding of acceptor substrate is inhibited in the same concentration range, but even to a somewhat greater extent than fragment reaction. The same antibiotics decrease the binding of the donor substrate only at concentrations which are ten to hundredfold higher than those required to inhibit binding of acceptor substrate. But in both cases the activity of the turimycin components increases with the length of the side chain R_2.

Table 3. The effect of the turimycins A_5, A_3 and A_2 on the CACCA–[^{3}H]Phe and CACCA–ac[^{14}C]Leu binding to 70S ribosomes.

	Conc. (μM)	CACCA–[^{3}H]Phe		Conc. (μM)	CACCA-ac[^{14}C]Leu	
		cpm	%		cpm	%
Control	—	1296	100	—	727	100
TM–A_5	0.5	932	72	1	549	75
	1.0	190	15	10	411	57
	2.0	62	5	100	81	11
TM–A_3	0.5	975	75	1	595	82
	1.0	601	46	10	460	63
	2.0	272	21	100	396	54
TM–A_2	0.5	1082	84	1	829	114
	1.0	834	64	10	538	74
	2.0	541	42	100	486	67

The reaction mixtures for assay of CACCA–Phe binding contained in 150 μl: 50 mM Tris-acetate (pH 7.2), 40 mM $MgCl_2$, 400 mM KCl, 100 mM NH_4Cl, ribosomes (525 μg), CACCA–[^{3}H]Phe (9438 cpm) and 30 μl ethanol. Reaction mixtures were incubated at 24°C for 20 minutes, filtered through Millipore filters and measured in toluene scintillation fluid.

The reaction mixtures for assay of CACCA–acLeu binding contained in 150 μl: 13 mM magnesium acetate, 270 mM KCl, 40 mM Tris–HCl (pH 7.4), ribosomes (525 μg), CACCA–ac[^{14}C]Leu (5900 cpm) and 75 μl ethanol. Mixtures were incubated at 0°C for 60 minutes and then centrifuged. Aliquots of the supernatant were measured with BRAY's scintillation fluid.

Discussion

In the present study we attempted to clarify the role of various functional groups of the turimycin molecule at a molecular level. The studied turimycin components differ in the type of the lactone ring as well as in the substituent R_1 of the lactone and the substituent R_2 of the mycarose.

It has been known that the acyl groups in 4″-position of mycarose play an important role in the antibacterial activity of leucomycins[10]. The removal of this acyl group results in decrease of antibacterial activity, while the activity increases with the length of this aliphatic side chain. SAITO *et al.*[11] described that leucomycin acylated with isovaleric acid on mycarose and leucomycin without acyl group on mycarose differ only in antibacterial activity but not in the inhibition of cell-free polyphenylalanine synthesis. From these results it was concluded[11,12] that the acyl groups on mycarose are

merely needed for the transport of the antibiotic into the cell and are not engaged in the action on ribosomes.

In contrast to these results we could demonstrate that acylated and deacylated turimycins differ in antibacterial activity as well as in their effect on peptidyltransferase reaction. This means that the acyl groups on mycarose take part in the action on the target site of the ribosome and are not only concerned with the penetration of these antibiotics into the bacterial cell. In agreement with the antibacterial activity inhibition of peptidyltransferase increases with the length of the side chain R_2. This dependence is demonstrated for all 3 groups of turimycins which differ in R_1. The differences are most pronounced in the turimycin A group (Fig. 2b).

Generally, among the turimycins with the same substituent R_2 the compounds which lack substitution in 3-position are most active against *B. subtilis* and in peptidyltransferase reaction. Acylation in 3-position of the lactone decreases antibacterial activity as well as the effect on peptidyltransferase reaction. From these results it can be concluded that this part of the molecule is involved in the action on the target site on the ribosome, too. Comparable results were obtained by PESTKA *et al.*[13], who demonstrated that leucomycin A_5 containing a hydrogen as R_1 binds to ribosomes with a greater affinity than leucomycins acylated in 3-position.

Inhibition of fragment reaction corresponds to inhibition of acceptor substrate binding as is shown for the compounds turimycin–A_2, turimycin–A_3 and turimycin–A_5, while the binding of donor substrate is inhibited only at higher antibiotic concentrations (Table 3). Therefore inhibition of peptidyltransferase can be deduced on interaction of turimycins with the acceptor binding site of the ribosome. The primary effect of turimycins on acceptor substrate binding is in agreement with the effect of other macrolides as spiramycin and carbomycin, as was described in previous papers[9,14].

Our experiments have further shown that presence of an epoxy group in 12~13-position of the lactone ring (lactone type II) changes neither antibacterial activity nor activity in peptidyltransferase reaction.

The described correlations between the effects of different turimycins on peptidyltransferase and the inhibition of bacterial growth strongly support the theory that the biological action of this group of antibiotics is due to an effect on peptidyltransferase.

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References

1) KNÖLL, H.; G. BRADLER, R. FÜGNER, P. KRAMER, H. PRAUSER, W. FORBERG, E. STRUMPF, H. FRICKE, W. EFFENBERGER & H. THRUM: Verfahren zur Gewinnung eines Makrolid-Antibiotikums. DDR-WP 84850 12. 9. 1971
2) ŌMURA, S. & A. NAKAGAWA: Chemical and biological studies on 16-membered macrolide antibiotics. J. Antibiotics 28: 401~433, 1975
3) PESTKA, S.: Inhibitors of ribosome functions. Ann. Rev. Microbiol. 25: 487~562, 1971
4) LESSARD, J. S. & S. PESTKA: Studies on the formation of transfer ribonucleic acid-ribosome complex. XXII. Binding of aminoacyl-oligonucleotides to ribosomes. J. Biol. Chem. 247: 6901~6908, 1972
5) MONRO, R. E.; J. ČERNÁ & K. A. MARCKER: Ribosome-catalyzed peptidyl transfer: substrate specificity at the P-site. Proc. Natl. Acad. Sci., U.S.A. 61: 1042~1049, 1968
6) PESTKA, S.: Methods in Enzymology. XX. Part C, pp. 502~507, Academic Press, New York, 1971
7) RYCHLÍK, I.; J. ČERNÁ, S. CHLADEK, J. ŽEMLIČKA & Z. HALADOVA: Substrate specifity of ribosomal peptidyl transferase: 2′(3′)-*o*-aminoacylnucleosides as acceptors of the peptide chain on the amino acid site. J. Mol. Biol. 43: 13~24, 1969
8) CELMA, M. L.; R. E. MONRO & D. VAZQUEZ: Substrate and antibiotic binding sites at the peptidyl transferase centre of *E. coli* ribosomes. FEBS Lett. 6: 273~277, 1970
9) PESTKA, S.: Studies on the formation of transfer ribonucleic acid-ribosome complexes. XI. Antibiotic effects on phenylalanyloligonucleotide binding to ribosomes. Proc. Natl. Acad. Sci., U.S.A. 64: 709~714. 1969

10) ŌMURA, S.; M. KATAGIRI, I. UMEZAWA, K. KOMIYAMA, T. MAEKAWA, K. SEKIKAWA, A. MATSUMAE & T. HATA: Structure-biological activities relationships among leucomycins and their derivatives. J. Antibiotics 21: 532~538, 1968
11) SAITO, T.; M. SHIMIZU & S. MITSUHASHI: Macrolide resistance in staphylococci. Ann. New York Acad. Sci. 182: 267~278, 1971
12) RAKHIT, S. & K. SINGH: Structure activity relationship in sixteen-membered macrolide antibiotics. J. Antibiotics 27: 221~224, 1974
13) PESTKA, S.; A. NAKAGAWA & S. ŌMURA: Effect of leucomycins and analogues on binding [^{14}C]erythromycin to *Escherichia coli* ribosomes. Antimicr. Agents & Chemoth. 6: 606~612, 1974
14) ČERNÁ, J. & I. RYCHLÍK: The effect of antibiotics on the substrate binding of the acceptor and donor site of ribosomal peptidyltransferase of an erythromycin resistant mutant of *Escherichia coli*. Biochim. Biophys. Acta 287: 292~300, 1972