Structure-activity Studies of Tylosin-related Macrolides

STEVEN A. FISH and ERIC CUNDLIFFE*

Department of Biochemistry, University of Leicester,
Leicester LE1 7RH, U.K.

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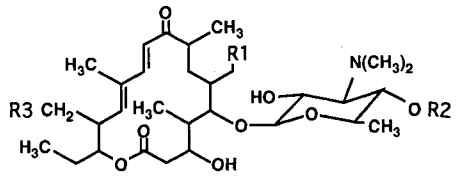
The effects of tylosin-related macrolide antibiotics were examined in cell-free protein synthesis (using a coupled transcription-translation system derived from *Streptomyces lividans*) and against whole cells of that organism. Anti-ribosomal potency was determined primarily by the number and nature of the glycosyl substituents, and was not significantly influenced by lactone ring oxidation or sugar methylation. In contrast, uptake of the drugs into *S. lividans* was influenced, either positively or negatively, by each of these structural parameters. The presence of *erm* type I or *erm* type II resistance genes in *S. lividans* markedly affected the resistance phenotype and studies involving ribosomes from such strains revealed differences in macrolide activity that were not otherwise apparent.

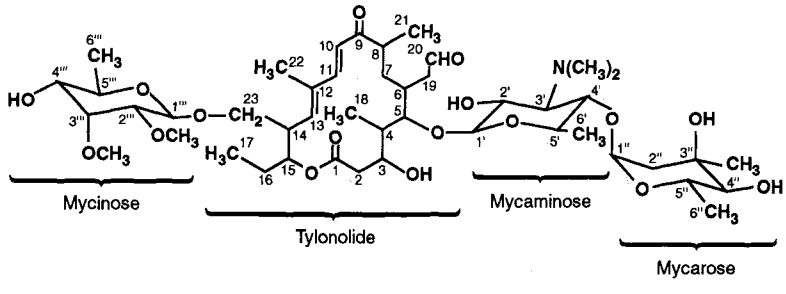
Macrolide antibiotics bind to the 50S subunit of the bacterial ribosome, thereby inhibiting protein synthesis[1]. Since cell-free protein-synthesising systems programmed by polyU are relatively insensitive to certain macrolides (such as erythromycin), the action of such drugs has previously been examined against polyA-directed synthesis of polylysine[2]. Alternatively, the anti-ribosomal activities of macrolides have been examined by following their respective abilities to compete for ribosomal binding sites with radiolabelled erythromycin[3~5] or leuco-

Fig. 1. Structures of tylosin-related macrolides.



	R_1	R_2	R_3
Tylactone (5-OH; * lacks mycaminose)	CH_3	*	H
20-Dihydro, 23-deoxy-OMT	CH_2OH	H	H
23-Deoxy-OMT	CHO	H	H
O-Mycaminosyl-tylonolide (OMT)	CHO	H	OH
Demycinosyl-tylosin (DMT)	CHO	mycarose	OH
Demycinosyloxy-tylosin	CHO	mycarose	H
Desmycosin	CHO	H	*O*-mycinose
O-Demethyl-lactenocin	CHO	H	*O*-deoxyallose
O-Demethyl-macrocin	CHO	mycarose	*O*-deoxyallose
Macrocin	CHO	mycarose	*O*-[2‴-*O*-demethyl mycinose]
Relomycin	CH_2OH	mycarose	*O*-mycinose
Tylosin	CHO	mycarose	*O*-mycinose

(mycinose is 2‴,3‴-*O*-methyl-deoxyallose)



Tylosin

mycin[6]. Assumptions inherent in such studies were that the various macrolides were competing for the same target site(s) and that ribosomal binding affinity measured in that way was directly related to the efficiency of ribosomal inhibition. Here, the ability of tylosin-related macrolides (Fig. 1) to inhibit ribosomal function has been measured directly in a coupled transcription-translation system, the closest cell-free model for intracellular protein synthesis. Macrolides do not inhibit RNA synthesis, therefore this assay system could be used specifically to monitor translation.

Since macrolides are mainly active against Gram-positive bacteria, coupled transcription-translation systems were prepared using fractionated extracts of *Streptomyces lividans*[7,8]. The strain used (OS456) had been specifically engineered to be macrolide-sensitive by insertional inactivation of a tandem pair of indigenous resistance genes[9]. We also generated derivatives of OS456 harbouring an *erm* type I gene (*tlrD*, from *Streptomyces fradiae*) or an *erm* type II gene (*ermE* from *Saccharopolyspora erythraea*). These encode, respectively, methyltransferases that mono- or di-methylate 23S ribosomal RNA thereby conferring the MLS-I or MLS-II resistance phenotypes[9]. "MLS" refers to macrolides, lincosamides, and streptogramin B type antibiotics, and one objective of the present work was to expand those phenotypes to include the tylosin-related macrolides. Ribosomes from strain OS456 and from the *erm* type I and type II strains ("erm-I" and "erm-II" ribosomes, respectively) were also incorporated into coupled transcription-translation systems. The aim here was twofold: to examine the consequences of structural variations in tylosin-related macrolides for drug-target interactions, and to observe how these were perturbed by modification of the ribosomes.

Materials and Methods

Growth and Manipulation of Organisms

Streptomyces lividans OS456 was maintained on NEF agar with the addition of hygromycin B (200 μg/ml; Boehringer Mannheim). The medium contained per litre: 5 g glucose; 1 g yeast extract (Oxoid, Basingstoke, U.K.); 0.5 g beef extract (Oxoid "Lab-Lemco" powder); 1 g casamino acids (Difco) and 20 g agar. Before autoclaving, the pH was adjusted to 7.2 using KOH. Protoplasts of OS456 were transformed with pLST912 carrying *tlrD*[10] or pLST391 carrying *ermE*[11] using standard methods[12]; these plasmids were maintained using lincomycin (50 μg/ml) or thiostrepton (5 μg/ml), respectively. Liquid cultures were grown in YEME medium[12] supplemented with 5 mM $MgCl_2$ and 0.5% (w/v) polyethylene glycol 6000.

Antibiotics and MIC Determinations

Tylosin-related macrolides used in this study were kindly provided by Dr. HERBERT KIRST, Lilly Research Laboratories, Greenfield, Indiana, Ind. MICs were determined by applying fresh spores of *S. lividans* strains to NEF agar containing antibiotics, followed by incubation at 30°C for 3 days.

Cell-free Protein Synthesis

A coupled transcription-translation system was used to assay cell-free protein synthesis. Salt-washed ribosomes were prepared from *S. lividans* OS456 and from the *tlrD*$^+$ and *ermE*$^+$ strains, and used together with protein synthesis initiation factors plus postribosomal 100,000 × *g* supernatant (S100), both from strain OS456. These materials were prepared as described elsewhere (THOMPSON *et al.*, 1984; CALCUTT and CUNDLIFFE, 1989). Plasmid DNA (pUC18; prepared using a Qiagen column) was added as the template for transcription (the S100 contains RNA polymerase), and translation of the resultant mRNA was measured as the incorporation of [^{35}S]methionine into newly synthesized protein.

Results and Discussion

Cell-free Protein Synthesis

Monoglycosidic macrolides, with mycaminose attached at C5-OH of the lactone ring, displayed low levels of activity against ribosomes from *S. lividans* OS456 or the *tlrD*$^+$ strain (*erm* type I), with no detectable inhibition of ribosomes from the *ermE*$^+$ (*erm* type II) strain (Table 1). Although these levels of anti-ribosomal activity were low, their significance was evident from the respective MIC values (Table 2); effects of similar magnitude were also observed previously with other MLS antibiotics[9]. Macrolides containing a neutral sugar in addition to mycaminose were dramatically more active than the monoglycosides against control or erm-I ribosomes, and even erm-II particles were sensitive to the triglycosides, albeit at somewhat higher concentrations. Since the diglycoside, demycinosyl-tylosin, and the bis-glycoside, demycarosyl-tylosin (desmycosin), displayed similar activities, each of the two neutral sugars contributed to enhanced potency, which was even further enhanced by their combined presence. When other macrolides (*i.e.* not of the tylosin series) were examined under similar conditions, differences in their anti-ribosomal activities were also attributed to the relative disposition of glycosidic residues around the polyketide lactones and not, for example, to the relative sizes of the lactone rings[9]. None of the other structural variations

Table 1. Inhibition of cell-free protein synthesis, in a coupled transcription-translation system, by tylosin-related macrolides.

Antibiotic	% Inhibition of synthesis (drug concn) with ribosomes from[a]:		
	Control strain	*erm* type I strain (*tlrD*$^+$)	*erm* type II strain (*ermE*$^+$)
Tylactone	Zero (100)	Zero (100)	Zero (100)
20-Dihydro, 23-deoxy OMT	10~15 (100)	0~5 (100)	0~5 (100)
23-Deoxy-OMT	0~5 (100)	0~5 (100)	0~5 (100)
OMT	0~5 (100)	0~5 (100)	0~5 (100)
DMT	85 (0.5)	40 (0.5)	0~5 (100)
Desmycosin	55 (0.5)	40 (0.5)	0~5 (100)
O-Demethyl-macrocin	90 (0.5)	80 (0.5)	35 (10)
Relomycin	90 (0.5)	45 (0.5)	35 (10)
Tylosin	90 (0.5)	70 (0.5)	55 (10)

[a] Calculated for 40 minutes time points. The synthesis rate was approximately linear over this period. Drug concentrations are in μg/ml. All other components of the coupled transcription-translation system were derived from control strain OS456.

OMT: *O*-mycaminosyl-tylonolide; DMT: demycinosyl-tylosin.

Table 2. MICs of tylosin-related macrolides for various strains of *S. lividans*.

Antibiotic	MICs (μg/ml) for:		
	Control strain OS456	*erm* type I strain (*tlrD*$^+$)	*erm* type II strain (*ermE*$^+$)
Tylactone	>5000	>5000	>5000
20-Dihydro, 23-deoxy OMT	10~25	250~500	1000~2500
23-Deoxy-OMT	1~5	100~250	1000~2500
OMT	10~25	250~500	2500~5000
DMT	25~50	500~1000	>5000
Desmycosin	1~5	5~10	1000~2500
O-Demethyl-macrocin	25~50	250~500	>5000
Macrocin	1~5	10~25	>5000
Relomycin	1~5	10~25	>5000
Tylosin	0.1~0.5	1~5	1000~2500

OMT: *O*-mycaminosyl-tylosin; DMT: demycinosyl-tylosin.

assessed here produced anti-ribosomal effects as dramatic as those attributable to the patterns of glycosylation. Clearly, the action of macrolide antibiotics at the level of the ribosome is determined primarily by the number and nature of their glycosyl substituents, as first suggested by others[2].

Antibacterial Activities of Tylosin-related Macrolides

Tylosin was significantly more potent than macrocin and at least 50-fold more potent than *O*-demethyl-macrocin against the control and *erm* type I strains, revealing the importance of methylation of the deoxyallose moiety, especially at 2‴-OH. A less dramatic, but still important, variable was the level of oxidation at C20; an aldehyde group at that site (as in tylosin or 23-deoxy-OMT) conferred greater potency than a primary alcohol (as in relomycin or 20-dihydro, 23-deoxy-OMT, respectively). A hydroxyl group at C23 was also disadvantageous (*e.g.* OMT was less potent than 23-deoxy-OMT) but substitution of the C23-OH with mycinose (converting OMT to desmycosin) significantly enhanced the activity (an effect seen most clearly with the *erm* type I strain). Demethyl-lactenocin, which differs from desmycosin in containing deoxyallose rather than mycinose, was not available to us. However, the pronounced effects of 2‴- and 3‴-*O*-methylation described above suggest that demethyl-lactenocin would have been much less potent than desmycosin against *S. lividans*, as seen previously with Gram-positive cocci[13].

Ribosomal Binding and Drug Uptake

Structural changes in drug molecules that affect their uptake and/or intracellular accumulation may have no effects at all on ribosomal binding affinity, but the converse situation is more interesting. Bacterial cells or mycelial units contain tens of thousands of ribosomes, which occupy a significant fraction of the intracellular volume and afford a potential "sink" effect that could promote the uptake of anti-ribosomal drugs. Such considerations do not apply to less abundant drug-targets, such as RNA polymerase, DNA gyrase *etc.* It is therefore possible that changes in drug molecules that change their affinity for ribosomes might have consequential effects on their uptake. Moreover, small changes in ribosomal affinity could easily lead to large changes in uptake. This point is best made by comparing the effects of specific drugs on strains that differ only in the state of their ribosomes. For example, a barely detectable reduction in ribosomal affinity (resulting from the *erm* type I mechanism) can cause the MIC value to increase dramatically, as seen here (Table 1) with several drugs, especially 23-deoxy-OMT, OMT and *O*-demethyl-macrocin. The present work exploits this phenomenon to reveal otherwise latent differences in the action of tylosin-related macrolides.

Contribution of Glycosyl Moieties to Macrolide Activity

Biological activity is conferred upon tylosin-related macrolides by substitution of the amino sugar, mycaminose, at C5-OH of the otherwise inert polyketide lactone, tylactone. However, the consequences of further glycosylation with neutral sugars have been variously interpreted. When assayed against Gram-positive cocci, the presence of mycinose and/or mycarose had little effect on antibacterial activity, so that tylosin, desmycosin, DMT and OMT displayed similar MIC values[13]. In contrast, desmycosin was less active than tylosin against *Bacillus subtilis* but was more active in a ribosomal binding assay, suggesting that the mycarose moiety might be more important for uptake or intracellular accumulation of tylosin than for drug-target interaction[5]. In our hands, the addition of mycarose to OMT (generating DMT) raised the MIC against *S. lividans* OS456 and the *erm* type I strain, despite the much greater activity of DMT against their ribosomes. Similarly, tylosin and desmycosin gave similar MIC values with the *erm* type II strain, although tylosin was vastly more potent than desmycosin against erm-II ribosomes. These data imply a marked negative influence of the mycarose moiety on drug uptake and/or accumulation in *S. lividans*. They also reveal a positive influence of the mycarose moiety on anti-ribosomal activity that is most readily seen with the refractory erm-II ribosomes.

It was also proposed[6] that the mycinose moiety aids uptake of tylosin-related macrolides rather than anti-ribosomal affinity, since OMT was more active than desmycosin against *Escherichia coli* ribosomes, but not against whole cells of various strains. In our hands, desmycosin was vastly more potent than OMT against control or erm-I ribosomes from *S. lividans* and tylosin was much more active than DMT against erm-II ribosomes, revealing an important role for the mycinose moiety in ribosomal binding. Our data also support the proposed involvement of the mycinose moiety in drug uptake, since tylosin and DMT were both extremely active against control or erm-I ribosomes, whereas the intact organisms (especially the *erm* type I strain) were much more sensitive to tylosin.

Changing the Oxidation Level at C20

Enhanced antibacterial potencies of tylosin compared with relomycin, and 23-deoxy-OMT compared with 20-dihydro, 23-deoxy-OMT (Table 2), while not as pronounced as those seen with other organisms[6,13], were not associated with enhanced anti-ribosomal activities (Table 1). These data are compatible with previous reports[5,6] that the primary influence of the C20 aldehyde group (as opposed to a primary alcohol) is at the level of drug uptake or accumulation rather than ribosomal binding.

Substitution at C23

Here and in previous reports[6,13], 23-deoxy-OMT was significantly more potent than OMT against intact bacteria. However, the anti-ribosomal activities of the two drugs were quite similar (present work; also[6]). The simplest explanation of these results is that hydroxylation at C23 impairs the uptake and/or intracellular accumulation of macrolide monoglycosides, with little or no effect on their anti-ribosomal activities. As discussed earlier, glycosylation at C23-OH reduces activity even further, unless followed by 2‴/3‴-*O*-methylation[13]. Interestingly, the negative influence of C23-OH was not apparent with diglycosidic macrolides; DMT and 23-demycinosyloxy-tylosin displayed similar antibacterial activities[14].

2‴/3‴-Methylation

Prior to the present work, the effects of this parameter on anti-ribosomal activity had not been investigated, although the contribution to antibacterial activity has long been known[13]. Since methylation of the 2‴- and 3‴-OH groups made virtually no contribution to anti-ribosomal potency (compare data obtained with *O*-demethylmacrocin and tylosin in Table 1), the primary effect must be to enhance drug uptake.

Ribosomal Binding and Inhibition of Protein Synthesis

Earlier studies focused on the abilities of macrolides to compete for ribosomal binding sites with radiolabelled erythromycin[3~5] or leucomycin[6]. Here, the inhibition of ribosomal function has been measured directly in a coupled transcription-translation system. Such differences in methodology and the use of different strains presumably underly variance between the respective results. For example, OMT and 23-deoxy-OMT competed as efficiently as tylosin in binding studies involving radiolabelled leucomycin and *E. coli* ribosomes[6], whereas tylosin was at least 2 orders of magnitude more potent than OMT or 23-deoxy-OMT against protein synthesis in extracts of *S. lividans* (Table 1). Another feature of the present work was the use of *erm* type strains. In addition to documenting the resistance phenotypes of such organisms with respect to the tylosin series of macrolides, the use of erm-II ribosomes in particular

amplified or revealed differences in inhibitory actions that were otherwise not apparent. For example, the dramatic anti-ribosomal superiority of tylosin and the other triglycosides over DMT and desmycosin was not obvious with wild type ribosomes. Such differences were not necessarily confined to the ribosomal affinities of the drugs, since the use of modified ribosomes can also reveal subtle differences in modes of action, as demonstrated elsewhere[15].

Acknowledgements

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