

Inhibition of Protein Synthesis on the Ribosome by Tildipirosin Compared with Other Veterinary Macrolides

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Tildipirosin is a 16-membered-ring macrolide developed to treat bacterial pathogens, including *Mannheimia haemolytica* and *Pasteurella multocida*, that cause respiratory tract infections in cattle and swine. Here we evaluated the efficacy of tildipirosin at inhibiting protein synthesis on the ribosome (50% inhibitory concentration [IC₅₀], 0.23 ± 0.01 μM) and compared it with the established veterinary macrolides tylosin, tilmicosin, and tulathromycin. Mutation and methylation at key rRNA nucleotides revealed differences in the interactions of these macrolides within their common ribosomal binding site.

Recent approval has been given in Europe and the United States for the use of tildipirosin (20,23-dipiperidinyl-mycaminosyl-tylonolide; Zuprevo) in combating bovine and swine respiratory tract infections. Tildipirosin is a derivative of the natural compound tylosin with two piperidine rings and no mycarose sugar (Fig. 1). Despite its wide use as a veterinary macrolide, tylosin is not particularly effective at penetrating the outer membrane of Gram-negative pathogens (Table 1), and the substitutions in tildipirosin were made to improve efficacy against *Mannheimia haemolytica* and *Pasteurella multocida*, which are the two main etiological agents of bovine respiratory disease (8, 22). Tildipirosin has additionally proven effective against *Histophilus somni*, *Bordetella bronchiseptica*, *Actinobacillus pleuropneumoniae*, and *Haemophilus parasuis* (6), which can also be associated with animal respiratory diseases. Different arrangements of hydrophobic and basic substituents are present in an earlier tylosin derivative, tilmicosin (20-dimethylpiperidinyl-mycaminosyl-tylonolide; Micotil) and the 15-membered triamilide tulathromycin (Draxxin) (Fig. 1), which are used for similar indications.

Here we quantified the inhibitory activity of tildipirosin (MSD Animal Health) on protein synthesis and compare it with tylosin, tilmicosin (Sigma), and tulathromycin (extracted from Draxxin [Pfizer]). The concentration of each macrolide that inhibits 50% of protein synthesis (IC₅₀) was determined in an *in vitro* transcription/translation assay, and the effects of methylations and mutations at rRNA nucleotides within the macrolide binding site were evaluated in cell cultures. The data have been visualized using computationally calculated models of the binding site and reveal subtle differences in macrolide contacts, indicating how changes in the rRNA target have distinct effects on drug efficacy.

An *in vitro* transcription/translation system based on cell extracts containing susceptible, wild-type *Escherichia coli* ribosomes (Promega) was adapted to translate the 27-kDa green fluorescent protein (GFP). GFP mRNA was transcribed from 3 μg of plasmid pIVEX (Roche) in 50 μl of 100 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES)-KOH (pH 7.9), 5 mM MgCl₂, 0.5 mM CaCl₂, 100 mM KCl, 5 mM NH₄Cl, 1 mM dithiothreitol, and 1 mM spermidine and translated to produce [³⁵S]methionine-labeled protein. Synthesis was followed over 155 min with macrolide antibiotics at up to 2 μM, and full-length GFP was quantified by phosphorimager scanning (Typhoon; GE Healthcare) of poly-

TABLE 1 MICs of macrolide antibiotics that prevent growth of Gram-negative strains^a

Strain	MIC (μg/ml) of macrolide			
	Tylosin	Tilmicosin	Tildipirosin	Tulathromycin
<i>M. haemolytica</i> 11935	64	4	0.5	2
<i>P. multocida</i> 4407	32	4	1	0.5
<i>E. coli</i> ATCC 25922	512	128	16	16
<i>E. coli</i> AS19rlmA ^I	1	2	0.5	1

^a *P. multocida* 4407 and *M. haemolytica* 11935 are susceptible strains that contain none of the macrolide resistance determinants (4, 19). *E. coli* ATCC 25922 also lacks any resistance determinants and is a wild-type strain with regard to cell wall/membrane structures. The hyperpermeable strain AS19rlmA^I is described in Table 2. Quality controls were included as previously described (19).

acrylamide sodium dodecyl sulfate gels. Rates of protein synthesis were calculated from the initial linear portions of the slopes (GFP band intensity plotted against time) and normalized against reactions in the absence of drug. All the macrolides were effective inhibitors of GFP synthesis, with IC₅₀ values of 0.36 ± 0.02 μM for tilmicosin, 0.26 ± 0.05 μM for tulathromycin, 0.31 ± 0.05 μM for tylosin, and 0.23 ± 0.01 μM for tildipirosin.

The IC₅₀ values largely match previous estimates for tilmicosin (0.39 ± 0.04 μM) (7), tulathromycin (0.37 ± 0.02 μM) (7), and tylosin (0.25 μM) (23); tildipirosin had not been studied previously. Measurement of radioisotope incorporation, as opposed to GFP fluorescence (23), enabled quantification of aborted and extended products formed by translational drop-off and stop codon read-through, respectively. None of the drugs tested here aborted peptide chains longer than 10 residues, consistent with observations for other macrolides blocking synthesis after addition of 2 to

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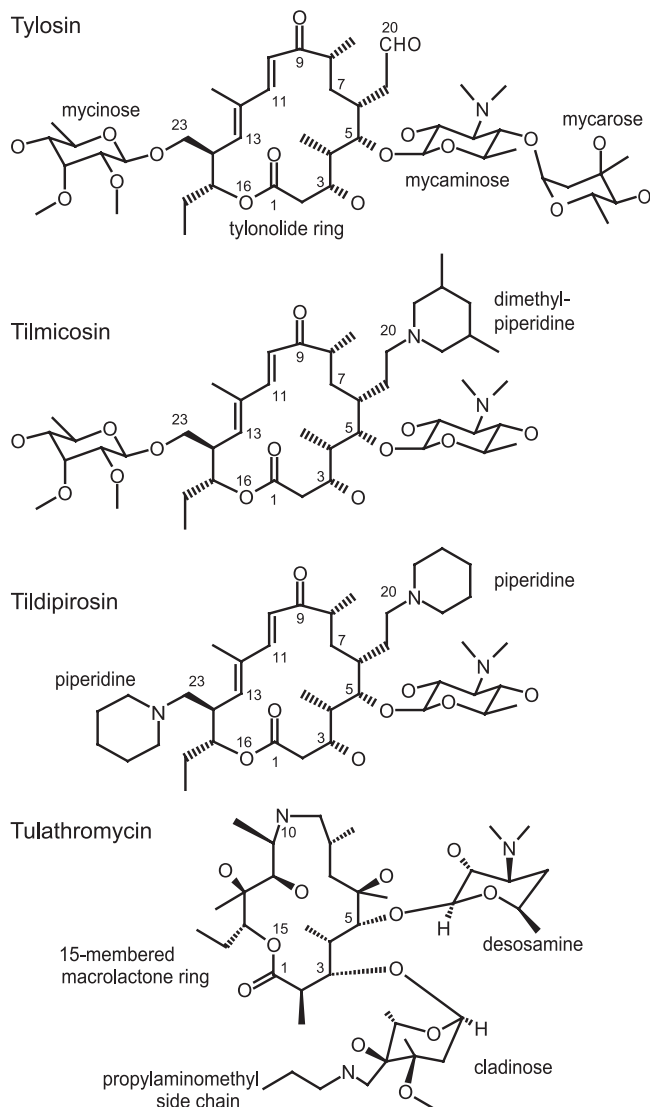


FIG 1 Chemical structures of the macrolides used in the study. Tilmicosin and tildipirosin are derivatives of tylosin and have retained the 16-membered macrolactone (tylonolide) ring and the 5-mycaminose amino sugar. The 16-membered macrolides are distinguished by the mycarose sugar in tylosin, the 20-dimethylpiperidine in tilmicosin, and the 20- and 23-piperidines in tildipirosin. The 15-membered triamilide tulathromycin is chemically closely related to azithromycin (1).

8 residues (13, 24). None of the drugs detectably enhanced UAA stop codon read-through.

The main inhibitory contacts of macrolide antibiotics are within the ribosomal tunnel at 23S rRNA nucleotide A2058 (5, 9, 20), and tylosin-like compounds make additional interactions around nucleotide G748 (9, 12). Mutations and methylations known to reduce macrolide binding were introduced at these and neighboring nucleotides in *E. coli* AS19rlmA^I (11). Strain AS19rlmA^I allows greater intracellular accumulation of drugs, resulting in macrolide MICs that are 16- to 500-fold lower than those for wild-type *E. coli* (Table 1).

MICs were measured by diluting overnight cultures of cells 10⁵-fold and added to microtiter wells containing 2-fold dilution steps of the macrolides between 0.5 μg/ml and 2,048 μg/ml. Mutation or methylation of 23S rRNA nucleotide G745, G748, or A752 caused modest increases in the MICs in a manner that is typical of macrolide drugs (2, 15, 26). More severe increases in the MICs were brought about by substitution or methylation at nucleotide A2058 on the other side of the macrolide site (Table 2). We note that such rRNA mutations were introduced primarily to study drug interaction and are unlikely to arise in the field in bacteria with multiple *rrn* operons (25). However, methylation at nucleotide A2058 has been seen to confer macrolide resistance in *P. multocida* and *M. haemolytica* isolates and is added by the *Pasteurellaceae*-specific monomethyltransferase Erm(42) (3, 10, 14, 19). A2058 monomethylation causes a similar resistance profile in *E. coli*, while dimethylation at A2058 confers high resistance to all macrolides, including tylosin (Table 2). No Erm dimethyltransferase has so far been observed in *P. multocida* or *M. haemolytica*.

Although, on their own, monomethylation of G748 and that of A2058 have only a minor effect on tylosin binding, in combination they function synergistically to confer resistance (12). A similar synergistic effect is found here for tilmicosin and tildipirosin but not for tulathromycin. This difference is explained by the binding models (Fig. 2), where all the drugs are seen to make their primary ribosome interaction at nucleotide A2058, but in contrast to tilmicosin and tildipirosin, tulathromycin is too small to span the ribosomal tunnel to contact G748.

These models, while explaining much of the genetic data, nevertheless represent static pictures of ribosome-drug interaction and leave a number of questions unanswered. For instance, despite the lack of any obvious contact, individual mutations and methylation around G748 have small but significant effects on

TABLE 2 MICs for recombinant *E. coli* AS19rlmA^I with rRNA methylation or mutation at the macrolide site^a

Macrolide	MIC (μg/ml) for <i>E. coli</i> AS19rlmA ^I recombinant strain with:												
	No change in 23S rRNA ^b	Methylations in 23S rRNA ^c						Mutations in 23S rRNA ^d					
		m ¹ G745	m ¹ G748	N ⁶ -A2058	m ¹ G745 N ⁶ -A2058	m ¹ G748 N ⁶ -A2058	N ⁶ , N ⁶ -A2058	A752U	Δ752	G745U	G748U	A2058G	A2058G Δ752
Tylosin	1	4	2	8	32	256	2,048	1	2	2	2	128	128
Tilmicosin	2	8	8	32	128	256	512	2	8	4	4	256	256
Tildipirosin	0.5	4	4	128	1,024	2,048	2,048	0.5	1	4	0.5	1,024	512
Tulathromycin	1	8	4	16	32	16	2,048	1	2	4	1	1,024	1,024

^a AS19rlmA^I was derived from the hyperpermeable strain AS19 (21) and lacks RlmA^I methylation at 23S rRNA nucleotide G745 (11). AS19rlmA^I was transformed with plasmids encoding either methyltransferases specific for 23S rRNA (11) or the *rrmB* operon with mutant versions of 23S rRNA (15). Cells were incubated at 37°C in triplicate, and MICs were scored after 20 h as the lowest concentrations at which no growth was observed.

^b Cells containing an empty plasmid.

^c Monomethylation at A2058 was added by the Erm(N) enzyme and conferred drug phenotypes identical to those of the *Pasteurellaceae* monomethyltransferase Erm(42) (3).

^d The wild-type bases are shown before the nucleotide numbers and the substituted bases after; nucleotide 752 is deleted in the Δ752 strain; the A2058G/Δ752 strain contains both these mutations.

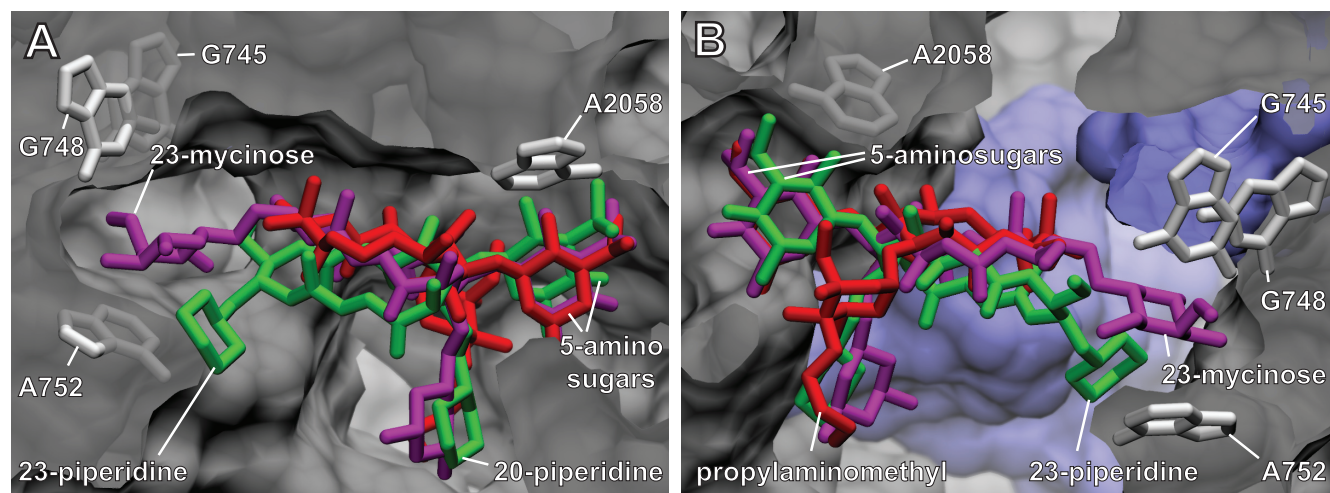


FIG 2 The macrolide site in the ribosome tunnel. (A and B) Two views of superimposed binding sites for tildipirosin (green), tilmicosin (magenta), and tulathromycin (red). Parameters for calculating tildipirosin and tilmicosin (and tylosin) binding using Schrödinger 2010(U1) Suite software were described recently (18). The model presented here for tulathromycin on the *E. coli* ribosome was calculated in a similar manner based on the *Thermus thermophilus* ribosome-azithromycin cocrystal structure PDB 3O11 (1) aligned against the *E. coli* 50S structure PDB 3OAT (5). Clear overlap in the positions of the 5-amino sugars mycaminose (tildipirosin and tilmicosin) and desosamine (tulathromycin) is evident. The 20-piperidine (tildipirosin), the 20-dimethylpiperidine (tilmicosin), and the 3-cladinose propylaminomethyl side chain (tulathromycin) reach into the tunnel lumen with slightly different orientations. More distinct differences are seen in the locations of the 23-piperidine (tildipirosin) and the 23-mycinoses (tilmicosin), while tulathromycin has no equivalent structure. Ribosomal nucleotides interacting with the drug substituents are indicated as gray sticks; ribosomal proteins are shown in blue.

tulathromycin binding (Table 2). This could reflect the manner in which the drug is accommodated into its binding site. The similarly structured azithromycin binds in a two-step process (17) that could thus involve transient interactions with nucleotides other than those contacted in the crystal structure (1). Tylosin also binds to its ribosomal site in a two-step process (16), and the methylations at G748 and A2058 probably interfere with this process prior to impeding the final orientation of the drug in its binding site (9). In the case of tildipirosin, a 23-piperidine replaces the 23-mycinoses of tylosin and tilmicosin (Fig. 2), and this part of the drug has been calculated to be slightly further away from G748 (18). While the IC₅₀ and growth studies indicate that tildipirosin binds tightly to an unmodified ribosomal target site, the data (Table 2) also suggest that modification of nucleotides within or adjacent to this site might interfere with accommodation of the drug into its optimal binding conformation.

We note that the nascent peptide in the ribosome tunnel was by necessity left out of the modeling calculations (18) and remains an important parameter that could contribute to the antimicrobial efficacy of tildipirosin that has been observed in the laboratory and in the field.

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