VOL. 57 NO. 4, APR. 2004 THE JOURNAL OF ANTIBIOTICS pp. 280 — 288

In Vitro Microbiological Characterization of a Novel Azalide, Two Triamilides and an Azalide Ketal against Bovine and Porcine Respiratory Pathogens

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(Received for publication December 11, 2003)

Several novel 15-membered-ring macrolide agents (azalide **1**, triamilides **2** and **3**, and the azalide 3,6-ketal **4**) were identified as potential antibacterial agents against *Mannheimia* (formerly named as *Pasteurella*) *haemolytica*, *Pasteurella multocida*, *Haemophilus somnus* and *Actinobacillus pleuropneumoniae*, important etiological agents of bovine and porcine respiratory disease. Compound **3** is the major component of the antibiotic tulathromycin. Antibacterial activity against tilmicosin-resistant *P. multocida* field isolates was also tested. *In vitro* MIC 50/90 analysis revealed that the four newly synthesized compounds were more potent than tilmicosin against *M. haemolytica* (4∼8×), *P. multocida* (8∼16×), *A. pleuropneumoniae* (4×), *H. somnus* (2× and 16×), and tilmicosin-resistant *P. multocida* (32×). In time-kill kinetic studies, all four novel compounds and tilmicosin showed bactericidal activity against *M. haemolytica*, *P. multocida* and *A. pleuropneumoniae* at both 4× and 8× MIC. A functional assay using genetically defined mutants revealed that all four novel compounds were poorer substrates for the efflux pump, AcrA/B system, than tilmicosin. A pH study using LPS mutants indicated that the enhanced *in vitro* potency of the triamilides, particularly compound **3** was mainly due to better penetration of the molecule through the outer membrane. The third amine group at the C-4″ position of the triamilde molecules contributed to this increased membrane penetration by increasing overall basicity. These studies indicate that the four novel compounds have potential as antibacterial agents against bovine and porcine respiratory disease.

Bovine respiratory disease (BRD, shipping fever) continues to be a primary cause of morbidity and mortality in feedlot production. The cause of BRD is complex and involves the effect of stress and resultant depression of immune system functions, along with viral and bacterial infections. Gram-negative organisms including *Manheimia haemolytica*, *Pasteurella multocida*, and *Haemophilus somnus* are the main bacterial components of the BRD complex. Bacterial swine respiratory disease (SRD) is mainly caused by *P. multocida* and *Actinobacillus pleuropneumoniae*. Control of these Gram-negative pathogens generally resolves the illness.

Tilmicosin, a derivative of desmycosin (16-membered-ring macrolide), introduced into the US market in 1990, is effective against BRD when administered to cattle at 10 mg/kg as a single subcutaneous injection. Injection of tilmicosin into swine, however, is known to be fatal[5]. To identify a safer and more potent single injection agent for livestock than tilmicosin, we synthesized and profiled many novel antibiotics based on the 15-membered-ring macrolide template. From this program, several novel 15-membered-ring macrolides were identified as potential antibacterial agents against BRD and SRD. These compounds are azalide **1**, triamilides **2** and **3**, and azalide 3,6-ketal **4** (structures are shown in Fig. 1). Compound **3** is the major component (component A: USAN[25]) of the antibiotic tulathromycin. In this paper, we report *in vitro* minimum inhibitory concentration (MIC) 50/90 potency using a collection of field isolates, and time-kill kinetic analysis of these four compounds with tilmicosin as a comparator. A

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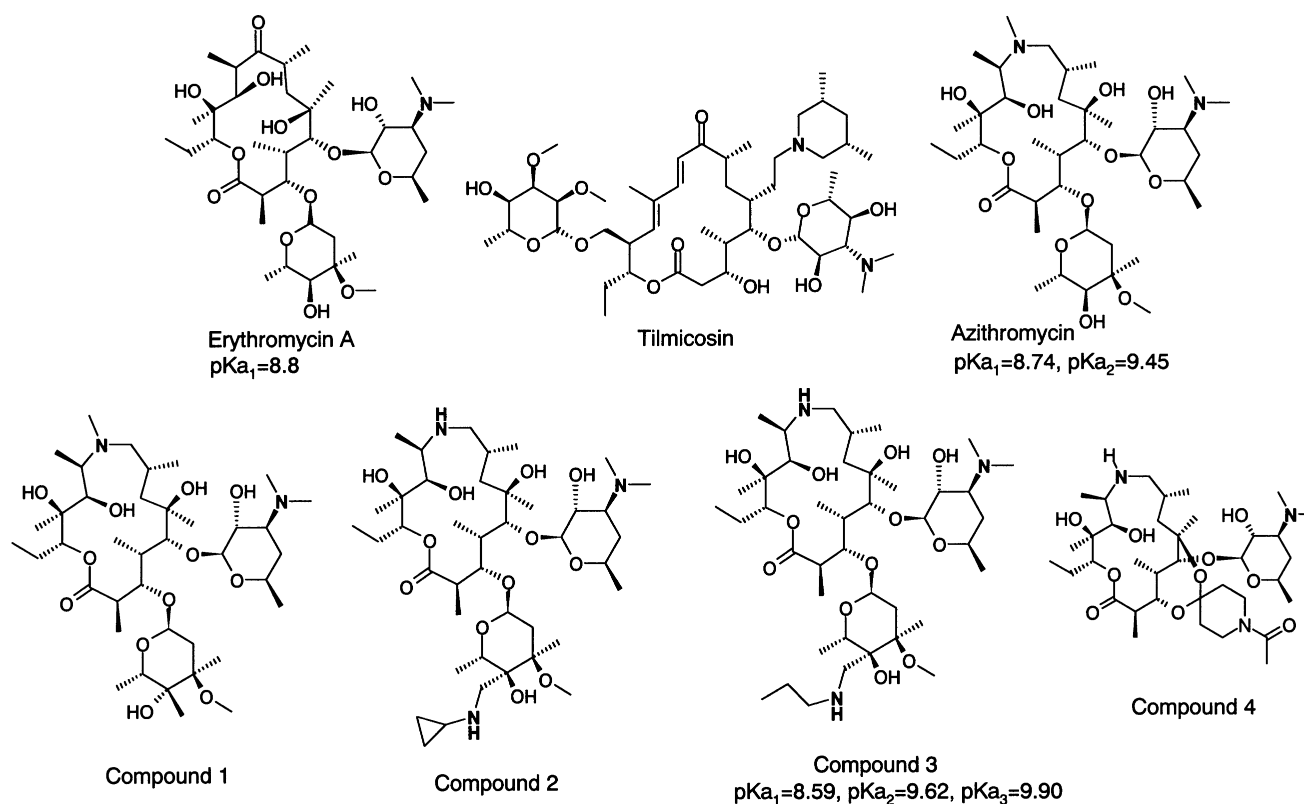
Fig. 1. Chemical structures of erythromycin A, tilmicosin, azithromycin, compound **1**, compound **2**, compound **3**, and compound **4**.



cell-free protein synthesis inhibition assay of erythromycin A, azithromycin, tilmicosin and compound **3** was carried out using purified ribosomes for the measurement of intrinsic activities. We also analyzed the effect of pH and lipopolysaccharide (LPS) mutations on *in vitro* activity of compound **3** (triamilide) compared with the control agents, erythromycin A and azithromycin.

Materials and Methods

Bacterial Strains

The bacterial strains used for MIC 50/90 analysis were collected from various veterinary diagnostic laboratories throughout the United States between 1992 and 1996.

The following organisms were used in the antibacterial assays and LPS mutation/pH assays: *Actinobacillus pleuropneumoniae* ATCC27088, *Escherichia coli* ATCC25922, *E. coli* W4680 (F, *ΔlacZ39*, *rpsL45*, *rpsL110*, *melB4*, from J. E. HEARST, UC Berkeley[13)]), *E. coli* WZM120 (same as W4680; *ΔacrAB*::Tn903Kan^r from J. E. HEARST, UC Berkeley[13)]), *H. somnus* ATCC43625, *M. haemolytica* ATCC14003, *P. multocida* ATCC15743, *Salmonella choleraesuis* ATCC19430, *S. typhimurium* ATCC13311, *S. typhimurium* SA 1355 (rfa^+ LPS phenotype smooth wild-type)[22)], *S. typhimurium* SL3749 (*rfaL446* LPS phenotype Ra)[22)], and *S. typhimurium* SL3789 (*rfaF511* LPS phenotype Rd_2)[22)].

The following strains were used for *in vitro* bactericidal kinetic studies[19)]: *M. haemolytica* 59B0046 (bovine lung origin), *P. multocida* 59A0067 (turkey origin), and *A. pleuropneumoniae* 44A0030 (swine lung origin).

Media and Antibiotics

Media used for these studies included Mueller-Hinton (MH) broth, MH agar (Difco), Brain Heart Infusion (BHI) broth (Difco), and BHI agar (Remel). For *A. pleuropneumoniae* strains, Haemophilus test agar (HTM), chocolate agar (Remel), and MH broth and agar (Difco and Remel) supplemented with β-NAD (15 μg/ml, Sigma) were used.

Cation adjusted Mueller-Hinton broth (CAMH, pH 7.3,

Ca^{2+} 25 mg/liter, Mg^{2+} 12.5 mg/liter) was used for MIC determinations under NCCLS guidelines[15]. For the LPS mutation/pH analysis, the pH of the CAMH was adjusted to 6.0, 7.0, and 8.0 by adding HCl or NaOH.

Antibiotics used in this study were erythromycin A, tilmicosin, azithromycin, azalide **1**, triamilides **2** and **3**, azalide 3,6-ketal **4**, and novobiocin. All antibiotics, except erythromycin A, were supplied from Pfizer's in-house collection. Erythromycin A was purchased from Sigma. The chemical structures of these macrolides are presented in Fig. 1. The synthetic methods for all four novel Pfizer compounds have been described[2,10,12].

In Vitro MIC Analysis

MICs (Minimum Inhibitory Concentrations) were determined using a broth microdilution method recommended by the NCCLS guidelines[15] and the detailed method has been described previously[8,18]. All susceptibility studies except LPS mutant/pH analyses were carried out in duplicate. LPS mutant/pH analyses were carried out in quadruplicate.

In Vitro MS2 Directed Polypeptide Synthesis Inhibition Assay

A cell-free translation system using S-150 derived from *E. coli* BL21 was previously described[4]. The macrolide resistant ribosomes were prepared from *E. coli* BL21 cells grown in the presence of azithromycin (for methylation of ribosomes) that harbors plasmid pVA838 encoding constitutive MSL_B gene. The experiment was carried out in duplicate and an average IC_{50} is reported.

Bactericidal Kinetics

Time-kill kinetic analyses were carried out by the method previously described[11,19]. Colony counts were performed with plates yielding 30 to 300 colonies. The lower limit of sensitivity of colony counts was 300 CFU/ml[20].

Results

Antibacterial Activities

The four novel compounds, azalide **1**, triamilides **2** and **3**, and azalide 3,6-ketal **4** were analyzed for their antibacterial activity against a collection of Gram-negative animal respiratory pathogens which included reference ATCC strains and a panel of field isolates (see Table 1-A). All four compounds showed improved *in vitro* antibacterial activity against these pathogens compared to tilmicosin.

The MIC 50/90 values for compounds **1**, **2**, **3**, and **4** against major livestock respiratory pathogens, *M. haemolytica* (bovine), *P. multocida* (bovine and swine), *A. pleuropneumoniae* (swine), and *H. somnus* (bovine), suggested that they were four or more times more potent than tilmicosin (Table 1-A). In particular, all four novel compounds showed good activity against tilmicosin-resistant *P. multocida* field isolates (the breakpoint of these strains against tilmicosin is 32 μg/ml according to NCCLS guidelines[16]). There was no noticeable difference among MIC 50/90s of all four compounds against *M. haemolytica*, *P. multocida*, and *A. pleuropneumoniae*. However, azalide **1** (0.5/0.5 μg/ml) showed greater potency against *H. somnus* than compound **2** (1.0/4.0 μg/ml), compound **3** (2.0/4.0 μg/ml), and compound **4** (1.0/4.0 μg/ml).

Time-kill Kinetic Studies

Time-kill kinetic analyses of the four novel compounds and tilmicosin at 4× MIC and 8× MIC levels were carried out against three respiratory pathogens *M. haemolytica*, *P. multocida*, and *A. pleuropneumoniae*. The 4× and 8× results were identical and therefore the 4× results only are presented in Fig. 2. All five compounds showed bactericidal activity against *A. pleuropneumoniae*, *M. haemolytica*, and *P. multocida* after 24-hour incubation. All five compounds effectively killed *M. haemolytica* during an initial 3-hour incubation and there was no re-growth during the following 21-hour incubation in the presence of these antibiotics. The kill-kinetics of compounds **1**, **2**, and **3** for *A. pleuropneumoniae* were identical to those for *M. haemolytica*. The kill-kinetics of tilmicosin and compound **4**, however, were slightly different. Both compounds needed an additional 3 hours (total drug exposure=6 hours) to kill *A. pleuropneumoniae* under the assay detection limits. In contrast, *P. multocida* was gradually killed during the 24-hour drug exposure. At early time points (3 and 6 hours), bacterial cell numbers were reduced only one to one and a half $\log_{10}$. Among the compounds tested, compound **3** showed better killing kinetics at early time points. All compounds showed bactericidal activity after 24-hour incubation.

Efflux and LPS Analyses

E. coli WZM120 and W4680 are an isogenic mutant/parent pair[13]. The mutant strain contains a deletion of the efflux pump, AcrA/B. Due to the deletion mutation, substrates of this pump, which include antibiotics like novobiocin and macrolides show significantly improved

Table 1. Antibacterial susceptibilities of compounds **1**, **2**, **3**, **4**, and tilmicosin.

Bacteria species	Strain/ Comments	Compound 1 (μg/ml)	Compound 2 (μg/ml)	Compound 3 (μg/ml)	Compound 4 (μg/ml)	Tilmicosin (μg/ml)	Novobiocin ** (μg/ml)
Table-1-A. Antibacterial susceptibilities against reference and field isolates (MIC 50/90s) of live stock respiratory pathogens							
A. pleuropneumoniae	ATCC27088	1.0/2.0	1.0	1.0	2.0	4.0	ND
	Field isolates (N=24) MIC50/90	0.5/1.0	0.5/1.0	0.5/1.0	1.0/1.0	4.0/4.0	ND
H. somnus	ATCC43625	0.25/0.5	1.0	1.0	2.0/4.0	4.0	ND
	Field isolates (N=24) MIC 50/90	0.5/0.5	1.0/4.0	2.0/4.0	1.0/4.0	4.0/16	ND
M. haemolytica	ATCC14003	0.5	1.0	0.5	2.0	2.0/4.0	ND
	Field isolates (N=32) MIC50/90	0.25/0.5	0.5/0.5	0.5/1.0	0.5/1.0	4.0/4.0	ND
P. multocida	ATCC15743	0.25	0.5	0.25	0.5	4.0	ND
	Field isolates (N=33) MIC50/90	0.25/0.5	0.25/0.5	0.5/1.0	0.25/0.5	4.0/8.0	ND
Tilmicosin resistant *P. multocida**	Field isolates (N=11) MIC50/90	1.0/1.0	1.0/1.0	0.5/0.5	1.0/1.0	32/32	ND
Table-1-B. Antibacterial activities against genetic mutants and their reference strains.							
E. coli	ATCC25922	4.0	2.0	1.0	4.0	64	ND
	W4680 (acrA/B)	4.0	2.0	1.0	4.0	32	512
	WZM120 (ΔacrA/B)	2.0	1.0	0.25/0.5	0.5	2.0	2.0
S. choleraesuis	ATCC19430	2.0	1.0	0.5	4.0	128	ND
S. typhimurium	ATCC 13311	2.0	1.0/2.0	0.5/1.0	8.0	64/128	ND
	SL3789 RD^2- LPS deep rough mutant	0.25	0.25	0.25	1.0	8.0	ND

* According to the NCCLS, breakpoint (resistance) of tilmicosin against *P. multocida* is defined as ≥ 32 μg/ml [16]. These field isolates are considered as resistant against tilmicosin. ** Novobiocin was used as a control for only isogenic pair of parent/mutant strains for the efflux study.

activity against the deletion mutant compared to its parent strain[13]. This causes a large difference in the MICs between mutant and parent strains. In this study, novobiocin was used as a control, and showed 256-fold difference in its antibacterial activity (512 μg/ml *vs.* 2.0 μg/ml. Table 1-B). Tilmicosin showed a 16-fold difference (32 μg/ml *vs.* 2.0 μg/ml). Compounds **1**, **2**, and **3** showed only 2-fold differences, and compound **4** showed an 8-fold difference (Table 1-B). These results suggest that compounds **1**, **2**, and **3** have lower affinity for this efflux pump than tilmicosin and novobiocin.

S. typhimurium SL 3789 is an LPS deep rough mutant. Historically the large molecular weight (larger than porins) and/or hydrophobic antibiotics that directly penetrate the outer membrane of Gram-negative bacteria show improved activity against this strain over the wild-type *S. typhimurium* and *S. choleraesuis* strains[18,19]. Hydrophilic and small molecular size antibiotics, such as penicillin, that can enter the cell through porins, do not show any improved activity against this mutant strain. Most compounds tested here showed MICs that were 8 times lower than MICs against the wild-type *S. typhimurium* and *S. choleraesuis* strains, except compound **3**, which showed only 2- to 4-fold improvement (Table 1-B).

Cell-free Transcriptional/Translational Analyses

Triamilide **3** (a 15-membered tri-basic macrolide), was analyzed by *in vitro* transcription/translation assays to measure intrinsic potency. Erythromycin A (a 14-membered mono-basic macrolide), azithromycin (a 15-membered di-basic macrolide), and tilmicosin (a 16-membered di-basic macrolide) were used as controls. Compound **3** and the two controls (erythromycin A and azithromycin) share a very similar macrolide lactone-ring template: both the azalide and triamilide are derived from erythromycin A by a insertion of an amine to position 9a in the lactone-ring. In contrast, the 16-membered-ring macrolide tilmicosin is structurally distinct from erythromycin A and azithromycin (Fig. 1), and this structural difference affects certain biological activities such as inducible MLS_B type resistance[9].

The results of the cell-free transcription/translation assay with macrolide-sensitive and macrolide-resistant ribosomes are presented in Table 2. All four macrolides (erythromycin A, azithromycin, compound **3**, and tilmicosin) showed

Fig. 2. Time-kill kinetic study of compound **1**, compound **2**, compound **3**, compound **4**, and tilmicosin.

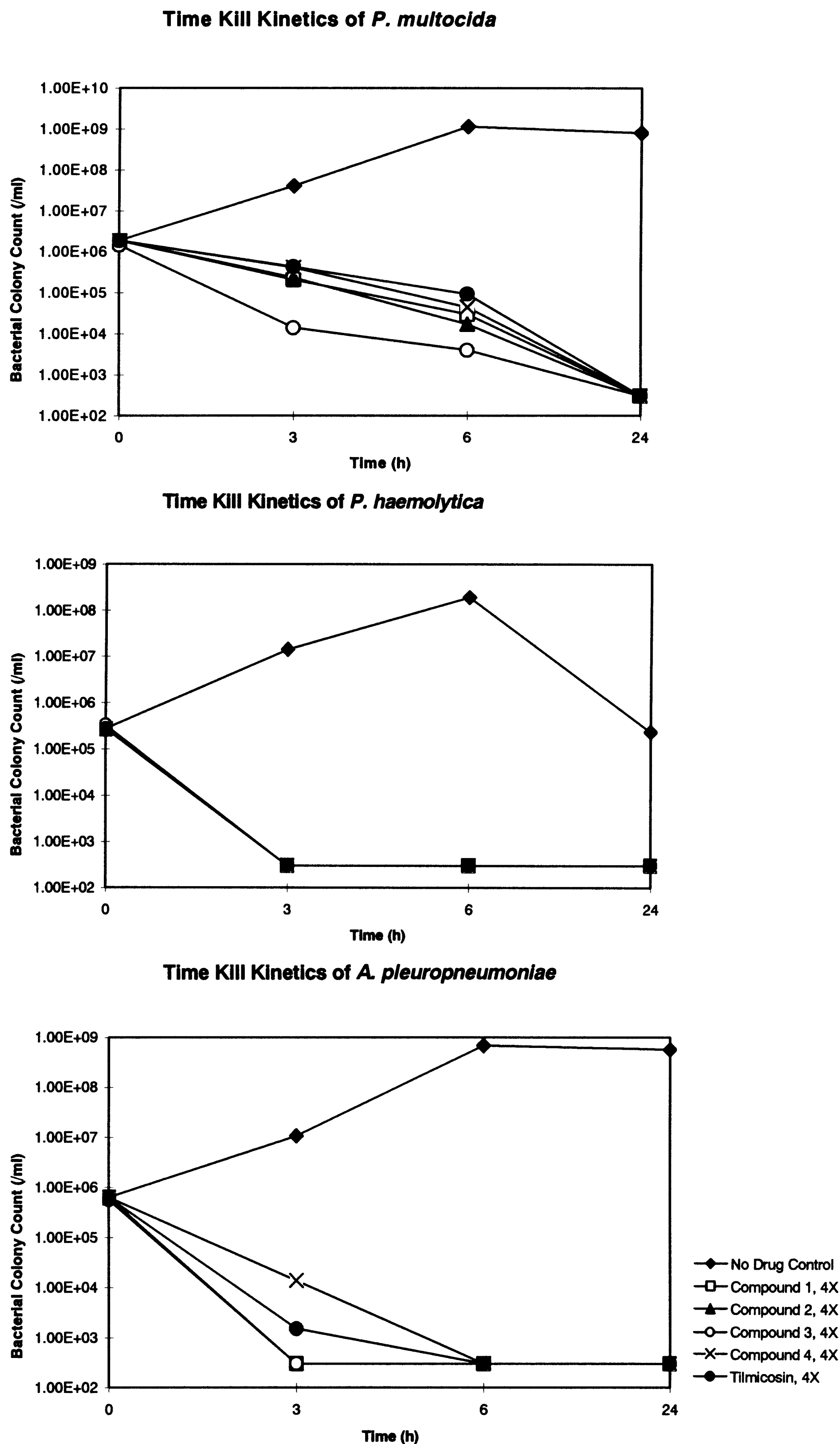


Table 2. Transcription/translation assay.

Antibiotics	Cell free transcription / translation inhibition assay	
	IC_{50} Macrolide-sensitive	IC_{50} Macrolide-resistant
Erythromycin A	0.43 µM	>150 µM
Azithromycin	0.10 µM	>150 µM
Compound 3	0.24 µM	>150 µM
Tilmicosin	0.39 µM	>150 µM

similar cell-free transcription/translation inhibition activities (0.1 to 0.4 µM) against macrolide-sensitive ribosomes but all were inactive against macrolide-resistant ribosomes (Table 2).

Outer Membrane Penetration of Macrolides

The *in vitro* potency of the tri-basic triamilide, compound **3** was further analyzed by pH assays using genetically defined LPS mutants to assess its bacterial outer membrane penetration ability, compared to the control agents, erythromycin A (mono-basic) and azithromycin (di-basic). In these experiments, pH change (acidic, neutral, and alkaline) was used as a tool to control ionized and neutral moieties of test molecules based on each compound's p*K*a. The p*K*a values of erythromycin A, azithromycin, and compound **3** are shown in Fig. 1. These values are already published[14] or obtained from a contract research laboratory[24]. The MIC results are presented in Table 3. A simplified LPS structure, the LPS mutation phenotype, and the corresponding mutant strain numbers are presented in Fig. 3[3].

The activities of the test macrolides were affected by the pH of the test medium; their potencies were significantly improved in alkaline conditions. With pH changes from acidic (pH 6.0) to alkaline (pH 8.0) conditions, the potency (MIC) of erythromycin A against the wild-type smooth LPS strain (SA 1355) increased 43-fold (MIC decreased from 2048 µg/ml to 48 µg/ml). Azithromycin potency improved 341-fold (MIC decreased from 512 µg/ml to 1.5 µg/ml), while compound **3** showed a 4000-fold increase in potency (MIC decreased from 512 µg/ml to 0.125 µg/ml). A similar pattern of increased activity at alkaline pH was observed in Ra and Rd_2 LPS mutants (Table 3). LPS structural changes due to mutation also affected *in vitro* potency. The susceptibility patterns of the Ra mutant to erythromycin A, azithromycin, and compound **3** were identical to those of the smooth wild-type strain. All three compounds, however, showed improved potency against the deep-rough mutant, Rd_2. At acidic conditions (pH 6.0), there was a minor improvement (2- to 3-fold) in the potency of azithromycin and compound **3**. In contrast, erythromycin A was 8 times more potent against the deep-rough mutant than the smooth wild-type and Ra mutant strains. At alkaline conditions (pH 8.0), both erythromycin A and azithromycin were 24 times more potent against Rd_2 compared to the smooth wild-type strain. Compound **3**, however, showed only a 4.5-fold improved potency against the Rd_2 strain compared to the wild-type smooth strain.

Discussion

The antibacterial activities against ATCC strains and MIC 50/90 analysis with field isolates indicate that the novel compounds azalide **1**, triamilides **2** and **3**, and azalide 3,6-ketal **4** are generally more potent against Gram-negative respiratory pathogens than tilmicosin, a drug frequently used in the treatment of BRD. The MIC 50/90s of all four novel antibiotics against major target pathogens of BRD (*M. haemolytica* and *P. multocida*) and SRD (*P. multocida* and *A. pleuropneumoniae*) were 4 to 16 times lower than those of tilmicosin (Table 1-A). All four novel compounds also showed good *in vitro* activity against tilmicosin-resistant *P. multocida* field isolates. Kill-kinetic studies indicated that all four novel compounds were equivalent to (compound **4**) or better than the comparator agent, tilmicosin, against *M. haemolytica*, *P. multocida*, and *A. pleuropneumoniae*.

In addition, the newly synthesized macrolides were poor substrates of the efflux pump AcrA/B (particularly azalide **1** and triamilides **2** and **3**). Many antibiotics, including macrolides (erythromycin A) and novobiocin, are known to be effectively pumped out of *E. coli* cells by this efflux system[4,13], which contributes to significantly higher MICs. Recently, the existence of AcrA/B in *S. typhimurium*[17,21]

Table 3. Effect of pH and LPS mutations on *in vitro* activity of erythromycin A (mono-basic 14-membered ring), azithromycin (di-basic 15-membered-ring) and triamilide, compound **3** (tri-basic 15-membered-ring).

Class	**Erythronolide** 14-membered ring (mono-basic)	**Azalide** 15-membered ring (di-basic)	**Triamilide** 15-membered ring (tri-basic)
S. typhimurium **mutant strain /pH**	Erythromycin A Mean MIC (μg/ml)**	Azithromycin Mean MIC(μg/ml)**	Compound 3 Mean MIC (μg/ml)**
SA 1355 (WT) Smooth LPS			
pH 6.0	2048	512	512
pH 7.0	224	12	6.0
pH 7.3	128	4.0	1.0
pH 8.0	48	1.5	0.125
SL3749 (mutant) Ra LPS			
pH 6.0	2048	512	512
pH 7.0	256	16	10
pH 7.3	128	4.0	1.0
pH 8.0	32	1.5	0.375
SL3789 (mutant) Rd_2 LPS			
pH 6.0	256	160	256
pH 7.0	16	1.0	4.0
pH 7.3	8.0	0.125	0.25
pH 8.0	2.0	0.0625	0.0274

**: The mean MIC values were obtained from quadruplicate MICs.

Fig. 3. Main features of *Salmonella* LPS structure, LPS phenotype and corresponding mutant strain number.

Abe ↓ **GlcNAc** ↓ **Hep** ↓ **P-P-EtN** ↓

[Man → Rha → Gal]n → Glc → Gal → Glc → Hep, P → Hep → [KDO → LipdA etc.]

LPS Phenotype: strain #

S (WT): SA 1355

Ra: SL 3749

Rd_2: SL 3789

* ***Salmonella*** **LPS structure and phenotype scheme was obtained from the literature [3].**

and its homologue in *Haemophilus influenzae*[23] was reported. These results indicate a wide distribution of the AcrA/B efflux pump system among Gram-negative bacteria.

In the present study, there was a 256-fold differential between the parent and *ΔacrA/B* mutant strains with novobiocin (Table 1-B). In contrast, tilmicosin showed only a 16-fold differential. This result indicates that tilmicosin is also a relatively poor substrate of this system. The azalide and triamilides, however, showed much lower affinity to the AcrA/B efflux pump system (2-fold differential) compared to tilmicosin. The significantly reduced efflux pump affinity, improved potency, and excellent kill-kinetics, suggest that the newly synthesized macrolides may have a significant advantages in treating livestock respiratory disease.

The results obtained here with the LPS mutant *Salmonella* strains raised interesting questions concerning

the impact and significance of the tribasic triamilide structure upon antibacterial potency.

It is well known that hydrophilic antibiotics below a certain size limit can cross the outer membrane of Gram-negative bacteria through the water-filled channels of porins[7]. Due to this size exclusion mechanism of the porins, macrolide antibiotics are generally inactive against Gram-negative bacteria, particularly *E. coli* and *Salmonella*, which have strong and rigid outer membranes. However, HANCOCK *et al.* suggested that the improved anti-*E. coli*/*Salmonella* activity of azithromycin originated from the two positively charged sites around the lactone-ring[6]. Both basic sites of azithromycin can interact with the LPS's negatively charged heptose-phosphate region that is stabilized by magnesium cations. This Mg^{2+} stabilized region gives extra strength to LPS as a permeability barrier. The disturbance of this region (*e.g.*, chelating Mg^{2+} by EDT, and/or replacing Mg^{2+} with polycationic compounds like polymixin B or azithromycin's two positively charged sites) causes improved outer membrane permeation of moderately hydrophobic bulky molecules like macrolide antibiotics, which then show improved potencies[6]. The LPS deep rough mutant strain (Rd_2) does not have this Mg^{2+} stabilized region, but the wild-type strain and rough mutant (Ra) does.

The MICs of the three macrolides tested against the wild-type smooth LPS strain and the Ra rough mutant strain were identical and improved significantly when the pH changed from neutral to alkaline conditions. In the same pH range, Rd_2 deep rough mutant's MICs were significantly lower than those of the wild-type and Ra strains.

The *in vitro* potencies of azithromycin and the triamilide compound **3** were not improved against Rd_2 deep rough mutant strain under acidic conditions (Table 3, pH 6.0). The basic amine groups of erythromycin A, azithromycin, and triamilide compound **3** are completely ionized at acidic conditions and are positively charged. In contrast, at neutral and alkaline pH, these amines are partially ionized and are less positively charged. The degree of neutralization depends on their dissociation constants (pKa). The fully positively-charged-amines in acidic conditions should give all three molecules an advantage as LPS disturbing agents over those partially charged amines under neutral and alkaline conditions. However, all three compounds were inactive at pH 6.0. This discrepancy may be explained by considering lipid solubility. It is well described in the literature[1] that "the un-ionized form is usually more lipid-soluble and can more easily diffuse across the cell membrane. In contrast, the ionized moiety is often virtually excluded from transmembrane diffusion because of its low lipid solubility". All three macrolides are fully ionized at pH 6.0, which maximizes their function as LPS disturbing agents, but minimizes their ability to pass through the outer and inner membranes due to their lipid insolubility. Therefore, those macrolides cannot reach their molecular target, the ribosomes. In alkaline conditions (pH 8.0), the percentage of neutralized molecules increases and these can reach the target by passing through the outer and inner membranes. However, the molecule's activities as an LPS disturbing agent, as observed in acidic conditions, are diminished. At neutral to physiological pH, the MICs observed are the result of the balance between LPS disturbing activity (positively charged residues) and lipid soluble transmembrane activity (neutral residue). In the deep rough mutant, the LPS permeability barrier is already eliminated and we mainly observe the lipid soluble transmembrane activity (and intrinsic protein synthesis activity). In this pH range, azithromycin is less ionized than compound **3**, which results in azithromycin's superior activity (Table 3).

In contrast to azithromycin and compound **3**, erythromycin A contains only one amine group on desosamine and its pKa_1 is 8.8. Although the majority of this amine is also ionized at pH 8, the single charged amine residue of erythromycin A lacks the effective outer membrane disturbing activity. Such membrane disturbing activity requires at least two positively charged groups around the macrolide lactone-ring. Therefore, erythromycin A is less effective at penetrating the outer membrane than the azalides and the triamilides. Our cell free transcription/translation inhibition assay indicated that the intrinsic activities of all three macrolides as protein synthesis inhibitors were practically identical (particularly between azithromycin and compound **3**). Therefore, the observed improved potency of compound **3** compared to azithromycin against the wild-type strain is probably caused by the different LPS disturbing activity of each molecule. The pKa_3 of the third amine on cladinose (not present in azithromycin) is 9.9. The majority of these amine sites are still positively charged at neutral-physiological pH which can fully interact with Mg^{2+} binding sites of LPS, together with the other positively charged NH group of the lactone-ring (pKa_2, 9.62). This unique nature of the third amine clearly differentiates triamildes from other macrolide antibiotics.

Acknowledgement

The authors wish to acknowledge Dr. ROBERT YANCEY, Dr. BURTON JAYNES (Veterinary Medicne Research &

Development, Pfizer), and Dr. TOM GOOTZ (Pfizer Global Research and Development), for their critical reading and discussion of this manuscript and N. VAMVAKIDES for his technical assistance.

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