In Vitro Microbiological Characterization of Novel Macrolide CP-163,505 for Animal Health Specific Use

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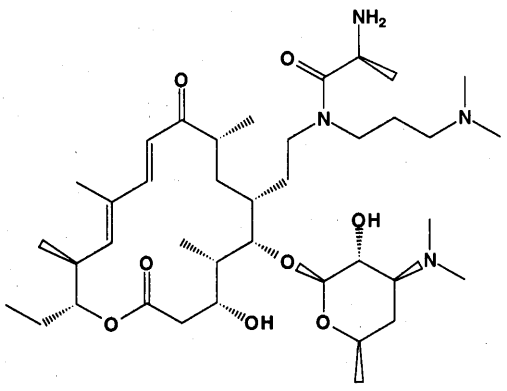
A novel 16-membered-ring macrolide agent (CP-163,505, a reductive amination derivative of repromicin) was identified as an antibacterial against *Pasteurella haemolytica*, *P. multocida* and *Actinobacillus pleuropneumoniae*, important etiological agents of livestock respiratory disease. *In vitro* $MIC_{50/90}$ analysis revealed that CP-163,505 was more potent (4×) than tilmicosin against *P. multocida*, and equivalent to tilmicosin against *P. haemolytica* and *A. pleuropneumoniae*. In time kill kinetic studies, CP-163,505 showed bactericidal activity against *P. haemolytica*, *P. multocida* and *A. pleuropneumoniae* and bacteriostatic activity against *E. coli* at 8 times its MIC. *In vitro*, CP-163,505 was more potent in alkaline pH (16~32×) and less potent in the presence of excess cations (Mg^{+2} and Ca^{+2}, 4×). EDTA and PMBN increased CP-163,505 potency against *E. coli* (4×) but not against the other species. Similar results were obtained with erythromycin A and tilmicosin, which were used as controls. From our data, we hypothesize that *Pasteurella* and *Actinobacillus* have an outer membrane significantly different from that of the typical enteric Gram-negative bacterium *E. coli*.

Bovine respiratory disease (BRD, shipping fever) is a major economic concern for the cattle industry[1]. Economic losses are due to unthrifty weight gains, mortality and cost of treatment. 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 bacteria, *Pasteurella haemolytica* and *P. multocida*, are the main bacterial components of the BRD complex. Bacterial swine respiratory disease is mainly caused by *P. multocida* and *Actinobacillus pleuropneumoniae*, which is also a Gram-negative bacterium. Control of these Gram-negative pathogens normally resolves the illness.

Tilmicosin, a derivative of desmycosin (16-membered-ring macrolide) introduced into the US market in 1990, is effective against BRD at 10 mg/kg as a single subcutaneous injection and has gained wide acceptance. Injection of tilmicosin to swine, however, has been shown to be fatal[2]. Semi-synthetic efforts were initiated to identify a safer and more potent single injection agent than tilmicosin. We initiated our studies with the relatively unexplored 16-membered-repromicin as a template by reductive amination[3~5]. A novel 16-membered-ring macrolide, CP-163,505 (Fig. 1) was identified as an antibacterial agent against BRD complex and swine respiratory disease. In the present paper, we report microbiological characterization of *in vitro* potency of CP-163,505 using tilmicosin and erythromycin A as controls. We also discuss potential structural differences in the outer membranes of *Pasteurella* and

Fig. 1. Structure of CP-163,505.



Actinobacillus compared to *E. coli*, based upon differential antibiotic sensitivity.

Materials and Methods

Bacterial Strains

The following bacterial strains were used in the antibacterial agar dilution assay: *A. pleuropneumoniae* 44A0004, *E. coli* 51A0538 (bovine isolate), *P. haemolytica* 59B0018 (bovine isolate), *P. multocida* 59A0006, *Salmonella choleraesuis* 58B0015 (swine isolate), and *S. typhimurium* LT2 SGSC230 (RD2 LPS deep rough mutant).

The following strains were used for *in vitro* characterization and bactericidal kinetic studies: *P. haemolytica* 59B0046 (bovine lung origin), *P. multocida* 59A0067 (turkey origin), *A. pleuropneumoniae* 44A0030 (swine lung origin) and *E. coli* 51A0150 (poultry lung origin).

Media and Antibiotics

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

The following antibiotics were used in this study: CP-163,505 and tilmicosin were supplied from our in-house compound collection. The synthetic method for CP-163,505 is described in Reference 3. Erythromycin A and fusidic acid were commercially purchased (Sigma).

In Vitro MIC Analysis

MICs (Minimum Inhibitory Concentrations) were determined using broth microdilution methods and agar dilution methods described previously[6].

Field Isolates Analysis ($MIC_{50/90}$)

Bacterial field isolates of *P. haemolytica*, *P. multocida*, *A. pleuropneumoniae*, *E. coli* and *Salmonella* spp. were obtained from veterinary diagnostic laboratories from various locations in the United States from 1988 to 1993. All of the bacteria except *A. pleuropneumoniae* were grown aerobically overnight on BHI agar plates at 37°C. *A. pleuropneumoniae* was grown on HTM agar plate at 37°C in the presence of 5% CO_2. MICs were determined using a broth microdilution method described previously[6].

Bactericidal Kinetics

Time-kill kinetics were determined, referring to published guidelines[7~9]. Erlenmeyer flasks (125 ml) were prepared containing 20 ml broth (supplemented with β-NAD for *A. pleuropneumoniae*) and 4 times and 8 times the MIC for each antibiotic. Bacteria were inoculated into the flasks for a final inoculum of $10^5 \sim 10^6$ cfu/ml. Flasks were incubated at 37°C with shaking. Samples (100 μl) were taken as indicated and serially diluted 1 : 10 in saline. Diluted samples (100 μl) were plated out on BHI or HTM agar plates in duplicate. Plates were incubated 24 hours at 37°C. Colony counts were performed with plates yielding 30 to 300 colonies. The lower limit of sensitivity of colony counts was 300 cfu/ml[10]. If a certain antibiotic showed $3 \log_{10}$ cfu/ml reduction of particular bacteria during 24 hours, it was considered to be bactericidal based on NCCLS guidelines[7].

Analysis for Various Factors Affecting *In Vitro* Potency

To evaluate potency in various types of liquid media and at various incubation points, MH, BHI and LB broths were used with MIC endpoints read at 18, 24, 42 and 48 hours. To determine the effect of pH on antibiotic activity, MH broth was adjusted to pH 6, pH 7 and pH 8 with 0.5 N HCl or 1 N NaOH. The influence of EDTA, a cation chelating agent (Sigma), and polymyxin B nonapeptide (PMBN), an outer membrane perturbing agent (Boehringer Mannheim), on the efficacy of the antibiotics was evaluated by adding various concentrations, independently, into MH broth. Fusidic acid (purchased from Sigma) was used as a positive control in the PMBN assay. The effect of cations (25 mg/liter Mg^{+2} and 50 mg/liter Ca^{+2}) and 40% calf serum (Gibco, heat inactivated 56°C~40 minutes) were examined by adding these components to the MH broth and BHI broth, respectively. The influence of incubation under 5% CO_2 in the presence of 40% heat inactivated calf serum was also evaluated.

In Vitro MS2 Directed Polypeptide Synthesis Inhibition Assay

A cell-free translation system using S-150 derived from *E. coli* BL21 has been described previously[11].

Results

Antibacterial Activity and $MIC_{50/90}$ Analyses

Antibacterial activity with seven Gram-negative aerobic animal pathogens and $MIC_{50/90}$s analyses of

Table 1. Antibacterial spectrum activity and MIC_{50}/MIC_{90} analysis of CP-163,505, erythromycin A and tilmicosin.

Bacteria	MIC (μg/ml)		
	CP-163,505	Erythromycin A	Tilmicosin
A. pleuropneumoniae 44A0004	≤0.39	1.56	1.56
Field isolates n=31			
$MIC_{50/90}$	3.13/6.25	3.13/6.25	6.25/6.25
Range	1.56~12.5	1.56~12.5	3.13~12.5
E. coli 51A0538	25	50	100
Field isolates n=27			
$MIC_{50/90}$	25/50	100/100	100/100
Range	12.5~>100	25~>100	25~>100
P. haemolytica 59B0018	0.78	1.56	1.56
Field isolates n=30			
$MIC_{50/90}$	3.13/3.13	3.13/3.13	3.13/3.13
Range	0.78~6.25	1.56~12.5	0.78~6.25
P. multocida 59A0006	≤0.39	0.78	3.13
Field isolates n=32			
$MIC_{50/90}$	1.56/3.13	3.13/6.25	6.25/12.5
Range	0.78~6.25	1.56~12.5	3.13~25
S. choleraesuis 58B0015	12.5	50	200
S. typhimurium LT2 SGSC230	3.13	3.13	25
Salmonella spp.			
Field isolates n=29			
$MIC_{50/90}$	25/50	100/>100	>100/>100
Range	12.5~>100	50~>100	100~>100

CP-163,505, tilmicosin and erythromycin A against recent field isolates are presented in Table 1. CP-163,505 showed similar antibacterial pattern against these pathogens with improved potency over tilmicosin and erythromycin A by 2-8 fold. The $MIC_{50/90}$ analyses indicated that CP-163,505 was more potent than tilmicosin and erythromycin A against *P. multocida* (4-fold and 2-fold, respectively) and had equivalent potency against *P. haemolytica* and *A. pleuropneumoniae*. CP-163,505 showed improved potency over tilmicosin and erythromycin A against *E. coli* and *Salmonella* spp. although the MICs against these two species are still too high to be considered susceptible to CP-163,505.

Bactericidal Kinetic Analysis

Time-kill kinetic studies of CP-163,505 were carried out at the levels of 4 times and 8 times the MICs using tilmicosin and erythromycin A as controls. The results of 8 times the MICs are presented in Fig. 2 to Fig. 4. All three macrolides were bactericidal against *P. haemolytica* 59B0046 and *A. pleuropneumoniae* 44A0030 at 8 times the MIC. CP-163,505 and tilmicosin were bactericidal for *P. multocida* 59A0067 but erythromycin A was bacteriostatic at 8 times the MIC. All three macrolides were bacteriostatic against *E. coli* 51A0150. CP-163,505 showed better kill kinetics in the early time points compared to tilmicosin and erythromycin A against *A. pleuropneumoniae* 44A0030 at 4 times the MIC (data not shown).

Effect of Incubation Time, Media and Cations

There was no difference in the MICs for CP-163,505, tilmicosin and erythromycin A in MH broth or BHI broth against four bacteria (Table 2). The MICs of all three macrolides in both broths were lower than in LB broth. Addition of cations (Ca^{+2}, 50 mg/liter and Mg^{+2}, 25 mg/liter) to MH broth decreased *in vitro* potency of CP-163,505, tilmicosin and erythromycin A by 4-fold against *E. coli* 51A0150 and *A. pleuropneumoniae* 44A0030. The activity of CP-163,505 against reference strains of *P. haemolytica* 59B0046 and *P. multocida* 59A0067 was also adversely affected by cations. In contrast, the potency of erythromycin A against *P. multocida* 59A0067 was unaffected by cations. The potency of erythromycin A against *P. haemolytica* 59B0046 and tilmicosin against *P. multocida* 59A0067 were reduced only two-fold in the presence of excess cations (Table 2). For each of the three growth media,

Fig. 2. CP163,505 time-kill kinetics.

--■-- *P. haem.* 0×, —■— *P. haem.* 8×, --▲-- *P. mult.* 0×, —▲— *P. mult.* 8×,
--●-- *A. pleur.* 0×, —●— *A. pleur.* 8×, --×-- *E. coli.* 0×, —×— *E. coli* 8×.

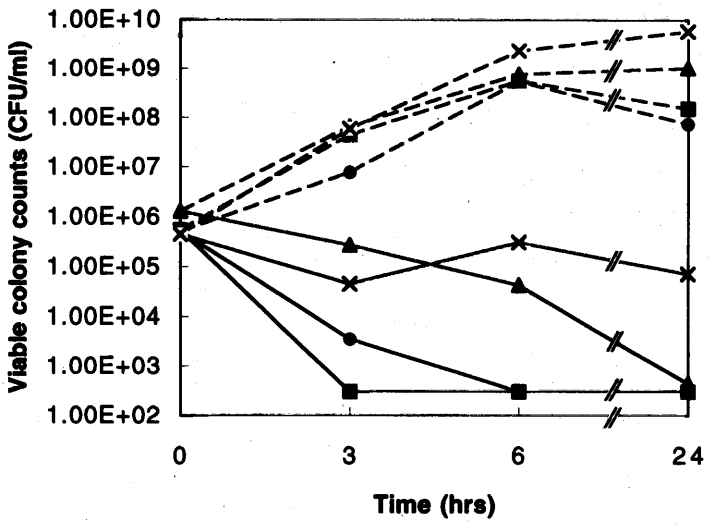
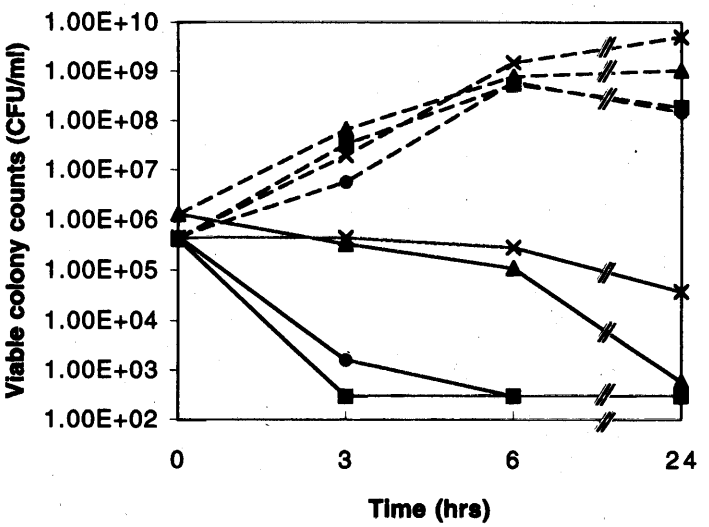


Fig. 3. Tilmicosin time-kill kinetics.

--■-- *P. haem.* 0×, —■— *P. haem.* 8×, --▲-- *P. mult.* 0×, —▲— *P. mult.* 8×,
--●-- *A. pleur.* 0×, —●— *A. pleur.* 8×, --×-- *E. coli.* 0×, —×— *E. coli* 8×.



the *in vitro* potency for the three macrolides was quite consistent at 18, 24, 42 and 48 hours incubation (data not shown).

Effect of Inoculum Sizes

Varying the inoculum size from 10^3 to 10^6 cfu/ml had almost no effect on the *in vitro* potency of CP-163,505, erythromycin A and tilmicosin against the four different bacteria tested in this study except erythromycin A against *P. multocida* 59A0067 and tilmicosin against both *E. coli* 51A0150 and *P. multocida* 59A0067 (data not shown). For these strains, a slight increase of MICs of both antibiotics was observed at 10^6 cfu/ml inoculum size. At an inoculum size of 10^7 cfu/ml, there was a 2~8 fold increase of MICs for three macrolides against *E. coli* 51A0150, *P. haemolytica* 59B0046 and *P. multocida* 59A0067; however, there was no difference against *A. pleuropneumoniae* 44A0030. In conclusion, it ap-

Fig. 4. Erythromycin time-kill kinetics.

--■-- *P. haem.* 0×, —■— *P. haem.* 8×, --▲-- *P. mult.* 0×, —▲— *P. mult.* 8×, --●-- *A. pleur.* 0×, —●— *A. pleur.* 8×, --×-- *E. coli.* 0×, —×— *E. coli* 8×.

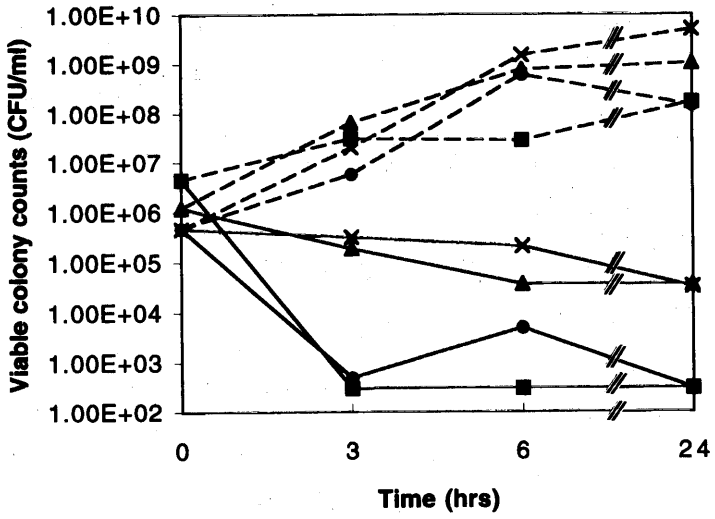


Table 2. Influence of media and cations on the *in vitro* potency of CP-163,505.

Bacteria	Microbiological media	MIC (μg/ml)		
		CP-163,505	Erythromycin A	Tilmicosin
E. coli (51A0150)	MH broth	6.25	25	25
	MH broth $+CA^{+2}$ and Mg^{+2}	25	100	100
	BHI broth	12.5	50	50
	LB broth	100	100	200
P. haemolytica (59B0046)	MH broth	1.56	3.13	1.56
	MH broth $+CA^{+2}$ and Mg^{+2}	6.25	6.25	12.5
	BHI broth	1.56	3.13	1.56
	LB broth	6.25	3.13	6.25
P. multocida (59A0067)	MH broth	0.78	3.13	3.13
	MH broth $+CA^{+2}$ and Mg^{+2}	3.13	3.13	6.25
	BHI broth	0.78	0.78	1.56
	LB broth	6.25	3.13	12.5
A. pleuropneumoniae (44A0030)	MH broth	0.78	1.56	1.56
	MH broth $+CA^{+2}$ and Mg^{+2}	3.13	6.25	6.25
	BHI broth	0.39	0.78	1.56
	LB broth	3.13	3.13	6.25

peared that inoculum variation had little effect on the MICs of these macrolides between 10^3 cfu/ml and 10^5 cfu/ml inoculum size.

Effect of pH

In vitro potency of CP-163,505, erythromycin A and tilmicosin against *E. coli* 51A0150 and *P. multocida* 59A0067 increased dramatically as the pH of the medium increased from pH 6.0 to pH 8.0 (Table 3). This phenomenon has been well documented as a typical characteristic of macrolides[12~14]. A similar potency increase at alkaline pH was observed for *P. haemolytica* 59B0046, however, the growth of this bacteria was significantly suppressed at pH 8.0 and could not grow at all at pH 6.0. *A. pleuropneumoniae* 44A0030 grew at only pH 7.0 and did not grow in either acidic or alkaline environments. These results indicate that both reference strains of *P. haemolytica* and *A. pleuropneumoniae* are extremely

Table 3. Effect of pH on *in vitro* potency of CP-163,505.

Bacteria	pH	MIC (μg/ml)		
		CP-163,505	Erythromycin A	Tilmicosin
E. coil (51A0150)	6	200	200	>200
	7	6.25	25	25
	8	≤0.2	3.13	1.56
P. haemolytica (59B0046)	6	No growth	No growth	No growth
	7	1.56	1.56	1.56
	8[a]	0.05[a]	0.2[a]	0.1[a]
P. multocida (59A0067)	6	6.25	12.5	25
	7	1.56	3.13	3.13
	8	0.1	0.39	0.39
A. pleuropneumoniae (44A0030)	6	No growth	No growth	No growth
	7	0.78	0.78	1.56
	8	No growth	No growth	No growth

[a] *Pasteurella haemolytica* barely grew at pH 8.

Table 4. Effect of EDTA on *in vitro* potency of CP-163,505.

Bacteria	EDTA (mM)	MIC (μg/ml)		
		CP-163,505	Erythromycin A	Tilmicosin
E. coil (51A0150)	0	6.25	25	25
	0.1	3.13	12.5	12.5
	0.5	1.56	3.13	3.13
P. haemolytica (59B0046)	0	0.78	1.56	0.78
	0.1	0.78	1.56	0.39
	0.5	No growth	No growth	No growth
P. multocida (59A0067)	0	0.78	1.56	1.56
	0.1	0.78	1.56	1.56
	0.5	No growth	No growth	No growth
A. pleuropneumoniae (44A0030)	0	0.78	0.78	0.78
	0.1	0.78	0.39	0.78
	0.5	No growth	No growth	No growth

sensitive to environmental pH changes.

Effect of EDTA

The addition of 0.1 mM and 0.5 mM EDTA increased the potency of CP-163,505 as it did with erythromycin A and tilmicosin, when tested against *E. coli* 51A0150 (Table 4). In contrast, there was no significant change in potency for *P. haemolytica* 59B0046, *P. multocida* 59A0067 and *A. pleuropneumoniae* 44A0030 with 0.1 mM EDTA. These three bacteria were sensitive to the higher levels of EDTA (0.5 mM) and did not grow.

Effect of PMBN

The addition of various concentrations of PMBN to the medium increased the potency of the three macrolides tested against *E. coli* 51A0150 (Table 5). However, the change in potency was not dramatic as observed with fusidic acid against *E. coli* 51A0150. PMBN showed antibacterial activity against *P. haemolytica* 59B0046 and *A. pleuropneumoniae* 44A0030 (MIC 3.13 μg/ml and 1.56 μg/ml, respectively). Therefore, the highest concentration of PMBN tested against these bacteria was 1.0 μg/ml. In contrast, *P. multocida* 59A0067 was more resistant to PMBN than *E. coli* 51A0150 (MIC 800 μg/ml and 200 μg/ml, respectively). Nevertheless, the MICs of *P. haemolytica* 59B0046, *P. multocida* 59A0067 and *A. pleuropneumoniae* 44A0030 were not affected by the addition of PMBN at sub-MIC levels.

Effect of Heat Inactivated 40% Bovine Serum

The addition of 40% heat inactivated calf serum to the assay system increased the potency of all three

Table 5. Effect of polymixin B nonapeptide (PMBN) on *in vitro* potency of CP-163,505.

Bacteria	PMBN (μg/ml)	MIC (μg/ml)			
		CP-163,505	Erythromycin A	Tilmicosin	Fusidic acid[a]
E. coil (51A0150)	0	3.13	25	25	>400
	5.0	1.56	0.78	1.56	12.5
	50	0.78	0.39	0.39	1.56
P. haemolytica (59B0046)	0	1.56	1.56	0.78	25
	0.5	1.56	1.56	1.56	6.25
	1.0	1.56	1.56	1.56	1.56
P. multocida (59A0067)	0	0.78	1.56	1.56	12.5
	5.0	0.78	1.56	1.56	25
	50	0.78	1.56	1.56	12.5
A. pleuropneumoniae (44A0030)	0	0.78	0.78	1.56	12.5
	0.5	0.78	0.78	1.56	12.5
	1.0[b]	—[b]	—[b]	—[b]	—[b]

[a] Fusidic acid was used as a positive control. PMBN MICs: *E. coli*=200 μg/ml, *P. haemolytica*=3.13 μg/ml, *P. multocida*=800 μg/ml and *A. pleuropneumoniae*=1.56 μg/ml.

[b] As expected, *A. pleuropneumoniae* growth was significantly reduced and was inconsistent at the concentration of 1.0 μg/ml PMBN.

Table 6. Effect of heat inactivated 40% bovine serum on *in vitro* potency of CP-163,505.

Bacteria	40% heat deactivated serum	MIC (μg/ml)		
		CP-163,505	Erythromycin A	Tilmicosin
E. coil (51A0150)	−	6.25	25	50
	+	0.39	6.25	3.13
	+ and 5% CO_2	6.25	25	25
P. haemolytica (59B0046)	−	1.56	3.13	1.56
	+	0.1	0.78	0.39
	+ and 5% CO_2	0.78	6.25	0.78
P. multocida (59A0067)	−	0.78	0.78	1.56
	+	0.05	0.39	0.39
	+ and 5% CO_2	0.78	0.78	1.56
A. pleuropneumoniae (44A0030)	−	0.39	0.78	0.78
	+	0.2	0.78	0.39
	+ and 5% CO_2	0.78	3.13	1.56

Pre-incubation pH, BHI 7.39 and 40% serum BHI 7.68. Post-incubation (20 hours) pH, BHI 7.88, 40% serum BHI 8.30, BHI under 5% CO_2 7.28, 40% serum BHI under 5% CO_2, 7.45. All pH values are an average of 7 wells, and were measured without bacterial growth.

macrolides when incubated against *E. coli* 51A0150, *P. haemolytica* 59B0046, and *P. multocida* 59A0067 (Table 6). The potency of CP-163,505 and tilmicosin increased slightly against *A. pleuropneumoniae* 44A0030 in the presence of serum while the value of erythromycin A was unchanged. The addition of serum to the growth medium shifted the pH towards alkalinity during 18 hours incubation (Table 7 legend), which is known to enhance macrolide potency[13,14]. However, this pH shift was neutralized by incubating plates under 5% CO_2 and consequently, the serum effect on macrolide *in vitro* potency disappeared.

MS2 Directed Polypeptide Synthesis Inhibition Assay

The translation IC_{50}s of CP-163,505, tilmicosin and erythromycin A with susceptible ribosomes were 4.0 μM, 3.5 μM and 0.16 μM, respectively. All three macrolides were inactive with MLS_B resistant ribosomes, and their IC_{50}s were >150 μM.

Discussion

CP-163,505, a novel 16-membered-ring macrolide was synthesized by the C-20 reductive amination of repromicin with 3'-dimethylamino-propylamine followed by coupling with an alanine residue[3] (Fig. 1). CP-163,505 showed improved *in vitro* potency against several animal pathogenic reference strains compared to tilmicosin, which is a current leading antibiotic in BRD treatment in the US (Table 1). This improved potency was also observed against recent field isolates (Table 1). MIC_{50} and MIC_{90} values of CP-163,505 against *P. multocida* were four times lower than those of tilmicosin. A similar pattern was observed against *E. coli* and *Salmonella* species. CP-163,505 showed equivalent potency against *P. haemolytica* and *A. pleuropneumoniae* compared to tilmicosin (Table 1). Bactericidal kinetic studies revealed that both CP-163,505 and tilmicosin showed an identical profile at the level of 8 times MIC. CP-163,505 showed better killing activity in the early time points against *A. pleuropneumoniae* than tilmicosin at the level of 4 times MIC (data not shown).

16-Membered-ring macrolides such as tilmicosin and CP-163,505 are structurally and biologically distinct from 14- and 15-membered macrolides (for example, erythromycin A and azithromycin, respectively). Erythromycin A and azithromycin are relatively flexible macrolide rings. In contrast, the 16-membered macrolide ring structure is relatively open, flat and rigid. This structural difference affects certain biological activities such as inducible MLS_B type resistance[15].

CP-163,505, tilmicosin and erythromycin A have been extensively analyzed in the present study using four species of animal pathogens to determine whether the structural differences discussed above influence the effects of environmental factors on *in vitro* potencies. The effects of environmental factors (media, pH, cations, serum, CO_2, inoculum size and time of incubation) on *in vitro* potency have been studied on azithromycin with human pathogens[14]. Very limited studies on the effects of pH, serum and inoculum size have previously been published on tilmicosin with animal pathogens[13]. Our profiling revealed that there was no significant difference on *in vitro* characteristics and properties of CP-163,505, tilmicosin and erythromycin A in spite of their structural differences. All three macrolides were more active in an alkaline environment, as expected for these basic compounds. The addition of 40% heat inactivated serum did not affect potency when the pH was held constant. This suggests that there is no highly significant protein binding of these macrolides. These results are consistent with the previous studies of azithromycin[14] and erythromycin A[16].

Erythromycin A and tilmicosin contain a monobasic site on desosamine and mycosamine, respectively. Azithromycin is a dibasic macrolide. CP-163,505 is a tribasic 16-membered-ring macrolide. The introduction of an extra basic site in the macrolide lactone ring of azithromycin significantly improved the activity against Gram-negative bacteria[17]. This improved anti-Gram-negative activity was due to the ability of two basic sites of azithromycin to interact with Mg^{+2} binding sites of lipopolysaccharide (LPS), and the self promoted uptake pathway was proposed[18]. It appears that the greater *in vitro* potency of CP-163,505 over tilmicosin, particularly against *E. coli* and *Salmonella*, may also be due to improved membrane transport, especially through the outer membrane by the existence of the extra basic sites on the C-20 side chain. This is because the IC_{50}s of CP-163,505 and tilmicosin in the cell free polypeptide synthesis inhibition assay were virtually identical (4.0 μM and 3.5 μM, respectively). As stated in the previous section, there is a significant tertiary structural difference between the 16-membered lactone ring and the 15-membered lactone ring. It is interesting to note that the addition of multiple basic sites around the macrolide lactone ring seems to increase the interaction with LPS and to improve the antibacterial activity against Gram-negative bacteria in spite of their structural difference. However, further detailed experimentation is necessary to support this statement.

It is known that the chelating agent, EDTA, eliminates Mg^{+2} and Ca^{+2} from the core region of LPS in the outer membrane of Gram-negative bacteria such as *E. coli* and *Salmonella*[19,20]. This cation elimination disturbs the outer membrane and affects the susceptibility of a wide variety of hydrophobic antibiotics, detergents and dyes[21]. PMBN is another type of outer membrane perturbing agent that increases the susceptibility of Gram-negative bacteria to hydrophobic agents[19,20]. The studies with pH, EDTA, and PMBN revealed that the animal respiratory pathogens, *P. haemolytica*, *P. multocida* and *A. pleuropneumoniae* are significantly more susceptible to these environmental changes than enteric bacteria such as *E. coli*. These results strongly suggest that the outer membrane structure of these respiratory pathogens, especially LPS, is significantly different from that of the typical enterics, which is well characterized. In fact, Conrad et al. reported that the LPS of *P. multocida* P-1581 is homogeneous and

predominantly of low molecular weight (estimated MW 5700 daltons), and there was no smooth-form LPS by overloaded SDS-PAGE analysis[22]. This report strongly suggests that LPS of *P. multocida* is different from enteric LPS. Detailed comparative studies of *P. multocida* as well as *P. haemolytica* and *A. pleuropneumoniae* will be needed.

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