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# *In Vitro* Microbiological Characterization of Novel Cyclic Homopentapeptides, CP-101,680 and CP-163,234, for Animal Health Use

LAURA J. L. NORCIA, ANNETTE M. SILVIA, JOHN P. DIRLAM, RODNEY C. SCHNUR†, JAY M. BERGERON, JAMES A. RETSEMA and SHIGERU F. HAYASHI*

Central Research Division, Pfizer Inc., Groton, CT 06340. U.S.A.

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Two cyclic homopentapeptides, CP-101,680 and CP-163,234 [6a-(3′,4′-dichlorophenylamino) analogs of viomycin and capreomycin, respectively], were identified as novel antibacterial agents for the treatment of animal disease, especially for livestock respiratory disease. The *in vitro* microbiological characterization of both CP-101,680 and CP-163,234 was carried out using their parent compounds, viomycin and capreomycin, as controls. This characterization included antibacterial spectrum, influence of media, inoculum size, pH, EDTA, polymixin B nonapeptide (PMBN), serum, cell-free protein synthesis inhibition, and time-kill kinetics. Our results indicated that the capreomycin analog, CP-163,234, showed slightly improved *in vitro* potency over the viomycin analog, CP-101,680. Both analogs showed very potent cell-free protein synthesis inhibition activity and were bactericidal against *Pasteurella haemolytica*, *P. multocida* and *Actinobacillus pleuropneumoniae* at the level of 4 times and 8 times MICs. CP-163,234 was bactericidal at the level of 4× and 8× MIC against *E. coli*, but re-growth was observed after 24 hours incubation at both concentrations of CP-101,680.

Viomycin (tuberactinomycin B), marketed as a tuberculostatic agent in the 1960's, is a cyclic homopentapeptide containing the unusual amino acids viomycidine, hydroxyl β-lysine, and β-ureidodehydroalanine. A more potent compound, capreomycin, discovered by Lilly[1] in 1959 from fermentation of *Streptomyces capreolus* has a similar structure but is produced as a four-component mixture, with capreomycin IA and IB present as major products, and IIA and IIB as minor products[2]. In recent years, the biosynthesis of capreomycin has been extensively studied by GOULD, *et al.*[3,4]. Viomycin and capreomycin are primarily potent against *Mycobacteria*[5], with little activity against other bacteria. It has been reported that viomycin inhibits protein synthesis primarily by blocking the translocation of peptidyl-transfer RNA from the A to the P site of the bacterial ribosome[6]. A 6a-(3,4-dichlorophenylamino) analog of viomycin (CP-101,680) was uncovered by a high throughput screen against the bovine pathogen *Pasteurella haemolytica*[7]. Subsequently, this structure served as a novel lead compound for both animal and human health infectious disease programs[7,8]. In the present paper, we describe *in vitro* microbiological profiling of both CP-101,680 and its equivalent capreomycin (IA and IB mixture) analog, CP-163,234, that was carried out as part of a program to assess clinical potential as antibacterial agents for livestock.

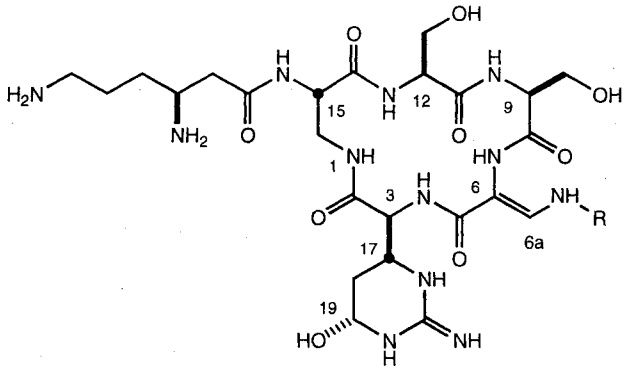
## Materials and Methods

### Bacterial Strains

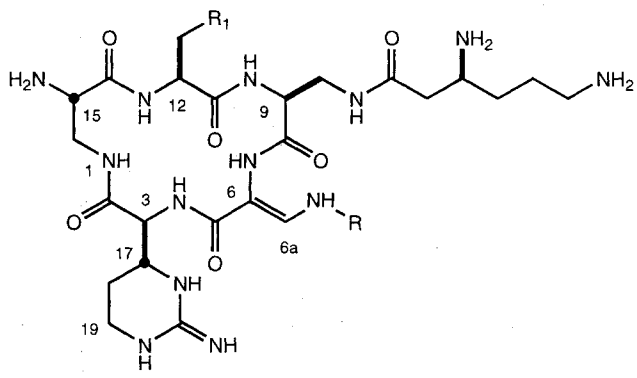
The following bacterial strains were used in the antibacterial spectrum assay: *Actinobacillus pleuropneumoniae* ATCC27088, *Bordetella bronchiseptica* ATCC19395, *Escherichia coli* ATCC25922, *E. coli* W4680 (F, *ΔlacZ39*, *rpsL45*, *rpsL110*, *melB4*, from J. E. HEARST, UC Berkeley[9]), *E. coli* WZM120 (same as W4680; *ΔacrAB*::Tn903*Kan*$^r$ from J. E. HEARST, UC Berkeley[9]),

† Deceased on May 19, 1996.

Fig. 1. Structure of viomycin, CP-101,680, capreomycin (IA, IB) and CP-163,234.



viomycin; R = $CONH_2$
CP-101,680; R = $C_6H_3$-3',4'-$(Cl)_2$



capreomycin IA; $R_1$ = OH, R = $CONH_2$
capreomycin IB; $R_1$ = H, R = $CONH_2$
CP-163,234; above mixture where R = $C_6H_3$-3',4'-$(Cl)_2$

*Haemophilus somnus* ATCC43625, *P. haemolytica* ATCC14003, *P. multocida* ATCC15743, *Salmonella choleraesuis* ATCC19430, *S. typhimurium* LT2 SGSC230 (RD2 LPS deep rough mutant[10]), *Staphylococcus aureus* ATCC29213 and *Streptococcus uberis* ATCC27958.

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 (Remel), and MH broth and agar (Difco, Remel) supplemented with $\beta$-NAD (15 $\mu$g/ml, Sigma) were used.

The antibiotics used in this study were viomycin, capreomycin, CP-101,680 [6a-(3′,4′-dichlorophenylamino) analog of viomycin], and CP-163,234 [6a-(3′,4′-dichlorophenylamino) analog of capreomycin]. All antibiotics were supplied from the in-house collection. The chemical structures of these compounds are presented in Fig. 1. The synthetic method for both CP-101,680 and CP-163,234 is described in reference[7].

### *In Vitro* MIC Analysis

MICs (Minimum Inhibitory Concentrations) were determined using a broth microdilution method described previously[11]. All experiments were carried out in duplicate.

### Anti-bacterial Spectrum Broth Microdilution Assay

The assay method was previously described[11]. All experiments were carried out in duplicate.

### Bactericidal Kinetics

Time-kill kinetic analysis was carried out by the method described[12~14]. Colony counts were performed with plates yielding 30 to 300 colonies. The lower limit of sensitivity of colony counts was 300 CFU/ml[12].

### Analysis for Various Factors Affecting *In Vitro* Potency

The influence of media, inoculum size, pH, EDTA, polymixin B nonapeptide (PMBN) and serum were carried out by the method described[13]. All experiments were carried out in duplicate.

### *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[15].

## Results

### Antibacterial Spectrum

The antibacterial spectrum results are presented in Table 1. Viomycin and capreomycin did not show any significant

Table 1. Antibacterial spectrum activity of viomycin, capreomycin, CP-101,680 and CP-163,234.

| Bacteria | Strain number | Comments | MIC (μg/ml) viomycin | CP-101,680 | capreomycin | CP-163,234 |
|---|---|---|---|---|---|---|
| *A. pleuropneumoiniae* | ATCC27088 | | >200 | 6.25 | 200 | 3.13 |
| *B. bronchiseptica* | ATCC19395 | | >200 | 25 | 200 | 6.25 |
| *E. coli* | ATCC25922 | | 100 | 6.25 | 50 | 12.5 |
| | W4680 | Parent of WZM120 wild-type of *acrAB* | 25 | 3.13 | 25 | 3.13 |
| | WZM120 | Mutant of W4680 with *ΔacrAB*::Tn903*Kan*$^r$ | 25 | 1.56 | 25 | 3.13 |
| *H. somnus* | ATCC43625 | | 200 | 1.56 | 100 | 1.56 |
| *P. haemolytica* | ATCC14003 | | 200 | 12.5 | 200 | 6.25 |
| *P. multocida* | ATCC15743 | | >200 | 12.5 | >200 | 6.25 |
| *S. choleraesuis* | ATCC19430 | | 50 | 3.13 | 50 | 3.13 |
| *S. typhimurium* | LT2 SGSC230 | LPS deep rough mutant RD2 | 50 | 6.25 | 25 | 6.25 |
| *S. aureus* | ATCC29213 | | 200 | 6.25 | 100 | 1.56 |
| *S. uberis* | ATCC27958 | | 200 | 3.13 | 50 | 1.56 |

antibacterial activity, with only moderate anti-enteric activity (MIC 25~100 μg/ml). In contrast, both of the 6a-(3′,4′-dichlorophenylamino) analogs of viomycin and capreomycin (CP-101,680 and CP-163,234, respectively) showed significant improvement in antibacterial activity against a wide spectrum of Gram-negative and Gram-positive animal pathogens (MIC range 1.56~25 μg/ml for CP-101,680 and 1.56~12.5 μg/ml for CP-163,234). CP-163,234 showed slightly better antibacterial activity than CP-101,680 against *A. pleuropneumoniae*, *B. bronchiseptica*, *P. haemolytica*, *P. multocida*, *S. aureus*, and *S. uberis* (2- to 4-fold). Both CP-101,680 and CP-163,234 did not show any differential in MICs against *ΔacrAB E. coli* strain (WZM120) and its isogenic parent (W4680). Both compounds also did not show any improvement in MICs against *S. typhimurium* LT2 strain compared to those against *E. coli* (ATCC25922) and *S. choleraesuis* (ATCC 19430).

## Bactericidal Kinetic Analysis

Time kill kinetics studies of CP-101,680 and CP-163,234 were carried out at the levels of 4 times and 8 times the MICs. The 8× MIC results are presented in Fig. 2 for CP-101,680 and Fig. 3 for CP-163,234. Both cyclic peptides were bactericidal against *P. haemolytica*, *P. multocida*, *A. pleuropneumoniae* and *E. coli* at both 4 times (data not shown) and 8 times the MIC. All four bacterial species were quickly killed during the initial 3 hour exposure to antibiotics. After 24 hours, no bacterial re-growth was observed, except *E. coli* against CP-101,680.

## Effect of Media, Cations and Inoculum Size

Both viomycin and capreomycin showed high MIC values (100~>200 μg/ml) in various broths against four bacteria (Table 2). CP-101,680 and CP-163,234 showed equivalent MICs in MH, BHI and LB broths and there was no significant media effect against bacterial strains tested except *E. coli*. MICs in MH broth against *E. coli* showed 4~8 times more potent activity than other broth media. Addition of cations (25 mg/liter $Ca^{2+}$ and 12.5 mg/liter $Mg^{2+}$) to MH broth showed no MIC change of either viomycin or capreomycin against the bacterial strains

Table 2. Influence of media on the *in vitro* potency of viomycin, capreomycin, CP-101,680 and CP-163,234.

| Bacteria | Microbiological Media | MIC (μg/ml) viomycin | CP-101,680 | capreomycin | CP-163,234 |
|---|---|---|---|---|---|
| ***E. coli*** (51A0150) | MH broth | 100 | 3.13 | 100 | 1.56 |
| | MH broth + $Ca^{2+}$ and $Mg^{2+}$ | 100 | 25 | 100 | 6.25 |
| | BHI broth | 200 | 12.5 | 200 | 6.25 |
| | LB broth | >200 | 25 | >200 | 6.25 |
| ***P. haemolytica*** (59B0046) | MH broth | 100 | 6.25 | 100 | 3.13 |
| | MH broth + $Ca^{2+}$ and $Mg^{2+}$ | 100 | 12.5 | 100 | 6.25 |
| | BHI broth | 200 | 6.25 | 200 | 3.13 |
| | LB broth | 200 | 12.5 | ≥200 | 6.25 |
| ***P. multocida*** (59A0067) | MH broth | 200 | 3.13 | 200 | 1.56 |
| | MH broth + $Ca^{2+}$ and $Mg^{2+}$ | >200 | 6.25 | >200 | 3.13 |
| | BHI broth | 100 | 1.56 | 100 | 0.78 |
| | LB broth | 200 | 1.56 | 100 | 1.56 |
| ***A. pleuropneumoniae*** (44A0030) | MH broth | 100 | 3.13 | 100 | 1.56 |
| | MH broth + $Ca^{2+}$ and $Mg^{2}$ | 100 | 3.13 | 100 | 1.56 |
| | BHI broth | 200 | 3.13 | 200 | 1.56 |
| | LB broth | 200 | 6.25 | >200 | 3.13 |

tested. CP-101,680 and CP-163,234 showed slight increase in MIC against *P. haemolytica* and *P. multocida* (two-fold) and no MIC change against *A. pleuropneumoniae* in the presence of additional cations. In contrast, the addition of cations into MH broth raised MICs of both CP-101,680 and CP-163,234 against *E. coli* by 4~8 times.

Varying the inoculum size from $10^4$ to $10^6$ cfu/ml was evaluated and showed no effect on the *in vitro* potency of viomycin, capreomycin, CP-101,680 and CP-163,234 against *E. coli*, *P. haemolytica* and *P. multocida* (data not shown). *A. pleuropneumoniae* showed a significant increase in sensitivity at the inoculum size $10^4$ cfu/ml for all four test compounds compared to those of $10^5$ and $10^6$ cfu/ml inoculum size (data not shown).

## Effect of pH

Viomycin and capreomycin showed significantly improved potency against four bacterial strains tested in alkaline pH (Table 3). *P. haemolytica*, and *A. pleuropneumoniae*, however did not grow in an acidic environment (Table 4) as described previously[13]. CP-101,680 and CP-163,234 were also more potent in alkaline conditions than in acidic conditions against *E. coli* and in neutral conditions against both *P. haemolytica* and *A. pleuropneumoniae*. MICs were highest, however, against *P. multocida* in neutral pH, followed by alkaline conditions with CP-101,680 and CP-163,234. All four compounds were most potent in alkaline conditions.

## Effect of PMBN and EDTA

PMBN showed antibacterial activity against *P. haemolytica* and *A. pleuropneumoniae* (MIC 3.13 μg/ml and 1.56 μg/ml, respectively[13]). Therefore, the highest concentration of PMBN tested against these bacteria was 1.0 μg/ml. In contrast, *P. multocida* and *E. coli* were more resistant to PMBN than *P. haemolytica* and *A. pleuropneumoniae* (MIC 800 μg/ml and 200 μg/ml, respectively[13]) and the highest concentration of PMBN tested was 50 μg/ml. The addition of various concentrations of PMBN to the culture medium at sub-MIC levels did not affect the potency of viomycin,

Table 3. Effect of pH on *in vitro* potency of viomycin, capreomycin, CP-101,680 and CP-163,234.

| Bacteria | pH | MIC(μg/ml) viomycin | CP-101,680 | capreomycin | CP163,234 |
|---|---|---|---|---|---|
| ***E. coil*** (51A0150) | 6 | >200 | 12.5 | >200 | 3.13 |
| | 7 | 200 | 6.25 | 200 | 1.56 |
| | 8 | 25 | 3.13 | 25 | 1.56 |
| ***P. haemolytica*** (59B0046) | 6 | NG | NG | NG | NG |
| | 7 | 100 | 6.25 | 100 | 3.13 |
| | 8 | 12.5 | 1.56 | 12.5 | 1.56 |
| ***P. multocida*** (59A0067) | 6 | 200 | 1.56 | 50 | 0.78 |
| | 7 | >200 | 3.13 | 100 | 1.56 |
| | 8 | 25 | 0.78 | 25 | 0.39 |
| ***A. pleuropneumoniae*** (44A0030) | 6 | NG | NG | NG | NG |
| | 7 | 100 | 3.13 | 100 | 1.56 |
| | 8 | 3.13 | <0.2 | 1.56 | <0.2 |

NG: No growth

Table 4. Effect of PMBN on *in vitro* potency of viomycin, capreomycin, CP-101,680 and CP-163,234.

| Bacteria | PMBN (μg/ml) | MIC (μg/ml) viomycin | CP-101,680 | capreomycin | CP-163,234 |
|---|---|---|---|---|---|
| ***E. coil*** (51A0150) | 0 | 50 | 3.13 | 50 | 3.13 |
| | 5 | 50 | 1.56 | 50 | 1.56 |
| | 10 | 50 | 1.56 | 50 | 0.78 |
| | 50 | 50 | 1.56 | 50 | 0.78 |
| ***P. haemolytica*** (59B0046) | 0 | 100 | 6.25 | 100 | 3.13 |
| | 0.5 | 200 | 6.25 | 200 | 3.13 |
| | 1.0 | 200 | 6.25 | 200 | 3.13 |
| ***P. multocida*** (59A0067) | 0 | 200 | 3.13 | 200 | 1.56 |
| | 5 | 200 | 3.13 | 200 | 0.78 |
| | 10 | 200 | 1.56 | 200 | 0.78 |
| | 50 | 200 | 3.13 | 200 | 0.78 |
| ***A. pleuropneumoniae*** (44A0030) | 0 | 100 | 3.13 | 100 | 3.13 |
| | 0.5 | 200 | 3.13 | 200 | 3.13 |
| | 1.0 | 100 | 3.13 | 100 | 3.13 |

Table 5. Effect of EDTA on *in vitro* potency of viomycin, capreomycin, CP-101,680 and CP-163,234.

| Bacteria | EDTA (mM) | MIC (μg/ml) | | | |
|---|---|---|---|---|---|
| | | viomycin | CP-101,680 | capreomycin | CP-163,234 |
| ***E. coil*** (51A0150) | 0 | 50 | 6.25 | 100 | 1.56 |
| | 0.1 | 50 | 3.13 | 100 | 1.56 |
| | 0.5 | 50 | 1.56 | 25 | 0.78 |
| ***P. haemolytica*** (59B0046) | 0 | 100 | 1.56 | 100 | 3.13 |
| | 0.1 | 200 | 3.13 | 200 | 6.25 |
| ***P. multocida*** (59A0067) | 0 | 200 | 1.56 | 100 | 1.56 |
| | 0.1 | 200 | 1.56 | 100 | 1.56 |
| ***A. pleuropneumoniae*** (44A0030) | 0 | 100 | 3.13 | 200 | 1.56 |
| | 0.1 | 100 | 3.13 | 200 | 1.56 |

capreomycin, CP-101,680 and CP-163,234 against *P. haemolytica*, *P. multocida* and *A. pleuropneumoniae*. In contrast, both CP-101,680 and CP-163,234 showed slightly increased potency against *E. coli* in the presence of sub-MIC levels of PMBN (Table 4).

The results of EDTA addition to the test medium are presented in Table 5. *P. haemolytica*, *P. multocida* and *A. pleuropneumoniae* were sensitive to EDTA at the concentration of 0.5 mM[13]. Therefore these bacteria were tested at 0.1 mM EDTA. This sub-MIC level of EDTA did not increase the potency of viomycin, capreomycin, CP-101,680 and CP-163,234 against *P. multocida* and *A. pleuropneumoniae*, and showed a slight increase in potency (2 fold) against *P. haemolytica*. The potency of CP-101,680 against *E. coli* increased in a dose dependent manner and a 4-fold increase in potency was observed in the presence of 0.5 mM EDTA. However, its parent compound, viomycin was not affected by the presence of EDTA (Table 5). In contrast to those, capreomycin and CP-163,234 showed 2- to 4-fold potency increase in the presence of 0.5 mM EDTA but this increase was not dose dependent (Table 5).

### Effect of 40% Heat Inactivated Bovine Serum

MIC results of the addition of 40% heat inactivated bovine serum to the assay system in the presence and absence of 5% $CO_2$ are presented in Table 6. The addition of serum to the growth medium shifted the pH towards alkalinity during 18 hours incubation[13]. The potency of viomycin and capreomycin against the four bacterial species tested was not affected by either the addition of serum nor the presence of 5% $CO_2$. However, MICs of CP-101,680 against all four bacterial species increased 2- to 4-fold with the addition of serum into the test medium. In the presence of 5% $CO_2$, these elevated MICs became much higher than in the absence of 5% $CO_2$. These results indicate that there is a significant serum binding effect for CP-101,680. MICs of CP-163,234 against the four bacterial species also increased in the presence of serum but no further MIC increase in the presence of 5% $CO_2$ was observed, except for *A. pleuropneumoniae*.

### MS2 Directed Polypeptide Synthesis Inhibition Assay

The translation $IC_{50}$s of viomycin, capreomycin, CP-101,680, CP-163,234, and an additional control antibiotic, erythromycin (Sigma) were 0.35 μM, 20 μM, 0.035 μM, 0.02 μM and 0.12 μM, respectively.

## Discussion

CP-101,680 and CP-163,234 showed significantly improved antibacterial activity against a wide spectrum of animal pathogens compared to their parent compounds, viomycin and capreomycin. In our spectrum analysis using several animal pathogens, viomycin and capreomycin showed very high MICs which would be considered to be

Table 6. Effect of heat inactivated 40% bovine serum on *in vitro* potency of viomycin, capreomycin, CP-101,680 and CP-163,234.

| Bacteria | 40% serum | MIC (μg/ml) viomycin | CP-101,680 | capreomycin | CP-163,234 |
|---|---|---|---|---|---|
| ***E. coil*** (51A0150) | - | 50 | 6.25 | 100 | 3.13 |
| | + | 50 | 25 | 50 | 6.25 |
| | + & 5% $CO_2$ | 100 | 50 | 200 | 6.25 |
| ***P. haemolytica*** (59B0046) | - | 100 | 6.25 | 100 | 1.56 |
| | + | 100 | 12.5 | 200 | 6.25 |
| | + & 5% $CO_2$ | 200 | 50 | 200 | 6.25 |
| ***P. multocida*** (59A0067) | - | 100 | 3.13 | 100 | 1.56 |
| | + | 100 | 6.25 | 100 | 6.25 |
| | + & 5% $CO_2$ | 200 | 25 | 200 | 6.25 |
| ***A. pleuropneumoniae*** (44A0030) | - | 100 | 3.13 | 100 | 1.56 |
| | + | 100 | 12.5 | 100 | 3.13 |
| | + & 5% $CO_2$ | 200 | 25 | 200 | 6.25 |

non-antibacterial (Table 1). However, the replacement of the urea group at the 6a position by 3,4 dichlororphenyl group drastically increased the antibacterial activity of these molecules. The improvement in antibacterial activity varied among bacterial species. For example, against the enteric Gram-negative species (*E. coli* and *Salmonella*) they showed 4 to 16 times improvement, while against *H. somnus* they showed 64 to 128 times improvement in MICs. As stated in the result section, both CP-101,680 and CP-163,234 showed extremely low $IC_{50}$s in cell-free protein synthesis inhibition assay, that is 0.035 μmole and 0.02 μmole, respectively. In contrast, the $IC_{50}$s of viomycin and capreomycin are 0.35 μmole and 20 μmole, much higher values than CP-101,680 and CP-163,234. These results suggest that the improved antibacterial activity originates from the improvement of the molecules' intrinsic activity rather than transport through the outer membrane. This conclusion was further confirmed by the MIC results against the *S. typhimurium* LPS mutant and the effect of PMBN, which are discussed later.

Cell-free protein synthesis inhibition experiments also showed that the $IC_{50}$ of viomycin is significantly lower (100 times) than capreomycin (0.3 μM *vs.* 20 μM). In contrast, both viomycin and capreomycin showed equivalent antibacterial activity against a panel of pathogens, although their MICs are very high (Table 1). This result seems to indicate that capreomycin has better transport through the inner (plasma) membrane than viomycin because neither the outer membrane transport or universal efflux pump are the rate limiting factors (discussed later). Viomycin has a hydroxylmethyl group at C-9, a hydroxy group at the C-19 position, and a β-lysine at the C-15 position. In contrast, capreomycin possesses a β-lysine at the C-9 position, an amino group at C-15, and hydrogens at the C-19 position (Fig. 1). This structural difference may contribute to better inner (plasma) membrane transport for capreomycin. However, the $IC_{50}$ difference was totally abolished by the replacement of the urea group with a 3,4-dichlororphenyl group at the 6a position.

The susceptibility of both the *E. coli* mutant strain WEZ120, lacking the universal efflux system (*ΔacrAB*) which pumps out many antibiotics and dyes from inside of cells, and its isogenic parent strain (W4680) were tested using CP-101,680, CP-163,234, viomycin, and capreomycin. Historically, antibiotics, which are a substrate of this universal pump, show significant differences in MICs (improvement of antibacterial activity) against the mutant strain compared to its parent[9,11,14]. Viomycin, capreomycin, CP-101,680, and CP-163,234 did not show any differential activity against WEZ120 and W4680 strains (Table 1). This

result indicates that these compounds are not substrates of the universal efflux pump AcrA/B and are not pumped out from inside of the cells.

The *S. typhimurium* LT2 SGSC230 strain in the antibacterial spectrum assay carries a mutation in lipopolysaccahride (LPS) synthesis and contains only lipid A and KDO, and lacks the O-antigen and heptose region[10]. Due to this disturbed LPS, SGSC230 shows hypersensitivity to hydrophobic antibiotics whose entry into the cells is usually blocked by complete LPS. Therefore, such antibiotics generally show significantly lower MICs against this strain compared to the wild-type *E. coli* and *S. choleraesuis* used in the spectrum assay[13,14]. However, viomycin, capreomycin, CP-101,680, and CP-163,234 did not show any differential activity against SGSC230 strain, which indicates that the outer membrane is not the limiting factor for entry of these compounds into the cell (Table 1). This conclusion is further supported by the demonstration that PMBN did not significantly decrease the MICs of these compounds against *E. coli* in the presence of sub-MIC levels (3.13 $\mu$g/ml to 1.56 $\mu$g/ml for CP-101,680 and 3.13 $\mu$g/ml to 0.78 $\mu$g/ml for CP-163,234). PMBN is known to be an outer membrane disrupting agent and at sub-MIC levels, the addition of PMBN in culture significantly enhances the activity of hydrophobic antibiotics such as erythromycin A, tilmicosin and fusidic acid (25 $\mu$g/ml to 0.39 $\mu$g/ml, 25 $\mu$g/ml to 0.39 $\mu$g/ml, and >400 $\mu$g/ml to 1.56 $\mu$g/ml, respectively)[13]. It is also known from previous work that the addition of sub-MIC levels of PMBN to a culture does not show any synergistic effect with hydrophobic compounds against *P. haemolytica*, *P. multocida* and *A. pleuropneumoniae*, probably due to the difference in the outer membrane structure[13]. The same conclusion was obtained in the present study using CP-101,680 and CP-163,234.

It is also known that overnight incubation in the presence of 40% heat inactivated bovine serum shifts the medium pH towards alkalinity and this pH shift significantly improves macrolide antibacterial activity. However, incubation in growth medium containing 40% bovine serum in the presence of 5% $CO_2$ neutralizes this pH shift and antibacterial activity of macrolides in alkaline conditions was also neutralized[13]. CP-101,680, CP-163,234, viomycin and capreomycin showed improved potency in alkaline conditions as observed with macrolides. In contrast to the macrolides, their antibacterial activity was less potent in the presence of 40% bovine serum. This decreased antibacterial activity was further diminished by incubating the assay plates in the presence of 5% $CO_2$. These results indicate that these compounds were bound to serum protein and this protein-binding diminished their antibacterial activity.

Both CP-101,680 and CP-163,234 showed bactericidal activity against *P. haemolytica*, *P. multocida* and *A. pleuropneumoniae* in time-kill kinetic analyses (Fig. 2 and 3). All three bacterial species were quickly killed during the initial 3 hour exposure to antibiotics at 8× MIC and no regrowth was observed. Identical results were obtained at 4× MIC (data not shown). These results indicate that both compounds are very potent bactericidal agents. The *E. coli* response to both compounds was slightly different from others. CP-163,234 showed similar bactericidal activity against *E. coli* at the level of both 4× and 8× MICs. *E. coli* was killed effectively during the initial 3 hours drug exposure and there was no regrowth during the following 21 hours (Fig. 3). In contrast, regrowth of *E. coli* was observed at 24 hours at both 4× (data not shown) and 8× MIC of CP-101,680, although CP-101,680 showed excellent killing during the initial 6 hours (Fig. 2). These results indicate that CP-163,234 is a more potent bactericidal agent than CP-101,680 against *E. coli*. Overall, the novel analogs CP-101,680 and CP-163,234 are potent bactericidal agents against the four pathogens tested. Due to the very high MICs, the time-kill kinetic study of the parent compounds, viomycin and capreomycin, were not tested for the present paper. However, bactericidality of capreomycin was reported against *Mycobacterium tuberculosis* with low MBC/MIC ratios[16]. *M. tuberculosis* is significantly sensitive to viomycin and capreomycin compared to other pathogenic bacteria.

In summary, our microbiological profiling of CP-101,680 and CP-163,234 reveals the following points: 1) both compounds demonstrated excellent *in vitro* antibacterial spectrum against animal respiratory, enteric and mastitis pathogens with very potent cell-free protein synthesis inhibition activity; 2) neither compound is a substrate of the universal efflux pump AcrA/B system and they can reach the target (ribosomes) without interference of the outer membrane barrier of Gram-negative bacteria; 3) both compounds are bactericidal agents with rapid killing activity; and 4) both compounds showed good *in vivo* activity in *E. coli* and *P. multocida* infection mice models by a subcutaneous (SC) route of administration (6~10 mg/kg)[7], in spite of their serum protein binding ability. These attributes seem to indicate that both CP-101,680 and CP-163,234 could be novel antibacterial agents for livestock infectious disease therapy.

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Fig. 2. Time-kill kinetics of CP-101,680.

---■--- *E. coli* control, —■— *E. coli* 8×, ---◆--- *P. haemolytica* control, —◆— *P. haemolytica* 8×, ---▲--- *P. multocida* control, —▲— *P. multocida* 8×, ---□--- *A. pleuropneumonia* control, —□— *A. pleuropneumonia* 8×.

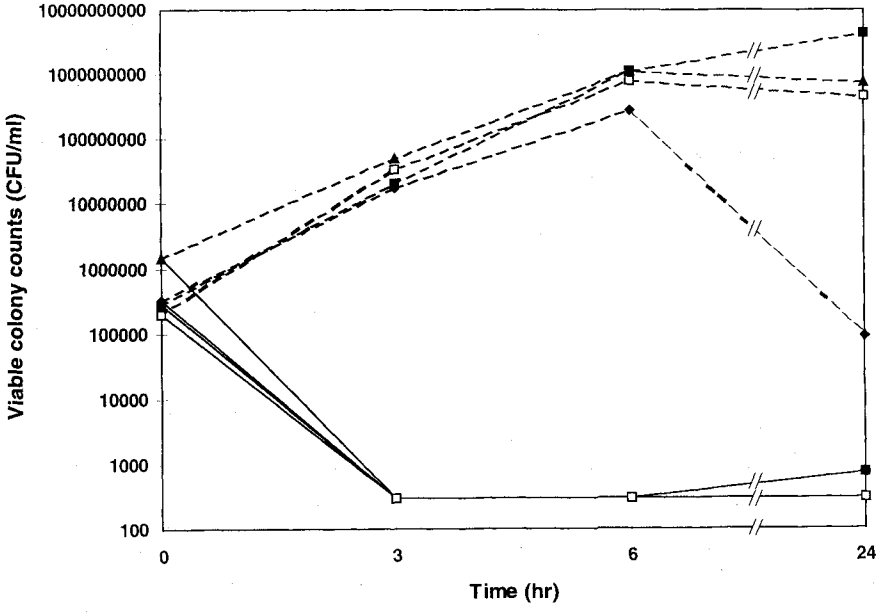
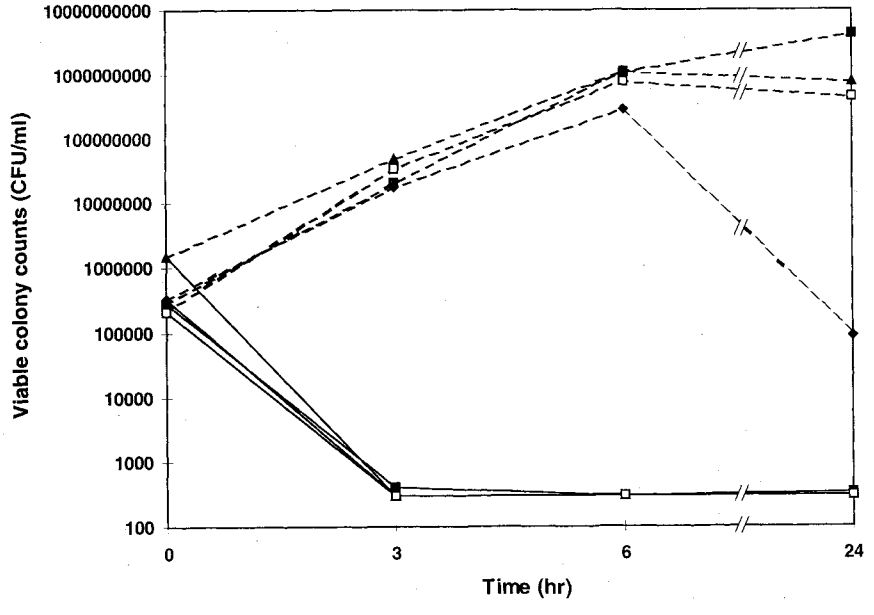



Fig. 3. Time-kill kinetics of CP-163,234.

---■--- *E. coli* control, —■— *E. coli* 8×, ---◆--- *P. haemolytica* control, —◆— *P. haemolytica*. 8×, ---▲--- *P. multocida* control, —▲— *P. multocida* 8×, ---□--- *A. pleuropneumonia* control, —□— *A. pleuropneumonia* 8×.



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