Friulimicin B Inhibits Cell Wall Biosynthesis through Complex Formation with Bactoprenol-Phosphate

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Revised Abstract

Background:
Friulimicin B (FRI), an acidic, cyclic lipopeptide, is intended for the treatment of severe infections caused by Gram-positive pathogens. FRI shows structural similarity with Daptomycin (DAP), however, its mode of action had not been elucidated previously.

Methods:
Whole cell assays with staphylococci and bacilli included the following: incorporation of radiolabelled precursors for biosynthesis of macromolecules, potassium ion leakage, membrane depolarization and intracellular accumulation of UDP-activated soluble cell wall precursors[1]. Cell free lipid II synthesis assays[2] were performed with *M. luteus* membranes as well as with isolated MraY and MurG, using bactoprenol-phosphate (C_{55}-P) and purified UDP-MurNAc-pentapeptide or lipid I as substrates.

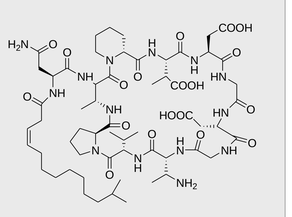
Results:
Whole cell assays did not yield evidence for a rapid "Nisin-like", gross membrane depolarization or pore formation for both FRI and DAP. FRI, unlike DAP, induced accumulation of UDP-MurNAc pentapeptide indicating cell wall biosynthesis inhibition as a putative mechanism. Lipid II synthesis assays showed that the MraY reaction was blocked through Ca^{2+}-dependent complex formation with the lipid carrier C_{55}-P. Moreover, C_{55}-P, but not lipid I or lipid II, was able to completely antagonize completely the activity of friulimicin B *in vitro* when added to conventional MIC determination assays. DAP was not active in any of the assays.

Conclusions:
The antibiotic activity of FRI is based on interruption of the cell wall precursor cycle through the formation of a Ca^{2+}-dependent complex with the bactoprenol-phosphate carrier. Since C_{55}-P also serves as a carrier in teichoic acid biosynthesis, it is likely that FRI blocks two pathways essential for a functional Gram-positive cell envelope.

Introduction

The lipopeptide antibiotic friulimicin B (FRI) is produced by the actinomycete *Actinoplanes friuliensis.* The lipopeptide is active against a broad range of Gram-positive bacteria, including antibiotic-resistant pathogens, like methicillin-resistant *Staphylococcus aureus,* enterococci[3-5] and obligatory anaerobic bacteria[6].
FRI (Fig.1) consists of a macrocyclic decapeptide core and a lipid tail interlinked by an exocyclic amino acid. FRI is water soluble and amphiphilic with an overall negative charge. Amphilicity is strongly increased in the presence of Ca^{2+} which is also indispensable for antimicrobial activity[7]. Regarding these physicochemical properties FRI is similar to daptomycin (DAP), which is supposed to kill Gram-positive bacteria through the formation of pores in the cytoplasmic membrane. We show here that FRI and DAP do not share a common mode of action mechanism.

Figure 1 : Chemical structure of Friulimicin B (FRI)



Methods

Susceptibility tests: Minimal growth inhibitory concentrations (MIC) were determined by standard broth microdilution methods in a polypropylene microtitre plate using cation adjusted Mueller-Hinton broth supplemented with Ca^{2+} ions (50 mg/L). Bacteria in the exponential growth phase were diluted to give a final inoculum of 10^5 CFU/ml. MICs were read after 16 h at 37°C.

Analysis of the cytoplasmic peptidoglycan nucleotide precursor pool: *S. simulans* 22 was grown in Mueller-Hinton broth to an OD of 0.5 and supplemented with 130 pg/ml of chloramphenicol. After 15 min, antibiotics (vancomycin [VAN]; FRI; DAP) were added at 10xMIC and incubated for 60 min. Cells were harvested and extracted with boiling water. The suspension was then centrifuged and the supernatant lyophilised. UDP-linked cell wall precursors were analyzed by HPLC and corresponding fractions were confirmed by mass spectrometry.

Incorporation of ^{3}H-glucosamine into peptidoglycan: was studied with *B. subtilis/ S. simulans* 22. Cells were grown to log-phase in CYG medium supplemented with Ca^{2+} (50 mg/l) and 1 µCi/mL of ^{3}H-glucosamine. Macromolecules were precipitated by addition of ice-cold TCA (10%) and passed onto glass microfibre filters. Filters were washed with 5 ml TCA (2.5%), dried and counted.

Potassium release from whole cells: *S. simulans 22* cells were harvested at an OD_{600} of 1.0-1.5, washed with cold choline-buffer (300 mM choline chloride, 30 mM Mes, 20 mM Tris, pH 6.5) and resuspended to an OD_{600} of 30. The concentrated cell suspension was kept on ice and used within 30 min. For each measurement the cells were diluted in choline-buffer (25°C) to an OD_{600} of about 3. Peptide induced leakage was plotted relative to the total amount of potassium release induced by addition of 1 µM Nisin (NIS).

***In vitro* peptidoglycan synthesis with isolated membranes:** *In vitro* lipid II synthesis was performed using membranes of *Micrococcus luteus* as described[2]. Membrane preparations were incubated in the presence of purified substrates, undecaprenylphosphate (C_{55}-P), UDP-*N*-acetylmuramic acid pentapeptide (UDP-MurNAc-pp) and [^{14}C]-UDP-GlcNAc for 1 h at 30°C. Bactoprenol containing products were extracted with butanol/ pyridine acetate (2:1; vol:vol; pH 4.2) and analyzed by TLC. Radiolabelled spots were visualized by iodine vapour, excised and counted. Peptides were added in molar ratios with respect to C_{55}-P.

***In vitro* peptidoglycan synthesis using purified recombinant proteins:** Synthesis and analysis was performed as described above except that purified, recombinant proteins (MraY; MurG) and substrates were tested. The formation of lipid II-Gly_1 by FemX was tested with [^{14}C]-glycine as described[2]. Polymerisation of lipid II by PBP2 was performed with 5 nmol lipid II and 7.5 µg PBP2 in 100 mM Mes buffer, 10 mM $MgCl_2$, 1.25 mM $CaCl_2$ at pH 5. Remaining lipid II was extracted and analyzed by TLC. Antibiotics were added in a 1:1 molar ratio with respect to the bactoprenol-bound substrates.

Results

- FRI inhibits the test strains used in this study, *S. simulans* 22 and *Bacillus subtilis* 168 at 0.078 mg/L → Table 1A
- Unlike DAP, the activity of FRI is antagonized by C_{55}-P → Table 1B
- Unlike DAP, FRI leads to the intracelllular accumulation of soluble UDP-linked cell wall precursors → Fig. 2
- FRI inhibits the incorporation of ^{3}H-glucosamine → Fig. 3
- Unlike Nisin, FRI does not cause a rapid leakage of intracellular K^+ → Fig. 4
- FRI inhibits the formation of lipid II by isolated cytoplasmic membranes from *M. luteus* → Fig. 5B
- FRI inhibits the MraY-catalyzed formation of lipid I (from C_{55}-P and UDP-MurNAc-pp) → Fig. 5C
- FRI does not inhibit:
  - MurG-catalyzed formation of lipid II → Fig. 5D
  - FemX-catalyzed formation of lipid II-Gly_1 → Fig. 5E
  - polymerisation of lipid II by PBP2 → Fig. 5F
- Data from Fig. 5B and 5C suggest a 1:1 stoichiometry of the FRI-C_{55}-P complex *in vitro*.

Results

Table 1 : Antibiotic Activity of FRI and DAP

A MICs (mg/L) of test strains

	FRI	DAP
S. simulans 22	0.078	0.039
B. subtilis 168	0.078	0.625

B Antagonism observed*

	C_{55}-P	C_{55}-PP	lipid I	lipid II	UDP-MurNac-pentapeptide	UDP-GlcNAc
FRI	+	-	-	-	-	-
DAP	-	-	-	-	-	-

* *S. simulans* 22 was incubated with 0.625 mg/L FRI and 0.31mg/L DAP, corresponding to 8 fold the MIC; antagonists were added in 5 fold molar excess with respect to the antibiotics.

Figure 2 : FRI causes the accumulation of UDP-MurNAc-pentapeptide

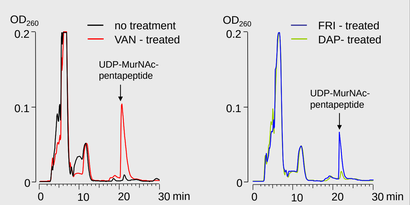


Figure 3 : FRI inhibits ^{3}H-glucosamine incorporation

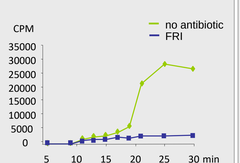


Figure 4 : FRI does not form Nisin-like pores

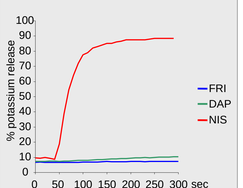
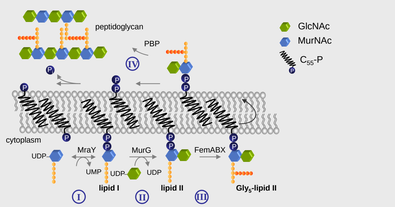


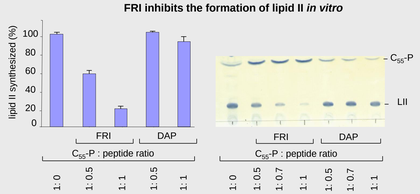
Figure 5 : Antibiotic activities of FRI and DAP in cell free biosynthesis pathways

A Staphylococcal cell wall biosynthesis and reactions tested



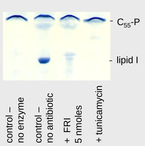
B Reactions (I) – (II) tested simultanously with isolated membranes

FRI inhibits the formation of lipid II *in vitro*



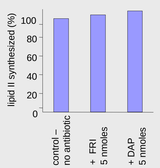
C Reaction (I) tested with purified MraY

FRI inhibits the MraY-catalyzed synthesis of lipid I *in vitro*



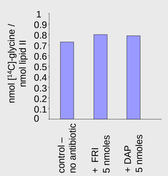
D Reaction (II) tested with purified MurG

FRI does not interfere with the MurG-catalyzed reaction



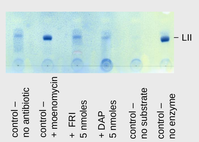
E Reaction (III) tested with purified FemX

FRI does not inhibit FemX



F Reaction (IV) tested with purified PBP2

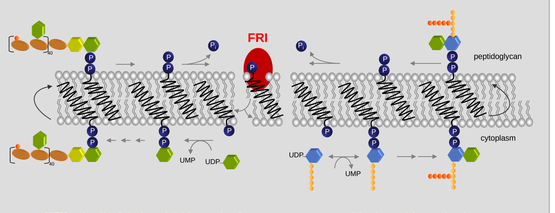
FRI does not inhibit PBP2



Conclusions

Figure 6 : Model for the antimicrobial activity of FRI

FRI forms a complex with the lipid carrier C_{55}-P, and blocks cell wall biosynthesis reactions



The antibiotic activity of FRI is based on the formation of a Ca^{2+}-dependent complex with bactoprenol-phosphate (C_{55}-P). C_{55}-P is the central carrier of the peptidoglycan precursor GlcNAc-MurNAc-pentapeptide and of the GlcNAc-ManNAc-PGro-(PGro-PGro)$_n$ precursor of wall teichoic acid (WTA). Abduction of the central carrier interrupts precursor cycling and blocks the synthesis of a functional cell envelope in Gram-positive pathogens (Fig. 6). All results clearly demonstrate that FRI and DAP do not share a common mode of action.

Literature

[1] H. Brötz et al., *AAC* 39 (1995) 714, 1995
[2] T. Schneider et al., Mol. Microbiol. 53 (2004) 675
[3] P. McGhee et al., Poster F1-1648, this ICAAC 2007
[4] M. Kresken, et al., Poster F1-1642, this ICAAC 2007
[5] H. Priefert, et al., Poster F1-1643, this ICAAC 2007
[6] R. Schaumann, et al., Poster F1-1644, this ICAAC 2007
[7] G.M. Eliopoulos et al., *AAC* 27 (1985) 357

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Distinct Mode of Action of the Lipopeptide Antibiotic Friulimicin B and the Lipodepsipeptide Daptomycin: A Proteomic Study

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Abstract

Background: Friulimicin B (FRI), a cyclic lipopeptide, is intended for the treatment of severe infections caused by Gram-positive pathogens. FRI shows structural similarities with daptomycin (DAP), both are acidic, amphipathic and require physiological calcium concentrations. We compared the mode of action of both compounds using a proteomic approach.

Methods: *B. subtilis* 168 wildtype was grown in Belitsky minimal medium and FRI / DAP was added (~1.3 and 3 x MIC for both compounds). 10 and 30 minutes after treatment the cells as well as untreated control cells were labelled with [^{35}S]-methionine for 5 minutes. Isolated protein extracts were separated in 2D gel electrophoresis. Proteins were visualized by autoradiography. Induction of the LiaRS two-component system for both antibiotics was investigated using a P_{liaI}-*lacZ* reporter system.

Results: Both antimicrobial compounds induced a set of proteins (including DltA, YceC, Ywfl, PtsH) that are also synthesized after addition of other cell envelope-damaging substances. The most prominent protein synthesized after DAP treatment was LiaH, known to be induced by compounds like bacitracin and vancomycin and other compounds involved in the lipid II cycle of cell wall synthesis, but not by other cell wall antibiotics, such as fosfomycin, D-cycloserine, or ß-lactams. FRI did not induce LiaH but other proteins (YceH, YoxD) described as marker proteins for cell envelope stress. In quantitative assays using P_{liaI}-*lacZ* reporter system, a strong LiaH induction was demonstrated for DAP at subinhibitory and MIC concentrations, but not for FRI in the concentration range from 0.01 - 50 µg/mL.

Conclusions: Although the proteomic induction patterns suggest some common response elements to disturbances of the cell envelope architecture, FRI and DAP have a different molecular mode of action.

Introduction

Friulimicin B (FRI, Fig. 1) is a novel lipopeptide antibiotic that is produced by *Actinoplanes friuliensis*. FRI is structurally similar with the lipodepsipeptide daptomycin (DAP) and displays a similar antibacterial activity spectrum to DAP addressing all important Gram-positive pathogens such as staphylococci, enterococci, pneumococci (including multi-resistant and DAP-resistant strains)[1-3] and obligatory anaerobic bacteria[4].

The purpose of the studies presented here, was to compare the mode of action (MOA) of FRI and DAP using both, a proteomic approach and the antibiotic sensing *B. subtilis* LiaRS two-component system.

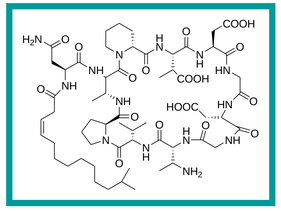

Fig. 1 Friulimicin B

Methods

Proteomic study:
B. subtilis 168 wildtype strain was grown aerobically at 37°C in Belitsky minimal medium[5] to mid-log phase. 10 and 30 minutes after addition of DAP or FRI (1.3 x and 3 x MIC, respectively) the cells as well as untreated control cells were labelled with 15 µCi/ml [^{35}S]-methionine. After 5 minutes incorporation of radioactive methionine was stopped by adding an excess of nonradioactive methionine and chloramphenicol to stop translation. Samples were taken and the harvested cells were sonicated to disrupt them. The isolated protein extracts were separated in 2D gel electrophoresis and radioactively labelled proteins were visualized by autoradiography. DECODON Delta2D software (DECODON GmbH, Greifswald, Germany) was used for warping and Dual Channel Imaging of autoradiographs.

LiaRS two component system:
Induction of the the *liaI* promoter, primary target of the *Bacillus subtilis* LiaRS two-component system, in the presence of FRI or DAP was quantified using P_{liaI}-*lacZ* reporter systems[6,7]. In semi-quantitative disk diffusion assays, 100 µl mid-log cultures of two *B. subtilis* reporter strains HB0961 (*liaI::pMUTIN*) and TMB016 (*amyE*::P_{liaI}-*lacZ*) were been added to 3 ml of 0.7% soft LB agar and poured onto LB agar plates containing 40 µg XGal/plate and 50 mg Ca^{2+}/l. Filter paper disks carried 5 µl of FRI and DAP stock solution (10 mg/ml each) Quantitative activation was measured by β-galactosidase assays. *B. subtilis* HB0961 was grown to an OD_{600} of 0.5 in 130 ml LB broth containing 50 mg Ca^{2+}/L and 1 mg/L of erythromycin. FRI and DAP in a concentration range of 0.01 to 50 mg/L were added to 6 ml aliquots of the culture to induce the system. For β-galactosidase assays 1 ml of each culture was harvested and analysed 30 min after induction[7].

Results and Discussion

Proteome analysis of *B. subtilis* in response to FRI and DAP
Addition of FRI and DAP to growing *B. subtilis* cells lead to a change in protein synthesis (Fig. 2). Induced proteins can be classified into three categories (Table 1). Category A compiled a set of four proteins (DltA, PtsH, YceC and Ywfl) which are induced by both, FRI and DAP. These proteins are known to be induced by other cell wall damaging antibiotics, like bacitracin and vancomycin, indicating that both compounds, FRI and DAP might target the cell envelope. LiaH (category B) is the only protein exclusively induced by DAP. The induction of this marker enzyme known to be induced by lipid II cycle inhibitors was not expected, as DAP seems to interfere with the membrane[8]. Proteins NfrA, TrxA, YceH and YoxD represent category C proteins which are exclusively induced by FRI. Some of these proteins are marker enzymes for cell envelope stress coinduced by cell wall antibiotics, such as vancomycin and bacitracin.

Analysis of LiaRS Two Component System in response to FRI and DAP
The *B. subtilis* LiaRS system is known to be a antibiotic-sensing system coordinating the response to several cell wall active compounds, like bacitracin, vancomycin, nisin and ramoplanin activating the P_{liaI} promoter[7]. Using a semi-quantitative disk diffusion assay (Fig. 3) and a quantitative β-galactosidase assay (Fig. 4), we could show that only DAP and not FRI is able to induce the P_{liaI}-*lacZ* reporter system. These results are in agreement with the proteomic response and underline that both related antibiotics have a distinct mode of action.

Results and Discussion

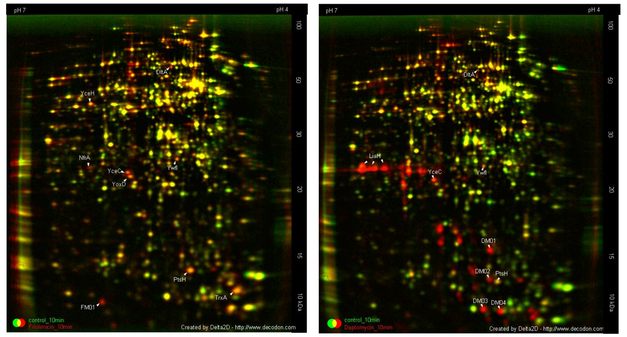

Fig. 2 Dual channel images of radioactively labelled proteins of *B. subtilis* 168 synthesised before (green image) and 10 min after exposure (red image) to FRI (left) and DAP (right)

A. Proteins induced by Friulimicin B and Daptomycin		
Protein	**Function / Functional category**	**Known Inducer**
DltA	D-alanyl-D-alanine carrier protein ligase involved D-alanylation of teichoic acids[9]	
PtsH	histidine-containing phosphocarrier protein of the PTS (HPr protein)[10]	Bacitracin[10]
YceC	unknown; similar to tellurium resistance protein	Bacitracin, Vancomycin[11]
Ywfl	unknown; similar to unknown proteins	
B. Proteins exclusively induced by Daptomycin		
Protein	**Function / Functional category**	**Known Inducer**
LiaH	Involved in maintenance of cell membrane integrity, induced by antibiotics that interfere with lipid II cycle[7]	Bacitracin[11]
C. Proteins exclusively induced by Friulimicin B		
Protein	**Function / Functional category**	**Known Inducer**
NfrA	FMN-containing NADPH-linked nitro/flavin reductase	
TrxA	Thioredoxin involved in membrane bioenergetics	
YceH	unknown; similar to toxic anion resistance protein	Bacitracin, Vancomycin[11]
YoxD	unknown; similar to 3-oxoacyl-acyl-carrier protein reductase	

Table 1 Marker proteins induced by FRI and/or DAP

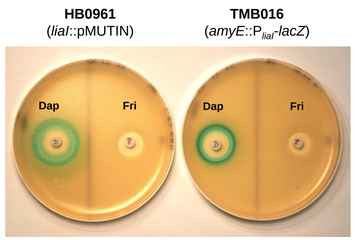

Fig. 3: Disk diffusion assay using P_{liaI}-*lacZ* reporter strains *B. subtilis* HB0961 and TMB016 after addition of DAP and FRI

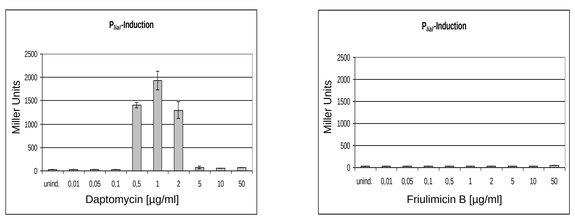

Fig. 4 β-galactosidase assay[7] using P_{liaI}-*lacZ* reporter strains *B. subtilis* HB0961 after addition of DAP and FRI

Conclusions

- Proteome induction patterns and the response of the P_{liaI}-lacZ reporter system suggest that both antibiotics, FRI and DAP, target the cell envelope but display a distinct molecular mode of action.
- These results are consistent with the described MOA of DAP (cell membrane interaction)[8] and the MOA of FRI (complex formation with bactoprenol-phosphate leading to the interruption of peptidoglycan and teichoic acid biosynthesis)[12].
- Based on the distinct molecular MOA for FRI and DAP one would not expect appearance of target based cross-resistance.

Literature

[1] P. Bremer, et al., *AAC* 47 (2003) 3025-3029.
[2] M. Kresken, et al., Poster F1-1642, this ICAAC 2007
[3] P. McGhee, et al., Poster F1-1648, this ICAAC 2007
[4] R. Schaumann, et al., Poster F1-1644, this ICAAC 2007
[5] J. Stülke, et al., *J. Gen. Microbiol.* 139 (1993) 2041-2045
[6] T. Mascher, et al., *Mol. Microbiol.* 50 (2003) 1591-1604
[7] T. Mascher, et al., *AAC* 48 (2004) 2888-2896
[8] J.A. Silverman, et al., *AAC* 47 (2003) 2538-2544
[9] M. Perego, et al., *J. Biol. Chem.* 270 (1995) 15598-15606
[10] M. Cao, et al., *Mol. Microbiol.* 45 (2002) 1267-1276
[11] J.E. Bandow, et al., *AAC* 47 (2003) 948-955
[12] T. Schneider, et al., Poster F1-1640, this ICAAC 2007

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Comparative In Vitro Activities of the Novel Antibacterial Friulimicin B and Other Antibacterial Agents Against Selected Aerobic Gram-positive Bacteria

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Revised Abstract

Background: Friulimicin B (FRI) is a novel lipopeptide antibacterial agent. The objective of the present study was to determine the *in vitro* activities of FRI against 242 Gram-positive cocci.

Methods: Organisms tested were methicillin-susceptible *S. aureus* (MSSA), methicillin-resistant *S. aureus* (MRSA), *S. epidermidis*, *E. faecalis* incl. 6 VREf, *E. faecium* incl. 8 VREm, *S. pneumoniae* incl. 26 PNSP, and *S. pyogenes*. Antibacterial agents tested were FRI, daptomycin (DAP), linezolid (LZD), vancomycin (VAN) and other drugs. The CLSI broth microdilution method was used to determine MICs for all bacterial isolates. MICs of FRI and DAP for 20 isolates were also determined using the CLSI agar dilution method. Furthermore, the impact of polysorbate 80 (0.002%) and human serum (50% v/v) on the MICs of FRI was investigated against 25 isolates.

Results: Applying the broth microdilution method, MICs of FRI ranged from 0.5 to 8 mg/L. $MIC_{50/90}$s (mg/L) are given in the Table.

	FRI	DAP	VAN	LZD
MSSA (n=25)	2 / 2	1 / 2	1 / 1	2 / 2
MRSA (n=27)	2 / 4	1 / 2	1 / 2	2 / 4
S. epidermidis (n=22)	2 / 2	1 / 1	2 / 2	1 / 2
E. faecalis (n=30)	8 / 8	2 / 4	2 / ≥128	2 /2
E. faecium (n=25)	4 / 8	4 / 8	1 / ≥128	2 / 2
S. pneumoniae (n=56)	1 / 2	0.5 / 1	0.5 / 0.5	1 / 1
S. pyogenes (n=57)	4 / 8	0.25 / 0.5	0.5 / 0.5	1 / 2

Adding polysorbate 80 to Mueller-Hinton broth gave 1 dilution step lower MICs of FRI for 8/25 strains, whereas the addition of human serum elevated the MICs of all strains by 1-3 dilution steps. MICs of both FRI and DAP were consistently lower when the agar dilution method was applied. Compared to DAP, FRI had a comparable spectrum of activity, but MIC_{90} values were nearly always 1 dilution step higher than those of DAP, except for *S. pyogenes* (4 dilution steps higher MICs of FRI).

Conclusions: FRI appears to be a promising new antimicrobial agent for the treatment of infections caused by Gram-positive bacteria including multi-resistant isolates.

Introduction

Friulimicin B (FRI) is a novel lipopeptide antibacterial agent, that exhibits potent activity against a variety of Gram-positive bacteria[1-4]. Its chemical structure is shown in **Figure 1.** FRI shows a structural similarity to the lipodepsipeptide daptomycin (DAP), but its molecular mode of action is different[5]. In contrast to the membrane interfering DAP, FRI inhibits late-stage cell wall synthesis[6-7].

The present study was performed to evaluate the comparative *in vitro* activities of FRI and other antibacterial agents against a panel of clinical isolates of major aerobic Gram-positive bacteria susceptible or resistant to conventional antibacterial agents. Furthermore, the impact of the surfactant polysorbate 80 (0.002%) and human serum (50% v/v) on the MICs of FRI was investigated.

Methods

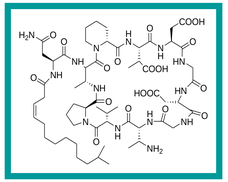
Bacterial strains

A total of 242 aerobic Gram-positive cocci collected from microbiology laboratories during various multi-centre studies conducted between 2001 and 2004 in Germany were tested: 30 *E. faecalis* (including six VRE), 25 *E. faecium* (including eight VRE), 52 *S. aureus* (25 MSSA, 27 MRSA), 22 *S. epidermidis* (11 MSSE, 11 MRSE), 56 *S. pneumoniae* (including 41 penicillin-non-susceptible and/or erythromycin-resistant strains), and 57 *S. pyogenes* (including 26 erythromycin-resistant strains).
All isolates were identified by routine laboratory methods and stored at -80°C until studied.

Antibacterial agents

Antibacterial agents tested were
- friulimicin B,
- ampicillin,
- daptomycin (DAP),
- erythromycin,
- linezolid,
- oxacillin,
- penicillin, and
- vancomycin.

Fig. 1: Friulimicin B



Determination of antimicrobial susceptibility

The CLSI broth microdilution procedure with geometric twofold serial dilutions in cation-adjusted Mueller-Hinton broth (CAMHB) purchased from Becton Dickinson (Heidelberg, Germany; BBL™ Cation Adjusted Mueller Hinton II Broth, lot no. 6317238, 20-25 mg Ca^{2+}/L) was used to determine MICs against all bacterial isolates[8]. MIC plates were prepared in-house. For testing of FRI and DAP, the calcium concentration of the test solution was adjusted to 50 mg/L Ca^{2+}.
MICs of FRI and DAP for 24 isolates were also determined using the CLSI agar dilution method[8]. Mueller Hinton agar purchased from Becton-Dickinson (Difco™ Mueller Hinton Agar, lot no. 6093202) was used as a nutrient medium. The calcium concentration of the test agar was adjusted to 50 mg/L Ca^{2+}. MICs of each strain were determined at least twice.
Furthermore, using the broth microdilution method, the effect of polysorbate 80 (0.002%) and human serum (50% v/v) on the MICs of friulimicin B was investigated against 25 isolates.
The accuracy of susceptibility testing was evaluated by MIC testing of four quality control organisms: *E. faecalis* ATCC 29212, *S. aureus* ATCC 29213, *S. aureus* ATCC 43300 (MRSA), and *S. pneumoniae* ATCC 49619 (**Table 1**).

Results

Comparative in vitro activity of friulimicin B using the broth microdilution method

Comparative activities of friulimicin B against the 242 aerobic Gram-positive cocci tested are shown in **Table 2.** Friulimicin B was consistently active against all pathogens, regardless of resistance to comparator agents. Overall, MIC values ranged from 0.5 to 8 mg/L.

In vitro activities of FRI and DAP using the agar dilution method

The *in vitro* activities of FRI and DAP were determined at least twice for five strains each of *S. aureus*, *S. epidermidis*, and *E. faecium* and *E. faecalis*. The results are shown in **Table 3**. MICs of both compounds were consistently lower than those determined with the broth microdilution method. Each compound inhibited all staphylococci at 1 mg/L and all enterococci at 4 mg/L.

Results

Impact of polysorbate 80 and human serum on the in vitro activity of FRI

Five strains each of *S. aureus*, *S. epidermidis*, *E. faecalis*, *E. faecium* and *S. pyogenes* were tested.
The effect of polysorbate 80, at a concentration of 0.002%, showed either no or a minor impact on the MICs of FRI (**Table 4**).
Sixteen and eight strains exhibited unchanged and one dilution step lower MICs, respectively, whereas there was one *S. epidermidis* strain with a two-fold increase in the MIC. The effect of normal human serum upon the activity of FRI against the 25 strains is also shown in **Table 4**. There was a decrease in activity in the presence of 50% (v/v) human serum against all strains, with MICs for 20 strains increasing more than fourfold.

Table 1: MICs of friulimicin B for quality control strains

Organism	MIC (mg/L)	
	Broth microdilution	Agar dilution
E. faecalis ATCC 29212	8	4-8
S. aureus ATCC 29213	2-4	1
S. aureus ATCC 43300 (MRSA)	2	0.5-1
S. pneumoniae ATCC 49619	0.25-1	not tested

Table 2: In vitro activity of friulimicin B in comparison to other antimicrobial agents against 242 aerobic gram-positive bacterial isolates using the CLSI broth microdilution method

Organism (Number of strains tested)	Antimicrobial agent	MIC (mg/L)		
		Range	MIC_{50}	MIC_{90}
Staphylococcus aureus, methicillin-susceptible (25)	Friulimicin B	1-4	2	2
	Daptomycin	1-4	1	2
	Linezolid	1-4	2	2
	Vancomycin	0.5-2	1	1
Staphylococcus aureus, methicillin-resistant (27)	Friulimicin B	1-4	2	4
	Daptomycin	1-2	1	2
	Linezolid	1-4	2	4
	Vancomycin	0.5-2	1	2
Staphylococcus epidermidis, methicillin-susceptible (11)	Friulimicin B	1-2	2	2
	Daptomycin	1	1	1
	Linezolid	1-2	1	2
	Vancomycin	1-2	1	2
Staphylococcus epidermidis, methicillin-resistant (11)	Friulimicin B	2	2	2
	Daptomycin	1	1	1
	Linezolid	0.25-2	1	1
	Vancomycin	1-2	2	2
Enterococcus faecalis (30)	Friulimicin B	4-8	8	8
	Daptomycin	1-4	2	4
	Linezolid	1-4	2	2
	Vancomycin	1 - ≥ 128	2	≥ 128
	Ampicillin	0.5-4	1	2
Enterococcus faecium (25)	Friulimicin B	2-8	4	8
	Daptomycin	2-8	4	8
	Linezolid	1-2	2	2
	Vancomycin	0.5 - ≥ 512	1	≥ 128
	Ampicillin	2-128	64	128
Streptococcus pneumoniae (56)	Friulimicin B	0.5-4	1	2
	Daptomycin	0.5-1	0.5	1
	Linezolid	0.25-2	1	1
	Vancomycin	0.25-0.5	0.5	0.5
	Erythromycin	0.031-> 64	32	≥ 64
	Penicillin	≤ 0.016-4	0.063	2
Streptococcus pyogenes (57)	Friulimicin B	1-8	4	8
	Daptomycin	0.125-0.5	0.25	0.5
	Linezolid	0.5-2	1	2
	Vancomycin	0.25-1	0.5	0.5
	Erythromycin	0.031-> 64	0.063	≥ 64
	Penicillin	≤ 0.016	≤ 0.016	≤ 0.016

Table 3: In vitro activity of friulimicin B and daptomycin against 20 aerobic bacterial isolates using the CLSI agar dilution method

Organism	Phenotype*	No. of tests	Mean MIC (mg/L)	
			Friulimicin B	Daptomycin
***Staphylococcus aureus* (n=5)**				
Staphylococcus aureus 710-1-22	MS	2	0.75	0.375
Staphylococcus aureus 710-1-23	MS	2	0.75	0.75
Staphylococcus aureus 710-1-11	MR	2	1.5	0.5
Staphylococcus aureus 710-1-49	MR	3	1	1
Staphylococcus aureus 710-1-78	MR	2	1	0.75
***Staphylococcus epidermidis* (n=5)**				
Staphylococcus epidermidis 720-1-34	MS	2	0.75	1
Staphylococcus epidermidis 720-1-40	MS	3	0.67	0.83
Staphylococcus epidermidis 720-1-42	MR	2	0.75	0.5
Staphylococcus epidermidis 720-1-2	MR	3	1	0.83
Staphylococcus epidermidis 720-1-1	MR	2	1.5	0.5
***Enterococcus faecalis* (n=5)**				
Enterococcus faecalis 810-1-36	VS	2	4	1
Enterococcus faecalis 810-1-6	VS	2	4	2
Enterococcus faecalis 810-1-28	VR	3	1.33	0.5
Enterococcus faecalis G10-65	VS	2	4	1
Enterococcus faecalis 810-1-65	VR	2	4	1
***Enterococcus faecium* (n=5)**				
Enterococcus faecium L7-10-70	VS	3	3.33	2.67
Enterococcus faecium L7-09-03	VS	3	2	4
Enterococcus faecium 820-1-4	VR	2	2	3
Enterococcus faecium 820-1-5	VR	2	2	3
Enterococcus faecium 820-1-14	VR	3	1	4

*MS, methicillin-susceptible; MR, methicillin-resistant; VS, vancomycin-susceptible; VR, vancomycin-resistant

Table 4: Effect polysorbate 80 (0.002%) and/or human serum (50% v/v) on broth microdilution MICs of friulimicin B

Organism	Phenotype*	MIC (mg/L)		
		Broth	Broth and polysorbate 80	Broth and human serum
***Staphylococcus aureus* (n=5)**				
Staphylococcus aureus 710-1-22	MS	2	1	8
Staphylococcus aureus 710-1-23	MS	4	2	16
***Staphylococcus aureus*, resistant to methicillin (n=3)**				
Staphylococcus aureus 710-1-11	MR	4	2	16
Staphylococcus aureus 710-1-49	MR	4	2	16
Staphylococcus aureus 710-1-78	MR	4	4	32
***Staphylococcus epidermidis*, susceptible to methicillin (n=2)**				
Staphylococcus epidermidis 720-1-34	MS	2	2	8
Staphylococcus epidermidis 720-1-40	MS	1	2	4
***Staphylococcus epidermidis*, resistant to methicillin (n=3)**				
Staphylococcus epidermidis 720-1-42	MR	2	2	8
Staphylococcus epidermidis 720-1-2	MR	4	2	8
Staphylococcus epidermidis 720-1-1	MR	2	2	16
***Enterococcus faecalis* (n=5)**				
Enterococcus faecalis 810-1-36	VS	4	4	32
Enterococcus faecalis 810-1-6	VS	8	8	32
Enterococcus faecalis 810-1-28	VR	4	2	32
Enterococcus faecalis 810-1-65	VR	8	4	32
Enterococcus faecalis 810-1-71	VR	8	8	32
***Enterococcus faecium* (n=5)**				
Enterococcus faecium L7-10-70	VS	4	4	32
Enterococcus faecium L7-09-03	VS	4	2	8
Enterococcus faecium 820-1-4	VR	4	4	16
Enterococcus faecium 820-1-5	VR	4	4	32
Enterococcus faecium 820-1-14	VR	4	4	16
***Streptococcus pyogenes* (n=5)**				
Streptococcus pyogenes 920-3-58	ES	1	1	4
Streptococcus pyogenes 920-2-9	ES	4	4	8
Streptococcus pyogenes 920-1-10	ER	4	4	8
Streptococcus pyogenes 920-1-16	ER	4	4	8
Streptococcus pyogenes 920-1-28	ER	1	1	4

*MS, methicillin-susceptible; MR. methicillin-resistant; VS, vancomycin-susceptible; VR, vancomycin-resistant; ES, erythromycin-susceptible; ER, erythromycin-resistant

Conclusions

- Overall, the spectrum of activity of FRI resembles that of DAP.
- Using the broth microdilution method, MICs of FRI were nearly always one dilution step higher than those of DAP, with MIC_{90} values of FRI for staphylococci, enterococci and streptococci ranging between 2 and 8 mg/L.
- Using the agar dilution method we found a trend towards lower MICs compared to the broth microdilution method and the differences in the MICs between FRI and DAP were less striking.
- Polysorbate 80 at a concentration of 0.002% had either no or a minor effect on the MICs of FRI.
- In the presence of 50% (v/v) human serum, a decrease in activity of FRI against all strains was observed.
- Activity of FRI was very uniform independent of resistance phenotype against marketed antibiotics.
- FRI appears to be a promising new antimicrobial agent for the treatment of infections caused by Gram-positive organisms including isolates that possess resistances to currently available drugs.

Literature

[1] Bemer P, Juvin ME, Bryskier A, Drugeon H. In vitro activities of a new lipopeptide, HMR 1043, against susceptible and resistant gram-positive isolates. Antimicrob Agents Chemother 2003;47:3025-9.
[2] Schaumann R, Adler D, Pelzer S, Labischinski H, Rodloff AC. Activity of friulimicin and five other antimicrobial agents against 179 gram-positive obligatory anaerobic bacteria. 47th ICAAC, Chicago 2007, Poster No. F1-1644
[3] McGhee P, Bogdanovic T , Credito K, Labischinski H, Appelbaum PC. Activity of friulimicin against glycopeptide and daptomycin non-susceptible *S .aureus*. 47th ICAAC, Chicago 2007, Poster No. F1-1648
[4] Schubert S, Dahlhoff A, Pelzer S, Labischinski H. Comparative Analysis of the bactericidal activities of friulimicin, daptomycin, tigecycline, and vancomycin against difficult to treat Isolates of *S. aureus* and *S. pneumoniae*. 47th ICAAC, Chicago 2007, Poster No. F1-1649
[5] Zuehlke D, Voigt B, Hecker M, Jordan S, Mascher T, Pelzer S, Labischinski H. Distinct mode of action of the lipopeptide antibiotic friulimicin and the lipodepsipeptide daptomycin: a proteomic study. 47th ICAAC, Chicago 2007 Poster No. F1-1641
[6] Silverman JA, Perlmutter NG, Shapiro HM. Correlation of daptomycin bactericidal activity amd membrane depolarization in *Staphylococcus aureus*. Antimicrob Agents Chemother 2003; 47: 2538-2544
[7] Schneider T, Gries K, Wiedemann I, Pelzer S, Labischinski H, Sahl HG. Friulimicin Inhibits Cell Wall Biosynthesis through Complex Formation with Bactoprenol-Phosphate. 47th ICAAC, Chicago 2007 Poster No. F1-1640
[8] National Committee for Clinical Laboratory Standards (NCCLS). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved Standard - 6th Edition. M7-A6. National Committee for Clinical Laboratory Standards, Wayne, Pa., 2003.

47th ICAAC,
Chicago 2007

F1-1643

Comparative *in vitro* Activity of the Novel Lipopeptide Friulimicin B with Daptomycin; the Effect of Inoculum, Pulmonary Surfactant and Calcium

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Revised Abstract

Background: Friulimicin B (FRI), an acidic, cyclic lipopeptide, is intended for the treatment of severe infections caused by Gram-positive pathogens. FRI shows structural similarities with the lipodepsipeptide daptomycin (DAP). We compared their *in vitro* activities against selected aerobic Gram-positive bacteria under different test conditions.

Methods: CLSI broth microdilution (BMD) method for DAP was used to compare the compounds using Mueller Hinton (MH) broth including 50 mg/L Ca^{2+} and 0.002% Tween 80. Ca^{2+} dependency, inoculum effect and influence of pulmonary surfactant (Survanta®) was measured using MH broth containing 50 mg/L Ca^{2+}.

Results: MIC determination of FRI is strongly influenced by the procedure and parameters used. The optimal procedure for MIC determination of FRI is BMD with MH supplemented with 50mg/L Ca^{2+} and addition of 0.002% Tween 80. MIC values against selected aerobic Gram+ bacteria were comparable to DAP (see Table 1). MIC of DAP against *S. aureus* increased 8-fold with increasing inoculum density from 4.7 x 10^3 to 4 x 10^7 CFU/mL. In contrast, the *in vitro* activity of FRI stayed constant across a similar range of inocula. The pulmonary surfactant had only a marginal effect on the activity of FRI against *S. aureus,* in contrast the activity of DAP was decreased 32-fold.

Conclusions: The *in vitro* activity of FRI against Gram+ pathogens is Ca^{2+} dependent and at least comparable to DAP using an optimized CLSI BMD method. In contrast to DAP, activity of FRI is not significantly inhibited by high inoculum or pulmonary surfactant.

Introduction

Friulimicin B (FRI, Fig. 1) is a novel lipopeptide antibiotic that is produced by *Actinoplanes friuliensis.* FRI is structurally similar with the lipodepsipeptide daptomycin (DAP), but has a distinct molecular mode of action[1]. In contrast to the membrane interfering DAP[2] FRI inhibits late-stage cell wall synthesis[3].

FRI displays a similar antibacterial activity spectrum to DAP addressing all important Gram-positive pathogens such as staphylococci, enterococci, pneumococci (including multi-resistant and DAP-resistant strains)[4-6] and obligatory anaerobic bacteria[7].

The purpose of the studies presented here, was to compare the influence of various test parameters, like the inoculum titre, the presence of Ca^{2+}, polysorbate 80 (Tween 80) and lung surfactant on the *in vitro* activity of FRI and DAP.

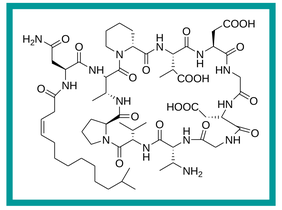

Fig. 1 Friulimicin B

Methods

Bacterial strains:
A total of 33 Gram-positive aerobes, comprising clinical isolates and quality control strains were used for the development and validation of MIC methods: 6 *E. faecalis* (including 2 VanB); 3 *E. faecium* (1 VanA, 1 VanB); 13 *S. aureus* (4 MSSA, 9 MRSA, 6 GISA); 8 coagulase-negative *Staphylococci* (1 hGISE); 2 *S. epidermidis* (1 FQR); 1 *S. haemolyticus* (hGIS).

Susceptibility tests:
MICs were determined by standard broth microdilution (BMD) in cation-adjusted Mueller-Hinton broth (CAMHB) according to CLSI guidelines[8]. For standard testing of FRI and DAP the calcium content of MHB was adjusted to a final concentration of 50 mg/L Ca^{2+} yielding $CAMHB_{50}$. For the FRI reference method $CAMHB^{50}$ was supplemented with 0.002% (v/v) polysorbate 80 (Tween 80). The influence of pulmonary surfactant on the antibacterial activity of FRI and comparator drugs was determined by BMD, supplementing $CAMHB_{50}$ with Survanta® (Abbott GmbH & Co. KG, Wiesbaden, Germany) to final concentrations of 0.25%, 0.5%, 1%, 5%, 10%, and 15% (v/v). Due to the turbidity of the medium at concentrations > 1% (v/v) Survanta®, MIC values were read as colour change from yellow to purple-blue, after the addition of thiazolyl blue tetrazolium bromide (MTT, final concentration 0.5 mg/ml) to the test wells and a further incubation at 35˚C for 45 min.
FRI was obtained from Combinature Biopharm AG, Berlin, Germany, and other drugs from their respective manufacturers.

Results and Discussion

Influence of Ca^{2+} concentrations on MIC
The antibacterial activity of FRI and DAP against *S. aureus* ATCC 29213 and *E. faecium* ATCC 19434 was strongly dependent on the presence of free Ca^{2+} ions. At Ca^{2+} concentration of 50 mg/L (corresponding to physiological concentration in blood), FRI displayed strong activity which reached its maximum at 100 mg Ca^{2+}/L (Table 1). For DAP, the highest activity against *E. faecium* was reached at 200 mg Ca^{2+}/L.

Bacterial Strain	Antibiotic	Ca^{2+} concentration [mg/L]				
		<20	20	50	100	200
S.aureus ATCC 29213	Friulimicin B	4	4	2	2	2
	Daptomycin	2	2	0.5	0.25	0.25
	Vancomycin	0.5	0.5	0.5	0.5	0.5
E. faecium ATCC 19343	Friulimicin B	8	8	4	2	2
	Daptomycin	16	16	4	2	1
	Vancomycin	0.5	0.5	0.5	0.5	0.5

Table 1: MICs (mg/L) at different Ca^{2+} concentrations

Optimal procedure for MIC determination of FRI
MIC determination of FRI is complex, since results are strongly influenced by the method (agar dilution showed a tendency to lower values against aerobic Gram-positive compared to BMD) and by the final concentration of Ca^{2+} and Tween 80. The optimal procedure (reference method) for determining the MIC of FRI is BMD with MH broth supplemented with Ca^{2+} of 50 mg/L and 0.002% (v/v) Tween 80[9]. Under these conditions FRI MIC values against 33 selected aerobic Gram-positive bacteria are comparable with DAP (Table 2).

Results and Discussion

Species	Organism	Comment	FRI	DAP
E. faecalis	EFE 763		1	4
E. faecalis	EFE 764		1	4
E. faecalis	EFE 94		2	1
E. faecalis	EFE 100	VanB	2	2
E. faecalis	EFE 101	VanB	1	4
E. faecalis	ATCC 29212		2	2
E. faecium	EFM 504		1	4
E. faecium	EFM 178	VanB	1	4
E. faecium	EFM 65	VanA	0,25	1
S. aureus	CDC 2161		0,25	0,125
S. aureus	CDC 2195		0,25	0,5
S. aureus	SCQ 21		0,25	0,5
S. aureus	ATCC 29213		0,25	0,5
S. aureus	SCP 504	GISA, MRSA	2	8
S. aureus	SCP 508		4	8
S. aureus	SCP 505		0,5	1
S. aureus	SCP 546	GISA	2	4
S. aureus	ATCC 43300		0,5	0,5
S. aureus	ATCC 700698		1	0,5
S. aureus	ATCC 700699	GISA, MRSA	2	2
S. aureus	New Jersey 992	hGISA, MRSA	2	1
S. aureus	Michigan 963	hGISA, MRSA	2	2
S. coag-	COR 29	MR	0,25	0,5
S. coag-	COR 30	MR	0,25	0,5
S. coag-	COR 31	MR	0,25	0,5
S. coag-	COR32	MR	0.5	0,5
S. coag-	COR 43	MR	0,5	0,5
S. coag-	COR 53	MR	0,5	2
S. coag-	COS 1		0,5	0,5
S. coag-	Wisconsin 759	hGISE	0,5	1
S. epidermidis	SEP 188		0,5	0,5
S. epidermidis	SEP 104	FQR	0,25	0,5
S. haemolyticus	Xiong 12	hGIS	0,25	0,25

Table 2: *In vitro* activity (MIC, mg/L) of FRI (BMD, reference method) compared to DAP (CLSI method)

Inoculum effect
MIC values of FRI and Vancomycin against *S. aureus* ATCC 29213 stayed constant at inoculum densities ranging from 4 x10^1 to 1 x 10^7 CFU/ml. However, an 8-fold increase in DAP MIC with increasing inoculum densities from 4.7 x 10^3 to 4 x 10^7 (Figure 2) was observed.

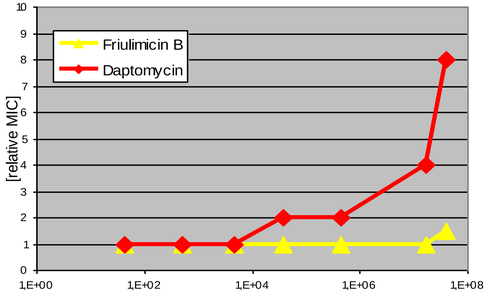

Fig. 2: Inoculum effect using *S. aureus* ATCC 29213

Influence of lung surfactant
The antibacterial activity of DAP is diminished by interaction with pulmonary surfactants which is the reason why DAP can not be used in community acquired pneumonia. The *in vitro* inhibition of antibacterial activity of FRI by pulmonary surfactant was much less pronounced than DAP which decreased 256-fold (Figure 3).

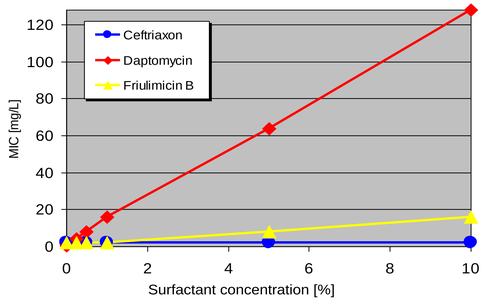

Fig. 3: MIC values against *S. aureus* ATCC 29213 in the presence of surfactant

Conclusions

- BMD using MH broth containing 50 mg/L Ca^{2+} and 0.002% (v/v) Tween 80 is the optimal procedure to determine MICs for FRI
- Use of FRI reference method gives MICs against 33 aerobic Gram-positive bacteria comparable to DAP
- In contrast to DAP, FRI is not significantly inhibited by high inoculum densities or the presence of high pulmonary surfactant concentrations

Literature

[1] D. Zuehlke, et al., Poster F1-1643, this ICAAC 2007
[2] J.A. Silverman et al., *AAC* 47 (2003) 2538-2544.
[3] T. Schneider, et al., Poster F1-1640, this ICAAC 2007
[4] P. Bremer, et al., *AAC* 47 (2003) 3025-3029.
[5] M. Kresken, et al., Poster F1-1642, this ICAAC 2007
[6] P. McGhee, et al., Poster F1-1648, this ICAAC 2007
[7] R. Schaumann, et al., Poster F1-1644, this ICAAC 2007
[8] National Committee for Clinical Laboratory Standards (NCCLS). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved Standard - 6th Edition. M7-A6. National Committee for Clinical Laboratory Standards, Wayne, Pa., 2003
[9] A. Engelhardt et al., Poster F1-1646, this ICAAC 2007

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Chicago 2007

F1-1644

Activity of Friulimicin B and Five Other Antimicrobial Agents against 179 Gram-Positive Obligatory Anaerobic Bacteria

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Revised Abstract

Background: Friulimicin B (FRI), an acidic cyclic lipopeptide, is intended for the treatment of severe infections caused by resistant Gram-positive pathogens. FRI shows structural similarities with daptomycin (DAP). We tested the activity of FRI compared with various other antimicrobials against 179 strains of Gram-positive obligately anaerobic bacteria.

Methods: FRI was compared with DAP, metronidazole (MET), moxifloxacin (MOX), linezolid (LIN) and vancomycin (VAN) against the following strains: *Clostridum difficile* (n=52), *Clostridium perfringens* (n=34), *Finegoldia magna* (n=14), *Peptostreptococcus* spp. (n=22), *Micromonas micros* (n=13), *Propionibacterium acnes* (n=19), *Lactobacillus* spp. (n=9), and *Eubacterium* spp. (n=16). MICs were determined employing the microdilution technique in Wilkens-Chalgren broth supplemented with vitamin K_1 and haemin. In addition, MICs of FRI were determined by agar dilution according to CLSI standard M11-A6.

Results: While the broth microdilution yielded reproducible results, the activity of FRI in agar medium was more erratic. The MIC_{50} and MIC_{90} values for broth microdilution are listed in Table 1.

Conclusions: FRI has promising activity against several pathogenic species of anaerobes.

Introduction

Friulimicin B (FRI, Figure 1), an acidic cyclic lipopeptide, is intended for the treatment of severe infections caused by resistant Gram-positive pathogens[1-4]. FRI shows structural similarities to daptomycin, a lipodepsipeptide (DAP) but has been shown to have a different mode of action[5-6].

We tested the activity of FRI compared with various other antimicrobials against 179 strains of Gram-positive obligately anaerobic bacteria.

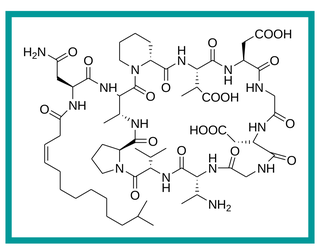


Figure 1 Friulimicin B

Methods

Bacterial Strains
179 Gram-positive anaerobes were taken from the culture collection from the Institute for Medical Microbiology and Epidemiology of Infectious Diseases, University of Leipzig, Germany. The strains were collected from clinical specimens at the Institute and from national and international studies and obtained in part from other laboratories. The following strains were used: *Clostridium difficile* (n=52), *C. perfringens* (n=34), *Finegoldia magna* (n=14), *Peptostreptococcus* spp. (n=22), (*P. anaerobius*, n=13; *P. asaccharolyticus*, n=6; *P. indolicus*, n=1; *P. prevotii*, n=2), *Micromonas micros* (n=13), *Propionibacterium acnes* (n=19), *Lactobacillus* spp. (n=9), (*L. acidophilus*, n=5; *L. casei*, n=2; *L. jensenii*, n=1; *Lactobacillus* spp. n=1), and *Eubacterium* spp. (n=16), (*E. aerofaciens*, n=5; *E. lentum*, n=9; *E. plautii*, n=1; *Eubacterium* spp. n=1). *S. aureus* ATCC 29213, *C. perfringens* ATCC 13124 and *E. lentum* ATCC 43055 were used as reference strains.

Antimicrobial Agents
Antimicrobial agents were obtained as laboratory powders of known potency from the manufacturers: FRI from Combinature Biopharm, Germany, DAP from Cubist Pharmaceuticals, Lexington, USA; metronidazole (MET) from Sigma Chemical Co., St. Louis, USA; moxifloxacin (MOX) from Bayer Vital GmbH, Leverkusen, Germany; linezolid (LIN) from Pharmacia & Upjohn Co., Kalamazoo, USA; and vancomycin (VAN) from Sigma Chemical Co., St. Louis, USA, respectively.

Broth microdilution MIC determinations
Tests were performed according to the recommendations of the Deutsches Institut für Normung (DIN) and standard DIN 58940-83[7]. The bacterial inocula were prepared by suspending growth from 24, 48 and 72 hour cultures grown on supplemented Columbia blood agar, respectively, (depending on the species) in Wilkins-Chalgren broth supplemented with vitamin K_1 and haemin. After semi-automated inoculation (Dynatech MIC-2000-inoculator, Dynatech Laboratories, Inc., Chantilly, USA) resulting in a final dilution of approximately 1.0×10^5 CFU/well (1.0×10^6 CFU/ml), plates were incubated for 48 h at 37°C in an anaerobic chamber. The MIC was defined as the lowest antibiotic concentration that inhibited visible growth. In addition, MICs of FRI were also determined by agar dilution according to CLSI standard M11-A6[8].

Results and Discussion

Organism (no. of strains tested)	FRI	DAP	MET	MOX	LIN	VAN
C. difficile (52)	0.125/0.25	0.25/1	0.125/0.5	1/16	0.5/2	0.5/1
C. perfringens (34)	1/2	0.5/2	2/4	0.5/1	2/2	1/1
F. magna (14)	0.25/1	0.5/1	0.5/4	0.125/2	2/2	0.25/0.5
Peptostreptococcus spp. (22)	0.25/1	0.25/1	0.25/1	0.125/1	1/1	0.5/0.5
M. micros (13)	1/2	0.5/2	0.25/>32	0.125/0.25	0.25/2	1/2
P. acnes (19)	1/2	1/2	>32/>32	0.25/0.25	0.5/1	1/1
Lactobacillus spp. (9)	2/4	2/8	>32/>32	1/2	4/8	4/>32
Eubacterium spp. (16)	8/>32	8/>32	1/32	0.5/2	4/32	4/16

Table 1 MIC_{50}/MIC_{90} (mg/L) of antimicrobials against anaerobes

Results and Discussion

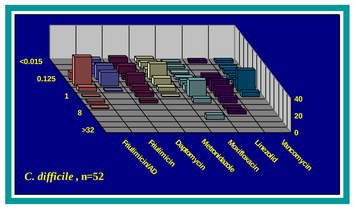
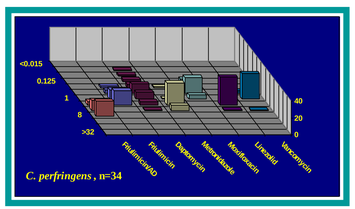


Figure 2



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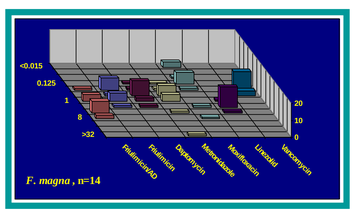


Figure 4

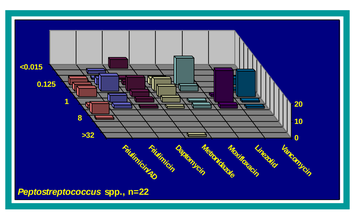
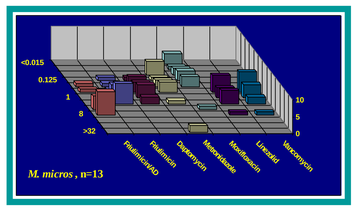


Figure 5



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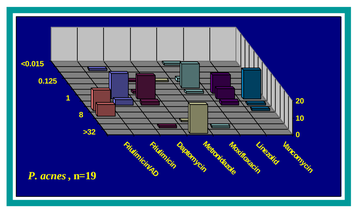

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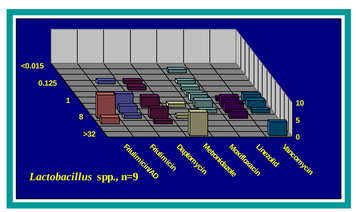

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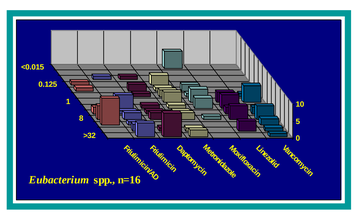

Figure 9

The figures 2-9 show the scatter histograms of MIC values obtained for FRI and the five other antimicrobial agents against 179 Gram-positive obligately anaerobic bacteria included in this study. The results obtained by testing in agar are indicated as Friulimicin/AD in each histogram.

FRI was particularly active against *C. difficile* (Figure 2), *F. magna* (Figure 4), and *Peptostreptococcus* species (Figure 5), where it was equal to or more active than DAP and metronidazole.

While results using the broth microdilution technique were reproducible, the activity of FRI in agar medium was more erratic.

Overall the *in-vitro* activity of FRI seems to be better than the *in-vitro* activity of DAP.

The MIC values against eubacteria (Figure 9) seem to display a bimodal distribution indicating that some strains are significantly less susceptible to FRI as well as to DAP.

Conclusions

- The novel lipopeptide friulimicin B has excellent activity against several pathogenic species of anaerobes.
- Friulimicin B has good activity against *C. difficile* and was more active than all 5 comparator drugs. *C. difficile* infections are now a major problem in many hospitals and institutions and friulimicin B could be a valuable agent for their treatment.
- Overall *in vitro* activity of friulimicin B compares favourably with that of daptomycin, metronidazole and moxi-floxacin.

Literature

[1] P. Bremer, et al., *AAC* 47 (2003) 3025-3029
[2] M. Kresken, et al., Poster F1-1642, this ICAAC 2007
[3] P. McGhee, et al., Poster F1-1648, this ICAAC 2007
[4] S. Schubert, et al., Poster F1-1649, this ICAAC 2007
[5] T. Schneider, et al., Poster F1-1640, this ICAAC 2007
[6] D. Zuehlke, et al., Poster F1-1641, this ICAAC 2007
[7] DIN, Deutsches Institut für Normung e.V. Medizinische Mikrobiologie und Immunologie: Diagnostische Verfahren. Berlin, Wien, Zürich: Beuth Verlag, 2004.
[8] Clinical and Laboratory Standards Institute (formerly NCCLS). Methods for antimicrobial susceptibility testing of anaerobic bacteria: Approved standard M11-A6. Wayne, PA, 2004

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Resistance Studies with Friulimicin B and Daptomycin

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Revised Abstract

Background: Friulimicin B (FRI), an acidic cyclic lipopeptide, is intended for the treatment of severe infections caused by resistant Gram-positive pathogens and shows structural similarities with daptomycin (DAP). We compared the *in vitro* emergence of resistance to both drugs using single-step and serial passage.
Methods: Spontaneous mutation frequencies were determined by plating 100µL of *Staphylococcus aureus*, *Enterococcus faecalis* and *Streptococcus pneumoniae* (1 - 4 x 10^{10} CFU/mL) onto agar containing 2 - 128x MIC of DAP or FRI. Mutants were isolated by serial passage (26 times) in liquid culture containing FRI or DAP at 0.5 - 4x MIC. 100µL of each passage was plated onto agar containing multiples of the MIC. Stability was confirmed after 3 passages on drug-free agar. Susceptibility testing was performed on all stable mutants in Mueller-Hinton containing 50mg/L Ca^{2+} and 0.002% Tween-80 (for FRI only) using CLSI guidelines.
Results: Mutation frequencies for *S. aureus* were < 8 x10^{-11} (FRI) and 5.8 x10^{-9} (DAP) and for *E. faecalis* < 1 x10^{-10} (to both) at 4x MIC after 24 h incubation. For *S. pneumoniae* resistance frequency to FRI was 8.1 x10^{-10}, no frequency for DAP could be detected due to confluent growth (4 - 16x MIC, 48 h). FRI mutants were selected between passages 4 - 14 with MICs 2 - 8x greater than the parent. DAP mutants with MIC increase of 2 - 16x were selected in the same period. Higher level DAP mutants (64 - 128x parent MIC) were isolated between 16-26 subcultures but none with FRI after 12 passages.
Conclusions: Selection of mutants to both drugs following the first 4 passages suggested resistance mechanism(s) involving cumulative events. Selection of DAP resistance occurred within a wider window than for FRI and proceeded to higher levels, suggesting that FRI has the lower potential for resistance development of the two drugs.

Introduction

Friulimicin B (FRI, Fig. 1) is a novel lipopeptide antibiotic that is produced by *Actinoplanes friuliensis*. FRI is structurally similar to the lipodepsipeptide daptomycin (DAP), but has a distinct molecular mode of action[1-2]. It displays good *in vitro* activity against a range of important Gram-positive pathogens such as staphylococci, enterococci and pneumococci[3-5], including multi-resistant strains.

Resistance frequencies and serial passage experiments can provide valuable insight into the potential for resistance development to an antibiotic of interest. Here, we report FRI mutation frequencies, determined in several Gram-positive pathogens and a detailed comparison of the kinetics of FRI and DAP resistance development in *S. aureus*.

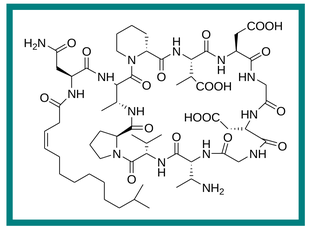

Fig. 1 Friulimicin B

Methods

Resistance frequencies were determined after plating concentrated cultures of test organism onto calcium (50mg/L) supplemented cation-adjusted Mueller-Hinton agar ($CAMHA_{50}$) containing test compound at multiples of the MIC.

FRI and DAP mutants of *S. aureus* ATCC 29213 were generated through daily serial passage in calcium (50mg/L) supplemented cation-adjusted Mueller-Hinton broth ($CAMHB_{50}$) containing test compound at multiples of the MIC and plating on agar containing the same drug concentration range. Stability was confirmed on drug containing agar following 3 passages on drug-free agar.

DAP MICs were determined by standard broth microdilution (BMD) according to CLSI guidelines[6]. FRI MICs were determined by E-test and (BMD) reference methods[7-8]. Minimum bactericidal concentrations (MBC) were determined by performing viable counts on non-growing wells from BMD MIC experiments. The MBC was defined as the minimum concentration of drug that killed 99.9% of the inoculum of 5 x10^5 CFU/ml.

Growth rates of mutants were determined in a Molecular Devices Versimax 96-well plate reader.

Thickness of cell walls in 40 cells were measured from digital transmission electron micrographs taken from osmium tetroxide stained samples of selected mutants.

Results and Discussion

Spontaneous resistance frequencies to FRI were lower than for DAP in *S. aureus* at 2x and 4x MIC (Table 1). These were undetectable in *E. faecalis* apart from at 2x MIC of DAP after 48h. Resistance frequencies to FRI were low in *S. pneumoniae* (8.1 x10^{-10}) at 4x MIC after 48h incubation but frequencies to DAP were not possible to calculate due to confluent growth on $CAMHA_{50}$ up to 16x MIC.

Figure 2 illustrates the changes in FRI and DAP susceptibility that occurred in *S. aureus* over 26 subcultures:

- Low-level (2 – 8-fold MIC increase) FRI mutants arose between 4 and 14 subcultures – no further increase in MIC was observed despite a further 12 subcultures.
- Low level DAP mutants arose between 3 -14 subcultures. This was followed by a period between 14-20 subcultures where DAP MIC against isolated mutants rapidly increased to 64 mg/L (256-fold MIC increase).

This step-wise decrease in susceptibility to DAP and FRI suggest that these events were cumulative and occurred with equal readiness throughout 14 subcultures. The decrease in susceptibility appeared to be limiting under FRI selection (no further MIC increase was observed) whereas DAP susceptibility continued to decrease under DAP selection pressure.

Microbiological characteristics of mutants isolated from different stages of the FRI subculture were investigated (Table 2). Some mutants produced smaller colonies but no decrease in gentamicin (GEN) susceptibility (which is an indicator of the small-colony variant phenotype) was observed.

Results and Discussion

		Incubation time					
		24 h			48 h		
Strain	Antibiotic	2x MIC	4x MIC	8x MIC	2x MIC	4x MIC	8x MIC
S. aureus ATCC 29213	FRI	4.5x10^{-10}	< 8x10^{-11}	< 8x10^{-11}	6.7x10^{-10}	< 8x10^{-11}	< 8x10^{-11}
	DAP	CG	5.8x10^{-9}	< 8x10^{-11}	CG	7.2x10^{-9}	< 8x10^{-11}
E. faecalis ATCC 29212	FRI	< 1x10^{-10}	< 1x10^{-10}	< 1x10^{-10}	< 1x10^{-10}	< 1x10^{-10}	< 1x10^{-10}
	DAP	< 1x10^{-10}	< 1x10^{-10}	< 1x10^{-10}	3.1x10^{-10}	< 1x10^{-10}	< 1x10^{-10}

		Incubation time 48h					
Strain	Antibiotic	4x MIC	8x MIC	16x MIC	32x MIC	64x MIC	128x MIC
S. pneumoniae ATCC 33400	FRI	8.1x10^{-10}	< 8.1x10^{-10}	ND	ND	ND	ND
	DAP	GC	CG	CG	1.68x10^{-8}	4.05x10^{-10}	< 8.1x10^{-10}

Table 1 Resistance rates of *S. aureus* ATCC 29213, *E. faecalis* ATCC 29212 and *S. pneumoniae* ATCC 33400 (48h only) for FRI and DAP at multiples of the MIC. (CG. confluent growth).

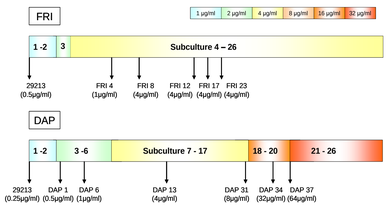

Fig. 2 Reduction in FRI (top) and DAP (bottom) susceptibility in *S. aureus.* Coloured bars represent the highest concentration of drug at which the indicated subculture could grow. Arrows show the point where MIC increases were detected. MICs were determined by broth microdilution. Those FRI mutants which were studied in more details are also highlighted.

Mutant	Subculture Number	Colony Size	FRI MIC (µg/ml)	DAP MIC (µg/ml)	VAN MIC (µg/ml)	FRI MIC / MBC ratio	GEN MIC (µg/ml)	Time taken to reach max growth rate (h)	Max growth rate (mOD600nm Units/h)
Parent	0	+++	0.25	0.125	0.75	1	0.25	2.7	70 ± 1.9
FRI 1	4	+++	1.5	1	3	2	0.25	4.2	64.2 ± 5.9
FRI 2	5	+++	1	0.5	1.5	1	0.25	3.1	102.1 ± 9.2
FRI 3	6	+++	1	0.5	2	2	0.25	3.4	98.7 ± 6.1
FRI 4	6	+++	1	0.5	2	1	0.25	3.2	97.8 ± 12.2
FRI 7	7	+++	1	0.75	2	1	0.25	3.1	90.2 ± 17.6
FRI 8	9	++	3	1.5	1.5	1	0.25	8.0	66.7 ± 5.5
FRI 11	9	+	4	1.5	1.5	1	0.25	7.7	68.6 ± 3.9
FRI 12	12	+	4	1.5	2	2	0.25	8.1	69.8 ± 6.8
FRI 15	12	++	2	1.5	2	2	0.25	5.6	76 ± 5.3
FRI 17	13	++	3	1.5	2	2	0.25	6.0	77.1 ± 5.8
FRI 19	13	+++	3	1.5	3	2	0.25	5.3	72 ± 2.3
FRI 20	14	+	3	1.5	3	2	0.125	9.4	46.3 ± 7.8
FRI 21	14	+++	3	1.5	1.5	1	0.25	5.0	78.7 ± 4
FRI 23	14	+++	3	1.5	3	1	0.25	5.1	78.1 ± 9.3

Table 2 Microbiological characteristics of FRI mutants isolated between subculture 4 -14. FRI, DAP and VAN MICs were determined by E-test. No distinct resistant colonies could be isolated at greater concentrations for a further 12 subcultures.

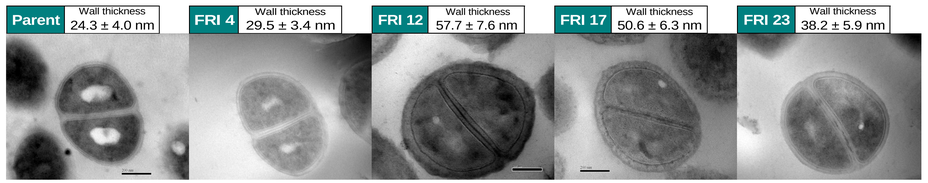

Fig. 3 Transmission electron micrographs of *S. aureus* 29123 and mutants with decreased FRI susceptibility. Wall thicknesses were averaged from 40 measurements

Several mutants had slightly raised FRI MBC/MIC ratios (increased from 1 to 2) but this did not indicate an increase in the level of tolerance to FRI (Table 2).

Transmission electron micrographs (Fig. 3) of these populations revealed that the mutants are surrounded by thicker cell walls than the parent.

Increased wall thickness (and reduced transglycosylation) has been observed in laboratory and clinical isolates of VISA and other strains of reduced VAN susceptibility[9-10]. The mechanism behind this peculiar phenotype (tentatively named HISA for intermediate susceptibility vs. high-molecular weight inhibitors against *S. aureus*[11]) could be reduced diffusion through thickened cell walls[12].

The FRI mutants described here demonstrated a slight reduced susceptibility to VAN and DAP (Table 2), resembling the HISA phenotype and were at the lower end of VISA classification (of VAN MIC ≥4µg/ml). This implies that additional mutational events may be required for generation of the full VISA phenotype which did not occur under selective pressure from FRI.

Conclusions

- Mutation frequencies in *S. aureus, E. faecalis* and *S. pneumoniae* were lower for FRI than for DAP
- Low-level mutants of DAP and FRI were selectable for 14 subcultures after which point higher-level DAP mutants were isolated whereas no further decrease in FRI susceptibility occurred
- The low-level FRI mutants that were isolated displayed a slight decreased susceptibility to DAP and VAN and had thicker cell walls than the parent (HISA phenotype)
- The low mutation frequency of FRI combined with the absence of high-level mutants in a population subcultured 26 times indicate that there is a low potential for FRI resistance development in *S. aureus*

References

[1] D. Zuehlke, et al., Poster F1-1641, this ICAAC 2007
[2] T. Schneider, et al., Poster F1-1640, this ICAAC 2007
[3] M. Kresken, et al., Poster F1-1642, this ICAAC 2007
[4] H. Priefert, et al., Poster F1-1643, this ICAAC 2007
[5] R. Schaumann, et al., Poster F1-1644, this ICAAC 2007
[6] National Committee for Clinical Laboratory Standards (NCCLS). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved Standard - 6th Edition. M7-A6. National Committee for Clinical Laboratory Standards, Wayne, Pa., 2003
[7] A. Engelhardt et al., Poster F1-1645, this ICAAC 2007
[8] A. Engelhardt et al., Poster F1-1646 , this ICAAC 2007
[9] K. Hiramatsu, et al., *AAC* 50 (2006) 428-438
[10] P. Pereira, et al., *AAC* EPUB (2007)
[11] P. McGhee, et al., Poster F1-1648, this ICAAC 2007
[12] L. Cui, et al., *AAC* 50 (2006) 1079-1082

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Activity of Friulimicin B Against Glycopeptide and Daptomycin Non-susceptible *S. aureus*

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Revised abstract

Background: Friulimicin B (FRI), an acidic cyclic lipopeptide intended for therapy of severe drug-R Gram-pos. infections, is structurally similar to daptomycin (DAP) with a different molecular target. We tested FRI, DAP, vancomycin (VAN), teicoplanin (TEC), quinupristin-dalfopristin (Q/D), linezolid (LIN) against a spectrum of 19 mostly difficult to treat *S. aureus* strains and one DAPR VRE.

Methods: Three DAPR clinical strains (1 from Hershey which developed from MRSA to VISA while the patient was on VAN) had DAP MICs 4-8 µg/ml and 6 lab DAPR clones obtained by multistep R selection had DAP MICs ≥16 µg/ml. VAN-non-S strains were 3 VRSA and 2 VISA. MIC was by CLSI agar dilution (+ Ca^{2+} for FRI, DAP). Time-kills were in CAMHB (CLSI), final inocula 5 x 10^5 to 5 x 10^6 cfu/ml. In addition a DAPR clinical *E. faecium* isolate was included in the analysis.

Results: MICs (µg/ml) against the 5 VAN-S MRSAs were: FRI, DAP, VAN, TEC, Q/D all 0.5-1 µg/ml, LIN 4 µg/ml. MICs (µg/ml) against 9 DAPR strains were 4-16 (FRI); 2-16 (VAN); 2-32 (TEC); 0.12-0.5 (Q/D); 1-4 (LIN). Against defined VISA strains, FRI and DAP MICs (µg/ml) were 8 and 4, resp., and against VRSA were 2-4 and 0.5-1, resp. VAN MICs were 4->32 µg/ml, TEC MICs 4->32 against VISA and VRSA. MICs of Q/D and LIN against VISA and VRSA were 0.25-0.5, 2-4. resp. Time-kills (MIC/2xMIC/4xMIC) showed that FRI and DAP were bactericidal (99.9% killing) at 4 x MIC after 24 h against 7/9 and 6/7 DAPR isolates, respectively, and against all 5 VAN-non-S strains. VAN and TEC were cidal against 1 isolate each. TEC, Q/D and linezolid were static against all strains. MICs (µg/ml) against the DAPR *E. faecium* isolate were 2 (FRM); 16 (DAP); >64 (VAN); 16 (TEC); 1 (Q/D); 1 (LIN). No killing at all was observed for TEC, Q/D, and LIN. DAP was bactericidal at 8 x MIC (128 µg/ml), a clinically non-relevant concentration.

Conclusions: FRI and DAP were very active against VRSA (0.5 - 4 µg/ml); MICs against DAPR- and VISA strains were 8-16, 4-8 µg/ml, resp. FRI was cidal against 7/9 of DAPR and reduced the CFU of the DAPR *E. faecium* isolate by 99% after 24 hrs at 16 µg/ml (8 x MIC).

Introduction

FRI (Fig. 1) is a novel lipopeptide antibiotic that is produced by *Actinoplanes friuliensis*. FRI is structurally similar to the lipodepsipeptide DAP, but has a distinct molecular mode of action. It displays good *in vitro* activity against a range of important Gram-positive pathogens such as staphylococci, enterococci and pneumococci[1-3], including multi-resistant strains.

Recent reports have described staphylococcal and enterococcal clinical strains with reduced sensitivity vs. DAP, and , in the case of VISA strains, a correlation between DAP, VAN and other high molecular weight antibiotics was observed[4-6]. Here we report on the susceptibility of such strains vs. FRI and other antibiotics using MIC and time kill-experiments.

Methods

Bacterial strains:
Organisms tested comprised 9 strains pre-qualified as DAP resistant (3 clinical, the remainder obtained by prior resistance selection studies), 2 pre-qualified as vancomycin-intermediate strains (VISA) (including one isolated in our hospital which developed vancomycin and DAP resistance after sequential treatment with both of these drugs[6]), 3 vancomycin resistant strains (VRSA) (including the Hershey strain) and 5 vancomycin susceptible methicillin-resistant strains (MRSA). The DAP resistant VRE strain *E. faecium* J4026[7] was kindly provided by J. H. Jorgensen, University of Texas. FRI was obtained from Combinature Biopharm AG. Berlin, Germany and other drugs from their respective manufacturers.

Susceptibility tests:
MICs were predetermined by macrodilution in cation-adjusted Mueller-Hinton broth (BBL Microbiology Systems, Cockeysville, MD) according to standard methodology. DAP and FRI susceptibility testing was in MH-broth adjusted to 50 µg/ml of calcium per standard methodology and glycopeptide MICs were read after 24 h. All strains were tested by the time-kill method with each compound alone as described previously[8].

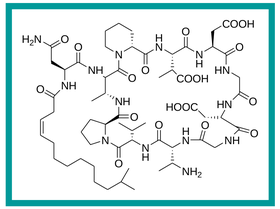


Fig. 1: Friulimicin B

Results

MICs (µg/ml) against the 9 DAP resistant isolates (Table 1) were 4-16 FRI; 2-16 vancomycin; 2-32 teicoplanin; 0.125-0.5 quinupristin/dalfopristin; 1-4 linezolid. Against VISA strains, FRI and DAP MICs (µg/ml) were both 4-8, and against VRSA they were 2-4 and 0.5-1, respectively. For the *E. faecium* VRE isolate FRI and DAP MICs were 2 and 16 µg/ml, respectively. Vancomycin MICs were 4->64 µg/ml and teicoplanin MICs were 4->32 against VISA, VRSA, and VRE. MICs of quinupristin/dalfopristin and linezolid against VISA and VRSA were 0.25-0.5 and 2-4 respectively. Based on these results, 11 out of the 19 *S. aureus* strains would have to be qualified as DAP resistant, 4 as VISA and 6 as VRSA. It should be realized that the classification of glycopeptide non-susceptible *S. aureus* strains is still in a state of flux and method-dependent, with a possible one dilution MIC difference potentially changing the classification of specific strains according to the method used.

Time-kill studies (Fig. 2, Table 2) on 14 strains for FRI and 12 for DAP (same strains but without strains Mut3 and Mut5) showed that FRI and DAP were bactericidal at 4 x MIC after 24 h against 7/9 and 6/7 isolates reported previously as DAP resistant isolates, respectively, and against all 5 vancomycin-non-susceptible strains.

Results

Strain	Pre-qualified resistotype	Determined resistotype	FRI	DAP	VAN	TEC	Q/D	LIN
SA212	MRSA	VSSA	1	0.5	1	0.5	1	4
SA238	MRSA	VSSA	0.5	1	0.5	0.5	0.5	4
SA487	MRSA	VSSA	0.5	0.5	1	0.5	0.5	4
SA490	MRSA	VSSA	1	1	1	0.5	0.5	4
SA495	MRSA	VSSA	1	0.5	1	1	0.5	4
SA506	MRSA, VISA	VISA, DAPR	8	4	4	8	0.25	4
SA507	MRSA, VISA	VISA, DAPR	4	4	4	4	0.5	2
SA509	MRSA, VRSA	VRSA	2	1	16	32	0.5	4
SA510	MRSA, VRSA	VRSA	4	0.5	32	16	0.5	4
SA512	MRSA, VRSA	VRSA	2	0.5	>32	>32	0.5	4
SA555	MRSA, DAPR	VISA, DAPR	8	4	8	32	0.125	4
SA560	MRSA, DAPR	VSSA, DAPR	8	2	2	4	0.125	4
SA562	MRSA, DAPR	VSSA, DAPR	8	2	2	2	0.125	4
SA Mut1	MRSA, DAPR	VRSA, DAPR	16	32	16	32	0.25	1
SA Mut3	MRSA, DAPR	VISA, DAPR	16	>32	8	8	0.5	4
SA Mut4	MRSA, DAPR	VSSA, DAPR	4	64	2	2	0.25	2
SA Mut5	MRSA, DAPR	VSSA, DAPR	8	64	2	8	0.125	4
SA Mut8	MRSA, DAPR	VRSA, DAPR	16	16	16	32	0.5	2
SA Mut9	MRSA, DAPR	VRSA, DAPR	16	32	16	16	0.5	1
EFJ4026	VRE, DAPR	VRE, DAPR	2	16	>64	16	1	1

Breakpoints used: vancomycin (S, I, R) ≤ 2, 4-8, ≥ 16, daptomycin (S, R) ≤1, >1 (for *S. aureus*).

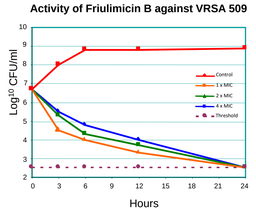
Table 1: MICs (µg/ml) of all agents against strains tested

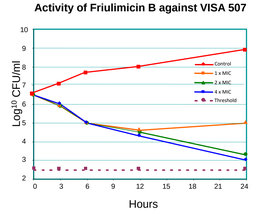
Drug	3 h			6 h			12 h			24 h		
% killing	90[a]	99[a]	99.9[a]	90[a]	99[a]	99.9[a]	90[a]	99[a]	99.9[a]	90[a]	99[a]	99.9[a]
Friulimicin B												
4 x MIC	5[b]	1	0	12	4	0	13	12	7	13	13	12
2 x MIC	4	1	0	11	4	0	13	11	3	13	13	11
MIC	2	1	0	10	3	0	12	6	2	12	6	6
Daptomycin												
4 x MIC	10	4	2	12	10	8	12	12	12	12	12	12
2 x MIC	7	3	1	11	9	6	12	12	9	12	12	11
MIC	4	0	0	10	7	2	12	9	6	10	7	4
Vancomycin												
4 x MIC	0	0	0	0	0	0	5	0	0	7	5	1
2 x MIC	0	0	0	0	0	0	5	0	0	8	5	1
MIC	0	0	0	0	0	0	4	0	0	7	4	1
Teicoplanin												
4 x MIC	1	0	0	1	1	0	6	2	1	10	2	1
2 x MIC	1	0	0	1	1	0	6	2	1	10	2	1
MIC	1	0	0	1	0	0	3	1	0	9	0	0
Q/D												
4 x MIC	2	0	0	5	0	0	7	2	0	10	2	0
2 x MIC	0	0	0	4	0	0	7	2	0	9	2	0
MIC	0	0	0	1	0	0	6	1	0	5	1	0
Linezolid												
4 x MIC	0	0	0	0	0	0	3	0	0	7	1	0
2 x MIC	0	0	0	0	0	0	2	0	0	6	1	0
MIC	0	0	0	0	0	0	1	0	0	2	1	0

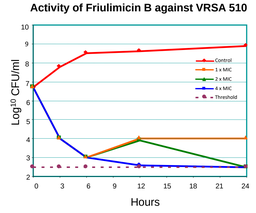
a: % killing, b: No. of strains killed

Please note that 14 *S. aureus* strains were tested against friulimicin B and 12 strains (same strain set, but without strains Mut3 and Mut5) in case of daptomycin

Table 2: Results of time-kill experiments







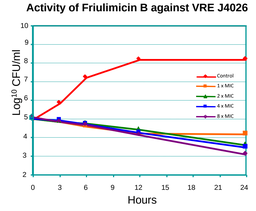


Fig. 2: Selected kill curves

Vancomycin and teicoplanin were bactericidal against 1 isolate each. Teicoplanin, quinupristin/dalfopristin and linezolid were bacteriostatic against all isolates.

The current study shows that FRI MICs fell in at least 3 distinct categories when compared to DAP:

i) Classical MRSA strains (marked in red in Table 1) with low FRI MICs, essentially identical to those of DAP

ii) VISA and other strains with reduced vancomycin sensitivity in which, as a rule all high molecular weight compounds show reduced susceptibility (marked in yellow). The mechanism behind this peculiar phenotype (tentatively named HISA for intermediate susceptibility vs. high-molecular weight inhibitors against *S. aureus*) could be reduced diffusion through thickened cell walls[9-11]

iii) Strains with strongly reduced sensitivity to either DAP or FRI, but no cross-resistance, as expected for compounds of distinct mode of action (marked in blue)

Conclusions

- Standard MRSA strains have good susceptibility to friulimicin B and to daptomycin.
- Strains ("HISA") with reduced susceptibility to all high molecular weight inhibitors irrespective of molecular mode of action were observed
- The mechanism behind HISA could be thickened cell walls as first described by Hiramatsu, Cui, and co-workers
- Kill kinetics of friulimicin B and daptomycin were similar, relative to MICs
- Friulimicin B at 8 x MIC led to a 24 hrs CFU reduction by 99% for a daptomycin resistant VRE isolate

Literature

[1] M. Kresken, et al., Poster F1-1642, this ICAAC 2007
[2] H. Priefert, et al., Poster F1-1643, this ICAAC 2007
[3] R. Schaumann, et al., Poster F1-1644, this ICAAC 2007
[4] L. Cui, et al., *AAC* 50 (2006) 1079-1082
[5] J. B. Patel, et al., *CID* 42 (2006) 1652-1653
[6] K. Julian, et al., *AAC* (2007) in press
[7] J.S. Lewis II, et al., *AAC* 49 (2005) 1664-1665
[8] C. L. Clark, et al., *AAC* 42 (1999) 2295 – 2298
[9] W. Stubbings, et al., Poster F1-1647, this ICAAC 2007
[10] G. Sakoulas, et al., *AAC* 50 (2006) 1581-1585
[11] L. Cui, et al., *AAC* 50 (2006) 428-438

47th ICAAC,
Chicago 2007

F1-1649

Comparative Analysis of the Bactericidal Activities of Friulimicin B, Daptomycin, Tigecycline, and Vancomycin against Difficult to Treat Isolates of *S. aureus* and *S. pneumoniae*

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Revised Abstract

Background: Friulimicin B is a lipopeptide antibiotic with a novel mode of action; it is active against multidrug-resistant (MDR) Gram-positive bacteria including MRSA, VISA, and MDR- *S. pneumoniae*. We studied the bactericidal kill constants of friulimicin B (FRI) in comparison with daptomycin (DAP), vancomycin (VAN), and tigecycline (TIG) against *S. aureus* (Sa) and *S. pneumoniae* (Spn).
Methods: Six strains with defined resistances against methicillin, and/or VAN, linezolid, ciprofloxacin (Sa) or penicillin, macrolide, ciprofloxacin (Spn) and susceptible or resistant ATCC reference strains were used. All strains were grown in cation-adjusted MH-broth plus 0.002% Tween 80 + 50mg/L Ca^{2+} under batch-culture conditions and were exposed to multiples of their individual MICs (1, 4, 8, 16 times). Cultures were inoculated with approx $1X10^6$ CFU/ml. Samples were taken at 0, 1, 2, 4, 6, 8, 24h to determine CFUs. During the initial log-linear phase of CFU-decline single point kill rates and times needed for 3log- and 6log- kill were calculated. Drug-free cultures served as controls.
Results: Phenotypically, FRI and DAP exerted the most pronounced bactericidal effect against Sa and Spn. The single point kill rates of FRI and DAP increased over the entire concentration range. The bactericidal activity was independent of the resistance genotype and MDR-phenotype. FRI exerted a concentration-dependent 6log kill against Sa within 10-24h. Activity against Spn was not concentration dependent. DAP eliminated Sa more rapidly but was less active against Spn. TIG and VAN were only bacteristatic.
Conclusions: The bactericidal activity of FRI against selected difficult to treat or MDR *S. aureus* and *S. pneumoniae* is independent of methicillin-, vancomycin-, linezolid-, quinolone-, penicillin-, or macrolide resistance. These characteristics may make FRI attractive for the therapy of infections in patients with critical illnesses.

Introduction

Friulimicin B (FRI, Fig. 1) is a natural compound produced by *Actinoplanes friuliensis;* it belongs to a novel class of lipopeptide antibiotics. It has structural similarities with daptomycin (DAP) but has been shown to have a different mode of action [1,2].

FRI inhibits the late stage of cell wall synthesis producing a potent and broad spectrum activity against all relevant Gram positive pathogens, while no DAP cross resistance occurs. Thus it is destinated for the treatment of infections caused by Gram-positive bacteria including but not limited to MRSA and VRE.

The aim of this study was to determine the *in vitro* bactericidal activity of FRI against a panel of Gram-positive bacteria susceptible or resistant to comparator drugs. This was done by determining time-kill curves with selected indicator strains with clinically relevant susceptibility/ resistance patterns (i.e. ß-lactam-, vancomycin-, linezolid- and quinolone resistance). Thus, the strains studied - apart from the susceptible reference strains used as controls - represent a number of pathogens which are difficult to treat in the clinic.

Methods

Test strains and susceptibility to standard drugs
Time-kill experiments were performed with the following panel of 6 strains:
Staphylococcus aureus (Sa) ATCC 29213: Methicillin-susceptible (MSSA) wild type reference strain for resistance testing, (vancomycin-, linezolid- and ciprofloxacin-susceptible)
Staphylococcus aureus (Sa) ATCC 33593: Methicillin-resistant (MRSA) reference strain,
(vancomycin-, linezolid- and ciprofloxacin-susceptible)
Staphylococcus aureus (Sa) NRS 119: Methicillin-resistant isolate (MRSA), (vancomycin susceptible, linezolid- and ciprofloxacin-resistant)
Staphylococcus aureus (Sa) VISA Mu 50: Vancomycin-intermediate (VISA), methicillin-resistant clinical isolate (vancomycin-intermediate, linezolid-susceptible, ciprofloxacin-resistant)
Streptococcus pneumoniae (Sp) ATCC 49619: Reference strain, clinical isolate, (penicillin-, vancomycin-, linezolid- and ciprofloxacin-susceptible)
Streptococcus pneumoniae (Sp) Bay 19397: Fluoroquinolone resistant laboratory strain (penicillin-resistant, vancomycin- and linezolid-susceptible, ciprofloxacin-resistant, *gyrA* mutation)
The strains were stored frozen at – 80°C in a volume of 100 µl.

Antibacterial agents tested
The agents tested in comparison with FRI (supplied by Combinature Biopharm AG, Berlin) were DAP (lot CDF 002/8, Novartis Pharma GmbH, Basel, CH), TIG (lot 24715, Wyeth-Lederly Pharma GmbH, Muenster, D), and VAN (lot 41354 TB 21,Hikma Pharma GmbH).

MIC determinations
MIC testing was done using a microdilution method according to CLSI (formerly NCCLS) guideline [3]. MICs were determined in Ca-supplemented (50mg/L) MH-broth plus 0.002% Tween 80 (CAMHB-50 + tween).

Time-Kill curve kinetics
Kill curve kinetics were done by using a slightly modified method according to CLSI (formerly NCCLS) guidelines [4] and as described by [5].

Mathematical models used for the calculation of kill-kinetics
The bactericidal effect was analyzed by using models as described recently by Schaper *et al* [6]. The basis is the assumption of a first-order exponential decrease with time of the number N of viable cells (CFU/mL) exposed to a drug at a certain concentration C (using multiples x of the MIC: C=x*MIC with x=1, 4, 8, 16, and 32). Single point kill rates (k) were calculated by k= -(ln(N/No))/t; in addition, the concentrations needed for 3log kill (C3log) were calculated (t½ = (ln2) / k). For clinical microbiologists the more relevant time to obtain a decrease in the initial number of CFU from No to No/1,000 can be calculated by the more general equation t½ = (ln f) / k with f = 1,000.

Results and Discussion

MICs (Table 1)

In CAMHB-50 + tween the VISA-strain was inhibited by 0.5 mg/L of FRI, the two reference strains by 0.25 to 1.0 mg/L, and the MDR strain NRS119 by 0.5 mg/L. The two *S. pneumoniae* strains were inhibited by FRI concentrations of 1.0 and 0.5 mg/L. The MICs of the comparators were within the same range.

Results and Discussion

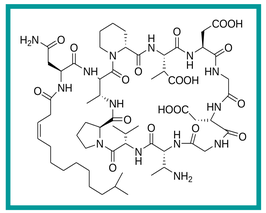

Fig. 1 Friulimicin B

Organism/Strain	MIC mg/L			
	FRI	DAP	VAN	TIG
Sa – MRSA ATCC 33593	0.5-1	0.5	1	0.5
Sa – MSSA ATCC 29213	0.25-0.5	0.5	1	0.5
Sa – VISA Mu 50	0.5	1	2	1-2
Sa – MRSA NRS 119	0.5	0.5	0.13	0.25
Sp – ATCC 49619	1	0.13	0.13	0.25
Sp – Bay 19397	0.5	0.5	0.5	4

Table 1 MIC values of compounds against the test strains (2 experiments)

Calculated single point kill rates (Table 2)
The single point kill rates calculated for FRI clearly demonstrate that the kill rates increase concentration dependently. The viable counts and thus the kill rates, too, of two strains only, *S. pneumoniae* ATCC 49619 and *S. pneumoniae* 19397 were independent of concentration.
Likewise, the single point kill rates calculated for DAP increase concentration dependently for all strains tested.
The VAN single point kill rates are small, thus indicating an almost bacteriostatic action in particular against *S. aureus.*
TIG kill rates are small, with the exception of *S. pneumoniae* BAY 19397, and almost concentration independent.

	Multiple of MIC			
	1	4	8	16
Friulimicin B (1 - 4 h)				
Sa ATCC 33593	1.61	1.91	1.96	2.11
Sa ATCC 29213	1.42	1.20	1.57	1.91
Sa VISA Mu50	1.32	1.54	1.40	1.62
Sa NRS 119	0.43	0.55	0.66	0.71
Sp ATCC 49619	1.14	1.01	0.86	0.87
Sp BAY 19397	1.48	1.19	1.23	1.33
Daptomycin (0 - 2 h)				
Sa ATCC 33593	0.6	1.87	2.64	3.74
Sa ATCC 29213	0.92	2.86	3.50	3.77
Sa VISA Mu50	0.566	2.32	2.422	4.20
Sa NRS 119	0.458	0.222	1.169	3.090
Sp ATCC 49619	1.738	2.93	2.88	3.594
Sp BAY 19397	1.908	1.87	2.14	2.328
Tigecycline (1 - 6 h)				
Sa ATCC 33593	0.016	0.019	0.030	0.098
Sa ATCC 29213	0.067	0.11	0.078	0.18
Sa VISA Mu50	0.27	0.36	0.26	0.25
Sa NRS 119	+ 0.15	0.14	0.03	0.09
Sp ATCC 49619	0.67	0.75	0.75	0.89
Sp BAY 19397	0.75	1.03	1.10	1.40
Vancomycin (1 - 6 h)				
Sa ATCC 33593	0.37	0.46	0.49	0.49
Sa ATCC 29213	0.47	0.60	0.54	0.56
Sa VISA Mu50	0.16	0.20	0.002	0.16
Sa NRS 119	0.75	0.76	0.77	0.82
Sp ATCC 49619	0.09	0.48	0.61	0.58
Sp BAY 19397	0.08	0.72	0.88	0.90

Table 2 Calculated Single point kill rates

	Multiple of MIC			
	1	4	8	16
Friulimicin B				
Sa ATCC 33593	4.28	3.61	3.53	3.27
Sa ATCC 29213	4.86	5.75	4.39	3.61
Sa VISA Mu50	5.22	4.48	4.93	4.26
Sa NRS 119	16.05	12.50	10.45	9.72
Sp ATCC 49619	6.05	6.83	8.02	7.93
Sp BAY 19397	4.66	5.79	5.61	5.18
Daptomycin				
Sa ATCC 33593	11.5	3.69	2.61	1.85
Sa ATCC 29213	7.5	2.41	1.97	1.83
Sa VISA Mu50	12.19	2.97	2.85	1.64
Sa NRS 119	15.06	31.08	5.90	2.23
Sp ATCC 49619	3.97	2.35	2.39	1.92
Sp BAY 19397	3.61	3.69	3.22	2.96
Tigecycline				
Sa ATCC 33593	> 24	> 24	> 24	> 24
Sa ATCC 29213	> 24	> 24	> 24	> 24
Sa VISA Mu50	> 24	19.16	> 24	> 24
Sa NRS 119	> 24	> 24	> 24	> 24
Sp ATCC 49619	10.29	9.20	9.20	7.75
Sp BAY 19397	9.20	6.69	6.27	4.93
Vancomycin				
Sa ATCC 33593	18.65	15.0	14.08	14.08
Sa ATCC 29213	14.68	11.5	12.78	12.32
Sa VISA Mu50	> 24	> 24	> 24	> 24
Sa NRS 119	9.20	9.08	8.96	8.41
Sp ATCC 49619	> 24	14.37	11.31	11.89
Sp BAY 19397	> 24	9.58	7.85	7.67

Table 3 Time (hours) for a 3log reduction in viability

Calculated periods of time needed for 3 log kill (Table 3)

The times needed for a reduction of viable counts of the four *S. aureus* test strains by 3log titres decrease with increasing concentrations of FRI; the times needed for a reduction of *S. pneumoniae* by 3 log titres are independent from the FRI concentration.
Likewise, the times needed for a reduction of viable counts of all six indicator organisms by 3log titres decrease with increasing DAP concentrations.
However, the times needed for 3log kill of the four *S. aureus* test strains at 1xMICs of FRI and DAP, respectively, are significantly different. FRI at 1x MIC reduced the viable counts of *S. aureus* much more rapidly than DAP.
VAN and TIG reduced the viable counts of the 6 indicator organisms by 3log titres very slowly, if at all.

Summary

The bactericidal activity of FRI was concentration-dependent against these difficult to treat or MDR *S. aureus* and *S. pneumoniae* and is independent from methicillin-, vancomycin-, linezolid-, quinolone-, penicillin-, or macrolide resistance.
At a concentration of 16 x MIC of FRI a regrowth of the test strains, except *S. aureus* ATCC 33593, was prevented. Therefore, resistance to FRI cannot develop under these test conditions.
DAP also had a concentration dependent bactericidal effect. The DAP kill rates were higher than those for FRI, indicating a stronger bactericidal effect BUT the 2 *S. pneumoniae* strains were not eliminated, so that in principle development of resistance may occur.
The bactericidal activities of FRI and DAP, differed significantly at 1xMIC. FRI exhibited a stronger bactericidal effect against *S. aureus* at low drug concentrations than DAP did. The time needed for a reduction of viable counts of *S. aureus* by 3 orders of magnitude (except the multi-resistant *S. aureus* NRS 119) were 2- 3 times smaller upon exposure to FRI than following an exposure to DAP.

Conclusions

- The concentration-dependent bactericidal activity of FRI against selected difficult to treat or MDR *S. aureus* and *S. pneumoniae* is independent of methicillin-, vancomycin-, linezolid-, quinolone-, penicillin- or macrolide resistance
- The pronounced bactericidal activity of FRI as opposed to the moderate activity of DAP **at low drug concentrations** may be of clinical relevance. Both agents are relatively highly protein bound. Therefore, free and thus antibacterially active drug concentrations may be close to the MICs of the relevant pathogens
- FRI appears to be a promising new antimicrobial agent for the treatment of infections caused by Gram-positive organisms, including isolates that are resistant to currently available drugs

Literature

[1] T. Schneider, et al., 47th ICAAC, Chicago 2007, Poster No. F1-1640
[2] D. Zuehlke D, et al., 47th ICAAC, Chicago 2007, Poster No. F1-1641
[3] Performance Standards for Antimicrobial Susceptibility Testing 11th Informational Supplement, Vol. 21 No 1, M100-S11, Jan. 2001. NCCLS
[4] Methods for Determining Bactericidal Activity of Antimicrobial Agents; Approved Guideline M26-A, Vol. 19 No.18, 1999. NCCLS
[5] Antibiotics in Laboratory Medicine. V. Lorian, Williams & Wilkins fourth Edition 1996.
[6] K.J. Schaper, et al., *Infection* 2005; 33 (Suppl 2): 3-14

47th ICAAC,
Chicago 2007

F1-1650

Determination of the Predictive PK/PD Parameter for the Efficacy of Friulimicin B in a Murine Staphylococcal Thigh Muscle Infection

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Revised Abstract

Background: Friulimicin B (FRI) is a novel lipopeptide antibiotic and is intended for the treatment of severe infections caused by Gram-positive pathogens. To determine the predictive PK/PD parameters for efficacy, FRI was evaluated in a murine thigh muscle infection caused by *Staphylococcus aureus*.

Methods: Mice were rendered neutropenic by injection of cyclophosphamide. Mice were inoculated into the right hind leg with $5x10^5$ *S. aureus* DSM 11823/mouse. Treatment with FRI was initiated 0.5 h post infection. Mice (n=3) were treated subcutaneously (s.c.) for 24 h with various divided doses (1, 2, 4 or 8). 24 h after start of therapy thigh muscles were removed aseptically and CFUs were determined by plating. Plasma concentrations were also determined after s.c. administration of various doses. For each dose and time point (n=3), the FRI concentration was determined using LC/MSMS. The PK/PD parameters were determined according to AAC 50: 243, 2006. The correlation between efficacy and each of the three PK/PD parameters (AUC/MIC, Cmax/MIC, T>MIC) was determined.

Results: After s.c. administration to mice, FRI showed dose proportional pharmacokinetics. In the thigh muscle infection, FRI demonstrated concentration-dependent killing of *S. aureus* DSM 11823 with a static dose of 9 mg/kg and a maximum reduction in CFU of 4 logs. FRI showed good correlation between CFU-reduction per thigh muscle and AUC/MIC (R-squared value 0.881) as well as Cmax/MIC (R-squared value 0.817) and no correlation to T> MIC. The best predictor for efficacy was AUC/MIC.

Conclusions: The best predictive PK/PD-parameter for efficacy was AUC/MIC while with Cmax/MIC, a slightly less strong relationship with efficacy was seen and no correlation was found to T>MIC.

Introduction

Friulimicin B (FRI) is a novel lipopeptide antibacterial agent that exhibits potent activity against a variety of Gram-positive bacteria[1-4]. It is structurally similar to daptomycin but has a distinct mode of action[5-6]. Its chemical structure is shown in Figure 1.
The aim of the present study was to determine the pharmacokinetic and pharmacodynamic parameters and to identify the predictive PK/PD-parameters for efficacy using a murine thigh muscle infection model.

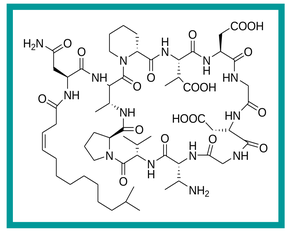

Fig. 1 Friulimicin B

Methods

Bacterial strain: A clinical isolate of *S. aureus,* strain DSM 11823, from the culture collection of Bayer HealthCare AG, Germany was used. The MIC of FRI determined by broth microdilution was 0.425 mg/L.

Antibiotic: FRI provided by Combinature Biopharm AG was used and dissolved in 0.9% NaCl. The specified dosages were related to the free friulimicin acid.

Animals and neutropenia: Female CFW-1 mice (18-20 g body weight) were used (Harlan-Winckelmann, Germany). The animals were kept under conventional housing conditions and rendered neutropenic by injecting intraperitoneally 150 mg/kg and 100 mg/kg of cyclophosphamide on days -4 and -1 before infection or administration of FRI.

Pharmacokinetic studies: FRI was administered subcutaneously (0.1 ml per mice) by single and divided doses (1, 2, 4 h). Samples were collected from 3 mice/time point by cardiac puncture and the concentration of FRI determined by HPLC coupled to a tandem mass spectrometer (LC/MSMS). The assay was linear over the range 100 – 100,000 µg/ml using a volume of 1 µl plasma.

Infection model: Neutropenic mice were infected with 100 µl of a *S. aureus* suspension in physiological saline ($5x10^5$ CFU/mouse) into the thigh muscle of the right hind leg (i.m.). Treatment started 0.5 h post infection (p.i.). Viable bacterial counts in the homogenized thigh muscles were determined at 24 h p.i. by plating serial tenfold dilutions onto Columbia blood agar plates. Bacterial colony forming units (CFU) were counted after overnight incubation of the plates at 37°C.

PK/PD parameter determinations: The PK/PD parameters were determined according to Andes & Craig[7] using 3 mice/time point. Mice were treated for 24 h with 29 varying dosing regimens with total doses of between 1.33 to 170.04 mg/kg in 1, 2, 4, or 8 doses.
For the analysis, the sigmoid dose-effect model was used. The correlation between efficacy (CFU reduction at 24h) and each of the three PK/PD parameters (AUC/MIC, Cmax/MIC, T>MIC) was determined.

Results and Discussion

Pharmacokinetic studies:
Pharmacokinetics of FRI administered subcutaneously to mice by single or multiple dosing were dose proportional between 1.594 and 102.02 mg/kg. There were no differences in the pharmacokinetic profiles after single or multiple administration and a good fit between measured and predicted concentrations using a one compartment model could be demonstrated (Figure 2). Therefore, the pharmacokinetic parameters could be calculated with high precision (Table 1). The predicted PK parameters were then used for the simulation of all concentration vs. time profiles after administration of the different doses and using the different dose regimens. Furthermore, AUC/MIC, Cmax/MIC and T>MIC were calculated from the simulated concentration vs. time profiles.

Infection model - Dose finding study:
Even a low dose of 1.06 mg/kg FRI resulted in a significant reduction of the bacterial load in the thigh muscle infection. Bactericidal killing, a 3 log CFU reduction, was achieved with 5.31 mg/kg s.c. (data not shown).

PK/PD determinations:
In the thigh muscle infection, FRI resulted in a dose dependent reduction of bacterial load (data not shown). A maximum CFU reduction was reached with doses equal or higher than 21.25 mg/kg/24h. Over the whole dose range tested, the efficacy was independent of the dosing frequency (Figure 3).

Results and Discussion

There was a good correlation between Δ-log10 CFU per thigh muscle and AUC/MIC (Fig. 4) as well as between Δ-log10 CFU per thigh muscle and Cmax/MIC (Fig. 5) and no correlation between Δ-log10 CFU per thigh muscle and T> MIC.
The best predictor for efficacy in the thigh muscle is AUC/MIC.
Calculation of kill rate: The AUC/MIC ratios that are necessary to produce units of killing as related to CFU at 24h in untreated controls were calculated according to the formula:

$$AUC / MIC = \sqrt[S]{\frac{ED_{50}^{\ S} \times E}{E_{max} - E}}$$

The following results were obtained:

log units killing	AUC/MIC ratio	Dose [mg/kg]
1	227	~ 3.4
2	408	~ 6.2
3.65*	1520	~ 23

*corresponds to 1 log unit killing as related to the initial inoculum

Maximum efficacy amounts to ~4 log units reduction of CFU after 24 h. Half maximum efficacy was reached at an AUC/MIC ratio of about 401 corresponding to a dose of ~ 6.1 mg/kg in mice.

The bacteriostatic activity (equivalent to a 2.65 log unit reduction of CFU), related to the initial infection inoculum, was achieved at an AUC/MIC ratio of about 586 which corresponds to a dose of 8.8 mg/kg.

		Estimate	CV[%]
AUC	[hr*mg/L]	716	4.72
AUC_{norm}	[kg·h/L]	28.1	4.72
C_{max}	[mg/L]	146	6.02
$C_{max,norm}$	[kg/L]	5.74	6.02
Tmax	[hr]	0.641	13.1
$t_{1/2}$	[hr]	2.91	9.76
V_F	[L/kg]	0.150	8.35
CL_F	[L/hr/kg]	0.0356	4.72

Table 1 Predicted pharmacokinetic parameters (using a one compartment model) after multiple (every 6h) subcutaneous administration of 25.5 mg/kg FRI to female CFW 1 mice

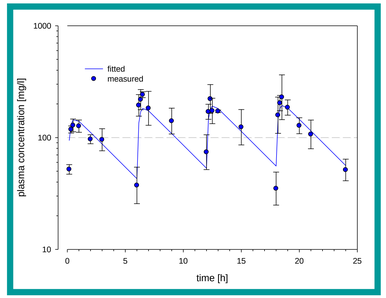

Fig. 2 Measured and fitted concentrations in plasma after multiple (every 6h) subcutaneous administration of 25.5 mg/kg FRI to female CFW 1 mice.

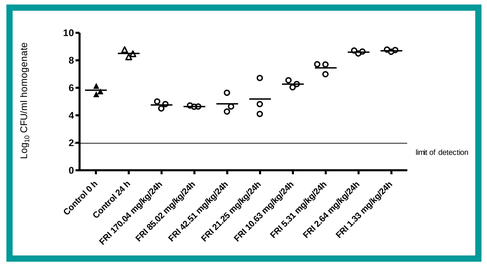

Fig. 3 Effect of eight doses of FRI on numbers of staphylococci in mouse thigh tissue (N=3). Inoculum 9.4 X 10^5

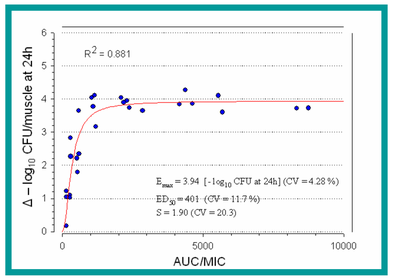

Fig. 4 Relationship between Δ-log10 CFU per thigh muscle and AUC/MIC.

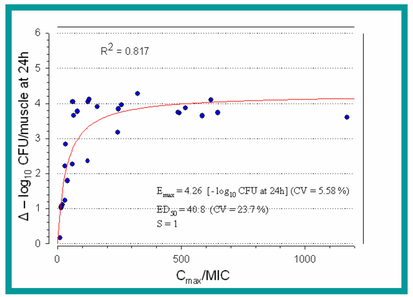

Fig. 5 Relationship between Δ-log10 CFU per thigh muscle and Cmax/MIC

Conclusions

- In a mouse thigh muscle staphylococcal infection, the best predictor for efficacy of friulimicin B was the AUC/MIC. With Cmax/MIC, a slightly less strong relationship to antibacterial efficacy was seen. No correlation was found with T>MIC
- The AUC/MIC ratios that are necessary to produce 1 or 2 units killing or the maximum kill rate were calculated and the corresponding doses were deduced

Literature

[1] P. Bremer et al., *AAC* 47 (2003) 3025-9
[2] R. Schaumann et al., 47th ICAAC, Chicago 2007, Poster No. F1-1644
[3] P. McGhee et al., 47th ICAAC, Chicago 2007, Poster No. F1-1648
[4] S. Schubert et al., 47th ICAAC, Chicago 2007, Poster No. F1-1649
[5] T. Schneider et al., 47th ICAAC, Chicago 2007, Poster No. F1-1640
[6] D. Zuehlke et. al. 47th ICAAC, Chicago 2007, Poster No. F1-1641
[7] D. Andes et al., *AAC* 50 (2006) 243-249

47th ICAAC,
Chicago 2007

F1-1651

Friulimicin B Shows Good Efficacy in a Staphylococcal Murine Abscess Infection

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Revised Abstract

Background: Friulimicin B (FRI) is a novel lipopeptide antibiotic and is intended for the treatment of severe infections caused by Gram-positive pathogens. The compound shows structural similarities with daptomycin (DAP). The present study compared FRI with DAP and vancomycin (VAN) in the mouse granuloma pouch model using a MSSA strain. This model is regarded as an example of a SSTI.

Methods: The infecting strain used was *S. aureus* DSM 11823. Pouches were induced by injection of air and croton oil into loose subcutaneous tissue of the backs of mice. Three days later, air was evacuated and substituted by soft agar (0.25 %). On day 5, pouches were inoculated with a staphylococcal suspension (3 x 10^5 CFU/mouse). Infected pouches were treated IV bid for two days. Therapeutic efficacy was assessed by reduction of CFUs in the pouch exudates determined 18h after the last treatment.

Results: In the untreated control group, bacteria grew to a density of ~ 6 x 10^8 CFU/ml of pouch exudates on day 3 after infection. Both FRI and DAP were more effective than VAN.

Conclusions: In this study, FRI shows good efficacy in a mouse model of SSTI, the granuloma pouch, caused by MSSA.

Introduction

Friulimicin B (FRI) is a novel lipopeptide antibacterial agent that exhibits potent activity against a variety of Gram-positive bacteria[1-4]. It is structurally similar to the lipodepsipeptide antibiotic daptomycin (DAP) but has a distinct mode of action[5-6]. Its chemical structure is shown in Figure 1.
The aim of this study was to investigate the potential of FRI in experimental skin and soft tissue infections (SSTI). The efficacy of FRI in the murine granuloma pouch model using a susceptible *Staphylococcus* strain was determined in comparison with that of vancomycin (VAN) and DAP. This model is regarded as an example of an SSTI[7].

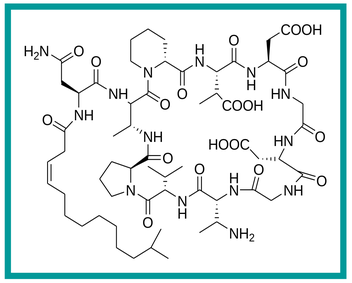

Fig. 1 Friulimicin B

Methods

Test compounds:
The test compounds were obtained from the following sources: FRI (Combinature Biopharm AG, Germany), VAN (Lilly Deutschland GmbH, Germany), DAP (Novartis Pharma GmbH, Germany).

Bacterial strain:
The staphylococcal strain DSM 11823, a clinical isolate, was taken from the culture collection of Bayer HealthCare AG, Germany.

MIC values for FRI, VAN and DAP were determined by agar dilution method. Mueller-Hinton agar substituted with 184 mg/l $CaCl_2$*H_2O was used. Agar plates were inoculated with bacterial spots using a Denley multipoint inoculator, containing ~1 - 5 x 10^4 colony forming units (CFUs) each. Plates were incubated for 16-20 hrs. at 37°C under 5% CO_2. The lowest antibiotic concentration yielding no growth was read as the MIC.

The organisms for the inoculum were grown overnight in Thioglycollate broth under anaerobic conditions at 37°C. The inoculum was prepared from a subculture in Columbia broth after 4-5 h incubation at 37°C under anaerobic conditions. The final bacterial suspension for the inoculum was adjusted in Columbia broth with 0.25% agar.

Animals:
For all experiments described, female CFW-1 mice (18-20 g body weight) were used (Harlan-Winckelmann, Germany). The animals were kept under conventional housing conditions.

Abscess Model: Granuloma Pouch (Figure 2)
Pouches were formed in mice by injecting 5 ml of air and 0.5 ml of 0.1% croton oil in olive oil under the skin of the back. After 72 h, the air was replaced by 1 ml of 0.25% agar in saline. A bacterial suspension (500 µL; ~10^5 CFU/mouse) was injected into the pouch an additional 48 h later (5 days after inducing the granuloma pouch).

Six mice were used in each treatment group. In a few mice a pouch did not develop.

Antibiotics were given intravenously at doses of 1.25, 2.5, 5 and 10 mg/kg at 0.5, 4, 24 and 32 h post-infection (i.e. BID for 2 days). The viable bacterial load in the pouch exudates was determined at 48 h post-infection by plating serial tenfold dilutions on sheep blood agar plates. Bacterial CFUs were counted after overnight incubation of the plates at 37°C.

Statistical significance of differences in the numbers of organisms in the pouches of the different treatment groups was determined by the Mann and Whitney test.

This model is regarded as a good example of a skin and soft tissue infection.

Friulimicin B	Vancomycin	Daptomycin
0.5	0.5	0.25

Table 1 MIC values (mg/L) of test compounds against *Staphylococcus aureus* DSM 11823

Results and Discussion

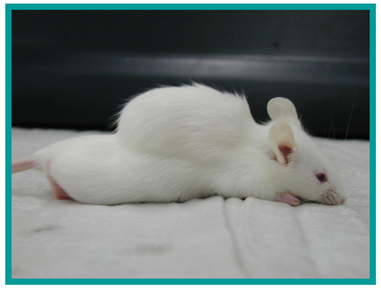

Fig. 2 Murine granuloma pouch model

The MIC values for the three test compounds are shown in Table 1.

The therapeutic efficacy of compounds in the granuloma pouch model was measured by the reduction of the numbers of staphylococcae in the exudates.

Figure 3 shows the counts in the individual granuloma pouches of each group at 2 days post infection and Table 2 shows the change in the numbers of viable organisms compared with control animals receiving no therapy.

The growth of the infecting organism in the control group was consistent and reached approximately 6 x 10^8 CFU/ml of homogenate by 2 days post-infection.

All test compounds showed a dose-dependent reduction of the bacterial load. DAP was more active than the comparators. Compared with VAN, the bactericidal efficacy of FRI was higher over the whole dose range (1.25 - 10 mg/kg). Both FRI and DAP produced a 6 log fall in bacterial numbers at the highest dose. FRI reduced the count by 1.12 logs at the lowest does, in contrast to VAN which only reduced the count by 0.4 logs.

A CFU-reduction of about 4 log units was achieved with 1.25 mg/kg of DAP while 5 mg/kg of VAN or 2.5 – 5 mg/kg of FRI were needed for the same efficacy.

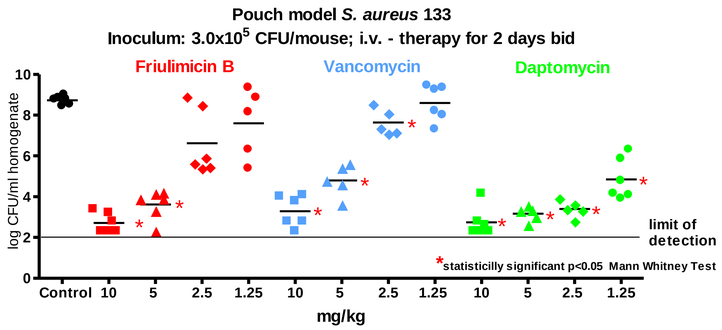

Fig. 3 Numbers of organisms in granuloma pouches following treatment with FRI, VAN or DAP

Dose	Friulimicin B		Vancomycin		Daptomycin	
mg/kg	Δ log CFU/ml homogenate	P value	Δ log CFU/ml homogenate	P value	Δ log CFU/ml homogenate	P value
1.25	-1.12	0.5368	-0.14	1.000	-3.88	0.0022
2.5	-2.11	0.0649	-1.09	0.0087	-5.34	0.0043
5	-5.11	0.0022	-3.93	0.0043	-5.57	0.0043
10	-6.02	0.0022	-5.44	0.0022	-5.99	0.0022

Table 2 Reduction in viable counts in granuloma pouches sampled two days post infection

Conclusions

- Using an experimental model of an SSTI – the mouse granuloma mouse model infected with a methicillin susceptible strain of *Staphylococcus aureus,* friulimicin B demonstrated excellent efficacy
- A range of doses (1.25, 2.5, 5.0 and 10 mg/kg) was administered intravenously BID for 2 days post infection and the highest two doses (5 and 10 mg/kg) of friulimicin B produced a dramatic bactericidal effect
- Overall friulimicin B showed a higher reduction in the bacterial counts in the exudates than vancomycin, but was less potent than daptomycin at the lower doses

Literature

[1] P. Bremer et al., *AAC* 47 (2003) 3025-9
[2] R. Schaumann et al., 47th ICAAC, Chicago 2007, Poster No. F1-1644
[3] P. McGhee et al., 47th ICAAC, Chicago 2007, Poster No. F1-1648
[4] S. Schubert et al., 47th ICAAC, Chicago 2007, Poster No. F1-1649
[5] T. Schneider et al., 47th ICAAC, Chicago 2007, Poster No. F1-1640
[6] D. Zuehlke et al., 47th ICAAC, Chicago 2007, Poster No. F1-1641
[7] Experimental models in antimicrobial chemotherapy; Vol. 1; Academic Press 1986

47th ICAAC,
Chicago 2007

F1-1652

Friulimicin B, a Cyclic Lipopeptide, Exhibits Potent Efficacy in a Murine Pneumococcal Pneumonia Model

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Revised Abstract

Background: With the emergence of resistance to currently available antibiotics, the development of novel antibiotics has become of major importance. Friulimicin B (FRI), a cyclic lipopeptide, is intended for the treatment of severe infections caused by Gram positive pathogens. We studied the efficacy of FRI compared with ceftriaxone (CRO), daptomycin (DAP) and linezolid (LIN) in an intranasal lung infection caused by *Streptococcus pneumoniae* L3TV.

Methods: A pulmonary infection model was used (JID, 2005:191, 2149 - 2152). Isoflurane anaesthetised mice were inoculated intranasally with 1 x 10^6 *S. pneumoniae* L3TV/mouse. Mice (n = 5) were assigned to treatment with either antibiotics or vehicle. Treatment started at 1 h post infection (p.i.) with a further dosing at 4 h p.i.. Bacterial counts in lungs from infected mice were determined at 24 h p.i.. Lungs were removed aseptically, weighed, homogenised and plated. CFUs were counted after overnight incubation at 37°C under 5% CO_2.

Results: The therapeutic efficacy in the murine pneumococcal pneumonia was measured by reduction of the CFUs in the lung. With low doses of FRI (1.25 or 5 mg/kg), CFUs in the lung were lower than with DAP or LIN therapy. In the presence of a formulation agent, the efficacy of FRI was slightly higher. DAP and LIN were less active and CRO slightly more active.

Conclusions: FRI was highly efficacious in reducing the viable counts of *S. pneumoniae* L3TV in a murine pulmonary infection model and was more effective than DAP or LIN.

Introduction

Friulimicin B (FRI) is a novel cyclic lipopeptide antibacterial agent with potent activity against *S. pneumoniae* including multidrug-resistant strains. The compound also has good activity against a variety of other Gram-positive bacteria[1-4]. It is structurally similar to the lipodepsipeptide antibiotic daptomycin but has a distinct mode of action [5-6]. No cross resistance is shown with daptomycin. Its chemical structure is shown in Figure 1.

Streptococcus pneumoniae is the most frequently isolated pathogen in community acquired pneumonia, and is a significant cause of morbidity and mortality in humans.

In the present work, we report the *in vivo* activity of FRI in comparison with ceftriaxone, daptomycin and linezolid in an *in vivo* mouse model of acute pneumonia caused by *Streptococcus pneumoniae*.

Methods

Test compounds:
The test compounds were obtained from the following sources: ceftriaxone (Hexal AG, Germany), daptomycin (Novartis Pharma GmbH, Germany), linezolid (Pharmacia GmbH, Germany), FRI (Combinature Biopharm AG, Germany).

Bacterial strain:
The pneumococcal strain L3 TV (serotype 3), a clinical isolate, was taken from the culture collection of Bayer HealthCare AG, Germany.

Susceptibility testing:
The MICs of FRI and DAP were determined by agar dilution method. Mueller-Hinton agar substituted with 184 mg/l $CaCl_2*H_2O$ was used. Agar plates were inoculated with bacterial spots (Denley multipoint inoculator) containing ~1-5 x 10^4 colony forming units (CFUs) each. Incubation was for 16-20 hrs. at 37°C under 5% CO_2. The lowest antibiotic concentration yielding no growth was read as the MIC. The MICs of the other test compounds were determined by micro dilution method using CLSI-guidelines[7].

Animals:
For all experiments described, female CFW-1 mice (18-20 g body weight) were used (Harlan-Winckelmann, Germany). The animals were kept under conventional housing conditions.

Pneumococcal pneumonia:
Isoflurane-anaesthetized mice were inoculated intranasally (i.n.) with 20 µl of physiological saline producing an infective dose of 1 x 10^6 CFU/mouse of *S. pneumoniae* strain L3TV i.n. The animals were treated with FRI, DAP, LIN or CRO intravenously, 1 and 4 h post infection (p.i.). All four drugs were adminstered at doses of 1.25, 5 and 20 mg/kg to groups of five mice.

The bacterial counts in the lung were determined at 24 h p.i. For determination of the viable bacterial load in the lungs, the lungs were removed aseptically and homogenised with an Ultra-Turrax (IKA-Werk, Germany) in sterile saline. Diluted samples were spread on blood agar plates and the resultant CFUs were counted after overnight incubation at 37°C in the presence of 5% CO_2.

Results and Discussion

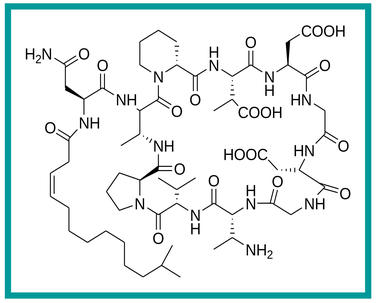

Fig. 1 Friulimicin B

Results and Discussion

The susceptibility of the infecting strain *S. pneumoniae* is detailed in Table 1. It was highly susceptible to ceftriaxone (MIC 0.03 mg/L). DAP was slightly less active than FRI and both were markedly more active than linezolid.

Compound	MIC [mg/L]
Friulimicin B	≤ 0.125
Daptomycin	0.25
Linezolid	2
Ceftriaxone	0.03
Ampicillin	0.25
Erythromycin	0.125

Table 1 Susceptibility of *S. pneumoniae* L3TV against test compounds

The therapeutic efficacy in the mouse model of pneumococcal pneumonia was measured by reduction of the CFUs in the lung at 24 h p.i.. The mean values (and standard deviation) for each group are illustrated in the histogram (Figure 2). Individual counts comparing the FRI and DAP treatment are illustrated in Figure 3, with a line indicating the mean value.

For all dosage groups tested FRI was more active than either DAP or LIN. CFUs in the lung of the FRI treated mice were at least 1 log-unit lower than with DAP or LIN therapy at all doses.

Overall cetriaxone was slightly more active than FRI, reflecting its good activity *in vitro*.

In spite of having activity *in vitro* that was close to that of FRI, the activity of DAP *in vivo* was markedly inferiour.

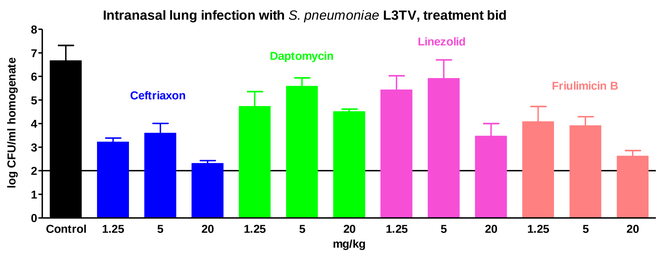

Fig. 2 Efficacy in the mouse model of *S. pneumoniae* pneumonia. The viable bacterial load in the lungs of antibiotic-treated mice compared with untreated control animals (mean values - n = 5 mice)

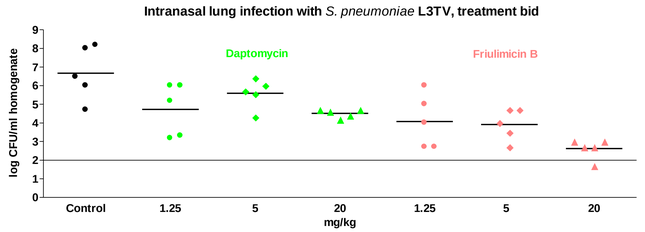

Fig. 3 Comparative efficacy of FRI and DAP in mouse model of pneumococcal pneumonia. Viable bacterial loads in the lungs of single mice compared with control animals

Conclusions

- Treatment with friulimicin B is highly effective in an animal model of pneumococcal pneumonia
- Friulimicin B demonstrated superior efficacy to daptomycin or linezolid as measured by a reduction in CFUs in the lungs
- Friulimicin B appears to be a promising new antimicrobial agent for the treatment of respiratory tract infections caused by Gram - positive organisms

Literature

[1] P. Bremer P, et al., AAC 47 (2003) 3025-9
[2] R. Schaumann, et al., 47th ICAAC, Chicago 2007, Poster No. F1-1644
[3] P. McGhee, et al., 47th ICAAC, Chicago 2007, Poster No. F1-1648
[4] S. Schubert, et al., 47th ICAAC, Chicago 2007, Poster No. F1-1649
[5] T. Schneider, et al., 47th ICAAC, Chicago 2007, Poster No. F1-1640
[6] D. Zuehlke D, et al., 47th ICAAC, Chicago 2007, Poster No. F1-1641
[7] National Committee for Clinical Laboratory Standards (NCCLS). - 7th Edition. M7-A7. National Committee for Clinical Laboratory Standards, Wayne, Pa., 2006