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

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Revised Abstract

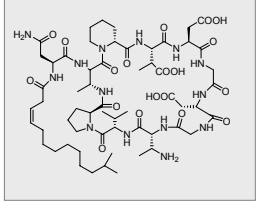
Background:
Friulimicin B (FRI), an acidic, cyclic lipopeptide, is intended for the treatment of severe infections caused by Gram-positive pathogens. FRI shows structural similarity with Daptomycin (DAP), however, its mode of action had not been elucidated previously.
Methods:
Whole cell assays with 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 depolariziation 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. MICs were read after 16 h of 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[3]. 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.
- 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

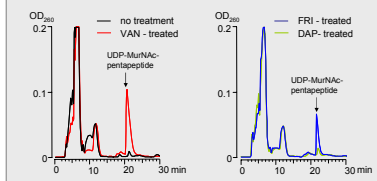


Figure 3 : FRI inhibits ^{3}H-glucosamine incorporation

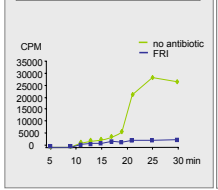


Figure 4 : FRI does not form Nisin-like pores

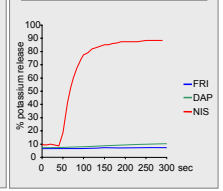
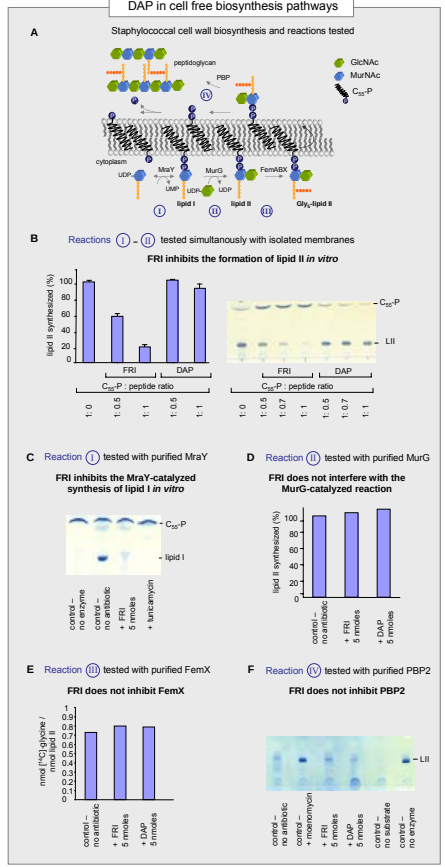
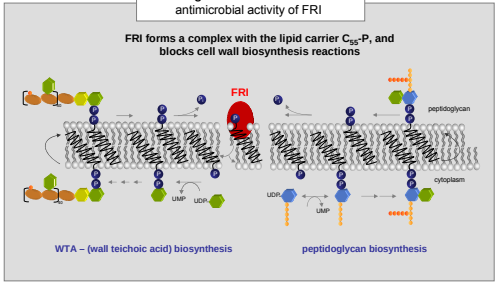


Figure 5 : Antibiotic activities of FRI and DAP in cell free biosynthesis pathways



Conclusions

Figure 6 : Model for the antimicrobial activity of FRI



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

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