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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*.

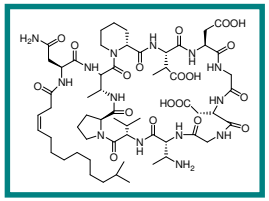

Fig. 1 Friulimicin B

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			Incubation time 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).

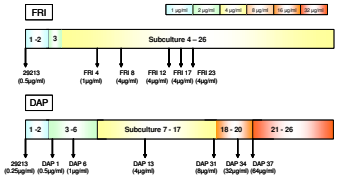

Fig. 2 Reduction in FRI (top) and DAP (bottom) susceptibility in *S. aureus*. Coloured bars represent the highest concentration of drug at which the indicated subculture could grow. Arrows show the point where MIC increases were detected. MICs were determined by broth microdilution. Those FRI mutants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

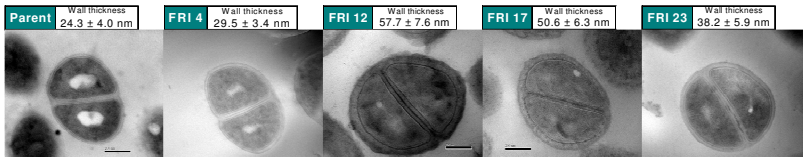

Fig. 3 Transmission electron micrographs of *S. aureus* 29123 and mutants with decreased FRI susceptibility. Wall thicknesses were averaged from 40 measurements

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*

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