Low Propensity of Resistance Development *in vitro* in *Staphylococcus aureus* with Lysin CF-301

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Introduction

CF-301 is a recombinantly-produced bacteriophage-derived lysin (cell wall hydrolase) and is the first agent of this class to enter Phase 2 of clinical development in the US for the treatment of bacteremia including endocarditis due to *S. aureus*.

To understand the potential for resistance to CF-301 in *S. aureus*, we previously performed a serial passage study using MRSA strain MW2 in MHB (1). While resistance did not appear, there was a stable 2-fold increase in MIC; the reduced susceptibility did not, however, proceed beyond 2-fold. Further, sub-MIC CF-301 suppressed resistance to conventional antibiotics when used in combination in serial passage studies. To extend our work, serial passage studies were next performed in different media types (MHB, CAMHB, CAMHB-HSD [AST medium recently developed for CF-301] and human serum [HuS]) using both MW2 and MSSA strain ATCC 29213. Resistance to CF-301 was again not observed and sub-MIC CF-301 acted to suppress resistance to oxacillin (OXA) in MSSA when used in combination. As before, a stable 2-fold increase in the CF-301 MIC was observed that did not proceed to resistance. In accordance with FDA guidance (2) a series of phenotypic and genotypic studies were performed to characterize the 2-fold MIC-shifted variants.

Objective

To investigate the potential for decreased susceptibility of *S. aureus* to CF-301 and to characterize the phenotypic and genotypic changes associated with MIC shifts.

Methods

Resistance assays were based on a standard serial passage method (1,3). Three lineages of each strain were exposed to a 1.1-fold dilution series of the indicated antimicrobials for 21-26 days. On a daily basis, intermediates growing in the highest concentration of each agent were recovered, sub-cultured, and analyzed for a final MIC determination. Serial passages were performed in MHB, CAMHB, CAMHB-HSD (CAMHB with 25% horse serum and 0.5mM DTT), and HuS. For OXA, strains were passaged in MHB+2% NaCl with and without CF-301 (1/16th MIC). Mutations causing a change in CF-301 susceptibility were identified by whole genome sequencing (WGS) in a comparison to both the MW2 reference genome and to strains passaged without antimicrobials. Phenotypic analysis of growth rate (in 0.25x LB) and cell wall ultrastructure (by transmission electron microscopy [TEM]) were included. The suicide plasmid, pHoss1, was used to construct *S. aureus* mutants by two-step allelic exchange (4). The *pHoss1* plasmid was kindly provided by Dr. Attila Karsi at Mississippi State University.

References

1. Schuch, *et al.,* 2014. J Infect. Dis. 209:1469-78
2. Microbiology Data for Systemic Antibacterial Drugs – Development, Analysis, and Presentation, Guidance for Industry (FDA-CDER, 2016)
3. Berti, *et al.,* 2012 AAC 56:5046-63.
4. Abdelhamed, *et al.,* 2015. Plasmid 81:1-8
5. Yang *et al.,* 2010. AAC 54:3161-9.
6. Rotolo *et al.*, 2016 (ASM Poster)

Study Outline

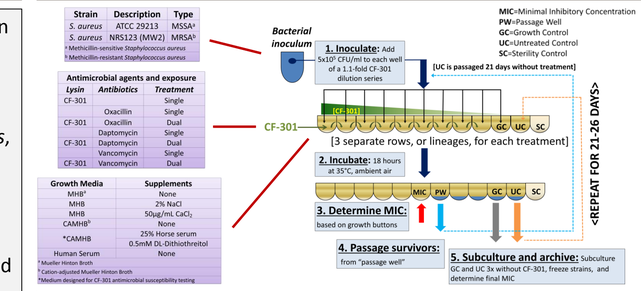
Strain	Description	Type
S. aureus	ATCC 29213	MSSA[a]
S. aureus	NRS123 (MW2)	MRSA[b]

[a] Methicillin-sensitive *Staphylococcus aureus*
[b] Methicillin-resistant *Staphylococcus aureus*

Antimicrobial agents and exposure		
Lysin	*Antibiotics*	*Treatment*
CF-301		Single
	Oxacillin	Single
CF-301	Oxacillin	Dual
	Daptomycin	Single
CF-301	Daptomycin	Dual
	Vancomycin	Single
CF-301	Vancomycin	Dual

Growth Media	Supplements
MHB[a]	None
MHB	2% NaCl
MHB	50µg/mL $CaCl_2$
CAMHB[b]	None
*CAMHB	25% Horse serum 0.5mM DL-Dithiothreitol
Human Serum	None

[a] Mueller Hinton Broth
[b] Cation-adjusted Mueller Hinton Broth
*Medium designed for CF-301 antimicrobial susceptibility testing

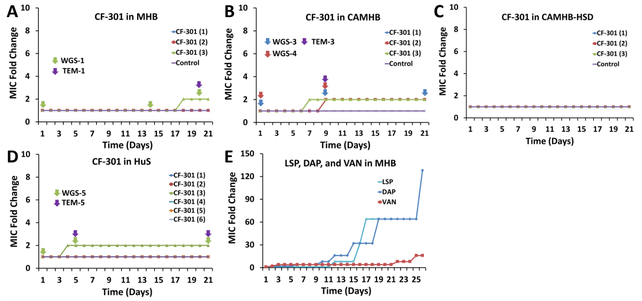


1. Serial passage resistance studies:
- a. CF-301 and comparators against MRSA strain MW2
- b. Suppression of DAP and VAN resistance
- c. CF-301 and comparator against MSSA strain ATCC 29213
- d. Increase in OXA susceptibility for MRSA (Seesaw effect)
- e. Effect on growth and cell wall ultrastructure

2. Genetic analysis of decreased CF-301 susceptibility:
- a. SNP/INDEL analysis based on WGS data
- b. Reconstruction of MIC-shifted variants in MSSA strain RN4220

1a. Serial passage with MRSA

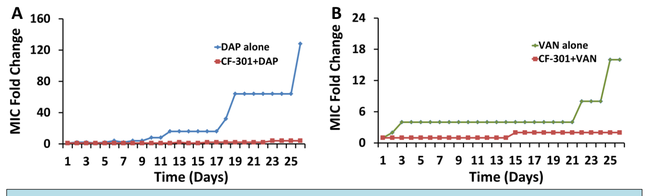
Analysis of CF-301 in MHB **(A)**, CAMHB **(B)**, CAMHB-HSD **(C)** and HuS **(D)**. A comparator lysin-like enzyme (lysostaphin) and two antibiotics (DAP and VAN) were also examined **(E)**. Each curve is a distinct lineage. Controls were passaged for 21 days in the absence of any antimicrobial agent. Arrows indicated passage isolates that were analyzed by WGS or TEM.



- **The CF-301 MIC <u>never</u> increased by more than 2-fold in serial passage**
- **Comparator agents demonstrated high-level (up to 128-fold) resistance**

1b. Suppression of DAP and VAN resistance

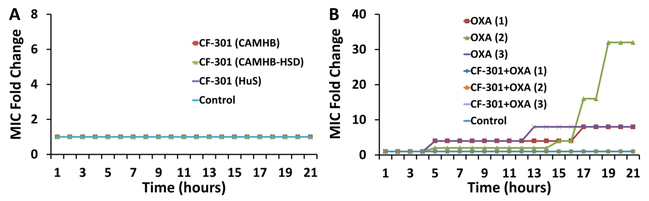
A) DAP MICs for MW2 passaged with and without 1/32 MIC CF-301 and **B)** VAN MICs for MW2 passaged with and without 1/16th MIC of CF-301.



- **CF-301 suppressed resistance to DAP and VAN in MRSA**

1c. Serial passage with MSSA

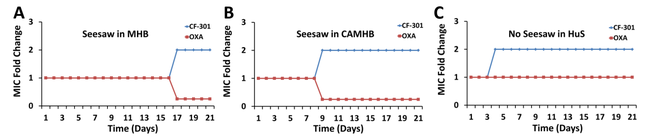
MSSA strain ATCC 29213 was passaged with CF-301 and/or OXA. (**A**), MICs for CF-301 (alone) passaged strains, (**B**) OXA MICs in the presence of a fixed (1/16th MIC) amount of CF-301 (**B**). Controls were passaged in the absence of antimicrobial agent.



- **The CF-301 MIC <u>did not</u> increase in any media type tested**
- **Resistance to OXA can be suppressed by sub-MIC CF-301**

1d. OXA susceptibility in MRSA

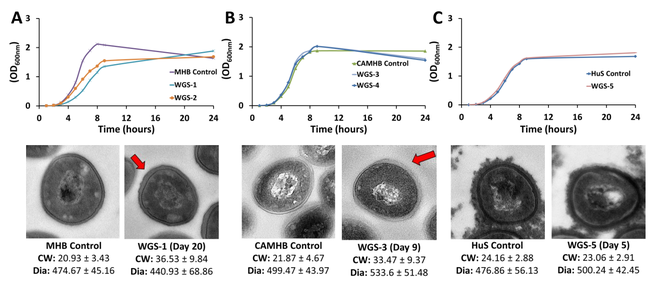
A seesaw effect was previously described whereby MIC increases for certain antistaphylococcal antibiotics (i.e, VAN and DAP) were associated with a concomitant decreases in MIC for semi-synthetic penicillins (5). Because of a potential "seesaw" effect, all intermediaries from CF-301-passaged MRSA strain MW2 were examined for OXA susceptibility. Representative lineages are shown for passages in MHB **(A)**, CAMHB **(B)**, and HuS **(C)**.



- **There is a seesaw effect for CF-301 and OXA in MHB and CAMHB (but not in HuS or CAMHB-HSD [data not shown])**
- **No effect was observed for DAP or VAN (data not shown)**

1e. Growth and cell wall ultrastructure

CF-301 MIC-shifted mutants derived from passages in **A)** MHB, **B)** CAMHB, and **C)** HuS were examined for growth and cell wall ultrastructure. Growth was examined in 0.25x LB at 37°C over 24 hours. Ultrastructure was examined using TEM. Average cell wall thickness (CW) and diameter (DIA) were measured based on analysis of 30 individual cells at x13,000 magnification. Arrows indicate cell wall thickening.



- **MIC-shifted mutants from MHB passages had altered growth kinetics**
- **Mutant from MHB and CAMHB passages had thicker walls**

2a. SNP/INDEL analysis

CF-301 MIC-shifted mutants from passages in MHB, CAMHB, and HuS were analyzed by WGS to identify mutations associated with decreased susceptibility. Comparisons were made to both the MW2 reference genome and controls passaged in the absence of antimicrobial agents.

Table 1. SNP/INDEL discovery of isolates generated by serial passage over 21 days

Laboratory passage	Isolation Day	Reference Position(s)[a]	Overlapping annotation[b]	Count	Coverage	Frequency	Type	Ref	Allele	AA change	MIC Fold Change
WGS-1 (MHB)	Day 14	868552	MW_RS04215	105	105	100	MNV	ATT	TGA	N3S	2
	Day 20	496743	near *rrf*	11	11	100	Insertion	-	T		
		496744		11	11	100	SNV	C	G		
	Day 20	2658191	*oatA*	79	79	100	SNV	C	A		
WGS-2 (MHB)	Day 3	1654553	*near rpsU*	51	51	100	SNV	C	G		2
	Day 3	1660899	*dnaK*	118	119	99.2	SNV	G	A	S14L	2
	Day 19	398246	near *PhoE*	37	37	100	SNV	A	G		2
	Day 19	2180250	*murA*	81	90	90	Insertion	-	C	V222fs	2
		2180252		72	80	90	Insertion	-	A	G221fs	
WGS-3 (CAMHB)	Day 9	1713882	*apt*	2918	2921	99.9	SNV	T	C	T123A	2
		2403752	*lyrA*	4265	4309	99	SNV	C	A	Y245*	
WGS-4 (CAMHB)	Day 9	1713882	*apt*	2918	2921	99.9	SNV	T	C	T123A	2
		2403752	*lyrA*	4265	4309	99	Deletion	C	-	L94fs	
		2429791	*hssS*	2653	2673	99.3	SNV	C	A	N139K	
WGS-5 (HuS)	Day 5 (-21)	378199	MW_RS01730	305	305	100	SNV	T	G	Q41P	2
	Day 5	1005808	MW_RS04895	51	62	82.3	Insertion	-	TTAT	D156fs	

[a] Position in the reference genome of *Staphylococcus aureus* MW2 (GeneBank accession number:NC_003923.1)
[b] Annotated open reading frames overlapping computationally predicted polymorphisms

- **Unique mutation sets were identified, including lesions in or proximal to loci encoding cell wall modifying enzymes (i.e., *oatA*, *murA*, and *lyrA*)**

2b. Confirmation of polymorphisms

A two-step (markerless) allelic exchange method was used to begin introducing mutations (in a step-wise manner, if needed) into MSSA strain RN4220. After construction, the effect of each mutation on MICs in MHB, CAMHB and HuS were/will be examined to confirm recapitulation of mutant phenotypes observed in the MRSA background. This work is in progress.

Table 2. Recapitulation of mutant phenotypes in RN4220

			MW2 MIC Fold Change			RN4220 MIC Fold Change[a]		
			CF-301			CF-301		
Laboratory passage	Overlapping annotation	Reference Position(s)	MHB	CAMHB-HSD	HuS	MHB	CAMHB-HSD	HuS
WGS-1	near *rrf*	496743	2	2	2	na[b]	na[b]	na[b]
		496744						
	MW_RS04215	868552				1	1	1
	oatA	2658191				2	2	2
WGS-5	MW_RS01730	378199	2	2	2	1	1	2
	MW_RS04895	1005808				1	2	2

[a] MICs of final constructs containing the mutation were compared to RN4220 wild-type
All mutations were verified by sequencing of the region containing the mutation

- **Individual mutations were identified conferring MIC changes**
- **Ongoing studies will definitively confirm mutation(s)**

Conclusions

- Genetic and phenotypic changes resulting from serial passage exposure to CF-301 only result in a ≤2-fold change in MIC
- The 2-fold shifted MIC variants will remain susceptible to the clinical CF-301 dose of 0.25 mg/kg, based on previously presented exposure target attainment studies and PK modeling (6)
- The propensity for CF-301 resistance in *S. aureus* remains low