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Effects of AFN-1252 on *In Vitro* and *In Vivo Staphylococcus aureus* Virulence Gene Expression

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Abstract

Background: AFN-1252 (AFN), a novel antibiotic currently in clinical development for staphylococcal infections, blocks type 2 fatty acid synthesis (FAS II) by inhibiting enoyl-ACP reductase in *S. aureus*. The current study describes the effects of AFN on bacterial gene expression in *S. aureus* lab cultures, and the pharmacokinetics (PK) and efficacy of AFN in a mouse granuloma (MG) model of *S. aureus* infection.
Methods: Affymetrix gene array and qRT-PCR were used to determine gene expression changes in AFN treated *S. aureus* cultures. Exponential-phase laboratory cultures of *S. aureus* were treated with either solvent control or 50 ng/mL of AFN for 15 minutes, and total RNA was extracted from the cells for analysis. In vivo experiments involved inoculating *S. aureus* into 5-day-old granulomas that were formed in the subcutaneous area of CD-1 mice, and orally dosing 100 mg/kg of AFN at 2 hours or at 2, 24, and 48 hours after inoculation. Granuloma fluid was collected at multiple time points over a 24- or 96-hour period following AFN treatment for CFU counting, mRNA profiling and determining AFN concentration.
Results: Exposure of AFN in *S. aureus* cultures resulted in the anticipated upregulation of genes involved in the FAS II pathway associated with the FapR regulon and the unpredicted downregulation of virulence genes that are controlled by the SaeRS two-component regulator. In the MG infection model, the relative exposure (AUC) of AFN in granuloma fluid when compared to plasma ranged from 69% - 75%, with a calculated Tmax of 4 hours. A single dose of AFN at 100 mg/kg at 2 hours post-infection resulted in mean $\log_{10}$ CFU reductions of 2.9 - 3.1 between 24 - 48 hours, while consecutive doses of AFN (100 mg/kg) at 2, 24, and 48 hours resulted in a maximal $\log_{10}$ CFU reduction of 5.3 at 72 hours.
Conclusions: AFN exposure had the unexpected effect of decreasing the expression of *S. aureus* genes encoding virulence factors that belong to the SaeRS regulon. In the MG model, AFN had favorable penetration in granuloma fluid and high efficacy against fluid-associated *S. aureus*.

Introduction

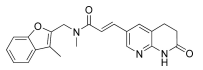
Staphylococcus aureus has the ability to produce a number of virulence factors (toxins) that are thought to be important during the infection process and resulting disease state in the host. *S. aureus* coordinates the expression of virulence factors through a network of regulators that includes *agr*, *sar*, and the two-component regulator, *saeRS*. Other investigators have explored ways to disrupt this coordinated expression in *S. aureus*, which have included evaluating a number of antibiotic classes in their ability to modulate the expression of several staphylococcal virulence factors. Antibiotic classes previously investigated for this modulating effect have included beta-lactams, glycopeptides, fluoroquinolones, and oxazolidinones. The results from these studies suggest that antibiotics could impact the severity of disease by modulating virulence factor expression in *S. aureus*, even at sub-inhibitory levels.

The focus of our work was to evaluate the modulating effects of AFN-1252, a novel fatty acid synthesis inhibitor, on *S. aureus* virulence factor expression in lab cultures and in a subcutaneous granuloma pouch animal model infected with *S. aureus*. Here, we describe the results from the gene expression studies, as well as the pharmacokinetic (PK) and efficacy results of AFN-1252 in the granuloma pouch model.

Methods and Materials

Bacterial strains. *S. aureus* strain RN4220 employed for *in vitro* work was obtained from the American Type Culture Collection (ATCC), and strain PDJ22 (*saeR*) was constructed by the insertion of an intron into the *saeR* gene. The USA300 and Wood46 strains were obtained through the Network of Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) program supported under NIAID/NIH Contract #HHSN272200700055C, and the Newman strain was kindly provided by Dr. Mark Hart (NCTR, Jefferson, AR).
Culture conditions and inoculum preparation. *For in vitro studies, S. aureus* strains were grown in nutrient-rich broth (TB) to mid-log phase, and then split into 2 aliquots for treatment with solvent control (DMSO) or treatment with formulated AFN-1252, cerulenin, or clarithromycin. *For in vivo studies, S. aureus* Wood46 was cultured overnight on TSA (trypticase soy agar), and plate growth was suspended in TSB (trypticase soy broth) to generate an infecting inoculum of 8.0 $\log_{10}$ CFU/mL.
Affymetrix array analysis. The abundance of gene transcripts was analyzed using the *S. aureus* Affymetrix array technology. RNA was isolated from control and treated bacteria; cDNA was prepared, labeled and hybridized to the chip. The complete dataset is deposited under accession number GSE19400 in the NCBI GEO database.
Measurement of mRNA levels. mRNA levels were quantified by RT-PCR using gene-specific primer sets that a yielded linear response across the range of mRNA concentrations encountered.
Mouse subcutaneous granuloma pouch model (MGP). Subcutaneous (SC) air pockets were aseptically formed on the dorsal aspect of anesthetized female, CD-1 mice (6-8 weeks of age) 5 days prior to infection. Air pockets were immediately injected with 0.4% croton oil (irritant), and 1 mL of sterile IV saline solution was injected into pouches 3 days later. Five-day old pouches were infected with 7.1 – 7.5 $\log_{10}$ CFU of Wood46, and animals were orally (PO) with 100 mg/kg AFN-1252 or linezolid (Zyvox®) 2, 26, or 50 hours after infection. AFN-1252 was formulated in a 1 % poloxamer solution, and linezolid (Zyvox®) was formulated in WFI per product insert instructions. Pouch fluid and heart blood was collected from animals at defined time points post-dosing and transferred into tubes for RNA extraction, CFU enumeration, or LCMS analysis.

Panel 1: Chemical Structure of AFN-1252

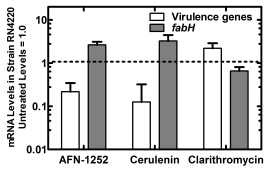


Panel 2: Affymetrix® Expression Array, AFN-1252 Decreases the Transcription of Virulence Factors Gene Governed by SaeRS

Virulence-Related Genes Downregulated by AFN-1252

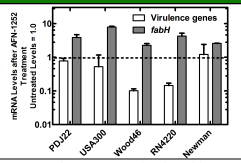
Locus Tag	Gene	Fold	Function
SA1752		8.18	β-Hemolysin
SA2208	*hlgB*	4.99	γ-Hemolysin
SA2209	*hlgC*	4.78	γ-Hemolysin
SA0662	*saeQ*	4.94	Hypothetical
SA0663	*saeP*	4.40	Hypothetical
SA2207	*hlgA*	4.21	γ-Hemolysin
SA2206	*sbi*	3.72	IgG binding protein
SA1811	*hlb*	3.63	β-Hemolysin
SA1000	*ehp*	3.63	Fibrinogen binding protein
SA1000	*efb*	3.49	Fibrinogen binding protein
SA1812		2.72	γ-Hemolysin
SA0661	*saeR*	2.45	2-Component regulator
SA1750		2.05	Map-w
SA0660	*saeS*	1.77	2-Component regulator
SA1007		1.70	α-Toxin
SA2462	*icaC*	1.61	Intracellular adhesion
SA0309	*geh*	1.54	Lipase
SA2461	*icaD*	1.54	Intracellular adhesion

Panel 3: Depression of Virulence Factor mRNA Levels in *S. aureus* Treated with Fatty Acid Synthesis Inhibitors



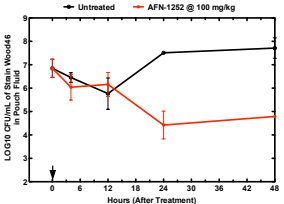
-Strain RN4220 was grown to mid-log phase in rich broth & treated with either AFN-1252, cerulenin or clarithromycin for 15 min.
-RNA was extracted and the average levels of virulence factor mRNAs (*saeP*, *ehp*, *efb* & *hlgC*) & *fabH* determined by quantitative RT-PCR.
-Standard errors were calculated from triplicate determinations derived from triplicate experiments.

Panel 4: Levels of Virulence Factor & *fabH* mRNA in *S. aureus* Strains Treated with AFN-1252



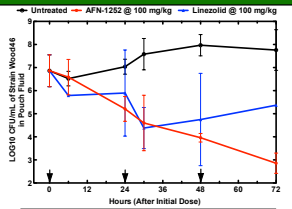
-Expression levels of virulence genes (*ehp*, *efb*, *hlgC*, *saeP*) & *fabH* were measured by qRT-PCR using *gmk* as the internal control.
-Triplicate determinations for each gene were averaged.

Panel 5: Single Dose of AFN-1252 Reduces *S. aureus* CFU in Mouse Pouches for up to 48 hrs



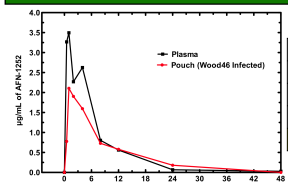
-Arrow respresents a single PO dose of AFN-1252 administered 2 hours after granuloma pouches were infected with 7.5 $\log_{10}$ CFU of Wood46. (LOD=2.05)

Panel 6: Multiple Doses of AFN-1252 Reduce *S. aureus* CFU in Mouse Pouches for up to 72 hrs



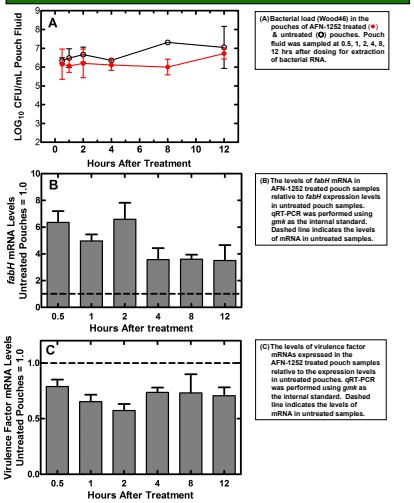
-Arrows respresent 3 separate PO doses of AFN-1252 or linezolid administered 2, 26, & 50 hours after granuloma pouches were infected with 7.1 - 7.5 $\log_{10}$ CFU of Wood46. (LOD=2.05)

Panel 7: Pharmacokinetics of AFN-1252 in the Mouse Pouch versus Plasma Following a Single PO Dose at 100 mg/kg



	Plasma	Pouch
Half-Life (hour)	4.41	4.9
Tmax (hour)	1.0	1.0
Cmax (µg/mL)	3.5	2.1
AUC_{0-48} (hr*µg/mL)	22.5	19.9
Relative AUC	1.0	0.85

Panel 8: Virulence Factor & *fabH* Expression Levels Following Treatment of Infected Pouches with AFN-1252



(A) Bacterial load (Wood46) in the pouches of AFN-1252 treated (●) & untreated (○) pouches. Pouch fluid was sampled at 0.5, 1, 2, 4, 8, 12 hrs after dosing for extraction of bacterial RNA.

(B) The levels of *fabH* mRNA in AFN-1252 treated pouch samples relative to *fabH* expression levels in untreated pouch samples. qRT-PCR was performed using *gmk* as the internal standard. Dashed line indicates the levels of mRNA in untreated samples.

(C) The levels of virulence factor mRNAs expressed in the AFN-1252 treated pouch samples relative to the expression levels in untreated pouches. qRT-PCR was performed using *gmk* as the internal standard. Dashed line indicates the levels of mRNA in untreated samples.

Summary and Conclusions

- In lab cultures of *S. aureus* RN4220, AFN-1252 inhibition of fatty acid synthesis results in the strong induction of *fabH* transcription (Panel 3). AFN-1252 regulation of *fabH* is tied to the activation of the FapR transcription factor.
- The Affymetrix® array data identified a marked repression of a group of virulence factor genes in lab cultures of RN4220 after AFN-1252 treatment (Panel 2). Specifically, the array data identified virulence genes that are governed by the 2-component regulator, SaeRS.
- qRT-PCR quantification of 4 virulence genes (*saeP*, *ehp*, *efb*, and *hlgC* identified a noticeable repression in lab cultures of RN4220 and Wood46 after treatment with AFN-1252 (Panel 4).
- The relative exposure (AUC) of AFN-1252 in Wood46 infected granuloma pouches was 85% when compared to plasma levels (Panel 7). AFN-1252 penetration into pouches occurs relatively fast (T_{max} = 1 hr, C_{max} = 2.1 µg mL) and its half-life in pouch fluid appears to be slightly longer than its plasma half-life (4.9 hrs vs. 4.4 hrs).
- When compared to untreated controls, a single dose of AFN-1252 resulted in a ~3.0 $\log_{10}$ reduction in pouch fluid CFUs at 24 and 48 hours after dosing (Panel 5). Multiple doses of AFN-1252 (2, 26, 50 hrs after infection) resulted in the reduction of pouch fluid CFU to near detection limits within 72 hours of the initial dose (Panel 7).
- qRT-PCR analysis of pouch fluid RNA extracts revealed that AFN-1252 strongly stimulated *fabH*, but the repression of virulence factor mRNA levels was less pronounced as compared to untreated controls (Panel 8).
- The results of this investigation indicate that AFN-1252 modulates *S. aureus* virulence gene expression both *in vitro* and *in vivo*, suggesting that AFN-1252 could alter disease outcome by affecting the virulence of *S. aureus* during the infection process.

References

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Acknowledgments

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