

Pharmacokinetics, pharmacodynamics and efficacy of novel FabI inhibitor AFN-1252 against MSSA and MRSA in the murine thigh infection model

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AFN-1252, a new antimicrobial agent, specifically and potently inhibits fatty acid synthesis in *Staphylococcus aureus*. We characterized *in vivo* pharmacokinetic and pharmacodynamic profiles of AFN-1252 administered orally to neutropenic mice inoculated in thighs ($\sim 10^6$ CFU) with methicillin-susceptible *S. aureus* (MSSA) ATCC 29213. Efficacy was also assessed in mice inoculated with MSSA, hospital-acquired Methicillin-resistant *Staphylococcus aureus* (HA-MRSA) or community-acquired (CA)-MRSA, and administered AFN-1252 or linezolid orally. Bacterial density was determined after 24 hours and efficacy defined as the change in CFU/thigh versus untreated controls at time 0. With MSSA, antibacterial reductions of ≥ 1 log were observed at ≥ 20 mg/kg doses, with $fAUC$ /minimum inhibitory concentration (MIC) best describing the pharmacodynamic profile of AFN-1252. The 80, 50 and 5% maximum effects were observed with $fAUC/MIC$ values of 22.3, 17.0, and 9.6, respectively. Similar values were obtained for CA-MRSA and HA-MRSA. AFN-1252 was 4–40 fold more effective than linezolid against CA-MRSA and HA-MRSA. These data demonstrate the excellent *in vivo* potency of AFN-1252 against phenotypically diverse *S. aureus*.

Keywords: AFN-1252, *S. aureus*, Efficacy, PK/PD, Murine

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) has long been recognized as a major nosocomial pathogen in healthcare facilities. Over the past decade, new forms of MRSA fully capable of causing catastrophic disease in persons without established risk factors for hospital-acquired (HA)-MRSA, have been frequently transmitted in the community.^{1–4} The success of *S. aureus* as a pathogen rests partly with its broad virulence characteristics, its ability to cause a wide range of infections and its increasing resistance to many commonly utilized antimicrobial agents.⁵

AFN-1252 is a new antimicrobial with a new and unique mechanism of action based on the inhibition of bacterial fatty acid synthesis. It is currently in clinical development by Affinium Pharmaceuticals (Toronto, ON, Canada and Austin, TX, USA), as an oral and intravenous agent directed at both drug-susceptible and -resistant staphylococci.^{6,7}

The pharmacokinetics pharmacodynamics and efficacy of AFN-1252 against *S. aureus* in the murine thigh infection model has been investigated. The first objective of these studies was to define the *in vivo* pharmacokinetic and pharmacodynamic profiles of AFN-1252 with methicillin-susceptible *S. aureus* (MSSA) strain ATCC 29213. The second objective was to assess the quantitative efficacy of AFN-1252 in a comparative single-dose response study with HA-MRSA and community-acquired MRSA (CA-MRSA).

Materials and Methods

Antimicrobial agents

AFN-1252 (Affinium Pharmaceuticals, Toronto, ON, Canada) as tosylate anhydrate (lot GJP-F-49(5)) was used in Study 1 and as tosylate monohydrate (lot A280-10-2) in Study 2. All AFN-1252 doses and concentrations were calculated, analyzed and reported as free base equivalents based on the relevant certificate of analysis. For the *in vitro* studies [determinations of minimum inhibitory concentrations (MICs) and protein-binding], AFN-1252 was solubilized in 100% dimethyl sulfoxide (DMSO) and

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further diluted in DMSO until the desired AFN-1252 stock solutions were achieved. The final diluent was cation-adjusted Mueller Hinton broth for MIC testing, and mouse plasma and plasma ultrafiltrate for protein-binding studies. For the pharmacokinetic and efficacy studies *in vivo*, a vehicle of 80% polyethylene glycol 400 (PEG 400 and water in a 4:1 ratio) was used. Commercially available linezolid (Pharmacia & Upjohn Company, Kalamazoo, MI, USA) was reconstituted in sterile water according to the manufacturer's specifications and the suspension administered orally as the comparator compound.

Bacterial isolates and susceptibilities

MSSA ATCC 29213 was used for *in vitro* and *in vivo* testing in both studies. Affinium Pharmaceuticals supplied one strain each of CA-MRSA (No. 32, CDC type USA300, PVL-positive) and of HA-MRSA (No. 63, CDC type USA100, PVL-negative) for MIC determination and efficacy assessment in Study 2. For all three isolates, MICs of a panel of antimicrobials were determined in triplicate by broth microdilution in accordance with CLSI guidelines.⁸

Protein-binding studies

Plasma protein-binding studies for AFN-1252 were conducted in triplicate using the ultrafiltration method as follows. A master stock solution of AFN-1252 was prepared in 100% DMSO, and secondary stock solutions were prepared in 50% DMSO at 200 times the final concentrations required for the assay. A final 1:200 dilution was performed in freshly collected mouse plasma to give concentrations of 2, 1, 0.75, 0.5, and 0.1 µg/ml. Each of the plasma/drug solutions was heated in a shaking water bath at 37°C for 10 minutes. Exactly 0.9 ml of the plasma samples were transferred in triplicate to the Amicon Centrifree® Micropartition devices (30 000 MW cut-off filter; Millipore, Bedford, MA, USA) and the filters centrifuged at 1000g at 10°C for 30 minutes. Non-specific binding of AFN-1252 to the filter device was determined with the ultrafiltrate matrix at 0.75 µg/ml. Concentrations of AFN-1252 in the plasma solutions and ultrafiltrates were determined by a validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) method.

Thigh infection model

Specific pathogen-free female ICR mice weighing approximately 25 g were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN, USA). The animals were maintained and utilized in accordance with National Research Council recommendations and were provided food and water *ad libitum*.⁹ Mice were rendered neutropenic by intraperitoneal injections of cyclophosphamide at 150 mg/kg 4 days before inoculation and 100 mg/kg 1 day before inoculation.¹⁰ A bacterial suspension of approximately 10⁶ CFU/ml in

0.9% sodium chloride was prepared from a 24-hour blood agar plate of each isolate. Final inoculum concentrations were confirmed by serial dilutions and plating techniques. Thigh infection was produced by injecting 0.1 ml of the inoculum into each mouse thigh 2 hours before starting antimicrobial therapy.

Efficacy as assessed by bacterial density

At 0 hour, i.e. 2 hours after injection of *S. aureus* into the thighs, 0.2 ml of AFN-1252 suspension or linezolid solution was administered by oral gavage to the animals (three mice per treatment group). For each experiment, one control group of three mice received drug-free vehicle in the same volume and schedule as the active drug regimen. At the same time, a group of three untreated control mice were killed for determination of bacterial counts.

In Study 1, AFN-1252 was given in 20 different treatment regimens, ranging from 5 to 400 mg/kg given 1 to 4 times daily, to animals inoculated with the MSSA strain. Additionally, to assist with the determination of the best pharmacodynamic parameter, dose fractionation studies were undertaken. Six treatment groups were administered total daily doses of 10 or 20 mg/kg as either a single, twice daily or four times daily regimen.

For studies with CA-MRSA and HA-MRSA, AFN-1252 and linezolid as a comparator were administered as single doses by oral gavage of 1, 2.5, 10, 40, 100, or 300 mg/kg (Study 2).

Untreated controls and treated animals were killed at 24 hours after dosing (the first dose if multiple dosing). Animals were euthanized by CO₂ inhalation and cervical dislocation. After the animals were killed, both thighs were removed and individually homogenized in normal saline. Serial dilutions were plated on trypticase soy agar with 5% sheep blood for CFU determinations. Efficacy (change in bacterial density) was calculated by subtracting the mean log₁₀ CFU per thigh of the untreated control mice at 0 hour from the log₁₀ CFU per thigh of each treated mouse at the end of 24 hours of therapy.

Pharmacokinetics

The pharmacokinetic studies of AFN-1252 were performed in mice inoculated with the MSSA strain as described above. In Study 1, single oral doses of AFN-1252 at 2.5, 5, 10, 30, and 100 mg/kg in 0.2 ml volumes were administered by gavage at 0 hour (i.e. 2 hours after inoculation). In Study 2, AFN-1252 was administered in the same manner with doses of 2.5, 5, 10, 20, 75, and 100 mg/kg. Blood samples were collected by intracardiac puncture from six mice per time point at eight time points (0.25, 0.5, 1, 2, 4, 8, 12, and 24 hours) for each regimen. Blood samples were placed in tubes containing sodium heparin and separated; plasma samples were stored at -80°C until assayed.

AFN-1252 concentrations in the murine plasma samples were determined by Charles River Laboratories Preclinical Services Montreal Inc., using a fully GLP-validated LC-MS/MS method. The internal standard was provided by Affinium Pharmaceuticals. The reference standards covered a range from 2 to 1000 ng/ml. Quality control samples of 6, 600, and 900 ng/ml were prepared in blank mouse plasma. The coefficient of variation for the calibration standards was $\leq 5\%$.

Pharmacokinetic parameters (AUC_{0-24} , C_{max} , T_{max} and $t_{1/2}$) for the composite data (i.e. six mice at each time point) of each dose were calculated using a one-compartment, first-order elimination model (WinNonlin Professional version 5.0.1, Pharsight Corporation, Mountain View, CA, USA). Compartmental selection was based on visual inspection of the fit, correlation between observed and calculated concentrations, and the numerization of the Akaike's goodness of fit criteria. Parameters obtained from the single dose studies were utilized to predict the drug exposures of the multiple dose regimens for that dosage. For doses without a confirmed pharmacokinetic profile, the exposure was estimated using the mean parameter values (Table 2) of the dose above and below the dose of interest. For doses of <2.5 mg/kg or >100 mg/kg, exposure estimates were constructed using the parameters derived from the closest dose undertaken in the pharmacokinetic studies. The free concentration–time profile for each regimen was calculated using a protein-binding value of 98.5%.

Table 1 MICs of *S. aureus* strains to AFN-1252 and other antimicrobials tested

Antibiotic	MIC ($\mu\text{g/ml}$)		
	MSSA	CA-MRSA	HA-MRSA
AFN-1252	0.008	0.004	0.004
Linezolid	2.0	2.0	2.0
Vancomycin	1.0	1.0	1.0
Erythromycin	1.0	>32	1.0
Clindamycin	0.125	>16	0.125
Levofloxacin	0.25	0.5	0.25
Doxycycline	0.5	0.5	0.5
Trimethoprim/ sulfamethoxazole	0.125/2.4	0.25/4.8	0.06/1.9

Note: Testing performed in triplicate – median values reported.

Table 2 Pharmacokinetics of free AFN-1252 in MSSA-infected neutropenic mice

AFN-1252 (mg/kg)	$fAUC_{0-24}$ (ng hour/ml)	fC_{max} (ng/ml)	T_{max} (hour)	$t_{1/2}$ (hour)
2.5	51.4	5.02	0.72	7.30
5	113	12.32	0.48	6.30
10	141	12.32	0.81	7.97
30	160	13.23	1.70	7.60
100	285	22.14	2.70	8.13

Note: $fAUC_{0-24}$: area under the plasma concentration–time curve at steady state over 24 hours for unbound drug.

fC_{max} : maximum plasma concentration of unbound drug.

T_{max} : time at which C_{max} occurs.

$t_{1/2}$: terminal half-life; time taken for concentration levels to fall to 50% of their value.

Pharmacodynamics

The pharmacodynamic profile (i.e. $fAUC$, fC_{max} and $fT_{>MIC}$) of each dose was constructed for each of the test organisms. The change in bacterial density in thighs, expressed as change in \log_{10} CFU, for both treated and untreated animals were reported using descriptive statistics. Spearman's rank correlation coefficient was used to evaluate the relationship between $fAUC$, fC_{max} and $fT_{>MIC}$ values and bacterial eradication. The relationship between these variables was further evaluated on WinNonlin with PD Model 108 inhibitory effect sigmoid E_{max} model. Visual inspection of the predicted line, correlation coefficient and numerization of Akaike's criteria factored in selection of weight of the data. The same weight was utilized for each pharmacodynamic index. The pharmacodynamic index that best correlated with efficacy was determined by the highest coefficient of determination (r^2) from composite plots of the free drug pharmacodynamic indices, as well as the dose fractionation studies.

Results

In vitro susceptibility

AFN-1252 exhibited MICs of 0.004 to 0.008 $\mu\text{g/ml}$ against the three strains of *S. aureus* (Table 1). This level of activity was at least 32-fold greater than other antibacterials tested and at least 500-fold greater than linezolid.

Protein binding and pharmacokinetic studies

The protein-binding of AFN-1252 (Study 1) was $98.5 \pm 0.35\%$ over a concentration range of 0.1 to 2 $\mu\text{g/ml}$. These studies revealed no concentration-dependent binding for this compound. Non-specific protein binding to the filter apparatus was not observed.

The PK profile displayed non-linear exposures as emphasized by the displayed $fAUC$, fC_{max} and T_{max} , while the half-life remains constant (Table 2, Fig. 1). The pharmacokinetic profile of AFN-1252 determined in Study 2 was similar (data not shown).

Efficacy as assessed by bacterial density with the MSSA strain (Study 1)

For the dose exposure and fractionation studies of AFN-1252, there was excellent recovery of the MSSA strain from infected thighs. At the initiation of

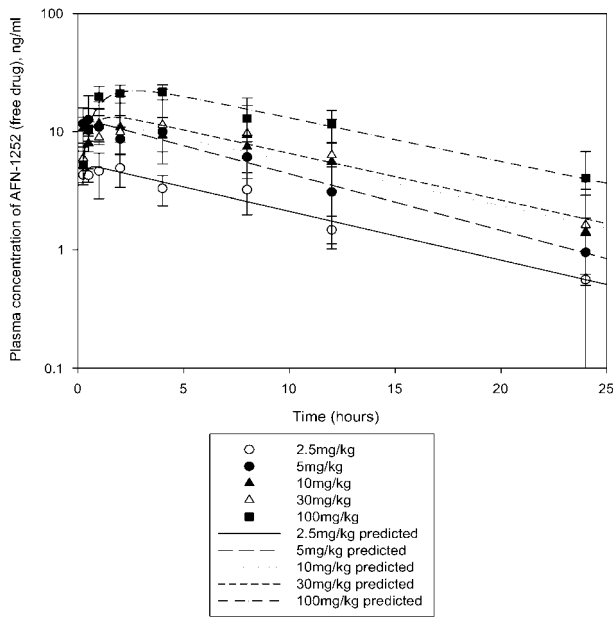


Figure 1 Pharmacokinetic profile of free AFN-1252 following single oral doses in immunocompromised MSSA-infected mice.

treatment (0 hour) bacterial densities ranged from 5.67 to 5.87 log CFU/thigh. The mean increase in bacterial density among untreated control animals at 24 hours was 2.40 ± 0.11 log CFU/thigh.

The dose-response relationships for AFN-1252 with the MSSA strain are presented in Fig. 2. A reduction in MSSA growth rate began at 5 mg/kg. Reductions in total bacterial count of 1 log and greater, compared to time 0, were observed at doses of ≥ 20 mg/kg. For dose ranging studies with MSSA, significant correlations (r^2 values of 0.7917 to 0.9692) were observed for the $fAUC/MIC$, $fT_{>MIC}$ and fC_{max}/MIC (Figs. 3–5). While the $fAUC/MIC$ had the highest correlation, $fT_{>MIC}$ was also very well correlated.

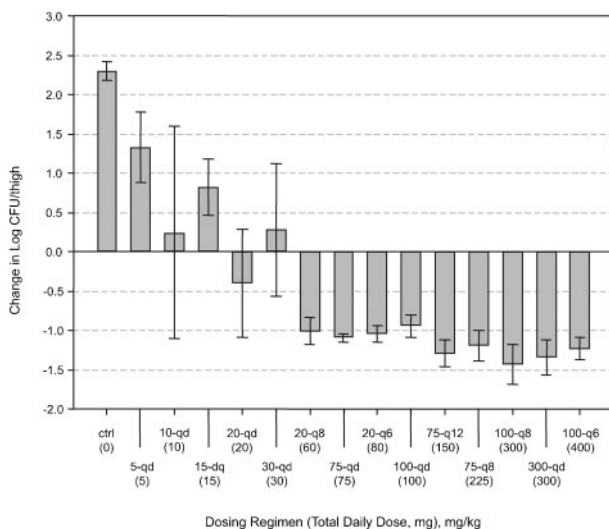


Figure 2 Summary of the dose-response relationship for AFN-1252 in MSSA-infected immunocompromised mice at 24 hours. Error bars represent standard deviations.

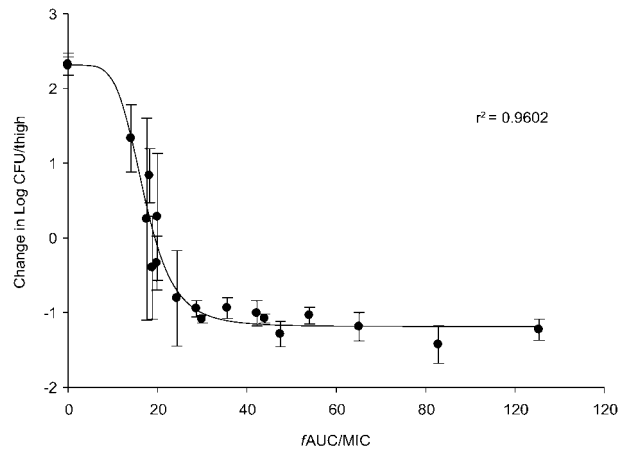


Figure 3 Relationship between $fAUC/MIC$ and change in bacterial density at 24 hours following treatment with AFN-1252 in MSSA-infected mice.

In an attempt to further determine the pharmacodynamic driver of efficacy, dose fractionation studies were undertaken. Dose fractionation of the 10 and 20 mg/kg doses demonstrated antibacterial effects with no clear advantage of increased fC_{max}/MIC (range studied 1.5–2.4) or $fT_{>MIC}$ (range studied 24–92), thus further suggesting that $fAUC/MIC$ (range studied 18–44) is the predominant parameter related to efficacy (Fig. 6). While the achievement of wide ranging fC_{max}/MIC values is hampered by the solubility of the compound, the composite curve (Fig. 3) displays the enhanced fit with $fAUC/MIC$. The 80% (ED_{80}), 50% (ED_{50}) and 5% (ED_5) maximally effective exposures were observed with $fAUC/MIC$ values of 22.3, 17.0, and 9.6, respectively.

Efficacy as assessed by bacterial density with CA-MRSA and HA-MRSA (Study 2)

At the beginning of treatment (0 hour), the bacterial densities ranged from 5.69 to 5.78 CFU/thigh for CA-MRSA and from 5.75 to 5.76 CFU/thigh for HA-MRSA. The mean increase in bacterial density among untreated control animals at 24 hours was

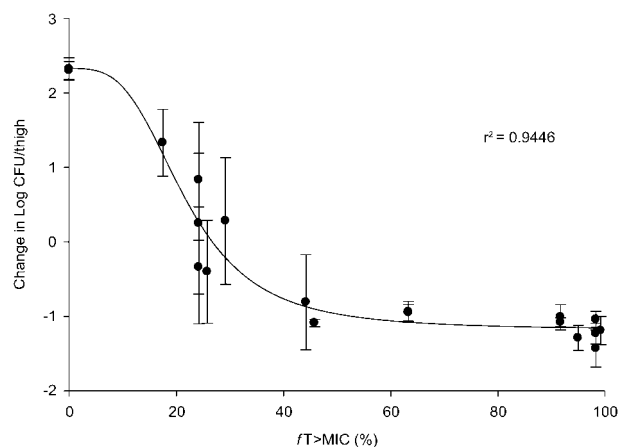


Figure 4 Relationship between $fT_{>MIC}$ and change in bacterial density at 24 hours following treatment with AFN-1252 in MSSA-infected mice.

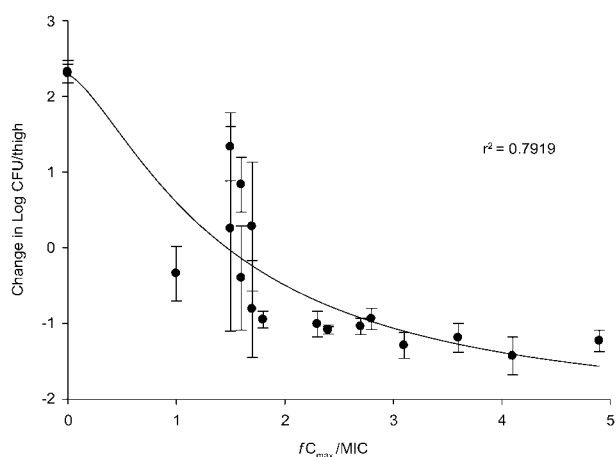


Figure 5 Relationship between fC_{max}/MIC and change in bacterial density at 24 hours following treatment with AFN-1252 in MSSA-infected mice.

1.93 to 2.43 CFU/thigh for CA-MRSA and 2.17 to 2.33 for HA-MRSA.

With CA-MRSA, AFN-1252 growth suppression began at the 2.5 mg/kg dose. Marked reduction in bacterial count, relative to time 0, was seen at a dose of ≥ 10 mg/kg. With linezolid, some suppression of growth was seen at 10 and 40 mg/kg, but doses of ≥ 100 mg/kg were required to achieve consistent reductions in CFU (Fig. 7). Statistical differences in CFU between the two compounds were seen at doses of 2.5 and 40 mg/kg.

With HA-MRSA, the AFN-1252 antibacterial effects were comparable to that observed with CA-MRSA. Reductions in CFU/thigh of >1 log were noted with AFN-1252 doses ≥ 10 mg/kg, whereas doses of ≥ 300 mg/kg were required for similar effect by linezolid (Fig. 8). Statistical differences in log CFU between the test compounds were seen at doses of 1, 10, 40, and 300 mg/kg.

The 80, 50 and 5% maximally effective mg/kg doses for the CA-MRSA and HA-MRSA isolates were

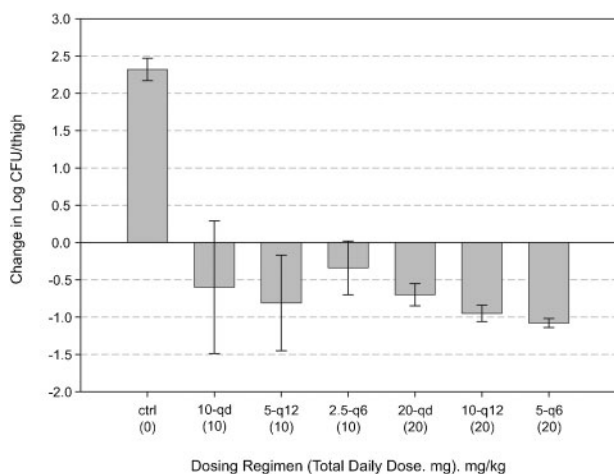


Figure 6 Summary of the dose fractionation studies of AFN-1252 in MSSA-infected immunocompromised mice at 24 hours. Error bars represent standard deviations.

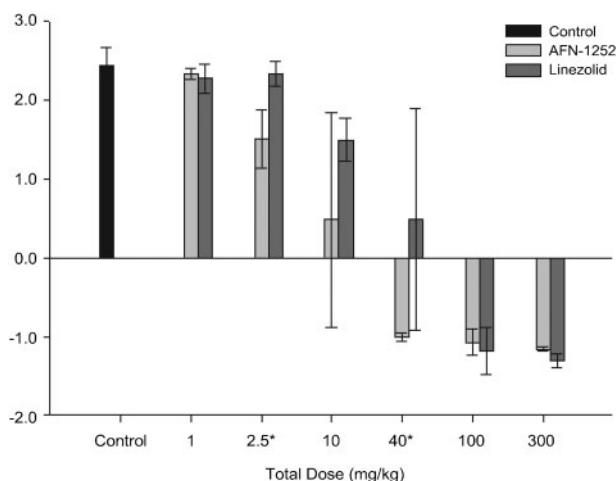


Figure 7 Dose-response relationship for single oral doses of AFN-1252 and linezolid in CA-MRSA-infected immunocompromised mice at 24 hours. Error bars represent standard deviations. *Denotes statistical differences in CFU between the two compounds.

between 4 and 20 times lower for AFN-1252 compared with linezolid. Similar to the pharmacodynamic correlations reported for the MSSA using the full dose-response curve, $fAUC/MIC$ also appears to be the best correlated index for CA-MRSA and HA-MRSA (data not shown). Table 3 summarises the $fAUC/MIC$ of AFN-1252 required to achieve various efficacy assessments for CA-MRSA, HA-MRSA and MSSA.

Discussion

The literature is replete with evidence of the clinical sequela associated with skin infection due to *S. aureus*; moreover the emergence of antibiotic resistance in a great many isolates derived in both the hospital and community setting continues to challenge the clinician.¹⁻⁴ As a result of this organism's

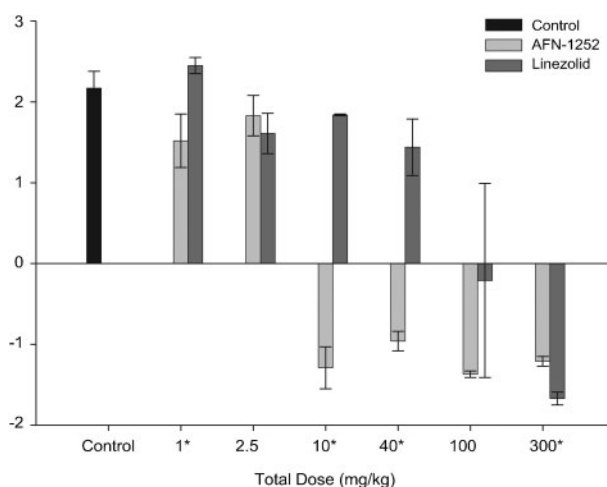


Figure 8 Dose-response relationship for single oral doses of AFN-1252 and linezolid in HA-MRSA infected immunocompromised mice at 24 hours. Error bars represent standard deviations. *Denotes statistical differences in CFU between the two compounds.

Table 3 Relationship between *f*AUC/MIC and various AFN-1252 efficacy assessments for MSSA, CA-MRSA and HA-MRSA at 24 hour^a

Isolate	ED ₈₀	ED ₅₀	ED ₅	Stasis	-1 Log kill	ΔLog CFU/thigh
MSSA	22.3	17.0	9.6	19.4	23.6	3.50
CA-MRSA	69.3	32.3	6.4	43.0	55.9	3.67
HA-MRSA	32.0	29.4	4.6	30.0	33.67	2.63

Note: ^aValues represent the *f*AUC/MIC at various percentages (80, 50 or 5%) of the effective dose, stasis and 1 log kill as determined by a sigmoid E_{max} model.

evolutionary propensity to finding new and frequent means of acquiring antibiotic resistance and the prominence of this etiologic agent in a wide range of infections, new antimicrobials with novel mechanisms of action are required.

A one-compartment model best characterized the free concentration-time profile of AFN-1252 in murine plasma (Study 1). AFN-1252 is characterized as a Class 2 compound on the Biopharmaceutics Classification System, possessing high permeability but low aqueous solubility.^{11,12} Following oral administration of the AFN-1252 suspension, rapid absorption, which is limited by the rate of compound dissolution, occurs along the entire gastrointestinal tract. As higher doses of the drug are administered, a smaller proportion of soluble drug is available, resulting in non-linear exposures as was noted in the PK profile with increasing doses. While non-linearity has been observed due to the nature of the compound under study, this was taken into account in the pharmacokinetic parameter estimates (see the section on 'Pharmacokinetics') and therefore these parameters can be reliably utilized in PD profiling.

Herein, we report the high *in vitro* potency of a novel antimicrobial, AFN-1252, in comparison with other agents commonly utilized against *S. aureus*, including methicillin-resistant strains. Multiple studies have shown potent and specific-spectrum anti-staphylococcal activity of AFN-1252 *in vitro* against clinical isolates of MSSA, MRSA, and methicillin-susceptible and -resistant *Staphylococcus epidermidis* (MSSE and MRSE), with MIC₉₀ values of ≤0.008–0.015, ≤0.008–0.015, 0.03–0.06 and ≤0.008–0.03 µg/ml, respectively.^{13,14}

Our current investigation demonstrates the excellent and equivalent *in vivo* potency of AFN-1252 against all strains tested, including both PVL-positive and -negative MRSA strains. In addition, when similar mg/kg doses of AFN-1252 and linezolid were administered orally, AFN-1252 produced greater antibacterial effects at lower dose levels. Lastly, this study suggests that the *f*AUC/MIC is predictive of efficacy and has also provided important evidence regarding the *in vivo* exposures of AFN-1252 required for antibacterial effects. AFN-1252 may well in the

future become an important addition to the physician's armamentarium in the treatment of staphylococcal infections.

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