

In Vitro Characterization of API-1252, a Novel Inhibitor of Bacterial Fatty Acid Biosynthesis, Against Drug Resistant Staphylococci

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ABSTRACT

Background API-1252 is a clinical candidate being developed as an oral and intravenous anti-staphylococcal agent with a novel mechanism of action targeting the essential enzyme enoyl-ACP reductase (FabI). In this study API-1252 was characterized by *in vitro* potency, resistance selection, time-kill profiles and mechanism of action.

Methods MIC testing was performed according to CLSI guidelines. Standard procedures were used for time-kill and resistance selection studies. Mechanism of action was determined by assessing inhibition of macromolecular synthesis. Strains used were methicillin-susceptible *Staphylococcus aureus* (MSSA), methicillin-resistant *S. aureus* (MRSA) including linezolid-resistant and vancomycin-intermediate strains, methicillin-susceptible *S. epidermidis* (MSSE) and methicillin-resistant *S. epidermidis* (MRSE). MIC₉₀ values were determined with 62 recent staphylococcal methicillin-susceptible and -resistant clinical isolates.

Results An MIC₉₀ of 0.016 µg/ml was obtained against all tested clinical strains of MRSA, MSSA, MRSE and MSSE. Time-kill studies showed a time-dependent mechanism of killing with a decrease of 1 to 3 log CFU/ml at 24 hours compared to time 0. Frequencies of resistance were 10⁷ to 10¹⁰ at 4X the MIC and were below the level of detection (<5x10⁻¹⁰) at higher inhibitor concentrations. In 24-day multiple passage resistance selection studies API-1252 MICs increased only 4-fold compared to 16 to 64-fold with ciprofloxacin. Mechanism of action studies demonstrated that API-1252 selectively inhibited fatty acid biosynthesis.

Conclusion The high potency, low level of resistance and novel mechanism of action highlight the potential therapeutic application of API-1252 as a novel, specific spectrum, oral and intravenous anti-staphylococcal agent in proven challenging staphylococcal infections or in empiric combination therapy.

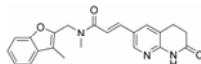
INTRODUCTION

The rapid increase in bacterial drug resistance has created an urgent need for new antibiotics with novel mechanisms of action. The bacterial FASII fatty acid synthesis pathway represents an attractive target for novel antibiotic development. Fatty acid biosynthesis is essential for bacterial growth and survival, forming the building blocks for the bacterial membrane and the acyl substrates for acylation of proteins, membrane-derived oligosaccharides and biotin. The essential enzyme FabI catalyzes the final step in the chain elongation process reducing enoyl-ACP to acyl-ACP. Inhibition of FabI prevents growth of the acyl chain and disrupts both saturated and unsaturated fatty acid biosynthesis.

METHODS

Test compounds API-1252 (Figure 1) was synthesized at Affinium Pharmaceuticals. Other antibiotics used were from Sigma-Aldrich or other commercial sources

Figure 1. Chemical structure of API-1252



Bacterial strains were from the American Type Culture Collection, Manassas, VA, or from the Affinium bacterial strain collection, or from the Micromyx Inc. strain collection

MIC testing was performed according to CLSI guidelines using the microdilution method in 96-well plates.

Macromolecular synthesis inhibition studies were performed with *S. aureus* ATCC 29213. Logarithmic phase cells were adjusted to an OD₆₀₀ of 0.25 and incubated with shaking in Mueller-Hinton broth at 37°C. Cultures were then treated with API-1252 (0.016 µg/ml; 2X the MIC) for 15 minutes. Tritium-labeled precursors were then added, and incubation continued for 20, 40 or 60 minutes. Experiments were terminated by treating the cultures with ice-cold 10% TCA for 2 hours. Precipitates were collected by filtration, washed and analyzed in a MicroBeta Trilux scintillation counter. Experiments were performed in duplicate and the results are expressed as the average % radioactive precursor incorporation relative to non-treated (no drug) control. Results with control antibiotics vancomycin (GlcNAc – cell wall), levofloxacin (thymidine – DNA), rifampin (uridine – RNA), chloramphenicol (AA mix – protein) and triclosan (acetate – lipids) were consistent with their specific mechanisms of action.

Frequency of resistance was determined following single selection on tryptic soy agar or Mueller-Hinton agar plates. Bacterial cells were spread to a density of 1 – 5 x 10⁷ CFU per plate on ten plates or to a density of 1 – 9 x 10⁷ CFU on a single plate. Resistant colonies were scored following 48 – 72 hour incubation at 35°C, and resistant phenotypes were confirmed by subculture on compound containing plates. Frequency of resistance was calculated by dividing the total number of resistant colonies by the total number of viable cells for each strain x compound concentration combination.

Multiple passage resistance selection was performed in 96 well plates using CLSI guidelines for microdilution MIC testing. Following 24 hour incubation, inocula for fresh MIC plates were prepared from the highest concentration of drug supporting visible growth (typically 1 dilution below the MIC). This process was repeated for 24 consecutive days.

Time kill experiments were performed according to CLSI guidelines.

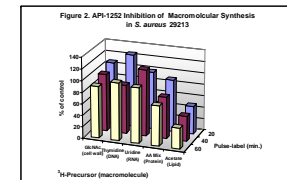
Table 1. Primary panel MICs of API-1252 and comparators*

Bacterial strain	MIC (µg/ml)		
	API-1252	VAN	LZD
<i>S. aureus</i> 29213	0.008	1	4
<i>S. aureus</i> 43300 MRSA	0.004	1	2
<i>S. aureus</i> 934335 TnR	0.016	2	2
<i>S. epidermidis</i> 1024939	0.008	2	2
<i>S. epidermidis</i> 1024961 Tn R	0.016	1	2
<i>S. pneumoniae</i> 49619	>32	0.25	2
<i>E. faecalis</i> 29212	>32	>2	4
<i>H. influenzae</i> 49247	0.25	>32	16
<i>M. catarrhalis</i> 49143	0.016	>32	4
<i>E. coli</i> AG100	>32	>32	>32
<i>E. coli</i> AG100A acfAB	0.016	>32	8
<i>P. aeruginosa</i> K767	>32	>32	>32
<i>P. aeruginosa</i> K1119 mexAB-oprM	>32	>32	>32

* VAN - vancomycin; LZD - linezolid

Primary panel MICs. API-1252 exhibited high potency against all staphylococcal strains tested including methicillin- and triclosan-resistant strains (Table 1). API-1252 was 50 to 500 times more potent against staphylococci than the comparators vancomycin and linezolid. The spectrum of activity was consistent with the presence of the essential FabI target in the susceptible species.

Table 2. Population MICs of API-1252 and Linezolid



Mechanism of action studies Inhibition of macromolecular synthesis in *S. aureus* (Figure 2) demonstrated that the primary mechanism of action of API-1252 is via inhibition of lipid biosynthesis. This inhibition was selective for acetate incorporation and also increased with time showing 52% inhibition after 20 minutes and 75% inhibition at 60 minutes.

Table 2. Population MICs of API-1252 and Linezolid

	API-1252				Linezolid			
	Range	MIC ₅₀	MIC ₉₀	MIC ₉₅	Range	MIC ₅₀	MIC ₉₀	MIC ₉₅
<i>S. aureus</i> (21)	<0.002 - 0.125	0.008	0.016	0.032	0.5 - 4	2	4	4
MRSA (10)	<0.002 - 0.016	0.004	0.016	0.032	0.5 - 4	2	4	4
MSSA (11)	0.004 - 0.125	0.008	0.016	0.032	2 - 4	2	4	4
<i>S. epidermidis</i> (41)	0.004 - 2	0.016	0.016	0.032	0.5 - 8	2	2	2
MRSE (21)	0.004 - 2	0.016	0.016	0.032	1 - 8	1	2	2
MSS (20)	0.008 - 0.5	0.016	0.016	0.032	0.5 - 8	2	4	4

Table 3. Staphylococcal frequencies of resistance with API-1252 and comparators*

Strain	Phenotype	API-1252			Rifampicin		Linezolid	
		4xMIC	16x - 128xMIC	>32xMIC	4xMIC	16xMIC	4xMIC	16x - 128xMIC
<i>S. aureus</i> 29213	MSSA	6.8E-10	<3.3E-10	<3.3E-10	Not tested	<3.3E-10	<3.3E-10	
<i>S. epidermidis</i> 1024939	MSSE	1.1E-09	<1.1E-09	<1.1E-09	Not tested	<3.3E-10	<3.3E-10	
<i>S. aureus</i> 43300	MRSA	1.2E-09	<3.0E-10	<3.0E-10	Not tested	<2.9E-10	<2.9E-10	
<i>S. aureus</i> 1137	MRSA	<1.3 E-10	<1.3 E-10	TNTC	0.0E-08	<1.3 E-10	<1.3 E-10	
<i>S. aureus</i> 2293	CA-MRSA	5.7E-10	<8.8 E-11	TNTC	5.07E-08	<1.3 E-10	<1.3 E-10	
<i>S. aureus</i> 1725	LRSA	1.3E-09	<2.0E-10	Rif ^R	LZD ^R	<1.3 E-10	<1.3 E-10	
<i>S. aureus</i> 2012	VISA	2.4E-09	5.6E-10	TNTC	8.5E-08	<1.3 E-10	<1.3 E-10	

*MICs for susceptible strains were 0.008, 0.004 and 1 - 4 µg/ml for API-1252, rifampicin and linezolid, respectively

**S. aureus* strain 1137, 2293, 1725 and 2012 were tested at 16X the MIC (0.12 µg/ml) and the remainder strain at 128X the MIC (1 µg/ml)

Frequencies of resistance. API-1252 showed frequencies of resistance of 10⁹ to 10¹⁰ at 4X the MIC (0.032 µg/ml) against the majority of the strains tested including MRSA, CA-MRSA, vancomycin intermediate and linezolid resistant strains (Table 3). At 16x to 128x the MIC (0.12 to 1 µg/ml) the frequencies of resistance were generally below the level of detection of ~1x10⁻¹⁰. In comparison, strains tested with rifampicin were highly resistant at 4X the MIC and showed frequencies of resistance approaching 10⁷ at 16X the MIC. With linezolid, all strains showed frequencies of resistance below the level of detection at all concentrations tested.

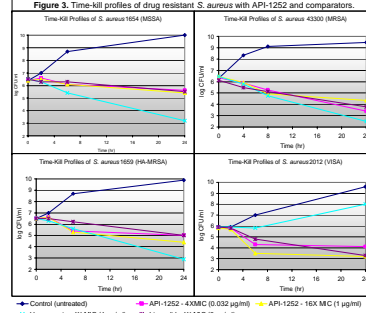
RESULTS

Table 4. Multiple passage resistance selection with API-1252 and comparators

Bacterial Strain	MIC (µg/ml)											
	API-1252			Vancomycin			Ciprofloxacin					
	Day 1	Day 24	X MIC increase	Day 1	Day 24	X MIC increase	Day 1	Day 24	X MIC increase	Day 1	Day 24	X MIC increase
<i>S. aureus</i> 29213 MSSA	0.004	0.016	4	1	2	2	1	16	16	16	16	16
<i>S. aureus</i> 43300 MRSA	0.004	0.016	4	1	2	2	0.25	16	64	64	64	64
<i>S. epidermidis</i> 1024939	0.004	0.016	4	2	4	2	0.25	16	64	64	64	64
<i>S. epidermidis</i> 1024961 TnR	0.016	0.064	4	1	2	2	0.25	8	32	32	32	32

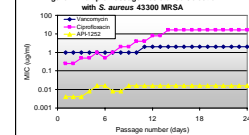
Multiple passage resistance selection. The increase in API-1252 MICs at day 24 vs. day 1 was 4 fold for all strains tested, similar to the results with vancomycin (Table 4). In contrast ciprofloxacin caused an increase of 16 to 64 fold in MICs at day 24 vs. day 1. The 4 fold API-1252 increase in MICs stabilized at day 10, and did not increase further up to day 24 (Figure 2).

Figure 3. Time-kill profiles of drug resistant *S. aureus* with API-1252 and comparators.



Time-kill studies (Figure 3) show that API-1252 has a time-dependent mechanism of killing that results in a 1 to 3 reduction in log viable counts at 24 hours compared to time 0. The API-1252 time-kill profiles were similar to those of linezolid. For strain *S. aureus* 2012 (VISA) vancomycin was tested at 16 µg/ml (1/2X the MIC).

Figure 2. Multiple Passage Resistance Selection with *S. aureus* 43300 MRSA



CONCLUSIONS

In vitro studies on API-1252 demonstrated the following features:

- Unique spectrum of activity
- Highly potent activities against staphylococci
- Equal potency against MRSA and MSSA strains
- Highly active against vancomycin intermediate *S. aureus*
- Low to non-detectable frequencies of resistance
- Time-kill profiles comparable to linezolid
- Novel mechanism of action

These properties support the further development of API-1252 as an oral and intravenous agent for challenging staphylococcal infections

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