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Antimicrobial Activity of Non-Hydroxamate LpxC Inhibitors

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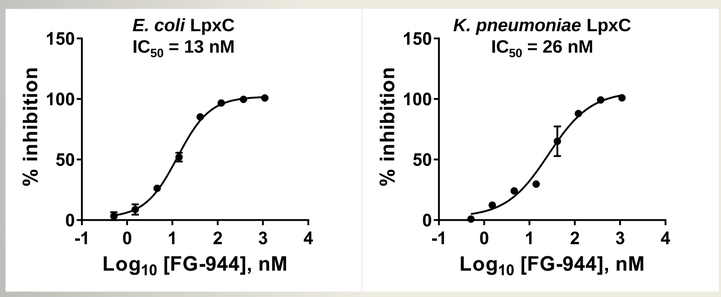
Abstract

Background: LpxC is a zinc-dependent deacetylase responsible for the biosynthesis of lipid A, an essential component for Gram-negative bacteria. LpxC inhibitors have been shown to be cidal to several key Gram-negative pathogens. Presented herein is the *in vitro* characterization of Forge's non-hydroxamate LpxC inhibitors including enzymatic and antimicrobial activity, time-kill studies, frequency of resistance profiling, and off-target effect screening.

Methods: LpxC inhibitors were screened in an enzymatic assay using the RapidFire/MS system and a fluorescent thermal stability assay with Sypro Orange Dye. The antimicrobial activities of Forge LpxC inhibitors were measured by minimum inhibitory concentration (MIC) determination using CLSI methods against a broad panel of Gram-negative and Gram-positive reference strains as well as MDR strains and clinical isolates, which included extended-spectrum beta-lactamases (ESBL), carbapenem-resistant *Enterobacteriaceae* (CRE), and *E. coli* harboring mcr-1. Development of resistance by *Enterobacteriaceae* was studied by standard spontaneous mutation frequency method. Bactericidal kinetics against Gram-negative organisms were determined by time-kill assays. Potential off-target activity was assayed using commercially available fluorescence-based enzyme assay kits and cytotoxicity assay in several human cell lines.

Results: LpxC Inhibitor FG-944 has nanomolar IC_{50} values for *E. coli* LpxC enzyme, increases thermal stability of *E. coli* LpxC protein by 14º C and has MIC values less than 1µg/mL against wild type and MDR *Enterobacteriaceae* while having no activity towards Gram-positive organisms such as *S. aureus*. Resistance frequencies and time-kill kinetics are comparable to historical values for hydroxamate-based LpxC inhibitors. Compounds show no cytotoxicity against human cell lines and no activity against other metalloenzymes in enzymatic assays at levels > 50X *in vivo* efficacious free drug concentrations.

Enzyme Assay



Inhibition of *E. coli* and *K. pneumoniae* LpxC activity by FG-944. IC_{50} values were determined using a mass spectrometry-based assay to measure de-acetylation of LPS substrate by LpxC proteins. Shift in *E. coli* LpxC stabilization upon binding of FG-944 is 14ºC, as determined by StepOnePlus instrument using recombinant *E. coli* protein and Sypro Orange Dye.

Frequency of Resistance

		4x MIC	8x MIC
E. coli ATCC 25922	FG-944	9.09×10^{-8}	5.11×10^{-8}
	CHIR-090	1.33×10^{-8}	1.33×10^{-8}
	PF-5081090	8.27×10^{-8}	1.73×10^{-8}
P. mirabilis HM752	FG-944	5.2×10^{-8}	4.0×10^{-9}

Frequency of Resistance (FoR) studies in *E. coli* and *P. mirabilis* confirm FG-944 has similar resistance profile to historical LpxC inhibitors. To confirm that the reduced susceptibility to FG-944 was stable, the resistant mutants were passaged five times in the absence of selection before being subjected to further susceptibility testing with levofloxacin, polymyxin B, FG-944, CHIR-090 and PF-5081090. The results showed that reduced susceptibility to FG-944 was retained for every isolate tested, and the increase in MIC was in the range 8- to 64-fold (mode 16-fold); similar increases in MIC were also observed for CHIR-090 and PF5081090 (cross-resistance), but no MIC change for levofloxacin and polymyxin B. Preliminary data indicates that resistant clones carry mutations in *FabZ* gene and have slower growth rates *in vitro*.

Antimicrobial Activity

Antimicrobial activity of FG-944 and comparators against a panel of MDR and XDR *Enterobacteriaceae spp.* clinical isolates including ESBL and CRE strains.

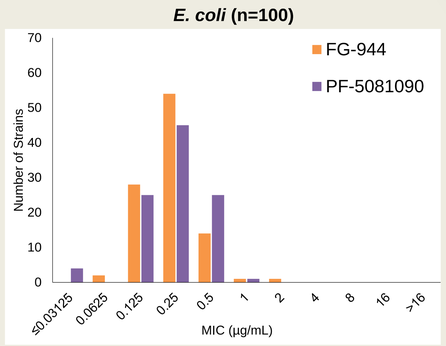
	FG-944			PF-5081090			Levofloxacin			Meropenem		
	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range
***E. coli* (n=100)**	0.25	0.5	0.06 to 2	0.125	0.25	≤0.03 to 0.5	>8	>8	≤0.016 to >8	≤0.016	8	≤0.016 to >8
***K. pneumoniae* (n=100)**	1	4	0.25 to 8	0.5	2	0.06 to 8	1	>8	0.03 to >8	4	>8	0.03 to >8
***Enterobacter* spp. (n=81)**	1	4	0.25 to 8	0.25	2	0.125 to 2	0.25	>8	≤0.016 to >8	0.125	4	≤0.016 to >8
***Proteus mirabilis* (n=42)**	2	8	1 to 8	1	2	0.5 to 8	0.06	4	≤0.016 to >8	≤0.125	0.5	≤0.125 to 16

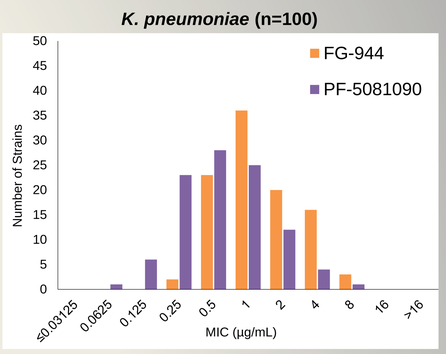
Antimicrobial activity of FG-944 and comparators against a panel of selected Gram-positive bacterial species. LpxC enzyme is found only in Gram-negative bacteria, hence no antimicrobial activity is expected against Gram-positive bacteria.

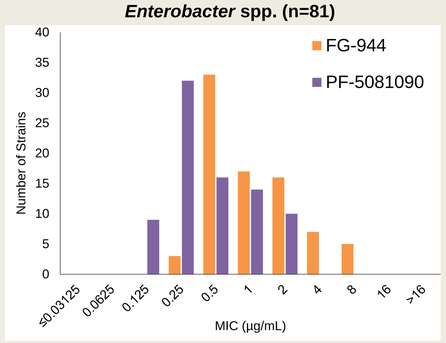
Strain		FG-944	Meropenem	Levofloxacin	PF-5081090
S. aureus	**ATCC 29213**	>64	0.125	0.25	>16
Enterococcus faecalis	**ATCC 29212**	>64	>1	1	>16
Streptococcus pyogenes	**ATCC 12384**	>64	0.008	0.5	>16
Bacillus thuringiensis	**ATCC 35646**	>64	0.06	0.125	>16
Lactobacillus rhamnosus	**ATCC 53103**	>64	>1	1	>16
Staphylococcus epidermidis	**ATCC 35984**	>64	>1	0.125	>16
Bifidobacterium breve	**HM 412**	>64	>1	4	>16
Clostridium difficile	**ATCC 700057**	>64	1	4	>16
Clostridium sordellii	**ATCC 9714**	>64	0.015	1	>16
Peptostreptococcus anaerobius	**DSM 20357**	>64	>1	0.5	>16
Streptococcus pneumoniae	**ATCC 49619**	>64	0.06	1	>16
Corynebacterium jeikeium	**NCTC 11914**	>64	>1	0.5	>16
Propionibacterium acnes	**ATCC 6919**	>64	0.06	0.5	>16
Listeria monocytogenes	**ATCC 7644**	>64	0.125	1	>16
Nocardia cyriacigeorgica	**N3295**	>64	>1	8	>16
Bacteroides fragilis	**ATCC 25285**	>64	0.06	0.5	>16

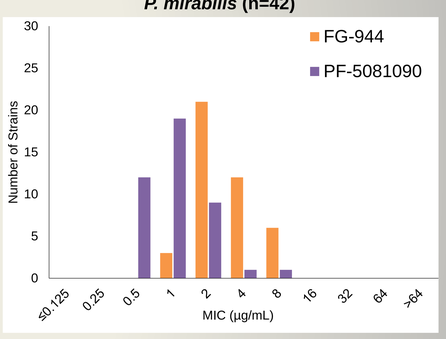
Antimicrobial activity of FG-944 and comparators against six strains of *Bacteroides fragilis,* one of the dominant species within the lower human intestinal tract.

B. fragilis	FG-944	Metronidazole	Levofloxacin	CHIR-090	PF-5081090
ATCC 25285	>16	0.5	1	>16	>16
HM709	>16	0.5	8	>16	>16
HM710	>16	0.5	4	>16	>16
HM711	>16	0.5	1	>16	>16
HM 714	>16	2	1	>16	>16
HM 20	>16	0.25	4	>16	>16

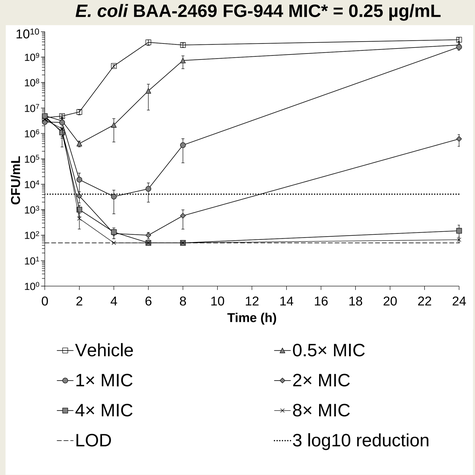


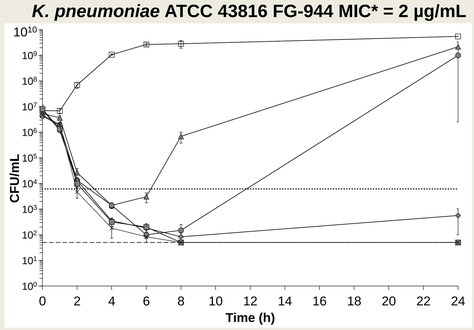


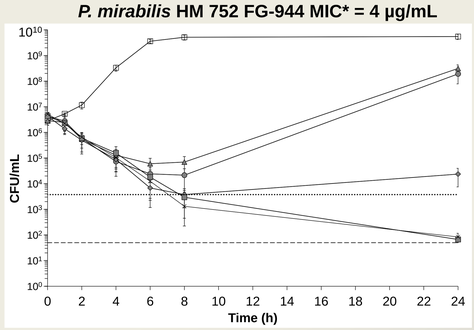




Time-Kill Experiments

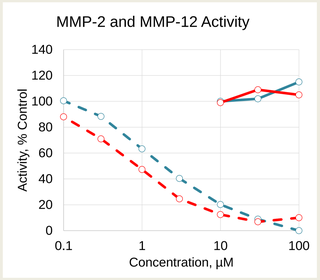






FG-944 achieved bactericidal activity against *E. coli* BAA-2469, *K. pneumoniae* ATCC 43816 and *P. mirabilis* HM 752 with rapid concentration-independent killing rates.
MIC* values determined by microdilution method. Bactericidal activity refers to ≥3 $\log_{10}$ CFU/mL (horizontal dotted line on the graph) reduction in viability relative to the starting inoculum after 24 h exposure to FG-944. The limit of detection (horizontal dashed line on the graph) for these assays was 50 CFU/mL.

Off-target Activity Assays



Inhibition of MMP-2 (blue) and MMP-12 (red) by FG-944 (solid lines) and PF-5081090 (dotted lines). Assays were run in triplicate using commercially available fluorescence-based enzyme assay kits.

FG-944 showed no activity in assays with other human metalloproteins (ACE1, HDACs, CAII), ion channel, CYP, and receptor panel screens and cytotoxicity assays with several human cell lines (>30 µM IC_{50}).

Summary and Conclusions

Forge has identified non-hydroxamate LpxC inhibitor, FG-944 that:

- demonstrates *in vitro* on-target activity
- not cross-reactive with other tested Zn(II) metalloenzymes.
- shows excellent activity against a variety of Gram-negative bacteria, including clinical isolates harboring plasmids containing the resistance genes *mcr-1*, ESBL, KPC, and NDM
- shows rapid bactericidal activity against *E. coli, K. pneumoniae* and *P. mirabilis*
- inactive against Gram-positive organisms
- has FoR rate on par with hydroxamic acid based LpxC inhibitors
- efficacious in mouse infection models (*data presented in poster 644*)

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