Identification of Novel Inhibitors of *Clostridioides difficile* Enoyl-ACP Reductase II (FabK) by High-Throughput Virtual and Experimental Screening

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A Overview

- *Clostridioides difficile* is categorized as an '*urgent*' drug-resistant threat by the CDC with 500,000 hospital-acquired infections and 29,000 deaths per year.[1]
- Infection recurrence occurs in ~25% of patients treated with metronidazole or vancomycin; sporulation contributes to high relapse rates.
- *Enoyl-Acyl Carrier Protein (ACP) reductase* is a promising target for narrow-spectrum anti-difficile agents with demonstrated essentiality and druggability.
- Species-specific and mechanistically distinct enoyl-ACP reductase isozymes (FabI, FabL, FabV, FabK) allow for narrow-spectrum *C. difficile* targeting with decreased microbiome impact.
- Inhibitors of *C. difficile* FabK (*Cd*FabK) have been characterized with promising in vitro and in vivo anti-difficile and anti-sporulation activity.[2, 3, 4]
- These studies report work done to identify novel inhibitors with increased potency.

B Background

Table 1. Enoyl-reductase distribution in gut flora.

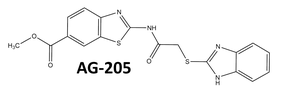
Organism	FabI	FabK	Repressor
Bifidobacteria[a]			
Lactobacteria	+	±	FabT
Propionobacteria	+		FabT
Peptostreptococci		+	Not Found
E. faecalis	+	+	FabT
E. faecium	+		FabT
Bacteriodes	+	+	Not Found
Staphylococci	+		FapR
Streptococci[b]		+	FabT
C. difficile		+	FapR
Enterobacteria	+	±	FapR

[a] *Yeast-like Fatty Acid Synthase*
[b] *Known to use endogenous FA's to bypass FAS-II*

- The FabK isozyme is present in *C. difficile*, while many non-pathogenic gut organisms express FabI.
- The FabI isozyme is not affected by FabK inhibitors.
- The FabT repressor allows for bypass of bacterial fatty acid synthesis (FAS-II) inhibition using host fatty acids; the FapR repressor does not.[5]
- Two known classes of FabK inhibitors exist - benzothiazoles and phenylimidazoles.
- The benzothiazole class does not possess whole-cell activity against *C. difficile.*
- The phenylimidazole inhibitor class possesses in vitro and in vivo activity, but poor ADME properties and divergent activity across species.

C Known FabK Inhibitors

- *Benzothiazole scaffold*

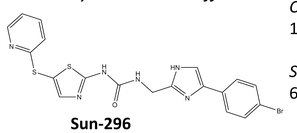


AG-205

*Cd*FabK IC_{50} 3.06 µM

*Sp*FabK IC_{50} 5.32 µM

- *Phenylimidazole scaffold*



Sun-296

*Cd*FabK IC_{50} 1.27µM

*Sp*FabK IC_{50} 67nM

Table 2. ADME Properties of Known Inhibitors

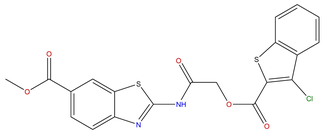
Compound	Protein Binding (%)	Solubility (µM)	Metabolic Stability (t½)	Caco-2 (nm/s)
AG-205	99.6 (±0.3)	0.001	1.01 H	66.6 (A/B)
Sun-296	99.77 (±0.2)	0.01	1.37 H	21.65 (A/B

Table 3. MIC's of Sun-296 against various bacteria (µM)

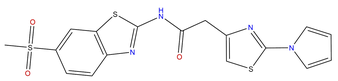
Organism	Isozyme	- palmitic acid	+ palmitic acid
C. difficile CD630	FabK	16	16
C. difficile R20291	FabK	8	2
S. pyogenes 19615	FabK	<0.0625	4
C. perfringens HM310	FabV	>64	>64
B. ovatus HM222	FabI & FabK	>64	>64
S. aureus Newman	FabI	>64	>64

Methods & Results - Virtual Screening

Methods: 1. *High-throughput molecular docking* of commercial chemical libraries into a comparative model of the *Cd*FabK (built from *S. pneumoniae* FabK structure). ~2.4M compounds docked and scored. 24 top scoring compounds ordered & tested in-house - no appreciable inhibitory activity. **2.** *Ligand-based virtual screen* - Shape & electrostatic matching search of same libraries using active site conformations of known inhibitors. 93 compounds ordered & tested in-house - *2 confirmed hits*.

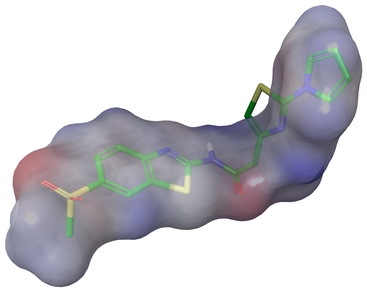


KH-58 - 80% *Cd*FabK inhibition @ 20 µM
*Cd*FabK IC_{50} = N/D



KH-70 - 92% *Cd*FabK inhibition @ 20 µM
*Cd*FabK IC_{50} = 0.35 µM

D

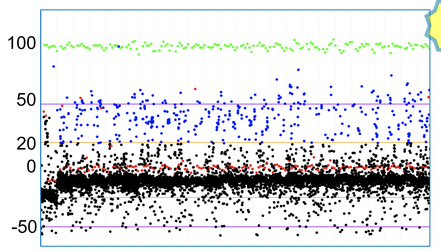


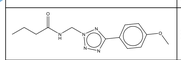
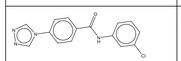
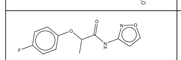
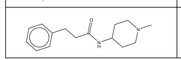
Shown above is the hit compound **KH-70** as it matched the AG-205 shape/electrostatic ligand-based search. *KH-70 is the first sub-micromolar inhibitor of CdFabK to be characterized to date.*

Methods & Results - High-Throughput Screening

Methods: 1. *Primary Assay* was a newly designed luminescence-based, biochemical assay that followed consumption of the NADH cofactor during the enzymatic reaction. **2.** *Secondary assay* was a continuous, fluorescence-intensity assay that followed consumption of NADH cofactor (ex 340, em 460). **3.** A lead-like (<300 kDa) library of 10K compounds was screened at SJCRH (*shown below*).

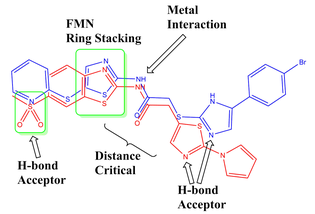
E



Compound	*Cd*FabK Inhibition (@ 100 µM)
	99.8%
	83.4%
	76.0%
	72.7%

Discussion

S.A.R. Analysis: Analysis of hits from both screening campaigns along with follow up analog testing have allowed the development of a Structure Activity Relationship hypothesis. Key points are shown in the figure below.
X-ray Crystal Structure: A 1.7Å co-crystal structure with KH-70 bound to CdFabK has been solved, allowing additional insight into S.A.R. and key binding determinants.



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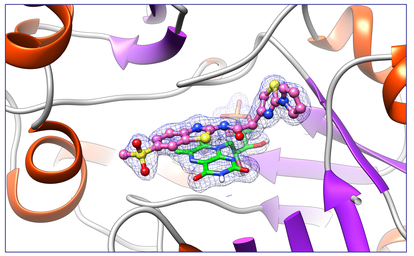

S.A.R. analysis of 296 (blue) and 70 (red).

Conclusions & Future Directions

- Experimental and high-throughput compounds screening have led to the identification of several low micromolar and one sub-micromolar inhibitor of *Cd*FabK. Activity testing of hits and hit analogs has led to an S.A.R. hypothesis that will guide future chemical modifications.
- Future studies will focus on attaining whole-cell and in vivo activity in the KH-70 series by incorporation of HTS hit features and bioisosteric modification of labile features (amide group).

Citations

1. CDC. **2019**. DOI: http://dx.doi.org/10.15620/cdc:82532.
2. Ozawa, T., et. al. *Bioorg. Med. Chem*., **2007**, *15*, 7325–7336.
3. Marreddy R.K.R., et al. *ACS Infect Dis*. **2019**, *5*, 208-217.
4. Jones, J.A., et al. *ACS Chem. Biol.* **2019**, *14*, 1528-1535.
5. Parsons, J. B., et. al. *PNAS.* **2011**. *108*, 15378–15383.

Acknowledgements

This work was supported by NIH/NIAID (R21AI126755). Molecular graphics were generated using the UCSF Chimera (NIGMS P41-GM103311). Use of LS-CAT Sector 21 (grant 085P1000817) at the Advanced Photon Source (US Department of Energy, Office of Science, Office of Basic Energy Sciences under Contract No. DE-AC02-11357) is acknowledged. JJ and RW gratefully acknowledge fellowship and scholarship support from the American Foundation for Pharmaceutical Education.