A novel benzoxaborole works by targeting FabI in *Escherichia coli*

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Abstract

To combat the looming crisis of antimicrobial-resistant infections, there is an urgent need for novel antimicrobial discovery and drug target identification. The benzoxaborole series were previously identified as inhibitors of mycobacterial growth. Here, we demonstrate that a benzoxaborole is also active against the Gram negative bacterium, *Escherichia coli* in vitro. We isolated resistant mutants of *E. coli* and subjected them to whole genome sequencing. We found mutations in the enoyl acyl carrier protein FabI. Mutations mapped around the active center site located close to the co-factor binding site. This site partially overlaps with the binding pocket of triclosan, a known FabI inhibitor. Similar to triclosan, the interaction of the benzoxaborole with FabI was dependent on the co-factor NAD^+. Identification of the target of this compound in *E. coli* provides scope for further development and optimization of this series for Gram negative pathogens.

Introduction

Antimicrobial resistance is a growing problem and at current rates of increase, it is estimated by the year 2050 ~10 million people will die due to infections caused by resistant bacteria (1). In order to tackle this crisis, we need to identify and characterize novel scaffolds for development as new antimicrobial agents. We previously identified AN11527, a benzoxaborole based compound as a potent inhibitor of *Mycobacterium tuberculosis* growth (2). This is an attractive series, since it has good antibacterial activity against extracellular and intracellular bacteria but lacks cytotoxicity. The target for this series is unknown, although resistant mutants had single nucleotide polymorphisms in three genes (Rv1683 Rv3068c and Rv0047c) (2). Rv0047c is involved in inositol metabolism, Rv3068c encodes a non-essential protein involved in glucose metabolism (PgmA) and Rv1683 encodes a bifunctional protein involved in triglyceride metabolism with both lipase and synthetase activity (3).

In this study we show that AN11527 is active against *Escherichia. coli*. We identified the cellular target of AN11527 in *E. coli* as the enoyl-acyl carrier protein reductase FabI. We determined a potential binding pocket for AN11527 which is located at the active center site and close to the cofactor binding site. We also determined that the binding interactions of AN11527 partially overlaps with that of the NAD^+-dependent inhibitor Triclosan, but not that of the co-factor independent 4-hydroxy-2-pyridine compound NITD-916.

Materials and methods

Determination of antibacterial activity

Mycobacterium smegmatis was grown in Middlebrook 7H9 medium supplemented with Middlebrook ADC enrichment (Becton Dickinson), 0.5% v/v glycerol and 0.05% w/v Tween80. *E. coli* was cultured in LB medium. *Staphylococcus aureus* was cultured in MH broth. We determined MICs against *M. smegmatis* mc^2155, *E. coli* JW5503 and *S. aureus* using a broth microdilution assays. The compound was tested as a two-fold serial dilution at a starting concentration of 100 µM (final DMSO concentration of 2%). Growth was measured by OD and MIC_{90} was recorded at the lowest concentration of compound which inhibited growth by 90% compared to controls.

Isolation of AN11527-resistant mutants

E. coli JW5503 was plated on LB agar containing 4X and 8X MIC AN11527 at 8 $x10^8$ to $2x10^9$ CFUs. Single colonies were isolated after overnight incubation at 37°C and cultured in 5 ml LB for genomic DNA isolation. Genomic DNA from resistant isolates was isolated and prepared for sequencing using the Nextera XL library kit. Whole genome sequencing was carried out using 2 X 150 paired-end reads at the Colorado State University whole genome sequencing facility. The *fabI* gene was PCR-amplified using

primers 5'- ATGGGTTTTCTTTCCGGTAAGCGCATTC -3' and 5'-TTATTTCAGTTCGAGTTCGTTCA -3'and sequenced.

Cloning and purification of *E. coli* FabI

E. *coli FabI* gene was PCR-amplified using primer pair 5'-CATATG ATGGGTTTTCTTTCCGGTAAGCGCAC-3 and 5′- GATCC TTATTTCAGTTCGAGTTCGTTC-3 primers. The PCR product was cloned in to pET15(b) using sites NdeI and BamHI to obtain the plasmid pET15b-FabI expressing FabI with an N-terminal His tag. FabI was expressed in BL21 (DE3) cells as follows: cells were grown to an OD of 0.4 to 0.6, 1 mM IPTG was added and protein was purified from the soluble fraction using a Ni- NTA column with 50 mM HEPES pH 8.0, 500 mM NaCl, 5% glycerol, 1 mM EDTA, 1 mM DTT. The purified protein was concentrated and stored in buffer containing 50mM HEPES pH 8.0, 500 mM NaCl, 50% glycerol, 5 mM DTT. FabI A197G was constructed by site directed mutagenesis using primers (5' CCGTACTCTGGCGGGATCCGGTATCAAAG 3') and (5' CTTTGATACCGGATCCCGCCAGAGTACGG 3').

Thermal shift assays

We determined protein melting temperatures using the Prometheus NT.48 instrument (NanoTemper Technologies). Purified FabI was diluted to a concentration of 25 µM in 50 nM HEPES, 200mM NaCl. Compound was added from 1.56 to 200 µM; NADH and NAD^+ were used at 250 µM. The reaction mixture was incubated at 25 C for 10 mins, 10 µL sample was loaded into the capillaries and placed on the sample holder. A temperature gradient from 20 °C to 80 °C at 0.5 or 1°C per min was applied. Intrinsic protein fluorescence at 330 and 350 nm was recorded and the Tm was calculated using Nano Temper Technologies software.

Results and Discussion

The benzoxaborole AN11527 is active against *E. coli*

We determined the activity of a novel benzoxaborole against *M. smegmatis*, *S. aureus* and *E. coli*. We used a *tolC* mutant strain of *E. coli* (JW5503). Although the compound showed good potency against *M. tuberculosis* (2), it had no activity against the non-pathogenic organism *M. smegmatis* (MIC >200 µM). There was no activity against the Gram positive species, *S. aureus* (MIC >200 µM). However, surprisingly there was good activity against the Gram negative organism, *E. coli* with an MIC of 6.25 µM.

Since *E. coli* was sensitive, we isolated resistant mutants in order to identify the target or mechanism of resistance. We plated 10^8-10^9 CFU on agar plates with 25 or 50 µM (4X and 8X MIC) compound and isolated resistant mutants. We confirmed that strains were resistant by streaking onto solid medium with 25 µM AN11527. Resistance was further confirmed in liquid broth and all mutant strains exhibited high-level resistance with > 32-fold shift in MIC (Table 1).

We conducted whole genome sequencing on two resistant-mutants and the wild-type strain with more than 96% coverage and we identified mutations in *fabI*. Previous work has demonstrated that similar compounds target mycobacterial InhA (4) an ortholog of FabI, and diazaborines are known to inhibit FabI (5). FabI and InhA are involved in the synthesis of fatty acids, although InhA is a enoyl acyl carrier protein reductase involved in the synthesis of mycolic acids which are specific to mycobacteria (6). Thus, it seemed possible that FabI is the target in *E. coli.* We selected 21 AN11527-resistant strains and sequenced *fabI* (Table 1). All of the strains had SNPs in *fabI* covering 9 different amino acid residues with 12 different mutations (11 SNPs and 1 deletion) (Table 1).

106

107 The crystal structure of FabI in *E. coli* has been determined (9,12). We mapped the amino acid residues
108 from our resistant isolates onto the three-dimensional structure. The mutations form a cluster large
109 enough to encompass a molecule of AN11527 and are located around the active center site (Figure 1).
110 Several of the residues lie close to the NADH/NAD^+ binding site (Figure 2) and are involved in the co-
111 factor binding. The binding pocket for AN11527 overlaps with other FabI inhibitors including a 4-
112 hydroxy-2-pyridine (NITD-916), isoniazid, and triclosan (7–9) (Figure 3). Therefore, we determined
113 whether the mutations also conferred resistance to triclosan. We observed a range of resistance for the
114 different mutations, with SNPs in I92, I192, F203 all conferring >4-fold resistance (Table 1). The I92L
115 allele conferred high level resistance (>80-fold), whereas mutations in F203 had varying resistance from
116 4-16-fold. Interestingly, the A116T allele conferred susceptibility to triclosan.

117

118 **Mycobacterial InhA inhibitors are active against *E. coli***

119 Previous work suggested that the 4-hydroxy-2-pyridine compound NITD-916 is active against *M.*
120 *tuberculosis*, but not *E. coli* (7). Since the benzoxaborole was active against *E. coli* and there is good
121 structural similarity between FabI and InhA, we tested it for activity against the TolC mutant strain. We
122 found that NITD-916 had good activity (MIC = 6.25 µM; Table 1) and thus its lack of activity is likely
123 due to efflux and lack of access to the target, as has been demonstrated for other FabI inhibitors in *E. coli*
124 (10). We tested our mutant isolates for cross-resistance to NITD-916; only two mutants showed low level
125 resistance (A21V and I192F) (Table 1). Both of these residues interact with the co-factor and this may
126 have an indirect effect on AN11527 and NITD-916 binding. However, several mutants were
127 hypersensitive (A197G, F203V and F203S). The equivalent of FabI F203 in M. *tuberculosis* is I215
128 which is involved in NITD-916 binding. However, in *E. coli* the $InhA_{F203}$ mutant strain did not exhibit

129 cross-resistance or hypersensitivity to NITD-916 (Figure 4). Thus, even though the binding pocket of
130 NITD-916 and AN11527 overlaps, the lack of cross-resistance suggests differences in binding.

131

132 **AN11527 binding to FabI is co-factor dependent**

133 FabI is a fatty acid reductase which uses cellular NADH as a co-factor to reduce the enoyl carbon of fatty
134 acids (8). FabI inhibitors can be divided in two groups based on whether their binding is co-factor
135 dependent or independent. Depending upon the mechanism of interaction bacterial FabI inhibitors are
136 categorized in two groups, one that requires a co-factor (NAD^+/NADH) to bind and another class which
137 binds directly (11).

138

139 We determined whether benzoxaborole binding was co-factor dependent using thermal shift assays.
140 NAD^+-dependent binding of triclosan resulted in a large thermal shift (Table 2). No changes in T_m were
141 observed when incubated with AN11527 alone or AN11527 plus NADH. However, AN11527 in the
142 presence of NAD^+ resulted in a shift. We confirmed this shift was dose-dependent (Table 3). We also
143 tested the $FabI_{A197G}$ allele for binding. As expected, no thermal shift was seen in the presence of AN11527
144 even in the presence of NAD^+, confirming loss of binding. Triclosan was still able to bind to the mutant
145 protein in the presence of NAD^+ as expected from the lack of resistance of the strain. These data further
146 support that FabI is the target of AN11527 in *E. coli*.

147

148 **Conclusion**

149 FabI is an essential enzyme in many bacterial species, as it catalyzes a key step in the biosynthesis of fatty
150 acid pathway as part of the FAS II complex. It is interesting to note that although the substrates and
151 products for FabI and the mycobacterial ortholog InhA are different, a number of inhibitors are active

against both enzymes (11). A wide range of FabI/InhA inhibitors have been identified including the frontline tuberculosis drug isoniazid and the antibacterial agent triclosan, as well as series under development such as the 4-hydroxy-2-pyridine compound NITD-916. We demonstrate that a benzoxaborole compound and NITD-916 are both active against the *E. coli* target and that resistance maps to FabI. We demonstrate that AN11527 binds to InhA in a co-factor dependent manner which is abrogated in mutant proteins.

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198 **Tables**

199 **Table 1. Characterization of resistant mutants.**

FabI allele	Number of isolates	AN11527		NITD-916		Triclosan	
		MIC (µM)	Fold-change	MIC_{90} (µM)	Fold-change	MIC_{90} (µM)	Fold-change
wild-type		6.25	n/a	6.3	n/a	0.16	n/a
A21V	1	>200	>32	50	4	0.062	0.4
I92L	1	>200	>32	12.5	2	12.5	80
G93S	4	>200	>32	6.3	1	nt	nt
A116T	1	>200	>32	6.3	1	0.0097	0.06
P191S	1	>200	>32	3.1	0.50	0.31	2
I192F	1	>200	>32	50	4.0	0.63	4
A197G	2	>200	>32	1.6	0.24	0.16	1
F203L	6	>200	>32	6.3	1.0	0.63	4
F203V	1	>200	>32	0.78	0.12	2.5	16
F203C	1	>200	>32	3.1	0.50	0.63	4
F203S	1	>200	>32	0.39	0.12	1.3	8
V213del	1	>200	>32	nt	nt	nt	nt

200

201 Minimum inhibitory concentrations (MIC) were determined in liquid medium against *E. coli* JW5503 and

202 AN11527-resistant *E. coli* JW5503. Data are from two independent runs.

203

204

205

206 **Table 2. Interaction of FabI with AN11527**

Allele	Molecule	Concentration	NAD^+	Tm	Shift
$FabI_{wt}$	None		-	52.1	
	AN11527		-	52.1	
	None		+	52.1	
	AN11527		+	56	3.9
	Triclosan		+	71.9	19.8
$FabI_{A197G}$	None		-	51.4	
	AN11527		-	51.2	
	None		+	51.3	
	AN11527		+	52.2	0.9
	Triclosan		+	65	13.7

207

208 FabI melting temperatures were measured by nanoDSF. Each sample contained 25 nM FabI plus
209 compound at 200 µM and NAD+ at 250 µM. where stated. The inflection point in ^oC was recorded as the
210 melting temperature; shifts are the difference in Tm. Data are representative of two independent
211 experiments.

212

Allele	Molecule	Concentration	NAD+	Tm	Shift
$FabI_{wt}$	AN11527	200 µM	+	56	3.9
	AN11527	50 µM	+	54.9	2.8
	AN11527	12.5 µM	+	53	0.9
	AN11527	3.2 µM	+	52.1	0

Table 3. Dose-dependent thermal shift of FabI with AN11527

FabI melting temperatures were measured by nanoDSF. Each sample contained 25 µM FabI plus compound and 250 µM NAD^+. The inflection point in ^oC was recorded as the melting temperature; shifts are the difference in Tm. Data are representative of two independent experiments.

Figure 1. Crystal structure of FabI (1D8A).

(A) Residues that confer resistance to AN11527 are shown in red and NAD^+ in green. The catalytic residue Y156 is in pink. Residues that confer resistance to AN11527 are highlighted in red and are located around the catalytic residue, the active center and close to the NAD+ binding site.

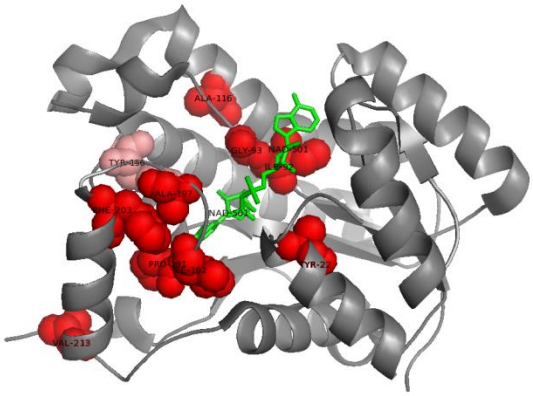
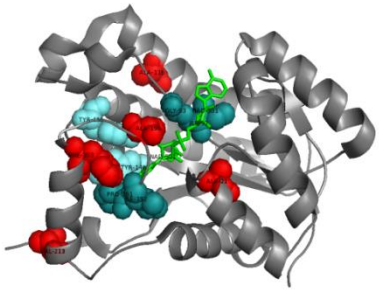
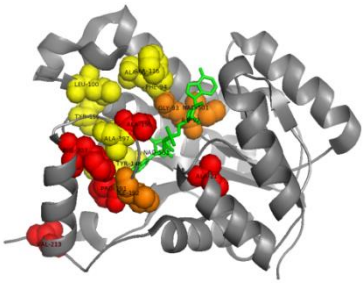


Figure 2: Comparison of the binding pockets for FabI inhibitors.

The proposed binding pocket of each inhibitor occupies the same general area around the active center site and is close to the NAD+ binding location (FabI crystal structure 1D8A). Residues that confer resistance to AN11527 are in red. NAD^+ is shown bound. For isoniazid and NITD-916 interacting residues are selected based on FabI and InhA homology. (A) Residues that confer resistance to isoniazid are light green; residues that confer resistance to both isoniazid and AN11527 are in dark green. (B) Residues that confer resistance to triclosan are light yellow; residues that confers resistance to both isoniazid and AN11527 are dark orange. (C) Residues that confer resistance to NITD-916 are blue; residues that confer resistance to both NITD-916 and AN11527 are purple.





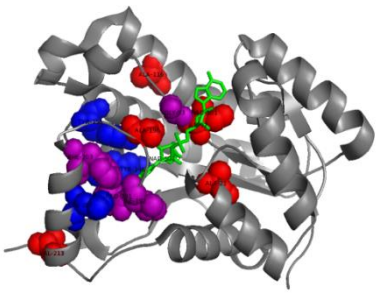


Figure 3: Location of FabI F203 and InhA I215

Superimposition of FabI and InhA structures; *E. coli* FabI with triclosan in grey and *M. tuberculosis* InhA with NITD-916 in cyan. In yellow and orange are the catalytic residue Y156 and in red and purple are the residue FabI F203 and InhA I215 respectively.