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Mechanisms of resistance against NITD-916, a direct inhibitor of *Mycobacterium tuberculosis* InhA

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Summary

Isoniazid inhibits *Mycobacterium tuberculosis* InhA and is a key component of drug regimens that treat tuberculosis. However, the high rate of resistance against isoniazid is a contributing factor to the emergence of multi-drug resistance strains of *M. tuberculosis*. The 4-hydroxy-2-pyridine NITD-916 is a direct inhibitor of *M. tuberculosis* InhA that has comparable efficacy to isoniazid in mouse models of TB infection but a lower frequency of resistance. To characterize resistance mechanisms against NITD-916 we isolated resistant mutants in H37Rv (Euro-American lineage) and HN878 (East-Asian lineage) strains of *M. tuberculosis*. The resistance frequency was similar in both strains. Mutations were identified in residues within or near to the active of InhA or in the *fabG1inhA* promoter region. All mutants were resistant to NITD-916 but were not cross resistant to isoniazid, despite homology to SNPs identified in isoniazid resistant clinical isolates.

Keywords

Mycobacteria; Antimicrobial; Resistance; Isoniazid; InhA

Isoniazid (INH) is a key component in tuberculosis drug regimens against drug susceptible strains of *Mycobacterium tuberculosis.* INH inhibits the enoyl-ACP reductase, InhA, of *M. tuberculosis* following pro-drug activation by the catalase-peroxidase enzyme, KatG (1). However, a high rate of resistance to INH driven by mutations in the non-essential KatG is a contributing factor to the emergence and spread of drug resistant strains of *M. tuberculosis.* The proven druggability of InhA in the treatment of *M. tuberculosis* has prompted research into direct inhibitors of InhA that overcome such INH liabilities as the requirement for KatG activation and the high rate of resistance (2). Several direct InhA inhibitors have been identified, including the thiadiazoles (GSK693) (3), 2-(o-tolyloxy)-5-hexylphenol (PT70) (4), and the 4-hydroxy-2-pyridines (NITD-916 and NITD-113) (3–5). Unlike the INH-NAD adduct that competes with NADH binding to InhA, NITD-916 forms a ternary complex with InhA and NADH to block access to the fatty acyl substrate binding pocket (5). NITD-916 inhibition of InhA reduces the synthesis of mycolic acids and results in cell death (5).

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NITD-916 has a lower frequency of resistance *in vitro* than INH (1×10^{-8} compared to 1×10^{-5} for INH), is active against INH resistant clinical isolates with KatG mutations, and has efficacy in both acute and established mouse models of TB infection (5). Combined these results demonstrate that NITD-916 overcomes a key liability of INH and has promise as a novel TB therapeutic. Structural and biochemical studies have provided insights into the NITD-916 mode of action. However, there is limited information on the possible routes to resistance that might emerge in either an *in vitro* or clinical setting with only three single nucleotide polymorphisms (SNPs) being previously identified in *M. tuberculosis* (5). The aim for this current study was to expand upon our understanding of resistance mechanism(s) against NITD-916 *M. tuberculosis*.

We determined the minimum inhibitory concentration (MIC) of NITD-916 against *M. tuberculosis* H37Rv on Middlebrook 7H10 agar containing 10% v/v OADC supplement (oleic acid, albumin, dextrose, catalase; Becton Dickinson) (7H10-OADC); the concentration that prevented growth completely was 0.08 μM. We isolated resistant mutants by plating late log phase *M. tuberculosis* on 7H10-OADC containing 5X or 10X MIC of NITD-916 (0.4 μM and 0.8 μM respectively). Colonies were streaked onto 7H10-OADC containing 5X MIC NITD-916; 33 of 52 colonies were confirmed as resistant. The frequency of resistance was between 8.5×10^{-7} and 2.6×10^{-7}. This rate of resistance is higher than previously reported, although still significantly lower than the frequency of resistance for INH (5; 6). The frequency of resistance to INH is high, since it is a prodrug, and mutations or deletions in the non-essential, activating enzyme KatG occur at a high frequency. We amplified and sequenced the *fabG1-inhA* genomic region using primers; TB-FabG1-MMF1 (TCAATACACCCGCAGCCA), InhA_F2(TTCATAGGTTCGGTCTCC) and InhA-R1 (GTGATACCCCACCGAAATGC). All isolates, except one, contained a mutation in *inhA* (Table 1); there were 19 different SNPs at 17 residues (Table 1). The remaining strain (LP-0532543 -RM30) had a C-T mutation upstream of the *fabG1-inhA* operon at position −15 to the translational start site (Table 1). This mutation is observed in approximately 19% of INH resistant clinical strains and leads to increased expression of InhA (7; 8), and would account for the resistance phenotype seen to NITD-916.

We determined the level of resistance for all strains on solid medium. Strains were arrayed into a 96-well plate and grown in Middlebrook 7H9 supplemented with 10% v/v OADC and 0.05% w/v Tween 80 (7H9-OADC-Tw) at 37°C for 7 days in duplicate. Cultures were diluted 1/100 in medium and 5 μL spotted onto rectangular agar plates containing 4X and 8X MIC. Plates were incubated for 3–4 weeks and spots were scored as growth or no growth. To confirm resistance, the IC_{90} was determined in liquid medium (7H9-OADC-Tw) as described (9). Bacterial growth was measured after 5 d at 37°C and the IC_{90} was defined as the concentration at which 90% of growth was inhibited. With the exception of I21V, strains were >4-fold resistant to NITD-916 in both solid and liquid media (Table 1).

Many of the SNPs we identified map to residues that are in the active site or in close proximity and several residues are known to interact with other direct inhibitors of InhA. M103 and I215 are active site residues, whilst I21 and F41 play a role in stabilizing NADH with which NITD-916 forms a ternary complex (10; 11). M103 and I215 interact with NITD-916 (5), whilst M161 and I202 form interactions with GSK625 and PT70 (3; 4). R195

is in close proximity to the active site residues G192 and G193, and Q214 is close to I215 (5). G205 and A206 are in close proximity to residues I202, V203 and L207 which interact with direct InhA inhibitors (12). The majority of the mutations we identified are novel, except S94, D148 and M161 which are known to provide resistance against NITD-916, and M103 which confers resistance to GSK693 (3; 5). These results highlight the overlapping binding sites of different InhA inhibitors. Our data indicate that mutations in the *inhA* coding region prevent the formation of the InhA-NADH-NITD916 ternary complex (either directly or indirectly through conformational changes), or confer resistance by increased expression of InhA.

Mutations in the InhA coding region, specifically in I21, I47 and S94, are seen in approximately 2% of INH^R clinical isolates (7). Structural and biochemical studies suggest that I21V, I47T and S94A alter the active site of InhA and reduce affinity for the INH-NAD adduct (8; 13–15). Consistent with previous publications, the S94A mutations conferred resistance to INH on solid medium (Table 1) (8; 16). The S94A strain was also cross-resistant to ethionamide (Table 1). Mutations at S19, I21, R195, Q214 and L269 also conferred resistance to INH on solid medium (Table 1). Interestingly, none of the mutations conferred resistance against INH in liquid culture (Table 1). The lack of resistance for S94A against INH has previously been observed in liquid medium (5). The discordance in resistance phenotypes under different conditions requires further investigation. The one exception was the promoter mutant, which had a five-fold increase in IC_{90} (Table 1). The increased IC_{90} for the promoter mutant is consistent with the prevalence of this mutation in approximately 19% of INH resistant clinical isolates (7).

Over-expression of InhA mutant alleles can confer increased resistance to INH and NITD-916 as compared to over-expression of the wild-type allele (5). The C-15T promoter mutation results in a 20-fold increase in InhA (8). InhA coding region SNPs are often found together with *inhA* promoter mutations in clinical isolates (7; 17). The lack of resistance to INH for the isolated InhA SNPs in this current study questions the contribution of InhA mutations in isolation to INH^R in clinical populations. We hypothesized that the InhA SNPs observed in clinical isolates are expressed at higher levels due to secondary C-15T *fabG1inhA* mutations (17). In order to investigate this further, we isolated resistant mutants in the strain carrying the promoter mutation. A total of 14 resistant mutants were isolated on 7H10-OADC containing 4.0 μM NITD-916 at a frequency of 6.2×10^{-8}. All isolates contained a secondary mutation in *inhA* and demonstrated high level resistance to NITD-916 (36-fold increase relative to wild-type) (Table 2). Strains containing double mutations showed higher levels of resistance than the corresponding strains with a single mutation - for example, strains with mutations I47, M161 and G205 had increased MICs compared to the single *inhA* mutants (7-fold, >28-fold, and 12-fold respectively) (Table 2). This confirmed that overexpression of mutant allele resulted in increased resistance. In contrast, resistance to INH was not increased (Table 2), even in strains with mutant alleles previously shown to have reduced affinity for INH-NAD (13; 14).

Strains from the East Asian geographic lineage of *M. tuberculosis*, including the Beijing sub lineage, acquire resistance against rifampicin, isoniazid and ethambutol at a higher rate than strains from the Euro-American lineage (18). To determine if resistance mechanisms

differed between geographic lineages, HN878 (Beijing lineage) resistant isolates were isolated using NITD-916; 19 confirmed resistant isolates were obtained on medium containing 5X and 10X MIC at a frequency of 9.1×10^{-7} and 2.1×10^{-7} respectively, similar to that seen in H37Rv. All isolates contained SNPs in *inhA*, with one SNP (R49H) not previously observed (Table 3). The C-T promoter mutation at position −15 was also observed in HN878 (Table 3). Consistent with results in H37Rv, all HN878 isolates had >4-fold resistance to NITD-916, but were not resistant to INH (Table 3).

The reduced frequency of resistance to NITD-916 overcomes a major liability associated with the use of INH in current TB regimens. Although previous work has provided insights into the mechanism of inhibition, there was a limited understanding on the potential mechanisms of resistance against this promising class of compounds. This study demonstrates that mutations in a variety of residues within or neighboring the InhA active site are the primary route to resistance within different geographical lineages of *M. tuberculosis*. Mutations that increase the level of InhA expression also provided resistance against NITD-916. Although the increased expression of InhA provided cross-resistance to INH, there was a surprising lack of cross-resistance to INH for mutations in the InhA coding region. It is anticipated that the results from this current study will be useful in guiding the development of improved hydroxy-pyridines and other direct InhA inhibitors.

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Table 1

Genotypes and phenotypes of *M. tuberculosis* H37Rv mutants resistant to NITD-916

Strain	InhA	Number of isolates	Solid medium MIC[A]				Liquid medium IC_{90} (fold shift vs WT)[B]	
			NITD-916	INH	ETH	TRI	NITD-916	INH
H37Rv	**WT**		**0.08 µM**	**0.8 µM**	**6.3 µM**	**50 µM**	**0.1 µM**	**1 µM**
LP-0532543-RM18	S19W	1	8x	4x	-	-	12x	-
LP-0532543 RM28	I21M	1	8x	4x	-	-	4x	-
LP-0532543-RM34	I21V	1	4x	4x	-	-	3x	2x
LP-0532543-RM13	F41L	1	8x	-	-	-	13x	-
LP-0532543-RM19	I47L	1	8x	-	-	-	7x	-
LP-0532543-RM1	S94A	2	8x	8x	8x	-	10x	2x
LP-0532543-RM6	M103T	2	8x	-	-	-	10x	-
LP-0532543-RM2	D148E	1	8x	-	-	-	>10x	-
LP-0532543-RM4	M161L	3	8x	-	-	-	>28x	-
LP-0532543-RM41	R195G	1	8x	8x	-	-	>10x	2x
LP-0532543-RM9	I202F	1	8x	-	-	-	9x	-
LP-0532543-RM7	G205A	2	8x	-	-	-	12x	-
LP-0532543-RM11	G205S	1	8x	-	-	-	13x	-
LP-0532543-RM3	A206E	4	8x	-	-	-	19x	-
LP-0532543-RM44	210	1	8x	8x	-	-	ND	ND
LP-0532543-RM14	G212D	1	8x	-	-	-	24x	-
LP-0532543-RM5	Q214P	1	8x	8x	-	-	ND	ND
LP-0532543-RM16	I215S	1	8x	-	-	-	9x	-
LP-0532543-RM37	L269R C-15T	6	8x	8x	-	-	11x	-
LP-0532543-RM30	upstream of *fabG1inhA*	1	8x	4x	4x	-	6x	5x

[A] MIC for WT was determined on solid medium. For resistant isolates, strains were plated onto 4x and 8x MIC for each compound on solid medium and growth scored.

[B] IC_{90} was determined in liquid medium. For resistant isolates, IC_{90}, data are presented as the fold-change compared to WT.

- denotes no shift

ND - not determined

INH - isoniazid ETH –Ethionamide TRI - Triclosan

Table 2

Genotypes and phenotypes of *M. tuberculosis* H37Rv double mutants

Strain	SNP		Number of isolates	IC_{90}(fold-shift)[A]	
	fabG1inhA promoter	InhA		NITD-916	INH
H37Rv	**WT**	**WT**		**0.032 μM**	**0.46 μM**
LP-0532543-RM30	C-15T	WT		6x	5x
LP-053254 -RM314	C-15T	I47M	1	>36x	5x
LP-0532543-RM301	C-15T	N159K	2	>36x	3x
LP-0532543-RM311	C-15T	M161V	2	>36x	5x
LP-0532543-RM304	C-15T	T162M	5	>36x	4x
LP-0532543-RM318	C-15T	M199L	1	>36x	4x
LP-0532543-RM313	C-15T	G205D	1	>36x	2x
LP-0532543-RM320	C-15T	G208D	1	>36x	5x

[A] IC_{90} was determined in liquid medium. For resistant isolates, data are presented as the fold-change compared to WT.

INH - isoniazid

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Table 3

Genotypes and phenotypes of *M. tuberculosis* HN878 mutants resistant to NITD-916

Strain	SNP		Number of isolates	IC_{90} (fold shift)[A]	
	fabG1inhA promoter	InhA		NITD-916	INH
HN878	**WT**	**WT**		**0.14 μM**	**0.31 μM**
HN-0532543-RM18	WT	R49H	1	4x	-
HN-0532543-RM10	WT	M103T	1	>7x	2x
HN-0532543-RM7	WT	M161L	1	>7x	-
HN-0532543-RM2	WT	T162M	10	>7x	2x
HN-0532543 -RM14	WT	G205R	1	4x	-
HN-0532543-RM9	WT	G212D	1	>7x	-
HN-0532543-RM17	C-15T	WT	3	5x	8x

[A] IC_{90} was determined in liquid medium. For resistant isolates, data are presented as the fold-change compared to WT.

- denotes no shift

INH - isoniazid