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# Transfer of a point mutation in *Mycobacterium tuberculosis inhA* resolves the target of isoniazid

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**Isoniazid is one of the most effective antituberculosis drugs, yet its precise mechanism of action is still controversial. Using specialized linkage transduction, a single point mutation allele (S94A) within the putative target gene *inhA* was transferred in *Mycobacterium tuberculosis*. The *inhA*(S94A) allele was sufficient to confer clinically relevant levels of resistance to isoniazid killing and inhibition of mycolic acid biosynthesis. This resistance correlated with the decreased binding of the INH-NAD inhibitor to InhA, as shown by enzymatic and X-ray crystallographic analyses, and establishes InhA as the primary target of isoniazid action in *M. tuberculosis*.**

Isoniazid (INH), whose bactericidal activity against *Mycobacterium tuberculosis* was discovered in 1952 (ref. 1), has become the foundation of modern chemotherapy for active and latent tuberculosis because of its excellent activity, low cost and relatively low toxicity. The mode of action of INH has been controversial, with early reports suggesting that INH affected cell permeability, inhibited DNA biosynthesis, altered NAD metabolism or inhibited mycolic acid biosynthesis. The mycolic acid biosynthesis inhibition hypothesis was validated by the demonstration that INH-induced inhibition of mycolic acid biosynthesis correlated with cell death[2], and the putative target of INH was postulated in 1975 to be a desaturase, a cyclopropanase or an enzyme involved in fatty-acid elongation[3].

The development of plasmid genomic library transfer systems for mycobacteria in 1994 (ref. 4) and the completion of the *M. tuberculosis* genome sequence in 1998 (ref. 5) spurred further progress. By transferring *M. tuberculosis* genes on multicopy plasmids in *M. smegmatis*, the *inhA* gene, encoding an NADH-specific enoyl-acyl carrier protein (ACP) reductase, was identified in 1994 as a putative target for both INH and the related drug ethionamide (ETH)[4]. The β-ketoacyl ACP synthase (KasA)[6] and, more recently, the dihydrofolate reductase (DHFR)[7] have also been proposed as targets of INH, based on INH binding studies. Moreover, mutations in *inhA* and *kasA* have also been found in *M. tuberculosis* INH-resistant clinical isolates[6,8]. The determination of a clinically relevant drug target, however, requires the ability to transfer such a single point mutation within a gene that putatively encodes a drug target and demonstrate that this transfer is sufficient, by itself, to confer drug resistance.

We performed a specialized linkage transduction[9] to introduce the *inhA*(S94A) point mutation, previously associated with INH resistance[4], linked to a gene conferring hygromycin resistance, into *M. tuberculosis* (**Fig. 1a**) or *M. bovis* BCG (**Supplementary Methods** online). Two different types of hygromycin-resistant recombinants could be obtained, depending on the site of recombination with respect to the S94A mutation, resulting in INH resistance or susceptibility (**Fig. 1b**). In *M. tuberculosis*, 51% of the hygromycin-resistant transductants were INH resistant (**Fig. 1c**). We screened the transductants for the absence or presence of the *inhA*(S94A) allele using sequence analysis or a hairpin-shaped primer assay and found a 100% correlation with INH resistance, ETH resistance and the presence of the *inhA*(S94A) allele. As predicted, the introduction of the S94A mutation was sufficient for conferring at least a fivefold resistance to INH and ETH in *M. tuberculosis* and *M. bovis* BCG (**Supplementary Table 1** and **Supplementary Fig. 1** online). We also introduced three *kasA* mutations (G269S, G312S and F413L), found in INH-resistant *M. tuberculosis* clinical isolates[6], into *M. tuberculosis* or *M. bovis* BCG using specialized linkage transduction to test whether these mutations conferred INH resistance. The presence of mutated *kasA* alleles was confirmed by sequencing. None of the transductants obtained showed any detectable level of resistance to INH (**Supplementary Table 1** online). These results indicate that no single *kasA* mutation was sufficient, by itself, to confer INH resistance in *M. tuberculosis*.

The bactericidal activity of INH correlates with the inhibition of mycolic acid biosynthesis[2]. Therefore, we analyzed two *inhA* isogenic strains carrying either the wild-type *inhA* allele ($mc^2$4910) or the *inhA*(S94A) allele ($mc^2$4911) for their resistance to mycolic acid biosynthesis inhibition in the presence of increasing concentrations of INH. At 0.25 μg/ml INH, mycolic acid biosynthesis was fully inhibited in INH-resistant $mc^2$4910, but not in INH-resistant $mc^2$4911 (**Fig. 1d**). Complete inhibition of mycolic acid biosynthesis in both *M. tuberculosis* isogenic strains occurred at twofold the minimum inhibitory concentration (MIC), in agreement with the bacteriocidal MIC activity.

As INH resistance can be mediated by overexpression of *inhA*[10], we compared the *inhA* mRNA levels between $mc^2$4910 and $mc^2$4911 at different concentrations of INH, using a molecular beacon RT-PCR assay. The mRNA levels were similar in both strains even at high concentrations of INH (**Fig. 1e**), and this corresponded to InhA protein levels (**Fig. 1f**). Therefore, INH resistance in $mc^2$4911 was not caused by *inhA* overexpression. In contrast, $mc^2$4914, an INH-resistant

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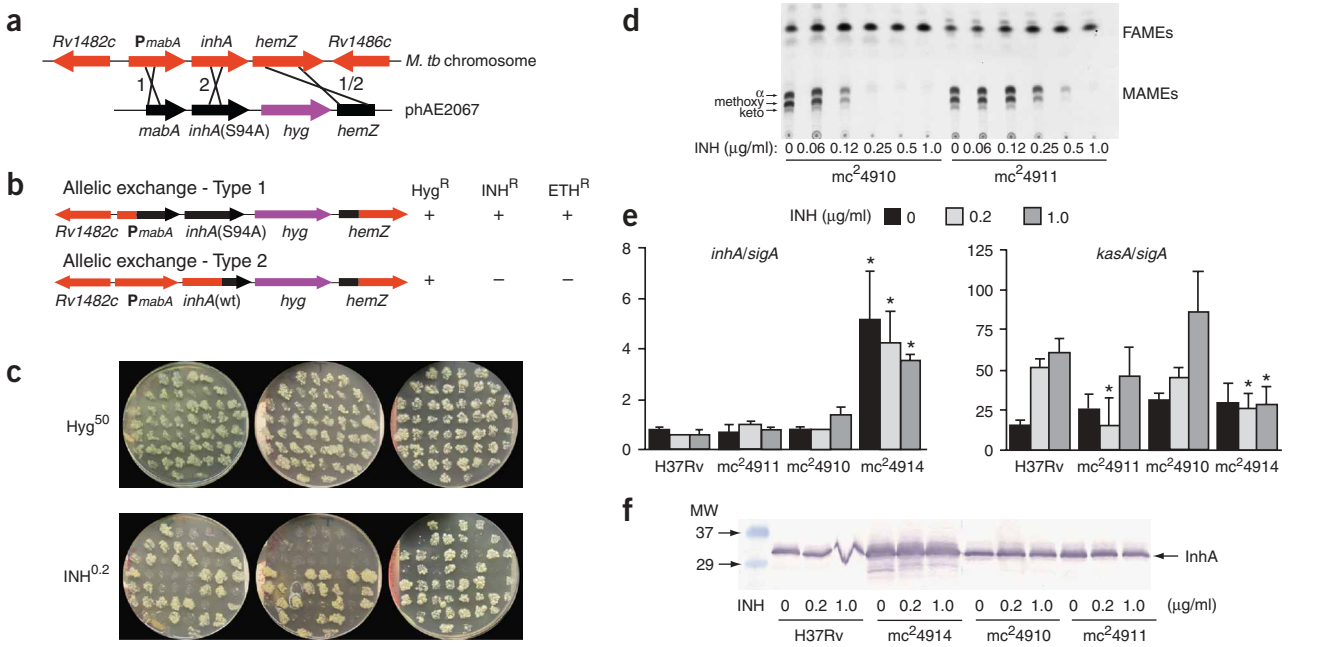


**Figure 1** Construction and analysis of *M. tuberculosis inhA*(S94A) (**Supplementary Methods**). (**a**) Schematic representation of the specialized transducing phage. A replicating shuttle phasmid phAE2067 containing *mabA, inhA* carrying the S94A mutation, a *hyg* resistance cassette and *hemZ* was used to transduce *M. tuberculosis* (*M. tb*). The two possible sites of recombination are marked 1 and 2. (**b**) The recombination can occur either before the point mutation (crossover type 1), resulting in an INH-resistant and ETH-resistant recombinant carrying the S94A mutation, or after the point mutation (crossover type 2; the strain contains a wild-type *inhA* gene). (**c**) Individual *M. tuberculosis* H37Rv *inhA*(S94A) transductants ($n = 150$) were screened by picking and patching onto plates containing either hygromycin (50 μg/ml) or INH (0.2 μg/ml). (**d**) Fatty acid methyl esters (FAMEs) and mycolic acid methyl esters (MAMEs) profiles of mc$^2$4910 (INH-sensitive) and mc$^2$4911 (INH-resistant). The *M. tuberculosis* strains were treated with different concentrations of INH for 4 h and labeled with [1-$^{14}$C]-acetate. $^{14}$C-labeled FAMEs and MAMEs were separated by thin-layer chromatography and detected by autoradiography. (**e**) Analysis of *inhA* and *kasA* mRNA levels of *M. tuberculosis* strains by RT-PCR. The *M. tuberculosis* cultures were treated with INH for 4 h before measuring the *inhA* and *kasA* mRNA levels. *inhA* and *kasA* values were normalized using *sigA* levels. Means ($n = 3$) ± s.e.m. (*$P < 0.05$). (**f**) Western blot analysis of InhA protein levels of *M. tuberculosis* strains treated with INH for 4 h. InhA was detected in total protein extract using rabbit antibodies to InhA raised against the *M. tuberculosis* InhA protein.

*M. tuberculosis* spontaneous mutant carrying the *inhA* expression region mutation (C–15T), which had been proposed as inducing overexpression of *inhA*, showed a 20-fold increase in *inhA* mRNA levels (**Fig. 1e**) and subsequent InhA protein levels (**Fig. 1f**). It has been suggested that this mutation maps to the ribosomal initiation site, so it was unclear whether it affected transcription or translation. This analysis shows that the C–15T *inhA* promoter mutation mediates enhanced transcription of *inhA* mRNA levels, resulting in INH resistance.

It was previously shown that *kasA* is induced in mycobacteria treated with INH[6]. In agreement, we found that *M. tuberculosis* strains H37Rv and mc$^2$4910 have increased *kasA* mRNA levels when treated with INH (**Fig. 1e**). In contrast, mc$^2$4911 and mc$^2$4914, which are resistant to INH, showed no increase in *kasA* mRNA levels when treated with INH at or below the MIC. An increase in the level of *kasA* mRNA was observed in mc$^2$4911 at 1.0 μg/ml INH. This suggests that *kasA* mRNA levels were induced only when InhA was inhibited, and that the increase in *kasA* expression does not correlate with INH resistance, a fact that is consistent with the observation that *kasA* overexpression in fast- and slow-growing mycobacteria does not increase resistance to INH[10].

It has been shown that InhA is inhibited by a covalent INH-NAD adduct[11], the product of activated INH with $NAD^+$ (ref. 12). To determine whether the InhA(S94A) enzyme is resistant to inhibition by the INH-NAD adduct, we measured the inactivation of wild-type and mutated InhA by this adduct in a dose-dependent fashion. The InhA(S94A) enzyme was 17 times more resistant to inhibition by the INH-NAD adduct ($IC_{50}$, 323 ± 41 nM for the InhA(S94A) enzyme versus 19 ± 10 nM for the wild-type enzyme), and showed a 30-fold increase in the $K_i$ value for the INH-NAD adduct ($K_i$ for the S94A mutant protein is 172 ± 22 nM; $K_i$ for the wild-type protein is 5 ± 3 nM). To determine the molecular basis for the reduced inhibition of the INH-NAD adduct to the InhA(S94A) enzyme, we co-crystallized the InhA(S94A) protein with the INH-NAD adduct (**Fig. 2a**) and compared it to the wild-type protein structure. Although the position and orientation of the INH-NAD adduct was nearly identical in the active sites of the wild-type (**Fig. 2b**) and InhA(S94A) (**Fig. 2c**) proteins, the loss of the serine residue resulted in the movement of an ordered water molecule that disrupted the hydrogen bonding network, which probably decreases the binding of the adduct to the InhA(S94A) protein.

Elucidating the mechanism of action of INH has been a complex endeavor because of: (i) the prior inability to transfer point mutations in *M. tuberculosis*; (ii) the clinically irrelevant binding of INH to proteins[6,7]; (iii) the fact that INH is a prodrug that requires modification before becoming active[13]; (iv) the 100-fold difference in susceptibility between *M. smegmatis* and *M. tuberculosis*; and (v) the initial inability to explain the accumulation of saturated $C_{26}$ fatty acid upon INH treatment[14]. Although a drug may bind to a number of different enzymes *in vitro*, the validation of a proposed target requires *in vivo* data. *In vivo* binding can be established by identifying amino acid substitutions within the target enzyme that reduce binding or by

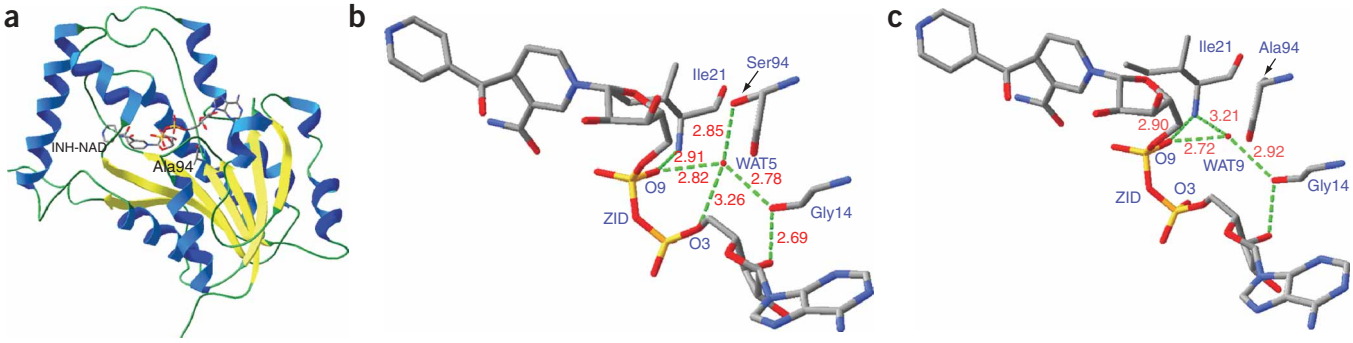


**Figure 2** X-ray crystallographic analysis of the INH-NAD adduct bound to wild-type InhA or InhA(S94A) protein. (**a**) Crystal structure of the S94A mutant protein with the INH-NAD adduct. (**b**) In the InhA–INH-NAD structure, the oxygen O9 of the phosphate of the INH-NAD adduct forms one hydrogen bond with the main-chain nitrogen atom of Ile21 and one hydrogen bond with a well-ordered water molecule. This water molecule is part of a hydrogen-bonding network formed by interactions between the side-chain oxygen atom of Ser94 (2.85 Å), the main-chain oxygen of Gly14 (2.78 Å) and the oxygen atoms O3 (3.26 Å) and O9 (2.82 Å) of the INH-NAD adduct. The same water molecule is within hydrogen-bonding distance of the main-chain nitrogen atoms of Ala22 (2.9 Å) and Ile21 (3.3 Å). (**c**) In the InhA(S94A)–INH-NAD structure, this hydrogen-bonding network is disrupted by the loss of the hydroxyl group in the S94A substitution. Although the water molecule is still visible in the same position, it is 2.92 Å from the main-chain oxygen of Gly14 and out of range of hydrogen bonding interaction with the oxygen atom O3 (3.54 Å). The water molecule is 0.2 Å and 0.1 Å closer to the adjacent residues Ala22 and Ile21, which form hydrogen bonds with their main-chain nitrogen atoms, respectively.

showing target overexpression, both of which confer clinically relevant drug resistance.

The coupling of specialized linkage transduction with enzymatic analyses and X-ray crystallography has enabled us to establish the precise molecular mechanism by which INH inhibits InhA, and supports the premise that *inhA* encodes the primary target of INH. Furthermore, the observations that *inhA* modification or overexpression[10], or altered NADH/NAD ratios[15], confers co-resistance to ETH support the hypothesis that InhA is a common target of both INH and ETH.

In addition to identifying the actual drug target of INH, the INH story has unveiled a new paradigm of drug action, in which INH functions by covalently modifying an enzyme cofactor. This paradigm provides important insights that may be valuable in developing new drugs against multidrug resistant and extensively drug-resistant *M. tuberculosis* (http://www.cdc.gov) as well as against other pathogenic mycobacteria like *M. ulcerans* and *M. leprae*.

*Note: Supplementary information is available on the Nature Medicine website.*

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AUTHOR CONTRIBUTIONS

C.V. performed specialized transductions of *inhA* in *M. tuberculosis* and BCG and analyzed the transductants for their inserts, INH and ETH resistance and biochemical resistance to INH, and wrote most of the manuscript with W.R.J. F.W. prepared the INH-NAD adduct, performed the InhA enzymological analyses and X-ray crystallography. M.A. constructed the phages for transduction and performed and analyzed the kasA allelic exchanges in *M. tuberculosis* and *M. bovis* BCG. T.R.W. sequenced the transductants. M.H.H. and R.C. conducted the hairpin-shaped primer assays and mRNA experiments. L.K. provided the pMV261::*kasA* constructs and did the western blot analysis. D.A., J.C.S. and W.R.J. contributed to the design of the study, data analysis and data interpretation.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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