

REVIEW ARTICLE

Isoniazid, The Frontline of Resistance in Mycobacterium tuberculosis

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ABSTRACT Tuberculosis is an ancient disease that has held close association with humans for millennia. Through persistence, this remarkably successful organism has managed to infect an estimated third of the world's population. Declining rates of tuberculosis in developed nations have masked an emerging epidemic of drug resistant cases that have been reported in almost every country under scrutiny. The recent completion of the genome sequence of *Mycobacterium tuberculosis* has mandated more efficient control and management of this disease. The momentum for this public health imperative will come from information gleaned from advances in genomics and related technologies towards deciphering molecular mechanisms of mycobacterial drug resistance.

INTRODUCTION

Tuberculosis, a disease of great antiquity holds lineage to saprophytic soil organisms whose later introduction as a human pathogen likely coincided with the domestication of cattle approximately 10,000 years ago. Throughout history, tuberculosis has been classified by many names, from Phthisis ("to waste") by the ancient Greeks, to consumption in the 1800's. In 1882 Robert Koch isolated the causative agent, *Mycobacterium tuberculosis* from crushed lung tubercles. Currently, M. tuberculosis is known as the world's leading cause of death from a single infectious agent, with a global prevalence of greater than 1.6 billion persons. (1,2)

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Despite overall declines in TB incidence in industrialized countries during the past three decades, mostly due to conscientious public health measures, there has recently been an important rise in the incidence of TB. (3,4) However, the contention that this is a "new" epidemic may not be entirely accurate when one considers the natural history of this disease follows secular trends that epicycle over the course of a human lifespan. What is "new" about this increasing disease burden is the widespread emergence of multi-drug resistant strains (MDR), which by definition, are strains resistant to at least the major frontline drugs: isoniazid and rifampin. This increase is due, at least in part, to the discontinuation of long course multi-drug treatments combined with patient non-compliance (5). In addition, the predilection of M. tuberculosis for the impoverished has further compounded this problem (6). As well, *M. tuberculosis* exhibits an important synergy with HIV and the role of the latter as a cofactor in TB disease has proven to be a major impediment to the control of both AIDS and Tuberculosis. (4,7,8)

Nonetheless, it is apparent there has been an increased incidence of MDR TB in both developing and

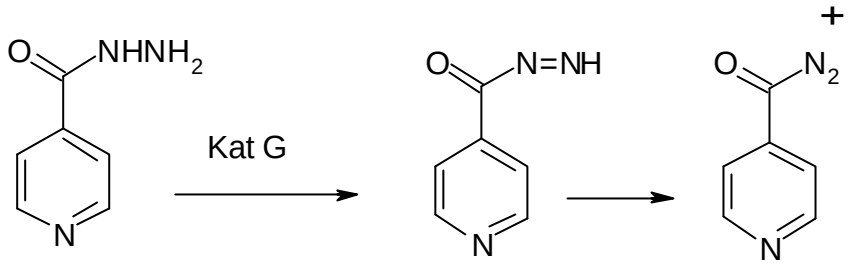

Figure 1. Potential metabolic activation mechanism for Isoniazid. Kat G mediates 2 electron transfers to produce an activated Isoniazid intermediate(s). It is this reactive intermediate that is capable of intracellular acylation of nucleophiles in *M.tuberculosis*, thereby facilitating toxic effects.

industrialized countries, despite the availability of directly observed therapy (DOTS) and BCG vaccine. These recent developments underscore the urgent need not only for new drugs and more efficacious vaccines, but more importantly, a concerted effort into devising methods for timely vaccine and chemotherapeutic development. In light of this urgent need, it may be serendipitous that we are seeing enormous leaps in proteomic and genomic technologies as well as the complete published sequences of the *M. tuberculosis* (H37RV) genome (9,10).

Therefore, this review is intended to consider previous work in light of recent advances using the major frontline antitubercular drug, isoniazid, as a paradigm for mycobacterial drug resistance. Following an abridged historical development of isoniazid, the current understanding of its mechanism of action and mechanism(s) of resistance and the rationale for the exquisite sensitivity of *M. tuberculosis* to isoniazid will be discussed. From there, the current directions of TB research in conjunction with present genomic based approaches will be considered.

ISONIAZID AND THE ROLE OF KatG

Isoniazid (INH), or isonicotinic acid hydrazide (Figure 1), is a synthetic bactericidal agent that was first produced in the early 1900's but was not utilized as an antitubercular agent until 1952. Presently, it is the prophylaxis of choice due to its low cost per dose, relatively low frequency of hepatotoxicity, (11,12) and reasonable bioavailability (13). In conjunction with Rifampin and Pyrazinamide it forms the major front line therapy worldwide (14).

INH enters mycobacterial cells via passive diffusion across the bacterial envelope (15). The minimal inhibitory concentration (MIC) for susceptible strains ranges from 0.02-0.05 mg/ml and is equally effective in *M. tuberculosis* and *M. tuberculosis* complex (*M. bovis, M. microti, M. africanum*) members. Surprisingly, INH exhibits little or no inhibitory activity against other mycobacteria and most prokaryotic pathogens. The reasons for this, and the primary mechanism of action of INH have been the subject of considerable investigation. Much of the current understanding of the in vivo mechanism of isoniazid has been extrapolated from in vitro work; almost exclusively focusing on relatively few bacterial enzymes associated with decreased susceptibility profiles. The first mechanistic insight of INH was revealed in 1954 when Middlebrook and others noted an inverse relationship to catalase-peroxidase activity and INH resistance (16,17).

This implicated the catalase-peroxidase enzyme, or KatG of *M. tuberculosis*. KatG is a hemeB containing dimer with only one functional domain. The other is apparently inactive (18). Its physiological role is protective, combating the low pH found during the "oxidative burst" in human phagocytes, where liberated O_2 radicals are converted to H_2O_2 within the phagosome. KatG activity eliminates this via a "deceptively simple reaction" (18);

$$2\,H_2O_2 \rightarrow 2\,H_2O + O_2$$

Which is in fact a 2-step prosthetic group mediated process:

$$\text{Heme-Fe[III]} + H_2O_2 \rightarrow \text{Heme-Fe[IV]} + H_2O$$
$$\text{Heme-Fe[IV]} + H_2O_2 \rightarrow \text{Heme-Fe[III]} + H_2O + O_2$$

It is clear that KatG plays a pivotal role in virulence and has been found to be essential for persistence in mouse and guinea pig models (18,19).

Curiously, it is this same protective enzyme that is implicated in susceptibility to INH. Specifically, INH is a prodrug that requires cellular activation by KatG producing a reactive species with antimicrobial action. The postulated reaction is shown in Figure 1. There is also evidence that INH effect is potentiated by the presence of peroxide, typically found in activated macrophages (20,21).

An elegant series of experiments by Zhang *et al*. demonstrated a key role for KatG in the action and resistance to INH. This group sought to identify the gene(s) responsible for INH resistance. A molecular

genetic approach was taken using a lab adapted *M. smegmatis* strain normally susceptible to moderate concentrations of INH (32 mg/ml). From this, a mutant strain was isolated that was viable in concentrations up to 500 mg/ml INH, this particular mutant was transformed with a cosmid library representing the entire genome of the INHs lab strain H37RV. Selection with INH allowed isolation of a hypersensitive clone, that displayed a marked catalase activity, thereby co-locating these two functions to a single genomic fragment (22).

Restriction mapping and Southern blotting localized the INH susceptibility to a smaller 2.9 Kb fragment, which in comparison with *E. coli* was shown to contain sufficient coding capacity for KatG. In addition, Zhang *et al.* provided evidence that over expression of this product could confer a susceptible phenotype in a dose dependent manner to a naturally INH^R *E. coli* strain.

To determine the clinical relevance of this result, a series of eight INH^R clinical isolates were assayed by southern analysis confirming that high resistance of two strains was due to deletion of the KatG gene. It is worth noting at this point that gene deletion is a relatively unusual mechanism of resistance in contrast to what is typically observed in bacterial systems where active efflux, (23) altered affinities of enzymatic targets, (24) and antibiotic inactivation, (25) represent norms in resistance acquisition.

Therefore, although convincing, it is likely that the studies conducted by Zhang *et al*. suffered from errors in sampling since this mechanism of resistance appears in a distinct minority of cases.

A more representative sample was analysed by Heym *et al*. (26). This group used 39 clinical isolates from diverse locales to determine whether mutations in KatG were associated with INH^R. A PCR-SSCP (PCR-single stranded conformational polymorphism) strategy was used, in which the target is first amplified by PCR. Products are then denatured to single -stranded form and run on high-resolution polyacrylamide gels. Sequence alterations can be inferred by altered mobilities in comparison to reference standards. Mutations are then typically confirmed by sequencing (For detailed reviews see 27, 28).

This methodology allowed rapid screening of resistant and control isolates in 12 arbitrary overlapping intervals. Aberrant mobilities were sequenced and indicated that 21 of the 36 resistant isolates contained mutations within KatG with an Arginine to Leucine mutation at position 463 predominating in seven isolates. Five others carried a Serine to Threonine mutation at position 315. An additional three isolates contained a deletion mutation at residues 120-123. The final six mutations in KatG were represented at a singular frequency.

Similar, but less convincing work was reported by Pretorius *et al.*, using a lower resolution PCR-SSCP methodology (29, 28). However, the strength of this work was in the relatively large and geographically diverse samples from Africa, the US and Switzerland. Results of this study and a later one show a high proportion (52%) of African isolates with Serine 315 Threonine (G-C) mutations at codon 315, and additional mutations at codons Thr 275 Ala, Arg 409 Ala, Arg 463 Leu and Asp 695 Ala. Overall, 64% of the observed INH resistance was attributable to mutations within KatG, again suggesting a complete KatG deletion was a rare event. An interesting additional finding of this report was that of a fully INH susceptible control isolate mutated at codon 463, which will be discussed later.

Table 1. Relative activities and isoniazid minimal inhibitory concentrations (MICs) of Bacillus Calmette Guerrin BCG transformants expressing KatG mutants versus reference lab strains.

Strain	Peroxidase (A405)	Catalase (A240)	Isoniazid MIC (mg ml -1)
VC	0.0	0.0	>500
TB-KatG	1.0	1.0	0.5
MAC-KatG	1.9	1.2	1.0
R104L	0.1	0.2	>500
H108Q	0.1	0.0	>500
N138S	0.1	0.0	>500
L148R	0.1	0.2	>500
H270Q	0.1	0.0	>500
T275P	0.3	0.0	>500
W321G	0.3	0.3	>500
D381G	0.0	0.0	>500
S315T	0.6	0.4	90
S140N	1.6	1.5	0.5
A350T	1.3	1.0	0.5
R463L	1.9	1.4	1.0
R463G	0.9	1.3	0.5
L587M	1.3	1.4	0.5

Adapted from Rouse *et al.* (1996). Of note is the S315T mutation that, while conferring only moderate resistance to isoniazid (˜ 90mg ml -1), allows retention of significant levels of associated peroxidase and catalase activities. **MIC**, minimal inhibitory concentration.

Numerous recent investigations have led to similar conclusions. Martila *et al.* found 22 of 24 (91.7%) INH resistant isolates carried a Ser 315 Thr mutation, of which 12 also carried a 463 mutation, all originating from the St. Petersburg area in Russia (30). Clonal spread in this case was not markedly noted by the authors in this study. However, due to the genetic similarity of isolates, it would have been interesting if some evidence had been presented regarding the transmission of drug resistant strains. Unfortunately, studies of clonal transmission of resistant bacteria in this area were not investigated.

The mutational spectrum of KatG may also be a factor of geography as the aforementioned author several years previous found a low prevalence, (three of fifty-four isolates containing mutations at codon 315) in Finnish patients (31). A similar result was also obtained by Rouse *et al*. in a clinical study of 26 INH^R isolates, nineteen of which were from Korea with only a single isolate mutated at codon 315 (33). However, it appears the study may have been limited by the use of isolates exhibiting rather low inhibitory concentrations of ~1 mg/ml. In contrast, an opposite

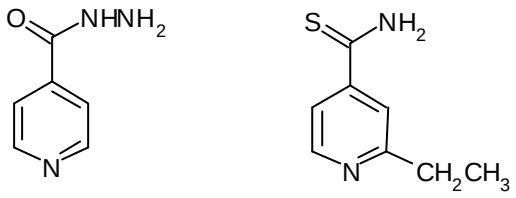

Figure 2. Structures of Isoniazid (INH) and Ethionamide (ETH/ETA)

finding was noted in studies in the Netherlands that showed distinctly higher levels of clinical isolates carrying 315 mutations (32).

Overwhelmingly, the predominance of the 315 mutations in clinical INH r is supported in several other large and well-conducted studies. Haas *et al*. (34) which concluded 64% of KatG mediated resistance was due to mutation of codon 315, as well as an older but extremely convincing study by Musser *et al*. (35) indicating 75% of INH^R isolates contained the 315 or 463 codon mutations.

In considering previous studies, several investigators noted that the majority of mutations seen clinically were of the missense type. This indicated the importance of maintaining some, albeit reduced, KatG function in vivo. The rationale behind this is that even low KatG activity still confers a selective advantage versus nonsense mutations that result in truncated products.

Rouse *et al.* validated this hypothesis in a well-designed study that investigated the impact of specific missense mutations on KatG function. This was accomplished by introducing fourteen genetically defined mutations at thirteen different codons (36). The relative effects of both in vitro enzyme assays and *ex vivo* INH resistance were compared as shown in Table 1. This data reveals the benefit of the Ser 315Thr mutation in contrast to other mutations, which abrogate catalase activity or result in relatively insignificant increases in MIC's. However, this last point may be confounded by the use of the BCG as a transformation target. BCG in general is comprised of a phylogenetically distinct group of attenuated species, harboring a series of mostly uncharacterized deletions (37, 38). This may have some effect on the validity of studies. However, relative concordance between data using this and other laboratory adapted species is reassuring.

Overall, Rouse's data is in agreement with recently published data by Wengenach *et al*. (39). Her report included a thorough biochemical analysis of the properties of the Ser 315Thr mutation in comparison to wild type. The results show a six folds drop in catalase activity but only a two folds reduction in peroxidase activity. Taken together, these studies add credence to the hypotheses that the Ser315Thr KatG mutant is a competent catalase peroxidase harboring reduced affinity for INH.

One of the most commonly seen mutations associated with INH resistance in a clinical setting is the Arg 463 Leu or Arg 463 Ala. However, the prevailing opinion is that this mutation does not appear to confer any selective advantage (39, 40). In particular, Johnsson *et al.* found no difference between purified wild type and the R463L KatG mutant in terms of activity or ability to discriminate isoniazid substrate (40). Despite being completely in vitro, this study combined with others (26,32,33,34,41), concludes the most plausible explanation is that the 463 mutation is a frequent and possibly geographically isolated polymorphism. However, it should be stated there might be a marginal decrease in INH susceptibility between the KatG 463 leu KatG 463 glu, since the 463 leu naturally occurs in M. Bovis, which does show slightly higher MIC. However, evidence of this sort is weak (36).

In conclusion, considerable evidence has shown that INH acts as a prodrug that requires activation by KatG. Approximately 60 - 70% of all observed INH resistance can be directly linked to defects in KatG. As mentioned, this indicates the likelihood of additional resistance effectors, and raises an important question with the exact target(s) of the activated INH product.

InhA AND FATTY ACID SYNTHESIS

As stated, mutations in KatG account for approximately two thirds of INH resistance. Therefore, several groups have postulated additional downstream targets of activated INH in mediating resistance (33, 35, 44). This was initially based on phenotypic evidence that INH affects cell wall synthesis. Also, at a low frequency of bacterial isolations (10-7), INH resistance was not correlated with loss of catalase activity but rather with co-acquisition of ethionamide (ETH) resistance. ETH is a structural analog to INH, indicating a possible common target (Figure 2).

In an effort to identify this target, Banerjee *et al.* utilized a lab derived spontaneous INH/ETHr mutant to construct a genetic library for complementation studies (42). Two ORFs (open reading frames) were identified, termed orf1 and inhA. Subsequent subcloning studies, using both genomic fragments from *M. bovis* and *M. tuberculosis* H37Rv cloned into the *M. smegmatis* MC2155 strain revealed several interesting results. The first is that the InhA product alone was sufficient to cause an INH resistant phenotype. Second, in comparison to the *M. smegmatis* gene the, *M. bovis* and *M. tuberculosis* InhA genes appear to be located within an operon that includes Orf1. Also, the intergenic region for both *M. bovis* and *M. tuberculosis* H37Rv was considerably shorter than that of *M. smegmatis* and may lack complete promoter sequences.

Sequence alignment of InhA showed marked conservation across all mycobacterial strains and

significant homology (40% identity) to the *E. coli* ENV M protein known to be involved in fatty acid biosynthesis. Sequence comparisons also revealed a single nucleotide change at position 94 from serine to alanine as well as a putative NAD+/NADH binding site. The basis of INH resistance was determined by cell free mycolic acid synthesis assays. In the presence of INH wild type InhA was inhibited in a rough dose dependent manner. In contrast, the S94A mutant strain showed 20 folds greater activity under similar conditions. These data are quite consistent with the supposition that KatG activated INH targets mycolic acid biosynthesis. This group also proposed a hypothetical mechanism based on *E. coli* ENV M resistance to diazaborine, (42) that was discarded, based on the crystallization and functional analysis of InhA by Dessen *et al*. (43).

This group confirmed several unresolved questions. First, was the confirmation of the InhA function in mycolic acid biosynthesis. The role of this enzyme was in the catalysis of the NADH specific reduction of 2-trans-enoyl acyl carrier protein (ACP), an essential step in fatty acid elongation. Second, it was shown by microcalorimetry that neither unmodified isoniazid nor ethionamide bound to InhA supporting a role for activation of these prodrugs by KatG. The salient feature of this paper was that while the K_m and V_{max} values of the enoyl substrate for the wild type and S94A mutant didn't differ significantly, the K_m for NADH was 5 fold higher in the S94A mutant.

This indicated the resistance mechanism was related to specific interplay between the enzyme and cofactor, not INH. Crystallization data from the WT and S94A mutant indicated that perturbations in hydrogen-bonding within the NADH binding site impaired its affinity for NADH. As tantalizing as these results were the complete picture was left to a later report by the same group where an attractive mechanism was put forth (44).

In the WT condition there is a preference for NADH to bind first to InhA, followed by an acyl-ACP substrate, initially leaving the InhA-NADH complex available for attack by activated isoniazid. Due to the higher affinity, the WT InhA-NADH-INH complex would result in permanent inhibition of mycolic acid synthesis. Conversely, the decreased affinity of the S94A mutant would promote acyl-ACP substrates to bind first before NADH thereby protecting the enzyme. Furthermore, in a NADH-INH bound condition the lower affinity may promote release of the inhibitory complex.

Although of enormous academic interest, the clinical relevance of the S94A mutant is questionable since the clinically observed mutations in inhA do not include S94A (44). Rather in this study they are reported at residues 16, 21, 78 and 95. The fact that these are still physically near the NADH binding domain is encouraging (Figure 4). As added proof to the proposed mechanism, a paper by Lei *et al*. on isolation of the InhA inhibitor complex showed complete inhibition of InhA by the presence of a tightly bound (K_D< 0.4nm) INH product that had been activated by KatG (45).

Table 2. Resistance-associated mutations and amino acid substitutions in the Kas A codon. Adapted from Mdluli *et al.*, 1998. Depicted diagrammatically in Figure 3.

KasA Codon	Nucleotide D	Amino Acid D
66	GAT -- AAT	D -- N
269	GGT -- AGT	G -- S
312	GGC -- AGC	G -- S
413	TTC -- TTA	F -- L

THE ROLE OF ß-KETOACYL ACP SYNTHASE (KASA) AND INH

There seems to be considerable dispute within the literature as to additional targets of INH (46). Mudluli *et al*. has investigated accumulations of a saturated hexacosanoic acid (C26:0) found under INH treatment (47). This saturated fatty acid was found linked to acyl carrier protein (AcpM) that normally runs at 12 KD in SDS-page gels, however a second 80 KD complex with the same amino terminus was found to be a covalent complex of ß-ketoacyl ACP (KasA) INH and ACPM. Automated sequence analysis of four INH^R clinical samples revealed four different mutations at the kasA loci (Table 2 and Figure 3). Notably, two samples with the mutations G269S and F413L carried no other mutations, adding strength to the association. The remaining two strains carried additional KatG 315 mutations.

By comparison to the crystal structure of the *E. coli* homolog, three of the amino changes were within the catalytic center, the fourth was found to be located at the carboxy terminus and purported to alter protein-protein interactions (47). The clinical importance of KasA in INH resistance still needs to be fully established. A study by Lee *et al.* may assist in this regard (48). A series of 160 INH resistant isolates were sequenced and 10% carried mutations in KasA involving R121K, G312S, G387D, and previously reported G269S. However, 6 out of 32 susceptible strains also contained the G312S alteration, a seemingly similar situation to the KatG 463 mutation. Nevertheless, the possibility of KasA constituting a resistance mechanism should not be ruled out completely, since the R121K and G387D mutations have yet to be reported in a susceptible strain, and mutations in KatG and InhA do not account for all INH^R seen clinically.

Therefore, it seems possible that geographically distinct polymorphisms between epidemiologically disparate strains may be commonplace. One should also consider that both INH action, as well as resistance is likely pleotrophic in nature. This line of thought is supported by a recent study that utilized specific inhibitors of Inh A and KasA to demonstrate that INH

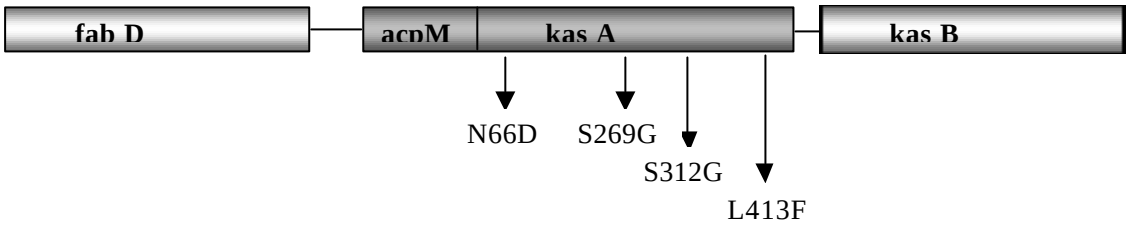

Figure 3. Diagrammatic representation of the kasA codon. Adapted from S. Ramasaswamy et al.,1998.

affects both targets simultaneously albeit by independent mechanisms, thereby arguing convincingly for a role of both targets in INH resistance (49).

THE OXIDATIVE STRESS PARADOX: THE OxyR-AhpC REGULON

The study of both KatG and InhA has provided valuable clues to the action of INH but has also introduced some unresolved questions. The exceptional sensitivity of *M. tuberculosis* to INH lacks rationale in comparison with other bacteria (54). To persist and flourish pathogenic mycobacteria must be able to withstand high oxidative stresses found in macrophages. In gram-negative organisms such as *E. coli* and S. *typhimurium*, the oxidative stress response is controlled by the OxyR protein. OxyR can upregulate several genes including AhpC (encoding acyl hydroperoxidase reductase), Dps (19KD starvation protein), OxyS (a divergent product of oxyR), GorA (glutathione reductase), and most importantly KatG (53). Sherman *et al.* investigated this response in *M. tuberculosis* and several other mycobacterial members (50). Metabolic labeling experiments were conducted under peroxide challenge and 2D protein gels indicated an OxyR like protective response was upregulated by only *M. smegmatis*; all *M. tuberculosis* and *M. tuberculosis* complex members elicited only an upregulated KatG. Deretic *et al.* and others have investigated the oxidative stress regulon in *M. tuberculosis* to determine the basis for these altered expression patterns (51, 52, 54). They specifically sequenced the AhpC gene and its putative regulator OxyR. In the laboratory adapted H37RV strains, both of the OxyR and AhpC genes were divergently transcribed, but the OxyR gene was found to be inactivated by multiple lesions including frameshifts , deletions and stop codons that ablated any gene function.

This result was confirmed in all *M. tuberculosis* strains tested and in all members of the *M. tuberculosis* complex. As added proof, inactivation of the OxyR or AhpC in *E. coli* will also confer INH susceptibility to this otherwise insensitive bacteria (55). When considering the above evidence, it appears that *M. tuberculosis* relies heavily on the defense afforded by their novel cell wall, having essentially eliminated most of the oxidative stress response (except KatG) from their genome.

Naturally, one must consider that the major mutations associated with INH resistance also cause reduced catalase-peroxidase activity in KatG, this, according to Sherman *et al.* is paradoxical since KatG is intimately involved in the survival and pathogenesis of *M. tuberculosis* (53). This group found a strong synergistic effect of H2O2 and INH in KatG positive BCG strains. They conducted a series of expression studies and a pre-induced KatG was capable of increasing survival 35 fold. To determine the compensatory mechanism in KatG (catalase negative) situations, Shermin examined 8 clinical INH^R KatG mutants for altered expression profiles. All 8 isolates expressed a 22 KD protein at considerably higher levels than H37RV controls. This protein was found to be AhpC. Its hyperexpression was a result of point mutations within its promoter region that apparently abrogate the requirement for OxyR regulatory control. The temporal appearance of this mutation was investigated and appears to be the result of a second *in vivo* selection event after KatG mutation. Several groups proposed that the increased AhpC expression could play multiple roles *in vivo* (53, 54). First, it could directly counteract INH effect, or simply compensate for loss of KatG activity thereby increasing overall viability. Sherman reported that while AhpC upregulation provides substantial benefit against peroxide insult it does not detoxify INH. Conversely, Zhang *et al.* supports an independent role for AhpC mutations in the emergence of low INH resistance (54).

The relevance of this mutation should also be questioned; particularly in light of two recent clinically based studies (56, 57). The first, sequenced the AxyR-AhpC region of 229 *M. tuberculosis* isolates recovered from infected humans and animals, where the KatG and InhA regions had been sequenced and reported previously (56). The most important feature of this study was that most INH^R strains carrying substantially reduced activity of KatG (ie. 315 mutation) lacked any alterations in AhpC or OxyR-AhpC intergenic regions. The second study analysed 57 clinical isolates. Here, 8 compensatory AhpC promoter mutations were identified in 8 catalase negative KatG defective strains whereas the corresponding region of 25 catalase positive INH^R isolates were unaltered (57). Taken together, these results show little evidence for an independent role for

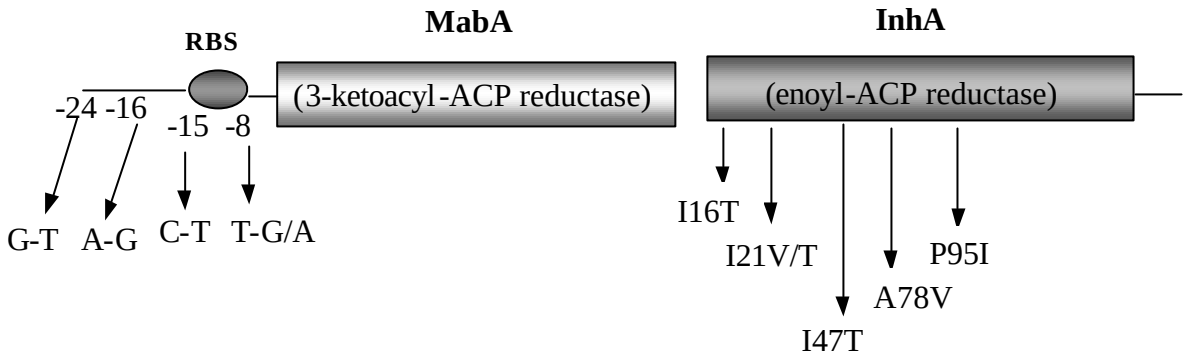

Figure 4. InhA and upstream regulatory element mutations implicated in INH resistance Adapted from S. Ramasaswamy et al 1998.

AhpC in INH resistance which appears at a low frequency similar to what is found in catalase negative strains. These strains may be of clinical significance.

THE "GLOBAL" APPROACH TO ISONIAZID RESISTANCE

Collectively, it is apparent there is controversy over the identification of the molecular correlates of resistance of MTB to isoniazid. There is also some lack of agreement concerning several mutations and/or polymorphisms being causally linked and clinically relevant to INH resistance. The main problem appears to be that previous genotypic studies of *M. tuberculosis* may have incorrectly estimated the importance of specific drug mutations due to errors in sampling, as well as the inclusion of MDR isolates and inappropriate controls. Also, the biochemical characterization of many proposed targets (resistant or susceptible) has inevitably used non-representative lab strains or non-pathogenic species for ease and safety, which may not accurately reflect that seen in the infected individual.

These errors, combined with a reductionist approach to drug resistance, has only revealed a fragmented and convoluted picture of *M. tuberculosis* resistance to isoniazid. To alleviate further discrepancies, a more holistic or "global" approach should be taken that makes full use of genomic databases, tools and methods. A comprehensive review of this subject can be obtained from several sources (58-65).

Several recent studies have undertaken this approach and have yielded a wealth of information. The first study by Wilson *et al.* uses micro-array hybridization to determine changes in expression patterns under INH exposure (66).

The basic methodology is summarized elsewhere, however in brief, at time zero (pre INH) and each successive time point (post INH), mRNA was isolated pelleted mycobacterial culture. These RNA species were differentially labeled with fluorochrome tags in a reverse transcriptase reaction (RT) to produce cDNAs that were used as probes for microarray hybridization. The resulting pattern is analysed via software and intensity changes allow discrimination between relative changes in mRNA expression.

This enabled the documentation of a highly induced gene cluster encoding components of the FAS II fatty acid operon. Several of these genes (acpM and kasA) have been previously reported, corroborating previous studies, and the methods used in this study. The induction of these genes were observed as early as twenty minutes post- treatment. Three additional INH induced proteins were also reported, the first of which is FbpC which is an abundant exported protein involved in mycolic acid maturation in the outer wall. FbpC has also been previously reported (67,68) since it constitutes one of the 3 highly homologous proteins comprising the 85 C Ag complex. As an added note, this complex has been proposed as the site of interaction with human fibronectin and therefore is a primary target for rational drug design and vaccines.

Several novel proteins lacking characterization were also found, 2 acetyl-CoA dehydrogenases (FADE23, FADE24) and an efflux protein EfpA as well as a subunit of AhpC. Further investigation of the relevance of transcriptional responses was analyzed in INH^R strains under conditions of INH and ETH treatment. The first results of this experiment showed a similar upregulation pattern by ETH as observed by INH. The second experiment of INH^R strains revealed no significant alteration of gene expression upon INH insult. Taken together, these results agree with previous literature yet provide a more complete view of all the players involved and the inclusion of several new therapeutic and prophylactic targets.

A similar approach was employed by Allard using a differential expression customized amplification library (Decal) (69). One important feature of this method is the ability to resolve four-fold differences in mRNA expression without confounding from constitutively expressed mRNA (housekeeping genes). Although beyond the scope of this paper, an additional method described by Brenner *et al.* allows the resolution of "a

few tens of mRNA copies per cell" (70). Both methods are quite amenable to bacterial systems as they do not rely on poly adenylation of mRNA transcripts and both require no previous sequence knowledge.

The Alland paper was directed specifically towards the effect of isoniazid on the genomic expression of Mycobacterium tuberculosis, similar to the work of the Wilson group. Their results showed an upregulation of three previously unknown isoniazid-induced genes, IniA, IniB and IniC, all of which have putative functions in cell wall synthesis or a hypothesized protective role in response to cell wall destruction. A later report has characterized the rather unique ini BAC promoter. A molecular genetic analysis described a regulatory region and putative repressor that was specifically induced by a variety of cell wall inhibitors, but only in actively replicating cells (71,74).

The final report by Piatek *et al.* is an excellent example of the use of a molecular epidemiological approach to drug resistance (72). The work utilized a PCR based molecular beacon assay. Briefly, this assay utilizes specifically paired fluorogenic PCR primers which allows real time monitoring of multiple PCR products in a single reaction (i.e. multiplex PCR), The assay itself is relatively quick and results can be analyzed within several hours (72, 73).

The populations used in this study were distinct. The first was from a reference lab in Spain. This population was known to have an overrepresentation of MDR clinical isolates. The second study population was from a community medical center in New York. The use of these two populations was to contrast and thereby preclude many previous mistakes in assessing the clinical relevance of mutations. The study was also used to characterize the assay and it was found to be particularly effective as a predictive screen for INH^R with a sensitivity and specificity of 85% and 100% respectively for detection of mutations in the KatG, InhA and AhpC loci. Results of stratified analysis of the sample populations showed dramatic differences in the ability to discriminate INH^R. Ninety-four percent of isolates from Spain contained mutations associated with INH^R versus only seventy-six percent of New York isolates. Analysis to exclude the confounding of MDR revealed 94% and 96% of isolated from Madrid and New York respectively contained INH resistance mutations. Restriction to New York isolates only showed a strong correlation of INH^R and MDR (94%) compared to only 44% of single drug resistance (i.e. INH^R) mutations.

The authors then investigated the possibility of the KasA mutation accounting for the discrepancies. No mutations were found in codons (66, 312, 413) previously reported to be associated with INH r (48). One previously reported mutation, G269S, was found in 10 isolates but were equally distributed between resistance and susceptible controls, suggesting no association with INH^R. As previously mentioned for KatG, this likely constitutes a polymorphism.

The authors went on to address the possibility of INH^R being the end result of a temporal sequence of mutations that have previously been unassociated with resistance. The hypothesis of a series cumulative mutations (i.e.."a genetic barrier") imparting resistance while interesting, is not a novel one, and in this particular situation it remains yet unfounded.

FUTURE DIRECTIONS

To resolve the previously stated issues, in the interests of public health will require accurate assessment of resistance mutations, polymorphisms, and their relevance (i.e. those being both sufficient and necessary to effect drug resistance) in a clinical setting. Although the primary target(s) of INH are elements of the FASII system, necessary for mycolic acid synthesis, the predominance of mutations seen in patients seem to be localized to KatG.

Further study of these and related problems using burgeoning new technologies, and information focusing on molecular pathogenesis should provide important new insights into mycobacterial metabolism, the genetic basis of mycobacterial drug resistance, and host response. This approach will inevitably lead to new targets, and drug leads. These will hopefully translate to prophylactic and therapeutic interventions in the next several years.

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