Tuberculosis and the Tubercle Bacillus
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Chapter 8

Mechanisms of Drug Resistance in *Mycobacterium tuberculosis*

YING ZHANG, CATHERINE VILCHÈZE, AND WILLIAM R. JACOBS, JR.

INTRODUCTION

Drug Resistance in *M. tuberculosis*

Despite the declaration of tuberculosis (TB) as a global emergency by the World Health Organization 10 years ago, the global problem of TB has worsened due to increased drug resistance and the human immunodeficiency virus (HIV) pandemic (39). Drug resistance in TB is a particular problem because the lengthy therapy of at least 6 months makes patient compliance very difficult, which frequently creates drug-resistant strains of *Mycobacterium tuberculosis*. The WHO reports the presence of drug-resistant strains of *M. tuberculosis* in 72 countries, with frequencies ranging from 3 to 41% (230). Multidrug-resistant TB (MDR-TB) caused by *M. tuberculosis* strains resistant to two or more drugs, usually isoniazid (INH) and rifampin (RIF), caused several fatal outbreaks worldwide and is an increasing threat to global TB control programs (39). While drug-sensitive TB can be effectively treated with four drugs, INH, RIF, ethambutol (EMB), and pyrazinamide (PZA), in a 6-month regimen called short-course chemotherapy, treatment of a case of MDR-TB can exceed 2 years, thus increasing the costs and side effects significantly. In the absence of any new treatment and with the growing epidemic of HIV infection, which weakens the host immune system and allows easier transmission of TB and the drug-resistant form, there is increasing concern about the control of the disease. In view of the problem of drug-resistant TB and with the advent of modern molecular biology tools, there has been great interest in understanding the molecular mechanisms of drug resistance and drug action in *M. tuberculosis*. Tremendous progress has been made in this area in the last decade. This chapter provides an update on genes associated with drug resistance and our current understanding of mechanisms of drug resistance and drug action in *M. tuberculosis*.

The Phenotype of Drug Resistance

Resistance is a phenotype, the ability of a bacterial cell to survive the presence of a drug at a concentration that normally kills or inhibits growth. Resistance is caused by mutation and can be differentiated from tolerance, which is a conditional phenotype mediated by the physiological state of the bacilli. For example, *M. tuberculosis* cells are resistant to INH when the cells are in stationary phase (135). For this reason, drug susceptibility of *M. tuberculosis* is measured by testing cells in the exponential phase of growth. The phenotype of drug resistance is determined in liquid medium containing the drug, as in the BACTEC method (184), or in solid medium, as in agar plate dilution analysis in medium containing various concentrations of the drug. The MIC is thus defined as the minimum concentration of the drug which kills 99% of cells. The MIC of INH can vary dramatically depending on the strain and the mutation conferring INH resistance. *M. tuberculosis* is exquisitely sensitive to INH, with INH MICs in the range of 0.02 to 0.2 μg/ml. Resistance levels show two distinct phenotypic ranges. High-level resistance can occur in a single step at ranges between 10 and 100 μg/ml (200- to 2,000-fold above normal). Low-level resistance occurs at 0.25 to 2.0 μg/ml (5- to 100-fold above normal). As described later in this chapter, many of the genetic bases for these differences have been defined. However, it must be emphasized that the in vitro MIC does not necessarily reflect the drug concentration required to kill or inhibit tubercle bacilli in vivo. A pertinent example is PZA, which has a MIC of 100 μg/ml by the BACTEC method that is considerably higher than its effective concentration in vivo. Drug resistance in *M. tuberculosis* can be either intrinsic (or natural) or acquired. Intrinsic resistance refers to nonsusceptibility due to unique characteristics of *M. tuberculosis* such as its natural resistance to penicillin or clarithromycin.

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Acquired resistance refers to susceptible *M. tuberculosis* becoming resistant to drugs as a result of mutations. In some rare situations, drug-resistant mycobacteria can become drug dependent; that is, they grow only in the presence of the drug. For example, streptomycin (SM)-dependent resistant *M. tuberculosis* strains have been found in some cases that have characteristic changes or mutations in the ribosome (75) (see the section on SM resistance [below]).

Identification of Genotypes Conferring Drug-Resistant Phenotypes

The emergence of drug resistance in bacteria is one of the easiest demonstrations of the "survival of the fittest" concept of Darwin's theory of evolution. From the time when drugs were first used to treat bacterial infections, drug resistance was observed (2). This is because the large number of bacterial cells in populations allows for the selection of mutants that are resistant to the drugs (96). Resistance is thus due to a change in the genotype resulting in a drug-resistant phenotype of a bacterium, which can be passed on to subsequent generations. This is in contrast to tolerance, or phenotypic resistance, another phenomenon that is common to *M. tuberculosis* and other bacterial species, in which changes in the metabolic or physiological status of the cell induce temporary drug resistance as seen in stationary-phase, starved, or dormant bacteria. Knowledge about the mutations conferring drug resistance not only leads to understanding of the mechanisms of drug resistance and drug action but also facilitates rapid detection of drug resistance by molecular means. To date, the definitive method for identification of mutations that confer drug resistance is the transfer of genes conferring drug resistance or drug susceptibility to, or from, drug-resistant strains. This represents the fulfillment of Koch's molecular postulate (49). For the broad-spectrum antibiotics, RIF, SM, and fluoroquinolones, the mechanisms of drug action and resistance had been elucidated previously by using genetically tractable organisms. Analogous mutations could thus be identified by DNA sequence analysis of drug-resistant strains of *M. tuberculosis*. However, the lack of gene transfer systems for mycobacteria had prevented the discovery of the drug-resistant alleles for any of the *Mycobacterium*-specific drugs. Thus, as of 1992, no drug resistance alleles had been identified for INH, ethionamide (ETH), EMB, PZA, or *p*-aminosalicylic acid (PAS). With the development of plasmid transformation systems for mycobacteria, for the first time it was possible to clone and transfer the minimal in identifying mycobacterial drug resistance alleles, as summarized in Table 1.

It is important to note that the mutations that confer drug resistance can be either recessive or dominant with respect to the wild-type gene. The characteristic of recessive or dominant behavior is a direct consequence of the merodiploid state (i.e., the introduction of a wild-type or mutant allele into a strain with a mutated gene or the wild-type gene). Recessive mutations represent the loss of some function. For example, Middlebrook had isolated INH-resistant mutants of *M. tuberculosis* and showed that the majority of strains had lost their catalase peroxidase activity (128). Zhang et al. cloned a DNA fragment that encoded the catalase peroxidase gene and demonstrated that the transfer of this wild-type gene to an INH-resistant strain restored INH susceptibility (235). Thus, the mutations in the INH-resistant mutants were recessive to the wild-type gene. In contrast, the introduction of plasmids which caused overexpression of the wild-type *inhA* gene, by virtue of multicopy plasmids or strongly expressed promoters, led to a dominant phenotype with respect to the wild-type *inhA* allele in the recipient strain (12, 95) and to INH resistance. The transferred alleles for drug resistance are characterized in terms of recessive or dominant behavior with respect to the wild-type genes.

Drug Resistance Mechanisms

Bacteria can become resistant to antibiotics or antibacterial agents by a number of common strategies, including target modification, target overexpression, barrier mechanisms, drug-inactivating enzymes, inactivation of drug-activating enzymes, and drug extrusion mechanisms. The first strategy described for bacteria was that of mutations in the target, which cause either reduced drug binding or overexpression of the drug target. Similar mechanisms exist for both prokaryotic and eukaryotic cells with the drug trimethoprim or methotrexate and the gene encoding the target of dihydrofolate reductase.

Since drugs must enter bacterial cells to be active, bacteria can become resistant by preventing entry of the drug. Barrier mechanisms can be mediated by decreased uptake, such as that caused by the porin (PenB) mutation in *Neisseria gonorrhoeae*, resulting in resistance to β-lactams and tetracycline (58), or by increased removal of antibiotics from the cell, such as the enhanced efflux for tetracycline mediated by efflux proteins TetA to TetE, TetG, TetH, and TetZ (121). Although mycobacteria have porins (150), they have

Table 1. Mechanisms of drug action and resistance in mycobacteria

Drug[a]	MIC (μg/ml)	Gene(s) involved in resistance	Gene function	Role	Mechanism of action	Mutation frequency (%)	Allele type
Isoniazid	0.02–0.2	*katG*	Catalase-peroxidase	Prodrug conversion	Inhibition of mycolic acid biosynthesis and other multiple effects on DNA, lipids, carbohydrates, and NAD metabolism	20–80	Recessive
		inhA	Enoyl ACP reductase	Drug target		15–43	Dominant
		ndh	NADH dehydrogenase II	Modulator of INH activity		10	Recessive
		ahpC	Alkyl hydroperoxidase	Marker of resistance		10–15	
<u>Rifampin</u>	0.5–2	*rpoB*	RNA polymerase	Drug target	Inhibition of transcription	96	Dominant
Pyrazinamide	16–50 (pH5.5)	*pncA*	Nicotinamidase/ pyrazinamidase	Prodrug conversion	Acidification of cytoplasm and de-energized membrane	72–97	Recessive
5-Chloro-pyrazinamide	8–32	*fasI*	FASI	Drug target	Inhibition of FASI	NA[b]	Dominant
Ethambutol	1–5	*embCAB*		Drug target	Inhibition of arabinogalactan synthesis	47–65	Dominant
<u>Streptomycin</u>	2–8	*rpsL*	S12 ribosomal protein	Drug target	Inhibition of protein synthesis	52–59	Recessive
		rrs	16S rRNA	Drug target		8–21	Dominant
<u>Amikacin/ kanamycin</u>	2–4	*rrs*	16S rRNA	Drug target	Inhibition of protein synthesis	76	Dominant
<u>Quinolones</u>	0.5–2.5	*gyrA*	DNA gyrase subunit A	Drug target	Inhibition of DNA gyrase	75–94	Dominant
		gyrB	DNA gyrase subunit B	Participates in drug binding?		In vitro	
Ethionamide	2.5–10	*etaA/ethA*	Flavin monooxygenase	Prodrug conversion	Inhibition of mycolic acid biosynthesis	37	Recessive
		inhA		Drug target		56	

[a]Rifampin, aminoglycosides, and fluoroquinolones (underlined) are broad-spectrum antibiotics, whose mechanism of resistance in *M. tuberculosis* is the same as in other bacteria.
[b]NA, not available.

to be mediated by efflux pumps that block drug uptake (104). Another mechanism of resistance is that of drug inactivation mediated by drug-degrading or inactivating enzymes (e.g., cleavage of penicillin by β-lactamase). Typically, resistance mediated by drug-inactivating enzymes is present at much higher levels than the resistance mediated by target alterations. The common theme for all these mechanisms is that they confer the phenotype of resistance of the drug to the recipient cell.

Prodrug Activation: a Unique Mycobacterial Phenomenon

M. tuberculosis is unique to date among bacteria because three of its drugs require activation to become inhibitory. The drugs INH, ETH, and PZA all require activation for activity against *M. tuberculosis*, as discussed in subsequent sections. Thus, resistance can be mediated by mutations that eliminate the activation step. Such inactivation has been demonstrated for KatG (catalase-peroxidase) in INH resistance (235), PncA (nicotinamidase/pyrazinamidase) in PZA resistance (181), and EtaA/EthA (flavin adenine dinucleotide [FAD]-containing monooxygenase) in ETH resistance (15, 42, 53, 208). The *M. tuberculosis* genome contains single copies of *katG* and *pncA*, which cause INH and PZA resistance, respectively, when mutated. It is interesting that *M. tuberculosis* has over 60 homologues of the ETH activator EthA. These monooxygenase-like enzymes are involved in detoxification in other systems, but no other organism has as many such enzymes in their genome. Metronidazole and nitrofuran are nitro-substituted prodrugs whose activation relies on nitroreductases in anaerobic bacteria. For example, resistance to metronidazole in *Helicobacter pylori* has recently been shown to be caused by mutations in *rdxA*, encoding NADPH nitroreductase (60), or in *frxA*, encoding NAD(P)H flavin oxidoreductase (93). Although it has been known for some time that nitrofuran resistance in *Escherichia coli* is accompanied by loss of nitroreductase enzyme (114), the genetic basis of nitrofuran resistance was identified more recently and was shown to be due to mutations in nitroreductase NfsA or NfsB, which is involved in activation of nitrofuran (220). It is noteworthy that the first molecular study to elucidate this type of resistance mechanism was that of KatG mutations in INH resistance (234, 235). Surprisingly, the mechanism of resistance due to loss of drug activating enzymes is not presented in most reviews or book chapters on this topic (160, 213). We would like to emphasize the importance of muta-

Defining a Drug Target

The first step in understanding the mechanism of drug action is to identify the drug target. At the outset, it is useful to define the basic concept of a drug target, which we define as a substrate, typically an essential enzyme, to which a drug binds and inactivates, possibly leading to cell death. Genetically, drug targets are defined by two types of mutations in the gene encoding the target that either alter its structure or cause overexpression. Mutations that alter the target prevent binding of the drug. Mutations that cause overexpression of the drug target mediate resistance by the excess copies titrating the drug, thereby reducing the effective drug concentrations. For example, drugs that target essential metabolic pathways, such as the sulfonamides or trimethoprim, were found to bind to and inhibit the essential enzyme dihydropteroate synthase or dihydrofolate reductase, respectively. For both these cases, resistance is mediated by target overexpression or modification and the mutant alleles would confer a resistance phenotype dominant to the wild-type allele. Nevertheless, overexpression of a gene is not sufficient evidence to prove that a gene encodes a target. For example, a drug-inactivating enzyme such as the arylamine *N*-acetyltransferase Nat can confer INH resistance when overexpressed, but *nat* is not the target of INH (155). Target verification also requires that mutations in the structural gene causing target alteration be identified. Thus, allelic exchange needs to be performed, in which a linked gene is cotransformed with a putative drug resistance allele to provide an unselected marker, thereby ascertaining if the allele confers resistance by target modification. Such strategies have been used to demonstrate that alleles of *rpoB*, *gyrA*, or *rpsL* confer resistance to RIF, fluoroquinolones, or SM, respectively. In addition to these genetic tests, biochemical and X-ray crystallographic studies revealing drug binding and inhibition can further confirm if an enzyme is a target. Defining a drug target is the key first step in elucidating the mechanism of action of a drug.

Mechanisms of Drug Action

Drugs can be either bacteriostatic or bactericidal. Once a drug enters and binds the target, the cell's metabolism is altered, which first leads to growth arrest and/or a cidal event. Bacteriostatic drugs simply inhibit a metabolic pathway and the cell stops growing. In contrast, drugs that induce cell death are bactericidal. The anti-TB drug PAS is thought to inhibit

activity of PAS on *M. tuberculosis* reduced its efficacy as an anti-TB agent as drug resistance rapidly emerged. Nevertheless, the introduction of PAS led to an important concept for modern chemotherapy, namely, that the use of two or more drugs in combating bacterial infections prevents the development of drug resistance. The addition of PAS to SM monotherapy greatly reduced the frequency of emergence of drug resistance (124). The use of two or more drugs for effectively treating mycobacterial infections remains a standard therapy for mycobacterial infections and, indeed, for treating other infections such as those caused by HIV and *H. pylori*. Despite these attempts, the improper use of drugs has led to the emergence of MDR-TB throughout the world today.

An example of a bactericidal drug is penicillin, which targets peptidoglycan biosynthesis, leading to lysis of actively growing cells. Penicillin is effective only on actively growing cells as the cell growth leads to the subsequent lysis of the bacterial cell (73). The discovery of penicillin by Fleming, and its application by Florey and Chain, were indeed landmarks in microbiology, since penicillin remains a highly effective bactericidal agent to this date.

SM, an antibacterial agent discovered by Schatz and Waksman in 1944, was hailed as the first broad-spectrum antibacterial and was even active against *M. tuberculosis* (178). This drug targets a specific ribosomal protein (S12 of the small ribosomal subunit encoded by the *rpsL* gene), thereby inhibiting protein biosynthesis. Inactivation of protein biosynthesis is a bactericidal event that leads to cell death, but, in contrast to penicillin, this does not trigger cell lysis.

Lack of R-Factor-Mediated Resistance

MDR was first detected in *Shigella* in 1955, when it was demonstrated that *Shigella* strains acquired resistance to numerous antibiotics such as sulfonamides, SM, ampicillin, chloramphenicol, and tetracycline through R plasmids (48, 215). Plasmids, transposons, or integrons are known to mediate drug resistance in various bacterial species (J. E. Davies, *Abstr. Ciba Found. Symp.*, abstr. 41a, p. 15–35, 1997; R. M. Hall, *Abstr. Ciba Found. Symp.*, abstr. 65a, p. 192–205, 1997) and in the fast-growing mycobacterium *M. fortuitum* but not in *M. tuberculosis* (110, 111). Instead, drug resistance in *M. tuberculosis* is caused by mutations in chromosomal genes (69). Typically, no single pleiotropic mutation mediates the MDR phenotype in *M. tuberculosis*, although coresistance to INH and ETH can occur with mutations in the target gene *inhA* (12, 142). Thus, the MDR phenotype is caused by sequential accumulation of mutations in different genes involved in resistance to individual drugs as a result of inappropriate treatment or poor adherence. Mechanisms of resistance to TB-specific drugs, such as INH and PZA, are unique to *M. tuberculosis*. On the other hand, mechanisms of resistance to broad-spectrum antibiotics such as SM, RIF, and fluoroquinolones in *M. tuberculosis* are the same as in other bacteria. Mycobacteria are naturally resistant to many antibiotics due to a highly hydrophobic cell envelope acting as an effective permeability barrier (27, 80), drug efflux systems (e.g., the major facilitator family and numerous ABC transporters) (37), and hydrolytic enzymes (e.g., β-lactamases and aminoglycoside acetyltransferases) (37, 94). Most of this chapter deals with drug resistance caused by genetic mutations. However, because of the increasing awareness of the problem of phenotypic resistance, the last section of this review briefly discusses this topic. The chemical structures of the most commonly used antituberculous drugs are shown in Fig. 1.

MECHANISMS OF DRUG ACTION AND RESISTANCE

Isoniazid

Isoniazid (isonicotinic acid hydrazide, INH) is an important first-line TB drug, and was introduced in 1952 (18, 52, 151). INH is highly active against *M. tuberculosis*, with a MIC in the range of 0.02 to 0.2 μg/ml. It has a simple structure containing a pyridine ring and a hydrazide group, and both moieties are essential for its high activity against *M. tuberculosis*.

Mechanism of action

The mode of action of INH is highly complex. The discovery of INH as a potent TB drug arose from two independent observations that nicotinamide had certain activity against *M. tuberculosis* in guinea pigs and mice (35, 120), and that the sulfa drug thiosemicarbazones had activity against *M. tuberculosis* (151). Synthesis of nicotinamide analogs and reshuffling of chemical moieties of thiosemicarbazones led to the discovery of INH. Several early studies reported that INH affected DNA biosynthesis (56), NAD biosynthesis (16, 191), and NAD metabolism by incorporation into NAD through exchange with nicotinamide (233) or by activation of NAD glycohydrolase by removing its repressor leading to NAD depletion (16) as possible mechanisms of action. INH is active against growing tubercle bacilli but not resting bacilli (135, 176). Oxygen plays an important role in INH

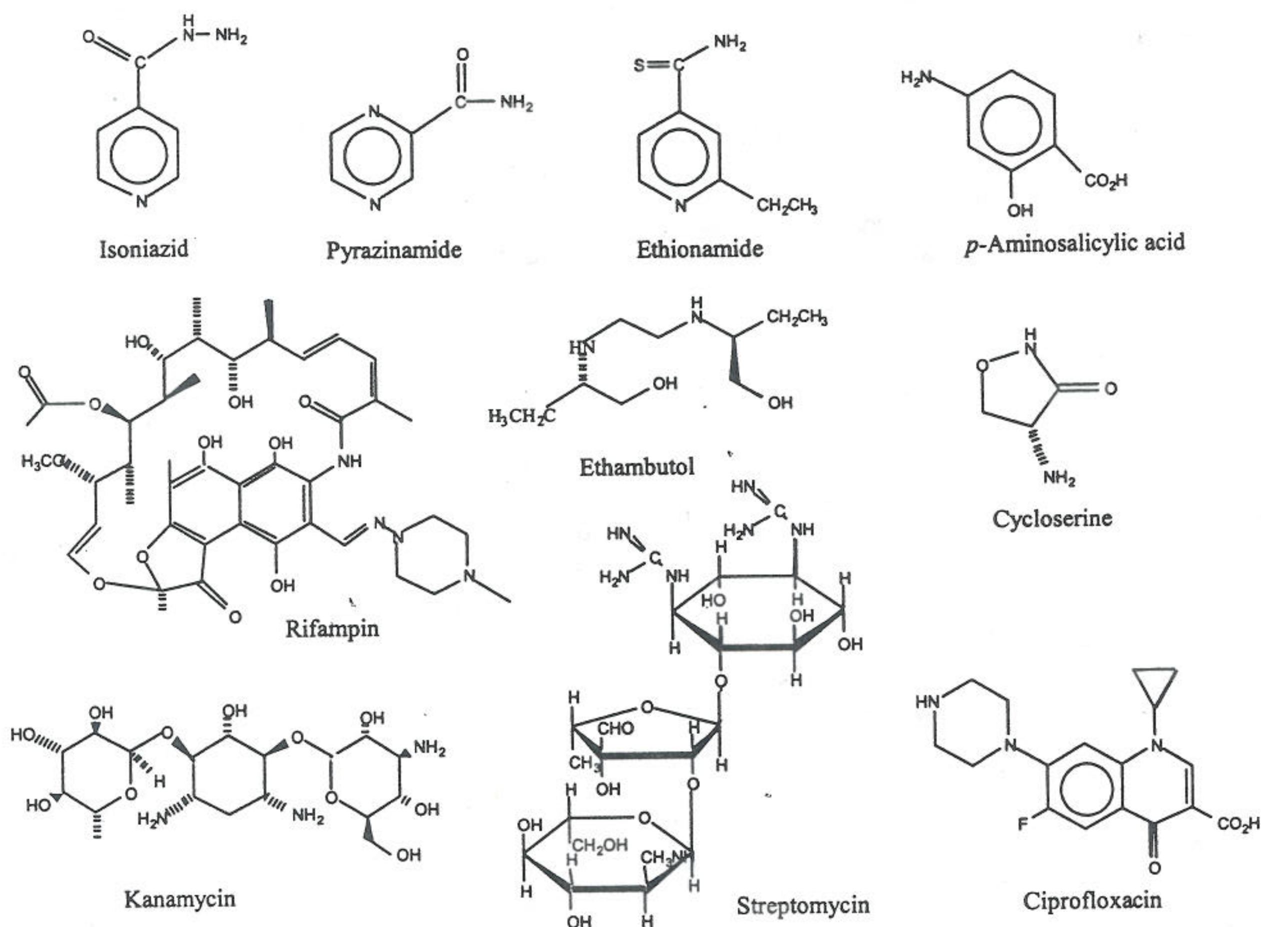


Figure 1. Structures of first-line and some second-line TB drugs.

action, since INH has no activity against *M. tuberculosis* under anaerobic conditions (135). The first report that INH inhibited the synthesis of mycolic acids, the long-chain (C_{70} to C_{90}) α-branched β-hydroxy fatty acids of the mycobacterial cell envelope, was published by Winder et al. (228). Takayama et al. extended this work (197) to demonstrate that the inhibition of mycolic acid synthesis led to a characteristic disruption of membranes and cell death (198). Furthermore, they demonstrated that INH treatment of *M. tuberculosis* led to the accumulation of a saturated C_{26} fatty acid and postulated that the enzyme that was inhibited was either a desaturase, a mycolic acid elongation enzyme, or a cyclopropanase (196).

Zhang et al. showed that INH was activated by the catalase-peroxidase KatG (235) to generate a range of reactive (oxygen and organic) species, which then attack multiple targets in the tubercle bacillus. One of these species, the isonicotinic acyl radical, was shown to attack the nicotinamide group of NAD^+ to

different isomeric INH-NAD adducts, the open form and cyclic form (149, 223). Questions are raised about whether the open or cyclic form, or both, is the active species (29). The KatG-mediated INH activation can also be achieved with manganese (Mn^{2+}) (101). Mn^{2+} facilitates the INH activation by enhancing the production of the INH-NAD adduct by both wild-type and mutant KatG enzymes (216). Although *M. tuberculosis* KatG itself is sufficient for INH activation, *M. smegmatis* KatG cannot directly activate INH and requires Mn^{2+} for activation (216). This may provide a likely explanation for the relative nonsusceptibility of *M. smegmatis* to INH. In fact, the introduction of the *M. tuberculosis katG* gene into wild-type *M. smegmatis* increases the susceptibility of *M. smegmatis* to INH by 50-fold (47). Superoxide plays a role in KatG-mediated INH action (30, 214). Treatment of *M. tuberculosis* H37Rv strain or a strain carrying the S315T KatG mutation with plumbagin and clofazimine, two compounds capable

Defining the precise target of action of INH required the development of plasmid transformation systems and led to the discovery of *inhA*, a gene from *M. tuberculosis* that conferred coresistance to INH and ETH when expressed in *M. smegmatis* (12). In these studies, a common missense mutation causing a substitution of an alanine for the serine at position 94 (S94A) was found in INH-resistant mutants of *M. bovis* and *M. smegmatis*. Mycolic acid biosynthetic assays from cell lysates from either the overexpressed *inhA* recombinants or the S94A mutant were resistant to INH inhibition compared to the parental strain. These studies led to the conclusion that *inhA* encoded the target for both INH and ETH.

The *inhA* gene encodes an NADH-specific enoyl-acyl carrier protein (ACP) reductase (165), part of the fatty acid synthase type II (FASII) system (109), and its three-dimensional structure was determined with NADH bound (45). The S94A mutant was shown to bind NADH five times less efficiently than the wild-type InhA protein did, and the X-ray crystallographic data revealed that replacing serine with alanine resulted in reduced hydrogen bonding of NADH to InhA (45). Quemard et al. were the first to demonstrate that KatG-activated radioactive INH bound to InhA but that this was inhibited by high concentrations of NADH (164). Johnsson and Schultz demonstrated that KatG-activated INH inhibited the InhA activity and that the S94A mutant was resistant to this inhibition (83). Basso et al. found that numerous InhA proteins from INH-resistant clinical isolates resisted KatG-activated INH inactivation and were all defective for NADH binding (14).

Mdluli et al. proposed that the β-ketoacyl-ACP synthase, KasA, also part of the FASII system, was the primary target of INH (123). They found that INH (1 μg/ml) induced two *M. tuberculosis* proteins involved in fatty acid and mycolic acid synthesis, the ACP AcpM and KasA, resulting in the formation of a complex containing KasA, AcpM, and INH (123). Since the inhibition of a β-ketoacyl-ACP synthase could explain the accumulation of a saturated C_{26} fatty acid on INH treatment of *M. tuberculosis*, as described by Takayama et al. (196), the authors concluded that KasA was the primary target of INH. Later, Kremer and colleagues showed that the KasA-AcpM-INH complex does not contain INH and that this complex is not induced by inhibition of KasA but is induced when InhA is inhibited (90). In the same study, the authors also demonstrated that activated INH does not inhibit purified KasA in vitro (90). Therefore, it remains to be demonstrated how the activated form of INH reacts with KasA or AcpM and if these interactions play any role in INH bactericidal activity.

Despite the efforts to establish that *inhA* encoded the primary target of INH, several significant questions remain unanswered. First, blocking enoyl reductase activity should result in the accumulation of a monounsaturated fatty acid, and yet several workers found the accumulation of a saturated C_{26} fatty acid on INH treatment of *M. tuberculosis* (123, 196). Second, transformation of *M. tuberculosis* with a multicopy plasmid library did not yield any clones that conferred resistance to INH or ETH (122). Third, it was unclear how a mutation that altered NADH binding had any relationship to INH resistance.

Vilchèze et al. hypothesized that the accumulation of a saturated C_{26} fatty acid did not result directly from a blockage of InhA but, rather, from the blockage of the FASII system resulting in accumulation of the end product of FASI (211). Mycobacteria, unlike most other bacteria, possess two different systems for generating fatty acids. Most bacteria make C_2 to C_{16} fatty acids by using the independent, ACP-based FASII system. In mycobacteria, FASII is used to extend C_{18} or C_{26} fatty acids to C_{56} while the eukaryote-like FASI system, first described by Bloch and coworkers (21, 28), makes C_2 to $C_{24/26}$ fatty acids. By isolating a temperature-sensitive mutation in *inhA*, Vilchèze et al. were able to demonstrate that thermal inactivation of InhA did result in the accumulation of a saturated FASI end product (211). Moreover, the thermal inactivation of InhA in *M. smegmatis* led to death kinetics and lysis that was very similar to that induced by INH (211). In addition to explaining the accumulation of a saturated FASI end product, this work established that inactivation of InhA was sufficient for cell lysis, fulfilling another drug target criterion.

The failure of the multicopy library to confer INH resistance on *M. tuberculosis*, observed by Mdluli et al. (122), was confirmed by E. Dubnau and W. R. Jacobs, Jr. (unpublished observation). However, the introduction of the same *M. tuberculosis* library into *M. smegmatis* yielded plasmids containing the *inhA* operon (12). Furthermore, the introduction of an *M. smegmatis* library into *M. bovis* BCG yielded an *inhA*-containing plasmid (12). The basis of this inconsistency was hypothesized to result from the *M. tuberculosis inhA* operon being tightly regulated in *M. tuberculosis* and *M. bovis* BCG. To address this possibility and to test if overexpressed *inhA* conferred resistance to INH and ETH in *M. tuberculosis*, the *inhA* gene from *M. tuberculosis* was fused to two independent promoters. Both resulting constructs conferred coresistance to INH and ETH in *M. tuberculosis*, *M. bovis* BCG, and *M. smegmatis* (95).

The relationship between INH and NADH was resolved with the surprising X-ray crystallographic

visualization of an INH-NAD adduct in the InhA binding pocket (172). This result suggested that a mutant of InhA that was defective in binding NADH (like S94A) should also be defective (hence resistant) in binding the INH-NAD adduct. Moreover, it also suggested that activated ETH might also form a similar adduct to inhibit InhA. Evidence supporting this observation comes from the discovery that mutations in NADH dehydrogenase II which result in increased $NADH/NAD^+$ ratios mediate coresistance to INH and ETH (see below).

In summary, the current model for the mode of action of INH is as follows (Fig. 2). INH is a prodrug that requires activation by the *katG*-encoded catalase-peroxidase (KatG) (235). The active species, an isonicotinic acyl radical, attacks the nicotinamide group of NAD^+ to form a covalent INH-NAD adduct (172), which inhibits InhA, resulting in a blockage of FASII activity. The blockage of FASII leads to accumulation of FASI end products. Unable to obtain mycolic acids required for the synthesis of the cell wall, the mycobacterial cells lyse.

Mechanisms of INH resistance

Loss of the *katG*-encoded catalase peroxidase. Middlebrook reported the isolation of spontaneous INH-resistant mutants of *M. tuberculosis* in the laboratory shortly after the discovery of the drug (129). Interestingly, he made the observation that INH-resistant isolates often lost catalase activity and also became attenuated for virulence in guinea pigs (128). The role of catalase in INH resistance remained unclear for many years, since not all INH-resistant strains lost catalase activity and since it was not known whether the loss of catalase was the cause of

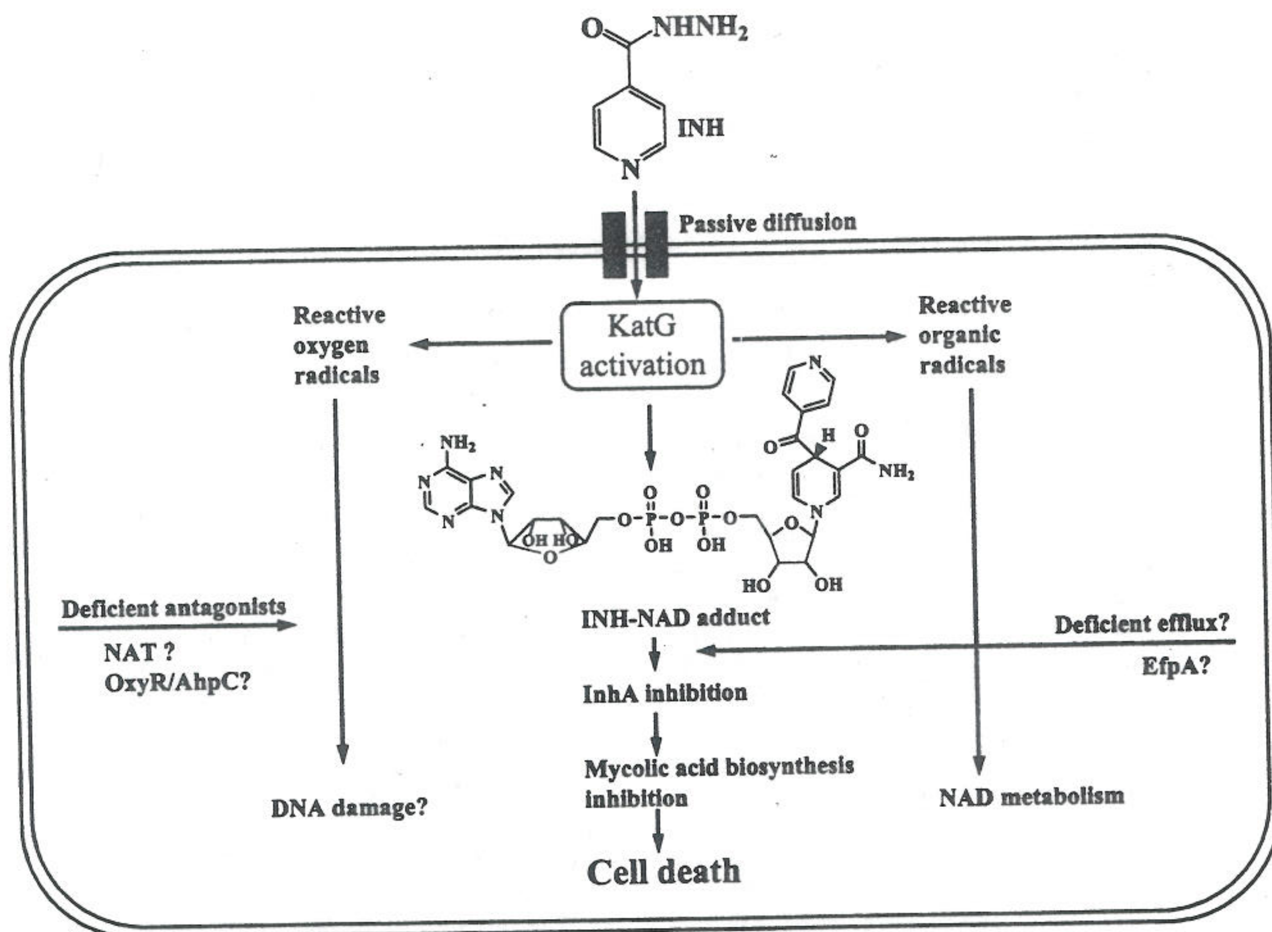


Figure 2. Mode of action of INH. INH enters tubercle bacilli by passive diffusion and is activated by KatG to a range of reactive species. These reactive species or radicals, which include both reactive oxygen species (hydrogen peroxide, superoxide, peroxynitrite, and hydroxyl radical) and organic radicals attack multiple targets, e.g., mycolic acid synthesis, DNA damage, and NAD metabolism in the cell. The isonicotinoyl acyl radical reacts with NAD^+ to form an INH-NAD adduct, which in-

INH resistance or just an accompanying event. Winder hypothesized that the loss of catalase-peroxidase activity may indicate that the enzyme was an activator for INH (227). Later, Middlebrook's observation led to the cloning of the catalase-peroxidase gene (*katG*) and identification of *katG* mutations as the cause of INH resistance in *M. tuberculosis* by gene transfer (234, 235). Analysis of three INH-resistant strains in the original study (235) showed that *katG* deletions were found in two highly resistant strains. Subsequent studies have shown that *katG* point mutations are more frequent than deletions in INH-resistant strains (85). Restoration of INH susceptibility to KatG-defective, INH-resistant strains (with either *katG* deletion or point mutations) by transformation with a functional *katG* gene demonstrated that *katG* mutations caused INH resistance (234). Loss of KatG function due to mutations in *katG* reduces the ability of KatG to activate the prodrug INH, thus leading to INH resistance, and this represents a novel mechanism of drug resistance. Between 20 and 80% of INH-resistant *M. tuberculosis* strains contain a mutation in the *katG* gene, depending on the geographical region of the clinical isolates (1, 4, 69, 85, 86, 98, 138, 143, 144, 147, 158, 168, 169, 171, 203).

Among the various mutations in KatG, the S315T mutation is the most common, occurring in about 50 to 93% of INH-resistant clinical isolates carrying a *katG* mutation (1, 4, 11, 65, 68, 86, 113, 138, 158, 168, 171, 182). The KatG S315T mutation reduces the catalase and peroxidase activity by 50% (174, 218, 231) and is associated with relatively high levels of INH resistance (MIC, 5 to 10 μg/ml) (209). The S315T mutation affects the binding of INH to KatG, resulting in less activated INH (107, 217, 231). The KatG R463L substitution is found in *M. bovis, M. africanum*, and *M. microti* (54), but this enzyme is as capable at activating INH as is the wild-type KatG (82) and is generally considered to not contribute to INH resistance (85). The *katG* gene forms an operon with the upstream gene *furA* in various mycobacteria including *M. tuberculosis* (44, 153). FurA is a negative regulator of *katG*, and its removal causes overexpression of KatG and hypersensitivity to INH whereas overexpression of FurA reduces KatG expression (232). Mutations in *furA* may cause INH resistance through repression of KatG expression, but this has not been observed in INH-resistant strains. The *katG* gene is situated in a highly variable region of the genome (241) containing repetitive DNA, and this, in turn, may contribute to the high frequency of *katG* mutations in INH-resistant strains.

Overexpression or alterations in the INH target, InhA. When *inhA* was overexpressed from a multicopy plasmid, the MIC of INH increased by 20- to 80-fold above the MICs of three independent *M. tuberculosis* strains (95). Mutations in INH-resistant clinical isolates of *M. tuberculosis* have been mapped to the promoter region and the *inhA* structural gene. These mutations occur in 15 to 43% of the INH-resistant *M. tuberculosis* strains and are usually associated with a low level of INH resistance (MIC, ≤1 μg/ml) (12, 14, 69, 85, 86, 98, 143–145, 158, 168, 171). A recent study, analyzing ETH-resistant *M. tuberculosis* isolates, revealed that 14 of 41 strains had promoter mutations in *inhA*, with no mutations in *katG* or *ethA* (142). All of these strains were coresistant to INH. These data are consistent with the premise that overexpression of *inhA* mediates coresistance to INH and ETH and that *inhA* encodes the target of INH and ETH.

In addition to overexpression of the InhA target, the same missense mutation, S94A, was found in the structural gene of *inhA* for an INH-resistant mutant of *M. smegmatis* and *M. bovis* (12). Allelic exchanges were performed with a kanamycin resistance gene linked to the *inhA* gene to prove that the S94A mutation was sufficient to mediate coresistance to INH and ETH. Interestingly, another recent study identified three clinical isolates of *M. tuberculosis* that possess the S94A mutation and all are coresistant to INH and ETH (142). In addition to this mutation, four independent I21T or I21V mutations in InhA were found, all of which confer coresistance to INH and ETH. Numerous additional studies have identified structural mutations in *inhA* as well (85, 86, 144, 168, 171, 182). Resistance to INH and ETH can be mediated by mutations that cause overexpression of *inhA* or cause alterations in the InhA protein.

Loss of NADH dehydrogenase II activity: a modulator of INH-NAD and ETH-NAD formation or binding. The NADH dehydrogenase type II gene, *ndh*, was shown to be involved in INH resistance by complementation of an INH-resistant, temperature-sensitive *M. smegmatis* mutant with a genomic library of *M. tuberculosis* (130). *ndh* mutations conferred coresistance to INH and ETH in *M. smegmatis* by lowering the rate of NADH oxidation. In a recent study, *ndh* mutations were detected in 8 (9.5%) of 84 INH-resistant *M. tuberculosis* clinical isolates, 7 of which had the same R268H mutation and 1 of which had the T110A mutation (98). The eight *M. tuberculosis* strains with *ndh* mutations were resistant to at least 0.1 μg of INH per ml, but the exact level of resistance was not reported. A more comprehensive study has been recently completed, demonstrating the analysis of 26 new *M. smegmatis* and *M. bovis* BCG *ndh* mutants. The mutations map over the entire

length of the NdhII protein, and all result in diminished NdhII activity (C. Vilchèze, T. R. Weisbrod, B. Chen, L. Kremer, M. H. Hazbón, F. Wang, D. Alland, J. C. Sacchettini, and W. R. Jacobs, Jr., submitted for publication). Moreover, the measurement of NADH/NAD^+ ratios demonstrates a striking increase between the mutants and the parental strains. A recent study showed that an increased amount of NADH can protect InhA from the inhibition by the INH-NAD adduct (149) by either competing with the INH-NAD adduct for binding to the active site of InhA or promoting displacement of the INH-NAD adduct from InhA. Since all of the *ndh* strains are coresistant to INH and ETH, the data support the hypothesis that altered NADH/NAD^+ ratios would mediate resistance by competitively inhibiting the binding of the INH-NAD adduct. By analogy, the coresistance to both INH and ETH suggests the existence of an ETH-NAD inhibitor for InhA, although this remains to be demonstrated experimentally.

Another gene that also complemented the temperature sensitivity and INH resistance phenotype in *M. smegmatis ndh* mutants was *mdh*, which encodes a malate dehydrogenase (130). Mdh catalyzes the NADH-dependent interconversion of oxaloacetate and malate in the tricarboxylic acid cycle. Although *M. smegmatis* does not possess the *mdh* gene, it was postulated that Mdh allows the restoration of the Ndh enzymatic activity by combining with the *M. smegmatis* Mqo (malate:quinone oxidoreductase) enzyme (140). However, mutations in *mdh* have not yet been found in INH-resistant *M. tuberculosis* isolates.

Alterations and overexpression of KasA. Mdluli et al. first reported mutations in *kasA* in INH-resistant clinical isolates of *M. tuberculosis* but did not perform any gene transfer experiment to prove that these mutations confer the INH resistance phenotype (123). Subsequent studies have revealed that three of the four mutations in *kasA* reported by Mdluli et al. were found in INH-resistant as well as in INH-susceptible isolates (97, 168), suggesting that these mutations do not play any role in conferring INH resistance.

Slayden and Barry have reported that overexpression of *kasA* conferred low-level resistance to INH in *M. tuberculosis* (185). In addition, these studies showed that overexpressed *kasA* confers resistance to thiolactomycin, a drug which has been shown to inhibit both KasA and KasB in *M. bovis* BCG (89). In another study by Kremer et al., it was shown that while the overexpression of *kasA* did confer resistance to thiolactomycin, it did not increase resistance to INH in BCG (89). To address these discrepancies, the various *kasA* and *inhA* overexpression plasmids (89, BCG, and three different strains of *M. tuberculosis* and analyzed by four independent laboratories (95). The results consistently revealed that overexpression of *kasA* conferred no increased resistance to INH or ETH whereas overexpressed *InhA* increased resistance to INH and ETH by 20- to 80-fold (97). Further biochemical and structural studies are needed to elucidate the role of KasA in INH action and resistance.

Other genes potentially involved in INH resistance. Although most INH-resistant strains may be accounted for by mutations in the above genes, some catalase-positive low-level INH-resistant clinical isolates do not have mutations in *katG, inhA, ndh*, or *kasA* (86, 98, 158, 168, 203), indicating that additional, unknown genes may be involved in INH resistance. The *mdh* gene (130) and the INH-inducible genes with unknown functions identified in a microarray analysis (224) could be candidates. In fact, some of the genes identified by the array analysis have recently been shown to contain mutations in some INH-resistant clinical isolates (168), although the genetic transfer experiments that confirm their role in INH resistance remain to be performed. One candidate gene is the efflux protein EfpA, which was shown to be induced by INH (224). Although no mutation resulting in increased EfpA expression has yet been found to cause INH resistance, one recent study using sequential exposure of an INH-sensitive strain to increasing levels of INH seems to show induction of transient high-level resistance to INH through induction of a reserpine-sensitive efflux mechanism (212). In addition, the arylamine *N*-acetyltransferase (NAT) enzyme, which is present in humans as two isoforms, NAT1 and NAT2, and can acetylate arylamines and hydrazines and inactivate INH, could be a candidate. NAT homologs occur in *M. tuberculosis* and *M. smegmatis*, and the purified enzyme converts INH to *N*-acetyl-INH in vitro (155, 156, 207). Overexpression of *nat* from *M. tuberculosis* in *M. smegmatis* caused a three-fold increase in resistance to INH (155). An *M. smegmatis nat* knockout mutant had a slightly increased susceptibility to INH (156). Eighteen percent of clinical isolates of *M. tuberculosis* contained a single point mutation (G207R) in the *nat* gene (207). The NAT G207R mutation appeared to correlate with a very slight decrease in INH susceptibility, at 0.02 μg/ml in two strains that harbor the mutation compared with 0.005 μg/ml in the sensitive control strain H37Rv (207). It will be of interest to determine the level of *nat* gene expression and the intrinsic enzymatic activity of the NAT protein in *M. tuberculosis* in comparison with other less susceptible mycobacterial species, as well as to investigate

moter up-mutations might be involved in INH resistance in *M. tuberculosis*.

Rifampin

Mechanism of action

RIF is a broad-spectrum rifamycin derivative that interferes with RNA synthesis by binding to bacterial RNA polymerase, an oligomer consisting of a core enzyme, formed by four chains ($\alpha_2\beta\beta'$), that associates with the σ subunit to specifically initiate transcription from promoters. There is evidence positioning the RIF binding site shortly upstream of the catalytic center (146), in keeping with the model that RIF plugs the product exit channel (116). Analysis of RIF-resistant mutants has contributed to a more precise definition of a priming nucleotide site overlapping with a larger site holding the RNA product in the active center during elongation. RIF is active against growing tubercle bacilli and also stationary-phase bacilli with reduced metabolism. The activity against the latter bacterial population is thought to be important in shortening the duration of treatment (134).

Mechanism of resistance

In *M. tuberculosis*, resistance to RIF occurs at a frequency of 1 in 10^7 to 10^8 bacilli. As in other bacteria, mutations in a defined region of *rpoB* have been found in >95% of RIF-resistant clinical isolates of *M. tuberculosis* (204). Resistance to RIF in *M. leprae* follows the same mechanism (74). Although *rpoB* mutations have been found in RIF-resistant *M. avium* (222), many isolates from the *M. avium-intracellulare* complex present a significant level of natural resistance to RIF, probably due to decreased permeability (61, 79). The role of *rpoB* mutations in causing resistance has been confirmed by genetic transformation experiments (132, 221). Mutation in *rpoB* generally results in high-level resistance (MIC, >32 μg/ml) and cross-resistance to all rifamycins. However, specific mutations in codons 511, 516, 518, and 522 are associated with lower-level resistance to RIF and rifapentin but retained susceptibility to rifabutin and the new rifamycin KRM1648 (22, 137, 221). Low-level resistance (MIC, 4 μg/ml) has been associated with an L176F mutation in a separate region of RpoB (R. Rossau, W. Mijs, G. Jannes, K. de Smet, D. van Heuverswijn, H. Traore, and F. Portaels, *Abstr. 20th Annu. Conf. Eur. Soc. Mycobacteriol.*, abstr. OC27, 1999), corresponding to *E. coli* L146F, a codon mutated in 2% of RIF-resistant *E. coli* strains (81, 183). Ribosylation, a degradative mechanism of resistance to RIF, has been found in the rapidly growing mycobacteria such as *M. smegmatis, M. chelonae, M. flavescens*, and *M. vaccae* (40, 163), but not in *M. tuberculosis*.

Pyrazinamide

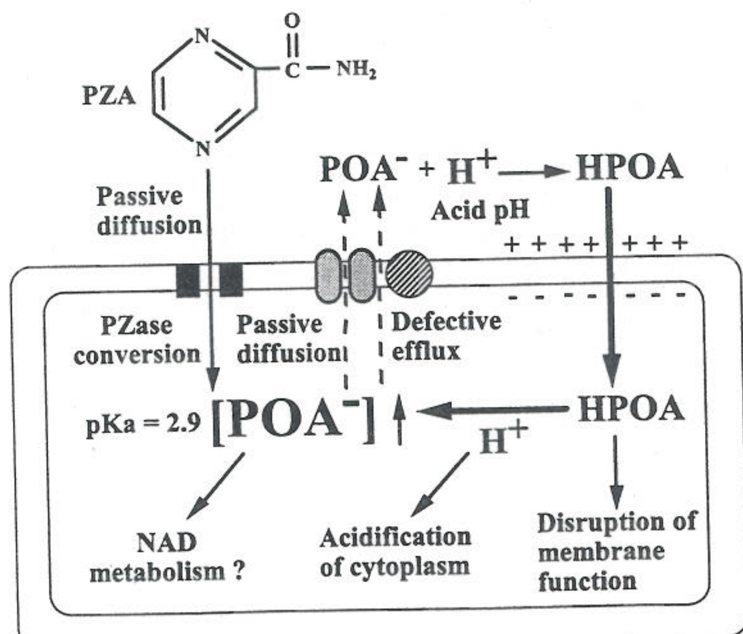
PZA is an unconventional and paradoxical TB drug that has high in vivo activity but poor in vitro activity (236). The discovery of PZA followed independent observations made by Chorine and Huant, who found that nicotinamide had activity against mycobacteria in vivo in totally different settings (35, 78). This led to the subsequent discovery of not only PZA (108) but also INH (52). The nicotinamide activity on *M. tuberculosis* was rediscovered by McKenzie et al. at Lederle Laboratories (120), where analogs of nicotinamide were synthesized, and at Merck. PZA was found to be the most active agent (92, 108, 186). What is remarkable is that the screening was performed directly on infected mice without in vitro susceptibility testing. Luckily, with hindsight, it is now appreciated that if the screening had been performed first under normal culture conditions in vitro, PZA would never have been discovered. The discovery of PZA is a telling story that the current drug screening using growing bacteria under normal culture conditions has significant limitations. Not only was the discovery of PZA unconventional, but also its mode of action is unusual and has puzzled investigators for decades.

Despite its powerful in vivo sterilizing activity, as demonstrated by its ability to shorten chemotherapy both in animal models and in humans (13, 192), PZA has no activity in vitro under normal culture conditions (202), but is active only at acidic pH in vitro (119). Even then, PZA has a high MIC (50 to 100 μg/ml) at pH 5.5 to 6.0, and unlike other TB drugs (e.g., INH and RIF), PZA kills *M. tuberculosis* cells only slowly and incompletely over a period of 2 weeks (66). Unlike conventional antibiotics, which are active mainly against growing bacteria, PZA kills nongrowing tubercle bacilli more effectively than metabolically active growing bacilli (237). PZA can show bactericidal or bacteriostatic activity depending on the metabolic status of the tubercle bacilli, pH, and drug concentration (236). In vivo, PZA has an achievable peak concentration in serum of 30 μg/ml, which is lower than its MIC. The high in vivo activity but poor in vitro activity of PZA presumably reflects differences between these environments. Indeed, the discovery of the acid-pH requirement for PZA activity by McDermott and Tompsett in 1954 was based on this reasoning (119). We have recently found that the increased local iron concentrations during active inflammation and the hypoxic conditions present in granulomatous lesions enhance the activity of PZA (187, 212a), providing an additional explanation for the high in vivo

activity and poor in vitro activity of PZA. The ability of PZA to shorten therapy is thought to be related to its bactericidal activity on a population of semidormant bacilli residing in an acidic environment (e.g., during inflammation) that are not effectively killed by other drugs (134). Besides acidic pH, various other factors such as metabolic status, age of the bacilli, albumin, inoculum size, and energy inhibitors (240) affect PZA activity (236, 237).

Mechanism of action

PZA is a prodrug that is converted to the active form, pyrazinoic acid (POA), by bacterial pyrazinamidase (PZase)/nicotinamidase (88, 181). Based on our recent studies (181, 238, 240, 242), we proposed the following model for the mode of action of PZA (Fig. 3): PZA enters the bacilli through passive diffusion and is converted into POA (a strong weak acid with pK_a of 2.9) by the cytoplasmic PZase. However, it is worth noting that PZA uptake and conversion to POA in *M. tuberculosis* are much slower than in the naturally resistant nontuberculous mycobacteria or *E. coli*, presumably due to higher PZase activity in other bacteria (238). POA is then excreted from the cell by passive diffusion and a weak efflux mechanism in *M. tuberculosis* (238). Once POA is outside the cell, if the extracellular pH is acidic, a small proportion of POA becomes the uncharged conjugate acid HPOA, which permeates through the membrane easily. However, acidic pH does not facilitate the uptake of PZA. The acid-facilitated POA influx is stronger than the weak POA efflux, and this causes accumulation of POA in *M. tuberculosis* cells. The HPOA brings protons into the cell, and this could eventually cause cytoplasmic acidification, such that vital enzymes are inhibited, as a potential mechanism of POA action. In addition, HPOA could potentially deenergize the membrane by collapsing the proton motive force and affect membrane transport as a possible mechanism of POA action (239). At neutral or alkaline pH, little POA was found in the tubercle bacilli, because over 99.9% of POA was in the charged anionic form. This observation explains why PZA is active at acidic pH but not at neutral pH (202) and also explains the correlation between the MIC of PZA and low acid pH (175, 237). While the process of acid-facilitated uptake of a weak acid is nonspecific, the unique activity of PZA against *M. tuberculosis* appears to be related to a deficient POA efflux mechanism (238) that is unable to counteract the effect of acid-facilitated POA influx, which could lead to eventual acidification of the cytoplasm, deenergized membranes, and a generally poor ability to maintain membrane energetics, especially in nongrowing tubercle bacilli at acid pH. Indeed, we have recently obtained the following data that further support the above hypothesis (240, 242): (i) acidic pH decreases the membrane potential, and this is reduced further by POA; (ii) POA inhibits the transport of nutrients such as serine, methionine, and uracil in *M. tuberculosis* at acidic pH through disruption of the membrane potential; (iii) the activity of PZA or POA is significantly enhanced by energy in-



hibitors such as DCCD (F1F0-ATPase inhibitor), rotenone (NADH dehydrogenase I-Complex I inhibitor), and azide (cytochrome *c* oxidase inhibitor) (240); (iv) PZA activity is significantly higher under hypoxic or anaerobic conditions than under atmospheric conditions with ambient oxygen (212a) (the preferential activity of PZA against tubercle bacilli under hypoxic conditions may result from low energy production); and (v) tubercle bacilli have a generally weak-acid-susceptible phenotype (240). Thus, the currently available data suggest that POA/PZA targets the membrane and affects membrane bioenergetics as a mechanism of action. In addition, this model explains best the various peculiar features of PZA, i.e., the requirement of acidic pH for activity, the preferential activity of PZA against old, nongrowing bacilli, and its higher activity under hypoxic conditions.

The target of PZA or POA has been suggested to be FASI in a study showing that PZA inhibits fatty acid biosynthesis in *M. tuberculosis* and from the finding that the multicopy *fas1* gene from *M. avium, M. bovis* BCG, or *M. tuberculosis* confers resistance to the PZA analog 5-chloropyrazinamide (5-Cl-PZA) in *M. smegmatis* (243). However, no mutations in FASI have been found in PZA-resistant *M. tuberculosis* strains. The proposition that FASI is the target of PZA in *M. tuberculosis* has recently been questioned. Boshoff et al. showed that while FASI is the target of 5-Cl-PZA, it is not the target of PZA (24). The available data do not support the presence of a specific cellular target for POA (239), although this possibility cannot be excluded. First, no POA-resistant mutants can be isolated (180). Furthermore, among clinical isolates of *M. tuberculosis* resistant to PZA, none are resistant to POA (Y. Zhang, unpublished data). However, it is unclear whether the inability to obtain a POA-resistant mutant is because the mutated target influences viability or because POA affects multiple cellular targets such that no specific mutant can be selected. Second, POA does not appear to bind to any cellular components in *M. tuberculosis* (Zhang, unpublished). Third, if there was a specific cellular target for POA, which is produced from PZA in the bacilli at both acidic pH and neutral pH (238), POA would have bound and shown an inhibitory effect even at neutral pH, which is not the case. The possibility remains that POA, as an analog of nicotinic acid, might be incorporated into NAD and affect NAD function, but preliminary results do not support this hypothesis (Zhang, unpublished).

Mechanism of resistance

M. tuberculosis strains are uniquely susceptible to PZA, which has an MIC of about 50 μg/ml at pH 5.5, whereas nontuberculous mycobacteria and other bacteria are intrinsically resistant to PZA; e.g., the MIC for *M. smegmatis* and *E. coli* is >2,000 μg/ml (23, 239). In *M. tuberculosis*, PZA susceptibility correlates with the presence of PZase activity. Like INH-resistant *M. tuberculosis* strains that lose catalase-peroxidase activity (128), *M. tuberculosis* strains lose PZase/nicotinamidase activity when they develop PZA resistance (88), and there is a good correlation between loss of PZase activity and PZA resistance (115, 133, 206). Defective PZase activity resulting from *pncA* mutations is the major mechanism for PZA resistance in *M. tuberculosis* (180), as confirmed by numerous other studies (71, 72, 77, 99, 102, 112, 127, 141, 154, 173, 189).

The *pncA* mutations identified are largely missense mutations causing amino acid substitutions and in some cases nucleotide insertions or deletions, nonsense mutations in the *pncA* structural gene, or nonsense mutations in the putative promoter region. A frequently occurring mutation at −11 (putative promoter region) was found in several studies (34, 99, 127, 154, 180, 189). The *pncA* mutations are highly diverse and are scattered along the gene with some degree of clustering that affects three regions (regions 3 to 17, 61 to 85, and 132 to 142) of the PncA protein (102, 180) that are likely to contain catalytic sites. The crystal structure of the *Pyrococcus horikoshii* PncA (37% identity to *M. tuberculosis* PncA) has provided some structural basis for understanding the *pncA* mutations in *M. tuberculosis* that cause PZA resistance (46), since the three regions where they cluster correspond to three of the four loops that contribute to the scaffold of the active site. Mutations at C138, D8, K96, D49, H51, and H71 modify the active-site triad and metal binding site. Residues F13, L19, H57 (position of the characteristic mutation of H57D in *M. bovis*), W68, G97, Y103, I113, A134, and H137 line the active site, and mutations at these positions are also predicted to cause loss of enzyme activity. Mutations at Q10, D12, S104, and T142 are predicted to disrupt the hydrogen-bonding interactions between the side-chain and main-chain atoms. Loss of PZase activity due to mutations at other sites can be attributed to potential perturbation of the active site or disruption of the protein core (46). These predictions need to be confirmed when the structure of the *M. tuberculosis* PncA is determined. The diverse nature of *pncA* mutations is unique to the PZA resis-tance, and other drug resistance genes usually do not show this degree of diversity. Although the basis for this is unclear, it is likely that since *pncA* is not an essential gene in *M. tuberculosis* (34), there is no selective pressure such that mutations can be tolerated anywhere in *pncA*.

Although most PZA-resistant *M. tuberculosis* strains have mutations in *pncA* (34, 180), there are

some resistant strains that do not, and these include PZase-negative strains (34, 102, 112) with a high level of resistance. This indicates that mutations in an undefined *pncA* regulatory gene may be involved in PZA resistance. Another type of such strain has low-level resistance and positive PZase activity, presumably due to an alternative mechanism of resistance.

While acquired PZA resistance in susceptible *M. tuberculosis* is due to *pncA* mutations, the natural PZA resistance in other mycobacteria or bacteria is not due to *pncA* mutations, with the exception of *M. bovis*, a member of the *M. tuberculosis* complex. Strains of *M. bovis* including BCG are known to be naturally resistant to PZA and lack PZase, and these features are commonly used to distinguish *M. bovis* from *M. tuberculosis*. The natural PZA resistance in *M. bovis* and BCG is due to a single point mutation of C to G at nucleotide position 169 of the *pncA* gene, causing amino acid substitution H57D (181). This single point mutation is the cause of the defective PZase activity and natural resistance to PZA in *M. bovis* and BCG and can be a useful marker for rapid differentiation of *M. bovis* from *M. tuberculosis* (179). Thus, *M. bovis* can be considered a special case of PZA-resistant *M. tuberculosis*. In a similar manner, as a result of PZase activity being affected, *M. kansasii*, which is naturally resistant to PZA (MIC, 250 μg/ml), also has very weak PZase activity with positive nicotinamidase activity (67). The natural PZA resistance in *M. kansasii* is due to reduced PZase activity, as shown by transformation studies with the *pncA* gene from *M. tuberculosis* or *M. avium* (193). However, the correlation between PZase activity and PZA susceptibility is not true for other naturally PZA-resistant mycobacterial species. For example, we have found that the natural PZA resistance in *M. smegmatis* is not due to defective PZase activity. On the contrary, *M. smegmatis* has two highly active PZase enzymes, PzaA (23) and PncA (63), and yet, it is highly resistant to PZA (MIC, >2,000 μg/ml) at acid pH (5.5) due to a highly active POA efflux mechanism. This efflux mechanism is also likely to be the cause of the intrinsic PZA resistance in many other bacterial species such as *M. avium*, *M. vaccae*, and *E. coli* (193, 238). In contrast, *M. tuberculosis* has a weak or deficient POA efflux mechanism as an underlying mechanism of its unique susceptibility to PZA and POA (238).

Ethambutol

Mechanism of action

EMB [(*S,S'*)-2,2'-(ethylenediimino)-di-1-butanol] inhibits the biosynthesis of arabinogalactan, the ma- (195). EMB interferes with the polymerization of cell wall arabinan of arabinogalactan and of lipoarabinomannan (131) and induces the accumulation of β-D-arabinofuranosyl-*P*-decaprenol, an intermediate in arabinan biosynthesis (100, 229). Arabinosyltransferase, an enzyme involved in the synthesis of arabinogalactan, has been proposed as the target of EMB (17). The enzyme is encoded by *embB*, which is part of an operon comprising the *embA* and *embB* genes in *M. avium* (17) and the *embC*, *embA*, and *embB* genes in other mycobacteria such as *M. smegmatis* (7, 205). The Emb proteins are about 65% identical to each other and are predicted to be integral membrane proteins with 12 transmembrane-spanning domains (205). However, the precise mechanism of how EMB inhibits EmbB is not known.

Mechanism of resistance

Mutations in the *embCAB* operon were identified in up to 65% of EMB-resistant clinical isolates of *M. tuberculosis*. Mutations at codon 306 of *embB* occur most frequently (167, 190, 205), but mutations at amino acid residues Asp328, Gly406, and Glu497 are also found (167, 190). The codon 306 region is highly conserved among the various Emb proteins and among different mycobacteria (7, 103, 205), although the EMB-resistant *M. leprae*, *M. chelonae*, and *M. abscessus* display variant amino acids at this position. Genetic transfer experiments involving these *emb* alleles supported the role of this region in determining natural resistance to EMB (7). However, a recent study from Russia has found that EmbB306 mutations were detected not only in 48.3% of EMB-resistant strains but also in 31.2% of EMB-susceptible strains (139), raising some doubts about the significance of *embB* mutations in EMB resistance. Additional mutations in the *embC-embA* intergenic region have been found in strains that also had resistance-associated amino acid substitutions in EmbA or EmbB (167), and these may play a secondary or compensatory role in resistance. An *embR* homologue, which is located 2 Mb from the *embCAB* locus in *M. tuberculosis* rather than immediately upstream of the *embAB* genes in *M. avium*, has recently been shown to contain mutations (a Q379R replacement and an A insertion at position 137 upstream of the *embR* start codon) associated with EMB resistance in *M. tuberculosis* (167). A mutation 24 bp upstream of the start codon of the *Rv0340* gene, which precedes the INH and EMB-inducible *iniBAC* operon (8), was associated with EMB resistance (167). Mutations in *rmlD* (S257P and T284L, or a G-to-T nucleotide change at position 71) and *rmlA2* (D152N), both of

were found to be associated with EMB resistance (167). Despite more new genes being identified as involved in EMB resistance, about 24% of EMB-resistant *M. tuberculosis* strains do not have mutations in any of the genes described above (167). Further genetic and biochemical studies are needed to confirm the role of the above genes in EMB resistance.

Streptomycin

Mechanism of action

SM is an aminoglycoside antibiotic that primarily interferes with protein synthesis but has some other effects such as damage to the cell membrane (9), inhibition of respiration, and stimulation of RNA synthesis (55). In addition, SM can cause misreading or miscoding of the genetic code (41). The site of action of SM is the 30S subunit of the ribosome, specifically at ribosomal protein S12 and the 16S rRNA (57). In *E. coli*, SM binds the bases between positions 903 and 910 of 16S rRNA and interferes with protein S12 in the translation process (136). The knowledge of how SM kills bacteria is derived largely from numerous studies carried out primarily with *E. coli*. The mode of action of SM in *M. tuberculosis* is presumed to be the same as in *E. coli*, as shown by the presence of mutations in the same target, i.e., ribosomal S12 protein (encoded by *rpsL*) and 16S rRNA (encoded by *rrs*).

Mechanism of resistance

M. tuberculosis becomes resistant by mutating the target of SM in the ribosome. The principal site of mutation is the *rpsL* gene, encoding ribosomal protein S12 (51, 75, 84, 148, 188). As in *E. coli*, residues 42 and 88 are the most important in the development of SM resistance. In mycobacteria, rates of selection for potentially nonrestrictive and restrictive mutations may vary from in vitro to in vivo circumstances. While restrictive mutations are selected in vitro at rates similar to that for the nonrestrictive K42R, they are rarely observed in vivo (26). This suggests that such mutants are under a strong negative pressure and that compensatory mutations fail to retain those mutants in the global pool. A second mechanism of resistance in *M. tuberculosis* is in *rrs*, the gene encoding 16S rRNA. While most bacteria have multiple copies of *rrs*, *M. tuberculosis* and other slow-growing mycobacteria have a single copy whereas rapidly growing mycobacteria have two copies (25). Mutation in the loops of 16S rRNA, the highly conserved 530 loop and on the adjacent 915 region (51), that interact with the S12 protein constitute an easily selected resistance site. An SM-dependent mutant of *M. tuberculosis* contained an insertion of cytosine in the 530 loop as a likely cause of its SM dependence (76). A previously described nucleotide change at position 491 of the *rrs* gene, in two clinical isolates resistant to SM (125, 188), is a polymorphism that is not associated with SM resistance (210).

Mutations in *rpsL* and *rrs* structures are identified in 50 and 20% of SM-resistant clinical isolates, respectively, and result in intermediate (MICs, 64 to 512 μg/ml) or high-level (MICs, >1,000 μg/ml) resistance. A third mechanism accounting for low-level resistance (MICs, 4 to 32 μg/ml) remains unidentified, but it may involve changes in drug uptake (38). Genes for aminoglycoside-modifying enzymes are present in the chromosome of *M. tuberculosis* and other mycobacteria (5, 37), but their role in resistance is not clear. The expression of the aminoglycoside 2′-*N*-acetyltransferase *aac(2′)-Ic* and *aac(2′)-Id* genes in *M. smegmatis* has been studied, and only *aac(2′)-Id* is correlated with aminoglycoside resistance (5).

Resistance to Other Drugs

Studies with other bacteria (50) have shown the presence of quinolone resistance mutations in (i) the DNA gyrase (composed of subunits GyrA and GyrB), (ii) topoisomerase IV, and (iii) the cell membrane proteins that regulate the intracellular concentration of the drug by mediating drug permeability and efflux. Stepwise accumulation of mutations in several of these genes can result in high levels of resistance. In *M. tuberculosis*, *gyrA* mutations cause resistance to ciprofloxacin (200) and cross-resistance to other fluoroquinolones (e.g., ofloxacin) (32). Amino acid sequences in GyrA may determine the level of susceptibility to quinolones (62). Although recognized as contributing to resistance in *E. coli*, mutations in *gyrB* have been identified only in laboratory mutants of *M. tuberculosis* (87). Interestingly, the genome of *M. tuberculosis* does not contain a definitive topoisomerase IV homologue (ParC or GrlA) (37), which causes quinolone resistance when mutated in other bacteria. Although the efflux pump, LfrA, confers low-level quinolone resistance in *M. smegmatis* (106, 199), such a mechanism has not been demonstrated to cause quinolone resistance in *M. tuberculosis*.

Kanamycin and capreomycin, like SM, inhibit protein synthesis through modification of ribosomal structures at the 16S rRNA. Mutations at *rrs* position 1400 are associated with high-level resistance to kanamycin and amikacin (6, 194, 201). Cross-resistance may be observed between kanamycin and capreomycin or viomycin (6, 194, 201).

The mechanism of resistance to PAS is unknown. Interference with folic acid biosynthesis and inhibition of iron uptake have been proposed as two possible mechanisms of action for PAS (227).

ETH is also a prodrug and inhibits mycolic acid biosynthesis. Mutations in *inhA* confer cross-resistance to INH and ETH (12, 142). Two independent studies identified a gene, *etaA* (42), also called *ethA* (15), which encodes an enzyme involved in activation of ETH. EtaA is an FAD-containing enzyme that oxidizes ETH to the corresponding S oxide, which is further oxidized to 2-ethyl-4-amidopyridine, presumably via the unstable oxidized sulfinic acid intermediate (208). This flavoenzyme also oxidizes thiacetazone, thiobenzamide, isothionicotinamide, and probably other thioamide drugs (208). Mutations in EtaA were found in all MDR-TB strains examined, and these strains displayed cross-resistance to thiocarbonyl-containing drugs including thiacetazone and thiocarlide (42). A more recent study of ETH-resistant *M. tuberculosis* clinical isolates showed that 95% of the strains were also INH resistant and that the ETH-resistant and INH-resistant phenotypes in 51% of the strains were due solely to mutations in the *inhA* gene and/or its promoter region whereas mutations in *ethA* were found in 37% of the strains (142).

Cycloserine inhibits the synthesis of peptidoglycan by blocking the action of D-alanine racemase and D-alanine:alanine synthase. The D-alanine racemase enzyme, encoded by *alrA*, has been cloned from *M. smegmatis*, and its expression from a multicopy vector in *M. smegmatis* or *M. bovis* BCG results in resistance to D-cycloserine (31). Inactivation of *alrA* in *M. smegmatis* caused increased sensitivity to D-cycloserine (33). In a recent study, mutation in an open reading frame with homology to the *E. coli* penicillin binding protein 4 (PBP4) gene was found to cause D-cycloserine resistance in *M. smegmatis* (157). However, the genetic basis of cycloserine resistance in *M. tuberculosis* remains to be identified.

FITNESS AND VIRULENCE OF DRUG-RESISTANT ORGANISMS

Different fitness phenotypes of *M. tuberculosis*, as manifested in differences in replication kinetics, mutation rate, and infectivity, may translate into differences in virulence in vivo. The issue of diminished fitness in the presence of resistance mutations needs to be examined carefully because of the different degree of fitness or virulence even among drug-susceptible strains. Use of isogenic strains is essential for comparing the relative fitness of drug-resistant organisms in experimental-animal models of infection. However, this issue is even more complex in human infections, due to differences in both bacterial and host factors. The W-Beijing family strains are associated predominantly with the MDR phenotype, which prompted speculation that these strains may have a higher frequency of mutations (10, 91). Mutations were found in the putative mutator genes (*mutT2* and *Rv3908*) in some W-Beijing families of *M. tuberculosis* strains, which could potentially cause increased mutation frequency and better adaptability to stress conditions, leading to increased spread of such strains (166). However, in a separate study, drug-susceptible W-Beijing strains of *M. tuberculosis* were no more prone to develop RIF resistance than were non-W-Beijing strains (219). It would be of interest to determine if the mutator mutations contribute to a more fit in vivo phenotype and higher frequency of developing drug resistance in W-Beijing family strains. Future studies using isogenic mutator strains and genetic complementation are required to address these issues.

Resistance to INH is probably the only case where acquisition of drug resistance has been shown to affect the virulence of the bacilli. It is well known that INH-resistant strains often lose not only catalase activity but also virulence for guinea pigs (128). There is generally a good correlation between loss of catalase activity and attenuation of virulence in guinea pigs and the level of INH resistance (126, 227). Attenuation of virulence in INH-resistant, catalase-deficient strains is less easily demonstrated in the mouse model. With the identification of *katG*, encoding catalase-peroxidase (235), it was shown that reintroducing the *katG* gene restored not only INH susceptibility but also virulence for INH-resistant, catalase-deficient strains in a guinea pig model (105, 226). If a particular mutation in *katG* eliminates catalase-peroxidase activity, the mutation is expected to attenuate virulence. In contrast, if some enzyme activity is retained, it would still be virulent to some degree. For instance, an INH-resistant strain containing the frequently occurring S315T KatG mutation still produced active catalase-peroxidase and was virulent in the mouse model (162), whereas the P275T KatG mutation, which completely eliminates catalase-peroxidase activity, was not (162). In a recent study in Holland, INH-resistant strains with the S315T KatG mutation were found to cause secondary TB cases as often as INH-susceptible organisms did (209). The mechanism of INH resistance could influence the virulence of the organisms. INH-resistant strains with mutations in genes other than *katG*, such as *inhA* or *ndh*, are expected still to be

strains of *M. bovis,* loss of virulence for guinea pigs was associated with loss of KatG activity but not with mutations in *inhA* (226). While mutation in the promoter region of *ahpC,* leading to overexpression of AhpC, is important for restoring peroxide homeostasis in the KatG-deficient organism, it does not appear to contribute to increased virulence in mice (70). However, blocking the expression of AhpC by using the antisense-RNA strategy appeared to attenuate the virulence of *M. bovis* in the guinea pig model (225). This discrepancy could be due to the different animal models used.

Resistance to other TB drugs is usually not associated with loss of virulence for *M. tuberculosis* isolates. We have found active transmission of TB due to PZase-negative, PZA-monoresistant strains exhibiting the same characteristic *pncA* mutation (an 8-bp deletion and an R140S mutation) and almost identical IS*6110* profile in Quebec, Canada (34). Mutations in RpoB could modify the enzymatic activity and the fitness of *M. tuberculosis* in vitro (20). Competitive coculturing of a parental strain of *M. tuberculosis* with various isogenic *rpoB* mutants identified various patterns of mutation-specific loss of replicative fitness in culture, but results of in vivo studies are not yet available. The S351L mutation, the most prevalent substitution in RIF-resistant isolates, conferred the smallest reduction of replication efficiency in vitro, whereas bacilli with the less frequently encountered mutations H526Y, H526D, and H526R, displayed a more profound diminution of fitness. Analysis of SM-resistant *M. tuberculosis* clinical isolates suggests that the nonrestrictive (more fit) *rpsL* mutation, K42R, is much more prevalent than the restrictive mutations found in SM-resistant mutants in vitro (26). There is a need for molecular epidemiology studies that combine precise genetic characterization of drug-resistant strains and strain typing, to investigate the issues of fitness and virulence of MDR-TB organisms. At present, most MDR-TB outbreaks have involved HIV-positive individuals. A real concern is that specific MDR-TB clones would become epidemic in the general population. The particularly successful strain W (Beijing family), which caused the MDR-TB outbreak in New York City and spread across the United States and abroad (3), is capable of causing active transmission of the disease in HIV-positive individuals. It is worth noting that strain W, which is resistant to INH (containing the common *katG* mutation S315T), still retains some catalase activity (19). It is likely that defective immune responses during HIV infection may underlie the transmission of the disease caused by this strain, which may be less easily transmitted in healthy individuals.

PHENOTYPIC RESISTANCE

Resistance in Stationary-Phase, Persistent, and Dormant Bacilli

Antibiotics usually act against actively growing bacteria but not against nongrowing forms. The lack of susceptibility of the nongrowing bacteria to antibiotics is due to changes in bacterial metabolism or physiological state and is therefore called phenotypic resistance. It is well known that when bacteria grow into the stationary phase they become nonsusceptible or phenotypically resistant to many antibiotics even though the bacteria are fully viable. Another type of phenotypic resistance, or drug tolerance, relates to the phenomenon of "persisters," a small number of surviving bacteria, from actively growing cultures, that are not killed after exposure to antibiotics. This is why the MIC is commonly defined as the lowest concentration of antibiotic that kills 99% of bacterial populations but never 100%. It remains to be determined if the very observation of persisters in the presence of antibiotics in vitro underlies mycobacterial persistence in vivo.

Phenotypic resistance is a major problem for antibiotic therapy, especially for TB. Nongrowing bacteria can be divided roughly into two different types depending on whether they grow immediately on subculture into a defined fresh medium. The nongrowing forms are a constant source of confusion for people who study bacterial persistence or dormancy. This confusion stems largely from the fact that very little is known about bacterial life-styles (especially those of the nongrowing bacteria), the definition of dormancy, and the definition of viability and death. It also stems partly from semantics. The term "persistent bacilli" often refers to in vivo-derived bacteria, and these can be either colony-forming or non-colony-forming bacteria, whereas "dormant bacilli" refers to bacteria that do not grow immediately on plates but can be resuscitated under certain specific conditions in liquid medium (with or without "resuscitation factors") to grow on plates. Despite this distinction, both persistent and dormant bacilli are phenotypically resistant to antibiotics.

The presence of dormant tubercle bacilli is best demonstrated in the Cornell mouse model (117), where mice infected with *M. tuberculosis* were treated with INH and PZA for 3 months. At the end of treatment, no bacilli could be demonstrated in the infected organs by colony formation on agar plates. However, 3 months after cessation of the chemotherapy, one-third of the mice relapsed with TB, and almost all mice relapsed if immunosuppressant steroids were given. The bacilli recovered from the relapsed mice

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