[Frontiers in Bioscience 9, 975-994, January 1, 2004]

MECHANISMS OF DRUG RESISTANCE IN MYCOBACTERIUM TUBERCULOSIS

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1. ABSTRACT

Tuberculosis is a worldwide health problem posing increasing threat with the spread of HIV infection and drug resistant Mycobacterium tuberculosis strains. Consequently, control of this disease has become a significant challenge despite the availability of chemotherapy and BCG vaccine. Drug resistance for all first-line anti-tuberculosis agents and some second-line agents has been observed. Moreover, the occurrence of strains of M. tuberculosis resistant to multiple anti-tuberculosis drugs is increasing. Mechanisms of action and resistance of major anti-tuberculosis drugs are reviewed. In addition, the phenotypic drug resistance such as dormant or persistent tubercle bacilli and its importance are also emphasized. In order to combat the threat of drug resistant tuberculosis and to more effectively control the disease, an understanding of the mechanisms underlying drug resistance is necessary. This knowledge could be used for the development of molecular tests for rapid detection of drug resistant bacilli and future anti-tuberculosis drugs.

2. INTRODUCTION

Tuberculosis (TB) is an ancient infectious killer that still remains the leading cause of death by an infectious agent worldwide today. It is estimated that one-third of the world's population is infected with Mycobacterium tuberculosis, the causative agent of TB, with approximately 8 million new cases and 2 million deaths each year (1). TB has become an increasing health problem since the emergence of HIV and the increasing appearance of drug resistant strains (1). Although drug resistant TB was reported in the past before the emergence of HIV, the problem of drug resistant TB was not as severe as it is today. The HIV infection allows drug resistant TB strains to transmit and cause disease more easily because of the suppression of the immune system. Drug resistance has been observed for all five of the first-line anti-tuberculosis drugs isoniazid (INH), rifampin (RIF), pyrazinamide (PZA), streptomycin (SM) and ethambutol (EMB) and for several of the second-line anti-tuberculosis drugs. Although the most common form of drug resistance is resistance to only one drug, strains of TB resistant to multiple drugs such as isoniazid and rifampin (also called MDR-TB) has been reported and is of great concern (1). Due to the problem of drug resistant tuberculosis, there has been a great deal of research interest to understand the molecular mechanisms of drug resistance in M. tuberculosis. The outbreak of MDR-TB in New York City in the late 1980s and early 1990's has drawn much media attention. The first molecular study of drug resistance mechanisms in M. tuberculosis was that by Zhang and colleagues in 1992 on the mechanism of isoniazid resistance in M. tuberculosis (2). Subsequent studies identified mechanisms of resistance to other major anti-tuberculosis drugs such as rifampin (3), streptomycin (4-6), target of isoniazid InhA (7), pyrazinamide (8), and ethambutol (9,10). Several recent reviews on this topic are available (11-15). The purpose of this review is to provide an overview of the mechanisms of drug resistance in M. tuberculosis, with emphasis on new developments in this

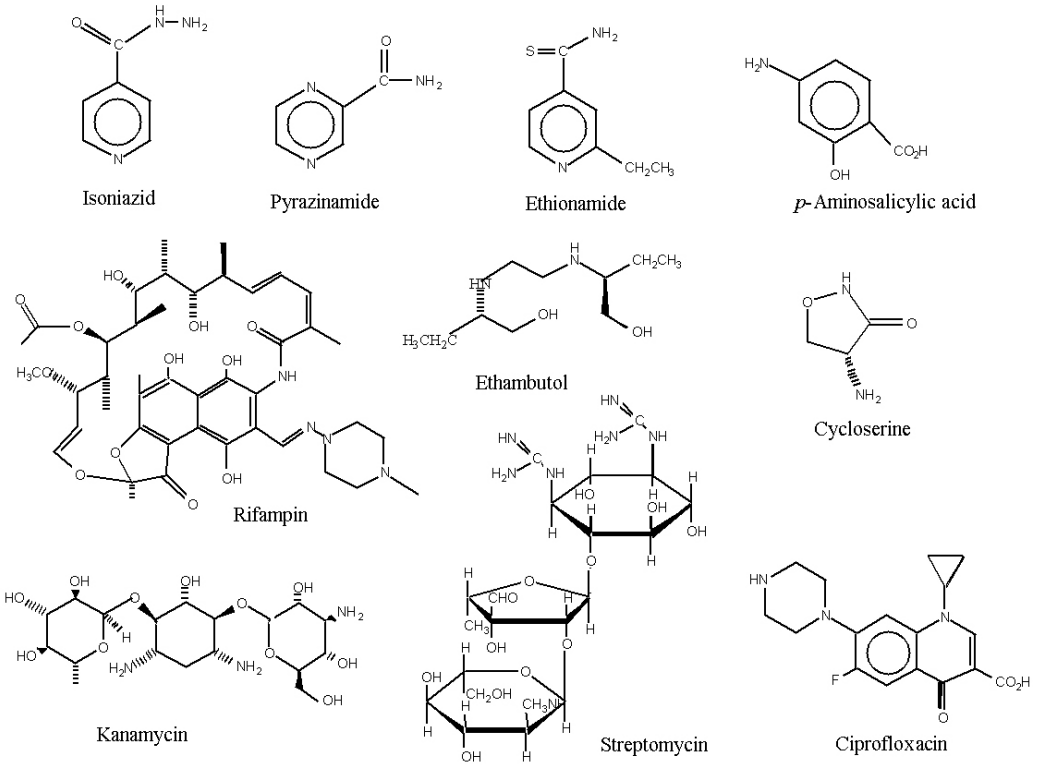

Figure 1. Structures of commonly used first-line and second-line TB drugs

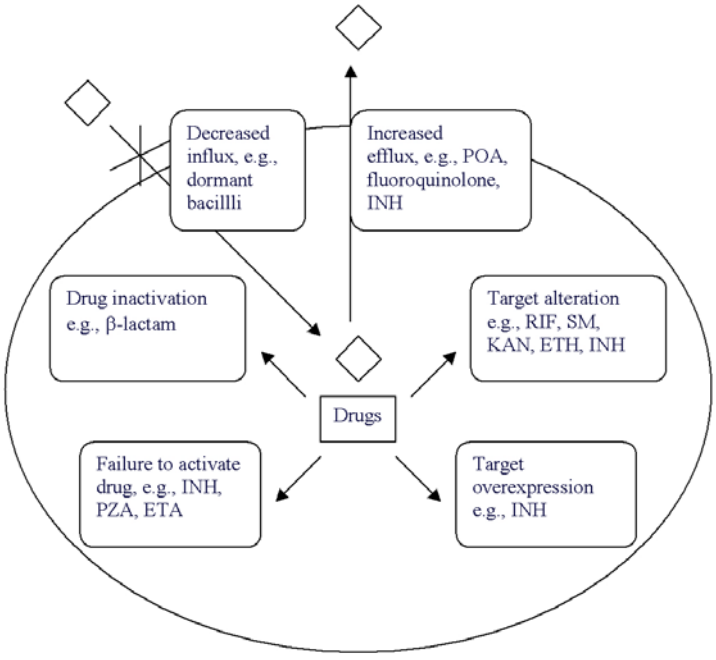

Figure 2. Mechanisms of drug resistance in mycobacteria. Diamond shape represents antimycobacterial drugs.

area. An improved understanding of drug resistance mechanisms in M. tuberculosis is important for rapid molecular detection of drug resistant strains and for the development of new anti-tuberculosis agents needed to combat this worldwide threat. A list of commonly used TB drugs is shown in Figure 1.

3. DRUG RESISTANCE MECHANISMS

3.1. General Mechanisms of Drug Resistance

Antibiotic resistance in bacteria can occur by a variety of mechanisms (11,12). These mechanisms are typically divided into five main categories (Figure 2): (i)

decreased uptake or impermeability, (ii) increased efflux, (iii) enzymatic inactivation and (iv) modification of the antibiotic target and (v) reduced pro-drug-activating enzyme activity. These mechanisms of resistance may be a result of intrinsic (natural) resistance or acquired means. Intrinsic or natural resistance refers to resistance that is not caused by any genetic alteration, while acquired resistance is due to a mutation or transfer of genetic material. M. tuberculosis has several means of intrinsic resistance. For example, M. tuberculosis has a very hydrophobic cell surface, which provides a permeability barrier for some antibiotics (16,17). Also M. tuberculosis possesses some enzymes such as beta-lactamase necessary to inactivate penicillin (18-20). Aside from natural resistance mechanisms, M. tuberculosis has acquired resistance mechanisms to anti-tuberculosis drugs by spontaneous mutations in chromosomal genes rather than by any type of gene transfer (11,12). A strain of M. tuberculosis is considered to be multi-drug resistant tuberculosis (MDR-TB) when it exhibits resistance to at least INH and RIF. The MDR-TB phenotype is due to an accumulation of mutations at different loci rather than by a single mutation (21). Typically poor patient adherence to treatment is thought to be responsible for the development of MDR-TB. Unlike many other bacterial species, plasmids or transposons play no role in drug resistance in M. tuberculosis (11,12), despite their presence in some other mycobacterial species (22,23). In a recent study, Boshoff and colleagues found an error-prone DNA polymerase DnaE2 in M. tuberculosis that appears to be involved in increasing the mutation frequency in the rpoB gene by causing increased number of RIF resistant mutants in the wild type strain than in the DnaE2 mutant strain, both *in vitro* and in mice (24). It will be of interest to see if M. tuberculosis DnaE2 is more prone to errors and has a higher mutation frequency to drug resistance than the corresponding enzyme from other bacterial species. However the clinical significance of this finding in terms of emergence of drug resistance in M. tuberculosis remains to be determined.

3.2. Isoniazid Resistance

Because of the importance of isoniazid in TB chemotherapy, its mechanisms of action and resistance have been reviewed many times in the past, including some more recent reviews (25-28).

3.2.1. Mechanism of action

Isoniazid is an important first-line anti-tuberculosis drug responsible for the initial dramatic decrease in actively metabolizing bacilli during treatment of TB. M. tuberculosis is highly susceptible to INH with MIC values in the range of 0.01-0.25 μg/ml (26). INH is active against growing tubercle bacilli in the presence of oxygen, but not active against resting bacilli under anaerobic conditions (29). The activity of INH is most obvious at 37^{o}C, but is greatly reduced at 4^{o}C (30), presumably a reflection of optimal temperature requirement for the KatG-mediated INH activation (see below).

INH is a pro-drug that requires activation by the M. tuberculosis catalase-peroxidase enzyme (KatG) to its active form (2,31-37). Upon activation reactive radicals are formed damaging multiple targets in the cell (11,26). The active species derived from INH activation by KatG include isonicotinic acyl radical, isonicotinic acyl species (37,38), in addition to reactive oxygen species (39,40). Besides KatG, Mn has also been shown to mediate similar INH activation as KatG (37,41). A recent study has shown that during Mn-mediated INH oxidation in the presence of (NAD+), a range of isomeric INH-NAD(H) adducts were formed, including the open form of isonicotinoyl radical found to be bound with InhA (37). This study raised the question as to whether only one form or other isomeric cyclic forms of isonicotinoyl radical is the active species (37). However, it remains to be determined if KatG-mediated INH activation also produces the same types of isomeric INH-NAD(H) adducts as Mn.

The most convincing molecular target has been shown to be InhA, an NADH-dependent enoyl acyl carrier protein (ACP) reductase, involved in mycolic acid synthesis (7, 42,43). Interestingly, InhA is also the target of triclosan (44,45) and ethionamide (7). A great deal of progress has been made concerning how INH inhibits the InhA enzyme through a series of crystallography (38,46) and functional studies (31-36, 47). During INH activation, the various reactive oxygen radicals produced (31,32,39,40) could cause damage to various cellular targets including DNA (48), carbohydrates and lipids (49). In addition, INH has been proposed to affect NAD metabolism by incorporating into NAD through exchange with nicotinamide (50), or by activating NAD glycohydrolase by removing its repressor leading to NAD depletion (51), as possible mechanisms of action. However, there is no recent work to confirm the effect of INH on the above aspects of NAD metabolism. The bactericidal activity of INH is most likely due to a combination of its multiple effects on the tubercle bacilli, though the relative importance of these effects in killing the bacilli needs further study.

M. tuberculosis is uniquely susceptible to INH (26). Despite the presence of KatG enzyme needed for INH activation and also InhA homolog, other mycobacteria such as M. smegmatis, M. phlei and M. avium are less susceptible with an MIC in the range of 10-100 μg/ml INH [26]. However, M. aurum, M. kansaii, M. gastri and M. xenopi (MIC=1-5 μg/ml) are more susceptible to INH than other non-tuberculous mycobacteria (26). Other bacterial species outside the mycobacterial genera are highly resistant to INH with MICs of at least 600 μg/ml (26). The reasons for this difference are complex and not well understood, but may have to do with the following: First, there is higher peroxidase activity of the KatG enzyme in M. tuberculosis than in other bacteria such as Escherichia coli and M. smegmatis or M. vaccae (26), which can lead to increased activation of INH in M. tuberculosis. Indeed, a recent study has shown that M. smegmatis KatG cannot directly activate INH and requires manganese for activation (52), which may be related to the less INH susceptible phenotype of M. smegmatis (52). Second, the sensitive target(s) such as InhA involved in mycolic acid biosynthesis may be more sensitive to INH-derived reactive

species in M. tuberculosis than their counterparts in other mycobacteria or bacteria. Third, M. tuberculosis may have a deficient efflux for INH-derived toxic radicals or isonicotinic acid generated during INH activation, compared with other mycobacteria or bacteria, as shown for pyrazinoic acid in the case of PZA susceptibility (53). It is quite remarkable that only the highly susceptible M. tuberculosis accumulates radioactive INH whereas naturally resistant mycobacteria or bacteria do not (54). This suggests that M. tuberculosis may have some deficiency in removing the INH derived radicals from the cell compared with non-tuberculous bacteria. It is of interest to note that the less susceptible M. smegmatis has a demonstrable efflux mechanism for INH (55). Although M. tuberculosis efflux protein EfpA is induced by INH (56), it will be of interest to see if M. tuberculosis INH efflux mechanism is less active than that in M. smegmatis. Fourth, M. tuberculosis may lack antagonists or adequate anti-oxidative defense for INH or its derivative. The KatE type (HP-II) heat-stable catalase is absent in M. tuberculosis, but present in other mycobacteria or bacteria (26). The KatE type catalase may remove the toxic peroxide produced during or needed for INH activation and may provide protection against INH in non-tuberculous mycobacteria or bacteria. However, overexpression of M. avium katE in M. tuberculosis did not appear to confer resistance to INH (57). The observation that overexpression of M. tuberculosis KatG conferred some degree of INH sensitivity to an E. coli katG/katE double mutant (2), led to examination of the role of OxyR in susceptibility to INH in E. coli (58). Mutation of OxyR (58) and KatG/AhpC (59) in E. coli caused increased susceptibility to INH. These findings led to the discovery of a defective OxyR (60,61) and the assessment of the role of AhpC in INH action in M. tuberculosis (60, 62,63). M. tuberculosis seems to be particularly susceptible to endogenously generated reactive oxygen or organic radicals, presumably a reflection of its deficient anti-oxidative defense and defective efflux mechanisms. Another potential antagonist arylamine N-acetyltransferase (NAT) involved in inactivation of INH (64,65) may be less active or poorly expressed in M. tuberculosis, but more active or highly expressed in other less susceptible mycobacteria or bacteria.

3.2.2. Mechanisms of resistance

INH resistance was noted shortly after its use as an anti-tuberculosis drug in the 1950's (66); however, it was not until the 1990's that the mechanisms underlying resistance were elucidated (2,67). Zhang *et al.* (2) were the first to show that mutations or deletions in the katG gene resulted in resistance to INH in clinical isolates of M. tuberculosis. Susceptibility could be restored upon transformation with a functional katG gene in resistant strains harboring katG mutations (67). It was also noted that INH-resistant strains that lose catalase activity had reduced virulence (66,68). Upon restoring the katG gene, not only INH sensitivity, but also virulence could be restored (69,70). Various mutations in the katG gene have been reported among INH-resistant isolates (19, 71-77), but the most common mutation is the Ser315Thr mutation, which is present in approximately 50% of all INH-resistant isolates and results in high-level resistance to INH (76,77).

However, the katG gene does not account for all INH-resistant strains. The gene inhA, encoding an NADH-dependent enoyl acyl carrier protein (ACP) reductase, has also been shown to be involved in INH resistance (7). Although mutations in the inhA structural gene were found initially to cause INH resistance (7), subsequent studies have shown that mutations in the promoter region of an upstream gene mabA, encoding 3-ketoacyl ACP reductase, which forms an operon with inhA, are more frequent than inhA structural gene mutations (74). The mabA promoter up-mutations will cause overexpression of target InhA, whereas mutations in InhA itself cause alterations of the InhA target, both of which cause INH resistance. However, overexpression of MabA alone did not appear to cause INH resistance in mycobacteria (78). The resistance conferred by inhA mutations is generally of low level, whereas katG mutations are more commonly associated with high levels of INH resistance (11). Mutations in InhA cause not only INH resistance, but also resistance to the structurally related second-line drug ethionamide (7) and the common antibacterial agent triclosan in M. smegmatis (44). Another gene kasA, encoding a beta-keto-acyl ACP synthase, a different enzyme involved in mycolic acid synthesis, was identified based on its induction by low levels of INH (79). Although kasA mutations were initially found in 4 INH-resistant strains (79), subsequent studies have reported that kasA mutations could also be found in INH susceptible strains (80,81). Recently, it was shown that overexpression of inhA increased resistance to INH, while kasA overexpression did not cause INH resistance, but caused thiolactomycin resistance (82). In addition, a recent study has shown that KatG-activated INH and triclosan inhibited InhA, but not KasA. Also, InhA inhibition induced the formation of KasA-AcpM complex that does not contain INH, suggesting that InhA, but not KasA is the primary target of INH (83). However, the role of KasA in INH resistance is not completely resolved and needs further study.

Mutations in ndh, encoding NADH dehydrogenase, initially identified in M. smegmatis as involved in INH resistance (84), have subsequently been found in some INH-resistant clinical isolates (85). Decreased activity of Ndh supposedly increases the NADH/NAD ratio, which could possibly compete for the binding of activated INH (isonicotinic acyl radical) to the target InhA, or may promote displacement of the isonicotinic acyl NADH from InhA (84). However, this remains to be confirmed. In KatG-negative INH-resistant strains, mutations in the promoter region of ahpC, encoding an alkylhydroperoxide reductase, have been observed as a compensation for the lack of catalase-peroxidase activity in such strains (61,86). However, overexpression of AphC did not appear to confer INH resistance (87), but can be a marker for INH resistance (88). Mutations in fadE24, Rv1592c, Rv1772, Rv0340, and iniBAC genes (89), which were induced by INH in a microarray analysis (90), were found to be present in some low level INH-resistant strains (91). However, the role of these newly identified genes in INH resistance needs to be confirmed by genetic complementation experiments. Among INH-resistant M. tuberculosis strains, mutations in katG is the most frequent

mechanism of resistance compared with other INH resistance genes such as inhA, ndh (11,26); however, the exact percentage of strains having katG mutations may vary according to different studies. Despite these advances, some INH-resistant strains, especially those of low to intermediate level resistance with positive catalase activity, do not have mutations in any of the above genes, which suggests unknown mechanisms of INH resistance.

3.3. Rifampin Resistance

Rifampin is another important first-line tuberculosis drug that kills log phase and to a great extent also stationary phase tubercle bacilli (92,93). RIF, a broad-spectrum rifamycin derivative, easily diffuses through the cell membrane due to its lipophilic nature where it acts by binding to the bacterial RNA polymerase, thereby inhibiting RNA synthesis (93). RIF has excellent sterilizing activity on populations of slowly metabolizing tubercle bacilli, and this property is thought to be important for shortening the duration of the treatment from previously 12 -18 months to 9 months (92). Resistance to RIF is one of the main causes of treatment failure (94). Resistance to RIF in M. tuberculosis is due to a single mutation in an 81 base pair region in the rpoB gene, a gene encoding the DNA-dependent RNA polymerase beta subunit (3). The most common mutations found in this region are associated with codons Ser531 and His526 (11). Mutations in these codons result in high-level resistance to RIF. Mutations in amino acid residues 511, 516, 518 and 522 are associated with low-level resistance to RIF and rifapentine, but strains with such mutations remain susceptible to rifabutin and the new rifamycin KRM1648 (rifalazil) (95-97). The single point mutations in rpoB, which cause resistance to RIF and also cross-resistance to many other rifamycin derivatives, account for about 96% of RIF-resistant M. tuberculosis strains (3) and are useful for diagnosing RIF-resistant TB (see the review by Parsons *et al.* in this Issue). Although ribosylation, a degradative mechanism of resistance to RIF, has been found in the rapid growing mycobacteria such as M. smegmatis, M. chelonae, M. flavescens, M. vaccae (98,99), such a mechanism has not been found in M. tuberculosis. Resistance to RIF in other bacterial species is also caused by a similar mechanism as that in M. tuberculosis.

3.4. Pyrazinamide Resistance

3.4.1. Mechanism of action

Pyrazinamide is an important first-line drug for the treatment of TB responsible for the shortening of TB therapy from the previous 9 to 12 months to the current 6 months due to its killing semi-dormant cell populations (92). PZA, an analog of nicotinamide, is a rather unique and unconventional antibiotic in that it is not active *in vitro* under normal culture conditions (100), but is only active at acidic pH with an MIC of 16-50 μg/ml at pH 5.5 (101) or an MIC of 100 μg/ml at pH 6.0 by the BACTEC method (102). Acid pH occurs in the *in vivo* environment during active inflammation due to the production of lactic acid by inflammatory cells (103). The activity of PZA is a function of pH [i.e., the lower the pH, the higher the PZA activity (103,104)] and the relationship between the PZA MIC and pH can be expressed by the Henderson-Hasselbach equation (105). Even at acid pH, PZA kills tubercle bacilli slowly and incompletely *in vitro* with no more than 76% of bacilli being killed after a two-week incubation (106). Another unusual feature of PZA is that it kills old, non-replicating bacilli with low metabolic activity more effectively than young, growing bacilli with high metabolic activity (105). This peculiar property may be underlying some of the discrepant results claiming that PZA is not effective against M. tuberculosis in tissue culture macrophages (107,108), but effective in some others (8,109). This property may be related to the ability of PZA to shorten the therapy by killing somewhat "sick" non-growing bacilli, not killed by other antibiotics. Indeed, PZA when used alone in the treatment of TB in the mouse model often gave poor efficacy (110), but its activity is greatly enhanced when PZA is used in combination with another companion drug such as INH (110), gatifloxacin (111), streptomycin or even a weak drug like PAS (112). Various factors besides pH can affect the activity of PZA, including inoculum size, age of the culture, BSA, efflux inhibitors (105), low oxygen (M. Wade and Y. Zhang, unpublished data) and iron (A. Somoskovi, M. Wade, Z. Sun and Y. Zhang, unpublished data) and energy inhibitors (113). For other details of PZA, please refer to the recent review by Zhang and Mitchison (103).

Despite its discovery over 50 years ago (114), its mechanism of action is poorly understood largely because of the unusual and paradoxical property of PZA. Although PZA and INH both are derived from nicotinamide, yet, unlike INH, which causes loss of acid-fastness due to its inhibition of mycolic acid synthesis, PZA by itself does not alter the morphology or change the staining property of the bacilli (Y. Zhang, unpublished observation). PZA is a pro-drug that requires activation to its active form, pyrazinoic acid (POA), by the enzyme pyrazinamidase (PZase) (8,115). Although the target of PZA has been proposed to be fatty acid synthase I (FAS-I) based on a study using the fast growing M. smegmatis and a related compound 5-Cl-PZA (116), this proposition has been recently questioned in a recent study by Boshoff *et al.*, who found that FAS-I is the target of 5-Cl-PZA, but not that of PZA (117). The available data so far also do not appear to support the presence of a specific cellular target for POA in M. tuberculosis (11). First, no POA-resistant mutants are isolated *in vitro* (118) or found among clinical isolates resistant to PZA (Y. Zhang, unpublished). Second, POA does not appear to bind to any cellular components in M. tuberculosis (Y. Zhang, unpublished observation). Third, if there were a specific cellular target for POA, POA, which is produced from PZA in the bacilli at both acid and neutral pH conditions (53), would have bound to the specific cellular target and shown inhibitory effect even at neutral pH, which is not the case. Although it is possible that POA as a structural analog of nicotinic acid may interfere with NAD metabolism by incorporating into NAD to make pseudo-NAD through the weak PncB activity in the NAD recycling pathway (Preiss-Handler pathway) (119) or by feedback inhibition of NAD synthesis, preliminary studies aimed at addressing these possibilities have not provided any supporting evidence for such proposition (Y. Zhang, unpublished).

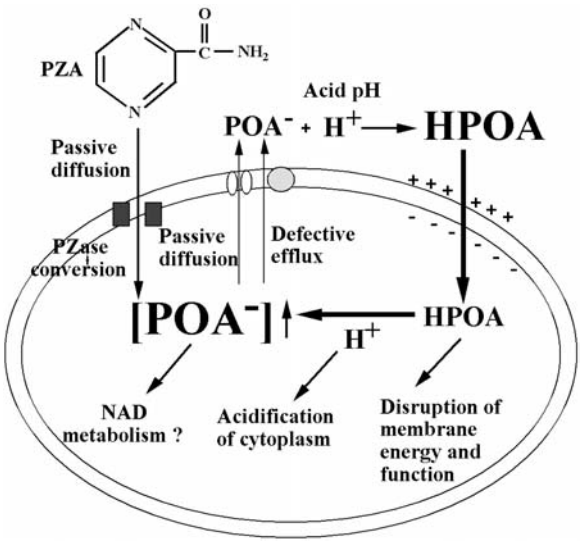

Figure 3. Mode of action of PZA. See text under Pyrazinamide Resistance for details of the model.

Zhang proposed a model for the mode of action of PZA (11,103) based on their studies (8,53,105,113) (Figure 3). PZA diffuses into the cell where it is converted to POA by PZase (8). POA will be in anion form (pKa=2.9) at close to neutral intracellular pH (53) and then diffuses out of the cell through passive diffusion and deficient efflux to the cell surface. Under acidic pH, POA anion will form protonated or uncharged HPOA, which will diffuse back into the cell. The acid-facilitated POA influx is stronger than the weak POA efflux and this causes accumulation of POA in M. tuberculosis (8). As HPOA enters the bacilli at acid pH, it brings in protons and over time could cause acidification of the cytoplasm and disruption of the proton motive force and depletion of energy (113). The disruption of proton motive force by POA then inhibits the membrane transport function (113). Another role of acid pH in addition to facilitating POA influx is to disrupt the membrane potential itself. Also, the presence of POA further decreases the membrane potential (113). It is worth noting that the membrane potential of old cells is less than that in young cells (113), and this observation explains why PZA is preferentially more active against old cells than young cells (105). In old bacteria with low metabolism, membrane potential is the sole source of ATP production required for viability (120, 121), and disruption of membrane potential by POA at acid pH will lead to depletion of energy. However, at neutral or alkaline pH, there is little POA found in the tubercle bacilli (53), because over 99.9% of POA will be in the charged anion form (105), which does not get back into cells easily and remains extracellular. This observation explains why PZA is active at acidic pH (101), but not at neutral pH (100) and also explains the correlation between the MIC of PZA and acidic pH values (104,105). Although the acid-facilitated uptake of weak acids is a non-specific process, the specificity of PZA for M. tuberculosis is conferred at the stage of POA transport, where M. tuberculosis has a deficient POA efflux mechanism (53) that is unable to counteract the effect of acid-facilitated POA influx, which could lead to eventual acidification of the cytoplasm and de-energized membrane, causing tubercle bacilli to die. Thus, the currently available data suggest that POA/PZA targets the membrane and affects the membrane bioenergetics and transport function as a mechanism of action. The unique susceptibility of M. tuberculosis to PZA is due to a combination of different factors including a deficient efflux mechanism for POA (53), inefficient ability to maintain proton motive force especially at acid pH (122) and slow metabolic activity (compared with other bacteria), especially in old non-growing bacilli with less energy reserve (113). This model best explains the various factors that influence the activity of PZA, such as the role of acid pH (53), the role of a deficient efflux mechanism in unique susceptibility to PZA (53), preferential activity of PZA against old, aged bacilli over young bacilli (105), the effect of energy inhibitors on enhancing PZA activity (113), and the preferential activity of PZA against tubercle bacilli under microaerophilic or anaerobic conditions (M. Wade and Y. Zhang, unpublished data). The observation that PZA is more active against tubercle bacilli at microaerophilic and anaerobic conditions than at aerobic conditions can be explained by the lower energy levels (in the forms of ATP and membrane potential) produced in the former, but higher energy produced in the latter conditions. The lower energy levels in bacilli under microaerophilic or anaerobic conditions provides a weak point for attack by HPOA, which further depletes the energy and eventually leads to cell death. This observation also provides another explanation as to why PZA is much more active against tubercle bacilli *in vivo* in the lesions (which is microaerophilic and even anaerobic in granulomas) than *in vitro* testing conditions (which is often aerobic and full of oxygen).

3.4.1. Mechanisms of resistance

Acquired resistance to PZA in M. tuberculosis has been reported and occurs concomitantly with loss of PZase activity (115,123-126). Scorpio and Zhang cloned the pncA gene encoding the PZase enzyme whose mutation is the major mechanism of PZA resistance (8), accounting for as high as 97% resistant strains (118). Mutations in the pncA gene resulting in resistance to PZA are highly diverse and scattered along the pncA gene (Table 2) (8,118,127-144), with some degree of clustering in three regions at amino acid residues 3-17, 61-85, 132-142 (118,134). These regions are likely to contain catalytic sites of the PZase enzyme. A recent study that determined the crystal structure of the P. horikoshii PncA (37% identity with M. tuberculosis PncA) has provided some structural basis for the pncA mutations in M. tuberculosis that cause PZA resistance (145). The three regions where pncA mutations appear to cluster correspond to three of the four loops that contribute to the scaffold of the active site. Mutations at C138, D8, K96, D49, H51, H71, modify the active site triad and metal binding site. Residues F13, L19, H57 (position of characteristic mutation of H57D in M. bovis), W68, G97, Y103, I113, A134, H137, line up 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

Table 1. Mechanisms of drug resistance in M. tuberculosis

Drugs	MIC (μg/ml)	Mechanism of action	Gene(s) involved in resistance	Role in resistance
Isoniazid	0.06-0.2	Inhibition of mycolic acid biosynthesis and other multiple effects on DNA, lipids, carbohydrates, and NAD metabolism	katG	Prodrug conversion
			inhA	Drug target
			kasA	Drug target?
			ndh	Modulator of INH activity
			ahpC	Marker of resistance
Rifampin	0.5-2	Inhibition of transcription	rpoB	Drug target
Pyrazinamide	16-50	Acidification of cytoplasm and de-energized membrane	pncA	Prodrug conversion
Ethambutol	1-5	Inhibition of arabinogalactan synthesis	embCAB	Drug target
Streptomycin	2-8	Inhibition of protein synthesis	rpsL	Drug target
			rrs (16S rRNA)	Drug target
Amikacin / Kanamycin	2-4	Inhibition of protein synthesis	rrs (16S rRNA)	Drug target
Fluoroquinolones	0.5-2.5	Inhibition of DNA gyrase	gyrA	Drug target
			gyrB	
Ethionamide	2.5-10	Inhibition of mycolic acid biosynthesis	etaA or ethA	Prodrug conversion
			inhA	Drug target

Table 2. pncA mutations associated with PZA resistance

Region	Amino acid or nucleotide	Comment	Region	Amino acid or nucleotide	Comment
Promoter *pncA*	-12 t->g -11 a->g,c del 11bp		**PncA (cont'd)**	F13S C14R,Y G17D L19P G23V A25E A26G A28D Y34S, stop Y41H,stop H43P V45G A46V,E T47A,S D49V,G,A H51Q,R,P D53A P54T,L H57D F58L,Tyr T61P P62R D63H,G Y64D S66P S67P W68L,R,G,S,stop P69R,L F80V H71E,R C72R T76P,I G78D F80V H82R L85P,R T87M S88stop I90S F94P K96N,T,E G97S,D,C Y99stop A102T,V Y103C,H,S,stop S104P,C T114P L116R N118T W119R,stop R121P G122stop R123L V125D V130G G132S,D,V I133T,N A134V T135P D136G,N,Y H137R,P C138T,S,Y V139L,A,M,G R140S Q141P,stop T142K,M,P,A A146V,T T153N R154G V155A,G L159R T160P A161P T168N A171P,T L172P V180F L182S	H57D is a characteristic polymorphism of *M. bovis*., causing its natural PZA resistance T87M plus del g436
	8 bp deletion @ start	nucleotide insertions or deletions resulting in frameshift			

***pncA* nucleotide ins/del**	ins tcg @23 ins c@28 234 bp del @56 ins g@70 ins g@71 del g@71 del g@77 delg@78 dela@79 del c@84 8 bp del @88 ins c@104 5.3 kb del @106 ins cg@107 del g@138 dela@143 80 bp del @151 del a@158 del c@161 ins g@162 ins t@162 ins a@192 ins 29bp@215 ins t@287 del t@287 ins g@288 ins a@301 ins c@306 5 bp del @307 ins ct@317 ins 1355bp@341 del c@341 del 11bp@379 AG ins @ 368 Del gg@381 AG ins@ 382 8bp del@382 18 bp ins@ 368 del 9bp@388 ins 9bp @ 388 ins g391 ins gg391 ins g392 ins t@393 ins gt@393 ins 16bp@395 del t@397 del tt@398 del c@403 CC insertion at 403 del g@406 ins g@414 ins tg@415 del 68bp@419 ins gc@420 ins g@443 ins 8bp@446 del t@452 ins t@465 del c@475 ins tgac@480 del gc@513 del 5bp@518 ins c@532	ins 1355bp@341 corresponds to transposition of IS*6110* into *pncA*			
PncA protein	M1I A3P L4S,W I5S Ile6T V7I,F,G,D D8Y,G,E V9G,A Q10P,R,K,stop D12A,N				

attributed to potential perturbation of the active site or disruption of the protein core (145). These predictions have to be confirmed when the structure of the M. tuberculosis PncA is determined. The pncA mutations are primarily missense mutations, however, insertions or deletions and nonsense mutations do occur as well (11). The diverse nature of pncA mutations is unique to PZA resistance, and other drug resistance genes usually do not show this degree of diversity. Although the basis for this high diversity is unclear, it is likely that since the pncA gene is not an essential gene (127) there is no selective pressure such that mutations anywhere in the pncA are tolerated. Although resistance to PZA occurs primarily through mutations in the pncA gene, a small number of PZA-resistant strains with low levels of resistance (about 2 fold higher than the MIC) also occur that lack pncA mutations (118, 127). However,

the exact molecular mechanisms underlying this type of resistance and the clinical significance of such strains remain unclear. M. bovis strains including BCG are naturally resistant to PZA and lack PZase activity due to a single characteristic point mutation (C->G at nucleotide 169) in the pncA, causing His57Asp substitution (8,118, 146). Other mycobacteria or bacteria are naturally resistant to PZA most likely due to highly active POA efflux pumps (103).

3.5. Streptomycin Resistance

Streptomycin was the first antibiotic used in the treatment of TB in 1944 (147). Consequently, SM was often given alone and resistance to the drug developed quickly as a result. SM inhibits protein synthesis by binding to the 30S subunit of the bacterial ribosome causing misreading of the mRNA message during translation (148). The site of action of SM is the small 30S subunit of the ribosome at the ribosomal protein S12 and the 16S rRNA (149). SM is thought to kill actively growing tubercle bacilli at neutral or alkaline pH conditions with an MIC of 2-8 μg/ml (150), but inactive against non-growing or intracellular bacilli (92). Resistance to the aminoglycoside SM is typically through mutations in S12 (rpsL) and 16S rRNA (rrs). Mutations in the rpsL and in rrs account for about 50% and 20% of SM-resistant strains (4-6,151-153). The most common mutation in rpsL is a substitution in codon 43 from lysine to arginine (151-153), causing high-level resistance to SM. Mutations in codon 88 is also common (4-6, 151-153). Mutations of the rrs gene occur in the loops of the 16S rRNA (4-6) and are clustered in two regions around nucleotides 530 and 915 (4-6). The interesting phenotype of streptomycin-dependence in an SM-resistant M. tuberculosis strain appears to be caused by a "C" insertion in the 530 loop (154). A polymorphism of C-to-T change at position 491 of the rrs gene identified in M. tuberculosis strains predominant in South Africa is not involved in streptomycin resistance (155). Mutations in the rrs gene usually account for low-level resistance. Together, mutations in the rpsL and rrs genes account for approximately 75% of all SM-resistant isolates. Therefore, alternate mechanisms of resistance must exist to account for the remaining 25% of SM-resistant strains. One such possible mechanism may be alterations in drug uptake, which have been thought to contribute to drug resistance (153). Although aminoglycoside modifying enzymes are present in M. tuberculosis and other mycobacteria (20,156,157), their role in SM resistance is not known.

3.6. Ethambutol Resistance

Ethambutol is a first-line drug used in the treatment of TB. EMB is only active against growing bacilli with an MIC of 1-5 μg/ml (150), but has no activity against non-growing bacilli (158). EMB inhibits synthesis of arabinogalactan, a major cell wall component of mycobacteria (159). Subsequent studies showed that EMB inhibits the polymerization of arabinan in arabinogalactan and lipoarabinomannan, causing rapid accumulation of decaprenyl-P-arabinose, an intermediate in the biosynthesis of the arabinan of cell wall arabinogalactan and arabinomannan (160-162). Genetic studies indicated that the target of EMB is EmbB, arabinosyl transferase, involved in synthesis of arabinogalactan (9,10). The Emb proteins EmbA, EmbB, and EmbC are about 65% identical and are predicted to be integral membrane proteins with 12 transmembrane-spanning domains (10). Genetic and biochemical studies indicated that EmbC is involved in LAM (lipoarabinomannan) synthesis (163), whereas EmbA and EmbB are involved in forming the terminal hexaarabinofuranoside motif in arabinogalactan synthesis (164). However, the precise mechanism of how EMB inhibits EmbB is still unclear.

Mechanisms of resistance to this anti-tuberculosis agent are primarily associated with point mutations in the embCAB operon (165,166), encoding various arabinosyl transferase enzymes necessary for cell wall biosynthesis. One of the most common mutations in this operon is associated with amino acid substitutions at codon Met306 of the embB gene (10,165,166). Mutations in the embB gene result in high-level resistance in M. tuberculosis (165); however, mutations in this gene only account for approximately 70% of resistant strains in one study (165). However, a recent study from Russia has shown that Met306 mutations were not only detected in 14 (48.3%) of 29 EMB-resistant strains, but also in 48 (31.2%) of 154 EMB-susceptible strains (167), raising some doubts about the significance of embB mutations in EMB resistance. Additional mutations in the embC-embA intergenic region have also been found in strains that also had mutations in EmbA or EmbB (166). These intergenic region mutations may play a secondary or compensatory role in resistance. The embR homologue, located 2 Mb from the embCAB locus in M. tuberculosis rather than immediately upstream of the embAB genes in M. avium, also contained mutations (Gln379Arg replacement and an A insertion at position −137 upstream of the EmbR start codon) associated with EMB resistance (166). In addition, mutations at the −24 position of the Rv0340 gene, which is upstream of the iniBAC operon and transcribed in the same direction and originally identified by induction by both INH and EMB (89), was associated with EMB resistance (166). Mutations in rmlD and rmlA2, both of which are involved in modification of rhamnose, were also found to be associated with EMB resistance (166). Despite additional new genes being identified having involvement in EMB resistance, some 24% of EMB-resistant M. tuberculosis strains do not have mutations in any of the genes described above (166). Future studies are needed to confirm the role of the above new genes in EMB resistance using genetic and biochemical approaches.

3.7. Fluoroquinolone Resistance

Fluoroquinolones are important second-line drugs used for the treatment of drug resistant TB. Fluoroquinolones are quite active against M. tuberculosis with MICs of 0.25-3 μg/ml (150). Among the fluoroquinolones used today are ciprofloxacin, ofloxacin, levofloxacin, and more recently the newer 8-methoxyquinolone gatifloxacin and moxifloxacin. These agents target DNA gyrase, which is responsible for DNA supercoiling and consists of two A and two B subunits encoded by gyrA and gyrB, respectively (168).

Fluoroquinolones bind to DNA gyrase, thereby inhibiting DNA supercoiling (168). While fluoroquinolone drugs are primarily active against growing tubercle bacilli, in a recent study, it was shown that ofloxacin and levofloxacin had some activity against a 100-day-old culture, while moxifloxacin and gatifloxacin had the highest activities (169). In particular, moxifloxacin was found to kill non-growing RIF-tolerant tubercle bacilli and was suggested to be able to shorten the TB therapy (169).

The increased use of these drugs has led to the emergence of drug resistant strains. In M. tuberculosis resistance to fluoroquinolones such as ciprofloxacin or ofloxacin is associated with mutations in a 40 amino acid region in gyrA (170- 172), which accounts for 40-70% of fluoroquinolone resistant isolates. Mutations in codons 90, 91 and 94 have been reported; however, resistance does occur in strains lacking mutations in these codons suggesting alternative mechanisms of resistance. Mutations in GyrA can cause cross-resistance to different fluoroquinolones (171,173). Higher levels of resistance can have mutations in both gyrA and gyrB (172). In M. smegmatis, increased expression of the LfrA efflux pump has been shown to cause resistance to fluoroquinolones, ethidium bromide, acriflavine and rhodamine (174,175). However, LfrA deletion caused only a 2-fold increase in susceptibility to fluoroquinolones, but caused more susceptibility to ethidium bromide and acriflavine (176). However, there is no LfrA homolog in M. tuberculosis and indeed no such mechanism has been reported to cause fluoroquinolone resistance in M. tuberculosis. Although topoisomerase IV (ParC) mutations have been shown to cause quinolone resistance in other bacteria (168), no such homologs have been found in the genome of M. tuberculosis (20).

3.8. Ethionamide Resistance

Ethionamide (ETA) is a second-line drug used to treat MDR-TB (177). The MIC of ETA for M. tuberculosis is 0.6-2.5 μg/ml (150). Like INH, ETA is a pro-drug that is activated by a monooxygenase (Rv3854c) also called EtaA (178) or EthA (179) and then inhibits InhA involved in mycolic acid synthesis. This mechanism of action is consistent with the observation that InhA target mutations cause resistance to both INH and ETA (7). EtaA or EthA is an FAD-containing enzyme that oxidizes ETA to the corresponding S-oxide, which is further oxidized by EtaA to 2-ethyl-4-amidopyridine, presumably via the unstable doubly oxidized sulfinic acid intermediate (180). The final product of the activation is 4-pyridylmethanol, which is the same as that of INH (178-180). EtaA or EthA also oxidizes thiacetazone, thiobenzamide, and isothionicotinamide, which is probably responsible for the oxidative activation of other thioamide anti-TB agents. ETA-resistant M. tuberculosis strains had mutations in EtaA and were cross-resistant to thiacetazone and thiocarlide (178). The etaA or ethA is negatively regulated by EtaR or EthR (Rv3855), whose overexpression caused ETA resistance and inactivation led to ETA hypersensitivity (178,179). Besides alterations in EtaA or EthA, EtaR or EthR can cause ETA resistance. Mutations affecting the target InhA could also cause resistance, though the relative frequency of these possible mutations in clinical isolates is not yet known.

3.9. Resistance to Other Drugs

Kanamycin and capreomycin, like streptomycin, are inhibitors of 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 in M. tuberculosis (181-183) and other mycobacteria (184). Cross-resistance may be observed between kanamycin and capreomycin or viomycin (181-183). Although interference with folic acid biosynthesis and inhibition of iron uptake have been proposed as possible mechanisms of action for para-amino salicylic acid (PAS) (185), the mechanisms of action and resistance to PAS are not well understood. Cycloserine inhibits the synthesis of peptidoglycan by blocking the action of D-alanine racemase (AlrA) and D-alanine:alanine ligase (Ddl) in mycobacteria (186,187). Overexpression of D-alanine racemase enzyme (AlrA) or D-alanine:alanine ligase (Ddl) of M. smegmatis or M. tuberculosis on a multicopy vector in M. smegmatis or M. bovis BCG results in resistance to D-cycloserine (186,187). Inactivation of alrA in M. smegmatis caused increased sensitivity to D-cycloserine (188). In a recent study, mutations in an open reading frame with homology (24% identity and 37% similarity) to the E. coli penicillin binding protein 4 (PBP4) were found to cause D-cycloserine resistance and vancomycin resistance in M. smegmatis (189). However, the mechanism of cycloserine resistance in M. tuberculosis remains to be established.

4. PHENOTYPIC RESISTANCE

Antibiotics are usually active against actively growing bacteria, but not against non-growing bacteria. The lack of susceptibility of the non-growing bacteria to antibiotics is due to changes in bacterial metabolism or physiological state and is therefore called phenotypic resistance (190,191). It is well known that when bacteria enter 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", which are represented by a small number of surviving bacteria that are not killed upon exposure of a log phase culture to antibiotics. It remains to be determined if the "persisters" not killed by antibiotics in the culture underlie the mycobacterial persistence *in vivo* (191). The third type of phenotypic resistance is found in dormant tubercle bacilli as demonstrated in the Cornell mouse model (110), where the bacilli persist despite extensive chemotherapy. Yet, the bacilli recovered from the relapsed mice after chemotherapy were fully susceptible to INH or PZA, indicating that the dormant bacilli did not develop stable genetic drug resistance, but were phenotypically resistant (110). These findings suggest that dormant bacilli present in the tissues were not dead even though they failed to form colonies on plates. The presence of persistent and dormant TB bacteria is thought to be the cause for the lengthy TB chemotherapy, since the current TB drugs are not effective in eliminating persistent or dormant bacilli (92,191). There has been considerable interest in the study of mycobacterial persistence and dormancy in recent years [see Y. Zhang, this Issue], with the aim to better understand the mechanism of this phenomenon and devise therapeutic

strategies targeting the persistent or dormant organisms. More effective TB control to a large extent depends on whether more effective TB drugs that not only are active against MDR-TB, but also target dormant and persistent bacteria and shorten the TB therapy can be developed.

A fourth type of phenotypic resistance is caused by salicylate. Although salicylate was initially found to induce a multiple antibiotic resistance (Mar) phenotype in E. coli (192), subsequent studies have shown that salicylate can induce antibiotic resistance in a variety of bacterial species (193). We have recently shown that salicylate also induces resistance to multiple anti-tuberculosis drugs in M. tuberculosis (194). In the presence of salicylate (0.5 mM) the killing effect of INH, RMP, EMB, SM and PAS was reduced. When salicylate and the anti-TB agents were incorporated into 7H11 plates, salicylate-induced resistance was more pronounced for PAS, SM and EMB, but was not apparent for INH and RMP (194). The decreased killing of bacteria by antibiotics, which causes increased number of survivors in the presence of salicylate, has been shown to facilitate the emergence of genetically drug-resistant mutants (195). It remains to be determined if salicylate could facilitate emergence of drug-resistant M. tuberculosis mutants. Salicylate induces antibiotic resistance in E. coli by binding to MarR and activating the transcription of marA and marB. MarAB regulates a range of genes to confer a Mar phenotype, including down-regulation of the OmpF porin expression via micF antisense-RNA to limit the entry of antibiotics (196) and switching on efflux pumps such as AcrAB to more effectively extrude antibiotics from the cells (197). In addition, salicylate also induces antibiotic resistance through a Mar-independent pathway in E. coli, since a Mar deletion strain still showed resistance in the presence of salicylate (196). The mechanism of the salicylate-induced resistance in M. tuberculosis is unknown. One possible mechanism is that M. tuberculosis may have a Mar-like regulatory mechanism as in E. coli. Overexpression of the E. coli marA on a multicopy plasmid in the fast growing M. smegmatis mediates resistance to multiple anti-mycobacterial agents such as INH, RIF, EMB, indicating the presence of a mar-like regulatory system in this organism (198). Although two MarA homologs were reported to be present in the M. tuberculosis genome (198), preliminary studies showed that the mRNA for at least one of the homologs (Rv1931) was not induced by salicylate (Y. Zhang, unpublished observation), indicating that Rv1931 is unlikely to be responsible for the salicylate induced drug resistance in M. tuberculosis. On the other hand, salicylate may reduce permeability of the mycobacterial cell membrane as a possible mechanism. Using ^{14}C-INH it was found that the uptake of INH by M. tuberculosis H37Ra was reduced in the presence of salicylate (Y. Zhang, unpublished observation). The possibility of an involvement of efflux pumps in the salicylate-induced resistance in M. tuberculosis remains to be tested. Since salicylate is widely used in the prevention and treatment of diverse disease conditions at concentrations (as high as 2 mM) (199) that are known to induce resistance to various drugs in M. tuberculosis *in vitro*, it will be of interest to determine if salicylate could interfere with TB chemotherapy in animal models and in humans.

In a recent study, Wallis and colleagues found that different clinical isolates of M. tuberculosis had a differing ability to withstand the killing effect of antibiotics, a term the authors called drug tolerance (200). The tolerance is phenotypic as these isolates were apparently susceptible to antibiotics by conventional drug susceptibility testing (200). Tolerance to INH and EMB correlated with tolerance to RIF, and interestingly, the drug-tolerant isolates appeared to be associated with prolonged persistence or relapse, suggesting a nonspecific mechanism (200). It remains to be seen if drug tolerance might affect the outcome of TB therapy in a more systematic study.

5. PERSPECTIVE

Due to the emergence and rise of MDR-TB, the development of new anti-tuberculosis drugs is more important than ever. Currently the best method for TB control is the 6-month directly observed treatment, short-course (DOTS) that has been promoted by the World Health Organization (201). However, this strategy cannot control all outbreaks of MDR-TB. Given the current lengthy TB therapy and the potential difficulty in complete patient compliance to the therapy and the HIV pandemic, the drug-resistant TB problem is likely to continue. Unfortunately since TB is not a major health concern in the United States and other developed countries and TB drug development is viewed as unprofitable, the sense of urgency seems to be lacking by drug companies to develop new drugs against TB. Despite the perceived low priority by many large pharmaceutical companies, there is considerable interest and urgency to develop new TB drugs. A new organization “The Global Alliance for TB Drug Development” was recently established to facilitate new TB drug development (202). Hopefully with the greater understanding of the molecular aspects underlying drug resistance and with the availability of the M. tuberculosis genome (20) and new approaches for drug screening (see Mitchison in this Issue), new drugs that are not only active against MDR-TB, but also shorten the therapy can be developed. Given such new drugs are available, effective control of TB and drug-resistant TB requires a multifaceted approach that combines government commitment and support, improved socioeconomic conditions, rapid detection of drug-resistant TB, and above all, better adherence to the therapy.

5. ACKNOWLEDGEMENT

This work was supported by NIH grants AI44063 and AI49485 and the Potts Memorial Foundation to YZ. MMW was supported by NIH training grant T32 AI07608.

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Key Words: Disease, Infection, Microorganisms, Bacterium, Mycobacterium Tuberculosis, Anti-Tuberculosis Drugs, Mechanisms of Resistance, Tuberculosis, Review

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