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COMMENTARY

New Horizons in the Treatment of Tuberculosis

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ABSTRACT. The development of new chemotherapy for the treatment of tuberculosis has three major objectives: first, the development of faster-acting drugs to shorten the duration of treatment; second, the development of novel antimicrobials to counter the emergence of bacteria resistant to current therapies; and, third, the development of chemotherapeutics that specifically target dormant bacilli to treat the one-third of the world's population latently infected with tubercle bacilli. Strategies based upon optimizing the inhibition of known targets require an extensive knowledge of the detailed mechanism of action of current antimycobacterial agents. For many agents such as isoniazid, ethambutol, rifampin, and pyrazinamide such knowledge is now available. Strategies based upon the identification of novel targets will necessitate the identification of biochemical pathways specific to mycobacteria and related organisms. Many unique metabolic processes occur during the biosynthesis of mycobacterial cell wall components, and some attractive new targets have emerged. The development of targets specific to latency will require a detailed picture of the metabolism and biochemical pathways occurring in dormant bacilli. Recent evidence suggests that anaerobic metabolic pathways may operate in dormant bacilli, and the enzymes involved in such pathways may also provide significant new targets for intervention. The combination of the mycobacterial genome sequence that is anticipated to become available this year with an improved understanding of the unique metabolic processes that define mycobacteria as a genus offers the greatest hope for the elimination of one of mankind's oldest enemies. BIOCHEM PHARMACOL **54**;11: 1165–1172, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. tuberculosis; drug resistance; isoniazid; pyrazinamide; ethambutol; methyltransferase

Despite the existence of effective chemotherapy, more people died of tuberculosis in 1995 than in any other year in history [1]. Without immediate decisive action, 30 million people will die from tuberculosis in this decade, more than from AIDS, malaria, cholera, and other tropical diseases combined [2]. Current guidelines for the treatment of active disease call for initial treatment with isoniazid, rifampin, and pyrazinamide for 2 months with a continuation of the isoniazid and rifampin for 6 months [3]. This regimen is typically supplemented with ethambutol or streptomycin until drug susceptibility tests have been completed. This four-drug "short-course regimen" is generally effective against susceptible organisms in pulmonary infections of adults provided the patient completes the full course of antibiotic treatment. The failure of patients to complete therapy as well as inappropriate monotherapy has led to the emergence and distribution of strains of MTB† resistant to every available chemotherapy (MDR-TB) [4, 5]. Such organisms will not remain confined to the Third World or to the poor and indigent of developed countries. The recent documentation of the spread of a single clone of multidrug-resistant MTB (the "W" strain) throughout the continental United States and to Europe highlights the danger of an airborne pathogen in our global society [6].

In part, the difficulties with current treatment are related to the nature of the disease process, since an initial infection with aerosolized MTB is typically controlled but not completely eliminated by the host immune system [7]. The presence of latent bacteria within the host means the continuous threat of reactivation exacerbated by subsequent episodes of immunosuppression later in life. The AIDS pandemic has significantly worsened this situation; patients infected with tuberculosis alone have a 0.2% chance per annum of developing active disease, while patients coinfected with HIV and tuberculosis have a 5–10% chance of reactivation [2]. The long lag time between infection and disease further complicates treatment since dormant bacilli are refractory to most if not all currently used antimycobacterial agents [8]. In this country, for instance, the number of patients latently infected with the "W" strain virtually guarantees future epidemics of MDR-TB, which will occur unpredictably over the next few decades as these patients reactivate [6].

The challenge in developing future chemotherapy, therefore, lies in meeting these three goals: first, to improve the efficacy of current therapy and develop truly short-course antibiotic regimens to encourage patient compliance

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† *Abbreviations:* MTB, *Mycobacterium tuberculosis*; MDR-TB, multidrug-resistant TB; CMAS, cyclopropane mycolic acid synthase; MMAS, methoxy mycolic acid synthase; and Mb, megabases.

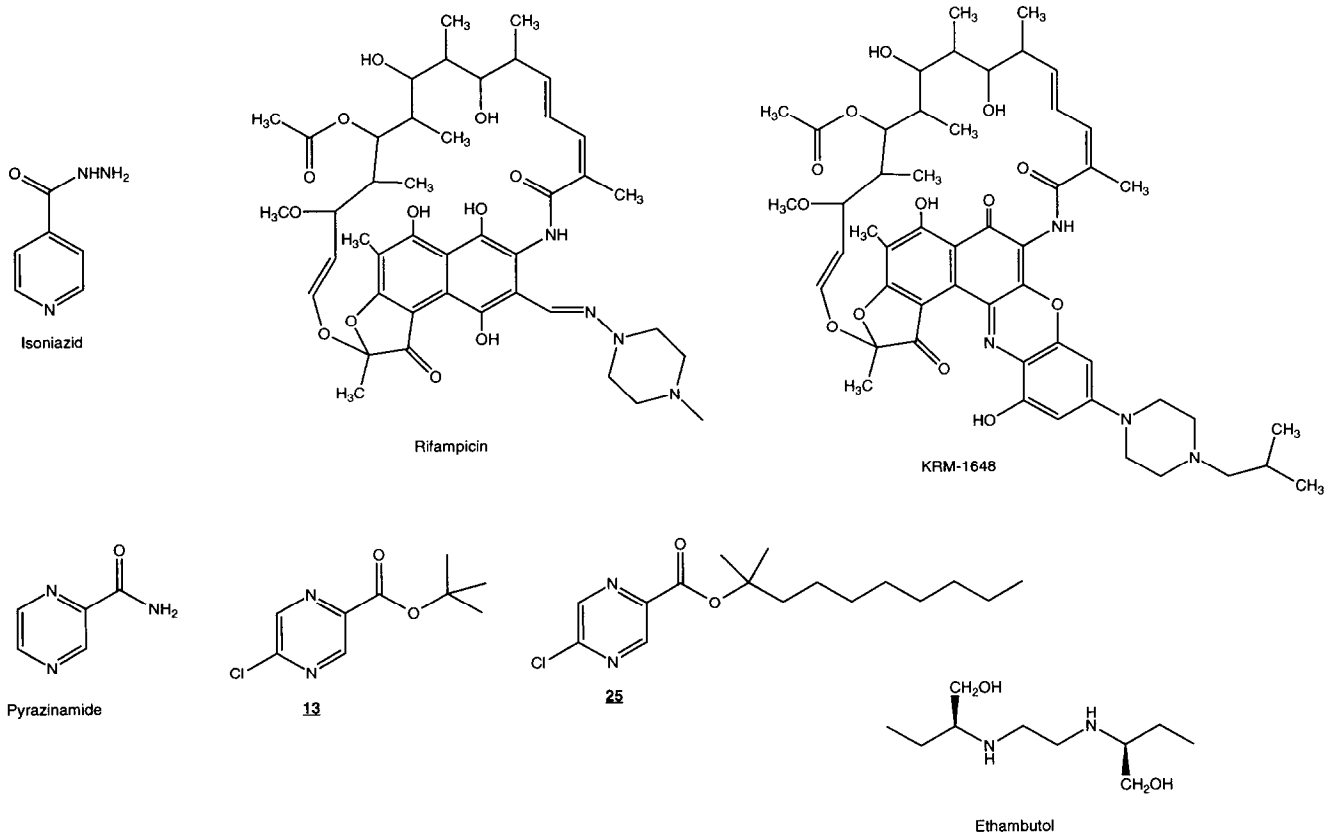

FIG. 1. Structures of antimycobacterial compounds.

and slow the development of drug resistance; second, to develop drugs with novel targets to treat patients already infected with MDR-TB; and third, to develop agents that specifically target latent organisms to treat the vast numbers of people at risk of developing active disease through reactivation. These goals will be facilitated by recent improvements in our understanding of the mechanism of action of current drugs, by recent advances in our understanding of the molecular biology and biochemistry of the tubercle bacillus, and by the mycobacterial genome project, which will better enable us to define biochemical pathways unique to the bacilli. This review is not intended to provide exhaustive coverage of the mechanisms of action of current antimycobacterials or an exhaustive review of potential antimycobacterial targets; the reader is pointed elsewhere for some excellent efforts in these directions [9–13]. Instead, this review is intended to highlight recent studies suggesting novel targets of high potential, which are sufficiently well characterized that the outline of future studies can be clearly defined.

REDESIGN OF EXISTING ANTIMYCOBACTERIALS

Isoniazid

Isoniazid (Fig. 1) demonstrates an amazing specificity for the tubercle bacilli with little or diminished activity against even closely related mycobacterial species and no activity against other unrelated bacteria [14]. Isoniazid is a pro-drug that requires activation by an endogenous mycobacterial enzyme, KatG, prior to exerting its bactericidal effect on a cellular target [15]. Isoniazid-resistant mutants frequently have an altered *katG* gene producing a mutant protein or lack KatG altogether [16]. Restoring *katG* to isoniazid-resistant strains defective in KatG production restores isoniazid sensitivity [17]. While the role of KatG in isoniazid resistance is well established, the actual function of KatG regarding isoniazid activation is unclear. The most informative work suggests that KatG serves to oxidize isoniazid to a highly reactive acyldiimide or acyldiazonium ion that could then react with a cellular nucleophile and thereby inactivate a specific target [18]. Recently, the conversion *in vitro* of isoniazid into isonicotinic acid and isonicotinamide was shown to proceed through the generation of an oxyferrous form of KatG by prior hydrazinolysis of a portion of the isoniazid [19]. This may not be an exclusive mechanism, however, as hydrogen peroxide and isoniazid toxicity are quite synergistic, suggesting that hydrogen peroxide may be a capable oxidant of isoniazid when present in sufficient quantity [20].

The definition of the actual active form of isoniazid seems unlikely due to the highly reactive nature of the intermediate and the extremely complex chemistry of the

acylpyridine nucleus. On the other hand, targeting oxidative stress response proteins such as KatG is a promising new direction in the development of antimycobacterial agents. In part, this is because the availability of isoniazid-induced *katG* mutants has resulted in a great deal of understanding of the biological function of these enzymes. Under normal conditions, KatG is the only catalase-peroxidase produced by MTB and is essential for growth of the organism inside macrophages but not *in vitro* [20, 21]. In most bacteria, *katG* is regulated by a transcription factor, called OxyR, which responds to oxidative conditions by up-regulating expression of the approximately 30 genes that comprise the OxyR regulon [22]. Recently, it was observed that MTB uniquely lacks a homologue of this transcription factor and has a severely circumscribed ability to affect gene regulation in response to oxidative challenge [23, 24]. The ability to increase KatG expression in the absence of OxyR may be related to another layer of catalase regulation mediated by the mycobacterial homologue of the iron-binding Fur protein, IdeR [25].

A gene related to KatG, which apparently conferred low-level isoniazid resistance on *Mycobacterium smegmatis* and whose promoter was mutated in isoniazid-resistant clinical isolates, was identified recently as *ahpC*, a mycobacterial thioredoxin-dependent alkylhydroperoxidase [26]. These and subsequent authors concluded that AhpC was contributing directly to isoniazid resistance by detoxifying toxic metabolites produced upon isoniazid activation [27]. A second interpretation of these results, that the observed AhpC up-regulation arose due to a compensatory mutation selected because of the loss of the required catalase-peroxidase function, was supported by the fact that in the absence of *katG* mutation massive AhpC overexpression does not confer isoniazid resistance on sensitive MTB [20]. Remarkably, this simple experiment continues to be ignored by some authors who claim that AhpC is somehow directly involved in detoxifying isoniazid in saprophytic M. *smegmatis* [28, 29]. Such studies have failed to account for KatG levels in these OxyR-proficient organisms and are undoubtedly simply measuring a decrease in isoniazid activation. What these experiments do make clear, however, is that KatG (or AhpC as a functional equivalent) is essential for the pathogenesis of MTB and that inhibitors of the catalase-peroxidase function (either as inhibitors of KatG in wild-type bacilli or as inhibitors of AhpC in KatG-deficient bacilli) represent a viable target for the development of new chemotherapy.

The second major area of research related to improving isoniazid is the identification of the target for the activated form of isoniazid to enable the design of analogues that would bypass the requirement for activation. Through extensive biochemical work, the target for isoniazid is known to lie in the biosynthetic pathway for the unique mycobacterial lipids, the mycolic acids [30]. These studies narrowed the search to a desaturase that produced tetracos-5-enoic acid [31–33]. Recently, a gene encoding an enoyl reductase was cloned by selecting for resistance to isoniazid in M. *smegmatis* an isoniazid-insensitive saprophytic mycobacterium [34]. This gene was shown to encode a protein called InhA, which has been subsequently crystallized and studied extensively [35, 36]. Unfortunately, this target conflicts with the wealth of biochemical information already in the literature, which suggests that the target in MTB was not an enoyl reductase but a desaturase. In fact, expressing InhA in the tubercle bacilli does not induce isoniazid resistance, and the failure of recent clinical studies to document any mutations in the InhA protein suggests that this protein is only an isoniazid target in M. *smegmatis* [37]. Obviously, the actual target for isoniazid in the mycolic acid biosynthetic pathway represents an attractive goal of future studies.

Rifampin

Rifampin (Fig. 1) is a key component of current antimycobacterial therapy, and resistance to this drug significantly lengthens treatment duration [12]. Rifampin acts by inhibiting the β-subunit of the DNA-dependent RNA polymerase encoded by the *rpoB* gene, and MTB, M. *smegmatis*, and M. *leprae* mutants resistant to rifampin are mutated in this gene [38–41]. The introduction of rifampin in the treatment of tuberculosis was instrumental in shortening therapy duration from 18 to 9 months, and these aspects have been reviewed recently [42].

Very active semi-synthetic derivatives of rifamycin have appeared recently, which promise some hope in further shortening the duration of chemotherapy and offer a proof of principle that improving drug affinity for current targets can shorten treatment duration. KRM-1648 (Fig. 1) is a benzoxazinorifamycin derivative, synthesized in 1991, which has been evaluated extensively in tuberculosis models [43–45]. *In vitro* studies were followed up quickly by studies in murine models in which KRM-1648 was shown to have potent antitubercular activity exceeding that of rifampin and rifabutin [46]. This activity was shown to be useful in combination therapy with isoniazid in mice and shown to have a significant advantage in the speed of clearance of organisms from infected animals [47, 48]. Organs from infected mice were sterile after 6 weeks of treatment with KRM-1648, while viable cells could still be observed even after 12 weeks of treatment with either isoniazid or rifampin. These results suggest that inclusion of KRM-1648 in standard combination therapy may shorten significantly the duration of such therapy [47]. Finally, although not effective against all isolates carrying mutant *rpoB* alleles, KRM-1648 is effective against some specific mutations that are clinically significant and has additional activity against atypical mycobacteria such as M. *avium* [49, 50].

Pyrazinamide

Pyrazinamide (Fig. 1) is an analogue of nicotinamide whose mode of action is remarkably analogous to that of isoniazid.

Like isoniazid, pyrazinamide is thought to be a pro-drug that requires deamidation by an endogenous mycobacterial enzyme, pyrazinamidase, to form pyrazinoic acid [51, 52]. Pyrazinoic acid is thought to be a toxic metabolite, but the precise cellular functions inhibited by this molecule have not been defined [53]. There have also been intriguing suggestions that pyrazinamide may have some efficacy against dormant or semi-dormant bacteria *in vitro*, although the molecular mechanisms involved remain obscure [54, 55]. Very recently, the requirement for activation of pyrazinamide via the action of a pyrazinamidase was proven by cloning the gene from MTB which encodes this enzyme (*pncA*). The *pncA* gene from a set of five clinical isolates resistant to pyrazinamide was examined and found to be mutated, correlating with a loss of pyrazinamidase activity. Transformation of strains with known *pncA* mutations with a wild-type *pncA* gene restored both pyrazinamidase activity and pyrazinamide sensitivity [56]. Also unexplained but of interest is the observation that pyrazinamide is only effective against bacilli at an acidic pH [57]. The possibility that the *pncA* gene is specifically responsive to acid pH will undoubtedly be resolved quickly and may offer further clues to the intracellular environment hosting MTB.

Knowledge of the mechanism of action of a prodrug may be helpful in designing out the requirement for activation and circumventing drug resistance. The intracellular production of pyrazinoic acid occurring by deamidation of pyrazinamide could also occur by hydrolysis of an ester of pyrazinoic acid by a non-specific cellular esterase [58]. Such esters have been shown to have potent activity not only against MTB but also against more difficult to treat atypical mycobacteria such as M. *avium* [59]. In addition recent compounds in the pyrazinoic acid ester series such as **13** and **25** have been shown to have 100-fold greater activity against MTB and 1000-fold greater serum stability [60]. These compounds offer considerable promise as potent new anti-tuberculars.

Ethambutol

The molecular mechanism of ethambutol action has undergone a renaissance in the recent literature, and the mode of action is now reasonably well defined. Early studies (reviewed in Ref. 9) suggested a target in the mycobacterial cell wall but were not successful in identifying the macromolecule that was directly affected. The observation by Takayama and Kilburn that arabinose incorporation into the cell wall arabinogalactan was inhibited [61] was the foundation for a series of experiments that resulted in the identification of arabinose decaprenylmonophosphate as the biogenetic source of arabinose in the cell wall arabinogalactan [62]. These studies demonstrated that arabinose incorporation into the cell wall was specifically inhibited, while arabinose incorporation into lipoarabinomannan was not as severely affected [63]. Chemical synthesis of the polyprenolphosphate arabinose donor allowed a direct demonstration of the effect of ethambutol in cell-free assays of arabinose transfer [64]. Mutants of M. *smegmatis* resistant to ethambutol show that the effects of the drugs also extend to the biosynthesis of lipoarabinomannan, pointing out a commonality between the biosynthetic incorporation of arabinose into these two macromolecules [65, 66]. In fact, the heterogeneity in the truncated forms of arabinan used in cell wall arabinogalactan and lipoarabinomannan together suggest that ethambutol may have many discrete arabinosyltransferases as its physiological targets with varying inhibition constants [66]. Recently, a three-gene operon from M. *avium* was identified (the *emb* region) which was sufficient to confer ethambutol resistance upon M. *smegmatis* when present on a high copy vector [67]. Two of the genes (*embA* and *embB*) encode apparent arabinosyltransferases and are homologous to each other, while the third gene is homologous to transcriptional repressors and may play a role in regulating operon expression. Ethambutol resistance was shown to be correlated with overexpression of the two arabinosyltransferases, and these activities were supported by *in vitro* assays. These results suggest that the *embAB* genes encode the enzymes responsible for the polymerization of arabinose into cell-wall bound arabinan, the primary target for ethambutol. As will be pointed out again later in the context of mycolate modification enzymes, targeting several enzymes that catalyze related chemical functions is a significant advantage in terms of slowing the development of drug resistance, since single mutational events in such targets would not confer resistance to the antibiotic. The availability of arabinosyl transferase *in vitro* assays and the appropriate arabinose donor for such enzymes, coupled with the ability to identify likely glycosyltransferases as they emerge from the mycobacterial genome sequence, suggest that such enzymes may become increasingly important in future efforts to develop effective new chemotherapy [68].

NOVEL TARGETS FOR FUTURE DEVELOPMENT

Mycolic Acid Methyltransferases

Mycolic acids represent a major constituent of the mycobacterial cell wall complex and form the inner leaflet of a terminal covalently attached asymmetric lipid bilayer [30, 69]. The properties of this bilayer appear to be a direct consequence of the various functional groups occurring in the longer chain of these complex α-alkyl-β-hydroxy fatty acids [70]. Maintenance of the proper viscosity in this membrane will be essential not just for ensuring appropriate diffusion rates of hydrophobic molecules but also for proper enzyme function in this extremely hydrophobic microenvironment. In pathogenic species of mycobacteria the most common modification is the introduction of *cis*-cyclopropanes at two positions in the α-alkyl chain. An enzyme (CMAS-1) involved in *cis*-cyclopropane formation was cloned in a saprophytic mycobacterial species that does not normally catalyze this transformation, and this functional group was thereby shown to play a role in the protection of

mycolic acids from degradation with peroxides [71]. This first enzyme had specificity for one of the two functionalized positions, and a second enzyme, CMAS-2, was cloned by homology and shown to have specificity for the other functionalized position [72]. This second *cis*-cyclopropane was shown to have an effect on the fluidity of the mycobacterial cell wall.

These two enzymes also showed high homology to a cluster of four enzymes whose functions were deduced recently by individually expressing each enzyme as well as expressing them pairwise. These four enzymes were sufficient to catalyze the formation of the second major series of mycolates occurring in the tubercle bacilli, methoxymycolates. Three of these four enzymes were shown to be involved in the formation of the *cis*-cyclopropane containing methoxymycolate series by catalyzing the following series of reactions: MMAS-2 catalyzes the introduction of a *cis*-cyclopropane into the oxygenated mycolates, MMAS-4 introduces an α-methyl hydroxy group, and MMAS-3 O-methylates the secondary alcohol to form the methyl ether [73]. The last gene in this cluster has been shown recently to catalyze the conversion of a *cis*-olefin into a *trans*-olefin with concomitant introduction of an allylic methyl branch (Yuan Y and Barry CE III, unpublished results). This enzyme family is highly homologous so that the least degree of identity between any two members is 52%, and all share an S-adenosyl-L-methionine binding site, which is absolutely conserved [73]. These features suggest the simplifying hypothesis that all of these enzymes function using a common chemical mechanism involving an initial methyl group addition to an olefinic mycolate precursor and that the fate of the cationic intermediate species thus formed determines the structure of the resulting mycolate (Fig. 2). As was noted earlier in this review, this makes these enzymes attractive drug targets since targeting of this common intermediate could be done in such a way as to affect the entire family of enzymes simultaneously. The essentiality of these targets is suggested by the fact that perturbations in the normal complement of mycolates induced in the various recombinant organisms has had profound effects on measurable macroscopic characteristics of the cell wall and the organism producing this wall.

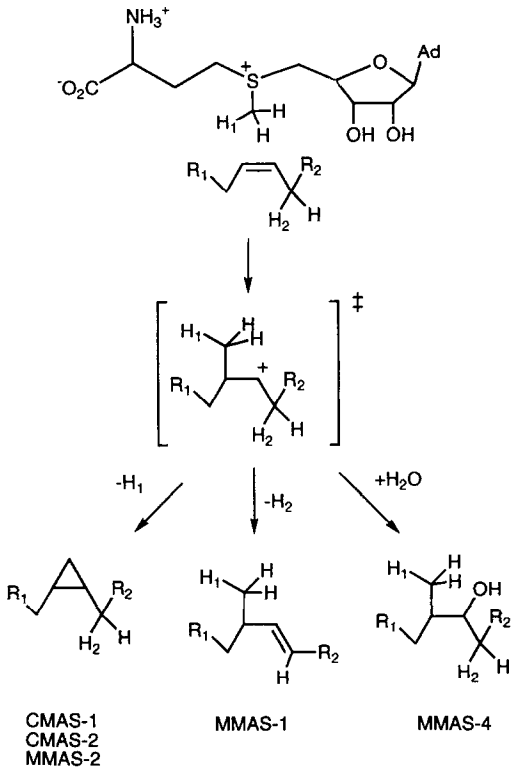

FIG. 2. The common cationic intermediate formed during mycolic acid modification.

EMERGING TARGETS IN LATENT BACILLI

The vast majority of tuberculosis cases arising in the United States and other developed countries are the result of reactivation, not initial infection, and as many as 10–15 million Americans have such infections. Lesions from such patients typically consist of completely blocked airways harboring bacilli that may or may not be cultivable [7]. MTB is a facultative aerobe that is known to possess various anaerobic metabolic capabilities, and latent bacilli are thought to inhabit a largely anaerobic microenvironment [74]. The ability of MTB to grow under reduced oxygen tension has been correlated with virulence, and fully virulent organisms metabolize glucose, for example, primarily through anaerobic glycolysis [75]. Understanding the anaerobic metabolic pathways involved in survival of latent bacilli has been facilitated by the development of *in vitro* model systems, which enable the phenotypic adaptation of bacilli to a condition allowing them to survive anaerobically [7, 76]. The development of such systems is allowing the definition of proteins essential for long-term survival of MTB. One such protein is the mycobacterial homologue of a family of small heat-shock proteins, called α-crystallins due to their initial discovery in vertebrate eyes. This protein appears to contribute to protein stability and diminished autolysis of stationary-phase organisms, and heterologous expression in the saprophyte M. *smegmatis* (which does not normally contain the gene for a corresponding protein) confers some of these properties on the recombinant organism [76]. Expression of the α-crystallin protein is not observed under aerobic growth conditions but is specifically triggered by lowered oxygen content during the transition to stationary phase. In fact, small changes in atmospheric oxygen content (from 10 to 2.5% oxygen in nitrogen) induce a dramatic up-regulation of expression of this protein, suggesting that the primary regulatory mechanism for expression of this protein is oxygen dependent [76] (Barry CE III and Sherman DR, unpublished results). Other proteins are known to be induced when the bacilli adapt to low oxygen, among them the enzymes isocitrate lyase and glycine dehydrogenase [77].

An exciting case has been made recently for the specific

action of a nitroimidazole compound, metronidazole, on dormant bacilli [78]. Metronidazole has long been known, and used, for the treatment of obligate anaerobes such as trichomonads, *Entamoeba histolytica*, *Giardia intestinalis*, and more recently against *Helicobacter pylori* in the treatment of gastroduodenal ulcers [79]. The action against anaerobic MTB presumably occurs through reduction of the nitroimidazole by one electron to the corresponding nitro radical anion, which could then decompose to give nitrite and an imidazole radical. This imidazole radical is then thought to inflict DNA damage upon the cell as the ultimate mechanism of action [80]. Any redox reaction in the cell with a reduction potential more negative than the nitroimidazole could conceivably cause activation of the drug. Typically, aerobes possesses only redox couples with higher potentials than that necessary to reduce such compounds and are therefore unaffected. The requirement for anaerobic bacilli could indicate that a unique protein system is up-regulated during the shift to anaerobiosis. This need not be the case, though, as activation of nitroimidazoles in the presence of oxygen results in regeneration of the nitro compound in a process known as futile cycling [81, 82]. Thus, the mycobacterial enzyme may be present during aerobic growth but would not appear to activate drug. The identity of the mycobacterial enzyme that activates metronidazole will be a valuable clue to anaerobic metabolism in the tubercle bacilli and may provide a novel target for the design of nitroimidazole analogues of increased potency.

SUMMARY

The preceding discussion on targets is an obviously biased appraisal of currently available options, but these are offered as proof that novel targets exist, and each in some way represents a potential pathway to the three goals described in the introduction. It is essential at this point not to underestimate the radical transformation that mycobacterial research will undergo in the next year with the completion of the mycobacterial genome project. An integrated map of the genome of MTB was reported early last year, and the cosmids from this map are being sequenced [83]. Currently, the genome project has produced about 3.33 Mb of sequence (the genome is estimated at 4.4 Mb) with about half of the sequenced DNA being fully annotated and deposited in the EMBL database (the sequence information and annotated cosmids are available directly online at "http://www.sanger.ac.uk/pathogens/"; this site also features a BLAST server specifically for the MTB sequences). In addition, an integrated mycobacterial database has been established and is being maintained covering this and related information [84]. ("http://kiev.physchem.kth.se/MycDB.html"). As mycobacteriologists begin attempting to fit this explosion of information into the context of the wealth of accumulated information about the biology of the organism, novel pathways and targets will emerge.

The sobering reality is that not a single antimycobacterial agent has been introduced into widespread use since rifampicin in the 1960s. The need for novel antimycobacterials is greater than ever, and it is only a question of time before increasing numbers of outbreaks of MDR-TB in developed countries force the reallocation of resources necessary to produce new pharmaceuticals. In only focusing on active disease, the potential market is simply too small to interest many pharmaceutical companies. Identification of targets that might be efficacious in treating tuberculin skin test positive individuals latently infected with anaerobically growing organisms could provide both an expanded market and some real hope for the global eradication of tuberculosis.

References

1. World Health Organization, *TB: Groups at Risk, WHO Report on the Tuberculosis Epidemic*. World Health Organization, Geneva, Switzerland, 1996.
2. Dolin PJ, Raviglione MC and Kochi A, Global tuberculosis incidence and mortality during 1990–2000. *Bull World Health Organ* **72:** 213–220, 1994.
3. Bass JB, Farer LS, Hopewell PC, O'Brien R, Jacobs RF, Ruben F, Dixie E, Snider J and Thornton G, Treatment of tuberculosis and tuberculosis infection in adults and children. *Am J Respir Crit Care Med* **149:** 1359–1374, 1994.
4. Snider DE Jr and Roper WL, The new tuberculosis. *N Engl J Med* **326:** 703–705, 1992.
5. Bloom BR and Murray CJL, Tuberculosis: Commentary on a reemergent killer. *Science* **257:** 1055–1064, 1992.
6. Bifani PJ, Plidaytis BB, Kapur V, Stockbauer K, Pan X, Lutfey ML, Moghazeh SL, Eisner W, Daniel TM, Kaplan MH, Crawford JT, Musser JM and Kreiswirth BN, Origin and interstate spread of a New York City multidrug-resistant *Mycobacterium tuberculosis* clone family. *JAMA* **275:** 452–457, 1996.
7. Wayne LG, Dormancy of *Mycobacterium tuberculosis* and latency of disease. *Eur J Clin Microbiol Infect Dis* **13:** 908–914, 1994.
8. Wayne LG and Hayes LG, An *in vitro* model for sequential study of shiftdown of *Mycobacterium tuberculosis* through two stages of nonreplicating persistence. *Infect Immun* **64:** 2062–2069, 1996.
9. Winder FG, Mode of action of antimycobacterial agents and associated aspects of the molecular biology of the mycobacteria. In: *The Biology of the Mycobacteria* (Eds. Ratledge C and Standford J), Vol. 1, pp. 353–438. Academic Press, London, 1982.
10. Young DB, Strategies for new drug development. In: *Tuberculosis: Pathogenesis, Protection, and Control* (Ed. Bloom BR), pp. 559–567. American Society for Microbiology, Washington, DC, 1994.
11. Young DB and Duncan K, Prospects for new interventions in the treatment and prevention of mycobacterial disease. *Annu Rev Microbiol* **49:** 641–673, 1995.
12. Musser JM, Antimicrobial agent resistance in mycobacteria: Molecular genetic insights. *Clin Microbiol Rev* **8:** 496–514, 1995.
13. Cole ST and Telenti A, Drug resistance in *Mycobacterium tuberculosis*. *Eur Respir J* **8** (Suppl 20): 701s–713s, 1995.
14. Takayama K and Davidson LA, Isonicotinic acid hydrazide. In: *Antibiotics: Mechanism of Action of Antibacterial Agents* (Ed. Hahn FE), Vol. I, pp. 98–119. Springer, Berlin, 1979.
15. Zhang Y, Heym B, Allen B, Young D and Cole S, The

catalase-peroxidase gene and isoniazid resistance of *Mycobacterium tuberculosis*. *Nature* **358:** 591–593, 1992.
16. Musser JM, Kapur V, Williams DL, Kreiswirth BN, van Soolingen D and van Embden JDA, Characterization of the catalase-peroxidase gene (*katG*) and *inhA* locus in isoniazid-resistant and -susceptible strains of *Mycobacterium tuberculosis* by automated DNA sequencing: Restricted array of mutations associated with drug resistance. *J Infect Dis* **173:** 196–202, 1996.
17. Zhang Y, Garbe T and Young D, Transformation with *katG* restores isoniazid-sensitivity in *Mycobacterium tuberculosis* isolates resistant to a range of drug concentrations. *Mol Microbiol* **8:** 521–524, 1993.
18. Johnsson K and Schultz PG, Mechanistic studies of the oxidation of isoniazid by the catalase peroxidase from *Mycobacterium tuberculosis*. *J Am Chem Soc* **116:** 7425–7426, 1994.
19. Magliozzo RS and Marcinkeviciene JA, Evidence for isoniazid oxidation by oxyferrous mycobacterial catalase-peroxidase. *J Am Chem Soc* **118:** 11303–11304, 1996.
20. Sherman DR, Mdluli K, Hickey MJ, Arain TM, Morris SL, Barry CE III and Stover CK, Compensatory *ahpC* gene expression in isoniazid-resistant *Mycobacterium tuberculosis*. *Science* **272:** 1641–1643, 1996.
21. Wilson TM, de Lisle GW and Collins DM, Effect of *inhA* and *katG* on isoniazid resistance and virulence of *Mycobacterium bovis*. *Mol Microbiol* **15:** 1009–1015, 1995.
22. Christman MF, Morgan RW, Jacobson FS and Ames BN, Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in *Salmonella typhimurium*. *Cell* **41:** 753–762, 1985.
23. Sherman DR, Sabo PJ, Hickey MJ, Arain TM, Mahairas GG, Yuan Y, Barry CE III and Stover CK, Disparate responses to oxidative stress in saprophytic and pathogenic mycobacteria. *Proc Natl Acad Sci USA* **92:** 6625–6629, 1995.
24. Deretic V, Philipp W, Dhandayuthapani S, Mudd MH, Curcic R, Garbe T, Heym B, Via LE and Cole ST, *Mycobacterium tuberculosis* is a natural mutant with an inactivated oxidative-stress regulatory gene: Implications for sensitivity to isoniazid. *Mol Microbiol* **17:** 889–900, 1995.
25. Dussurget O, Rodriguez M and Smith I, An *ideR* mutant of *Mycobacterium smegmatis* has derepressed siderophore production and an altered oxidative-stress response. *Mol Microbiol* **22:** 535–544, 1996.
26. Wilson TM and Collins DM, *ahpC*, a gene involved in isoniazid resistance of the *Mycobacterium tuberculosis* complex. *Mol Microbiol* **19:** 1025–1034, 1996.
27. Dhandayuthapani S, Zhang Y, Mudd MH and Deretic V, Oxidative stress response and its role in sensitivity to isoniazid in mycobacteria: Characterization and inducibility of *ahpC* by peroxides in *Mycobacterium smegmatis* and lack of expression in M. *ƒurum* and M. *tuberculosis*. *J Bacteriol* **178:** 3641–3649, 1996.
28. Zhang Y, Dhandayuthapani S and Deretic V, Molecular basis for the exquisite sensitivity of *Mycobacterium tuberculosis* to isoniazid. *Proc Natl Acad Sci USA* **93:** 13212–13216, 1996.
29. Deretic V, Pagan-Ramos E, Zhang Y, Dhandayuthapani S and Via LE, The extreme sensitivity of *Mycobacterium tuberculosis* to the front-line antituberculosis drug isoniazid. *Nature Biotechnol* **14:** 1557–1561, 1996.
30. Barry CE III and Mdluli K, Drug sensitivity and environmental adaptation of mycobacterial cell wall components. *Trends Microbiol* **4:** 275–281, 1996.
31. Takayama K, Wang L, and David HL, Effect of isoniazid on the *in vivo* mycolic acid synthesis, cell growth, and viability of *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* **2:** 29–35, 1972.
32. Takayama K, Schnoes HK, Armstrong EL and Boyle RW, Site of inhibitory action of isoniazid in the synthesis of mycolic acids in *Mycobacterium tuberculosis*. *J Lipid Res* **16:** 308–317, 1975.
33. Davidson LA and Takayama K, Isoniazid inhibition of the synthesis of monounsaturated long-chain fatty acids in *Mycobacterium tuberculosis* H37Ra. *Antimicrob Agents Chemother* **16:** 104–105, 1979.
34. Banerjee A, Dubnau E, Quemard A, Balasubramanian V, Um KS, Wilson T, Collins D, de Lisle G and Jacobs WR Jr, *InhA*, a gene encoding a target for isoniazid and ethionamide in *Mycobacterium tuberculosis*. *Science* **263:** 227–230, 1994.
35. Dessen A, Quenmard A, Blanchard JS, Jacobs WR Jr and Sacchettini JC, Crystal structure and function of the isoniazid target of *Mycobacterium tuberculosis*. *Science* **267:** 1638–1641, 1995.
36. Sacchettini JC and Blanchard JS, The structure and function of the isoniazid target in M. *tuberculosis*. *Res Microbiol* **147:** 36–43, 1996.
37. Mdluli K, Sherman DR, Hickey MJ, Kreiswirth BN, Morris S, Stover CK and Barry CE III, Biochemical and genetic data suggest that inhA is not the primary target for activated isoniazid in *Mycobacterium tuberculosis*. *J Infect Dis* **174:** 1085–1090, 1996.
38. Cole ST, Rifamycin resistance in mycobacteria. *Res Microbiol* **147:** 48–52, 1996.
39. Telenti A, Imboden P, Marchesi F, Schidheini T and Bodmer T, Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis*. *Lancet* **341:** 647–650, 1993.
40. Levin ME and Hatfull GF, *Mycobacterium smegmatis* RNA polymerase: DNA supercoiling, action of rifampicin and mechanism of refampicin resistance. *Mol Microbiol* **8:** 277–285, 1993.
41. Honore N and Cole ST, Molecular basis of rifampin resistance in *Mycobacterium leprae*. *Antimicrob Agents Chemother* **37:** 414–418, 1993.
42. Grosset J, Current problems with tuberculosis treatment. *Res Microbiol* **147:** 10–16, 1996.
43. Saito H, Tomioka H, Sato K, Emori M, Yamane T, Yamashita K, Hosoe K and Hidaka T, *In vitro* antimycobacterial activities of newly synthesized benzoxazinorifamycins. *Antimicrob Agents Chemother* **35:** 542–547, 1991.
44. Kuze F, Yamamoto T, Amitani R and Suzuki K, *In vivo* activities of new rifamycin derivatives against mycobacteria. *Kekkaku* **66:** 7–12, 1990.
45. Yamane T, Hashizume T, Yamashita K, Konishi E, Hosoe K, Hidaka T, Watanabe K, Kawaharada H, Yamamoto T and Kuze F, Synthesis and biological activity of 3′-hydroxy-5′-aminobenzoxazinorifamycin derivatives. *Chem Pharm Bull (Tokyo)* **41:** 148–155, 1993.
46. Klemens SP, Grossi MA and Cynamon MH, Activity of KRM-1648, a new benzoxazinorifamycin, against *Mycobacterium tuberculosis* in a murine model. *Antimicrob Agents Chemother* **38:** 2245–2248, 1994.
47. Klemens SP and Cynamon MH, Activity of KRM-1648 in combination with isoniazid against *Mycobacterium tuberculosis* in a murine model. *Antimicrob Agents Chemother* **40:** 298–301, 1996.
48. Yamamoto T, Amitani R, Suzuki K, Tanaka E, Murayama T and Kuze F, *In vitro* bactericidal and *in vivo* therapeutic activities of a new rifamycin derivative, KRM-1648, against *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* **40:** 426–428, 1996.
49. Tomioka H, Saito H, Sato K, Yamane T, Yamashita K, Hosoe K, Fujii K and Hidaka T, Chemotherapeutic efficacy of a newly synthesized benzoxazinorifamycin, KRM-1648, against *Mycobacterium avium* complex infection induced in mice. *Antimicrob Agents Chemother* **36:** 387–393, 1992.
50. Mohazeh SL, Pan X, Arain T, Stover CK, Musser JM and Kreiswirth BN, Comparative antimycobacterial activities of

rifampin, rifapentine, and KRM-1648 against a collection of rifampin-resistant *Mycobacterium tuberculosis* isolates with known *rpoB* mutations. *Antimicrob Agents Chemother* **40:** 2655–2657, 1996.
51. Konno K, Feldman FM and McDermott W, Pyrazinamide susceptibility and amidase activity of tubercle bacilli. *Am Rev Respir Dis* **95:** 461–469, 1967.
52. Butler WR and Kilburn JO, Susceptibility of *Mycobacterium tuberculosis* to pyrazinamide and its relationship to pyrazinamidase activity. *Antimicrob Agents Chemother* **24:** 600–601, 1983.
53. Heifets LB, Flory MA and Lindholm-Levy PJ, Does pyrazinoic acid as an active moiety of pyrazinamide have specific activity against *Mycobacterium tuberculosis*? *Antimicrob Agents Chemother* **33:** 1252–1254, 1989.
54. Mitchison DA, The action of antituberculosis drugs in short-course therapy. *Tubercle* **66:** 219–225, 1985.
55. Heifets L and Lindholm-Levy P, Pyrazinamide sterilizing activity *in vitro* against semi-dormant *Mycobacterium tuberculosis* bacterial populations. *Am Rev Respir Dis* **145:** 1223–1225, 1992.
56. Scorpio A and Zhang Y, Mutations in *pncA*, a gene encoding pyrazinamidase/nicotinamidase, cause resistance to the antituberculous drug pyrazinamide in tubercle bacillus. *Nature Med* **2:** 662–667, 1996.
57. McDermott W and Tomsett R, Activation of pyrazinamide and nicotinamide in acidic environments *in vitro*. *Am Rev Tuberc* **70:** 748–754, 1954.
58. Cynamon MH, Klemens SP, Chou TS, Gimi RH and Welch JT, Antimycobacterial activity of a series of pyrazinoic acid esters. *J Med Chem* **35:** 1212–1215, 1992.
59. Cynamon MH, Gimi R, Gyenes F, Sharpe CA, Bergmann KE, Han H-J, Gregor LB, Rapolu R, Luciano G and Welch JT, Pyrazinoic acid esters with broad spectrum *in vitro* antimycobacterial activity. *J Med Chem* **38:** 3902–3907, 1995.
60. Bergmann KE, Cynamon MH and Welch JT, Quantitative structure-activity relationships for the *in vitro* antimycobacterial activity of pyrazinoic acid esters. *J Med Chem* **39:** 3394–3400, 1996.
61. Takayama K and Kilburn JO, Inhibition of synthesis of arabinogalactan by ethambutol in *Mycobacterium smegmatis*. *Antimicrob Agents Chemother* **33:** 1493–1499, 1989.
62. Wolucka BA, McNeil MR, de Hoffmann E, Chojnacki T and Brennan PJ, Recognition of the lipid intermediated for arabinogalactan/arabinomannan biosynthesis and its relation to the mode of action of ethambutol on mycobacteria. *J Biol Chem* **269:** 23328–23335, 1994.
63. Deng L, Mikusova K, Robuck KG, Scherman M, Brennan PJ and McNeil MR, Recognition of multiple effects of ethambutol on metabolism of mycobacterial cell envelope. *Antimicrob Agents Chemother* **39:** 694–701, 1995.
64. Lee RE, Mikusova K, Brennan PJ and Besra GS, Synthesis of the mycobacterial arabinose donor β-D-arabinofuranosyl-1-monophosphoryl-decaprenol, development of a basic arabinosyl-transferase assay, and identification of ethambutol as an arabinosyl transferase inhibitor. *J Am Chem Soc* **117:** 11829–11832, 1995.
65. Khoo K-H, Douglas E, Azadi P, Inamine JM, Besra GS, Mikusova K, Brennan PJ and Chatterjee D, Truncated structural variants of lipoarabinomannan in ethambutol drug-resistant strains of *Mycobacterium smegmatis*. *J Biol Chem* **271:** 28682–28690, 1996.
66. Mikusova K, Slayden RA, Besra GS and Brennan PJ, Biogenesis of the mycobacterial cell wall and the site of action of ethambutol. *Antimicrob Agents Chemother* **39:** 2484–2489, 1995.
67. Belanger AE, Besra GS, Ford ME, Mikusova K, Belisle JT, Brennan PJ and Inamine JM, The *embAB* genes of *Mycobacterium avium* encode an arabinosyl transferase involved in cell wall arabinan biosynthesis that is the target for the antimycobacterial drug ethambutol. *Proc Natl Acad Sci USA* **93:** 11919–11924, 1996.
68. Maddry JA, Suling WJ and Reynolds RC, Glycosyltransferases as targets for inhibition of cell wall synthesis in *M. tuberculosis* and *M. avium*. *Res Microbiol* **147:** 106–112, 1996.
69. Brennan PJ and Nikaido H, The envelope of mycobacteria. *Annu Rev Biochem* **64:** 29–63, 1995.
70. Liu J, Barry CE III, Besra GS and Nikaido H, Mycolic acid structure determines the fluidity of the mycobacterial cell wall. *J Biol Chem* **271:** 29545–29551, 1996.
71. Yuan Y, Lee RE, Besra GS, Belisle JT and Barry CE III, Identification of a gene involved in the biosynthesis of cyclopropanated mycolic acids in *Mycobacterium tuberculosis*. *Proc Natl Acad Sci USA* **92:** 6630–6634, 1995.
72. George KM, Yuan Y, Sherman DR and Barry CE III, The biosynthesis of cyclopropanated mycolic acids in *Mycobacterium tuberculosis*: Identification and functional analysis of CMAS-2. *J Biol Chem* **270:** 27292–27298, 1995.
73. Yuan Y and Barry CE III, A common mechanism for the biosynthesis of methoxy and cyclopropyl mycolic acids in *Mycobacterium tuberculosis*. *Proc Natl Acad Sci USA* **93:** 12828–12833, 1996.
74. Segal W, Growth dynamics of *in vivo* and *in vitro* grown mycobacterial pathogens. In: *The Mycobacteria, A Sourcebook, Part A* (Eds. Kubica GP and Wayne LG), Vol. 15, pp. 547–573. Marcel Dekker, New York, 1984.
75. Ramakrishnan T, Indira M and Maller RK, Evaluation of the route of glucose utilization in virulent and avirulent strains of *Mycobacterium tuberculosis*. *Biochim Biophys Acta* **59:** 529–532, 1962.
76. Yuan Y, Crane DD and Barry CE III, Stationary phase-associated protein expression in *Mycobacterium tuberculosis*: Function of the mycobacterial α-crystallin homolog. *J Bacteriol* **178:** 4484–4492, 1996.
77. Wayne LG and Lin K-Y, Glyoxylate metabolism and adaptation of *Mycobacterium tuberculosis* to survival under anaerobic conditions. *Infect Immun* **37:** 1042–1049, 1982.
78. Wayne LG and Sramek HA, Metronidazole is bactericidal to dormant cells of *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* **38:** 2054–2058, 1994.
79. Edwards DI, Nitroimidazole drugs—Action and resistance mechanisms. I. Mechanisms of action. *J Antimicrob Chemother* **31:** 9–20, 1993.
80. Knox RJ, Edwards DI and Knight RC, The mechanism of nitroimidazole damage to DNA: Coulometric evidence. *Int J Radiat Oncol Biol Phys* **10:** 1315–1318, 1984.
81. Sealy RC, Swartz HM and Olive PL, Electron spin resonance-spin trapping detection of superoxide formed during aerobic reduction of nitro compounds. *Biochem Biophys Res Commun* **82:** 680, 1978.
82. Perez-Reyes E, Kalyanaraman B and Mason RP, The reductive metabolism of metronidazole and ornidazole by aerobic liver microsomes. *Mol Pharmacol* **17:** 239–244, 1980.
83. Philipp WJ, Poulet S, Eiglmeier K, Pascopella L, Balasubramanian V, Heym B, Bergh S, Bloom BR, Jacobs WR Jr and Cole ST, An integrated map of the genome of the tubercle bacillus, *Mycobacterium tuberculosis* H37Rv, and comparison with *Mycobacterium leprosy*. *Proc Natl Acad Sci USA* **93:** 3132–3137, 1996.
84. Bergh S and Cole ST, MycDB: An integrated mycobacterial database. *Mol Microbiol* **12:** 517–534, 1994.