Tuberculosis: Pathogenesis, Protection, and Control
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Chapter 32

Strategies for New Drug Development

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The emergence of strains of *Mycobacterium tuberculosis* resistant to existing drugs has focused attention on the urgent need for development of new antimycobacterial agents. Such agents have not been perceived as a high priority by pharmaceutical companies over the last 30 years, and a coordinated effort to screen general antimicrobial compounds developed during this time for activity against *M. tuberculosis* may well prove worthwhile. The recent development of genetic tools for monitoring the viability of *M. tuberculosis* provides a rapid approach for this type of screening (Jacobs et al., 1993). From a broader perspective, molecular genetic tools for study and manipulation of mycobacteria provide access to a vast amount of new information about the biochemistry and metabolism of *M. tuberculosis*, and exploitation of this information has important potential in the rational development of a new generation of antimycobacterial agents and perhaps in the design of improved strategies for use of existing drugs. This chapter focuses on the prospects for using a fundamental molecular approach to identification of novel lead compounds for new drug development. Further important steps in drug development, such as toxicity testing, optimization of pharmacokinetics, etc., are not addressed in this review.

In selecting targets for antimicrobial agents, it is clearly advantageous to avoid bacterial enzymes with closely related counterparts in mammalian cells. In addition, to avoid disruption of normal microbial flora during the prolonged course of tuberculosis therapy and to limit possible transfer of resistance factors from other bacteria! genera, it is preferable that new drug targets be specific for mycobacteria. Drugs must act on a target that is essential for bacterial survival, and ideally, they should be effective against bacteria throughout their growth cycle both inside and outside mammalian cells during infection. In this section, we first review existing and potential drug targets in *M. tuberculosis*. We then discuss distinctive features of mycobacteria relevant to drug design, and finally, we consider experimental approaches applicable to rational drug discovery programs.

DRUG TARGETS IN *M. TUBERCULOSIS*

Most antibacterial agents inhibit biosynthetic pathways involved in the production of macromolecules (proteins, nucleic acids, or cell wall polymers). Several of the broad-spectrum antibacterial agents are effective

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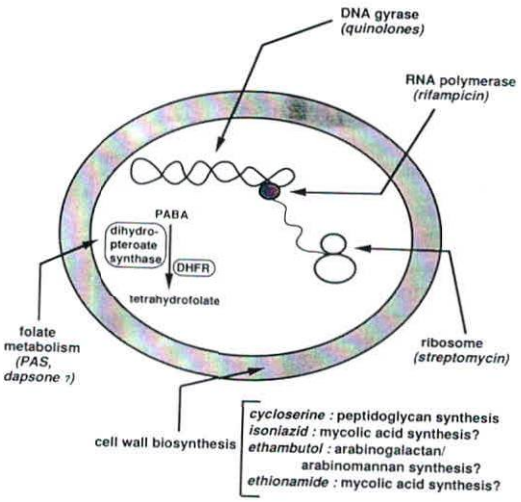

Figure 1. Sites of action of antimycobacterial agents. PABA, *p*-aminobenzoic acid; DHFR, dihydrofolate reductase; PAS, *p*-aminosalicylic acid.

against mycobacteria (Fig. 1), and the sites of action of these existing drugs clearly represent potential targets for new drug development.

Protein Synthesis

Streptomycin, the first antibiotic available for widespread use in treatment of tuberculosis, is a member of the aminoglycoside family that disrupts bacterial protein synthesis. As in other bacteria, streptomycin resistance in *M. tuberculosis* is conferred by mutations that alter the ribosomal protein S12 or the ribosomal 16S RNA molecule (Finken et al., 1993). Kanamycin, a related aminoglycoside with a similar mode of action, and its semisynthetic derivative amikacin are also used in tuberculosis therapy. While protein synthesis is clearly an important drug target in *M. tuberculosis*, other families of protein synthesis inhibitors (tetracycline, chloramphenicol, and the macrolides [e.g., erythromycin]) have no clinical use against tuberculosis. The intensive effort that has gone into development of protein synthesis inhibitors and the rather limited spectra of those agents found to be effective against tuberculosis suggest that the ribosome may not be a particularly attractive target for new antituberculous drug design.

Nucleic Acids

Sulfonamides, which were the first clinically effective antibacterial agents, are structural analogs of *p*-aminobenzoic acid that inhibit biosynthesis of tetrahydrofolic acid, thus blocking production of the purine and pyrimidine bases required for nucleic acid synthesis. The antituberculous drug *p*-aminosalicylic acid (Fig. 2) was initially designed as a competitive inhibitor of salicylic acid and may act on the tetrahydrofolate pathway as well as on the salicylate-dependent biosynthesis of mycobactins that are required for iron transport. An important strategy for enhancing the activity of sulfonamides against some bacteria has been their use in combination with trimethoprim, a drug that inhibits a subsequent step in the tetrahydrofolate pathway catalyzed by the enzyme dihydrofolate reductase. Although trimethoprim is not active against mycobacteria, a considerable amount of structural information is available concerning bacterial and mammalian dihydrofolate reductases, and a detailed study of *M. tuberculosis* enzymes from the tetrahydrofolate pathway may provide a

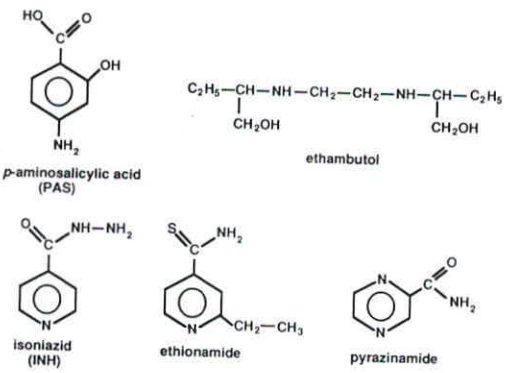

Figure 2. Structures of antimycobacterial agents.

basis for rational design of novel synergistic drug combinations.

The fluoroquinolones (ofloxacin, ciprofloxacin, sparfloxacin) are broad-spectrum antibacterial agents that disrupt the bacterial chromosome by inhibiting the supercoiling activity of DNA gyrase. The fluoroquinolones are increasingly important in treatment of mycobacterial diseases, and the genes encoding both subunits of the DNA gyrase enzyme of *M. tuberculosis* have been cloned (Takiff, personnal communication). Pharmaceutical companies have invested considerable effort in development of gyrase inhibitors, and it may be of interest to screen for evidence of specificity for the mycobacterial enzyme among such compounds.

Rifampin is a key drug in mycobacterial therapy that has a broad antibacterial spectrum and a well-defined target. In this case, transcription is inhibited by an interaction with the β subunit of the bacterial RNA polymerase molecule. In mycobacteria, as in other bacteria, resistance is conferred by point mutations in the *rpoB* gene (Telenti et al., 1993).

Cell Wall Biosynthesis

Broad-spectrum antibacterial agents

Biosynthesis of cell wall peptidoglycan provides targets for a large number of antibacterial agents. Cycloserine inhibits incorporation of D-alanine into the peptidoglycan precursor, while the glycopeptide drugs vancomycin and teicoplanin inhibit assembly of the precursors by binding to the terminal D-Ala–D-Ala residues. Members of the extensive family of β-lactams (penicillins and cephalosporins) inhibit a series of carboxypeptidases and transpeptidases (the penicillin-binding proteins) required for cross-linking of the peptidoglycan units. Among all of the agents, it is striking that only cycloserine (a drug associated with serious side effects) is effective in antimycobacterial therapy. The ineffectiveness of the other drugs is almost certainly due to their failure to get access to the appropriate target enzymes rather than to any fundamental difference in the core structure or biosynthesis of mycobacterial peptidoglycan (Jarlier et al., 1991). As discussed below, permeability is a crucial factor in determining the efficacy of antimycobacterial agents.

New cell wall targets

While the complex cell wall structure of mycobacteria probably confers the permeability barrier that underlies their resistance to many existing antibacterial agents, the same unique structure contains a series of potential targets for novel mycobacterium-specific inhibitors. A considerable amount of information concerning the polysaccharide and lipid structures that make up the mycobacterial cell wall is available. The peptidoglycan backbone is covalently attached to an arabinogalactan polymer (Daffe et al., 1990), and it is probable that inhibition of steps involved in arabinogalactan biosynthesis would prove lethal to the cell. The hydrophobic, wax-like character of the mycobacterial cell wall is conferred by a family of long-chain α-branched fatty acids (the mycolic acids) that are in turn covalently associated with the cell wall arabinogalactan. The mycolic acids are unique to the mycobacteria, and again, it can be envisaged that their synthesis and assembly into the cell wall entail a series of enzymes, each representing a potentially attractive target for antibacterial action. A further series of noncovalently associated components contributes to the cell wall structure. Lipoarabinomannan molecules are thought to traverse the cell wall in a manner analogous to lipoteichoic acids of the gram-positive bacteria and are able to trigger cytokine release by mammalian cells in a manner reminiscent of the action of gram-negative lipopolysaccharide (Chatterjee et al., 1992). Long-chain fatty acyl dial-

cohols (phthiocerol dimycocerosate) and sulfated and nonsulfated trehalose esters add further complexity to the outer surface of *M. tuberculosis* and may contribute both to bacterial permeability and to interactions with mammalian cells during infection. While the exact contribution of each of these components to mycobacterial viability is unknown, it is attractive to suggest that maintenance of this overall cell wall structure is a crucial factor in survival and pathogenicity of *M. tuberculosis* and that any drugs capable of disrupting synthesis and assembly of cell wall components may have some potential as antimycobacterial agents.

Ethambutol, isoniazid, and ethionamide

There are several indications that some existing antituberculous drugs may indeed act on the cell wall biosynthetic pathways. Identification of the precise targets of such drugs may allow design of novel inhibitors of the same enzyme or of related steps in the same pathway.

Ethambutol (Fig. 2) has a polyamine-like structure and was originally thought to interfere with RNA synthesis. More recent evidence from metabolic labeling experiments with ethambutol, however, suggests inhibition of glucose incorporation into arabinogalactan and arabinomannan polymers as an early event in drug action (Takayama and Kilburn, 1989). Although the biochemistry of such an activity is far from clear, the ability to transfer ethambutol resistance between mycobacterial strains by using cloned DNA fragments (Inamine, personal communication) will provide an important new approach to this problem.

Isoniazid (INH; Fig. 2) has a very high degree of specificity for *M. tuberculosis*, and there is a vast literature concerning its proposed mode of action (see Zhang and Young [1993] for a recent review). INH susceptibility is dependent on the presence of a catalase-peroxidase enzyme that may convert the drug to an activated intermediate within the bacterial cell. As in the case of ethambutol, metabolic labeling experiments monitoring the earliest detectable effects of INH provide evidence for action on cell wall biosynthesis, with mycolic acid synthesis being the most likely target (Winder and Collins, 1970). A point mutation in a locus termed the *inhA* gene is associated with INH resistance in *M. smegmatis* (Bannerjee and Jacobs, personal communication), and it is attractive to propose that this gene encodes an enzyme involved in mycolic acid synthesis. Interestingly, the same mutation confers resistance to ethionamide. Ethionamide, which is structurally related to INH (Fig. 2), may also inhibit mycolic acid biosynthesis, although in this case, the catalase-peroxidase step is not required, and some INH-resistant isolates show enhanced sensitivity to ethionamide (Winder, 1982).

ADDITIONAL FACTORS RELEVANT TO DRUG DESIGN

Permeability and Transport

As noted above, access of drugs to their target molecules appears to be a key factor in determining mycobacterial susceptibility and resistance. Strains of *M. avium-M. intracellulare* are significantly more resistant than *M. tuberculosis* to most antibacterial agents, for example, and this resistance is thought to reflect a general decrease in the organism's permeability to the drug. It has been proposed that the mycolic acids and surface-associated lipids of mycobacteria form a permeability barrier analogous to the outer membranes of gram-negative bacteria (Jarlier and Nikaido, 1990; see also chapter 22 of this volume), and discovering means of transporting drugs across this hydrophobic barrier may hold the key to improved antimycobacterial therapy. At present, we have only a few

hints concerning the nature of transport systems in mycobacteria. Trias et al. (1992) detected a small amount of a porin-like molecule in cell wall preparations from *M. chelonae*, siderophores (mycobactins) and exochelins required for iron acquisition have been found (Ratledge, 1982), and an *M. tuberculosis* lipoprotein resembling a periplasmic binding protein required for phosphate transport has been characterized (Andersen et al., 1990). Detailed analysis of the uptake mechanisms required for transport of nutrients across the putative outer membrane, the intervening cell wall region, and the inner bacterial cell membrane may yield valuable insights. Drugs could be designed to take advantage of active uptake by such transport systems, for example, and transporters specific for essential nutrients could themselves be targets for novel inhibitors.

Prodrugs

M. tuberculosis isolates with defects in the *katG* gene encoding a catalase-peroxidase enzyme develop resistance to INH, indicating a possible role for the enzyme in intracellular activation of the drug (Zhang et al., 1992). Similarly, resistance to pyrazinamide (Fig. 2) is generally associated with the loss of a pyrazinamidase enzyme, and it is probable that pyrazinoic acid is the active form of the drug within the bacteria (Konno et al., 1967). The concept of using a prodrug, which is subsequently converted to an active form within the bacteria, may represent a useful mechanism for achieving efficient drug uptake. Sensitivity to pyrazinamide is dependent on the conditions of bacterial growth. Growth at acidic pH is necessary to demonstrate in vitro susceptibility of *M. tuberculosis*, while in vivo susceptibility is thought to reflect conditions encountered within intracellular phagocytic vesicles (Mackaness, 1956; Crowle et al., 1991). For other bacterial pathogens, it is broadly appreciated that key phenotypic changes occur during adaptation to the host environment (Miller et al., 1989), and further analysis of the final target of pyrazinamide may provide useful insights into intracellular adaptation of *M. tuberculosis*. Features specific to the in vivo phenotype represent possible drug targets, and pyrazinamide provides a clear illustration of the importance of studying drug action in vivo as well as in simple bacterial cultures.

Drug Combinations

It has been demonstrated empirically that certain drug combinations (INH and pyrazinamide, for example) are synergistic, and it is attractive to propose rational strategies for the design of potentially useful combinations. Inhibition of sequential steps in a single pathway is a promising approach (e.g., sulfonamide and trimethoprim), and the association between INH resistance and loss of catalase activity suggests that drugs capable of generating oxidative radicals might usefully be combined with INH therapy. Inhibitors that disrupt some aspect of cell wall biosynthesis may not be lethal in themselves but might affect the permeability barrier in such a way as to increase the effectiveness of other drugs given in combination therapy. It has been suggested that ethambutol may have such an action in enhancing the susceptibility of *M. avium* to other drugs (Hoffner et al., 1987; Rastogi et al., 1990).

Outside the Cell Wall

An alternative strategy for circumventing the permeability barrier is to select targets that are present outside the cell wall. These could be hydrolytic enzymes or transport molecules required for bacterial nutrition or molecules involved in specific interactions with host cells. In culture, *M. tuberculosis* exports an array of proteins that are under intensive study in relation to their antigenic properties but are poorly understood in terms of biochemical function. Several fi-

bronectin-binding proteins have been identified (Abou-Zeid et al., 1991), but it remains to be determined whether these have a functionally important interaction with the mammalian extracellular matrix or an as-yet-undetected enzymatic role important for mycobacterial growth. Some of the surface and secreted proteins are found as lipoproteins (Young and Garbe, 1991), with additional evidence of glycosylation in some instances (Fifis et al., 1991; Garbe et al., 1993). Posttranslational modification probably occurs on the outer side of the cell membrane, and identification of the relevant enzymes could provide interesting targets for antimycobacterial agents that might affect transport systems and growth of *M. tuberculosis*. Although it lacks characteristic secretion signals, superoxide dismutase (SOD) is found in culture filtrates of *M. tuberculosis* (Zhang et al., 1991). It is proposed that extracellular SOD protects *Nocardia asteroides* from exogenous superoxide radicals generated within the phagolysosome (Beaman and Beaman, 1990), and extracellular SOD may similarly be required for intracellular survival of mycobacterial pathogens. Extensive conservation between bacterial SOD and the corresponding mitochondrial enzyme in mammalian cells represents a significant barrier in relation to drug targeting, although the availability of a crystal structure for the *M. tuberculosis* enzyme may allow development of such an approach (Cooper et al., in press). Supernatant fluid from *M. tuberculosis* cultures contains several proteins (SOD and the DnaK and GroES chaperones, for example) that are considered to be cytoplasmic proteins in *Escherichia coli* (Young et al., 1990). It remains to be determined whether the presence of these proteins is due to a signal peptide-independent system for protein export or simply reflects a limited degree of leakage from dead or damaged cells. Should *M. tuberculosis* prove to have a specific protein export system, it would be important to consider the possibility that there are additional efflux systems that behave synergistically with the permeability barrier in conferring drug resistance.

Dormancy and Persisters

The greatest challenge for development of new drugs against tuberculosis is to design strategies that will reduce the duration of treatment. Current therapies kill actively growing bacteria within a few days but have to be continued for many months in order to finally eliminate persisting bacteria, which are thought to survive either by reaching a site that is inaccessible to drugs or by entering a state of dormancy with much-reduced metabolic activity. It is widely recognized that when bacterial cultures are starved for nutrients, they enter a stationary phase in which cells stop dividing and develop an ability to survive under conditions that would be lethal during the actively growing phase (Matin et al., 1989). We know nothing of the physiological state of the persisting tubercle bacilli, but entry into such a state of generalized stress resistance would be consistent with the ability of some organisms to survive during drug therapy and then reactivate in a fully drug-susceptible form.

Detailed study of stationary-phase changes in *E. coli* and other bacteria has shown that stress resistance is not simply due to a reduced metabolic activity but actually requires the programmed synthesis of a set of stress proteins (Siegele and Kolter, 1992). In *E. coli*, this synthesis is achieved by changes in transcriptional patterns associated with particular RNA polymerase sigma subunits. In addition to the α and β subunits required for polymerization of the ribonucleotide units, the bacterial RNA polymerase contains a σ subunit that directs the enzyme to transcribe particular genes. Most *E. coli* genes are transcribed by an RNA polymerase carrying a 70-kDa σ subunit (RpoD), but in response to stress,

changes in the levels of minor σ subunits can direct an alteration in transcriptional patterns. Genes associated with stationary-phase stress resistance are controlled by a novel σ subunit, the product of the *rpoS* (or *katF*) gene (Hengge-Aronis, 1993). The critical importance of this form of transcriptional regulation in determining bacterial cell survival is dramatically demonstrated by the observation that bacteria carrying *rpoS* mutations can readily be selected on the basis of their ability to compete with wild-type cells during selection under starvation conditions (Zambrano et al., 1993). Elucidation of corresponding pathways in *M. tuberculosis* might allow us to think in terms of designing reagents that would interfere with development of generalized stress resistance by targeting specific σ subunits or perhaps by interrupting relevant signaling pathways, for example. While such a strategy is entirely speculative at present, it can readily be envisaged that the current intense interest in studying molecular mechanisms of mycobacterial virulence will open up quite novel opportunities for rational drug design.

EXPERIMENTAL STRATEGIES

From the above discussion, it is clear that an extensive list of potential drug targets in *M. tuberculosis* can be compiled with relative ease. Conversion of such a list into an actual drug discovery program will demand considerably greater effort and imagination. Progress will require a combination of biochemical and genetic skills, but for the purpose of outlining potential experimental approaches to rational drug design, we will address these two strategies independently.

Biochemical Approaches

Enzymes involved in synthesis of the complex cell wall components represent attractive potential drug targets. Although the structures of many of these components have been determined, the relevant biosynthetic pathways remain largely unknown. Isolation and characterization of biosynthetic intermediates represent an important strategy for defining such pathways and also provide an approach to drug discovery. By synthesizing structural analogs of these intermediates, it may be possible to identify inhibitory molecules that could provide lead compounds for new drugs. Development of cell-free systems for monitoring biosynthesis of cell wall components will play an important role both in elucidating the biochemical pathways and in screening for inhibitors. Identification of compounds that are active in cell-free systems could be followed by synthesis of related structures designed to enhance uptake into intact mycobacteria. At a further level of sophistication, purification of individual enzyme targets will simplify screening of large numbers of potential inhibitors, and resolution of three-dimensional enzyme structures will ultimately allow exploitation of the full power of rational drug discovery techniques.

Genetics and Sequencing

The recent development of molecular genetic systems for mycobacteria opens a range of novel opportunities for drug discovery. In the case of the existing antimycobacterial agents discussed above (INH, ethambutol, etc.), gene transfer experiments can be used to identify and clone the corresponding drug targets by monitoring appropriate transfer of drug resistance or susceptibility. In addition, the generation of extensive amounts of sequence information from mycobacterial genome projects will undoubtedly play an increasingly dominant role in identification of genes encoding potential drug targets. The key experimental challenge will be to design techniques to allow expression of genes or gene clusters in functional assays suitable for drug

screening. While conventional *E. coli* expression systems may be suitable for some enzymes, it is likely that expression in rapidly growing mycobacterial hosts will prove advantageous, particularly when multiple genes encoding sequential enzymes within a single pathway are to be studied. In addition to development of recombinant cell-free systems as discussed above, it can be envisaged that gene replacement technology could be used to construct rapidly growing mycobacteria that utilize specific *M. tuberculosis* enzymes to catalyze individual steps involved in key biosynthetic pathways. Such chimeric organisms could provide a basis for the development of novel drug screening assays.

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