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COMMENTARY

Use of Genomics and Combinatorial Chemistry in the Development of New Antimycobacterial Drugs

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ABSTRACT. With the completion of the genome of *Mycobacterium tuberculosis* comes the promise of a new generation of potent drugs to combat the emerging epidemic of multiply drug-resistant isolates. Translating this genomic information into realistic assays, valid targets, and preclinical drug candidates represents the next great hope in tuberculosis control. We propose a paradigm for exploiting the genome to inform the development of novel antituberculars, utilizing the techniques of differential gene expression as monitored by DNA microarrays coupled with the emerging discipline of combinatorial chemistry. A comparison of currently used antituberculars with the properties of other pharmaceuticals suggests that such compounds will have a defined range of physiochemical properties. In general, we can expect the next generation of antituberculars to be small, relatively hydrophilic molecules that bind tightly to specific cellular targets. Many current antimycobacterials require some form of cellular activation (e.g. the activation of isoniazid by a catalase-peroxidase). Activation corresponds to the oxidative, reductive, or hydrolytic unmasking of reactive groups, which occurs with many current antimycobacterial prodrugs. Understanding the mechanisms involved in activation of current antimycobacterial therapeutics also may facilitate the development of alternative activation strategies or of analogs that require no such processes. BIOCHEM PHARMACOL **59**;3:221–231, 2000. © 1999 Elsevier Science Inc.

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In spite of recent intensive funding and research, *Mycobacterium tuberculosis* remains the leading cause of human death and suffering due to a single infectious entity. In 1997, 7.25 million people developed tuberculosis, which ultimately proved fatal in 2.9 million cases. An estimated one-third of the world population currently is infected with the bacillus [1]. Declining overall case rates in the United States have obscured the emerging epidemic of drug-resistant tuberculosis. Studies in other developed countries such as Denmark verify an intuitive presumption: multiply drug-resistant tuberculosis is transmitted indiscriminately across national borders [2]. Drug resistance rates in many developing nations are alarmingly high and may reach 30% or greater in even some developed countries, such as the former Soviet states of Latvia, Lithuania, and Estonia [3]. In a recently completed survey by the World Health Organization, 100% of 35 countries surveyed worldwide (including the United States) have reported resistance to antituberculosis therapeutics ranging from 2 to 42% of all isolates [4]. Many such strains have mutations rendering them resistant to all front-line therapies.

Why have so many isolates become drug-resistant? Current front-line therapy consists of three drugs introduced in the 1950s: INH,† rifampin, and PZA for 2 months, followed by 4 months of follow-up therapy with INH and rifampin [5]. A strong contributor to the emergence of drug resistance is the relative ineffectiveness of the three front-line therapies, whose MIC values range from 150 nM to 800 μM. For all three of these drugs, the MIC is very close to the maximal serum concentration, which is limited by toxicity, resulting in a poor therapeutic index for each (Table 1). Serum concentrations of these drugs may oscillate between levels above and below the MIC over the course of daily administration. This phenomenon, combined with poor patient compliance over the course of this very long chemotherapy, has been proposed to be linked directly to the emergence of drug resistance [9]. The ineffectiveness of current therapies is, therefore, directly responsible for both the very long duration of therapy and the emergence of resistance to these drugs.

Although there has been much discussion of special populations of privileged bacteria that will be insensitive to any therapeutic agent that only affects dividing bacilli, the evidence for these special populations is incredibly weak. Instead, what seems clear is that a new therapeutic agent

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† *Abbreviations:* INH, isonicotinic acid hydrazide, isoniazid; PZA, pyrazinamide; MIC, minimal inhibitory concentration; ETA, ethionamide; and POA, pyrazinoic acid.

TABLE 1. Comparison of *in vitro* inhibitory activity and *in vivo* properties of common tuberculosis drugs

	In vitro MIC (μg/mL)	*In vivo* C_{max} (μg/mL)	*In vivo* $T_{1/2}$ in serum (hr)
Amikacin*†	1	30	2.3
p-Aminosalicylate‡§	1.5	7.5	1
INH†–§	0.02	7	1–3
Ciprofloxacin†	<1	4	3–4
Clofazamine†–§	0.15	3	10.6 days
Cycloserine †–§	10	35	10
Ethambutol†–§	1.5	5	3.3
ETA†–§	1.2	2	2.1
Capreomycin*‡§	5	3	2.5
Kanamycin*†§	6	30	2–3
Ofloxacin†	<1	10	7
PZA†–§	6.2	65	10–24
Rifabutin†§	0.5	10	45
Rifampin†–§	0.2	10	3.5
Streptomycin*†§	0.5	3	2.3
Thiacetazone†	0.4	3.2	12.9
Viomycin*‡	4	3	2.5

*Drug administered by injection.
†Data from Ref. 6.
‡Data from Ref. 7.
§Data from Ref. 8.

with a subnanomolar MIC and favorable pharmacokinetic and toxicological properties would both dramatically shorten the duration of therapy and suppress the emergence of drug resistance. The tools for the development of such novel therapeutic agents have become available recently, and the pathway towards their development has become clearer. This commentary seeks to outline the process for this development by integrating the various tools currently available to the tuberculosis research community.

WINNING THE TARGET LOTTERY: SCIENCE OR LUCK?

This decade has seen dramatic advances in our understanding of the metabolism and intracellular lifestyle of *M. tuberculosis*, culminating in the recent publication of the complete genomic DNA sequence of the virulent strain H37Rv [10]. Of the estimated 4000 encoded proteins, about 40% have known biochemical functions, another 44% have some sequence homology, and 16% are completely unknown. The emphasis of mycobacterial research now has shifted from gene hunting to interpretation of the biology of the whole organism in an effort to better define which activities are likely to be critical to survival and thus amenable to the development of new drugs. Rate-limiting steps in metabolic pathways that are unique to prokaryotes have been the traditional focus of anti-infectives, and many such targets are obvious from the genome sequence. Such candidates have been validated by the efficacy of currently prescribed medications [11]. Selecting targets whose inactivation would lead to either bacterial death or stasis is not difficult; discovering how to exploit such targets and develop new active molecules in a time- and resource-efficient manner represents a more challenging enterprise.

Amidst this wealth of sequence information, emerging new techniques for monitoring differential gene and protein expression offer valuable guidance in elucidating regulatory mechanisms of metabolic pathways and thereby pinpointing new drug targets [12–14]. DNA-based microarrays offer perhaps the most well developed of these new technologies. These arrays are composed of precisely distributed spots immobilized on a glass surface to which isolated RNA can be hybridized. These will be particularly important for uncovering metabolic pathways representative of *in vivo* bacterial populations, which may be markedly different from those in artificial culture media. The metabolism of the tubercle bacillus harvested from infected cells or granulomas excised from human patients may provide valuable clues to important new drug targets *in vivo*. The ability to examine simultaneously the expression of all 4000 genes in the genome will allow rapid analysis of metabolic pathways under many stress conditions. Such arrays can be used further to predict the mechanisms of action and secondary interactions of both known pharmaceuticals and unknown novel antibacterials (Fig. 1) [15]. DNA microarrays have already been applied to the identification of drug susceptibility patterns in mycobacteria [16].

Analysis of patterns of gene expression takes some of the guesswork out of target selection and validation. However, it must be noted that not all metabolic pathways are under transcriptional control. Some are controlled using post-translational modifications (e.g. phosphorylation) and may be missed in gene induction analyses. To compensate for this, proteomics, or two-dimensional protein mapping, also has been used for target selection and drug mechanism of action studies and, in some cases, gives more information than DNA-based expression arrays [17, 18]. As an example, in the study of the mechanism of INH action we recently described a covalent complex of the drug with two different but related proteins, AcpM and KasA [19]. Although both proteins were transcriptionally up-regulated and, therefore, would be expected to appear in a DNA array-based experiment, the actual molecular interaction between the two proteins and INH would not have been uncovered in the absence of the proteomic experiment.

TRANSLATING A POTENTIAL TARGET INTO A POTENT SCREEN

Unlike the information available from simply selecting a potentially lethal target from the mycobacterial genome, DNA and protein array experiments often provide an additional level of information about cellular processes. Such responses reflect genetic reactions to a specific deficit. For example, biosynthetic enzymes may be up-regulated in response to inhibitors of cell wall structures. Often such regulatory read-outs are extremely specific and are correlated *in vivo* to the activity of a particular enzyme or group

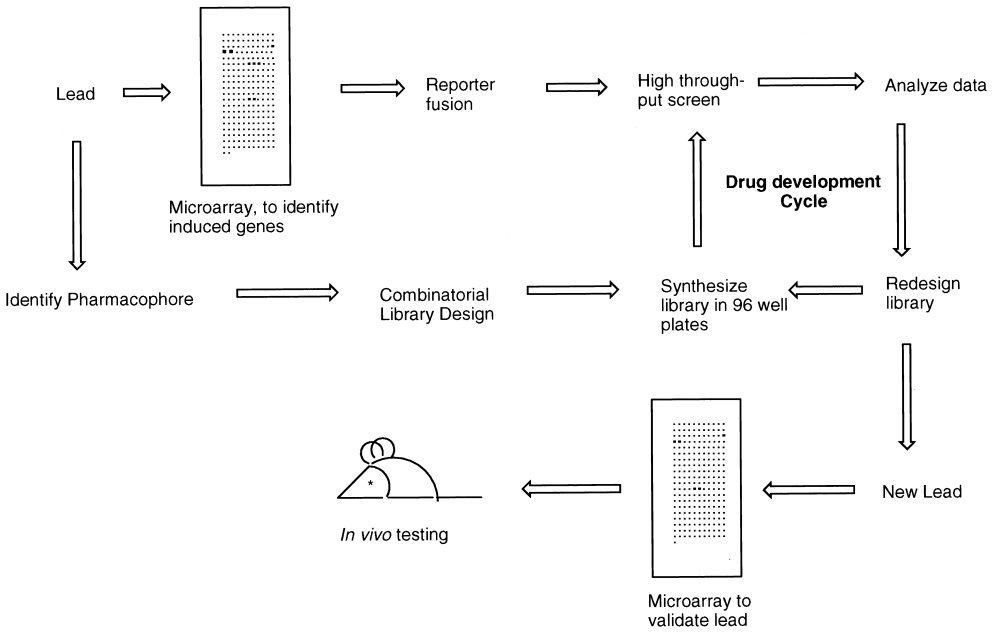

FIG. 1. Integration of microarray genomics, high-throughput screening, and combinatorial chemistry.

of enzymes. Such information can be translated directly into a screen for inhibitors of a specified enzyme or pathway. The most useful of such responses are positive in nature, when treatment with a given substance results in up-regulation of a gene or operon. These drug-sensitive promoters can be coupled directly to a reporter gene (e.g. luciferase) and transformed back into the bacterium, whereupon they demonstrate drug-sensitive reporter gene expression (Fig. 1). *In vivo* screens have many advantages over traditional enzyme activity-based assays, including (a) impermeable lead compounds are quickly eliminated from further consideration, and (b) multiple metabolic steps can be monitored simultaneously. The latter could also be seen as a liability for determining precise structure–activity relationships for inhibitors of a single enzyme, depending upon the specificity of the reporter strain used. For high-throughput screening for inhibitors of a metabolic pathway with known transcriptional regulation, such screens are ideal and can be followed up with enzyme-based screens of individual activities. For slow-growing organisms such as *M. tuberculosis*, such screens offer the additional substantial advantage of short assay times and rapid transcriptional responses (on the order of hours) compared to the very long times required for direct MIC determinations (typically several weeks).

INTEGRATING COMBINATORIAL CHEMISTRY WITH MYCOBACTERIAL GENOMICS

The concepts of combinatorial (split and pool) chemistry to synthesize large libraries of compounds that are extremely diverse, in combination with microassay techniques for high-throughput screening, have revolutionized drug discovery [20, 21]. New combinatorial chemical techniques have been developed to make structurally diverse compound libraries. Such libraries can possess elements intended to sample maximal structural diversity for random unbiased surveys or can be focused around an active lead molecule to attempt to maximize binding efficiency. Often, the survey of an unbiased library is followed by the production of a focused library in a cycle around the most active substructures to optimize binding efficiency. The chemical reactivity of this lead molecule then becomes the limiting factor and often predisposes the library to certain types of synthetic conditions. Ideally, the most complex libraries are built up on solid supports such as derivatized plastic beads, but the chemistry for all the steps must be high-yielding and must not interfere with the linker attaching library elements to the support. Such supports allow for split and pool synthesis, resulting in vast libraries containing discrete compounds on each bead. Solution-phase libraries are more convenient for parallel synthesis, which creates larger quantities of smaller single-compound libraries, often with higher functionality or reactivity not suitable for solid-supported chemistry.

In lieu of screening broad structural classes of molecules for active lead molecules, one strategy that integrates well with data from microarrays such as that described above is to begin from a known antimycobacterial agent with a defined target that is amenable to combinatorial analog construction. Virtually every antitubercular agent currently in use was developed in the 1950s; therefore, relatively few analogs were produced. Figure 1 summarizes the conceptual progression from lead compound through microarray profile to development of a high-throughput screen and parallel combinatorial library design. The cycle terminates with preclinical candidates with improved potency, whose pre-

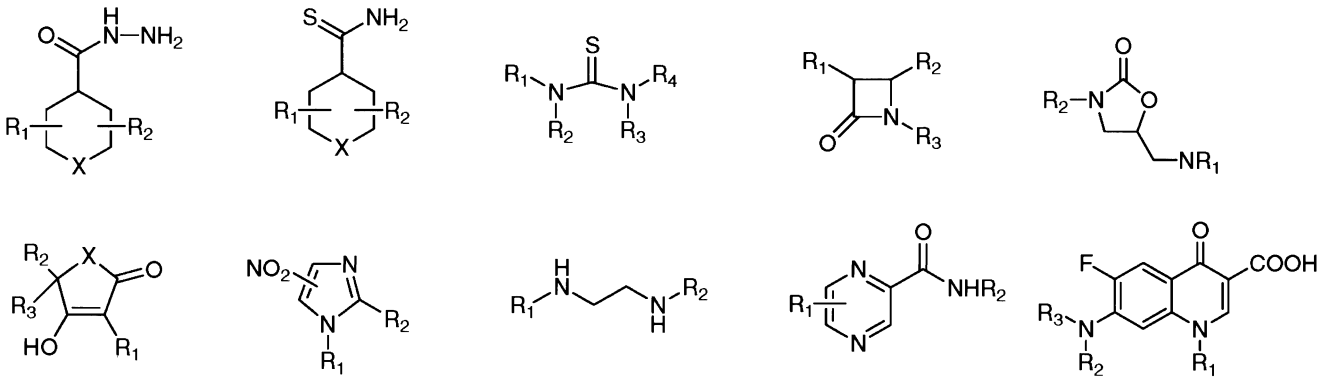

FIG. 2. Antimycobacterial pharmacophores. R groups indicate positions of potential diversity. From left to right and top to bottom, the core structures represent isonicotinic acid hydrazides, thioamides, thioureas, β-lactams, oxazolidinones, tetronic acids, nitroimidazoles, diamines, pyrazinoic acids, and fluoroquinolones.

sumed mechanism is verified by another round of differential gene or protein expression analysis before being moved into *in vivo* testing. Antimycobacterial pharmacophores suitable for such library construction are shown in Fig. 2 and include acylhydrazides such as INH, thioamides such as ETA, diarylthioureas such as Isoxyl (thiocarlide), β-lactams, small heterocycles including thiolactomycin, oxazolidinones, and nitroimidazoles, di-secondary amines such as ethambutol, and PZAs or POAs. Many so-called second-line antimycobacterial agents are extremely ineffective and could be improved dramatically by using such straightforward techniques and a few simple considerations in optimization.

PHYSIOCHEMICAL CONSIDERATIONS FOR NEW ANTITUBERCULARS

The availability of many simple monomers for combinatorial library construction (or even for normal synthesis of analogs) can quickly overwhelm even very robust screening techniques. In addition, activity *in vitro* often displays poor correlation with activity *in vivo*. The primary reason for such failure is the often conflicting balance between bioavailability and activity. Consideration of appropriate characteristics for an orally available pharmaceutical should occur very early in the development cycle, even as early as lead and monomer selection for libraries. Many current combinatorial libraries do not discriminate against compounds that may have inadequate oral activity, although early consideration of such factors has been advocated to improve ultimate success in drug identification [22, 23]. Such characteristics can be determined by a comparison of current antimycobacterial agents with the list of all known active drug molecules.

The bioavailability of a drug is dependent on three basic parameters: solubility, permeability, and metabolic half-life. These parameters affect intestinal absorption, the initial hurdle in oral drug delivery, as well as toxicity. Because of poor bioavailability, obtaining oral activity for a possible agent can quickly become the rate-limiting step of the drug discovery process. Oral efficacy is improved primarily through increased potency as guided by optimization during high-throughput screening, but improvements in solubility and/or permeability without effects on activity can contribute to ultimate success. As an aid in the design of drugs for oral administration, there have been a set of general guidelines outlined ("Lipinski's Rules") that provide an empirical definition of a drug-like molecule [24, 25]. These studies focused on large groups of bioactive drugs and were not restricted to anti-infectives. Mathematically predictive rules diagnostic of absorption on the bases of permeation and molecular size have been defined. In addition to formula weight, the number of atoms, molecular volume, and the molar refractivity reflect molecular size. The relationship of molecular weight and lipophilicity is not always predictable, however; in terms of antimicrobials, increased molecular weights are generally associated with increased lipophilicity. Overall, lipophilicity appears to be the primary intrinsic property related to absorption. The primary predictive index of lipophilicity is a computational value, Log*P*, which represents the octanol solubility of a given compound in the presence of aqueous solute. An estimate of lipophilicity can be easily predicted by simply counting NH and OH bonds, and Ns and Os. In general, then, desirable candidate attributes are a molecular weight below 500, with a preferable range of 230–390 (mean 360). For the number of atoms the preferred range is 30–55 (mean 48). For the molar refractivity the qualifying range is 40–130, with a preferred value range of 70–110 (mean 97). The preferred value for Log*P* is 1.3 to 4.1 (mean 2.3) within an acceptable range of -0.4 to 5.6. For the total number of hydrogen bond donors, OHs and NHs, the value should not exceed 5, and for hydrogen bond acceptors, Ns and Os, the number should not exceed 10 (see Table 2).

When clinically useful mycobacterial chemotherapeutic agents are compared to the battery of drugs available from the current databases in terms of physically predictable properties, size, and lipophilicity, they generally conform to the preferred parameters. The physical size for mycobacte-

TABLE 2. Molecular physiochemical characteristics of known mycobacterial drugs

Compound	MW	Molar volume	AMR	No. of atoms	ALog*P*	OH + NH	N + O	pK_{a_1}
Cycloserine	102	79	22	13	−1.84	2	2	7.40
PZA	123	94	31	14	−0.37	1	3	13.91
INH	137	110	36	17	-0.89	2	2	11.27
p-Aminosalicylate	153	102	40	20	-1.46	3	0	15.70
ETA	166	142	50	21	1.22	1	2	12.14
Ethambutol	204	207	58	38	−0.09	4	0	15.03
Thiacetazone	236	181	65	28	1.22	3	2	15.16
Salinazid	241	193	68	29	2.53	2	3	12.39
Ciprofloxacin	333	228	84	44	1.25	3	4	15.11
Ofloxacin	363	245	92	48	0.89	2	1	15.27
Sparfloxacin	394	275	98	52	2.81	4	5	15.1
Isoxyl	400	357	122	60	7.7	2	3	13.96
Clofazamine	473	366	136	67	7.62	1	2	8.04
Kanamycin*	484	297	111	69	−3.28	11	4	14.70
Streptomycin*	583	293	121	81	−4.27	16	4	15.37
Amikacin*	585	364	134	83	−4.47	13	1	15.01
Capreomycin*	668	381	150	91	−11.43	15	6	18.09
Viomycin*	682	408	161	94	−11.10	14	7	17.96
Rifamide	810	628	212	116	3.62	5	9	
Rifampin	822	611	213	117	2.95	6	9	
Rifabutin	847	632	222	123	3.53	5	9	
Rifapentine	877	648	229	127	4.45	6	9	
General characteristics	160–480 (230–390) $\bar{x}$ = 360		40–130 (70–110) $\bar{x}$ = 97	20–70 (30–55) $\bar{x}$ = 48	−0.4–5.6 (1.3–4.1) $\bar{x}$ = 2.3	<5	<10	
Mycobacterial characteristics	440* 393	310	102	61	0.06* 2.0	5.4	3.7	13.97

Calculations that represent the size of the molecule are expressed as molecular weight (MW), molar volume, molar refractivity (AMR), and number of atoms. Calculations that represent hydrophobicity are calculated Log*P* (ALog*P*), number of proton donors (OH + NH), and number of proton acceptors (N + O).

*Denotes drugs administered via injection and the averaged values calculated without these to get a more accurate value of orally available drugs.

rial drugs follows an abnormal distribution pattern, with a large number of synthetic compounds, such as INH, much smaller than the average and many natural products, such as rifampin, much larger than the average (Fig. 3A). Mycobacterial drugs fall in a number of classes primarily related to nicotinamide, quinolones, and rifampin pharmacophores. Of these, the nicotinamide analogs are the smallest, with an average molecular weight of 164, and the

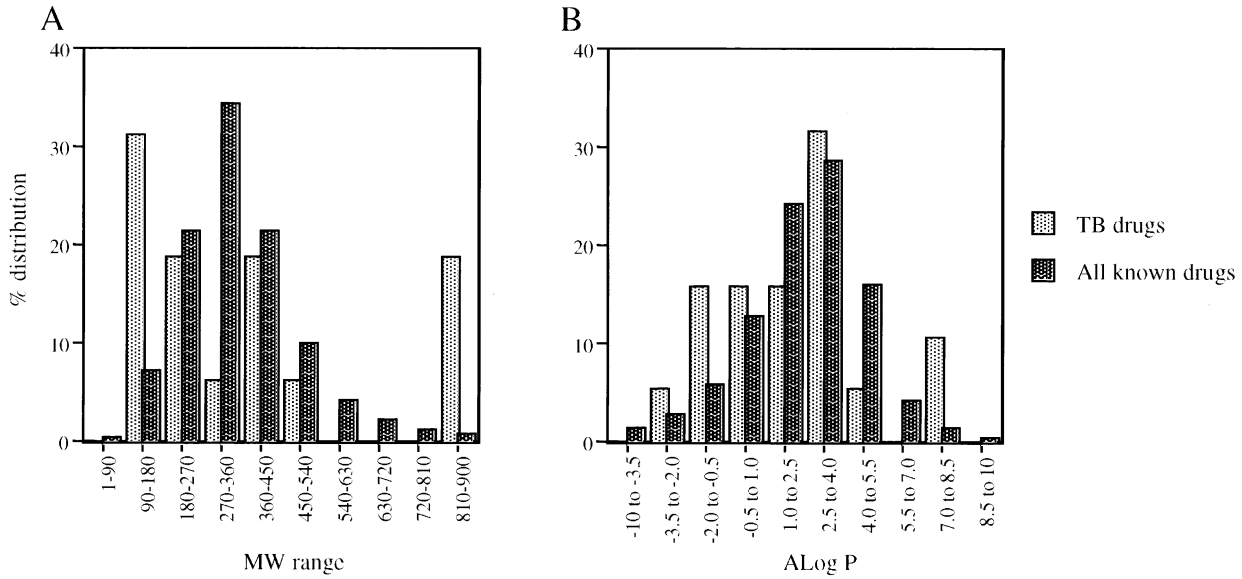

FIG. 3. Comparison of the pharmacokinetic properties of orally administered TB drugs to the CMC drug database [25]. (A) Molecular weight; (B) Log*P*. Percent distribution refers to the total percentage of entries falling into this category.

rifampin-based structures are the largest, with an average molecular weight of 840.

Conventional thinking driven by the physical properties of the tubercle bacillus suggests that drugs with more hydrophobic character would have an increased activity. Studies involving pyrazinoate esters have demonstrated that Log*P* values in the range of 2–4 have higher *in vitro* potency [26]. These data suggest that entry into mycobacterial cells is indeed positively affected by increasing lipophilicity. Although it sometimes may be confused by activation issues (*vide supra*), other studies looking at the effects of lipophilicity on *in vitro* activity support this Log*P* range. With analogs of quinolones [27–29], it was determined that antibacterial activity correlated to side-groups that were better electron donors with a higher hydrophobicity. However, lipophilic character placed with regard to the active pharmacological center did not correlate with increased antimicrobial activity. *In vitro* studies of thiosemicarbazone analogs offer perhaps the clearest demonstration of activity versus optimal hydrophobicity [30]. These studies afforded the same peak Log*P* range as that found with pyrazinoic acids, demonstrating that for *M. tuberculosis* and a variety of slow-growing mycobacteria the optimum Log*P* value was 4, whereas for a fast-growing strain such as *M. smegmatis* the optimum was 3. A positive *in vitro* relationship between Log*P* and antitubercular activity for a series of alkoxybenzoic acids also has been demonstrated, again finding an optimal Log*P* value of 4 [31]. Rifapentine, which was approved for use in the treatment of pulmonary tuberculosis in the United States in 1998, has a Log*P* value 1.5 units higher than rifampin (Table 2) [32]. This higher hydrophobicity again correlates with enhanced activity [33, 34]. Thus, the majority of current antituberculars conform to the general guidelines and fall into the category with Log*P* values ranging from 2.5 to 4.0 (Fig. 3B).

THE WILDCARD. WHY DO SO MANY MYCOBACTERIAL DRUGS REQUIRE ACTIVATION?

An unusually large proportion of drug resistance mechanisms in *M. tuberculosis* have been demonstrated to involve alteration of genes connected with drug metabolism or drug "activation" by mycobacteria. A "prodrug" is classically defined as an inactive compound that is converted to an active drug by a biotransformation or nonenzymic process such as hydrolysis, oxidation, or reduction. In *M. tuberculosis*, drug metabolism often seems to result in an intermediate of sufficient chemical reactivity to undergo covalent binding to cellular targets and exert a lethal effect on the bacilli (Fig. 4).

INH is perhaps the most important front-line antitubercular in use today and is the best characterized illustration of the concept of cellular activation to a reactive species. Almost immediately after INH was introduced into clinical use in the early 1950s, drug-resistant strains emerged [35], which frequently had altered or absent catalase-peroxidase activities [35, 36]. In the last decade, it has been revealed that an estimated 50% or more of INH resistance is caused by deletions or mutations in the *kat*G gene, which encodes the sole catalase-peroxidase activity of the cell [37–41]. KatG catalyzes the two-electron oxidation of INH to an acyl imine, which undergoes subsequent enzymatic or nonenzymatic transformation to a reactive intermediate capable of acylating nucleophilic groups in target proteins and causing INH toxicity in *M. tuberculosis*. Mutations or deletions in *kat*G effectively circumvent the toxic effect of activated INH on its molecular targets. The targets thus far include KasA, a β-ketoacyl synthase of *M. tuberculosis* [19], and InhA, the enoyl-ACP reductase of *M. smegmatis* [42], which both appear to function in mycolic acid biosynthesis.

ETA is a second-line antitubercular that, in common with INH, has been demonstrated to affect mycolic acid biosynthesis in *M. tuberculosis* [43, 44]. In the inhibition of KasA by INH, the covalent trimolecular complex of the drug, the acyl carrier protein AcpM, and KasA also is formed upon treatment with ETA.* A single mutation in *inh*A can confer resistance to INH and ETA in *M. smegmatis* [42]. The data implicate ETA or its metabolites in a shared role with INH on molecular targets involved in mycolic acid biosynthesis. In a clinical setting, a large number of INH-resistant isolates are still ETA susceptible, but a high percentage of ETA-resistant isolates are INH resistant. The large proportion of INH-resistant isolates that have deletions or mutations in *kat*G are generally the ones that retain their susceptibility to ETA, suggesting that ETA is metabolized or "activated" via another mechanism in *M. tuberculosis* [45].

ETA has demonstrated superior and more consistent activity *in vivo* than *in vitro*, and it has been shown that a major metabolite of ETA *in vivo* in humans and in a variety of animals is the sulfoxide [46]. The sulfoxide exhibits greater activity *in vitro* against *M. tuberculosis* than ETA [46], but as treatment with either leads to similar serum ETA to sulfoxide ratios, one may presume that there is no greater antitubercular efficacy gained by treatment with the sulfoxide [47, 48]. These observations suggest that ETA is a prodrug, which is metabolized *in vivo* in mycobacteria and possibly in mammals. Many toxic thiocarbonyl compounds including thioureas, thiobenzamides, and thioacetamide are thought to require metabolic activation *in vivo* via one or more enzymatic *S*-oxidations for expression of their toxicity. The binding of cellular nucleophiles appears to correlate with the biological injury produced [49]. In the case of thioacetamide, activation consisting of two successive *S*-oxidation steps leads to covalent modification of cellular nucleophiles [50, 51]. To test the hypothesis that ETA and its sulfoxide can be metabolized further to reactive electrophilic intermediates in mycobacteria, these drugs were oxidized with horseradish peroxidase and hydrogen peroxide in the presence of simple nucleophilic amines to give the corresponding amidines [45]. Taken together, the

* Mdluli K and Barry CE III, unpublished observations.

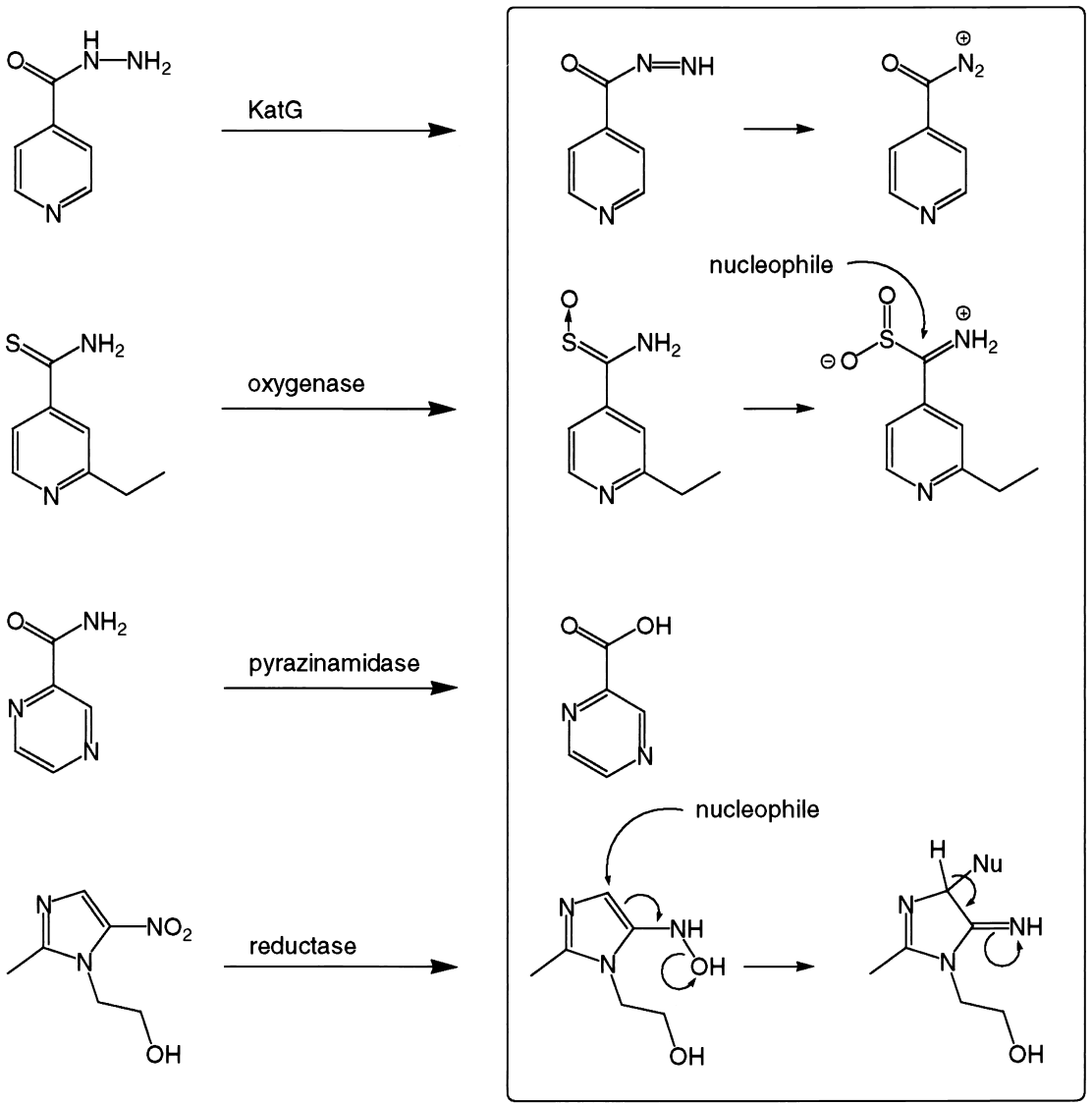

FIG. 4. Metabolic activation by *M. tuberculosis*. INH is activated by KatG through an acyl-imine to a reactive intermediate or intermediates, which undergo binding to a cellular nucleophile. ETA is postulated to be activated by an unknown oxygenase or oxygenases through its sulfoxide to a bi-oxygenated electrophilic intermediate. A mycobacterial pyrazinamidase activates PZA to POA or a reactive intermediate in POA formation. Metronidazole is thought to be activated by a reductase, which performs a four-electron reduction to produce reactive metronidazole metabolites.

preceding observations suggest that ETA is activated in *M. tuberculosis* via an enzyme (or enzymes) yet to be characterized, through its sulfoxide to a higher *S*-oxide. It is interesting to note the literature amassed on clinical cross-resistance connecting ETA, Isoxyl (thiocarlide), and thiacetazone observed in the *M. tuberculosis* complex [52–62]. All three drugs have a thiocarbonyl moiety in common, suggesting that a subset of the cross-resistant mutants may have defects in activating this moiety.

Yet another example of drug resistance caused by mutations in a gene linked with drug activation is found in the hydrolytic activation of PZA. PZA was discovered to have antitubercular activity in 1952 [63] and became a front-line drug in the 1980s; it has gained widespread use in combination with INH and rifampin [5, 64, 65]. There is a solid body of evidence demonstrating that mutations in the *pncA* gene of *M. tuberculosis* are a major mechanism of PZA resistance and that they can be correlated with loss of pyrazinamidase activity [66–68]. Pyrazinamidase metabolizes PZA to POA, which is suggested to be the toxic species in *M. tuberculosis* [69]. At acidic pH, PZA-resistant *M. tuberculosis* isolates are susceptible to POA, and PZA-sensitive isolates are commonly more susceptible to POA than PZA *in vitro*, although POA appears ineffective when given orally to mice infected with *M. tuberculosis* [69]. It has been proposed recently that a deficient efflux mechanism underlies the unique susceptibility of *M. tuberculosis* to PZA [70]. It was found that in *M. tuberculosis*, acidic pH

enhanced the intracellular accumulation of POA following hydrolysis of PZA. The saprophyte M. *smegmatis*, which is intrinsically resistant to PZA, converts PZA to POA but does not accumulate POA due to a very active efflux mechanism. The molecular mechanism of toxicity due to POA accumulation is unknown.

In an inventive attempt to bypass pyrazinamidase metabolism and still create POA or an active intermediate in the formation of POA intracellularly, an assortment of more lipophilic derivatives of PZA have been synthesized, which demonstrate promising antimycobacterial activity. These include derivatives of classical medicinal chemistry isosteres of pyrazinecarboxylic acids [71] and a variety of acyl pyrazinoates, which are more effective than POA *in vitro* in PZA-resistant and -sensitive M. *tuberculosis* isolates [26, 72, 73]. The acyl pyrazinoates are postulated to circumvent pyrazinamidase activation and to be metabolized by a mycobacterial esterase.

Using an *in vitro* system, metronidazole was shown to act on "dormant" M. *tuberculosis* in an oxygen-free layer of undisturbed cultures [74], and there are conflicting findings suggesting that combination therapy with metronidazole may be of value in eliminating "persisting" tubercle bacilli [75, 76]. Compounds of similar nitroimidazole structure have been shown to have potent antitubercular activity *in vitro* and *in vivo*, for example CGI 17341 [77] and PA 824. PA 824 is a nitroimidazopyran that appears to have a novel mechanism of action and is active against strains of multidrug-resistant M. *tuberculosis*.* Resistance to metronidazole and related nitroimidazoles has been studied extensively in anaerobes, and these compounds are reductively activated in the absence of oxygen to a variety of reactive nitrogen species [78, 79]. Presumably, metronidazole, CGI 17341, and PA 824 also must be activated by M. *tuberculosis*. PA 824-resistant mutants have been described that have lost the ability to metabolize or "activate" PA 824, in the now familiar scenario encountered time and again with antitubercular.

Why, then, has this scenario repeated so often with antituberculars? One possibility is that the oxidative activation is intimately associated with the heightened potential of an organism inhabiting an environment in which it may be constantly bombarded by defensive molecules aimed at its destruction. OxyR, a transcriptional regulator present in many bacteria that serves to up-regulate genes involved in defense against oxidative stress, including *kat*G, is not present in M. *tuberculosis* [80]. Instead of viewing this as a defective oxidative stress regulon, it may, in fact, represent the loss of the need to ever turn off such genes as a group, resulting in constitutive hyperexpression of KatG and sensitivity of the organism to oxidatively activated prodrugs. Another possibility is that the antimycobacterial drugs represent relics of the type of drugs available in the 1950s when their antimycobacterial activity was uncovered. The *in vitro* screening that was conducted may have selected for small neutral hydrophilic molecules that had the capacity to become reactive *in vivo*.

*Baker W, Hickey MJ, Towell, JA, Yuan Y, Barry, CE III, Sherman DR, Stover CK, unpublished observations.

CAN WE PREDICT THE PHYSICAL PROPERTIES OF THE NEXT GENERATION OF ANTITUBERCULOSIS DRUGS?

The characteristics of the next generation of effective antimycobacterials would represent both the properties needed for killing the bacillus and the properties that make drugs amenable to oral delivery. The hydrophobic character of antitubercular drugs is as diverse as the size range, but as mentioned above, in general, more hydrophobic character is better for potency at least *in vitro*. There is clearly a dichotomy between the need for antimycobacterial agents to have high serum concentrations and the need to permeate the bacillus. The average Log*P* value of all the antitubercular drugs is 0.06. However, when one excludes drugs administered by injection, the value moves to 2.0. Thus, for the next generation of antituberculars it seems favorable to produce relatively small molecules without unusually high or low Log*P* values, biased perhaps towards a high Log*P* value. Specifically, molecules generated via a combinatorial method should be in the MW range of 100–400 with Log*P* values of *ca.* 2.5 to 4.5.

The final issue has to do with the desirability of prodrugs as therapeutic candidates. While such "Trojan horse" strategies are appealing because of the possibility of masked chemical functionality, the necessity of unmasking them potentially provides an opportunity for the bacillus to evolve resistance more easily. It has been argued that prodrugs are less desirable from the perspective of increased opportunities for the development of resistance. In fact, the frequency of emergence of resistance to INH (the prototypical mycobacterial prodrug) is about one organism per 1,000,000 [81], whereas the frequency of emergence of resistance to rifampicin is about one organism per 100,000,000 [82]. It is important to note, though, that the frequency for INH is measured *in vitro* where loss of KatG activity is the overwhelming mutational event, not *in vivo* where the overwhelming majority of INH resistance is due to a single amino acid substitution of serine for threonine at position 315 of KatG [81, 83]. In cases where *kat*G is deleted, this is accompanied by a second mutational event, the up-regulation of a peroxidase gene, *ahp*C [84]. The cumulative frequency of these two events is certainly not 1 in 1,000,000 and is distinct from the mutations that occur *in vitro*. Thus, drug resistance may occur at more comparable frequencies *in vivo*, and there may be no real disadvantage to prodrugs in terms of mutational possibilities. On the other hand, the overwhelming majority of mutations that confer resistance to drugs requiring activation affect the activation process, not the actual target, suggesting that there is something to be gained by avoiding prodrugs. By nature, such compounds also pose a higher risk of toxicity if a homologous mammalian enzyme exists that also is

capable of activation. In any case, prodrug candidates also must be bioavailable and can be designed with the same predictions used for direct inhibitors. Indeed, as in the example of the POA esters cited above, understanding in detail the activation pathways of current antimycobacterial prodrugs may suggest alternative mechanisms for circumventing the normal activation pathway and may provide more opportunity for manipulation of pharmacokinetic parameters.

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