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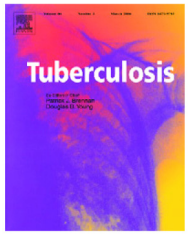

A philosophy of anti-infectives as a guide in the search for new drugs for tuberculosis

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Summary
How we develop antibiotics is shaped by how we view infectious disease. Given the urgent need for new chemotherapeutics for tuberculosis and other infectious diseases, it is timely to reconsider a view of infectious disease that is strongly supported by contemporary evidence but that has rarely been applied in antibiotic development. This view recognizes the importance of nonreplicating bacteria in persistent infections, acknowledges the heterogeneity and stringency of chemical environments encountered by the pathogen in the host, and emphasizes metabolic adaptation of the host and the pathogen during their competition. For example, efforts in our lab are guided by the perspective that *Mycobacterium tuberculosis* (Mtb) has co-evolved with the human immune response, with the result that Mtb turns host-imposed metabolic adversity to its own advantage. We seek chemotherapeutics that turn Mtb's adversity to the host's advantage.

Tooling up and taking stock

Mycobacterium tuberculosis (Mtb) infects about one-third of the human population and often persists for life. Tuberculosis (TB) is the leading cause of death from a single bacterial infection and the leading opportunistic infection in HIV-infected hosts.[1–3] Multiple and extensive drug resistance (MDR and XDR) in clinical isolates in many countries make ~425,000 cases per year treatable only at great cost in resource-poor settings or effectively untreatable.[4,5] The TB pandemic may spin further out of control as the incidence of obesity-related diabetes rises in areas of high TB prevalence, because this condition markedly increases the incidence and transmissibility of TB and reduces its response to chemotherapy.[6–8]

We face these challenges at a time when, as Arturo Casadeval notes, "Infectious disease is the only field of medicine that is becoming less effective".[9] The shrinking portfolio of effective antibiotics results from the inexorable retirement of those to which resistance is spreading, combined with a decline in development of new antibiotics by companies facing economic, regulatory and scientific obstacles.[10] After a hiatus of nearly 40 years, and with the help and encouragement of the Bill and Melinda Gates Foundation, the Global Alliance for TB Drug Development, Médecins Sans Frontières, the National Institutes of Health, the Wellcome Trust, the European Union, the Organization for Economic Cooperation and Development , BioVentures for Global Health, the Institute of Medicine of the National Academies of Science[11] and other agencies, society is

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beginning to address the widening gap between existing TB chemotherapeutics and clinical need.

Nonetheless, and despite the efforts of the Global Alliance, Tibotec, Novartis, GlaxoSmithKline, AstraZeneca and Otsuka, as well as several academic labs, it is not clear that there are enough lead compounds entering the TB drug pipeline to ensure that several new anti-TB chemotherapeutics will emerge in the next decade.[12] Multiple new agents are needed to treat XDR-TB. Monotherapy would only ensure rapid emergence of resistance, and we would be back where we are now, with nothing to offer an increasing number of patients afflicted by a deadly and contagious disease for which the major risk factor is sharing air.

At this critical juncture in the history of TB chemotherapy, it is timely to re-examine the philosophies that favor some anti-infective targets over others and thereby guide drug discovery. Historically, two such philosophies arose in parallel. One became dominant, while the other has been almost completely ignored. This needs to change, because both are useful, each complements the other, the need is great and time is short. Below we review these two philosophies, consider their pertinence to TB and the implications for chemotherapy, and list examples of targets to which the less-traveled road has led one laboratory.

Philosophies of infectious disease and antibiotic development

How one approaches the development of antibiotics depends on how one views infectious disease. The "germ theory" of infectious disease causation that arose in the late XIXth century encouraged a simplistic view of pathogenesis: clinical infectious disease could be viewed as what happens when microbes grow in a human test tube. In contrast to a glass test tube, humans are multi-compartmental, metabolically active, frequently noncompliant and occasionally litigious. Yet, at base, this view regards people as a culture medium in which bacterial pathogens flourish. It follows that the most efficient route to seek antibiotics is to start by finding compounds that kill bacteria in vitro in culture conditions that allow them to replicate quickly.

A powerful boost to this view of infectious disease was the origin of the first chemically defined anti-infectives from synthetic aniline dyes rather than from microbes themselves. As synthetic chemists, Ehrlich and Domagk opened a path that was soon festooned with Nobel Prizes: to Ehrlich who developed Salvarsan in 1908; to Domagk for prontosil in 1939 (he later discovered isoniazid); to Fleming, Florey and Chain for penicillin in 1945; and to Waksman for streptomycin in 1952. Fleming discovered the antibiotic action of one microbe, *Penicillium notatum*, toward others (Gram-positive bacteria),[13] but when Florey, Chain and Heatley purified penicillin and demonstrated its clinical utility, the beta-lactam could be understood as a chemical that happened to be a natural product, rather than as a mediator of inter-species competition.

In contrast, XXIst century science encourages us to see the pathogenesis of infectious disease as a competition between host and pathogen that involves both short-term adaptations and co-evolution of their genomes. Because the host and pathogen each exert selective pressure on the other, the environment in the host may differ markedly from that in the test tube. It follows that the physiology of the pathogen in the host may differ markedly from that in the test tube. This perspective invites the would-be developer of antibiotics to seek compounds that prevent bacteria from disabling the host or from surviving in the host environment. When screening for anti-infectives in vitro with this goal, one should try to mimic the host environment as closely as is practical. With respect to some populations of pathogens in some anatomic settings, the worst way to do this would be to use culture conditions that support the pathogen's rapid replication.

The notion of viewing infectious disease as inter-species competition for a niche arose in the interval between Fleming's discovery of the antibiotic action of a mold and the Oxford team's purification of penicillin as the active principle. In 1939, Rene Dubos isolated bactericidal substances (later resolved as gramicidin, tyrothricin and tyrocidine) from a soil microbe.[14] He viewed this as a lesson that species adapt and compete in a given niche. His view of the interactions among microbes served as a foundation for his later views of interactions among all species and provided the philosophical foundation of today's environmental movement.[15] Another natural product isolated by Dubos, working with Oswald Avery, was a bacterial enzyme that broke down the capsule of the pneumococcus. Evidently, Dubos and Avery were neither surprised nor discouraged that the enzyme had no discernible effect on the pneumococcus during culture in vitro, because in that environment the pathogen faced no competition. In contrast, in the vertebrate host, the enzyme was effective because it rendered the bacteria susceptible to the host environment, specifically, the host's antibodies, complement and neutrophils. In their words, "It is of interest that although neither the enzyme nor the specific antibody [that is, the host's anti-pneumococcal antibody] is by itself bactericidal or bacteriolytic, yet each by reacting specifically with the capsular substance exposes the virulent organisms to the phagocytic action of the body tissues. The enzyme, like the specific antibody, serves merely to initiate the protective reaction, the completion of which is ultimately dependent for its successful issue upon the effective cellular response of the host".[16]

Ironically, this approach was rendered clinically useless by the host's development of antibodies against the bacterial enzyme. Had the agent that sensitized the bacteria to host immunity been a small chemical rather than an immunogenic protein, this approach to antibiotic development might not have lain dormant for the next 70 years.

In 2005, Liu et al. identified a small molecule inhibitor of the synthesis of the eponymous gold pigment of *Staphylococcus aureus*.[17] The inhibitor, and deletion of the gene encoding its enzymatic target, had no effect

on the growth of the pathogen under standard conditions in vitro, but rendered the bacterium sensitive to reactive oxygen intermediates produced by host phagocytes and allowed mice to cure themselves.[17] This remarkable set of experiments answered a call[10] for anti-infectives that can sensitize pathogens to host immune responses by inhibiting pathogens' evolved defenses against those immune chemistries. Others have also called for this complementary approach to identifying anti-infective targets.[18]

Streptococcus pneumoniae and *S. aureus* are strikingly different from Mtb. Is the philosophy that stretches across the decades from Avery and Dubos[16] to Nizet and his colleagues[17] relevant to TB?

The critical impact of host-microbe interactions on the course of TB

Mtb has co-evolved with the immune response of humans. The pressures that human physiology and immunity bring to bear against Mtb alert and/or force the bacterium to reduce its replication. This host response is essential to avoid rapid death from miliary TB. However, the same host response — non-sterilizing containment — also serves the pathogen, because it preserves the possibility of transmission. Mtb's continued display and release of antigens and other substances that elicit chemotactic factors leads to progressive accumulation of host inflammatory cells. These may kill some Mtb, but over time in a proportion of hosts, the inflammatory response destroys enough lung tissue to allow resumption of bacterial replication, access to the airway, provocation of cough and generation of infectious aerosol, allowing Mtb to complete its host-to-host cycle. In this view, the challenge for anti-TB chemotherapy is similar to the challenge faced by Mtb in the host. Mtb turns its metabolic adversity to its own advantage. Chemotherapy should turn Mtb's metabolic adversity to the host's advantage.

To pursue this line of reasoning, we need to take into account the heterogeneous environments that Mtb faces in infected hosts, both in terms of Mtb's replication status and the host chemistries that affect it. We will consider each in turn.

During latent TB infection (LTBI), most Mtb are non-replicating, and during clinically active disease, some Mtb are non-replicating.[19–21] As noted by Levin and Rozen in describing "drug indifference," "the rate at which bacteria are killed by … [antibiotics] is directly proportional to their rate of growth… Bacteria that are not dividing… are partially or completely refractory to killing by antibiotics from most, if not all, the major classes."[22] In a related phenomenon, "bacterial persistence," "a small fraction of [even] a dividing bacterial population (the persistent cells) is refractory to killing by the antibiotic."[22] Thus, "The contribution of non-replicating (dormant) antibiotic-refractory, but genetically susceptible, subpopulations of bacteria… is the primary reason for the long duration of what is called 'short-course' tuberculosis chemotherapy."[22] According to Warner and Mizrahi, "… the dependence of the frontline anti-TB drugs on actively replicating cells for activity is probably the greatest limitation of current therapy."[23] The potential for non-replicating Mtb to cause active disease is illustrated by the "Cornell model," in which mice clinically cured of TB by chemotherapy, whose tissues contain no detectable mycobacteria capable of giving rise to colonies in vitro, relapse when broadly immunosuppressed.[24] In an immunologically specific version of the Cornell model, mice deficient in inducible nitric oxide (NO) synthase[25] relapse within 3 weeks of cessation of apparently curative chemotherapy (McKinney J, Bloom BR, MacMicking J, Nathan C and Jacobs WR Jr, unpublished observations). Thus, durable cure of TB requires eradication of both replicating and non-replicating Mtb.[24] Eradication of non-replicators is believed to be what demands that chemotherapy with current agents last from 6 months to 2 years to avoid relapse. Such prolonged treatment is difficult to sustain. Its interruption fosters the emergence of drug-resistant strains.

The heterogeneity of the microenvironments in which Mtb resides[26] may be another reason why chemotherapy of active TB requires prolonged administration of multiple drugs. The following conditions are thought to exist in the Mtb-containing phagosome within macrophages in immunocompetent mice: a pH of about 4.5;[27] NO diffusing from the cytosol and/or locally generated after acidification of its auto-oxidation product, nitrite;[28] reactive oxygen intermediates, as judged by heightened susceptibility to mycobacterial infection in people with chronic granulomatous disease caused by genetic disruption of the phagocyte oxidase;[29] lack of leucine and lysine;[30,31] minimal carbohydrate;[32] decreased oxygen;[33] and limiting iron.[34] These inferences are supported by gene expression profiles of Mtb recovered from macrophages in vitro[35] and from lungs of mice[34–37] compared to Mtb cultured in media altered in the specified manner, along with the inability of amino acid auxotrophs of Mtb to survive in macrophages.[30,31] Exposure to reactive nitrogen intermediates (RNI) alone elicits many of the same adaptive transcriptional responses in Mtb seen with depletion of amino acids, carbohydrates, iron or oxygen.[35] In vivo, some Mtb is likely to face a low pH[27] because pyrazinamide is effective and requires a low pH. The phagosome is likely to contain NO-derived species because Mtb-infected macrophages in humans have repeatedly (in at least 107 patients in at least 7 studies from around the world) been documented to be iNOS positive[38] notwithstanding the lack of iNOS in macrophages that differentiate in vitro from normal donors' monocytes. The avascularity of granulomas, the in vivo expression profiles of Mtb and the in situ reduction of pimonidazole[19,26] suggest that some degree of hypoxia may be commonplace in Mtb-infected sites, albeit not uniformly. These conditions are likely to be consequential for chemotherapy, because O_2-deprived Mtb became relatively resistant to isoniazid, rifampin and ciprofloxacin[39] and carbon-starved Mtb became relatively resistant to isoniazid, rifampin, ethambutol, *p*-aminosalicylic acid, ofloxacin, and cycloserine.[40] Conversely, two compounds that retained substantial efficacy in the Wayne model of non-replicative persis-

tence induced by hypoxia, rifampin and PA-824,[39] are both effective in mice.

Besides macrophages, Mtb also resides in dendritic cells[41] and to a lesser extent in other cells, including adipocytes,[42] but almost nothing is known about the milieu within those cells' mycobacteria-containing compartments. Moreover, Mtb may escape the phagosome to reside in the cytosol.[43] Again, the metabolic consequences for the bacterium are unknown. In some circumstances, Mtb is delivered to autophagous vacuoles,[44] where it may be exposed to peptides derived by endoproteolysis of ubiquitin that can be mycobactericidal in vitro,[45] but where metabolic parameters remain to be defined.

Finally, Mtb resides in diverse extracellular environments. Caseous material surrounded by calcification is likely to be anoxic. Caseous material in cavities that communicate with bronchi may be hyperoxic (equilibrated with 20% O_2 in the gas phase) with respect to healthy tissue (~5% O_2). In noncalcified granulomas or areas of highly cellular interstitial pneumonitis, large numbers of host cells may congregate in such a way as to displace the vasculature, such that there is likely to be a gradient of oxygen, with marked hypoxia toward the center.[19] The oxygen gradient is likely to be accompanied by other gradients of blood-borne nutrients, such as glucose and amino acids. Finally, cellular necrosis[46] may release Mtb into the extracellular fluid, whose composition is mimicked by Dulbecco's modified Eagle's medium (DMEM)-10% FBS, in which we found that Mtb survives but fails to replicate.[47]

Implications for chemotherapy

Over 20 years ago, a scientist at Lilly Labs bemoaned the diminishing returns in antibiotic research, noting, "…it is possible that the same kinds … of inhibitors are found because the same screening … methods are used… the future discovery of unique drugs to treat infectious diseases requires the use of novel screening strategies … (for) drugs that interfere with … pathogenesis... Compounds with this kind of activity would not necessarily have classical antibiotic activity…".[48] The call was echoed a decade later from a scientist at SmithKline Beecham: "… (To) provide a promising avenue for the discovery of new chemotherapeutic agents, (we should)… design new agents that would act against microbial genes expressed … during infection in vivo, as opposed to the 'housekeeping' functions expressed by micro-organisms growing in the artificial microenvironment of the Petri dish…".[49] Another decade, and the call came again: "Drug libraries should be screened against clinically latent bacteria…".[50]

In contrast, most antibiotics target biosynthetic processes that bacteria need to increase their biomass,[51] specifically, processes that are essential in rich, oxygenated medium in vitro for synthesis of protein, DNA/RNA, cell wall or folate. A philosophy of infectious disease that emphasizes metabolic adaptation of the host and the pathogen during their competition, the heterogeneity and stringency of chemical environments encountered by the pathogen in the host and the importance of nonreplicative persistence calls for the following additions to the standard paradigm. We can focus on more than just four types of targets, beginning with an appreciation that essentiality is conditional, such that Mtb genes essential in mice include many that are not essential in vitro.[52,53] We can recognize that synthesis is not the only feature of a molecule's life cycle vulnerable to inhibition; transport, repair and degradation of macromolecules are also potential targets. Moreover, folate is not the only small molecule whose synthesis can be a profitable target; we can also take aim at pathways that produce other cofactors, intermediary metabolites and ATP.[10,11,54] For example, inhibition of enzymes of intermediary metabolism can interfere with Mtb's energy production, ion transport, generation of cofactors and precursors, export of bioactive lipids and antioxidant and antinitrosative defenses.

Drugs that represent new chemophores active against new targets could provide a desperately needed therapeutic option in the treatment of MDR and XDR-TB. Treatment of LTBI, in which almost all the Mtb are nonreplicating, is a critical need in its own right, because latent Mtb infection sustains the pandemic: nearly 9 million latently infected people develop active TB per year, each of whom infects an estimated 10–15 other people per year before dying or responding to treatment.[55] Thus, it might be profitable to complement conventional anti-TB chemotherapy by inhibiting pathways Mtb depends on to survive adverse host conditions, including pathways of adaptation to metabolic limitations and macromolecular damage.

Examples of new targets

The following targets are the current focus of chemical biologic efforts of our lab together with our collaborators. These are just examples to illustrate a theme, and no effort is made to review similar approaches in other labs. However, it is important that such efforts are underway.[56]

Macromolecular repair: the Uvr system

A screen for genes required by Mtb to resist reactive nitrogen intermediates (RNI) identified hypersensitive mutants with disruptions in *uvrB*.[57] *uvrB* encodes a member of the nucleotide excision repair (NER) pathway, which in other bacteria repairs damaged nucleotides that distort the DNA helix. As RNI and reactive oxygen intermediates[29] are known mutagens, Mtb may require UvrB to maintain DNA integrity during its persistence in the nitrosative and oxidative environment of the phagosome in immunologically activated macrophages. Indeed, mice infected with *uvrB*-deficient Mtb lived much longer than mice infected with wild type Mtb or the complemented mutant.[58] In *E. coli* and other bacteria, NER pathway genes are upregulated as a part of the SOS response to DNA damage, and the bacteria

remain in a non-replicative state until all the DNA damage is repaired. Inhibition of the Mtb SOS response in general or NER in particular may prove to be a useful addition to combination chemotherapy for Mtb infection. There is precedent in that the quinolone family of DNA gyrase inhibitors are important agents in the treatment of MDR tuberculosis.

We have undertaken a phenotypic screen of a chemical compound collection for agents that prevent *M. smegmatis* from recovering after UV exposure, but do not inhibit the growth of unexposed *M. smegmatis*. The *M. smegmatis* is a recombinant strain in which the endogenous *uvrB* gene has been deleted and replaced with *uvrB* from Mtb. We are mindful that while humans lack the Uvr system of NER, the NER pathway as such is conserved throughout evolution. Loss-of-function mutations in a human homolog of UvrB called XPD cause xeroderma pigmentosa, a severe disorder of development with marked UV sensitivity and predisposition to cancer.

Macromolecular degradation: the proteasome

The diverse functions of proteasomes[59,60] include providing a rapid means of adaptation to changing conditions. Proteolytic degradation of inhibitors is a widespread mechanism for activating transcription factors (e.g., NF-κB) and kinases (e.g., cyclins). Another key function is to degrade irreversibly oxidized[61,62] or nitrosated[63] proteins to avoid toxic gain of function.[64] Yet another key function is to cannibalize low priority proteins to survive amino acid starvation.[65] Mtb appears to face amino acid starvation in vivo, based on the failure of amino acid auxotrophs to survive in mammalian hosts,[30,31] and protein synthesis is markedly suppressed in non-replicating Mtb in vitro.[66]

A genome wide screen identified enzymes whose disruption sensitized Mtb to oxidative/nitrosative injury.[57] Among the 10,100 mutants tested, 5 of 12 mutants identified had unique transposon insertions in 2 genes annotated as serving the proteasome.[57] Two highly specific proteasome inhibitors, a peptidyl boronate and epoxomycin, each prevented growth of Mtb and were mycobactericidal during recovery of Mtb from exposure to RNI.[57] Both compounds inhibited a chymotryptic activity in Mtb lysates. An enantiomer of the peptidyl boronate neither inhibited the chymotryptic activity, prevented growth nor killed Mtb.[57] We have expressed and characterized the recombinant Mtb proteasome structurally and biochemically.[67,68] S. Ehrt's lab has used a conditionally regulated allele of the genes encoding the proteasome core particle to demonstrate that Mtb dies out in the organs of mice after expression of the proteasome is suppressed during the chronic phase of infection[69] — the first time that an essential function has been ascribed to the proteasome of a prokaryote.

Thus, while the proteasome of Mtb is essential for infection of a host, it is not required for Mtb to grow in rich media (Middlebrook 7H9) in room air (20% O_2). The latter situation would exclude the Mtb proteasome as a drug target by conventional criteria. However, in nature, Mtb is likely to encounter similar conditions only in cavities that communicate with open airways. Known proteasome inhibitors and new ones we have discovered share the property of entering Mtb and killing it under pathophysiologically relevant conditions that preclude its replication, such as mild acidity (pH 5.5, the pH required by pyrazinamide, a clinically proven anti-TB agent),[69] carbohydrate- and amino acid-limited medium and exposure to reactive nitrogen intermediates. To give proteasome inhibitors a better chance of entering a pharmaceutical development pathway as TB chemotherapeutics, we are working to enhance their therapeutic index by increasing their selectivity for the proteasomes of Mtb over the proteasomes of the host. Success in this endeavor should provide the ability to inhibit bacterial protein metabolism — a well validated target for anti-infectives — at up to 3 different points concurrently: at the stage of transcription (e.g., with rifamycins); at the stage of translation (e.g., with aminoglycosides and capreomycin); and at the stage of turnover (e.g., with proteasome inhibitors). Multi-stage interference with protein metabolism in Mtb should have synergistic effects in a given bacterium and additive effects among different subpopulations of bacteria facing varied metabolic conditions.

Resistance to acid: a membrane protease

Acid damages macromolecules and interferes with biochemical reactions required for bacterial viability, and is considered to be a major antimicrobial defense of phagocytes. Acid also synergizes with other host defenses such as reactive oxygen and nitrogen intermediates and lysosomal hydrolases. Mtb is able to prevent acidification of phagosomes by arresting phagosome-lysosome fusion; however, activation of macrophages with interferon (IFN) γ relieves this block and phagosomes acidify.[27] Nonetheless, Mtb resists the low pH of this compartment.[70] To identify Mtb genes required for resistance to low pH we conducted a screen of 10,100 transposon mutants and isolated a mutant in a membrane-associated serine protease. The protease mutant was hypersensitive to acidified medium, but grew like wild-type Mtb at the near-neutral pH of growth medium *in vitro*. Further, unlike wild-type Mtb, the mutant was unable to maintain its intrabacterial pH inside activated macrophages and was killed. The mutant was also severely attenuated in both the acute and persistent stages of infection in mice. A deficiency in the membrane protease makes Mtb highly susceptible to the host environment and therefore it represents an attractive drug target. Studies to identify inhibitors of the membrane protease and other pathways involved in intrabacterial pH maintenance are underway.

Intermediary metabolism and resistance to reactive nitrogen intermediates: dihydrolipoamide acyltransferase and lipoamide dehydrogenase

Unlike other bacteria, Mtb possesses a unique defense system that links antioxidant and metabolic pathways.

It includes a peroxiredoxin, alkyl hydroperoxide reductase subunit C (AhpC); a thioredoxin-like protein, AhpD; dihydrolipoamide acyltransferase (DlaT); and lipoamide dehydrogenase (Lpd).[71] Together, the four enzymes function as an NADH-dependent peroxynitrite reductase and peroxidase. DlaT and Lpd are also E2 and E3 components of pyruvate dehydrogenase.[72] The dual functionality of DlaT and Lpd and exclusivity of their shared functions within the bacterial kingdom attracted our attention to both as targets. In accordance with DlaT's predicted functions, Mtb deficient in *dlaT* replicated poorly in vitro, was more susceptible to nitrosative stress, did not persist in mouse bone marrow macrophages and did not persist at the same numbers nor cause as much pathology as wild type Mtb in the mouse.[73] More strikingly, DlaT was essential for pathogenesis in a guinea pig model of tuberculosis.[47] We screened a chemical library for compounds that would inhibit Mtb's peroxynitrite reductase and identified rhodanines as time-dependent and irreversible, AhpD-competitive DlaT inhibitors. Improved analogs inhibited both of DlaT's activities and were nontoxic to mammalian cells but bactericidal to *M. bovis* BCG and Mtb H37Rv in culture and inside mouse bone marrow macrophages. Rhodanines had no effect on mycobacteria under regular growth conditions but potently killed bacteria that were not replicating in the following settings: culture medium at pH 5.5 in the presence of sublethal nitrite; culture medium at pH 6.6 in the presence of bacteriostatic NO-donor, DETANO; non-replicative persistence (NRP) phases 1 and 2 of the Wayne model;[39] and mammalian cell culture media. Rhodanines will have to be tested for their antitubercular activity in animal models but demonstrate that chemical compounds can selectively kill non-replicating bacteria.[47]

We expect that targeting or disrupting Lpd may result in a more severe phenotype than targeting or disrupting DlaT. Lpd is the only functional lipoamide dehydrogenase in Mtb.[74] In other organisms, it serves as a common component of α-ketoglutarate dehydrogenase, branched chain ketoacid dehydrogenase and the glycine cleavage system. Lpd may also have functions independent from these multienzyme complexes; it was reported to be secreted and bind coronin-1, preventing phagosomal maturation.[75] The crystal structure of Mtb's Lpd revealed differences between the Mtb and eukaryotic enzymes[76] that might permit selective inhibition of Mtb's Lpd. Trypanosomal Lpd is the known target of nifurtimox (Lampit), an agent used to treat Chagas' disease.[77]

Further targets in intermediary metabolism: other ketoacid dehydrogenases and decarboxylases

The Krebs cycle in Mtb is not canonical due to the lack of an active α-ketoglutarate dehydrogenase (KDH) complex.[72,78] We proposed that α-ketoglutarate decarboxylase (Kgd) (Rv1248c) would nonoxidatively decarboxylate α-ketoglutarate, generating CO_2 and succinic semialdehyde.[80] SSA could then be converted to succinate by the action of specific succinic semialdehyde dehydrogenases, connecting the oxidative and reductive branches of the Krebs cycle.[78] Recent work from our lab (unpublished results) indicates that Kgd's production of SSA is kinetically dissimilar to what is expected for a metabolic enzyme. While this analysis does not support the proposed mechanism, it may make Kgd an even more interesting target, because in addition to its potential essentiality,[52] its function is likely to be quite different from its human homolog. Kgd and its homologs in other *Actinomycetes* have a unique *N*-terminal insertion of ~200 amino acids bearing homology to the catalytic domain of dihydrolipoamide succinyltransferase,[81] raising the possibility that Kgd may be bifunctional. To understand Kgd's role we are generating a *kgd* knockout in a Tet-regulated background, and performing biochemical and structure analysis with the pure enzyme while developing inhibitors through target-templated click chemistry.[81]

Target validation

Not all the examples listed above will qualify as validated drug targets, nor can this be determined based solely on the phenotypes of their mutants in vivo. Complete disruption of the gene encoding an enzyme produces a deficiency that is rarely matched by chemical inhibition of the enzyme in vivo. More informative genetic approaches may involve regulated expression of the gene, as in the study of the proteasome discussed above.[69] Not all enzymes are amenable to potent inhibition by small compounds. Crystallography of the target is invaluable in evaluating the potential to achieve inhibition by drug-like molecules. An otherwise excellent microbial target may be disqualified by its homology to a critical host enzyme with respect to their active sites, even if the overall homology between the two proteins is so low that they would not otherwise be considered to be structurally related.

Out of the silos to join forces

From a purely scientific perspective, the prospects for new chemotherapeutics for TB should be bright. Many Mtb genes are essential in vivo, and the number would be far greater if we considered synthetic lethality and designed combination chemotherapy from the outset.[10] Moreover, phenotypic screens against intact mycobacteria have identified scores of compounds, many of them natural products, that inhibit mycobacterial growth.[82] Unfortunately, in most cases, their targets and toxicities are unknown. Numerous opportunities are not pursued because resources are lacking or are inaccessible to those motivated to use them. To come to grips with TB and with resurgent infectious diseases as a whole, we need more effective policies as well as more effective science.[10,12,83]

At the policy level, we need to realign incentives for antibiotic research and development so that pharmaceutical innovation is better matched to medical need.

We must better manage our existing antibiotics portfolio to put priority on slowing down the emergence of drug resistance, including by offering incentives to stop feeding half our antibiotic output to healthy food animals. We should reform the procedures of regulatory agencies for antibiotic approval so they apply special criteria to the evaluation of agents whose superiority may consist solely in avoiding the resistance that pathogens display toward existing antibiotics. Finally, there is urgent need for improved global coordination in monitoring and responding to infectious diseases.

At the scientific level, academic labs need to collaborate across disciplines, for example, by bringing together immunology, microbial genetics, structural and computational biology, enzymology and medicinal chemistry, just as biotechnology and pharmaceutical firms do in their drug development teams. At the same time, we need much more collaboration among sectors, to improve mutual access of academics and biotechnology and pharmaceutical companies to each other's resources and expertise.

Fortunately, from the WHO Intergovernmental Working Group on Public Health, Innovation and Intellectual Policy to the programs of the Gates Foundation and other agencies, recognition of these critical issues seems to be growing.

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