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Discovery and Validation of New Antitubercular Compounds as Potential Drug Leads and Probes

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SUMMARY

Increasing multidrug resistance in *Mycobacterium tuberculosis* continues to diminish the number of effective drugs available for treatment of active tuberculosis. Although there are four new products (representing three new chemical classes) in clinical development, an active, robust pipeline of new chemical entities is critical to discovery of medicines to dramatically improve or shorten length of therapy via new mechanisms of action. In the absence of major pharmaceutical industry activity in tuberculosis drug development, the National Institute of Allergy and Infectious Diseases (NIAID) has supported the development of a high throughput screen for growth inhibitors of *M. tuberculosis* using a chemically diverse commercial library, a compound library available through the NIH Roadmap, Molecular Libraries Screening Center Network, and other compound sources. The rationale for these screens and suggested approaches for follow-up studies to identify compounds for advanced preclinical studies and as chemical probes of critical functions in *M. tuberculosis,* are discussed.

Keywords

high-throughput screening; tuberculosis; chemical probes; drug development; mycobacteria

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The process of antiinfective drug discovery and development is simple: kill the bug, spare the host. Although all of basic assays are available to monitor compounds as they move forward in the development process, it is still difficult to efficiently reduce this simple paradigm to practice. The three major overlying and almost philosophical approaches used for discovering compounds that inhibit new targets in a pathogen are 1) whole cell growth inhibition screens, 2) isolated *in vitro* enzyme or pathway screens; and 3) the hybrid whole cell with enzyme or pathway-targeted readout screens. The advantages and disadvantages of each approach are given in Table 1.

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In deciding on the best approach, investigators must weigh the status of the technology available for screen design, and the resources available for follow up analysis and validation of hits. For example, the difficulty in performing genetic manipulations in an organism can limit the ability to design reporter-based whole cell screens and to construct over and under-producing strains for use in target identification. The availability of technologies such as microarrays and their interpretation also can bear on mechanism of action studies. Additional factors are the depth of knowledge of metabolic pathways in a given microorganism and stringent validation of essential biochemical targets. In this regard the NIAID tuberculosis screening programs have contributed to the design and implementation of several new assays useful in evaluating new compounds for antitubercular activity,[1-7] including several enzymes assays (see www.taacf.org).

Estimates of the global TB burden continue to worsen. In 2009 there will be over 8 million reported new cases, with 500,000 new cases of multidrug resistant TB (MDRTB) and 40,000 new cases of extensively drug resistant TB (XDRTB). As disturbing as these incidence figures are, they may be significantly underestimated as argued by Cohen et. al.[8] We know that XDRTB strains can arise in discrete stages by acquisition of drug resistance mutations within 7 years,[9] and that the evolutionary path can occur in many strain backgrounds.[10] While major strides have been made in the 20th century in reducing the burden and prevalence of TB infection, shortfalls in TB control and treatment programs has led to losses in the progress that was so sorely arrived at. The NIAID has and will continue to focus research efforts to address these trends in areas of patient care, health care delivery training, diagnostics, vaccines, and the quest for new and better therapeutic drugs and drug targets.

The NIAID-sponsored Tuberculosis Antimicrobial Acquisition and Coordination Facility (www.taacf.org) was designed to assist researchers, especially chemists, in their efforts to discover and develop new antitubercular drugs.[11] Screening began in the early 1990s under an interagency collaboration with the Health Resources and Services Administration's Gillis W. Long Hansen's Disease Center adopting assays developed by Dr. Scott G. Franzblau[2] as the most rapid approach to discovery of new drugs to treat *M. tuberculosis*. In the years that followed, the TAACF evolved to include medicinal chemists and microbiologists from Southern Research Institute, technology transfer experts from Research Triangle Institute, and experts in animal models of infection at Colorado State University.[4] Screening efforts have successfully used both whole cell growth inhibition approaches and *in vitro* enzyme or pathway assays to identify new antitubercular drug hits/leads. Two inhibitors of PanC were discovered by the latter approach with K_i values of 75 ±13 μM[12] and approximately 24 nM (www.pubchem.gov), however, in both cases these initial hits either did not enter cells, or were inactive on whole cells for other reasons (e.g. degradation, instability, sequestering). Other *in vitro* screens that have yielded interesting hits are dihydrofolate reductase (DHFR; hits selective for *M. tuberculosis* over the human enzyme and active as *M. tuberculosis* growth inhibitors), and malate synthase (work in progress). Over the years the TAACF has registered and screened over 90,000 submitted compounds by hundreds of investigators from over 40 countries worldwide for whole cell growth inhibition with over 250 resulting publications. More than 130 compounds were evaluated in animal models of infection leading to the identification of new lead series with in vivo efficacy, some of which have been published[13-15]. However, the contribution of the TAACF program extends further to ongoing drug development research involving *in vivo* efficacy studies for which final data is not yet been released by the investigators.

Since client-provided samples are covered by confidentiality agreements, the bulk of the data generated by the TAACF is not publically available. Thus, we initiated development of an efficient high throughput screening (HTS) approach in order to provide to the TB research community growth inhibition data on large compound libraries that could be publically

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released. A stacked-plate, serial 2-fold dilution, 384-well assay for growth inhibitors of virulent *M. tuberculosis* was developed and validated for high throughput mode. This assay has screened over 438,000 compounds and completed follow up analysis on 338,000 compounds. Included were a 100,000-compound commercial library from ChemBridge Corporation,[11, 16] 213,000 compounds from the NIH Molecular Libraries Screening Centers Network[17], a 100,000 internal screening set, and a 25,000 commercial kinase inhibitor-based compounds set. The MLSCN library is the first data set publically released in March of 2009 (see www.pubchem.gov). Data analysis yielded several structural classes with some clusters pointing to indications of structure activity relationships (SAR)[17] and has already stimulated interest by the TB research community. Testing of the Prestwick Library of known drugs (http://www.prestwickchemical.fr/index.php?pa=26) led to the identification of unexpected antitubercular activity[11] (data available at http://pubchem.ncbi.nlm.nih.gov/ and at www.taacf.org).

While our primary goal was to identify active compounds for preclinical evaluation as new anti-tubercular drugs, the use of new molecules as chemical tools in probing the biology of *M. tuberculosis* (e.g. identification of new essential genes required for cell growth or survival of non-replicating bacteria) may increase our understanding of the organism's probes have been of great value in unraveling the genetics and biochemistry of microbial DNA, RNA, cell wall and protein synthesis and there is no reason to assume that this will not continue, branching out to many specific biochemical targets and pathways (e.g. kinase signaling and other regulatory paths). Various approaches that could be used in M. tuberculosis to further characterize compounds include: 1) testing on strains over or under producing essential gene products; 2) testing for synthetic lethal interactions with other known compounds or specific genetic mutants; 3) testing against isolated enzyme targets *in vitro*; 4) testing for pathway perturbations via microarray expression analysis; 5) testing effects on macromolecular pathways (e.g. DNA, RNA, protein, and cell wall synthesis using specific metabolic radiolabeling); 6) *in silico* docking of structures to known essential Mtb targets; 7) large scale QSAR analysis of bulk screening data; 8) evaluation in whole cell reporter based assays,[18] and 9) selection of resistant mutants for identification of the target gene/protein. Other very novel detection systems can be designed to assay for compounds affecting essential regulatory circuits.[19] We have resupplied selected hits from the HTS campaign into microtiter plates in order to facilitate follow up evaluation of prioritized hits. Persons interested in obtaining a sample of these plated compounds should contact the Project Officer (rgoldman@niaid.nih.com) or the NIAID (blaughon@niaid.nih.gov).

Another approach to efficiently adding values to these data sets is to evaluate validated hits for antibacterial activity against other non-mycobacterial pathogens to identify any TB specific inhibitory compounds and to identify possible new leads for other ‘high need’ pathogens such as vancomycin resistant enterococci (VRE), methcillin resistant *Staphylococcus aureus* (MRSA), multidrug resistant Gram-negative bacteria, *Pseudomonas aeruginosa*, and biodefense related organisms such as *Bacillus anthracis*, *Burkholderia pseudomallei* and *B. mallei*, *Yersinia pestis* and *Acinetobacter baumannii*. Thus, these data could provide unique leads for evaluation as anti-tubercular drugs and as drugs for treating infections due to other bacteria for which our current pharmacopeia is lacking.

All screening data will ultimately be deposited in PubChem (www.pubchem.gov) and should represent a valuable resource for those who share our dedication to the discovery and development of new antitubercular drugs.

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Table 1

Characteristics of antibacterial assays for detecting new leads.
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Assay	Advantages	Disadvantages
Whole cell growth inhibition	Compound likely enters microbial cells: the cell entry problem is less of an issue Can immediately move to: whole cell mode of action analysis, evaluation of microbial spectrum	Usually a longer assay (many hours to days) Nonspecific compounds acting at the cell surface or intracellularly may be detected Compound could be unstable, sequestered or degraded by cells and thus inactive but analog could be a valid lead Mode of action unknown; may need to determine/validate for further development and to avoid optimizing nonspecific action or target drift
Isolated in vitro enzyme or pathway	Usually a rapid assay (minutes as opposed to hours or days) Target known in advance	Nonspecific compounds may be detected Compound may not enter cells, or could be sequestered or degraded If compound is active on whole cells, the mode of action needs to be confirmed
Hybrid whole cell with specific enzyme or pathway targeted readout	Compound likely enters microbial cells: the cell entry problem is less of an issue Can immediately move to: whole cell mode of action analysis; evaluation of microbial spectrum Target known in advance	Usually a longer assay (many hours to days) More difficult to design The mode of action needs to be confirmed to support further development and to avoid optimizing nonspecific action or target drift Unknown interactions can give false positives that are difficult to unravel Compound could be unstable, sequestered or degraded by cells and thus inactive but analog could be a valid lead