Tuberculosis: a persistent threat to human health

Tuberculosis (TB) has long plagued humans and is estimated to have caused approximately 1 billion deaths in the past 200 years. With the development of antibiotics, it was thought we had discovered the tools necessary to eradicate TB. However, TB remains a global health crisis and is the leading cause of death by an infectious disease worldwide, causing 1.8 million deaths last year. This raises the question: why have we failed to eradicate TB?

TB has been difficult to control for a variety of reasons, such as: a large human reservoir (~2 billion people have latent TB infections); TB spreads person-to-person via aerosols with an infectious dose as low as a single bacterium; the co-epidemic with HIV/AIDS promotes the spread of disease; currently available vaccines and diagnostics are poorly effective; and public health infrastructure is woefully inadequate throughout most of the world. However, perhaps the most serious problem with TB control is the inadequacy of current antibiotics. To cure TB, current treatment regimens require that a multidrug cocktail be consumed for 6 months. This long course leads to skipped pills and early cessation of treatment and is driving the evolution and spread of multidrug resistant (MDR)-TB. Treatment of MDR-TB takes >2 years, requires the use of expensive second-line antibiotics with serious side effects and is prone to failure. Anyone can catch TB, including drug resistant TB. Given the ease of global travel, the slogan "TB anywhere is TB everywhere" is true and worrisome. We need new TB drugs that act faster and work against drug resistant-TB.

If we simply develop new, traditional antibiotics, it is likely TB will not be eradicated in our lifetime. We already have several effective, inexpensive TB antibiotics, but they all suffer from the same shortcoming: they take too long to work. There are very few infectious diseases that require a 6 month long course of antibiotics. Some bacterial and fungal infections can be treated with a single dose of antibiotics, most others with 1-2 weeks of treatment. To expand upon the first question "why have we failed to eradicate TB?" a natural follow-up question is: why does it take so long to cure TB?

TB is caused by the bacterium *Mycobacterium tuberculosis* (Mtb). Our current understanding of Mtb biology holds that during infection Mtb presents as a spectrum of disease, where some bacteria are actively growing but others are dormant persisters. Mtb persistence is driven, in part, by the nutrient limited and hypoxic environment of the granuloma niche occupied by the pathogen during infection. The actively growing bacteria are reasonably easy to kill in the first two months of treatment with current antibiotics. However, the non-replicating persistent (NRP) bacteria are highly tolerant to antibiotics thus driving the long treatment courses that are required to kill all of the persistent bacteria. Therefore, if we can understand the molecular mechanisms by which Mtb establishes non-replicating persistence (*i.e.* dormancy) perhaps we can develop novel therapeutics targeting these pathways. Such novel therapeutics could revolutionize TB therapy as they promise to kill persisters, limit antibiotic tolerance and thus shorten the course of therapy.

The mission of my research program is to make basic and applied research discoveries that jumpstart the development of new drugs to treat TB. My research lab is a hybrid basic and applied research program. We are pursuing fundamental, "blue-skies" curiosity-driven research problems aimed at understanding the physiology of Mtb pathogenesis and persistence. Additionally, I have established a high throughput screening platform in my lab that enables my team to conduct large-scale chemical biology studies and academic drug discovery. We have invested significant effort into these chemical biology studies (we have screened >890,000 compounds against Mtb) and are one of the few academic labs in the world capable of conducting such high throughput drug discovery research on Mtb. My long-term vision is to define the molecular mechanisms by which Mtb establishes persistent infections and then translate this new knowledge to develop novel chemical therapeutics that reduce persistence, antibiotic tolerance and shorten therapy. Doing so will significantly improve clinical practice, patient outcomes and help meet the grand challenge of TB eradication.

Research Progress: Using fluorescent biosensors to illuminate Mtb pathogenesis

Research Focus #1: Hypoxia-driven adaptation and Mtb pathogenesis. Mtb alters its physiology in response to host immune pressures thus enabling the bacterium to remain viable in humans for decades. Mtb is an intracellular pathogen that resides within macrophages, a host immune cell that kills most other bacteria. The macrophage phagosome has access to nutrients via the recycling endosomal pathway and is an environment in which adapted Mtb can exist in a replicative, growing state. Following infection, the macrophage releases immune modulators that orchestrate the formation of a granuloma around the infected macrophage. The granuloma limits the availability of nutrients and oxygen to the bacterium and drives Mtb to realign its gene expression and physiology to support NRP.

A key genetic regulator of the hypoxia-driven NRP is DosRST, a bacterial two component regulator system (TCS) that governs the expression of ~50 hypoxia regulated genes. DosRST is required for Mtb persistence in animal models, therefore, compounds or genetic mutations that inhibit the DosRST network may hinder the ability of Mtb to maintain a persistent state and thus may reveal new drugs or drug targets that could be exploited to shorten TB therapy. A major barrier to studying DosRST-dependent persistence is that there is not a natural phenotype (*i.e.* a readily discernable trait) that can alert a scientist that the persistence pathway is induced. To address this problem, I engineered an Mtb biosensor strain that exhibits DosRST-dependent, inducible green fluorescence in response to hypoxia. This synthetic phenotype opened up new avenues to studying Mtb persistence, because we finally had a way of seeing when the bacterium was remodeling its physiology to establish dormancy.

This fluorescent biosensor enabled my lab to discover chemical probes that inhibit persistence. The idea is simple: if the bacterium requires DosRST to turn on fluorescence under hypoxia, we can find inhibitors by testing compounds for the ability to inhibit biosensor fluorescence. This targeted, whole cell phenotypic screening approach has received significant grant support including two grants from the Bill & Melinda Gates Foundation through the Grand Challenges Explorations program, and also an R21 grant from the NIH. Using the method of High Throughput Screening (HTS), my lab tested >540,000 compounds to discover inhibitors of DosRST signaling. [redacted] a graduate student in my lab, led this study. We successfully discovered six novel classes of compounds that inhibit DosRST and these results were published in the journal Nature Chemical Biology. In this study, we showed that inhibitors of DosRST could reduce Mtb survival during persistence inducing conditions and break antibiotic tolerance. Moreover, we defined the biochemical mechanism of two of the compounds, showing that they directly targeted the proteins DosS and DosT. This discovery is highly significant because it shows for the first time that DosS and DosT proteins are prone to chemical inhibition and that targeting these proteins inhibits persistence and drug tolerance.

Research Focus #2: pH-driven adaptation and Mtb pathogenesis. Environmental pH is another important cue required for pathogenesis and my lab is a leader in characterizing the mechanisms of Mtb pH-driven adaptation. As a post-doc, I discovered that a regulator called PhoPR is required to sense and adapt to acidic pH. Mutants of PhoPR are attenuated for virulence, therefore, understanding the role of pH and PhoPR in pathogenesis will reveal molecular mechanisms of Mtb virulence.

With the support of a career development grant from the NIH, we conducted a HTS campaign for chemical inhibitors of pH-driven adaptation. For this screen, we engineered an Mtb biosensor strain that exhibits PhoPR-dependent green fluorescence in response to acidic pH, macrophage infection and growth in mice. In a study led by graduate student [redacted] we screened a 273,000 compound library and discovered that ethoxzolamide (ETZ), an FDA approved carbonic anhydrase inhibitor, functions to suppress PhoPR-dependent pH-driven adaptation. In a variety of different assays, we showed that ETZ treatment leads to phenotypes similar to a *phoPR* mutant, including attenuating Mtb infection of macrophages and mice. This exciting finding, published in Antimicrobial Agents and Chemotherapy, has opened up new avenues of research exploring the link between carbonic anhydrases, *phoPR* and pH in Mtb pathogenesis. Comparison of the two biosensor screens also identified >40

compounds that selectively inhibit Mtb growth at acidic pH. We hypothesized these compounds may be targeting physiology that is selectively required for Mtb adaptation to acidic pH. A team led by post-doc Dr [redacted] characterized one such compound, called AC2P36, which revealed that Mtb is highly sensitive to thiol stress at acidic pH. Treating Mtb with AC2P36 at acidic pH chemically depletes free thiols, causes an ROS burst, kills the bacterium and potentiates antibiotics. This study was published in the journal Cell Chemical Biology. In support of the significance of this work, follow-up studies on the chemical biology of pH-driven adaptation has received R01 grant support from the NIH.

Another question my lab has examined is: Why does Mtb slows its growth at acidic pH? Slow growth is associated with Mtb persistence, therefore understanding the link between pH and growth may provide new mechanisms of persistence physiology and drug tolerance. In a study led by MD/PhD graduate student [redacted] we found that at acidic pH, Mtb growth requires host-associated carbon sources including cholesterol and its catabolites pyruvate and acetyl-CoA, whereas other tested sole-sources promoted the establishment of NRP. Furthermore, we found that PhoPR is required for acidic pH-induced slowing of Mtb growth and also functions to maintain redox homeostasis. This work was published in the journal Molecular Microbiology. Using forward and reverse genetic approaches, Jake has also defined specific metabolic enzymes and novel genes that are required to slow Mtb growth at acidic pH. Mutations in one of these genes make Mtb more susceptible to antibiotics at acidic pH. This study is complete and will be submitted for peer review imminently. Overall, my lab has become an established leader in the field of Mtb pH-dependent adaptations and we will continue to ask important questions related to this mechanism of bacterial pathogenesis.

Collaborative research

Progress in science benefits from the open sharing of ideas and collaboration. To enhance research projects central to my main research foci (hypoxia- and pH-driven adaptations), I have developed collaborations with medicinal chemists (Profs. [redacted] at Univ. of Michigan, [redacted] at MSU); scientists with expertise in pharmacokinetics (Prof. [redacted]s); and protein secretion (Prof. [redacted] at Notre Dame). My lab has also developed expertise in conducting RNA-seq transcriptional profiling and whole genome sequencing in Mtb. These techniques require significant bioinformatics expertise, which I have cultivated in my lab. I have collaborated with scientists at Notre Dame (Prof. [redacted]) and Colorado State University (Prof. [redacted]) where my expertise in genomics has supported published studies. To more broadly share our methods with the community, we have also published and made available open-source software, called SPARTA, to conduct processing and statistical analysis of bacterial RNA-seq data.

Michigan State University (MSU) is environment where collaboration is encouraged and I have had several productive collaborations with MSU researchers. Prof. [redacted] (Dept. of Chemistry) has developed novel imidazoline molecules that target the human proteasome via a novel mechanism. We found that these inhibitors also inhibit Mtb growth, likely by targeting the proteasome. This collaboration is ongoing and has been supported by an SPG grant from the MSU Foundation and an R21 from the NIH. Another collaboration is with Profs [redacted] (College of Veterinary Medicine) where we are studying the persistence of *M. bovis* (an animal pathogen related to Mtb) in silage. *M. bovis* is endemic in Michigan deer populations and its survival in silage may represent a means of transmission to Michigan livestock. This work has been supported by a grant from the Michigan Animal Agriculture Alliance and is an example of how my research can directly impact animal agriculture in Michigan. I am also collaborating with Prof. [redacted] (College of Veterinary Medicine) on a project studying *M. bovis* transmission in dairy products in Brazil. This international project was supported by an Endowed Research Fund award. In summary, I have actively collaborated with researchers at MSU and at other institutions, on projects where I benefitted from others expertise, or where I could share my expertise. These collaborative projects promoted interdisciplinary approaches and expanded my intellectual horizons and I intend to continue the pursuit of new collaborations.

Future Research and Funding Plans

pH-driven adaptation focus: The chemical biology of pH-driven adaptation project has received R01 support and we will continue studies proposed in this grant such as defining the link between PhoPR signaling and carbonic anhydrases. We are pursuing the hypothesis that PhoPR may use periplasmic pH as a proxy to sense host carbon dioxide. We are also characterizing the function of six additional pH-selective growth inhibitors. Excitingly, these inhibitors are converging on common pH-sensitive targets including thiol and iron homeostasis. We are making strong progress on this research and I will submit a R01 renewal proposal on this topic in 2020. I will also submit an R21 grant examining the genetics and physiology of Mtb pH-driven adaptation. There remain many unanswered questions regarding how Mtb regulates metabolism, respiration and nutrient acquisition at acidic pH and based on reverse and forward genetic studies we have identified candidate genes that are hypothesized to control this physiology.

Hypoxia-driven adaptation focus: The discovery that DosS and DosT proteins are vulnerable to endoperoxide bearing molecules (such as artemisinin) has led our team to discover that other unrelated synthetic molecules carrying an endoperoxide are also active against DosS/T. We plan to examine the use of synthetic endoperoxides to modulate Mtb persistence, study their biochemical mechanisms of action and define their utility to limit Mtb survival *in vivo*. We are also conducting mechanism of action studies and medicinal chemistry and pharmacokinetic optimizations of several other DosRST inhibitors, with the goal of examining their function against Mtb during animal infection. Notably, we have developed single cell imaging approaches that will enable us to use the fluorescent biosensor strain as a biomarker for drug exposure *in vivo* and to define in which lung microenvironments DosRST signaling is induced by immune cues or inhibited by small molecules. This project will be the subject of an upcoming, new R01 proposal.

New research directions: It is also important that I continue to explore new directions, because a diversified research portfolio is important for the long-term health of my lab. Studies are underway to define the mechanism of action of several new series of small molecules that inhibit Mtb growth inside macrophages (two new series HC2091 and AC4M01 have already been extensively characterized). We are also conducting studies testing the hypothesis that the Mtb regulators TcrXY and SigJ play a role in remodeling Mtb metabolism at acidic pH. I will continue to follow my long-term vision to define the molecular mechanisms by which Mtb adapts to immune cues and establishes persistent infections.

Concluding Remarks

My first five years at MSU have been busy! I have established a successful, independent lab focused on defining the mechanisms by which Mtb senses and adapts to host immune cues and have discovered small molecules and genes that target this facet of pathogenesis. Supporting the impact of my research, studies from my lab have been published in journals such as Nature Chemical Biology, Cell Chemical Biology, Molecular Microbiology and Antimicrobial Agents and Chemotherapy and have been supported by over $4 million of external grant support from the NIH, the Gates Foundation and others. Additionally, in recognition of my research progress, I have received college- and university-wide awards including the Zoetis Award for Veterinary Research, the Jean P. Schultz Biomedical Research Award and the Innovation of the Year Award. I have also established a national and international scientific reputation, as evidenced by my inclusion on several NIH and Dept. of Defense study sections, invitations to serve on journal editorial boards and to review manuscripts, and opportunities to present seminars at universities, national meetings and international meetings. I have also been a devoted teacher in the classroom and the lab (as detailed in the accompanying teaching statement). I have been committed to service at departmental, college and university-wide levels, having served on several faculty search committees, the Department Faculty Advisory Committee, the College Advisory Council and as an elected college representative to the Faculty Senate and University Council. I have dedicated myself to excellence in my research, teaching and service and I am grateful for the opportunity to be a member of the Michigan State University community.

Training a future generation of knowledgeable citizens and skilled scientists

My father was a high school mathematics teacher. He taught a variety of classes and towards the end of his career he taught senior level classes in calculus, algebra, and geometry. He was very satisfied with his teaching career and I remember him being most excited about his advanced classes, about him discussing the success of his students solving difficult problems or being admitted to mathematics programs at universities. I have been influenced by my father's positive experience as a teacher and have always wanted to pursue a career as an educator. During my time at MSU, I have dedicated myself to excellence in teaching and have found this effort highly rewarding.

When teaching in the classroom, one of my primary goals is to engage with the students. Although the detailed content of the material is important, if the students are not actively engaged in their learning, it is likely that they will simply forget the material at the end of the semester. Engaged learning, by contrast, has the potential to have a lasting impact. For example, for the undergraduate level class I teach, MMG461 Microbial Pathogenesis, I have found that getting to know the students encourages engagement. I usually show up early and stay late after class to talk with the students about their courses, career goals and the class material. These informal discussions make the classroom a friendly, open environment where ideas can be freely shared and difficult concepts explored. When lecturing, I have regular breaks for discussions and I design my lectures to provide this time without having to rush through the material. I also use many real world examples (including my own research experience) to place the sometimes abstract ideas into context. Microbial pathogenesis is my passion and I am enthusiastic about the topic and my goal is to help fuel this enthusiasm in my students. I believe this teaching approach has had a positive impact. For example, I received an e-mail from an MMG461 student who graduated from MSU and is currently attending veterinary school at Ohio State. He wanted to discuss the potential of using intracellular pathogens to deliver drugs to cancer cells. It was an excellent idea (with several practical issues) that showed to me that the student was engaged, after graduation, in creative scientific thought about microbial pathogenesis. I am hopeful that other students remain similarly engaged. Other evidence of engaging with the students is that several have asked me to write them letters of recommendation for graduate or professional school. I have consistently received excellent evaluations from student in MMG461 Molecular Pathogenesis. My most recent student evaluation scores show all of my instructor scores above 1.5, with an instructor involvement score of 1.2. A score of 1 is considered "superior" on a scale from 1 to 5. I love teaching undergraduates microbiology and it is a privilege to teach a topic directly related to my research interests.

I also teach microbial pathogenesis at the graduate level (MMG861). Teaching this class involves reading primary research papers and encouraging students to identify the hypotheses being tested and then interpret and discuss the data and conclusions. For my section of this class, I have been assigning papers that relate to the theme of metabolism during intracellular pathogenesis. We cover diverse pathogens (fungi, gram-positives and gram-negatives) and each section provides opportunities to compare and contrast the various mechanisms pathogens have evolved to survive inside host cells. The students have also been very receptive to my teaching with my instructor scores averaging 1.6.

Teaching professional students at the Colleges and Veterinary Medicine and Osteopathic Medicine has been an area where I have worked hard to improve my teaching. I teach mycology to veterinary students (4 lectures in MMG563) and I teach one lecture on tuberculosis to medical students (MMG532). I have always received excellent teaching scores from the students in these classes, but some comments suggested that the students wanted more objective-driven lectures with practical examples. One of my ongoing goals has been to improve teaching of professional students and I have done so by devising specific learning objectives, reorganizing material, providing additional clinical case studies as examples and generating interactive questions (that students can respond to on their computers). This past year for MMG563, across all categories, I had an average rating of 4.6/5 (five is the best), including numerous positive comments, suggesting the modifications to my teaching material are having a positive impact on student learning and satisfaction. Teaching at the undergraduate,

graduate and professional student levels requires very different strategies to engage the students and my goal is to keep improving my teaching abilities to better educate and engage with the students.

I am also deeply committed to mentoring of undergraduates and professional students in the lab. To date, I have trained 16 undergraduates in my lab, as well as 5 professional students (mostly veterinary medicine students). When an undergraduate or professional student joins the lab, I dedicate a significant amount of time personally training the students in basic molecular biology techniques and ensuring they feel comfortable in a new, challenging environment. Over time, each undergraduate is paired with a graduate student or post-doc to co-mentor the student on a specific project. Undergraduates who have trained in my lab have gone onto graduate school, medical school, dental school and research careers, and I am confident the training in my lab helped them achieve their goals. Students in my lab have also received numerous awards, including [redacted] who was awarded a national ASM Undergraduate Research Fellowship and [redacted] who received a travel award to attend the Annual Biomedical Research Conference for Minority Students. Moreover, the undergraduate and professional students make major impacts on our research projects and three such students have been authors on publications.

Another important aspect of teaching and training in my lab is my commitment to promoting diversity. It is essential that the scientists and medical professionals we train represent the diversity of our society and I have engaged with programs so that my teaching and research programs function to promote diversity. I have mentored four research students in my lab through the BRUSH and SROP diversity promoting programs. Other diversity promoting activities related to teaching and education I have conducted include seminars to SROP and AGEP groups at MSU, serving as a fellowship reviewer (since 2013) for the ASM Undergraduate Research Fellows program and serving on the umbrella graduate admissions committee for the BioMolecular Sciences program.

Mentoring graduate students currently represents the most significant aspect of my work, both in terms of time committed and importance for fulfilling my career goals as a researcher. I currently have three PhD graduate students in my lab who are conducting the bulk of my lab's research and I have graduated two PhD students so far ([redacted]. I have been extremely fortunate to have assembled a team of talented, dedicated and good-natured students. These students share my commitment to combatting the ongoing Mtb epidemic by conducting creative, high quality research. The team also shares my commitment to responsible conduct of research and attention to safe work practices in the lab. As a mentor, I help my students develop hypotheses, design experiments and analyze data. I also assist with writing papers, presentations and their professional development. Another important role as a mentor is to help guide the students in the art of doing science. For me, science is about discovery and about being willing to venture deep into the proverbial darkness of the unknown. I encourage students to pursue big ideas and creatively solve important problems, even if this means investing time developing new tools or pursuing a study with a high risk of failure. As long as an experiment is well designed and controlled, failure is embraced in my lab. It is a sign that we are pursuing ambitious goals. I believe this "dream big" approach will lead to high-impact discoveries and also keep the students (and myself) excited about the process of doing science. I have seen this mentoring approach succeed in the students I have trained. The students are making excellent research progress, and more importantly, developing into independent, creative thinkers. Observing this transformation of my students into productive scientists is enormously gratifying and is one of the real joys of being a professor.