Host-Factor Enhancement of Therapy for Tuberculosis

By

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A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Infectious Diseases and Immunity in the Graduate Division of the University of California, Berkeley

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Abstract

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Tuberculosis (TB) is a disease of major public health importance and improvements to its treatment could greatly benefit efforts aimed at eliminating the disease. Current treatment options for TB are limited in effectiveness and have numerous fundamental failings due to the necessarily lengthy duration of therapy and toxicity of the antimicrobial drugs deployed, among other issues. The studies described herein where undertaken with the goal of developing adjunctive treatments or modifications of existing treatments which could improve the treatment course, outcome, or both for standard TB antimicrobial chemotherapy.

Three areas of research are discussed beginning with adaptive immune augmentation through therapeutic vaccination, proceeding to investigations of innate immune adjuvant therapy and concluding with host environment mediated improvement of selectivity index of TB antimicrobial compounds.

A post-treatment, therapeutic vaccine was studied with the goal of developing a tool which could prevent relapse or reactivation disease. Though the project was a follow up to a study which demonstrated exceptional protection, the vaccine candidate did not demonstrate any detectable efficacy in three parallel murine infection experiments. Possible reasons and implications of this failure are discussed.

Because correlates of protection for adaptive immunity to TB are poorly understood and have not proven to be tractable for intervention, innate immune enhancement was investigated. Autophagy, a cell-intrinsic process with antimicrobial capabilities, was selected due to its well described tuberculocidal activity and pharmacologic manipulability. However, despite the apparent capacity of some test compounds to increase autophagic flux, none demonstrated robust restriction of mycobacterial growth in murine or human macrophages. That study did, however, lead to the serendipitous discovery that pH based drug partitioning can increase the selectivity index of antimicrobial drugs against M. tuberculosis inside cultured macrophages.
I dedicate this work to my parents, David and Elizabeth Schump – they made it all possible.
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TUBERCULOSIS – PATHOGEN AND DISEASE

Tuberculosis (TB) is an ancient disease of humans caused by the rod shaped bacteria of the *Mycobacterium tuberculosis* (*Mtb*) complex, which continues to be a prolific killer of humans to this day. *Mtb* kills more people each year than any other single infectious agent except HIV (Lawn and Zumla 2011). The origin of the disease is still the subject of debate (Bos, Harkins et al. 2014), but it has been known at least since the ancient Greek era when it was called phthisis (Pease 1940). Although *Mtb* and several related mycobacterial species are capable of infecting a wide range on animal hosts (O'Reilly and Daborn 1995), throughout this work TB will always refer to the disease of humans, and unless otherwise specified, the causative agent may be assumed to be *Mtb*.

In the medieval ages TB was called the White Plague or White Death and has been known more recently as Consumption. TB is a wasting disease most commonly characterized by coughing and weight loss, though all cell types and tissues can be infected (Harries and Dye 2006; Lawn and Zumla 2011). In advanced cases with severe pathology, hemoptysis is often observed. Practically all new infections occur by the inhalation of droplet nuclei containing *Mtb* expelled from an individual suffering from active TB. *Mtb* was proven to be the definitive cause of tuberculosis by Robert Koch in 1882 (Koch 1882).

In the absence of treatment, the case fatality rate is around 50% for a typical case of pulmonary TB while disseminated disease is practically always fatal (Mitchison 2005). Many people – probably the majority – who come into contact with the pathogen do not show signs of infection, and even among those who do, 90-95% of infected individuals will contain the infection and have what is known as a latent infection that never progresses to active disease (Harries and Dye 2006). The other 5-10% of individuals who carry a latent infection for a time will eventually have the infection reactivate and become symptomatic and transmissible. HIV/AIDS and other forms of immune suppression increase the likelihood of an infected person developing active disease dramatically; among latently infected people, HIV infected individuals have a risk of reactivation of around 8%-10% per year rather than 5-10% over a lifetime for HIV negative people (Gordin and Masur 2012). The impact of HIV/AIDS co-infection with TB is enormous and in the absence of well administered care, the prognosis is extremely bleak (Aaron, Saadoun et al. 2004).

EPIDEMIOLOGY

The most recent report on the global epidemiology of TB from the World Health Organization states that in 2013, “an estimated 9.0 million people developed TB and 1.5 million died from the disease, 360,000 of whom were HIV-positive (WHO 2014).” It is estimated that around one third of the world’s population is latently infected by *Mtb* (Dye, Scheele et al. 1999). Prevalence and incidence of disease vary markedly with geographic location as well as with socio-economic status in any one place (Dye and Williams 2010). See Figure 1.1 below which shows a map detailing the estimated incidence of tuberculosis worldwide in 2013. Within the United States, most cases are among foreign born individuals and are believed to be the result of reactivation of a latent infection acquired abroad (Gordin and Masur 2012). The highest burden countries in terms of overall number of cases are India, China, Nigeria and Pakistan. The highest incidence
rates of active disease are found in Swaziland, Lesotho, South Africa and Namibia (WHO 2014 pg. 33)

**FIGURE 1-1: GLOBAL ESTIMATED TB INCIDENCE RATES PER 100,000 POPULATION IN 2013**

![Map showing global TB incidence rates per 100,000 population in 2013.](image)

**SOURCE:** (WHO 2014)

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**TREATMENT AND PREVENTION**

The first major step toward preventing TB infections was the identification of the causative microorganism by Robert Koch in 1882. Once the etiology of the disease was understood and the primary mode of transmission (i.e., droplet nuclei expelled by coughing) known, the benefit of simple interventions such as isolation of patients and improvement of ventilation in relevant buildings was obvious. By the 1920’s, efforts to develop a vaccine had met with success and the only currently licensed vaccine for the prevention of TB, known as BCG, dates from this period (Liu, Tran et al. 2009). It is currently the most widely used vaccine worldwide. The efficacy of BCG has been studied in depth and found to be highly variable; estimates range from zero to eighty percent protection from pulmonary TB in adults, though efficacy preventing disseminated disease in children is much more robust (Brewer 2000). Vaccination as a control strategy for tuberculosis will be addressed in chapter two.
The first powerful treatments for halting the growth of \textit{Mtb} were para-amino salicylic acid, discovered by Jorgen Lehmann in 1943 and thiosemicarbazone discovered by Gerhard Domagk around 1945 (Daniel 2006), while the first antibiotic which could kill the bacterium was streptomycin (Schatz, Bugle et al. 1944). The activity of streptomycin was demonstrated by the clinical trials following the drug’s isolation which are also famous for being the first example of rigorous randomization in clinical trial design (BMJ 1948). Unfortunately, \textit{Mtb} began evolving resistance to streptomycin immediately and resistant strains were described before the end of the first trials (Youmans, Williston et al. 1946). Numerous other compounds were successfully developed in the middle of the twentieth century for the treatment of TB, but by the beginning of the twentieth century, resistance to exiting compounds was rapidly increasing while development of new TB drugs was lacking(Gandhi, Nunn et al. 2010). The need for new therapeutics and efforts to develop them, by modulating host functions and by leveraging elements of the host environment, are the subjects of chapters three and four, respectively.

MICROBIAL NICHE

\textit{Mycobacterium tuberculosis} generally gains access to its niche by inhalation into the lower respiratory tract where even a single bacillus can cause infection. The most commonly cited initial host cell is the tissue resident macrophage, but it has been shown that alveolar epithelial cells can also be infected (Bermudez and Goodman 1996; Lin, Zhang et al. 1998) and a protein which facilitates invasion of non-phagocytic cells by \textit{Mtb} has been identified (Arruda, Bomfim et al. 1993). Phagocytic host cells, such as macrophages and dendritic cells (Wolf, Linas et al. 2007) ingest bacteria with the help of several defined receptors (Ernst 1998). Prior to ingestion by phagocytes, though, the initial contact of the bacillus with alveolar fluid on the mucosal lining must be considered, and that fluid has been found to contain several important components which govern the subsequent encounter of \textit{Mtb} with immune cells including surfactants, hydrolases, and complement (Arcos, Diangelo et al. 2015).

In a naïve host, no adaptive response is present, and the bacteria grow relatively unrestricted, primarily within macrophages. While natural killer cells (Junqueira-Kipnis, Kipnis et al. 2003) and innate immune effectors are present, they are generally unable to contain the infection until the onset of adaptive immunity, which comes into force after two weeks of infection (Urdahl, Shafiani et al. 2011). Nevertheless, innate immune sensors and effectors shape the immune response (Korbel, Schneider et al. 2008). Interplay between bacillus and host is complex at this point and mycobacterial avoidance of innate immune recognition involves numerous evasion techniques including masking of immune-reactive bacterial components (Cambier, Takaki et al. 2014), effector molecule secretion (Stanley, Raghavan et al. 2003), and manipulation of chemokine signaling (Slight and Khader 2013).

Once an adaptive response is active, a balance of inflammation and tissue repair is necessary for the host to prevent unchecked bacterial growth on the one hand, while preventing excessive tissue pathology on the other (Saunders and Britton 2007). For some years, the TH1/TH2 paradigm was applied to TB in the way it had been demonstrated to govern the outcome of
leishmaniasis (Mougneau, Bihl et al. 2011) and was thought to be analogous. Specifically, it was thought that a TH1 response is protective while a TH2 response is deleterious (Demissie, Abebe et al. 2004). It has become clear, however, that this view is overly simplistic (Jung, LaCourse et al. 2002), and a more complicated balance of immune effectors appears to be necessary. Critical components of a protective response have mostly been demonstrated by increased susceptibility of gene knockout mice. In this way, interferon gamma, inducible nitric oxide synthase, tumor necrosis factor alpha, CD4 and CD8 T cells, as well as other immune components, have been demonstrated to be indispensable for control of mycobacterial infection (Flynn and Chan 2001).

When the full complement of immune cells are recruited to a focus of infection, they coalesce into a structure called a granuloma which consists of concentric rings of various cell types and debris. See Figure 1-2 for an overview of several commonly observed granuloma types. Other types of granulomas than those depicted in the figure occur, too, such as calcified lesions. Macrophages in the vicinity are often observed to increase in size and become laden with lipid droplets. Such giant cells are referred to as foamy macrophages and they, along with the infecting bacteria themselves, contribute a great deal of lipid rich material to the caseous core of typical granulomas in humans (Russell, Cardona et al. 2009). The core of human granulomas are often necrotic and hypoxic (Tsai, Chakravarty et al. 2006; Via, Lin et al. 2008). The kinetics of formation and disintegration of granulomas have been studied in some detail using modern imaging modalities (Egen, Rothfuchs et al. 2008).

While the structures themselves are reasonably well described, their function is a matter of some debate (Russell 2007; Paige and Bishai 2010). Granulomas were long thought to represent a host adaptation which limits infiltration, but they also allow for the persistence of bacteria, making it less clear which side of the host-pathogen interaction is benefited more. While dissolution of granulomas, or failure to form them in the first place, is associated with poor control, the bacteria have been observed to more efficiently replicate and infect newly recruited macrophages within established granulomas (Cosma, Humbert et al. 2008; Davis and Ramakrishnan 2009). Studies of granulomas in latently infected non-human primates have further underscored the polymorphic nature of these structures (Lin, Ford et al. 2013). Within an infected lung, granulomas can behave independently, with some expanding while some contract or stay static. Consistent with those observations are histologic examinations of human resected lung tissue from TB patients. Such studies have demonstrated that “the lung of a chronic TB patient contains a diversity of micro-anatomical niches created by the different immunological processes occurring independently at these sites.” (Kaplan, Post et al. 2003) Importantly, though, even in the areas with the greatest bacterial load –namely the luminal surface of cavities– bacteria are primarily observed inside of macrophages.
FIGURE 1-2: OVERVIEW OF COMPOSITION OF TYPICAL GRANULOMAS

(Tuberculous granulomas. There are several granuloma types that can be found among humans and non-human primates, even within the same individual. a | The classic tuberculous granuloma, found in active disease and latent infection, is the caseous granuloma, which is composed of epithelial macrophages, neutrophils, a cuff of lymphocytes (CD4+ and CD8+ T cells and B cells) and sometimes surrounded by peripheral fibrosis. The centre of this type of granuloma is caseous, a necrotic state that probably consists of dead macrophages and other cells. This area is hypoxic. Mycobacteria in this granuloma can be found in macrophages (either in contact with T cells or not) in the hypoxic centre or possibly even in the fibrotic rim; this provides the mycobacteria with different microenvironments. b | The non-necrotizing granuloma is usually seen in active disease and consists primarily of macrophages and some lymphocytes; this lesion can be seen in guinea pigs and mice, albeit with more lymphocytes. M. tuberculosis bacilli are within macrophages in this lesion. c | Fibrotic lesions are seen mostly in latent tuberculosis but also in active disease and are composed almost completely of fibroblasts, with a minimal number of macrophages. Although it is possible to culture bacilli from some fibrotic lesions, it is not clear where the bacilli reside (possibly in macrophages or in the fibrotic area) or what the microenvironment is like.

The intracellular environment experienced by *Mtb* within macrophages has been the subject of considerable research (Vergne, Chua et al. 2004; Ehrt and Schnappinger 2009; Cambier, Falkow et al. 2014). Mycobacteria are generally thought to reside in partially acidified phagosomes (Sturgill-Koszycki, Schlesinger et al. 1994) which are prevented from fusing with lysosomes (Armstrong and Hart 1971). That *Mtb* resides in this intracellular niche is counterintuitive given the primary role of macrophages in engulfing and destroying bacteria (Langermans, Hazenbos et al. 1994). Indeed, macrophages deploy several systems for generating reactive oxygen and nitrogen species (Bedard and Krause 2007) which have been demonstrated to be mycobactericidal (Zahrt and Deretic 2002). Autophagy, a cell intrinsic mechanism which has been reported to have anti-mycobacterial activity (Gutierrez, Master et al. 2004), is also inhibited by the bacteria (Deretic, Singh et al. 2006). The manipulation of autophagy in host cells is the subject of chapter three. There have been reports of *Mtb* found in the cytosol (van der Wel, Hava et al. 2007), but the bacteria are generally thought to reside within a membrane bound phagosome in an incompletely mature state which partially acidifies to around pH 6.4 (Pethe, Swenson et al. 2004; Rohde, Yates et al. 2007). Like many intracellular pathogens, *Mtb* actively competes with the host for acquisition of micronutrients such as iron (Agranoff and Krishna 2004).

After the establishment of granulomas, sufficient tissue destruction most occur for bacteria to spill back out into airways in order to facilitate onward transmission. Matrix metalloproteinases have been implicated in this progression (Parks, Wilson et al. 2004; Ong, Elkington et al. 2014) and the primary driver of this pathology is thought to be the host immune response. That this pathology is mediated by the host immune system is supported by the observation that HIV/AIDS patients are actually less likely to transmit infection (Cauthen, Dooley et al. 1996) despite evidence that they harbor higher bacterial loads (Diedrich and Flynn 2011). In the end, then, it appears that the host is manipulated into opening the door for the bacteria to exit and establish a new infection whereupon the cycle repeats itself.

The three chapters which follow describe efforts directed at breaking this cycle of transmission by modifying the host response to infection using exogenously supplied antigenic, immune-modulatory, or antimicrobial substances, respectively.


CHAPTER 2

TRIAL OF A POST-EXPOSURE, THERAPEUTIC VACCINE FOR TB BASED ON THE MYCOBACTERIAL MCE1A PROTEIN
TUBERCULOSIS – CONTROL BY VACCINATION

Vaccines are among the most powerful tools for infectious disease prevention. Many diseases for which effective vaccines exist have been drastically reduced in prevalence and one, smallpox, has been altogether eradicated ([CDC] 2011). Historically, vaccines have been prophylactic interventions to prevent infection by priming a host response in advance of exposure to a pathogen. However in the case of tuberculosis, due to the enormous pool of already infected individuals, estimated at one third of the global population (Dye, Scheele et al. 1999), a post-exposure vaccine holds great potential for preventing the development of disease among those already latently infected with the bacillus. Additionally, an effective therapeutic vaccine could hypothetically reduce the relapse rate after treatment (Wallis, Doherty et al. 2009).

Unfortunately, despite the enormous toll tuberculosis exerts on the human population, as described in chapter one, only one vaccine against it has been developed, Bacillus Calmette–Guérin (BCG). Although safe, unfortunately, the vaccine does not robustly protect adults from pulmonary tuberculosis (Brewer 2000), even if booster doses are given (Rodrigues, Pereira et al. 2005).

The protection afforded by BCG has proven to be highly variable depending on geographic location (Black, Weir et al. 2002) and has been suggested to be dependent on many factors such as concomitant helminth infection (Elias, Britton et al. 2008), which biases toward a non-protective TH2 T-cell response, and exposure to cross-reactive mycobacterial antigens from non-tuberculous mycobacteria (Lin, Reddy et al. 2009), which leads to underestimation of vaccine activity due to unequal baseline immunity in comparator groups or interference with BCG response (Brandt, Feino Cunha et al. 2002). Other factors such as the nutritional status of the recipient and latitude (possibly mediated by vitamin D levels) have been suggested (Fine 1995). The strain used for vaccination is yet another important variable, since deviations in handling have led to differences between strains used in different parts of the world (Liu, Tran et al. 2009). While BCG does protect against childhood forms of the disease and disseminated infection (Rodrigues, Diwan et al. 1993), these are not the source of most new infections, so protection afforded in that setting does little to avert future cases of TB. An additional layer of complexity is added by variability in the strain of Mtb actually encountered by individuals after vaccination which has been shown to lead to different immunologic manifestations (Ordway, Shang et al. 2011).

Vaccination against TB is particularly challenging, because unlike many other infectious diseases like chickenpox or yellow fever, a resolved episode of TB does not lead to lifelong -or even particularly powerful- immunity. Indeed, after successful treatment and resolution of TB infection, only about a one log reduction in bacterial load in the lungs is seen in animal models and infection rate is not affected (Russell, Barry et al. 2010). Therefore, the protection sought from a vaccine needs to be better than that induced by a natural infection. Still, it has been demonstrated that vaccination with subunits (i.e., part[s] of the bacterium) can impact immunity to the disease since culture filtrate confers similar resistance to BCG (Andersen 1994). Current approaches using whole cell, subunit, and viral vectored candidates have been reviewed elsewhere (Kaufmann, Hussey et al. 2010; da Costa, Walker et al. 2015). The exact nature of the necessary immune response to protect against clinical TB –much less asymptomatic Mtb infection– is poorly defined (Bhatt, Verma et al. 2015). While both CD4 and CD8 T cells are
believed to play important roles, and certain cytokines such as interferon gamma and tumor necrosis factor alpha have been shown to be involved in containment of *Mtb* infection in the host (Flynn and Chan 2001), the precise response that a highly effective vaccine must induce is not understood (Dietrich and Doherty 2009).

Despite all of these challenges, the potential benefit of a highly effective vaccine against TB is too great to ignore. Using a mathematical model of TB dynamics, the potential for effective vaccines to reduce transmission and subsequent disease rates have been estimated (Young and Dye 2006). Projections for various interventions involving pre- or post-exposure vaccines are depicted in Figure 2-1 using South Asia as a case study. Though the projections are profoundly dependent on the assumptions regarding vaccine effectiveness and deployment (in the model below, the combined coverage and efficacy yield 70% protection of the target population), such reductions in disease, if realized, would surely rank among the great public health triumphs of human history.
Figure 2-1: The figure shows the impact of vaccine strategies alone (A) or in combination with drug treatment (B) in a model which approximates the case of South Asia. It is estimated that mass vaccination of uninfected populations would reduce the annual incidence to 20 per 100,000 in 2050. A vaccine effective prophylactically as well as therapeutically would reduce the TB incidence rate to 14 per 100,000, theoretically. It is estimated that combining drug treatment and vaccination would reduce TB incidence to 8 per 100,000 in a single campaign.

Because as many as one third of the global population is already latently infected with \(Mtb\) and because a very large proportion of disease and transmission stems from reactivation of latent infection or relapse of incompletely sterilized infections (Lawn and Zumla 2011), a post exposure vaccine would be particularly promising for use alongside chemotherapy as a means of limiting TB disease and transmission.

**MCE1A POST-EXPOSURE VACCINE CANDIDATE**

The Mce1A vaccine candidate consists of a recombinant fragment of the Mce1A protein from \(Mtb\) which spans amino acids 51-454 and includes a motif which has cell penetrating properties (Arruda, Bomfim et al. 1993; Lu, Tager et al. 2006). The truncation of the protein was intended to decrease its hydrophobicity and facilitate production and purification of the recombinant protein in \(E. coli\). Because of its cell penetrating properties, which may facilitate antigen uptake and because it is known to be expressed in macrophages \(in-vivo\) (Uchida, Casali et al. 2007), Mce1a was selected as a vaccine antigen. In a published study, (Miyata, Cheigh et al. 2012), it was demonstrated that the Mce1a vaccine confers nearly completely sterilizing immunity in the lungs of mice when administered intraperitoneally after drug treatment in a modified version of the “Cornell mouse model.” The Cornell model is described below. The original study did not make use of any adjuvant or excipient and was delivered in phosphate buffered saline (PBS). Importantly, the challenge strain of \(Mtb\) in that study was a mutant with a targeted knock out of the repressor of the \(mce1\) operon (\(mce1R\) KO). Because the \(mce1R\) KO strain has been shown to overexpress proteins of the \(mce1\) operon (Casali, White et al. 2005), including \(mce1A\), the potential for the demonstrated efficacy to be an immunologic artifact due to overexpression of the vaccine antigen was considered. The present study was undertaken to ascertain whether similar protection would be generated against a wild type strain of \(Mtb\).

**CORNELL MODEL OF REACTIVATION/RELAPSE TUBERCULOSIS**

Essentially, the Cornell model is meant to mimic reactivation or relapse TB where an initial infection is brought under control though the use of antibiotics such that no viable bacteria can be isolated from the lungs of mice for a period of time (McCune, Feldmann et al. 1966). Subsequently, though, some mice spontaneously reactivate/relapse and have a measurable bacterial load in their lungs (or other organs, such as spleen). The period after drug treatment is intended to mimic the phenomenon of latency in humans, which is a state of low or undetectable bacterial load (Chan and Flynn 2004). Bacterial outgrowth afterward is meant to mimic reactivation, which can happen spontaneously in humans after a variable period of latency. In practice though, it is also a model of relapse, which occurs when incomplete sterilization of an infection leads to eventual treatment failure and bacterial recrudescence. Both of these are undesirable outcomes with regard to \(Mtb\) infection, so a vaccine which can protect against either of them would be valuable. A typical plot of bacterial load in the lungs and spleens of mice used in this model is displayed in Figure 2-2.
Figure 2-2: The figure depicts the typical course of infection in the Cornell model of reactivation/relapse TB in terms of bacterial load in the lungs at selected time points. The shaded portion indicates the antibiotic treatment period.


MATERIALS AND METHODS

Bacterial culture. Mycobacterium tuberculosis strain H37Rv was grown to mid-log phase for use in infection. Bacteria were grown in Middlebrook 7H9 broth (Difco, 271310) supplemented with 0.5% (v/v) glycerol, 0.05% (w/v) Tween 80 (Sigma, P1754), and 10% (v/v) albumin-dextrose-catalase enrichment. Cultures were maintained at 37°C in 125mL vented Erlenmeyer flasks with 100 RPM circular agitation.

Infection. Seventy five 8-week-old female BALB/c mice (Charles River) were infected with ~10^2 CFU of Mtb strain H37Rv using the Inhalation Exposure System (Glas-co., Terre Haute, IN) in three parallel groups. Infection was allowed to proceed for four weeks without treatment.

Drug treatment. The antimicrobial regimen was 100 µg/mL isoniazid and 15 mg/mL pyrazinamide provided ad libitum in drinking water for six weeks. Treatment spanned weeks four through ten post infection.
Vaccination regimen and treatment arms. Mice received three injections subcutaneously spaced three weeks apart after the drug treatment period and an additional one week washout period. The four treatment groups were: Mce1A protein alone (50 µg), Mce1A protein (5 µg) plus 5 µg glucopyranosyl lipid A adjuvant in stable emulsion (GLAS-SE), placebo alone (BSA, equimolar concentration to Mce1A), or placebo with GLA-SE. All injections were delivered in 200µL of PBS.

Assessment of bacterial load. Bacterial load was quantified by colony forming unit (CFU) counts of lung and spleen homogenates serially diluted in Middlebrook 7H9 medium with 0.05% Tween 80 and plated onto Middlebrook 7H11 agar plates. Agar plates were incubated for three weeks at 37°C in a humidified incubator before enumeration.

Time points. One day after infection, three mice from each infection group were sacrificed to confirm the bacterial inoculum delivered. At four weeks and ten weeks of infection, three mice were sacrificed for CFU enumeration. At 32 weeks post infection seven mice from each treatment group were sacrificed and bacterial quantified in the same way. Finally, at 52 weeks post infection eight mice from each treatment group were sacrificed and assessed for bacterial load.

RESULT - MCE1A VACCINE IN CORNELL MODEL WITH WILD TYPE MTB

The execution of the modified Cornell model was successful and the initial infection, four week peak of untreated bacterial load and post drug treatment CFU counts were comparable with the initial study. Disappointingly, however, bacterial loads in the lungs and spleens of mice at 35 and 52 weeks post infection were not different from placebo (BSA) control (all comparisons p >> 0.05 by unpaired, two tailed t-test). Time course graphs of the bacterial loads in the lungs and spleens of mice are displayed as Figures 2-3 and 2-4, respectively. The individual results by treatment arm at 35 and 52 weeks are given as Figures 2-5 and 2-6, for clarity (all relevant comparisons p > 0.5 by two tailed, unpaired t-test). Beyond average bacterial load, the rate of reactivation/relapse was also not different, as shown in Figures 2-7 and 2-8 which show the proportion of mice with any bacteria recovered by organ and by treatment arm at the two time points following vaccination. In all cases, the label “adj” denotes that a treatment group included GLA-SE while “non-adj” indicates that no adjuvant was present.
Figure 2-3: Balb/C mice were infected with $10^2$ CFU *Mtb*, left untreated for four weeks, treated for six weeks with isoniazid and pyrazinamide and then received three vaccinations (Mce1A) or sham injections (BSA) over the next six weeks. Bacterial load in lungs of mice by treatment group at time points post infection is displayed. ‘Adj’ indicates the presence of GLA-SE adjuvant while ‘non-adj’ denotes its absence.

Figure 2-4: Bacterial load in the spleens of mice at indicated time points post infection after treatment described above are displayed.
Figure 2-5: Bacterial load at 35 weeks post infection by organ and vaccine group of mice in Cornell model of reactivation/relapse disease. Vaccine and placebo treatments do not differ by unpaired, two tailed student’s t test (p > 0.05).

Figure 2-6: Bacterial load at 52 weeks post infection by organ and vaccine group of mice in Cornell model of reactivation/relapse disease. Vaccine and placebo treatments do not differ by unpaired, two tailed student’s t test (p > 0.05).
Figure 2-7: The proportion of mice from each treatment group (n = 7/group) from which any bacteria were recovered in lungs (left bars) or spleen (right bars) at 35 weeks of infection.

Figure 2-8: The proportion of mice from each treatment group (n = 8/group) from which any bacteria were recovered in lungs (left bars) or spleen (right bars) at 52 weeks of infection.
The weight of mice was tracked individually as a surrogate for overall health, but no major differences in the average weight of mice in different treatment groups were observed Figure 2-9. Error bars are omitted from Figure 2-9 for clarity; the range of standard deviation values across all treatment groups was from 0.9 to 2.8 grams or 3.8-10.6% of the measured weight. The lack of divergence of the groups by weight is consistent with the CFU data and supports the conclusion that the Mce1A vaccine candidate lacked any detectable efficacy in this setting.

Beyond tracking of weight, all mice were monitored by veterinary technicians from the office of laboratory animal care for general health. The work in this chapter was performed under animal use protocol #R228-1211B.

Figure 2-9: The average weight of mice from each treatment group over time is shown.
Only the 35 week time point is comparable, in terms of time-post-infection, with the original experiment described in (Miyata, Cheigh et al. 2012), since a 21 week time point was not conducted in the present study and the original study did not follow mice out to 52 weeks. The differing results in this experiment at 35 weeks post infection versus the previous study at 32 weeks are clear; nearly complete protection in that study versus no detectable effect in this study. While it cannot be known whether such a difference between this study and the original one existed at 21 weeks post infection, it seems unlikely that the protection observed in the original study would have been observed, given later results. Even if protection at 21 weeks had been observed in the present study, it would have represented transient protection, which would not be sufficient to drive interest in the vaccine candidate and would be of questionable relevance. Whether the protection observed in Miyata et al. would have persisted at 52 weeks cannot be known from available information.

There are numerous potential reasons for the discrepancy in the results presented here versus those published in Miyata et al. Differences in preparation or delivery of the antigen would be the first to consider, chronologically.

The same plasmid was used to facilitate production of the protein in both studies, so the encoded protein should be the same and was confirmed by SDS-PAGE of induced and uninduced (IPTG) bacterial lysates. The identity of the protein was confirmed by western blot using a rabbit polyclonal anti-Mce1A antibody. However, the exact nature of the protein purification was not the same for both preparations of vaccine. Both were captured using a polyhistidine tag and recovered in denaturing conditions, but the antigen for the current vaccine study had been lyophilized whereas the protein in the pilot study was not. In neither case was the exact quantity of LPS (lipopolysaccharide, also known as endotoxin) in the final vaccine preparation determined. In the absence of rigorous efforts to remove LPS, a large but unknown quantity would be expected to remain associated with any protein of bacterial origin. It seems possible that part of the apparent success of the vaccine in Miyata et al., or the failure of the current study, involved the unintentional co-administration of some quantity of LPS.

In fact, LPS stimulation of TLR4 has been suggested to be a promising target for adjuvant action in vaccine development (Casella and Mitchell 2008) and the adjuvant used in the study described here, GLA-SE, also acts by binding TLR4 and has demonstrated activity inducing expression of cytokines known to be important for containment of *Mtb* infection, such as interferon gamma and tumor necrosis factor alpha (Coler, Bertholet et al. 2011). One TB vaccine using this adjuvant, “ID93+GLA-SE,” is in clinical trials currently (da Costa, Walker et al. 2015). That is in contrast to TLR2, which is a natural target of binding by *Mtb* derived lipoproteins, but is dispensable for adaptive immunity to TB (McBride, Bhatt et al. 2011). However, the fact that the addition of TLR4 agonist adjuvant did not improve outcomes of those treatment arms where it was included relative to those where it was omitted suggests that either TLR4 signaling is not
beneficial in this context or that the threshold for such an effect was exceeded and addition of ligand above that level is of no, or deleterious, effect.

Among other potentially divergent aspects of the two studies could be the quantity of antigen delivered and its condition upon administration. Although both vaccines were produced in *E. coli* and purified yielding a final, denatured protein of the same truncated length, handling differences could leave varying quantities of protein adsorbed to various glass—and to a greater degree, plastic—ware used in the preparation of the vaccines, since proteins are known to adhere to such surfaces, reducing the quantity passed onward in each handling step (Andrade and Hlady 1986). This would be especially salient given the lack of any additional excipients in the described vaccines which could otherwise have decreased loss. Additionally, as mentioned above, the protein used in the current study had been lyophilized prior to preparation of the final vaccine formulation and loading into syringes. The composition of the syringes, duration of time the solutions were loaded in them, as well as temperature to which the vaccines were exposed, could all have potentially affected the physical composition of the vaccine at the time of delivery. The route of vaccination being changed could also potentially have been a factor in the differing results. Route of antigen delivery has been suggested to modify the response to TB vaccines due to the impact on the population of T cells that are exposed to the antigen (Horvath and Xing 2013). However, in Miyata et al, it is mentioned that subcutaneous vaccination was effective against the *mce1R* KO strain. It seems unlikely, then, that the subcutaneous route of administration was a major factor in the failure of the follow up study to recapitulate the protection seen in the earlier study.

The most likely explanation for the disparate results of the two trials is the difference in strain of *Mtb* used in the infections. The fact that the *mce1R* KO strain overexpresses the antigen which was vaccinated against in (Miyata, Cheigh et al. 2012), whereas the follow up experiment utilized an unmodified wild type strain, is probably the most meaningful difference between the two trials and likely the primary driver of the different outcomes. While the *mce1R* KO strain could potentially be used to test whole cell or subunit vaccines composed of parts of *Mtb* not encoded or substantially affected by the *mce1* operon, its use when the antigen of choice is so composed apparently gives results which are not reproducible against wild type *Mtb*.

**CONCLUSIONS**

Failure of post-exposure vaccination in the Cornell model has been described previously; both BCG and *M. vaccae*, another mycobacterial species, fail to protect against reactivation TB in the Cornell model (Dhillon and Mitchison 1994). One reportedly successful treatment of established *Mtb* infection using DNA vaccination after a similar setup to the Cornell model (Lowrie, Tascon et al. 1999) was challenged in a subsequent report by a group which was unable to reproduce those findings (Taylor, Turner et al. 2003) and actually observed worsening of pathology in vaccinated animals. Indeed, the potential for exacerbating, rather than improving, outcomes by vaccination of *Mtb* infected individuals has been a concern for some time (Anderson 1891; Daniel 2006). The mechanism of such reactions has been partially elucidated and is believed to be IL17 driven (Cruz, Fraga et al. 2010).
No vaccine has currently shown convincingly better efficacy than BCG in the nearly 100 years since that vaccine was produced by empirically testing serially passaged *M. bovis* until it lost sufficient pathogenicity to be used as a vaccine (Liu, Tran et al. 2009). A few candidates have shown modest protection similar to BCG in animal models (Bertholet, Ireton et al. 2010; Aagaard, Hoang et al. 2011) but such protection must be considered an incremental success. As of the time of writing, robustly predictive correlates of acquired protection have not been well defined (Ellner, Hirsch et al. 2000; Bhatt, Verma et al. 2015). The most advanced vaccine candidate to date, MVA85A, recently failed to prevent infection with *Mtb* as well as progression to TB disease in a large clinical trial (Tameris, Hatherill et al. 2013). Despite having been successful in animal studies and good evidence that it elicits the TH1/TH17 response previously thought to be most likely to offer protection against TB, the vaccine did not show efficacy against incident infection or disease. This underscores the gaps in understanding correlates of protection against tuberculosis and also suggests that current animal models of TB are imperfect.

Although the protection afforded by the Mce1A vaccine in (Miyata, Cheigh et al. 2012) was not replicated with a wild-type *Mtb* strain, the modified Cornell model gave the expected experimental parameters and the result obtained is believed to be valid. Refinement of this model using the six week antibiotic treatment period seems to have produced precisely the desired setup and should allow testing of vaccine candidates against the H37Rv wild type *Mtb* strain in BALB/c mice, although there is some suggestion that more resistant mice (for example C57BL/6) would generally show greater protection after vaccination (Dannenberg 2010).

Another potential for these studies to provide insight for future vaccine development is in the further analysis of the response generated by the Mce1A vaccine in the *mce1R* KO strain. That is, if the protection shown in Miyata et al. can be replicated against that strain, it may be worth characterizing that response— even if it is not directly reproducible in other strains—to glean some insight into what an effective response against *Mtb* infection might look like. Artificial as it may be, this protection is the most powerful described to date, so it is likely worthwhile to characterize it in detail in the pursuit of correlates of protection that could prove to be durable in other contexts.

Such a correlate of protection from reactivation or relapse would be exceedingly valuable since no other immune-modulatory approach to adjunctive TB treatment has shown such a profound effect. If a robust correlate of protection against relapse and reactivation disease were identified, it could be used as a biomarker for vaccine development which could lead to an effective vaccine to prevent disease in the enormous population of latently infected individuals. Such a vaccine could also potentially be given alongside conventional TB chemotherapy where it could theoretically reduce the rate of relapse upon completion of treatment.

Overall, the results presented here suggest that empiricism is still most likely the most powerful route for TB vaccine development. Either significant efforts must be directed to generating a better understanding of the correlates of immunity to TB, or sufficient resources must be allocated to generating an improved vaccine by some modern empiric or partially empiric testing method (for example, using numerous candidates with many based upon known attenuation mechanisms). Approaches could include testing myriad *Mtb* knockout strains, strains which overexpress potentially protective antigens or numerous candidate subunit vaccines with an
assortment of adjuvants. Even then, it is not clear that success in any of the available animal models would translate to improved protection afforded to humans.

Because such efforts are not feasible in a university setting without exceptional outside support, alternatives to vaccination for improving TB treatment were pursued. Specifically, the process of autophagy has recently been reported by several groups to be both capable of impacting *Mtb* growth in infected cells or animals (Gutierrez, Master et al. 2004; Singh 2006; Alonso, Pethe et al. 2007; Biswas, Qureshi et al. 2008; Kumar, Nath et al. 2010; Ponpuak, Davis et al. 2010; Zullo and Lee 2012), and also tractable for pharmacologic intervention (Floto, Sarkar et al. 2007; Sarkar, Perlstein et al. 2007; Zhang, Yu et al. 2007; Williams, Sarkar et al. 2008; Balgi, Fonseca et al. 2009; Rose, Menzies et al. 2010; Hundeshagen, Hamacher-Brady et al. 2011; Stanley, Barczak et al. 2014). Chapter three explores yet another approach to enhance host response to control or reduce latent infection. The application of pharmacologic autophagy induction as an immune-adjuvant treatment for tuberculosis is examined.


CHAPTER 3

EFFECT OF MTOR-INDEPENDENT INDUCERS OF AUTOPHAGY ON INTRACELLULAR *M. TUBERCULOSIS*
INTRODUCTION

The line of investigation described in this chapter was undertaken with the goal of developing an adjunctive immune-modulatory treatment for tuberculosis (TB). Because *Mtb* is most often observed inside of macrophages, even in high burden infection (Kaplan, Post et al. 2003), it was hypothesized that augmenting innate immune effectors in macrophages could lead to reduced bacterial load, reduced pathology or both. The approach pursued was based on the induction of increased flux through the autophagy pathway. The rationale of this approach was based on reports that autophagy is capable of restricting the intracellular growth of *Mtb* while simultaneously suppressing inflammation (Castillo, Dekonenko et al. 2012).

Such restriction of growth, if achieved, could lead to the development of interventions for preventing reactivation in latently infected individuals by boosting their anti-mycobacterial immune function, or to interventions to be deployed alongside conventional chemotherapeutic agents to prevent relapse disease in the same way. Autophagy induction has been described to both directly affect bacterial load and to boost the adaptive immune response to mycobacterial infection by upregulating antigen expression (Jagannath, Lindsey et al. 2009).

IMMUNE ADJUVANTS

The potential of host-directed, immune-modulatory treatment of TB has been demonstrated by several different approaches including cytokine supplementation, DNA vaccination, *M. vaccae* exposure, therapeutic subunit vaccination, and siRNA treatment (Condos, Rom et al. 1997; Lowrie, Tascon et al. 1999; Johnson, Kamy et al. 2000; Aagaard, Hoang et al. 2011; Rosas-Taraco, Higgins et al. 2011). Some of these approaches have also proven effective against drug resistant *Mtb* (Condos, Rom et al. 1997; Okada, Kita et al. 2009; Bertholet, Ireton et al. 2010). Host directed small molecule treatments have shown promise recently with inhibitors of phosphodiesterases (PE) type four (Koo, Manca et al. 2011), PE types three and five (Maiga, Agarwal et al. 2012), ABL kinase (Napier, Rafi et al. 2011), and HMG-CoA reductase (Parihar, Guler et al. 2013) all demonstrating reduction in bacterial load in various models of mycobacterial infection.

AUTOPHAGY

The process of autophagy is a bulk degradation pathway, the function of which is to remove constituents of eukaryotic cells which are too large for proteosomal degradation (Yang and Klionsky 2010). It is involved in numerous cell homeostatic processes, notably as the main pathway leading to lysosomal destruction of organelles and long lived proteins (Mizushima and Komatsu 2011). The removal of damaged mitochondria (*i.e.*, “mitophagy”) is another important function of autophagy (Youle and Narendra 2011). Metabolically, autophagy is a key mechanism because it is regulated by several sensors of cellular energy stores (*e.g.*, AMPK) and when induced, has the effect of liberating metabolizable biomolecules to provide energy for the cell (Mihaylova and Shaw 2011). In agreement with this function of autophagy is the well-
established observation that nutrient starvation induces autophagy (Mizushima, Yoshimori et al. 2010). The centrality of autophagy as a cellular homeostatic system is demonstrated by its evolutionary conservation back to yeast (Hughes and Rusten 2007). While autophagy has sometimes been suggested to be a mechanism of cell death, in the vast majority of cases it is likely an effect, and not a cause, of initiating cell death pathways (Kroemer and Levine 2008).

The rationale for manipulation of autophagy in the context of Mtb infection derived from studies in which that process was shown to reduce mycobacterial survival (Gutierrez, Master et al. 2004; Singh 2006; Alonso, Pethe et al. 2007; Biswas, Qureshi et al. 2008; Kumar, Nath et al. 2010; Ponpuak, Davis et al. 2010; Zullo and Lee 2012). The role of autophagy in resistance to TB has been underscored by genome wide association studies showing that mutations in autophagy related genes correlate with increased incidence of active TB in humans (Intemann, Thye et al. 2009; Che, Li et al. 2010). Beyond that, an siRNA screen in human cells for host factors that affect mycobacterial load discovered a dense network of interactions surrounding key autophagy regulators (Kumar, Nath et al. 2010). These findings are further supported by the observation that autophagy deficient (ATG5 cKO) mice are markedly more susceptible to TB (Castillo, Dekonenko et al. 2012; Watson, Manzanillo et al. 2012). Also consistent with an important role for autophagy in mycobacterial control is the observation that Th2 cytokines, long known to be deleterious to the control of TB, suppress autophagy (Harris, De Haro et al. 2007).

Autophagy induction subsequently allows for a more robust immune response by making additional pathogen derived peptides available for antigen presentation (Munz 2009). Importantly, this has been demonstrated with regard to mycobacteria specifically (Jagannath, Lindsey et al. 2009). To biologists familiar with the propensity of natural systems to optimize for multi-functionality, it should not come as a surprise that a host cell would turn a system capable of powerful catabolic activity on invading pathogens (Ponpuak, Davis et al. 2010). The resulting downstream augmentation of the adaptive immune response, taken together with its demonstrated direct bactericidal effect on mycobacteria (Gutierrez, Master et al. 2004; Singh 2006; Alonso, Pethe et al. 2007; Biswas, Qureshi et al. 2008; Kumar, Nath et al. 2010; Ponpuak, Davis et al. 2010; Zullo and Lee 2012), clearly indicates that autophagy is a potentially powerful target for immune-modulatory intervention. While there is evidence that Mtb has at least one mechanism for inhibiting autophagy in human primary macrophages (Petrucchioli, Romagnoli et al. 2012), this resistance may be indicative of evolutionary efforts to combat a potent selective pressure.

**MTOR INDEPENDENCE**

The standard method for autophagy induction, mTOR inhibition, is not an acceptable strategy in the context of infectious disease because even very specific inhibitors of this kinase, such as rapamycin, strongly suppresses the adaptive immune system (Araki, Ellebedy et al. 2011). This has been demonstrated both in animal models (Calne, Collier et al. 1989), and more recently by the clinical use of rapamycin after human organ transplantation to prevent rejection (Watson, Friend et al. 1999). Furthermore, mTOR antagonists have documented lung toxicity (Duran, Siu et al. 2006).
Pharmacologic induction of autophagy without detrimental effects on the adaptive immune system has been made possible by the discovery of mTOR independent inducers of autophagy (Sarkar, Floto et al. 2005; Sarkar, Davies et al. 2007; Zhang, Yu et al. 2007; Williams, Sarkar et al. 2008). Preliminary data suggested that mTOR independent autophagy may be capable of destroying intracellular mycobacteria (Floto, Sarkar et al. 2007).

A further advantage of many of the compounds screened for this effect in those studies is that they have safety records in humans, albeit in a different context. Many are FDA approved for other indications (Williams, Sarkar et al. 2008), are present in foods (resveratrol), or are pharmaceutical excipients (trehalose). See Appendix A for a summary of relevant characteristics of compounds which were considered for inclusion in these studies. Two of these drugs, carbamazepine (CBZ) and fluoxetine (FLX), were hits in screens for compounds that restrict mycobacterial viability inside macrophages (Sundaramurthy, Barsacchi et al. 2013; Stanley, Barczak et al. 2014). Unfortunately, CBZ has some known interactions with anti-TB drugs. However these interactions are not outright contraindications, but rather factors to be considered with regard to dosing (Zolezzi 2002). Oxcarbazepine, an analog of CBZ which has fewer metabolic side effects, was also a hit in the same screen as CBZ, albeit with a slightly lower phenoscore (Sundaramurthy, Barsacchi et al. 2013). There is also evidence that oxcarbazepine can exert effects through PI3K enzymes but in a way which apparently does not affect AKT, an mTOR pathway regulator (Simao, Zamin et al. 2009).

Several of the compounds identified by other groups and which the present studies utilized indirectly inhibit calpains as their mechanism for inducing autophagy (Williams, Sarkar et al. 2008). Calpain activity is necessary for hyper-secretion of inflammatory cytokines by macrophages in autophagy deficient mice in the context of mycobacterial infection (Castillo, Dekonenko et al. 2012), so this approach appeared to be particularly promising. Also, calpains mediate a switch from pro-autophagic to pro-apoptotic phenotype via ATG5 (Yousefi, Perozzo et al. 2006) so their blockade could be beneficial by preventing excessive host cell death, though this remains speculative.

Resveratrol, a polyphenol with a good safety profile, has been reported to induce autophagy (Mauth, Jacob et al. 2011) in an mTOR independent manner (Scarlatti, Maffei et al. 2008). Moreover, this has been shown to occur in vivo (Lekli, Ray et al. 2010).

Some of the selected mTOR independent autophagy inducing compounds are also voltage gated calcium channel inhibitors (see Appendix A). The channels they target have been reported to be active in modulating immune cells, including lymphocytes, dendritic cells, NK cells and neutrophils (Suzuki, Inoue et al. 2010), as well as affecting mycobacterial clearance in dendritic cells in vitro (Gupta, Salam et al. 2009). Additionally, rapamycin and its analogs have been reported to bind these channels, so some portion their effect against mycobacterial infection may be due to that interaction (Ruan, Pong et al. 2008). The role of calcium flux in Mtb persistence has been reviewed (Kusner 2005). Essentially, calcium flux is not necessary for phagocytosis, but is necessary for the respiratory burst and phagosomal fusion with lysosomes. Calcium flux appears to be inhibited by Mtb and its inhibition correlates with increased survival. This inhibition of calcium flux and its effects on Mtb bacterial load are reversible with ionophore treatment or calcium chelation (Malik, Denning et al. 2000).
The exact mechanism of action by which most of the drugs in this class induce autophagy is controversial; the role of calcium, in particular, is contested and has been reviewed elsewhere (Decuypere, Bultynck et al. 2011). The activity of hVps34 is reported to be calcium independent, however (Yan, Flinn et al. 2009), and calcium flux is not necessary for autophagy mediated by inositol triphosphate receptor inhibition (Criollo, Maiuri et al. 2007). This mechanism, which most of the drugs tested converge upon in their extended pathways (Renna, Jimenez-Sanchez et al. 2010), is the most commonly identified mTOR independent target in high throughput screens (Zhang, Yu et al. 2007; Williams, Sarkar et al. 2008). Because this induction is apparently upstream of, and dependent on, Atg5, Atg10, Atg12, Beclin-1 and hVps34, it is expected to operate along the canonical autophagy pathway (Criollo, Maiuri et al. 2007). The regulation of autophagy by inositol derivatives is discussed in a salient review (Criollo, Vicencio et al. 2007).

**ADJUNCTIVE THERAPY**

When drug susceptible TB is treated with an effective antibiotic regimen, most of the culturable bacteria are eliminated relatively quickly; a patient’s sputum is typically culture negative after two months when three effective antibiotics are used (Gelband 2000), but treatment of even uncomplicated cases is recommended to continue for a totally of six months (Mitchison 2005). The additional time beyond apparent sterilization is intended to lower the rate of relapse disease, or recrudescence of the infection (Gelband 2000). If the bacteria which persist within macrophages after the initial intensive phase of killing by antibiotics could be eliminated by autophagy induction, a reduction in relapse rates may be achieved. Alternatively, treatment duration may be reduced by co-treatment with autophagy inducing drugs, which would be expected to limit noncompliance and selection of drug resistance.

A study investigating antimicrobial tolerance phenotypes (Adams, Takaki et al. 2011) found that one drug reported to induce autophagy independently of mTOR, verapamil (Williams, Sarkar et al. 2008), also has the effect of limiting the development of antibiotic tolerance by mycobacteria. Suppression of antimicrobial tolerance in this way, in addition to direct killing of mycobacteria with antimicrobial agents, would hypothetically decrease relapse rates and drug resistance further than autophagy induction plus antimicrobials compounds alone, so treatment with that compound was pursued.

Additional synergy could be expected *in-vivo* from the generation of interferon gamma (IFN-γ) by T cells, a cytokine which has itself been shown to induce autophagy (Singh, Davis et al. 2006). Relatedly, the autophagic destruction of internalized mycobacteria has been shown to upregulate antigen presentation and T cell activation leading to the secretion of IFN-γ (Jagannath, Lindsey et al. 2009). It follows that the induction of autophagy and subsequent production of an autophagy inducing cytokine would generate a feed forward cycle.
MACROPHAGE CELL CULTURE. All experiments, unless otherwise noted, were performed on resting macrophages at a density of 5x10⁴/well in 200μL of medium in 96 well cluster plates. Macrophages used were the RAW 264.7 murine cell line (ATCC TIB-71), THP-1 human monocytic cell line (ATCC TIB-202) or primary murine bone marrow derived macrophages (BMDM). THP-1 cells were maintained in RPMI medium with 10% fetal bovine serum (FBS) while RAW and primary murine macrophages were maintained in DMEM with 10% FBS. When THP-1 cells were used, they were treated with 12-O-tetradecanoylphorbol 13-acetate (PMA) at 100nM for 48 hours before infection, after which time PMA was no longer used. For all macrophage media, glutamine was supplied as Glutamax (L-alanyl-L-glutamine, Invitrogen) at 2mM. Macrophages were maintained at 37°C, 5% CO₂ in a humidified incubator and handling steps were executed as quickly as safely possible to minimize exposure to ambient conditions.

AUTOPHAGIC FLUX ASSAYS. Fluorescence microscopy and western blot methods were used to monitor autophagic flux using LC3 as a marker [For a review of the interpretation of autophagic flux data, see (Mizushima, Yoshimori et al. 2010)]. For IF studies, RAW 264.7 macrophages carrying the pROW2 plasmid (encoding CMV-GFP-LC3) were used as described (Watson, Manzanillo et al. 2012). Confirmation of results was achieved using a well validated antibody directed against LC3 (L8918, Sigma) according to manufacturer’s instructions. Images were collected on an epi-fluorescence wide-field microscope (Zeiss Axio Observer D1) and scored using a script for ImageJ software from NIH. Western blots were conducted essentially as described (Menzies, Moreau et al. 2001) and quantified by ChemiDoc MP with ImageLab 4.0.1 software (Bio-Rad).

BACTERIAL CULTURE. All experiments were conducted with Mycobacterium tuberculosis strain Erdman. Bacteria were grown in 50mL of Middlebrook 7H9 broth supplemented with 0.5% (v/v) glycerol, 0.05% Tween 80 (w/v), and 10% (v/v) oleate-albumin-dextrose-catalase (BBL Middlebrook OADC Enrichment, catalog no. 212351). Cultures were maintained at 37°C in 1L roller bottles.

INFECTION. Bacterial cultures were grown to mid log phase for use in infections. Bacteria were washed thrice in PBS, resuspended and centrifuged at 50g to pellet clumps. Infection medium, consisting of appropriate cell culture medium for the macrophage type used but with horse serum substituted for FBS, was made to contain enough bacteria to achieve a multiplicity of infection of one in 100μL. For example, for a typical experiment with 5x10⁴ macrophages per well, 100μL of infection medium at 5x10⁵ CFU/mL would be applied. Phagocytosis was allowed to proceed for four hours, after which macrophages were washed twice with warm PBS and returned to 200μL of normal medium. For plating experiments, macrophages were lysed by the addition of 50μL of 0.5% Triton X-100, yielding a final concentration of 0.1% detergent. Serial dilutions were produced in complete 7H9 medium and plated on Middlebrook 7H10 agar plates containing 10% OADC and no antibiotics. Plates were enumerated after 17-21 days of incubation at 37°C in a humidified incubator.
**Drug treatment.** All compounds were purchased from Sigma Aldrich except rapamycin (Cayman Chemical Co. 13346). Drugs were dissolved in Hybri-Max DMSO (D2650, Sigma) to give stock solutions at 1000 fold their intended final concentration. Solvent controls contained 0.1% DMSO. All compounds for use in infections were filter sterilized using 0.2µm nylon syringe filters.

**RESULTS AND CONCLUSION**

Despite the preponderance of evidence supporting the theoretical basis for mTOR independent inducers of autophagy to restrict mycobacterial growth in infected macrophages, no compound investigated robustly affected bacterial load except for fluoxetine, which also did so in autophagy deficient (ATG5 cKO) cells (Figure 3-1), implying that its activity is not dependent on canonical autophagy. [For a review of the interpretation of autophagic flux data, see (Mizushima, Yoshimori et al. 2010).] Numerous infections with an auto-luminescent reporter strain carrying the bacterial LUX operon as well as experiments using the gold-standard measure of bacterial load, colony forming unit enumeration (CFU), failed to demonstrate a reproducible impact on bacterial growth.

![Bacterial Load in BMDM at 72HPI By CFU (Normalized to DMSO 0.1% Control)](image)

**Figure 3-1:** Fluoxetine restricts the growth of *Mtb* in autophagy deficient (ATG5 KO) bone marrow derived macrophages as well as in wild type (C57B/6) cells. In 10 µM and 20µM conditions, restriction is not statistically different between the two cell types (p > 0.05 by unpaired, two tailed student’s t-test).
The reason(s) for this failure are obscure. Some of the study compounds, notably carbamazepine, were found to apparently upregulate autophagy by western blot for LC3 (Figure 3-2), a marker of autophagy (Mizushima, Yoshimori et al. 2010), and by immuno-fluorescence microscopy for the same target, in resting macrophages. Results did not appear to depend on the macrophage type, solvent used (DMSO, ethanol), or time point post infection. The impact of handling, such as the amount of time that macrophages were outside of the incubator for drug application or other manipulation, was minimized but could have played a role in producing variability in results. For all experiments, the multiplicity of infection (MOI) was set to one. Clumping of the bacteria, however, could have affected the actual MOI achieved. Greater clumping could have reduced the quantity of bacteria in successfully modified phagosomes, since it has been reported that clusters of bacteria resist phagosomal maturation less effectively than single bacilli (de Chastellier, Forquet et al. 2009).

**Figure 3-2**: Western blot of LC3 protein from RAW264.7 murine macrophages treated as labeled (A). Quantification of LC3B expression relative to LC3A or β actin shows apparent increase in autophagic flux by addition of carbamazepine (CBZ) and chloroquine (Chlor.) versus either treatment alone. For a review of the interpretation of autophagic flux data, see (Mizushima, Yoshimori et al. 2010).

Drugs were selected based on their therapeutic index as gauged by the ratio of the concentrations at which they have been reported to induce autophagy to the concentrations at which they are used clinically, as well as their side effect profile and contraindications. The compounds were tested at a target concentration which was chosen based on serum levels achieved in humans and on the concentration at which they were reported to induce autophagy in the primary literature. They were also tested at tenfold higher and lower concentrations (denoted 0.1x, 1x and 10x). For the first set of experiments, the target concentrations were as follows: fluoxetine (FLX) 1µM, verapamil (VER) 1µM, rilmenidine (RIL) 0.01µM, carbamazepine (CBZ) 25µM, minoxidil (MNX) 1µM, and valproic acid (VPA) 500µM.
Rapamycin (“Rapa” in Figure 3-3) was used as a positive control for autophagy induction, but its activity was highly variable. Over longer incubation periods, such as the 72 hour experiment displayed in Figures 3-3, it did not restrict mycobacterial growth. It seems that reports in the literature which focus on very early time points such as two hours post infection (Gutierrez, Master et al. 2004) neglect to look at the longer term effects, which are of greater practical importance. Another potential confounder of other studies is the practice of washing macrophage monolayers before plating for CFU. Toxic compounds (as rapamycin, among others, seems to be at later timepoints) give spurious results because detached macrophages and the bacteria they contain are lost to the washes, artificially increasing the compound’s apparent effect. To avoid this distortion, no wash step was included in the experiments described here after the application of the medium containing the experimental compounds; for lysis, a 1/5th volume (50 µL) of fivefold concentrated (0.5%) triton X-100 was added to the experimental wells (200 µL each) and serial dilutions were made directly from the resulting lysate suspension. This modification was based partly on the methods reported in (Vogt and Nathan 2011).

**Figure 3-3:** The fold increase in luminescence as a measure of bacterial load is shown for THP-1 cells 72 hours after infection with autoluminescent *Mtb* in conditions as labeled. See appendix A for description of compounds tested (carbamazepine, CBZ, resveratrol, RES,
Rapamycin, Rapa, and fluoxetine, FLX). Drugs were applied immediately after infection (early), after an 18 hour delay (late) or in six hour treatment periods (pulsed).

Additionally, amiodarone was used in some experiments but its use was discontinued once it was realized that it is an mTOR inhibitor and has serious pulmonary side effects (see Appendix A). Resveratrol (“RES” in Figure 3-3) was not included in initial screens for autophagy induction, but was included in some later bacterial load experiments because of reports that it induces autophagy through an mTOR independent mechanism and is non-toxic (Scarlatti, Maffei et al. 2008; Lekli, Ray et al. 2010; Mauthe, Jacob et al. 2011) Nevertheless, it did not significantly reduce bacterial load in any experiment.

While none of the drugs were highly toxic as measured by propidium iodide uptake in RAW 264.7 cells after incubation for 18 hours (a representative experiment is depicted in Figure 3-4), among other assays such as lactate dehydrogenase measurement, only FLX and CBZ ever appeared to be active in terms of restricting mycobacterial growth. Subsequent experiments with CBZ, including a kinetic experiment to determine the outcome of applying drugs immediately after infection (“early”), 18 hours post infection (“late”) or in six hour pulses (“pulsed”) produced equivocal results (Figure 3-3) with regard to CBZ activity, and in the end only FLX had reproducible activity against intracellular Mtb.

**Figure 3-4:** RAW 264.7 cells were exposed to compounds at target concentration, as described in the text, or 10 fold higher or lower concentrations. Uptake of propidium iodide, a measure of plasma membrane integrity and a surrogate for cell health, was scored after 18 hours of exposure to experimental compounds or cycloheximide (100µM) positive control for cell death.
FLX, however, was shown to be active in autophagy (ATG5) deficient cells by both CFU enumeration (Figure 3-1, above). The FLX treatment was not statistically between ATG5 KO and wild type macrophages (p > 0.05 by unpaired two tailed t-test). The deficiency of the cells for ATG5 was confirmed by western blot (Figure 3-5). The shift in the band observed with the anti-ATG5 antibody in the KO cell lysates (left lane) is due to the excision of an exon flanked by loxP sites by the Cre recombinase under the control of the LysM promoter (Zhao, Fux et al. 2008). Additionally, FLX did not appear to increase autophagic flux because combining it with chloroquine did not increase the accumulation of LC3 puncta in resting bone marrow derived macrophages by immunofluorescence (Figure 3-6) or by western blot. It seems likely, given the chemical profile of FLX, that it acts in the same manner as chloroquine, a well validated inhibitor of autophagic flux (Mizushima, Yoshimori et al. 2010), and that the observed absolute LC3 increase with FLX treatment is due to the accumulation of LC3 rather than induction of autophagic flux. Both FLX and chloroquine are weakly basic compounds which have been shown to accumulate in acidic organelles within cells, which will be discussed in chapter four. For chloroquine, that accumulation is known to inhibit autophagy; for FLX the same effect seems likely.

Figure 3-5: ATG5 conditional knockout (A) or wild type C57B/6 bone marrow derived macrophages (B) were lysed and probed with an antibody against ATG5 (Sigma, A0731). The ATG5-12 complex should be detected just below the 70kD marker in cells with functional ATG5.
Figure 3-6: Bone marrow derived macrophages were exposed to experimental compounds as labeled and then analyzed by immunofluorescence for LC3 to determine the level of autophagic flux. Results are the average puncta count per cell with error bars showing the standard deviation.

In conclusion, none of the drugs pursued as mTOR independent autophagy inducers were able to restrict *Mtb* growth in infected macrophages except for FLX, which was subsequently shown not to act by modulating autophagy. Regardless of its mechanism of action, however, FLX showed robust activity against intracellular *Mtb*. The anti-mycobacterial activity of FLX was still of great interest because the long term goal of the project was always the development of an adjunctive treatment for TB, regardless of the mode of action. The activity of FLX and drugs with similar chemical profiles is the subject of chapter four.


de Chastellier, C., F. Forquet, et al. (2009). "Mycobacterium requires an all-around closely apposing phagosome membrane to maintain the maturation block and this apposition is re-established when it rescues itself from phagolysosomes." Cell Microbiol 11(8): 1190-1207.


CHAPTER 4

PARTITIONING OF ANTIMICROBIAL COMPOUNDS BY PH BASED ION TRAPPING CAN INCREASE INTRA-MACROPHAGE SELECTIVITY INDEX AGAINST MYCOBACTERIUM TUBERCULOSIS

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**ABSTRACT**

The efficacy of antimicrobial drugs against *Mycobacterium tuberculosis* (*Mtb*), an intracellular bacterial pathogen, is generally first established by testing compounds against bacteria in culture medium. However, inside of infected macrophages bacteria encounter an environment which differs substantially from broth culture and are subject to important host-dependent pharmacokinetic phenomena which can modulate drug activity. Here we describe how pH dependent partitioning drives asymmetric antimicrobial compound distribution in *Mtb* infected macrophages. Specifically, weak bases with moderate activity against *Mtb* were shown to accumulate intracellularly due to differential permeability and relative abundance of their ionized and un-ionized forms. Non-protonatable analogs of the test compounds did not show this effect. Neutralization of acidic organelles directly with ammonium chloride or indirectly with bafilomycin A1 partially abrogated the growth restriction of these drugs. We further demonstrated that the efficacy of a clinically used compound, clofazimine, is augmented by pH based partitioning. Because the parameters which govern this effect are well understood and are amenable to chemical modification, this knowledge may enable the rational development of more effective anti-infective drugs against tuberculosis.

**INTRODUCTION**

*Mycobacterium tuberculosis* (*Mtb*), the causative agent of tuberculosis (TB), ranks with HIV as the two pathogens which are the most prolific killers of humans (2015). As many as two billion people are estimated to be latently infected with *Mtb* (Dye, Scheele et al. 1999), despite the existence of effective antibitotics. Treatment of TB is challenging due to the long duration of chemotherapy needed to sterilize infection (Blumberg, Burman et al. 2003) and the emergence and spread of drug resistance (Gandhi, Nunn et al. 2010). The current standard of care for uncomplicated, drug-susceptible cases is a minimum of 26 weeks of antibiotics given first as eight weeks of an intensive, combined drug regimen and then a further 18 weeks of two drugs (Blumberg, Burman et al. 2003). Shortening of this regimen is a major goal of current drug development efforts while the development of drugs with novel mechanisms of action or particularly favorable pharmacokinetic profiles is another priority to combat drug resistance and improve compliance.

Standard TB treatment has been shortened before, from 9-12 months to the current 6 month ‘short-course’ period with the addition of pyrazinamide (PZA) (Blumberg, Burman et al. 2003; Zhang and Mitchison 2003). Strikingly, PZA is inactive *in-vitro* at the neutral pH used in most “whole-cell” screens and so would not have been identified by current approaches. It is only because PZA was administered in a murine model of TB that its potent *in-vivo* activity was discovered (Zhang and Mitchison 2003). This demonstrates the need for more robust systems for screening potential novel antimicrobial compounds in relevant pharmacokinetic environments. Screening compounds against *Mtb* in its natural niche of the macrophage phagosome is a step in this direction, and this study derives from a hit from such an intra-macrophage screen (Stanley, Grant et al. 2012).
Specifically, the antidepressant fluoxetine (FLX) was found to restrict \textit{Mtb} growth inside macrophages at concentrations much lower than those required for direct antibacterial effect against bacteria in broth. While this effect was originally thought to indicate a host-mediated effect, several experimental approaches suggest that direct action of the compound occurs due to accumulation inside the acidic compartment of exposed cells.

Because weakly basic compounds (pKa ~ 8) of moderate lipophilicity have been shown to accumulate in acidic organelles through a phenomenon called ion trapping (de Duve, de Barys et al. 1974; MacIntyre and Cutler 1988; Daniel and Wojcikowski 1997; Kaufmann and Krise 2007) and because \textit{Mtb} is known to reside in a relatively acidic compartment (Rohde, Yates et al. 2007; Vandal, Nathan et al. 2009), we hypothesized that this mechanism explains the increased activity of FLX and other chemically similar compounds when applied to infected macrophages relative to their activity against bacteria in culture. We will refer to this class of compounds as “acidic compartment accumulating” or ACA drugs. In addition to FLX, we examined two other drugs based on their pKa and logP values, which are consistent with an ACA profile. They are the local anesthetic dibucaine (DIB) and the antidepressant sertraline (SRT) (Mayer, Wong et al. 1988; Hiemke and Hartter 2000).

**MATERIALS AND METHODS**

**Macrophage cell culture.** All experiments were performed on resting primary murine bone marrow derived macrophages (BMDM) at a density of $5 \times 10^4$/well in 200µL of medium in 96 well cluster plates. BMDM medium was composed of DMEM supplemented with 10% FBS (Gibco, ref. 11960 and 10082), glutamine as Glutamax (Gibco, ref. 35050) at 2mM, and 10% 3T3-MCSF cell conditioned medium. Macrophages were maintained at 37°C, 5% CO2 in a humidified incubator and handling steps were executed as quickly as safely possible to minimize exposure to ambient conditions. For cytotoxicity measurement, CellTox Green was used according to manufacturer’s instructions (Promega, G8741).

**Bacterial culture.** All experiments were conducted with \textit{Mycobacterium tuberculosis} grown to mid-log phase. Bacteria were grown in 50mL of Middlebrook 7H9 broth (Difco, 271310) supplemented with 0.5% (v/v) glycerol, 0.05% (w/v) Tween 80 (Sigma, P1754), and 10% (v/v) oleate-albumin-dextrose-catalase enrichment (BBL Middlebrook OADC, catalog no. 212351). Cultures were maintained at 37°C in 1L roller bottles. The Erdman strain of \textit{Mtb} was used for all experiments except for those involving the Rv2930c::Tn mutant, for which the strain matched control was H37Rv.

**MIC experiments.** Minimum inhibitory concentration for study drugs were determined using a standard microtiter dilution protocol (Palomino, Martin et al. 2002).

**Infection.** Bacteria were washed thrice in PBS, resuspended and centrifuged at 50g to pellet clumps. Bacterial suspension was added to DMEM medium with 10% horse serum substituted for FBS to produce infection medium at $5 \times 10^5$ CFU/mL in 100µL per well. Phagocytosis was allowed to proceed for four hours, after which macrophages were washed twice with warm PBS and returned to normal medium.
**Drug treatment.** Immediately after infection, macrophages were returned to normal medium with or without experimental compounds as appropriate. For experiments involving neutralization of acidic organelles, macrophages were returned to 100µL of BMDM medium with or without neutralization agent for one hour followed by a further 100µL of medium containing 2X concentration of experimental compound or control dissolved in BMDM medium of the same composition as the first 100µL.

**Bacterial load quantification.** For plating experiments, macrophages were lysed by the addition of 50µL of 0.5% Triton X-100, yielding a final concentration of 0.1% detergent. Serial dilutions were produced in complete 7H9 medium and plated on Middlebrook 7H10 agar plates (Difco, 262710) containing 10% OADC and no antibiotics. CFU were counted after 17-21 days of incubation at 37°C in a humidified incubator. For luminescence experiments, macrophages were maintained in white 96 well cluster plates (Costar, 3610) with white vinyl sealing tape attached to the bottom (Thermo Scientific, 236272) and read at 32°C with a Spectramax L luminometer (Molecular Devices). Triplicate reads were averaged for each of three biological replicates at each time point.

**Reagents.** The following compounds were purchased from Sigma Aldrich: dibucaine (D0638), resazurin (R7017), ammonium chloride (A-9434), bafilomycin A1 (B1793), isoniazid (I3377), racemic fluoxetine (F132), serotonin (H9523), Triton X-100 (T8787) and clofazimine (C8895). Fluoxetine enantiomers and the quaternary amine analog were generous gifts of Eli Lilly and Co. Sertraline was from Enzo (BML-NS115) and the ketone analog was from Matrix Scientific (072144). Hoechst 33342 was from Anaspec (AS-83218). Drugs for use in infection experiments were dissolved in Hybri-Max DMSO (D2650, Sigma) to give stock solutions at 1000 fold their intended final concentration and filter sterilized using 0.2µm nylon syringe filters. Solvent controls contained 0.125% DMSO.

**Imaging.** BMDM were grown at a density of 1x10^5/well in eight chambered cover-glass culture slides (Nunc, 155411) and treated as labeled overnight. For image acquisition, macrophages were washed with warm PBS with calcium and magnesium and incubated at room temperature with Hoechst 33342 at 10µM for five minutes. Nuclear counter stain solution was removed and replaced with PBS at 4C and images were acquired promptly by wide field fluorescence microscopy (Zeiss Axio Observer D1). Images were processed and analyzed using ImageJ software (ImageJ, U. S. National Institutes of Health, Bethesda, MD).

**Statistics.** All statistical analysis was performed using Prism version 6.05 (GraphPad Software, San Diego, CA).
RESULTS

Mtb growth restriction inside macrophages by ACA compounds. All three compounds with predicted accumulation in acidic organelles (Figure 4.1a) were found to restrict the growth of Mtb after 72 hours of treatment in resting primary murine macrophages in a dose dependent manner with a bioluminescent reporter strain (Figure 4.1b). The level of restriction observed with FLX was comparable to the original description of its activity in this context (Stanley, Barczak et al. 2014) and with results achieved by CFU enumeration (Figure 4.1c).

Figure 4-1: Structures of study compounds (A). Intra-macrophage growth restriction of Mtb by luminescent reporter (n ≥ 3, ±SEM) (B) or colony formin unit (CFU) enumeration (n = 2, ± SEM)(C). All treatments show reduced relative luminescent units (RLU) or CFU compared to DMSO control ( p < 0.05, ANOVA with Bonferroni adjustment for multiple comparisons). Study compounds do not induce cell death by CellTox Green assay (D) (n = 2-4, ±SEM, all comparisons vs DMSO control non-significant except Triton X-100 positive control for membrane integrity loss p < 0.001). All conditions in each experiment were tested as triplicate biological replicates; CFU experiments were plated in triplicate from triplicate biological replicates.

Activity of ACA drugs is not due to host cell toxicity. In order to rule out the possibility that the observed effect was secondary to a cell death phenotype, we tested for macrophage cell death using the fluorescent dye CellTox Green, which is excluded from living cells but is highly
fluorescent when bound to the DNA of dead or dying cells. No significant cell death was observed relative to solvent control in ACA drug treatment conditions, while cells exposed to the detergent Triton X-100 had a strong fluorescent signal indicative of loss of plasma membrane integrity (Figure 4.1d).

**Neutralization of acidic compartments reduces the effect of ACA compounds.**

To determine whether neutralization of the pH gradient would reduce or eliminate accumulation of ACA drugs, we used ammonium chloride to directly neutralize acidic compartments of infected, resting macrophages just after infection (Poole and Ohkuma 1981). Separately, we used bafilomycin A1 to do the same indirectly through inhibition of the vacuolar H+ ATPase (Sullivan, Young et al. 2012). We titrated the concentrations such that the neutralization treatments alone did not affect bacterial load as measured by luminescence assay. Statistically significant reductions in the restriction of mycobacterial growth were observed with ACA compounds when applied with ammonium chloride (20mM) or bafilomycin A1 (5nM) (Figure 4.2) while an active antimicrobial drug (isoniazid, INH) which is not compatible with pH based partitioning, was not affected by neutralization.

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**Figure 4-2:** Neutralization of acidic organelles either directly with ammonium chloride or indirectly with bafilomycin A1 partially abrogates the growth restriction observed with FLX, SRT and DIB (10µM). The effect of low dose (0.02µg/mL) isoniazid (INH) is not reduced by neutralization with NH₄Cl. (* p<0.05, ** p<0.005, **** p<0.0001 ANOVA with Bonferroni adjustment for multiple comparisons, ns = not significant by unpaired, two tailed student’s t-test)
Non-protonatable analogs of ACA compounds are inactive against intra-macrophage bacteria.

To confirm that the secondary amines in FLX and SRT are the basic functional group governing the growth restriction observed, we tested the activity of close analogs lacking that group. Specifically, we tested an analog of FLX which has a quaternary amine rather than a secondary amine and is therefore positively charged at physiologic pH (referred to as QFLX, Figure 4.3a) and an analog of sertraline which has a ketone in place of the secondary amine (referred to as KSRT, Figure 4.3a). These compounds showed comparable extracellular growth inhibitory activity to the parent compounds (not more than 3 fold increased MIC values by growth curve or low inoculum MIC assay), but showed no restriction of mycobacterial growth inside primary murine bone marrow derived macrophages (Figure 4.3b). When applied to infected macrophages, the parent compounds were still active at 2 and 4 fold lower concentration than the analog concentrations which failed to restrict growth of *Mtb*. These data suggest that the slight decrease in extracellular activity does not account for the absent intra-macrophage activity of the analogs.

![Image of chemical structures](image)

**Figure 4-3:** Nonprotonatable analogs of study drugs (A) do not restrict mycobacterial growth (B) by luminescence assay for *Mtb* bacterial load (n ≥ 3, ±SEM). (****p<0.001, ANOVA with Bonferroni adjustment for multiple comparisons).

An *Mtb* mutant which resides in a more acidic intracellular environment is more sensitive to FLX and SRT within macrophages despite being no more sensitive when extracellular. If accumulation of compounds in this study by partitioning into acidic compartments is the mechanism of *Mtb* growth restriction, it should follow that *Mtb* mutants with greater exposure to
highly acidic environments inside of macrophages would be more sensitive to them. Since such mutants have been described, we tested one which has been reported to reside in a more mature phago-lysosome like intracellular environment (pH ~5.8, compared with pH ~6.4 for wild-type) without profound attenuation (Pethe, Swenson et al. 2004). This mutant (for this study, produced by transposon insertion into Rv2930c, referred to as Rv2930c::Tn) was found to have an indistinguishable MIC\(_{50}\) value by optical density growth curve as well as MIC\(_{99}\) by micro-titer broth dilution to wild type \(Mtb\) (H37Rv) (data not shown). The mutant was, however, more sensitive to both FLX and SRT when applied to infected macrophages (Figure 4.4). Dibucaine was not statically more effective versus Rv2930c::Tn (data not shown).

**Figure 4-4:** A transposon mutant of \(Mtb\), Rv2930c::Tn (empty bars), which resides in a more acidic compartment within macrophages, is more sensitive to FLX and SRT than wild type control (H37Rv, filled bars). (**p=0.004, ****p<0.001 unpaired, two-tailed t-test with Welch’s correction)

**Clofazimine, a clinical antimicrobial drug used in TB treatment, is subject to ion trapping.**

The chemical properties of clofazimine, a second-line TB drug (2008), are consistent with an ACA profile, so we investigated whether the compound’s activity was augmented by pH based partitioning. We established that a concentration of 0.5 \(\mu\)g/mL reduces bacterial load by approximately 46% (Figure 4.5a) relative to solvent control in infected macrophages. We found that this reduction was partially reversible with ammonium chloride or bafilomycin A1 treatment, to 65% and 78% of control, respectively. None of the treatments caused significant cell death by CellTox Green assay; a detergent control (Triton-X100, 0.1% final concentration) was the only treatment statistically different from solvent control (Figure 4.5b). Clofazimine has a fluorescence spectrum which allowed us to image the accumulation of the drug inside of presumed acidic compartments of the cell. Using wide field epi-fluorescence microscopy (excitation 550nm, emission 605nm), we visualized resting primary murine macrophages after overnight incubation with clofazimine alone (5 \(\mu\)g/mL) or in combination with ammonium...
chloride (20mM) or bafilomycin A1 (5nM) (Figure 4.5c). While a weak autofluorescence signal could be observed in the solvent control, and some amorphous staining was observed in the neutralization conditions, the clofazimine alone condition showed a much stronger overall fluorescence signal and a punctate staining appearance consistent with lysosomal accumulation (Figure 4.5c). Using a stringent threshold applied via a macro for ImageJ to objectively quantify puncta above a set brightness level, we found the clofazimine alone condition to contain significantly more such areas per cell than the other treatments (Figure 4.5d).

Figure 4-5: Acidic compartment neutralization reduces the efficacy of clofazimine in infected BMDM by luminescence assay for bacterial load (A) (n ≥ 3, ±SEM) without altering macrophage viability as determined by CytoTox Green assay (B). Triton X-100 positive control for membrane integrity loss, however, showed significant increase in fluorescent signal. Overnight incubation induces observable accumulation of clofazamine in BMDM (C), Ex/Em 550/605nm. Quantitation of > 3,300 cells, including at least 700 from each treatment using ImageJ software is displayed (D) (**p=0.002 ****p<0.001 ANOVA with Bonferroni adjustment for multiple comparisons).
It has long been known that weak bases are more membrane permeable in their protonated, non-ionized form (Milne, Scribner et al. 1958). This biophysical phenomenon, coupled with the imbalance of ionized forms across membranes driven by actively maintained pH gradients, has been shown to generate asymmetric distribution of weak bases. One such report used dibucaine—a compound used in this study—as a model (Mayer, Wong et al. 1988). In his seminal 1974 work on the subject, Christian de Duve proposed that antibiotics with ‘lysosomotropic’ properties might be more effective against intracellular pathogens (de Duve, de Barsy et al. 1974). Despite the passage of 41 years, this, to our knowledge, the first investigation of this theory with regard to mycobacterial infection. Although a few groups have looked at the impact of pH based partitioning on antibiotic pharmacokinetics (PK) at the cellular level (Lemaire, Van Bambeke et al. 2009; Togami, Chono et al. 2010), in the field of mycobacteriology it has been almost completely overlooked. Intracellular accumulation of drugs used in the treatment of TB has been observed (Hand, Corwin et al. 1984; Pascual, Tsukayama et al. 1987) but the basis for this accumulation was not determined.

Very few studies have investigated the activity of TB drugs in the intracellular environment. When compared with extracellular activity, intra-macrophage activities can differ markedly (Dhillon and Mitchison 1992; Rastogi, Labrousse et al. 1996) in mycobacterial infection models. Isoniazid was selected as a negative control in this study because its chemical properties are not consistent with an ACA profile and it has been shown to be present in similar concentrations inside and outside of macrophages (Hand, Corwin et al. 1984). Clofazimine, on the other hand, has been shown to be more active against intracellular bacteria in the context of Mycobacterium avium infection (Rastogi, Potar et al. 1987), and in light of the findings presented here, pH based partitioning is strongly supported as a causal mechanism. Physical accumulation, rather than some impact on the infected cells is further supported by numerous observations in the clinical literature (Hudson, Cox et al. 1988; Sandler, Ng et al. 1992; Silverman, Holter et al. 1993; Harbeck, Worthen et al. 1999) wherein crystals of drug have repeatedly been observed in lung tissue of treated patients, implying an accumulation phenotype. Interest in clofazimine has recently intensified due to its successful use against multi-drug resistant Mtb (Caminero, Sotgiu et al. 2010; Dey, Brigden et al. 2013), and the demonstration that it can shorten treatment in a murine model of TB (Tyagi, Ammerman et al. 2015). Still, an effect on host cells cannot be totally excluded and evidence exists to suggest that loading lysosomes with clofazimine impacts the expression of lysosomal enzymes (Sarracent and Finlay 1982).

The impact of ACA drugs on the endogenous functions of the structures they accumulate within is difficult to predict. For example, the partial neutralization of (phago)lysosomes by ACA drugs due to their basicity may represent a countervailing effect to the direct antimicrobial effect of the drugs themselves (Vandal, Nathan et al. 2009) and could account for the lack of additional effect against the Rv2930c::Tn mutant demonstrated by dibucaine in this study (given its relatively low antimicrobial activity but high accumulation). This effect, if present, would be expected to be less of a concern with ACA drugs with greater antimicrobial activity, though, since lower doses could be administered. Additionally, it has been shown that chloroquine, a known lysosomal
accumulator, does not decrease the activity of pyrazinamide, a drug only active at acidic pH, when applied to infected macrophages (Crowle and May 1990). This implies that strong alkalization of \textit{Mtb} containing structures does not occur with that compound and may be generalizable. That finding, and our results with ammonium chloride and bafilomycin treatment alone, which were similar to published results reported by another group (Sullivan, Young et al. 2012), suggests that acidic compartment neutralization alone does not profoundly impact the course of infection of macrophages with wild type \textit{Mtb}. So while mutants which traffic to more mature phagolysosomes may be saved by neutralization [our results and figure 7c in (Sullivan, Young et al. 2012)], wild type bacteria are less affected. An impact on autophagy, in particular, could be a concern given that bafilomycin is an inhibitor of that process and was shown to partially reverse the action of ACA drugs. A role for canonical autophagy is essentially ruled out, however, by our observations of unaffected activity of FLX and SRT in autophagy deficient (ATG5 conditional knockout) cells (see chapter 3, figure 3.1).

Conversely, host-beneficial effects may result from ACA drug deposition and subsequent effects on the host cell such as phopholipidosis (Mesens, Steemans et al. 2010), for example by interference with the close apposition of the bacterial cell wall to the host membrane which has been reported to be necessary for phagosomal maturation arrest (de Chastellier, Forquet et al. 2009), a key survival strategy of \textit{Mtb}. A multifactorial mechanism of restriction of mycobacterial growth by high-accumulating drugs seems likely.

The antimicrobial activities of fluoxetine and sertraline have been described before (Munoz-Bellido, Munoz-Criado et al. 2000; Coban, Tanriverdi Cayci et al. 2009; Bohnert, Szymaniak-Vits et al. 2011; Zhai, Wu et al. 2012) but only for cryptococcal infection has the therapeutic index of one of the drugs (\textit{i.e.}, SRT) been suggested to be compatible with clinical treatment (Zhai, Wu et al. 2012). Our contention here is not that FLX, SRT or DIB are viable drugs for the treatment of TB, but rather that they are exemplars of a phenomenon (\textit{i.e.}, ion trapping) which could increase the local concentration of antimicrobial compounds to which \textit{Mtb} is exposed intracellularly. Rational modification of antibiotics to endow them with an ACA profile has been demonstrated; Renard and colleagues modified penicillin G (an otherwise non-accumulating, predominantly extracellular drug) by removing a carboxylic acid moiety and replacing it with a basic, amine containing group (Renard, Vanderhaeghe et al. 1987). That alteration was sufficient to cause a shift toward accumulation of the drug in the acidic compartment – primarily lysosomes as identified by co-fractionation.

As an antidepressant, fluoxetine is thought to work by blocking serotonin uptake from synapses (Wong, Perry et al. 2005). Though murine macrophages may express the serotonin transporter (Jackson, Walker et al. 1988), the level of serotonin in culture medium did not impact the restriction of mycobacterial growth we observed in our infections at any physiologically plausible concentration, and medium made with dialyzed, serotonin-free fetal bovine serum (FBS) performed no differently from standard FBS (data not shown). Also, the concentration range used in this study was around three orders of magnitude greater than the Ki of the drug for the serotonin transporter (Koch, Perry et al. 2002), so essentially all binding sites should be saturated and a dose-wise response should not be observed. It remained possible that FLX was directly binding serotonin receptors, rather than the transporter, because such affinity has been reported (Koch, Perry et al. 2002). However, the enantiomers of FLX, which have differing
affinity for many of the serotonin receptors from one another, have the same ability to restrict the growth of *Mtb* in macrophages (Figure 4.6). These data suggest that specific binding of FLX to host targets is not the mechanism by which it restricts mycobacterial growth.

**Figure 4-6:** The (R) and (S) enantiomers of fluoxetine have equivalent growth restriction activity against intracellular *Mtb* as measured by relative luminescence at 72 hours post infection.

Compounds with good antimicrobial activity which are tailored to fit the ACA profile would be expected to be more effective against intracellular *Mtb* than unmodified versions, all else being equal. This knowledge may allow for the rational development of antimicrobial compounds for mycobacterial infection based on the insight into cellular pharmacokinetics presented here.
de Chastellier, C., F. Forquet, et al. (2009). "Mycobacterium requires an all-around closely apposing phagosome membrane to maintain the maturation block and this apposition is re-established when it rescues itself from phagolysosomes." Cell Microbiol 11(8): 1190-1207.


The studies described in this dissertation were embarked upon with the goal of improving the therapy of tuberculosis by changing the dynamics of the host-pathogen interface. Whether by shaping the adaptive immune response using a vaccine as in chapter one, by augmenting innate immune function by pharmacologic intervention, as in chapter two, or by leveraging characteristics of the intracellular niche occupied by *Mtb* to improve chemotherapy, as in chapter four, the goal was always to tip the outcome of infection in favor of containment and eradication of the pathogen.

The vaccine approach pursued in chapter two failed to protect against reactivation or relapse disease in a murine model of TB despite promising data from an earlier study which showed strong protection. After a thorough assessment of the outcomes and differences in execution of the two studies, it seems most likely that the cause of the disparate results was the change in challenge strain of *Mtb*. Because the subunit vaccine used in both studies was composed of a protein which is overexpressed by the strain of *Mtb* used in the original report, the protection demonstrated in that study was probably an artifact of antigen overexpression in the mutant strain. The failure to recapitulate the earlier result with wild type *Mtb* was disappointing, but further study of the resolution of infection seen in that system may still be worth pursuing in pursuit of correlates of protection against reactivation or relapse TB.

In chapter three, the application of compounds reported to upregulate autophagy, a host-cell process with reported mycobactericidal activity, was described. Despite a strong theoretical basis for expecting these compounds to restrict mycobacterial growth, none robustly did so. One compound, however, was confirmed to have reproducible activity against intracellular *Mtb*. That compound, fluoxetine, was found not to operate through canonical autophagy, but to act at least partially through an accumulation phenomenon which was described in depth in chapter four.

Finally, in chapter four, the accumulation of weakly basic antimicrobial drugs was demonstrated to be due to pH based ion trapping. The accumulation of several model compounds was shown to depend on intracellular accumulation in acidic organelles. This accumulation was reversible by neutralization of the acidic compartment and did not occur with analogs of the study drugs which were modified to have non-basic pKa values. It was further demonstrated that a mutant of *Mtb* which resides in more acidic phagosomes was more sensitive to two of the study drugs. A clinically used compound compatible with the pH based partitioning effect, clofazimine, was shown to benefit from ion trapping, as well, and due to its fluorescence spectrum, its accumulation was shown by fluorescence microscopy.

The findings described in chapter four may enable medicinal chemists to rationally develop new anti-infective drugs for TB to have more favorable pharmacokinetic profiles based on their cellular level distribution. This aspect of host-factor enhancement, namely asymmetric drug distribution due to pH gradients, was discovered serendipitously and represents the major contribution of this work to future development of therapies for tuberculosis.
### Appendix A

<table>
<thead>
<tr>
<th>Drug</th>
<th>Indication</th>
<th>Mechanism</th>
<th>Minimum Effective Dose&lt;sup&gt;i&lt;/sup&gt;</th>
<th>MRTD&lt;sup&gt;ii&lt;/sup&gt; (mg/kg/day)</th>
<th>Maximum tolerated</th>
<th>Half-life</th>
<th>Notes</th>
<th>Exp Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbamazepine</td>
<td>Anti-convulsant &amp; specific analgesic; trigeminal neuralgia</td>
<td>↓ inositol and IP3 levels</td>
<td>(exp)&lt;sup&gt;2&lt;/sup&gt; 50 μM, 3 μM&lt;sup&gt;1&lt;/sup&gt; (exp-in-vivo)&lt;sup&gt;1&lt;/sup&gt; 250 mg kg&lt;sup&gt;-1&lt;/sup&gt; (clinical) 17-51 μM&lt;sup&gt;iii&lt;/sup&gt;</td>
<td>26.7</td>
<td>&gt;60 μM</td>
<td>12-65 hours&lt;sup&gt;iv&lt;/sup&gt;</td>
<td>v vi</td>
<td>2,3</td>
</tr>
<tr>
<td>Sodium Valproate</td>
<td>Anti-epileptic</td>
<td>↓ inositol and IP3 levels</td>
<td>(exp) 500μM&lt;sup&gt;2&lt;/sup&gt; (clinical) 347–693 μM&lt;sup&gt;vii&lt;/sup&gt;</td>
<td>41.7&lt;sup&gt;viii&lt;/sup&gt;</td>
<td>&gt; 1041 μM</td>
<td>9-16 hours&lt;sup&gt;ix&lt;/sup&gt;</td>
<td>2</td>
<td></td>
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<tr>
<td>Lithium</td>
<td>Mania/bipolar</td>
<td>↓ inositol mono-phosphatase</td>
<td>(clinical) 0.6-1.2 mM&lt;sup&gt;3&lt;/sup&gt; (exp)10 mM</td>
<td>-</td>
<td>&gt;2 mM</td>
<td>12hours (1dose)-1.3days (plateau)&lt;sup&gt;xi&lt;/sup&gt;</td>
<td>xii xiii</td>
<td>1,4</td>
</tr>
<tr>
<td>Verapamil</td>
<td>Anti-arrhythmia&lt;sup&gt;xiv&lt;/sup&gt;</td>
<td>L-type Ca&lt;sup&gt;2+&lt;/sup&gt; channel antagonist</td>
<td>(exp) 5.2μM&lt;sup&gt;xv&lt;/sup&gt; 0.3, 1 &amp; 3 μM&lt;sup&gt;2&lt;/sup&gt; (clinical) Cmax R isomer 77.8 ng/ml, Cmax S isomer 16.8 ng/ml&lt;sup&gt;vi&lt;/sup&gt;</td>
<td>-</td>
<td>LD&lt;sub&gt;50&lt;/sub&gt;= 8 mg/kg (i.v. in mice)&lt;sup&gt;xvii&lt;/sup&gt;</td>
<td>2.8-7.4 hours&lt;sup&gt;xviii&lt;/sup&gt; XR up to 11hours&lt;sup&gt;xix&lt;/sup&gt;</td>
<td>xx xxii</td>
<td>5</td>
</tr>
<tr>
<td>Drug</td>
<td>Effect</td>
<td>Mechanism</td>
<td>Concentration</td>
<td>Clearance</td>
<td>Toxicity</td>
<td></td>
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<tr>
<td>Amiodarone</td>
<td>V-fib, V-tach</td>
<td>Inhibitor of mTORC1&lt;sup&gt;6&lt;/sup&gt;</td>
<td>(exp) 3.7 μM&lt;sup&gt;xxiii&lt;/sup&gt; 1 μM&lt;sup&gt;1&lt;/sup&gt; 50 μM&lt;sup&gt;6&lt;/sup&gt; (clinical) Steady state 1-2.5 mg/L oral&lt;sup&gt;xxiv&lt;/sup&gt; (up to 4 μM)&lt;sup&gt;xxv&lt;/sup&gt;</td>
<td></td>
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<tr>
<td></td>
<td>Na&lt;sup&gt;+&lt;/sup&gt;/Ca&lt;sup&gt;2+&lt;/sup&gt; exchange; ↓ intracellular Ca</td>
<td></td>
<td>26.7</td>
<td>IV mouse: LD&lt;sub&gt;50&lt;/sub&gt; = 178 mg/kg&lt;sup&gt;xxvi&lt;/sup&gt; Terminal: 40-55 days (range: 26-107 days)&lt;sup&gt;xxvii&lt;/sup&gt; 58 days (range 15-142 days)&lt;sup&gt;xxviii&lt;/sup&gt;</td>
<td>2,5</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Nimodipine</td>
<td>↓ Vaso-spasms after hemorrhage</td>
<td>L-type Ca&lt;sup&gt;2+&lt;/sup&gt; channel blocker</td>
<td>(exp) 1 μM&lt;sup&gt;2&lt;/sup&gt; (clinical) 27-53μg/L plasma&lt;sup&gt;xxix&lt;/sup&gt;</td>
<td>6.00</td>
<td>131.6 ±16.3 ng/mL&lt;sup&gt;xxxiv&lt;/sup&gt; (see note) 1.7-9 hours&lt;sup&gt;xxxv&lt;/sup&gt; 8-9 hours&lt;sup&gt;xxxvi&lt;/sup&gt;</td>
<td>2</td>
<td></td>
<td></td>
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<tr>
<td>Nitrendipine</td>
<td>Anti-hypertensive</td>
<td>L type Ca&lt;sup&gt;2+&lt;/sup&gt; exchange; ↓ intracellular Ca&lt;sup&gt;xi&lt;/sup&gt;</td>
<td>(exp) 1 μM&lt;sup&gt;2&lt;/sup&gt; (clinical) at least 4 ng/ml&lt;sup&gt;xli&lt;/sup&gt;</td>
<td>0.333</td>
<td>Mouse LD&lt;sub&gt;50&lt;/sub&gt;: IP 303mg/kg IV 34.5 mg/kg Oral 2540 mg/kg&lt;sup&gt;xlii&lt;/sup&gt; 10-22 hours&lt;sup&gt;xlili&lt;/sup&gt;</td>
<td>2</td>
<td></td>
<td></td>
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<tr>
<td>Trehalose</td>
<td>None (not FDA approved) Cleared for parenteral use however</td>
<td>Unknown but mTor independent</td>
<td>(exp.) 100mM, perhaps lower&lt;sup&gt;xliv&lt;/sup&gt;</td>
<td>-</td>
<td>N/A&lt;sup&gt;xlv&lt;/sup&gt; IV-short Lung-unknown but no trehalase expression&lt;sup&gt;xlvi&lt;/sup&gt;</td>
<td>7.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drug</td>
<td>Condition</td>
<td>Action</td>
<td>ED₅₀ (μM)</td>
<td>LD₅₀ (mg/kg)</td>
<td>Elimination</td>
<td></td>
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<tr>
<td>Perhexiline</td>
<td>Angina and Chronic Heart Failure</td>
<td>↓trans-mitochondrial carnitine “shuttle” (CPT1/CPT 2 inhibition) also Ca²⁺ &amp; K⁺ channel antagonist</td>
<td>(exp) 10 µM⁶ (clinical) 150–599 µg/mL¹⁰</td>
<td>Oral LD₅₀ rat: 2150 mg/kg Oral LD₅₀ Mouse: 2641 mg/kg</td>
<td>Variable &amp; non-linear; ~ 2-6 days, can be up to 30 days</td>
<td></td>
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<tr>
<td>Clonidine</td>
<td>Hyper-tension, ADHD with ex. release tablets</td>
<td>α₂-adrenergic agonist, imidazoline type 1 receptor</td>
<td>(exp) 0.3, 1 &amp; 3µM² (clinical) 1-3ng/ml at steady state</td>
<td>Oral LD₅₀ 150 mg/kg, Dog oral LD₅₀ 30 mg/kg</td>
<td>6-20 hours⁴¹⁴</td>
<td></td>
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<tr>
<td>Rilmenidine</td>
<td>Anti-hypertensive</td>
<td>imidazoline-1 R antagonist (exp-in-vivo) 10 mg/kg bw</td>
<td>-</td>
<td>-</td>
<td>8 hours⁴¹⁵</td>
<td></td>
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</tr>
<tr>
<td>Minoxidil</td>
<td>severe hypertension &amp; androgenic alopecia</td>
<td>ATP-K+ ch. activator (exp) 1 µM² (clinical) 35.2ng/ml (Single Dose 11.1)</td>
<td>-</td>
<td>-</td>
<td>4.2 hours⁴¹⁵</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Experimental References

Appendix A Notes

[Note: all hyperlinks current as of April 2012]

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i Abbreviations: “exp” denotes a value from the basic science literature while “clinical” denotes a value from clinical practice.

ii Maximum Recommended Therapeutic Dose – according to: http://www.fda.gov/AboutFDA/CentersOffices/CDER/ucm092199.html

ii Timing of serum samples: Absorption is slow, peak levels occur 6-8 hours after ingestion of the first dose; the half-life ranges from 8-60 hours, therefore, steady-state is achieved in 2-5 days

Therapeutic levels: 4-12 mcg/mL (SI: 17-51 μmol/L)

Toxic concentration: >15 mcg/mL; patients who require higher levels of 8-12 mcg/mL (SI: 34-51 μmol/L) should be watched closely.

Side effects including CNS effects occur commonly at higher dosage levels. If other anticonvulsants are given therapeutic range is 4-8 mcg/mL.

[http://www.merckmanuals.com/professional/lexicomp/carbamazepine.html]

iv Highly dependent on previous dosing - extended release available (keep in mind it autoinduces its own metabolism): “Following a single extended release dose of carbamazepine, the average half-life range from 35-40 hours and 12-17 hours on repeated dosing.”


v Isoniazid: May decrease the metabolism of Carbamazepine

[http://www.merckmanuals.com/professional/lexicomp/carbamazepine.html]

vi Carbamazepine may enhance the adverse/toxic effect of Lithium

[http://www.merckmanuals.com/professional/lexicomp/carbamazepine.html]

vii http://www.merckmanuals.com/media/professional/pdf/Table_214-5.pdf?qt=valproate&alt=sh

viii Valproic acid

Carbamazepine may enhance the adverse/toxic effect of lithium
[http://www.merckmanuals.com/professional/lexicomp/carbamazepine.html]

Oral: Treatment of hypertension; angina pectoris (vasospastic, chronic stable, unstable); supraventricular tachyarrhythmia (PSVT, atrial fibrillation/flutter [rate control])
I.V.: Supraventricular tachyarrhythmia (PSVT, atrial fibrillation/flutter [rate control])
http://www.merckmanuals.com/professional/lexicomp/Verapamil.html


Mean Cmax of the R isomer was 77.8 ng/ml and 16.8 ng/ml for the S isomer; AUC (0-24h) of the R isomer was 1037 ng•h/ml and 195 ng•h/ml for the S isomer.
http://www.rxlist.com/verelan_pm-drug.htm

Half life: Single dose: 3-7 hours, Multiple doses: 4.5-12 hours
Extended release ~11 hours, drug release delayed ~ 4-5 hours
Sustained release: 5.21 hours 7-9 hours
http://www.merckmanuals.com/professional/lexicomp/Verapamil.html

(Williams et al - 2008)
Rifamycin Derivatives: May increase the metabolism of Calcium Channel Blockers. This primarily affects oral forms of calcium channel blockers. Management: The labeling for some U.S. and Canadian calcium channel blockers contraindicate use with rifampin however recommendations vary. Consult appropriate labeling. *Risk D: Consider therapy modification*

http://www.merckmanuals.com/professional/lexicomp/Verapamil.html

Amiodarone Labeled Indications:
Management of life-threatening recurrent ventricular fibrillation (VF) or hemodynamically-unstable ventricular tachycardia (VT) refractory to other antiarrhythmic agents or in patients intolerant of other agents used for these conditions…

Steady-state amiodarone concentrations of 1 to 2.5 mg/L have been associated with antiarrhythmic effects and acceptable toxicity following chronic oral amiodarone therapy.


Therapeutic levels: 0.5-2.5 mg/L (SI: 1-4 μmol/L)

http://www.merckmanuals.com/professional/lexicomp/Amiodarone.html#N2B1DC

“Following chronic oral administration of the drug in humans, amiodarone and N-desethylamiodarone are distributed extensively into many body tissues and fluids, including adipose tissue, liver, lung, spleen, skeletal muscle, bone marrow, adrenal glands, kidneys, pancreas, testes, semen, saliva, lymph nodes, myocardium, thyroid gland, skin, and brain.”


Potentially fatal pulmonary toxicity is the most severe adverse effect associated with oral amiodarone therapy. More information is available:

Essentially irreversibly inhibits mTORC1 activity according to:


http://www.drugbank.ca/drugs,DB00393
http://www.rxlist.com/nimotop-drug.htm

Rifampin: From experience with the calcium antagonist nifedipine it is to be expected that rifampin accelerates the metabolism of nimodipine capsules due to enzyme induction. Thus, efficacy of nimodipine capsules could be reduced when concomitantly administered with rifampin.

Antiepileptic Drugs: A pharmacokinetic study in epileptic patients receiving long-term treatment has shown that concurrent administration of oral nimodipine and antiepileptic drugs (phenobarbital, phenytoin and/or carbamazepine) reduces the bioavailability of nimodipine by about 80%. In those patients receiving sodiumvalproate and oral nimodipine, the bioavailability of the nimodipine increased by about 50%. Therefore, the concomitant use of oral nimodipine and these antiepileptic drugs requires close monitoring and appropriate adjustment of the dosage of nimodipine.

A calcium channel blocker with marked vasodilator action. It is an effective antihypertensive agent and differs from other calcium channel blockers in that it does not reduce glomerular filtration rate and is mildly natriuretic, rather than sodium retentive.
http://www.drugbank.ca/drugs,DB01054
Nitrendipine, a dihydropyridine calcium-channel blocker, is used alone or with an angiotensin-converting enzyme inhibitor, to treat hypertension, chronic stable angina pectoris, and Prinzmetal's variant angina. Nitrendipine is similar to other peripheral vasodilators. Nitrendipine inhibits the influx of extra cellular calcium across the myocardial and vascular smooth muscle cell membranes possibly by deforming the channel, inhibiting ion-control gating mechanisms, and/or interfering with the release of calcium from the sarcoplasmic reticulum. The decrease in intracellular calcium inhibits the contractile processes of the myocardial smooth muscle cells, causing dilation of the coronary and systemic arteries, increased oxygen delivery to the myocardial tissue, decreased total peripheral resistance, decreased systemic blood pressure, and decreased afterload.

http://www.drugbank.ca/drugs/DB01054


http://www.mims.com/Philippines/drug/info/nitrendipine/nitrendipine?type=full&mtype=generic

100mM trehalose was about equal to 0.2µM rapamycin for autophagy induction (Sakar et al 2007)

In mice, oral LD50 was > 5,000 (mg/kg bw) Intravenous: > 1,000 (mg/kg bw) Atkinson & Thomas (1994)

“In general, the fate of ingested or parenterally administered trehalose corresponds to that of glucose since trehalose is rapidly hydrolysed to glucose by the enzyme trehalase.” http://www.inchem.org/documents/jecfa/jecmono/v46je05.htm

However, trehalase is only found in the kidney and small intestine according to T.J.Oesterreicher et al. I Gene 270(2001)211-220

(see Ashrafian et al 2007)

http://www.drugbank.ca/drugs/DB01074

http://www.drugbank.ca/drugs/DB01074

Seldom used in US – common in Australia and New Zealand. (Ashrafian et al 2007)

Serious adverse effects are generally only seen after 3 months or more of treatment. (Ashrafian et al 2007)

http://www.drugbank.ca/drugs/DB00575

(Rose et al - 2010)


The American Journal of Cardiology
Volume 61, Issue 7, 24 February 1988, Pages D47-D53
A Symposium: Rilmenidine-A Novel Alpha2 Agonist Antihypertensive Agent


(Rose et al - 2010)

http://www.drugbank.ca/drugs/DB00350


Shown to help clear protein aggregates (as in Huntington’s disease) See (Williams et al 2008)

Clonidine in small doses (0.2 to 0.8 mg daily) also helps to prevent the compensatory reflex effects produced by minoxidil, since clonidine suppresses sympathetic outflow from the brain.