Limited Activity of Clofazimine as a Single Drug in a Mouse Model of Tuberculosis Exhibiting Caseous Necrotic Granulomas

Scott M. Irwin1, Veronica Gruppo1, Elizabeth Brooks1, Janet Gilliland1, Michael Scherman1, Matthew J. Reichlen2, Rachel Leistikow2, Igor Kramnik3, Eric L. Nuernberger4, Martin I. Voskuil2, and Anne J. Lenaerts1#

(1) Mycobacteria Research Laboratories, Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, CO USA; (2) Department of Microbiology, University of Colorado School of Medicine, Aurora, Colorado, USA (3) Boston University School of Medicine, Department of Pulmonary, Allergy, Sleep and Critical Care Medicine, Boston University, Boston, MA USA; (4) Center for Tuberculosis Research, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

Running Title: Limited Clofazimine Activity in C3HeB/FeJ Mice

#Corresponding author. Mailing address: Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, CO 80523, USA. Phone: 970-491-3079. Fax 970-491-1815. E-mail: Anne.Lenaerts@colostate.edu.

Key words: clofazimine, animal models, C3HeB/FeJ, BALB/c, mouse model, aerosol infection, tuberculosis, therapeutic agents
Abstract

New drugs and drugs with a novel mechanism of action are desperately needed to shorten the duration of tuberculosis treatment, to prevent the emergence of drug resistance, and to treat multiple drug resistant strains of *Mycobacterium tuberculosis*. Recently, there has been renewed interest in clofazimine (CFZ). In this study, we utilized the C3HeB/FeJ mouse model possessing highly organized, hypoxic pulmonary granulomas with caseous necrosis to evaluate CFZ monotherapy in comparison to BALB/c mice which only form multifocal, coalescing cellular aggregates devoid of caseous necrosis. While CFZ treatment was highly effective in BALB/c mice, its activity was attenuated in the lungs of C3HeB/FeJ mice. This lack of efficacy was directly related to the pathological progression of disease in these mice, as administration of CFZ prior to the formation of hypoxic, necrotic granulomas reconstituted bactericidal activity in this mouse strain. These results support the continued use of mouse models of tuberculosis infection which exhibit a granulomatous response in the lungs that more closely resembles the pathology found in human disease.

Introduction

The Stop TB Partnership in conjunction with the World Health Organization have set a goal of reducing tuberculosis (TB) incidence to less than one case per million by 2050 (1). If we hope to achieve this goal, we must more effectively employ the current resources in our anti-TB armament, as well as develop more efficacious drugs, diagnostics, and vaccines. Such a
significant reduction in the incidence of TB can only occur by treatment of both active and latent
TB cases (2). The emergence and spread of multidrug resistant (MDR) and extensively drug
resistant (XDR) strains of TB poses a serious obstacle to meeting the 2050 goal. Drugs with a
novel mechanism of action and chemotherapeutic regimens effective against drug resistant TB
must be developed and tested if we hope to make substantive gains in reducing the worldwide
incidence of TB.

Almost 60 years ago, Barry et al. identified clofazimine (CFZ) as a compound that was
highly active against *Mycobacterium bovis* Ravanel as well as *Mycobacterium tuberculosis* (*M.
tuberculosis*) under *in vitro* conditions and in mice following intravenous infection (3). Initial
optimism faded; however, when further studies in guinea pigs (4, 5) and non-human primates (6)
failed to demonstrate significant therapeutic activity, although it should be noted that later
studies implicated poor absorption kinetics in these animal models as the primary cause of the
lack of efficacy (4). These failures in pre-clinical animal models, coupled with the identification
of the highly effective drug isoniazid and later rifampicin, shifted attention away from clinical
usage of CFZ to treat TB, although it has been used successfully to treat leprosy since the early
1960s. However the desire to shorten the duration of treatment necessary to prevent relapse and
the emergence of MDR and XDR TB have renewed interest in both CFZ as well as other related
riminophenazine compounds for the treatment of pulmonary tuberculosis (7-9). A recent
observational study in Bangladesh examined six combination regimens for efficacy against MDR
TB (10). Although all of the regimens contained CFZ during the intensive phase, the two
regimens with the highest rate of cure also contained CFZ during the continuation phase.
Similarly encouraging results were observed in BALB/c mice (11), demonstrating that the
addition of CFZ to a 9-month second-line regimen significantly increased the rate of killing and
decreased the relapse rate to levels comparable to that observed in the study by van Deun, *et al.*

CFZ has also recently been shown to have significant additive activity in BALB/c mice when administered with bedaquiline (TMC207) and pyrazinamide (12, 13). Therefore, the addition of CFZ to novel TB drug regimens may augment efforts to shorten the duration of TB chemotherapy, which is the ultimate goal of the Global Alliance for TB Drug Development and the Critical Path to TB Drug Regimens initiative (14).

CFZ is a poorly soluble, lipophilic drug that is highly protein bound and possesses an exceptionally long half-life of approximately 70 days in mice (15). The drug partitions to adipose tissue and due to its relative insolubility, it preferentially accumulates within macrophages (16, 17), and is known to have immunosuppressive properties (18). As a result of the highly elongated pharmacokinetic/pharmacodynamic profile, CFZ can accumulate to very high levels within tissues and can crystalize within cells, especially in the gastrointestinal tract. In addition, long-term use can result in coloration of the skin that while medically unimportant, can cause social stigmatization. Efforts are currently underway to synthesize and test CFZ analogs to find compounds with reduced side effects and more favorable pharmacodynamic properties (8, 9).

While many important insights have been gained from mouse models of TB infection, the discordance between the histological appearance of human and mouse pulmonary granulomas limits the practical usefulness of the mouse when modeling aspects such as drug penetration of fibrotic and caseous necrotic lesions, activity within hypoxic microenvironments, and activity against bacilli under different metabolic conditions. In addition, the bacilli within human pulmonary granulomas, especially those involved in cavitary disease and transmission, are believed to be primarily extracellular (19). However the bacilli within the lungs of traditional...
mouse models of infection are predominantly intracellular (20). Therefore the efficacy of compounds that preferentially accumulate within macrophages could be overestimated by testing in an animal model that lacks necrotic lesions containing abundant extracellular bacilli.

Igor Kramnik’s group discovered that the C3HeB/FeJ mouse strain (commonly referred to as the Kramnik mouse model) was highly susceptible to infection with *M. tuberculosis* (21). They also determined that this increased susceptibility was mediated to a large degree by functional inactivation due to naturally occurring mutations in the intracellular pathogen resistance 1 (Ipr1) isoform of the interferon inducible-75 (Ifi75) gene (22). Interestingly, following experimental aerosol infection, this mouse strain developed large, caseating granulomas in the lung that have been shown by our group as well as others to be hypoxic (23, 24). These granulomas contain large numbers of extracellular bacilli, and are often surrounded by a rim of foamy macrophages harboring intracellular bacilli (23). Over time many of these granulomas show evidence of continuous collagen remodeling and fibrotic encapsulation (23). These granulomas bear a striking resemblance to human pulmonary lesions, and may facilitate more realistic modeling of microenvironmental conditions not found within conventional mouse models.

Due to renewed interest in use of CFZ as an adjunct to novel chemotherapeutic regimens, we wanted to assess the efficacy of this drug in the Kramnik mouse model of infection to determine if CFZ is effective against extracellular bacteria, under hypoxic conditions, and against bacterial phenotypes exposed to environmental conditions believed to be more similar to what tubercle bacilli experience in human lung lesions.
Materials and Methods

Animals

Female specific pathogen-free C3HeB/FeJ and BALB/c mice aged 6-8 weeks were purchased from Jackson Laboratories, Bar Harbor ME. Mice were housed in a bio-safety level III animal facility and maintained with sterile bedding, water, and mouse chow. Specific pathogen-free status was verified by testing sentinel mice housed within the colony for 13 known mouse pathogens.

Bacteria and aerosol infections

*M. tuberculosis* Erdman strain (TMCC 107) was used for aerosol infections of mice for drug evaluations and prepared as previously described (25, 26). Briefly, the bacteria were originally grown as a pellicle to generate low passage seed lots (25). Working stocks were generated by growing to mid-log phase in Proskauer-Beck medium containing 0.05% Tween 80 (Sigma-Aldrich, St. Louis, MO) in three passages, enumerated by serial dilution on 7H11 agar plates, divided into 1.5 ml aliquots and stored at -80°C until use.

C3HeB/FeJ mice and BALB/c mice were exposed to a low-dose aerosol infection with *M. tuberculosis* in a Glas-Col inhalation exposure system, as previously described (26). The inoculum concentration was adjusted to yield ~50-75 CFU or ~400 CFU in the lungs of
C3HeB/FeJ mice or BALB/c mice, respectively. Five mice were sacrificed the following day to
determine the number of CFU implanted in the lungs.

Drug treatment and enumeration of bacterial load of lungs and spleens

For drug treated animals, CFZ (Sigma-Aldrich) was prepared by grinding with a mortar
and pestle and added to 0.05% agarose dissolved in sterile distilled water. Individual animals
were administered a 200 µl dose at 20 mg/kg daily for 5 days a week via oral gavage. For CFZ
treated animals, treatment was initiated either 3, 4, or 7 weeks following aerosol infection. Mice
were euthanized after 2, 4, and 8 weeks of treatment by CO₂ inhalation. At the time of sacrifice,
all lung lobes as well as the spleens were aseptically removed and disrupted with a tissue
homogenizer (Glas-Col Inc., Terra Haute, IN) in 4 ml of PBS. The number of viable organisms
was determined by plating serial dilutions of lung homogenate on Middlebrook 7H11 agar plates
supplemented with OADC (GIBCO BRL, Gaithersburg, MD) and 0.03 mg/ml cycloheximide
and 0.05 mg/ml carbenicillin. Due to the long half-life and high protein binding capacity of CFZ,
lungs and spleens from drug treated animals were homogenized in saline + 10% BSA, and plated
onto 7H11/OADC agar plates containing 0.4% activated charcoal to prevent carry-over (12).
Colonies were counted after at least 21 days of incubation at 37°C and at least 42 days for
charcoal containing plates (26). The viable bacterial counts of whole organs were calculated,
converted to logarithms [CFU counts were log-transformed as \( \log_{10}(x + 1) \), where \( x \) equals the
total organ CFU count]. The data were expressed as the mean \( \log_{10} \) CFU ± the standard error of
the mean for each group.

In vitro bacterial cultivation
All in vitro cultivation of *M. tuberculosis* Erdman was performed in Dubos Tween-albumin (DTA) media (27) in sterile 20 mm x 125 mm glass screw cap tubes containing 12 mm x 4 mm stir bars. Cultures were grown aerobically with high (aerobic) or low aeration (hypoxic) or under anoxic conditions using the Rapid Anaerobic Dormancy (RAD) model, as described by Leistikow, *et al.* where bacterial metabolism drives the oxygen concentration to undetectable levels utilizing rapid stirring, a small headspace volume, and tightly sealed screw cap tubes (28).

For aerobic growth under high and low aeration, cultures were diluted to an OD$_{600}$ of 0.05 and inoculated into sterile 20 mm x 125 mm glass screw cap tubes containing 12 mm x 4 mm stir bars at a final volume of 1 ml and 10 ml for high and low aeration respectively. The 10 ml culture has a 10-fold lower surface to volume ratio than the 1 ml high aeration culture. Cultures were given either CFZ at a final concentration of 50 µg/ml or DMSO as a vehicle control, sealed with loose fitting caps and incubated at 37°C with rapid stirring. The MIC was previously determined to be 0.24 µg/ml for broth, and 0.03 µg/ml for 7H11 agar plates for this strain. One, three, and five days later, bacterial cultures were serially diluted in DTA and plated on Dubos agar plates + OADC and 0.4% activated charcoal. Colonies were counted after at least 21 days of incubation at 37°C.

**Statistical analysis**

The viable CFU counts were converted to logarithms, which were then evaluated by a one-way analysis of variance, followed by a multiple comparison analysis of variance by a one-way Tukey test (SAS Software program, Research Triangle Park, NC). Differences were considered significant at the 95% level of confidence.
All lung lobes from individual mice were collected at necropsy and infused with 4% paraformaldehyde in phosphate buffered saline (PBS). Tissue sections were embedded in paraffin and cut to 5 μm thickness on a microtome. Subsequent tissue sections were mounted on glass slides, deparaffinized and stained with hematoxylin and eosin (H & E). Sections were visualized using a Nikon TE-I motorized microscope controlled by Nikon NIS Elements software (Nikon, Melville, NY).

Results

**CFZ was highly effective in the lungs of BALB/c but not C3HeB/FeJ mice**

BALB/c and Kramnik mice were aerosol infected with $2.53 \log_{10} \pm 0.03$ CFU and $1.8 \log_{10} \pm 0.03$ CFU, respectively. After four weeks, bacterial loads in the lungs reached $6.0 \log_{10}$ CFU in BALB/c mice and $7.8 \log_{10}$ CFU in Kramnik mice, at which point mice in treatment groups were administered 20 mg/kg CFZ via oral gavage five days per week. Treatment with CFZ was highly effective in the lungs of BALB/c mice (Figure 1A), producing a continuous and progressive decline, culminating in a $2.5 \log_{10}$ CFU reduction in bacterial numbers after four weeks of treatment ($p < 0.001$). Pulmonary bacterial numbers continued to decline in BALB/c mice, ultimately reaching $2.0 \log_{10}$ CFU, which amounted to a $4.0 \log_{10}$ CFU reduction after eight weeks of CFZ treatment.
In contrast to BALB/c mice, Kramnik mice treated with CFZ for four weeks showed no decrease in pulmonary CFU (Figure 1B). Although eight weeks of treatment resulted in a 1.0 log\(_{10}\) CFU decrease in bacterial numbers, this decrease was not statistically different than control (untreated) Kramnik mice at this time point.

CFZ treatment of BALB/c mice was also highly effective at reducing bacterial numbers in the spleen, ultimately achieving a 3.1 log\(_{10}\) CFU reduction after eight weeks of treatment (p < 0.001). Of importance, CFZ treatment was also highly effective at reducing bacterial numbers in the spleens of Kramnik mice. Eight weeks of CFZ treatment resulted in a 2.6 log\(_{10}\) CFU reduction, which was comparable to that observed in the spleens of BALB/c mice (p > 0.05).

The pathological progression of granuloma formation in Kramnik mice

The strikingly different pathological progression of disease in the lungs of Kramnik mice in comparison to BALB/c mice is likely responsible for the differential response to CFZ between these two strains of mice. To further understand the role that the pathological process of granuloma formation played in the observed attenuation of CFZ efficacy, we performed a comprehensive histological analysis to understand the pathological progression of granuloma development in the lungs of Kramnik mice to identify a time point prior to the formation of well-developed caseous necrotic granulomas and a time point where we reproducibly observed such well-developed granulomas.

At three weeks post-infection, the inflammatory lesions in the lungs of BALB/c mice were characterized as loosely organized cellular aggregates composed of macrophages,
epithelioid macrophages, and large numbers of lymphocytes that were primarily localized within
perivascular regions (Figure 2A), consistent with observations of Rhoades, et al. (29). Small
isolated pockets of neutrophils were only occasionally observed. Kramnik mice exhibited
markedly different cellular lesions composed predominantly of neutrophillic clusters interspersed
with epithelioid macrophages (Figure 2B). Few, if any lymphocytes were found within these
lesions. At this time point, only small foci of individual cellular necrosis were present in the
lungs of Kramnik mice, and large lesions containing caseous necrotic material were absent.

By seven weeks of infection, the pulmonary lesions in BALB/c mice were predominantly
composed of multifocal coalescing lesions. These lesions were composed of disorganized
clusters of macrophages, epithelioid macrophages, and increasing numbers of foamy
macrophages surrounded by large numbers of lymphocytes arranged as punctate clusters in
association with epithelioid and foamy macrophages (Figure 2C). In contrast, the lesions in the
lungs of Kramnik mice progressed to highly organized structures, which consistently displayed
large areas of central caseous necrosis and a peripheral rim of foamy macrophages, with or
without a well-defined collagen rim (Figure 2D).

Highly organized granulomas and caseous necrosis were not observed in the spleens of
BALB/c mice (Figure 2E) or Kramnik mice (Figure 2F), even though bacterial numbers in the
spleens of both mouse strains were comparable.

Pulmonary pathology was responsible for the differential effectiveness of CFZ
To determine the impact of the granuloma pathology upon the effectiveness of drug treatment with CFZ, Kramnik mice were infected with a low dose aerosol and CFZ treatment was initiated prior to the formation of well-defined granulomas (3 weeks post-infection) or after the formation of caseous necrotic granulomas (7 weeks post-infection). Three weeks following infection, bacterial loads in the lungs of Kramnik mice reached $7.1 \pm 0.11 \log_{10} \text{CFU}$ (Figure 3A). Four weeks of CFZ treatment reduced the pulmonary bacterial load in Kramnik mice by 5.8 log$_{10}$ CFU ($p < 0.001$). Seven weeks after infection, bacterial loads reached $7.7 \pm 0.36 \log_{10} \text{CFU}$. However, treatment with CFZ for four weeks after the development of extensive pulmonary pathology only resulted in a 1.6 log$_{10}$ CFU reduction in the lungs of Kramnik mice.

In the spleens of Kramnik mice where no necrotic granulomas were observed at all time points tested, bacterial loads reached $3.7 \pm 0.11 \log_{10} \text{CFU}$ three weeks after aerosol infection (Figure 3B). In response to four weeks of CFZ treatment initiated after 3 weeks of infection, bacterial numbers dropped 2.4 log$_{10}$ CFU. In mice infected for seven weeks, bacterial loads reached $5.1 \pm 0.10 \log_{10} \text{CFU}$ and treatment with CFZ for four weeks resulted in a 1.3 log$_{10}$ CFU reduction.

**CFZ was effective in immune compromised interferon-$\gamma$ knockout mice**

Since CFZ is known to have immunomodulatory properties, we wanted to ensure that the weakened immune status of the Kramnik mice was not responsible for the lack of CFZ efficacy observed in the lungs. We infected highly susceptible interferon-gamma knockout (GKO) mice with *M. tuberculosis* via the LDA route. After 13 days when the bacterial load was $7.1 \log_{10} \text{CFU}$ in the lungs and $4.6 \log_{10} \text{CFU}$ in the spleen, we initiated CFZ monotherapy in the experimental...
group of animals. After 10 days of treatment, the pulmonary bacterial load decreased 3.1 log\textsubscript{10} CFU (Figure 4A) and the bacterial load in the spleen decreased 4.0 log\textsubscript{10} CFU in the GKO mice (Figure 4B). This decrease was comparable to that observed in immune competent BALB/c mice (Figure 1A), indicating that the loss of CFZ activity in the lungs of Kramnik mice was not related to decreased immune function in these animals.

**CFZ was ineffective under anaerobic conditions in vitro**

We next wanted to compare the activity of CFZ under aerobic, hypoxic, and anaerobic conditions to determine the role of oxygen in CFZ activity. CFZ treatment of aerobic cultures (loose cap permitting free air exchange) of *M. tuberculosis* Erdman resulted in a 2.5 log\textsubscript{10} CFU reduction by three days post-treatment, and a 7.3 log\textsubscript{10} CFU reduction after five days (Figure 5A). CFZ activity decreased significantly when *M. tuberculosis* was cultured under hypoxic conditions (1:10 culture volume to headspace ratio), with only a 0.92 log\textsubscript{10} CFU reduction observed after three days and a 2.8 log\textsubscript{10} CFU reduction after five days (Figure 5B). We then utilized a derivation of the traditional Wayne model (30) known as the Rapid Anaerobic Dormancy (RAD) model (28) to culture *M. tuberculosis* Erdman under anaerobic conditions. In this culture system, oxygen is completely consumed after six days of culture using a 1:10 culture volume to headspace ratio. CFZ was administered in an anaerobic chamber at day 12 in the RAD model. After one day, a 0.58 log\textsubscript{10} reduction in CFU was observed (Figure 5C), which increased slightly to a 0.90 log\textsubscript{10} CFU reduction by eight days following treatment.
Discussion

Although CFZ has impressive in vitro killing kinetics and efficacy in traditional mouse models of TB infection, its lack of efficacy in guinea pigs and rhesus monkeys as well as its unusual pharmacokinetic properties and high lipophilicity have limited widespread clinical usage for the treatment of pulmonary tuberculosis. A major finding in the studies presented here is that CFZ activity is diminished in a mouse model with caseous necrotic granulomas compared to a traditional mouse model. We showed here that CFZ monotherapy was effective against the primarily intracellular bacilli located within the inflammatory lesions in the lungs of BALB/c mice. However, this activity was highly attenuated in the lungs of Kramnik mice possessing hypoxic, caseous necrotic lesions containing primarily extracellular bacilli. In contrast, CFZ was similarly effective in the spleens of both BALB/c and Kramnik mice, suggesting that the lack of CFZ activity observed in the lungs was not due to fundamental differences in pharmacokinetics of the drug in these two mouse strains. Preliminary pharmacokinetic analysis of CFZ showed similar drug levels in plasma for both M. tuberculosis infected BALB/c and Kramnik mice (data not shown).

In order to ensure that the diminished CFZ activity in the lungs was not due to a specific inherent characteristic of the C3HeB/FeJ mouse strain, we showed in a subsequent study using only Kramnik mice, that CFZ activity was observed in the lungs at early stages of infection in the absence of caseous necrotic lesions, whereas CFZ activity was highly diminished at time points when lung lesions became necrotic. Again, in contrast to the attenuated efficacy observed in the lungs, CFZ remained effective in the spleens of Kramnik mice throughout all stages of infection, showing in vivo efficacy similar to that of BALB/c mice. An examination of the histopathology
of the spleen tissue of Kramnik mice failed to show highly organized granulomas with evidence of caseous necrosis at all time points examined, similar to observations made by Pichugin, et al. (31). Therefore the consistent efficacy of CFZ throughout the course of infection in the spleens of Kramnik mice and the lack of advanced necrotic lesions in this organ further implicates the pathological response as being the predominant factor contributing to the attenuated efficacy of CFZ in the lungs.

Although Kramnik mice are not generally considered immunosuppressed, they nevertheless possess a specific immune defect related to functional inactivation of the Ipr1 genes (22). Due to the reported immunomodulatory effects of CFZ upon host cells, we wanted to ensure that the weakened immune status of the Kramnik mice was not responsible for the lack of CFZ efficacy in the lungs. CFZ has been shown to have pro-inflammatory effects, by stimulating the production of superoxide anion (32). CFZ also possesses anti-inflammatory effects such as inhibition of neutrophil motility and activation of phospholipase A2 in neutrophils, leading to the production of prostaglandin E2 as well as other anti-inflammatory mediators (32-34). In addition to its effect upon neutrophils, CFZ also inhibits the lymphocyte proliferative response to mitogens, which may be related to its binding to the Kv1.3 potassium channel and perturbation of calcium oscillations required for optimal T cell receptor signaling and T cell proliferation (34, 35). In order to ensure that the altered immune status of the Kramnik mouse model was not responsible for the lack of activity of CFZ we evaluated the drug in the GKO mouse model. Deletion of the interferon-gamma gene renders these mice highly susceptible to infection with M. tuberculosis by incapacitating the T-Helper type 1–IFN-γ axis of M. tuberculosis immunity (36). We found that CFZ was highly effective in both the lungs and spleens of GKO mice when administered for nine days. This efficacy was similar to that observed in BALB/c mice. These
results support the idea that the differential activity of CFZ in BALB/c and Kramnik mice is specifically related to the granulomatous pathology in the lungs of these mice and is not due to differences in immune function between mouse strains.

The pathological process resulting in the development of pulmonary granulomas is a highly regulated, coordinated immunological process involving multiple cell types, cytokines, and pro-inflammatory mediators. It has been hypothesized that the granuloma effectively walls off bacilli residing within an infectious focus, preventing further intrapulmonary as well as extrapulmonary dissemination (37). Conversely, this structure may also create an environment that facilitates bacterial persistence over long periods of time. Pulmonary granulomas in Kramnik mice have been shown by our group (23) as well as others (24) to be hypoxic, which may alter bacterial metabolism and promote a state of latency. The highly organized collagen layers surrounding the granuloma may also act as a barrier to immune cells, preventing eradication of the bacilli by host defense mechanisms. Lastly, this structure may represent a significant barrier to effective drug treatment by impeding drug penetration into mature lesions, resulting in subtherapeutic concentrations of drug within this microenvironment, and increasing the likelihood of the emergence of drug resistance.

Although the mechanism of action for CFZ is not entirely clear, the bacterial outer membrane and in particular electron transport are thought to be the primary target. In the original 1957 paper, Barry, et al. speculated that the high redox potential of CFZ suggests a mechanism of action where CFZ cycles between oxidized and reduced states, generating reactive oxygen species with antimicrobial activity (3). This hypothesis was supported by recent elegant work from Harvey Rubin’s group indicating that CFZ is able to siphon off electrons from the bacterial
electron transport chain by competing with the menaquinone pool for electrons donated by NADH to the oxidoreductase NDH-2 (38). In this model, CFZ is reduced upon interaction with NDH-2 and subsequently oxidized in the presence of molecular oxygen in a redox cycle which simultaneously generates superoxide and hydrogen peroxide and depletes intracellular ATP pools. Under hypoxic conditions, reduced availability of oxygen would slow the formation of reactive oxygen species (ROS), and limit the reoxidation of CFZ to its active form. However, recent work by Liu and Imlay (39) and by Keren, et al. (40) has cast doubt upon the idea that many antibiotics exert their bactericidal effects through a common mechanism of ROS production. As the granulomas within the lungs of Kramnik mice have been shown to be hypoxic, the proposed redox mechanism of action for CFZ is at least consistent with our in vivo data demonstrating attenuated CFZ activity in the Kramnik model of TB infection after the formation of well-developed, hypoxic granulomas.

To further examine the potential role of molecular oxygen in the attenuation of CFZ activity, we evaluated CFZ activity in *M. tuberculosis* cultures at various oxygen concentrations. For this purpose, we utilized the in vitro RAD bacterial culture model to generate hypoxic and completely anaerobic bacterial cultures (28). In this assay, the active metabolism of the bacilli drives the oxygen concentration in the headspace of sealed culture tubes over time to undetectable levels (28). In these studies, CFZ activity decreased significantly when cultured under hypoxic conditions, and the activity was further attenuated in the absence of oxygen. These results were somewhat surprising, as other researchers have reported that CFZ had significant anti-mycobacterial activity using different low oxygen in vitro culture systems (8, 41, 42). In our assay, the loss of oxygen was monitored using methylene blue as an indicator to demonstrate that the cultures were hypoxic (30). In addition, we harvested, manipulated, and...
plated the cultures under low-oxygen conditions within a sealed container inside of the biosafety cabinet to prevent alteration of the bacterial metabolic phenotype. Most other culture systems, such as the LORA assay, still have small residual amounts of oxygen (< 0.16%), which may allow redox cycling of CFZ (albeit at a reduced rate) and some anti-bacterial activity. Of importance, these very minute amounts of oxygen could be sufficient to preserve CFZ effectiveness. Also, the presence of oxygen during the 28 hour recovery phase of the LORA assay could potentially compromise the results and overestimate the efficacy of CFZ under these conditions. However, it should be noted that even when cultured under completely anaerobic conditions using the RAD model, CFZ still retained $\sim \log_{10}$ of bactericidal activity (as seen in Figure 5C) suggesting that other mechanisms in addition to molecular oxygen may potentially contribute to the observed killing. In that respect, we can speculate that other electron donors could be involved in the reduction of CFZ into the active form. Lastly, in the in vitro studies performed here, *M. tuberculosis* Erdman was used whereas most other investigators use *M. tuberculosis* H37Rv in the LORA assay and other anaerobic culture systems (8, 41). *M. tuberculosis* strain-specific differences in CFZ susceptibility under low oxygen conditions are now being further investigated to address this question.

It has been suggested that CFZ has improved activity when bacteria are intracellular due to the accumulation of the drug within immune cells (43, 44). In TB patients with active disease, it is thought that the majority of bacteria are extracellular in various lung compartments (in sputum, and in necrotic, fibrotic, and cavitary lung lesions). The pharmacokinetics of the drug are such that CFZ accumulates to high levels in the tissues and within macrophages (45-47), while serum concentrations are low. Together with the high protein binding of CFZ ($\geq \%$), plasma protein binding [Anna Upton, personal communication]), this partitioning may limit the
exposure of extracellular bacilli to biologically active drug concentrations (48). Of additional
importance, the granuloma structure itself may impede drug penetration, preventing exposure of
bacilli within caseous necrotic lesions to bactericidal concentrations of drugs and facilitating the
emergence of antimicrobial resistance. A recent report by Prideaux, et al., demonstrated that
moxifloxacin preferentially accumulates in immune cells surrounding the caseum, with
decreased penetration into the caseum of necrotic granulomas in a rabbit model of TB infection
(49). Experiments are currently in progress to quantify CFZ drug levels and to assess the
penetration of CFZ into caseous granulomas in Kramnik mice.

It is important to understand that while CFZ monotherapy showed limited activity in the
necrotic lung lesions of Kramnik mice, clinical usage of CFZ would always be in combination
with other drugs. Therefore the results presented in this study should be interpreted with caution.
CFZ may still have synergistic effects with other TB drugs and/or a non-overlapping spectrum of
activity targeting subpopulations of bacteria that are difficult to eradicate using available TB
drugs. In addition, as lung pathology begins to resolve due to the action of efficacious
companion drugs, the changing microenvironment may promote CFZ activity. Studies in our
laboratory are currently underway to investigate the addition of CFZ to combination drug
regimens in Kramnik mice.

Our results are in concordance with the recent clinical results of CFZ in a Phase IIa early
bactericidal activity (EBA) clinical study (NC-003) conducted by the Global Alliance for TB
Drug Development. A preliminary analysis of the NC-003 trial presented at the Union World
Conference on Lung Health in November, 2013 (50) showed no efficacy of CFZ monotherapy in
the first 14 days of treatment, and no additive effect when CFZ was added to bedaquiline and
PA-824 (51). Although EBA trials serve as an important starting point to determine appropriate dosing and define short-term clinical efficacy, the results obtained from EBA studies may not completely reflect the total spectrum of killing activity of all drugs (52). Long-term administration (> 14 days) of CFZ may still provide significant sterilizing activity that is not evident in an EBA study, and may prevent relapse of infection. Of importance, the plasma concentrations of CFZ observed in the trial were significantly lower than mathematical models predicted for that dosing regimen based upon prior data (53), potentially underestimating the CFZ efficacy. Higher doses may be needed to optimize the contribution of CFZ in multidrug regimens.

Another explanation for the inactivity of CFZ may relate to the nature of the bacterial subpopulation(s) found in sputum originating from cavitary lesions where oxygenation is limited. A better understanding of the metabolic state of bacterial populations in sputum samples would be required to answer that question.

The development of well-defined, hypoxic granulomas with abundant caseous necrosis and extracellular bacilli in Kramnik mice provides a low-cost, convenient animal model to evaluate the impact of such lesions on drug efficacy. Differential activity of CFZ in a traditional BALB/c mouse model and the Kramnik mouse model underscores the utility of the latter model in dissecting further the \textit{in vivo} activity of a drug. Animal models that more accurately reflect the spectrum of pathological complexity within the lung may improve the concordance between preclinical models and human clinical trials.
Acknowledgments

This work was supported by the Bill and Melinda Gates Foundation under grant ID numbers 1033596, “Evaluation of a New Murine Model for Testing Tuberculosis Chemotherapy” and 1037174 entitled “Qualification of C3HeB/FeJ mice for Experimental Chemotherapy of Tuberculosis” and National Institutes of Health grant number AI061505.

We acknowledge the staff of the Laboratory Animal Resources at Colorado State University for their animal care. We thank Dr. Phil Chapman (Colorado State University) for statistical support and Dr. Michael Lyons for helpful discussions.

Figure Legends

Figure 1. CFZ was highly effective at reducing bacterial CFUs in BALB/c mice (A) in the lungs (filled symbols) and in the spleen (open symbols). In contrast, CFZ activity was significantly attenuated in the lungs of C3HeB/FeJ mice (B), while comparable activity was observed in the spleen. Data represent mean log_{10} CFU counts ± SEM; detection limit = 50 CFU. * = p < 0.001.

Figure 2. Pathological progression of disease in BALB/c and C3HeB/FeJ mice. Cellular aggregates in the lungs of BALB/c mice at 3 weeks post-infection (A; 100x) were composed predominantly of macrophage cells with distinct regions of lymphocytic perivascular and peribronchiolar cuffing (arrows). C3HeB/FeJ mice at 3 weeks post-infection (B; 100x) exhibited cellular lesions composed predominantly of neutrophilic clusters (arrows) and epithelioid macrophages, with evidence of an early fibrotic response. By seven weeks (C; 40x), the cellular
aggregates in BALB/c mice formed loosely organized inflammatory granulomas lacking a well-defined collagen rim. In contrast, by seven weeks (D; 40x), highly organized granulomas had formed in the lungs of C3HeB/FeJ mice possessing a hypoxic neutrophillic central caseous necrotic core region (CN) and a layer of foamy macrophages (FM) delineated by a collagen rim (arrows) that encapsulated the granuloma structure. By seven weeks post-infection, no caseating necrotic granulomas were observed in the spleens of BALB/c (E; 40x) or C3HeB/FeJ (F; 40x) mice.

**Figure 3.** Attenuation of CFZ activity is related to pulmonary pathology. Administration of CFZ to C3HeB/FeJ mice prior to the formation of well defined pulmonary granulomas (A; 3 weeks post-infection) reconstituted bactericidal activity in the lungs (filled symbols). Initiation of CFZ treatment after the formation of well defined pulmonary granulomas with significant caseous necrosis (B; 7 weeks post-infection) resulted in significant attenuation of bactericidal activity in C3HeB/FeJ mice. CFZ exhibited comparable activity in the spleens (open symbols) of C3HeB/FeJ mice which lack well defined granulomas and caseous necrosis. Data represent mean log$_{10}$ CFU counts ± SEM; detection limit = 50 CFU. * = p < 0.001, ♦ = p < 0.05.

**Figure 4.** CFZ was effective in immunocompromised GKO mice. Interferon-γ gene disrupted mice were treated for nine days with 20 mg/kg CFZ beginning on day 13 post-infection. CFZ reduced bacterial loads in the lungs (A) by 3.1 log$_{10}$ CFU and in the spleens (B) by 3.95 log$_{10}$ CFU compared to untreated controls. Data represent mean log$_{10}$ CFU counts ± SEM; detection limit = 50 CFU. * = p < 0.001.
Figure 5. CFZ activity decreased as oxygen concentration decreased. CFZ was highly effective in vitro under high aeration (A). Under low aeration (B), CFZ had reduced but demonstrable activity despite similar bacterial growth. However CFZ activity was lowest under completely anaerobic conditions achieved in the Rapid Anaerobic Dormancy (RAD) culture model (C). CFZ was added at a final concentration of 50 mg/ml. Data represent mean log$_{10}$ CFU counts ± SD; detection limit = 50 CFU. * = p < 0.001.


tuberculosis and reduced potential for accumulation. Antimicrob Agents Chemother 
55:5185-5193.

M, Fu L, Hou Y, Gong N, Lv Y, Li C, Cooper CB, Upton AM, Yin D, Ma Z, Huang 
H. 2012. Identification of less lipophilic raminophenazine derivatives for the treatment of 

Short, highly effective, and inexpensive standardized treatment of multidrug-resistant 

11. Grosset JH, Tyagi S, Almeida DV, Converse PJ, Li SY, Ammerman NC, Bishai WR, 
Enarson D, Trebucq A. 2013. Assessment of clofazimine activity in a second-line 
regimen for tuberculosis in mice. Am J Respir Crit Care Med 188:608-612.

12. Tasneen R, Li SY, Peloquin CA, Taylor D, Williams KN, Andries K, Mdluli KE, 
regimens in a murine model of tuberculosis. Antimicrob Agents Chemother 55:5485-
5492.

RS, Mdluli KE, Nuernberger EL. 2012. Sterilizing activities of novel combinations 
lacking first- and second-line drugs in a murine model of tuberculosis. Antimicrob 
Agents Chemother 56:3114-3120.

14. Lienhardt C, Raviglione M, Spigelman M, Hafner R, Jaramillo E, Hoelscher M, 
Zumla A, Gheuens J. 2012. New drugs for the treatment of tuberculosis: needs, 


