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Clofazimine Analogs with Efficacy against Experimental Tuberculosis and Reduced Potential for Accumulation

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Running Title: Clofazimine analogs for TB

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ABSTRACT

The global tuberculosis crisis urgently demands new, efficacious, orally available drugs with the potential to shorten the long complex treatments of drug sensitive and drug resistant disease. Clofazimine, a riminophenazine used for many years to treat leprosy, demonstrates efficacy in animal models of tuberculosis via a novel mode of action. However, clofazimine's physicochemical and pharmacokinetic properties contribute to side effects that limit its use; in particular, an extremely long half-life and propensity for tissue accumulation together with clofazimine's dye properties lead to unwelcome skin discoloration. We recently conducted a systematic structure-activity study of over five hundred riminophenazine analogs for anti-*M. tuberculosis* activity. Here, we describe the characteristics of twelve prioritized compounds in more detail. The new riminophenazine analogs have enhanced *in vitro* activity compared to clofazimine against replicating *M. tuberculosis* H37Rv as well as panels of drug sensitive and drug resistant clinical isolates. The new compounds demonstrate at least equivalent activity compared to clofazimine against intracellular *M. tuberculosis* and in addition, most are active against non-replicating *M. tuberculosis*. Eleven of these more water-soluble riminophenazine analogs possess shorter half-lives than clofazimine when dosed orally to mice, suggesting they may accumulate less. Most importantly, the nine compounds progressed to efficacy testing demonstrate inhibition of bacterial growth in the lungs that is superior to the activity of an equivalent dose of clofazimine when administered orally for 20 days in a murine model of acute tuberculosis. The efficacy of these compounds coupled with their decreased potential for accumulation and therefore perhaps also tissue discoloration, warrants their further study.

INTRODUCTION

Despite global efforts, tuberculosis (TB) remains responsible for the second greatest number of deaths due to an infectious disease with 1.7 million deaths reported due to TB in 2009(45). Of particular concern, the increasing prevalence of TB caused by multi-drug resistant (MDR) and extensively drug resistant (XDR) strains of *M. tuberculosis* puts at risk hard-won gains to public health. MDR-TB treatment regimens, where available, comprise multiple expensive drugs with limited efficacy and significant toxicity that must be administered by both oral and parenteral routes, for up to 24 months (30). Treatment of drug-sensitive TB is also long and complex, requiring at least 6 months of a 4-drug regimen to achieve a stable cure. The first line TB drugs are poorly tolerated, reducing compliance and increasing the risk of resistance development. New TB drugs that are safe, orally available, have novel modes of action and efficacy sufficient to simplify and shorten the regimen required to cure TB would impact both patients and TB control by providing improved treatment for drug resistant TB and by providing a faster, more tolerable cure for drug sensitive TB enhancing treatment success and reducing further resistance acquisition.

In addition to screening for new chemotypes active against mycobacterial cells and against promising new TB drug targets, we and others active in TB drug discovery have investigated derivatives of proven anti-mycobacterial compounds that may not have been fully exploited. One such compound is clofazimine(CFM), a riminophenazine and marketed anti-mycobacterial drug used since 1962 in treatment of leprosy (16, 17) and more recently, in treatment of *M. avium* complex infection (16) and MDR-TB (43). CFM, a redox active compound, with dye

properties, was specifically designed as an anti-tubercular to accumulate within cells of the mononuclear phagocyte system (MPS) and kill intracellular bacteria (6-8). CFM and analogs have been shown to demonstrate activity *in vitro* against most mycobacteria (38) as well as some gram positive species (19). Against replicating *M. tuberculosis*, CFM demonstrates activity *in vitro* against both drug sensitive and resistant strains(8, 29) and activity against *M. tuberculosis* replicating inside macrophages (44). Although activity against replicating and intracellular bacteria is desirable in an anti-tubercular drug, it is widely believed that sub-populations of non-replicating or slowly replicating bacteria, which may be intracellular, extracellular, or both, exist during TB infection and are refractory to many antibiotics. These phenotypically drug tolerant "persister" *M. tuberculosis* may contribute to the extremely long treatment duration required to produce a stable cure for TB (18). *In vitro* assays are available that involve culture conditions thought to mimic aspects of the intracellular and extracellular host environments where *M. tuberculosis* resides and slow or halt bacterial replication; CFM exhibits potent activity in the low-oxygen-recovery assay (LORA), that is similar to the LORA activity of the potent sterilizing drug rifampicin (RIF) (10).

Efficacy of CFM was inconsistent in early *in vivo* models of TB, likely but not definitively due to inter-species variation in absorption (12); however, later studies have proven CFM and analogs efficacious as monotherapy in murine models of TB (1, 20, 27, 29, 37). The combination of *in vivo* efficacy in murine models of TB, clinical experience through the treatment of leprosy and activity against drug resistant *M. tuberculosis*, suggesting a novel mode of action, have led to renewed interest in this compound for the treatment of TB. However, the very physicochemical properties selected by its inventors to allow for accumulation in macrophages,

lead to suboptimal pharmacokinetic properties and consequent side effects that may limit its use. An excessively long half-life (greater than 70 days in humans) and propensity for accumulation and crystallization within fatty tissues and the tissues of the MPS cause gastrointestinal and ocular harm; moreover, these pharmacokinetic properties of the drug together with its dye properties lead to unwelcome skin discoloration (13, 14, 16, 21, 25, 26, 41).

Globally, work to discover riminophenazine analogs with anti-TB efficacy equal to or better than that of CFM, with improved physicochemical properties and fewer side effects has been ongoing, but development has not progressed beyond preliminary determinations of *in vivo* efficacy (20, 36, 37, 44). In an effort to perform a thorough evaluation of the potential of this class for anti-tubercular therapy, we systematically investigated the structure-activity relationship of over 500 new CFM analogs for anti-*M. tuberculosis* activity (Dali Yin, Haihong Huang, et al. 2011 unpublished). Here, we report the pharmacological properties of 12 prioritized compounds.

MATERIALS AND METHODS

Compounds. Rifampin (RIF) and isoniazid (INH) were purchased from Sigma and CFM was provided by Nanjing Liye (China). Novel riminophenazine analogs were synthesized by The Institute of Materia Medica(Dali Yin, Haihong Huang, et al. 2011 unpublished).

Bacterial strains. The *M. tuberculosis* strains used in these studies comprised the laboratory strain *M. tuberculosis H37Rv* (ATCC 27294; American Type Culture Collection, Rockville, Md.), and clinical isolates including drug susceptible and drug resistant strains. All isolates were obtained from the State Laboratory of Tuberculosis Reference of China.

Determinations of solubility and log P. To determine logP, water (2 ml) was added to 1ml 4 mg/ml solution of analyte in octan-1-ol. The mixture was shaken for 6 h at room temperature. After centrifugation and manual phase separation the aqueous phase was analyzed by HPLC. The log P was calculated using the formula:log P = log ($[analyte]_{octan\text{-}1\text{-}ol}/[analyte]_{water}$).To determine aqueous solubility 10 mg compound was dissolved in pH 7.0 buffer solution and in pH 1.0 buffer solution. Equilibration of test compound was achieved after 30 minutes by vigorous shaking for 30 seconds at a time over 5 minute intervals at 20±5°C.

Minimal inhibitory concentrations (**MICs).** MICs against replicating *M. tuberculosis* were determined by the microplate Alamar blue assay (MABA) (11). RIF, INH and CFM were included as positive controls. Riminophenazine analog stock solutions and the range of final testing concentrations were 64 µg/ml and 32 to 0.5 µg/ml. For the most active compounds, the stock concentration and final testing concentration range were lowered to 3.2 µg/ml and 2 to 0.015 µg/ml, respectively. *M. tuberculosis H37Rv* or a clinical isolate was grown to late log phase (70 to 100 Klett units) in Difco™ Middlebrook 7H9 Broth (Cat. No. 271310) supplemented with 0.2% (vol/vol) glycerol, 0.05% Tween 80, and 10% (vol/vol) albumin-dextrose-catalase (BBL™ Middlebrook ADC Enrichment, Cat. No. 212352) (7H9-ADC-TG). Cultures were centrifuged then washed twice, and resuspended in phosphate-buffered saline. Suspensions were then passed through an 8 µm-pore-size filter to remove clumps, and aliquots were frozen at -80°C. Twofold dilutions of riminophenazine analogs were prepared in 7H9-ADC-TG in a volume of 100 µl in 96-well, black, clear-bottom microplates (BD Biosciences, Franklin Lakes, N.J.). *M. tuberculosis* (100µl containing 2×10^5 colony forming units [CFU]) was added, yielding a final testing volume of 200 µl. The plates were incubated at 37°C; on the seventh day of incubation, 12.5 µl 20%

126 Tween 80 and 20 µl of Alamar blue were added to all wells. After incubation at 37°C for 16 to 24
127 hrs, fluorescence was read at an excitation of 530 nm and emission of 590 nm. The MIC was
128 defined as the lowest concentration effecting a reduction in fluorescence of ≥90% relative to
129 the mean of replicate bacteria-only controls. MICs against non-replicating *M. tuberculosis* were
130 determined using the low-oxygen-recovery assay (LORA) as described previously (10).

131 **Cytotoxicity**. Vero cells were cultured in RPMI1640 medium supplemented with 10% fetal
132 bovine serum (FBS). The cells were incubated in a humidified atmosphere of 5% CO_2 at 37°C.
133 Stocks of cells were cultured in 25 cm^2 tissue culture flasks and sub-cultured 2- to 3-times per
134 week.Cytotoxicity testing was performed in a transparent 96-well microplate. Outer perimeter
135 wells were filled with sterile water to prevent dehydration in experimental wells. The cells were
136 incubated at 37°C under 5% CO_2 until confluent and then diluted with culture medium to 4×10^5
137 cells/ml. Threefold serial dilutions of the stock solutions resulted in final concentrations of 64 to
138 0.26 µg/ml in a final volume of 100 µl. After incubation at 37°C for 48 hrs, medium was
139 removed and monolayers washed twice with 100 µl warm Hanks' balanced salt solution (HBSS).
140 One hundred microliters warm medium and 10 µl freshly made methyl thiazolyldiphenyl-
141 tetrazoliumbromide (MTT) were added to each well, then plates were incubated for 4hrs, after
142 which absorbance was determined at 492 nm.

143 **Macrophage assay.** J774 A.1 cells were grown to confluency in 75 cm^2 cell culture flasks in
144 DMEM medium containing 10% FBS. Using a cell scraper, the cells were detached and
145 centrifuged at 200 × g for 5 min at room temperature, and the pellet resuspended to a final
146 concentration of 3×10^5 to 6×10^5 cells/ml. One-milliliter aliquots of cell suspension were
147 distributed into 24-well plates (Corning/Costar), and the plates were incubated at 37°C in a 5%

148 CO_2 incubator for16 hrs. *M. tuberculosis H37Rv* frozen cultures were thawed, sonicated for 15
149 sec and diluted to a final concentration of 3×10^7 to 6×10^7 CFU/ml with DMEM, and 100 µL of
150 the dilution was dispensed to each well to give multiplicity of infection (MOI) about 10
151 bacteria/cell. The plates were incubated at 37°C for 4 hrs to allow for phagocytosis. The
152 supernatant was aspirated, and the cells were washed three times with DMEM medium
153 without FBS to remove extracellular mycobacteria. The contents of the wells were replaced
154 with DMEM containing 1% FBS and different concentrations of the compounds (1.0, 0.5, and
155 0.25 µg /ml). Control wells received drug-free medium. The medium with or without drugs was
156 replaced every day. After 3 days of incubation, monolayers were visually inspected under the
157 microscope to ensure they remained intact, then medium was removed and macrophages were
158 lysed with 200 µl 0.1% sodium dodecyl sulfate. After 10 min of incubation at 37°C, 800 µl fresh
159 medium was added. Twenty microliters of the lysates were inoculated into 96-well, black, clear-
160 bottom microplates, and 7H9-ADC-TG added, yielding a final testing volume of 200 µl. The
161 plates were incubated at 37°C; on the seventh day of incubation, 12.5 µl 20% Tween 80 and 20
162 µl Alamar blue were added to all wells. After incubation at 37°C for 16 to 24 h, the fluorescence
163 of the wells was read at an excitation of 530 nm and emission of 590 nm. Infected macrophages
164 incubated in drug-free control wells were designated 100% for $H_{37}Rv$ viability, while 7H9
165 medium controls were used to determine background fluorescence. The intracellular
166 mycobacterial growth inhibition activities of the compounds were calculated as the percentage
167 reduction in background corrected fluorescence compared to the 100% $H_{37}Rv$ viability positive
168 control fluorescence ($Inhibition\% = [1-(flu_{drug}-flu_{background})/(flu_{control}-flu_{background})]\times100\%$) .

Pharmacokinetics. Specific-pathogen-free (SPF) male BALB/c mice weighing 18-22 g were used with n = 3 mice per time point. Feed and water were freely given during acclimation with a 16-hr fasting period prior to dose administration. Compounds were prepared at 2 mg/ml in 0.5% carboxymethyl cellulose (CMC) and a 0.2 ml suspension was administrated orally to each mouse to achieve a 20 mg/kg body weight dose. Blood samples were collected via the fossa orbitalis vein at 0, 0.5, 1,2, 4, 8, and 24 hrs post administration and centrifuged at 2500 × g for 10 min to obtain serum which was then stored at –20°C. One-hundred microliters of the serum was added to 200 µl acetonitrile and the resulting solution was vortexed for 2 min and centrifuged at 2500 × g for 10 min to remove protein. The supernatant was removed to an Eppendorf tube and centrifuged at 11000 × g for 5 min. The supernatant was removed to a sample bottle and 40 µl injected for HPLC analysis (Waters 600 Controller, 2487 Dual λ Absorbance Detector, 717 plus Autosampler and Waters Empower Chromatogram Workstation). The analytical column was Waters SymmetryShield^{TM} RP_{18} (3.9×150 mm, 5 µm). The mobile phase was prepared by mixing acetic sodium buffer solution and methanol in various proportions determined in a pre-experiment. The flow rate was1ml/min, λ_{max}=495nm. All chromatograms obtained were evaluated by the peak area. Pharmacokinetic (PK) analyses of the plasma concentration-time relationships for the riminophenazines were performed using Drug and StatisticsVersion 3.0 (Bontz Inc., Beijing, China). A noncompartmental library model was used to calculate PK parameters including the maximum concentration of drug in plasma (C_{max}), elimination half-life, and area under the concentration-time curve from 0 to 24 h ($AUC_{0–24}$). PK data in Table 6 are reported as means± standard deviations (SD).

Aerosol model of acute infection. SPF BALB/c male mice, weighing 18-20 g were used in this study. Mice were infected via aerosol with a suspension of 5× 10^6 CFU/ml *M.tuberculosis*H37Rv using a Glas-Col inhalation system, to deposit 50-100 bacilli into the lungs of each animal. The course of infection was followed by plating homogenates of harvested organs [n = 3] on 7H11agar plates (7H11 plates containing 10% oleic acid-albumin-dextrose-catalase (OADC) enrichment and 50µg/ml cycloheximide, 200U/ml polymyxin B, 50µg/ml carbenicillin and 20µg/ml trimethoprim) and determining CFU on Day 3, Day 10 and Day 30 post-infection. Drugs and compounds were dissolved or suspended in 0.5% CMC and administered by oral gavage in a maximum volume of 200 µl such that a dose of 20 mg/kg body weight was achieved. Mice were treated 5/7 days per week during the acute phase of infection from Day 10 until Day 30. Each treated group was composed of 5 or 6 mice, while the control group, which received only CMC, was composed of 7 to 10 mice. Mice were sacrificed the day after the last day of treatment, organ weights determined, lungs removed, homogenized, and serially diluted in 10-fold steps in HBSS. One-hundred microliters were spread on 7H11 agar in duplicate. The plates were incubated at 37°C for 2-3 weeks. Data are expressed as the $\log_{10}$ (and as $\log_{10}$ reduction) provided by a given dose of the compound against the growth of the organism in the untreated control group. Mean $\log_{10}$ values were calculated from bacterial burden counts. Student's *t* test was used to compare means between test and control groups. A *P* value of $\leqslant 0.05$ was considered significant.

RESULTS

Riminophenazine analogs with enhanced potency compared to CFM against drug sensitive and drug resistant replicating *M. tuberculosis.* The 12 riminophenazine analogs possess MICs ranging from 0.011 to 0.038μg/ml with the most active exhibiting potency 10-fold better than CFM (MIC = 0.12μg/ml). In addition, the compounds have acceptable cytotoxicity against Vero cells with selectivity indices ranging from 18 to >5818 (Table 1). To further explore the *in vitro* activities of the compounds compared to CFM, MICs were determined using the Microplate Alamar Blue Assay (MABA) against a panel of 10 drug sensitive *M. tuberculosis* clinical isolates and against 10 clinical isolates with varying drug resistance profiles, including both MDR and XDR strains. The MICs for this set of riminophenazine analogs against the drug sensitive isolates range from 0.015 to 0.116μg/ml and most compounds are more potent than CFM (0.044-0.24μg/ml) (Table 2). Likewise, the new compounds demonstrated enhanced activity compared to CFM against the drug resistant panel with MICs ranging from 0.015 to 0.121 μg/ml compared to 0.108-0.24 μg/ml for CFM (Table 3). This result indicates the potential utility of these compounds against both MDR and XDR-TB including strains resistant to both fluoroquinolones and aminoglycosides. In addition, these data suggests a novel mode of action for this class compared with TB drugs in current use.

Riminophenazine analogs active against non-replicating *M. tuberculosis* and intracellular *M. tuberculosis.* The activity of CFM against intracellular *M. tuberculosis* has been well documented (20, 37, 44). It is presumed related to the tendency of this anti-bacterial compound to accumulate in phagocytic cells (4, 14). We investigated the activity of the new

riminophenazine analogs against *M. tuberculosis* replicating within J774A.1 macrophages using a recovery assay. We compared the extracellular recovery of bacteria following 3 days intracellular exposure to compounds at various concentrations. Following 7 days recovery, the bacterial titers for compound exposed wells, as detected via Alamar blue, were lower than for those of the no compound control wells. Dose-dependent activity was observed for all riminophenazine analogs and was at least equipotent to CFM (Table 4).Intracellular bacterial growth for the drug free control during the assay period was determined by CFU enumeration to be approximately 1 $\log_{10}$ (6.07 $\pm$ 0.04 $\log_{10}$ CFU on day 0 and 7.16 $\pm$ 0.11 $\log_{10}$ CFU on day 3). Therefore, the diminished bacterial recovery observed following exposure to the riminophenazine analogs versus the no drug control reflects either bacteriostatic or bactericidal activity exerted during the intracellular phase of the assay. Further studies directly enumerating intracellular bacteria versus duration of compound exposure will be useful to distinguish growth inhibition from bactericidal activity for compounds that are progressed.

We also determined the activities of the new compounds against low-oxygen-induced non-replicating *M. tuberculosis* using the LORA assay. These bacteria may reflect a sub-population of drug tolerant bacteria that contributes to the persistence of a TB infection in the face of prolonged chemotherapy. The majority of the riminophenazine analogs demonstrated activity in this assay with the MICs of TBI-161, TBI-678 and TBI-688 being in the range of that of CFM (Table 5). Of interest, these activities do not correlate with potency against replicating bacteria or intracellular bacteria, suggesting a complex mode of action for this class.

Less lipophilic riminophenazine analogs with decreased plasma half-lives compared to CFM. Riminophenazine analogs with lower lipophilicity and shorter half-lives compared to CFM can be expected to accumulate less in tissues including those of the MPS. We hypothesize that a consequence of reduced tissue accumulation and crystallization will be reduced skin discoloration. Therefore, compounds of interest for further investigation will demonstrate shorter half-lives and reduced lipophilicity compared to CFM while affording exposure necessary to efficacy when dosed orally. The logP values of the 12 new compounds are lower than those of CFM (logP= 5.34) and range from 3.51-4.82. No compound has aqueous solubility above the limit of detection (0.01 g/100ml) at pH7 but solubility at pH1 is enhanced for the new compounds (ranging from 0.2-10.4g/100ml) compared to CFM which exhibits solubility below the detection limit (Table 1). Through previous work with CFM, we demonstrated that the mouse is a suitable animal model for evaluation of the potential of riminophenazine analogs to accumulate and discolor tissue (28). We therefore determined primary PK parameters for each riminophenazine analog in mice, dosed once by the oral route (Table 6). All but one of the new compounds possesses a shorter plasma half-life and increased C_{max} and AUC in comparison to CFM. Shorter serum half lives in combination with lower lipophilicity may indicate a decreased potential for the new compounds to produce adverse effects due to drug accumulation.

Riminophenazine analogs that are efficacious against acute TB Infection in mice. A murine model of acute infection with *M. tuberculosis* H37Rv was used to assess the efficacy of the new riminophenazine analogs in comparison that of to CFM. Prior to conducting efficacy testing, a preliminary tolerability study was carried out in mice for all 12 compounds. Through this, it was determined that 3 of the compounds (TBI-161, TBI-678 and TBI-688) have LD_{50} values lower

than 600mg/kg. The remaining 9 compounds were therefore prioritized for efficacy evaluation. The 9 compounds demonstrated a range of efficacies against murine acute *M. tuberculosis* infection that were, in all cases, superior to that of CFM when administered at an equivalent dose for 20 days (Table 7). In this model of acute infection, the lung bacterial burden of the untreated control group increased from3.17 ± 0.39 $\log_{10}$ CFU to 8.53±0.32$\log_{10}$ CFU over the 20 day course of chemotherapy. At the end of the treatment period, mean lung CFU counts for all riminophenazine-treated groups (including CFM)were 3-5-log units lower than those for the untreated control group (P<0.001). Treatment with any of the new riminophenazine analogs resulted in mean lung CFU counts lower than those of the CFM-treated group (P<0.001). The lowest lung burdens were observed following treatment with TBI- 449, TBI -450 and TBI-416. Although the new riminophenazine analogs demonstrated efficacy superior to CFM in this model and at this dose, none of these compounds reduced the lung burden below that observed pre-treatment in this experiment. We conclude that, under the conditions selected, we observed primarily bacteriostatic rather than bactericidal activity for the riminophenazine analogs and CFM. Further studies are planned to evaluate the maximal efficacy achievable and bactericidal potential of selected compounds against murine TB. We have previously observed that CFM treatment of mice leads to discoloration of their internal organs, ears and feet in a dose and duration dependent manner(28). During the present study we observed discoloration of ears, internal organs and fat as seen previously, following dosing of CFM at 20 mg/kg for 20 days. However, the new riminophenazine analogs, administered at the same dose for the same length of time, caused a range of discoloration effects. Of interest, two of the most efficacious

295 compounds, TBI-416 and TBI-449, appeared to cause the least discoloration of tissues (data not
296 shown) in this preliminary assessment.

297 **DISCUSSION**

298 The riminophenazine CFM is one of very few compounds with demonstrated potential for
299 use as an oral drug in treatment of both drug sensitive and resistant TB. However, CFM,
300 administered at 100mg/day in the treatment of leprosy, induces well-documented and
301 unwelcome discoloration of the patient's skin. This discoloration is evident within weeks of
302 beginning treatment, fades months to years after cessation of treatment and occurs in a
303 significant percentage of patients (42, 16, 33). CFM-induced skin discoloration has also been
304 observed during treatment of *M. avium* complex and MDR-TB infections as well as during
305 clinical trials aimed to investigate the usefulness of CFM in inflammatory diseases, suggesting
306 this phenomenon is not leprosy specific (9, 16, 24, 43). The reports available suggest that the
307 intensity, duration and prevalence of skin discoloration are dependent on dose and duration of
308 treatment. Animal and clinical studies indicate that discoloration occurs in skin as well as in
309 many other tissues, especially fat and organs of the MPS, via intracellular accumulation and
310 crystallization of this bright red compound (15, 31, 32, 35, 41). CFM's 70 day half-life in man and
311 lipophilic nature are the likely primary drivers of this side effect as well as other dose and
312 duration-dependent side effects. Therefore we have hypothesized that more water-soluble
313 riminophenazine analogs with shorter half-lives may accumulate less and discolor tissues less.
314 Further, more potent riminophenazine analogs that are as efficacious as CFM at a lower dose
315 and/or possess rapidly sterilizing anti-*M.tuberculosis* efficacy may capture the potential efficacy

316 benefit of CFM without the problematic discoloration side effect. Hence, we aimed to discover
317 riminophenazine analogs with better *in vitro* potency than CFM; that demonstrate efficacy in a
318 mouse model of TB; and that exhibit lower lipophilicity and shorter plasma half-lives compared
319 to CFM.

320 Our *in vitro* activity studies reveal that all 12 compounds are more active than CFM against *M.*
321 *tuberculosis* replicating in culture or inside macrophages, and most are active against low-
322 oxygen induced non-replicating *M. tuberculosis.* However, there is a disconnect between the
323 ranked activities of the new compounds and CFM against replicating and non-replicating *M.*
324 *tuberculosis in vitro.* This is particularly interesting in light of the apparently multi-faceted anti-
325 tubercular mode of action of CFM. The mode of action of CFM has been difficult to study due in
326 part to the low frequency of resistance generation (38). Recent work suggests the bactericidal
327 activity occurs via reactive oxygen species (ROS) production following reduction of CFM by
328 mycobacterial NADH quinone-oxidoreductase(46). This finding links CFM to both a
329 pharmacologically validated *M. tuberculosis* target pathway, the electron transport chain (5)
330 and the established mechanism of ROS-driven bactericidal action (22). The differences we noted
331 between the rank order potencies of these analogs and CFM against extracellular replicating *M.*
332 *tuberculosis* and low-oxygen-induced non-replicating *M. tuberculosis* suggest a mode of action
333 that emphasizes different mechanisms under different environmental conditions. Further
334 complexity may be inherent in the activity of CFM against intracellular *M. tuberculosis*:
335 Interactions that may enhance macrophage-mediated, anti-microbial mechanisms have been
336 observed for CFM (2, 3), suggesting that a combination of host-directed and anti-bacterial
337 properties may affect growth inhibition of intracellular mycobacteria exposed to CFM. In

addition, the clinically apparent anti-inflammatory properties of CFM, currently under investigation for the treatment of several auto-immune disorders (23, 24, 39, 40) may also contribute to the *in vivo* action of this compound against TB. Our *in vitro* data suggest that, for most of these new analogs, the aspects of CFM's mechanism important to activity against extracellular and intracellular replicating bacteria and against extracellular non-replicating bacteria have been preserved. Study of the mode of action of these new compounds and correlation with their *in vitro* and *in vivo* pharmacological activities should improve our understanding of the anti-TB action of this class.

The efficacy evaluation we describe confirms that the 9 compounds tested are active *in vivo*, at least against *M. tuberculosis* rapidly replicating within the lungs of mice. In the model presented, all 9 new riminophenazine analogs demonstrate enhanced efficacy compared to CFM when administered for 20 days at 20mg/kg in an acute murine TB model. However, simple inspection of the *in vivo* activities of the compounds compared with their AUC/MIC ratios reveals no facile correlation between the two. As a result, we suggest caution when interpreting the potential of these compounds from the rank order efficacies exhibited in this experiment. The varied pharmacokinetic and *in vitro* activity profiles of the compounds recommend the collection of further *in vivo* activity data to permit a well-informed evaluation and comparison of the efficacy potential of these compounds.

Specifically, the variation in plasma half-lives of these compounds, from 5.54 to 41.25h may bias efficacy determinations following relatively short treatment periods towards those with shorter half-lives that more quickly reach steady state. Likewise, any correlation between tissue

359 accumulation and efficacy for these riminophenazines would be difficult to detect over a 20 day
360 course of treatment. Future studies evaluating efficacy of these compounds over longer courses
361 of treatment and at a range of doses are planned to permit clearer comparison of their
362 efficacies and better understanding of the PK/PD drivers for this class.

363 Further, the mouse model of TB infection used here simulates human acute infection in
364 which the host immune response has not yet fully developed and the bacteria introduced to
365 the lungs replicate unabated. Although the new compounds demonstrate activity in preventing
366 further replication of *M. tuberculosis* in this acute infection model, it will also be important in
367 future studies to investigate their bactericidal activity in a model of chronic TB infection. In a
368 murine chronic infection model, drug treatment commences following establishment of the full
369 host immune response and consequent slowing of *M. tuberculosis* replication and plateau of
370 the TB lung burden (34). If the slowly-replicating bacteria present during chronic murine TB
371 reflect a subpopulation of persister *M.tuberculosis* present during human infection, bactericidal
372 activity against chronic murine TB may predict the clinical treatment shortening potential of
373 these compounds. It has been suggested that activities of compounds against non-replicating *M.*
374 *tuberculosis in vitro* may reflect their bactericidal activity against slowly or non-replicating
375 bacteria *in vivo;* it will be interesting to study whether the new compounds with the best
376 activity against *in vitro* non-replicating *M. tuberculosis*, also exhibit the best efficacy in a model
377 of chronic infection. Future studies will establish the dose of each compound required to
378 produce CFM-equivalent efficacy as monotherapy and as part of drug combinations, against
379 chronic TB in mouse and other animal models.

In addition to the enhanced efficacy of the new compounds compared to CFM, their decreased lipophilicity and half-lives suggest a lower potential to accumulate in and discolor tissues. We have previously observed that CFM treatment of mice leads to discoloration of their internal organs, ears and feet in a dose and duration dependent manner(28). The range of discoloration effects we observed following administration of 20 doses of each compound evaluated for efficacy indicates that it may be possible to discover CFM analogs with varied tissue accumulation and distribution properties while retaining or improving efficacy. However, it is intriguing that the compounds producing the least discoloration of mouse tissues following 20 doses (TBI-416 and TBI-450) do not have the lowest logP and half-life values of the compounds studied. This suggests a more complex relationship between the physicochemical and pharmacokinetic properties of these compounds and tissue discoloration in mice than we hypothesized. It remains important to investigate the distribution and discoloration caused by the new compounds versus CFM at the dose and for the duration of dosing required for efficacy equivalent to that of CFM when administered as part of a drug combination for experimental TB. Future experiments to quantitatively assess tissue distribution and skin coloration will be necessary to our understanding of this phenomenon and to our assessment of the potential for induction of skin coloration by the new compounds in the clinic.

In conclusion, we describe new riminophenazine analogs with enhanced *in vitro* potency against *M. tuberculosis.* The compounds have lower lipophilicities and shorter plasma half-lives compared to CFM, suggesting a lower propensity to accumulate, precipitate and discolor mammalian tissue. The nine compounds tested are active *in vivo* and therefore merit further

investigation as potential components of novel treatment-shortening regimens for drug sensitive and drug-resistant TB treatment.

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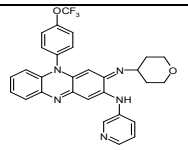
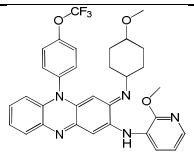
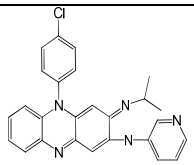
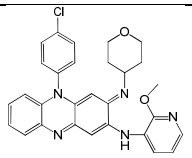
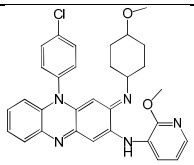
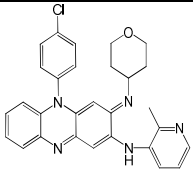
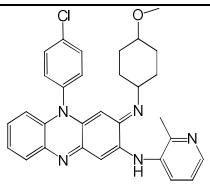
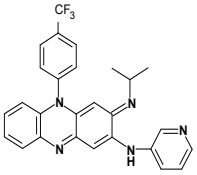
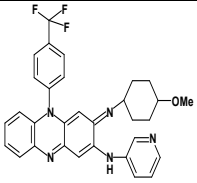
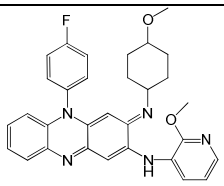
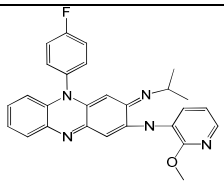
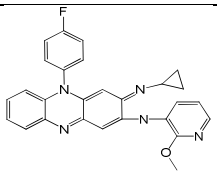
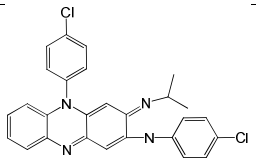
Table 1. Structure, logP, solubility, *in vitro* activity against *M. tuberculosis H37Rv* and selectivity of riminophenazine analogs

Compds	Structure	ClogP	LogP	Solubility (g/100ml)		MIC (μg/ml)	Vero IC50 (μg/ml)	SI[a]
				pH 1	pH 7			

TBI-161		5.50	4.30	1.18	Insol.*	0.03	>64	>2133
TBI-166		6.85	4.52	1.09	Insol.	0.016	>64	>4000
TBI-416		5.88	3.51	10.4	Insol.	0.03	0.54	18
TBI-443		5.47	4.15	1.00	Insol.	0.016	>64	>4000
TBI-444		6.50	3.74	1.80	Insol.	0.016	>64	>4000
TBI-449		5.17	4.81	1.10	Insol.	0.03	9.73	324
TBI-450		6.20	4.11	1.50	Insol.	0.016	50.88	3180

TBI-678		5.76	4.17	1.12	Insol.	0.03	19.66	655
TBI-688		6.04	4.29	1.04	Insol.	0.03	28.35	945
TBI-1002		5.93	4.82	0.20	Insol.	0.016	>64	>4000
TBI-1004		6.10	4.07	0.70	Insol.	0.038	>64	>1684
TBI-1010		5.85	4.37	1.00	Insol.	0.011	>64	>5818
CFM		7.7	5.34	Insol.	Insol.	0.12	68.62	572

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[a]SI = selectivity index IC_{50}/MIC (For IC50 values of >64 μg/ml, a value of 64 μg/ml was used for the SI calculation).*Limit of detection = 0.01g/100ml

Table 2. ***In vitro*** **activity of riminophenazine analogs against drug-sensitive clinical isolates of** ***M. tuberculosis***

compounds	MIC (μg/ml)		
	MIC range	MIC_{50}	MIC_{90}
TBI-161	0.014～0.07	0.029	0.057
TBI-166	0.014～0.085	0.057	0.061
TBI-416	0.015～0.059	0.047	0.058
TBI-443	0.015～0.086	0.057	0.062
TBI-444	0.015～0.063	0.058	0.061
TBI-449	0.023～0.120	0.06	0.11
TBI-450	0.015～0.062	0.054	0.057
TBI-678	0.015～0.080	0.055	0.073
TBI-688	0.015～0.062	0.029	0.059
TBI-1002	0.020～0.116	0.076	0.099
TBI-1004	0.015～0.119	0.082	0.109
TBI-1010	0.015～0.028	0.015	0.021
CFM	0.044～0.215	0.12	0.215
RIF	0.014～0.049	0.031	0.048
INH	0.020～0.090	0.046	0.048

MIC_{50} andMIC_{90} refer to MICs at which 50% and 90% of isolates are inhibited, respectively.

Table 3. ***In vitro*** **activity of riminophenazine analogs against drug-resistant clinical isolates of** ***M. tuberculosis***

compounds	MIC (μg/ml)		
	MIC range	MIC_{50}	MIC_{90}
TBI-161	0.029～0.081	0.052	0.054
TBI-166	0.027～0.095	0.058	0.080
TBI-416	0.025～0.076	0.037	0.060
TBI-443	0.030～0.061	0.059	0.060
TBI-444	0.031～0.060	0.056	0.060
TBI-449	0.050～0.114	0.082	0.104
TBI-450	0.031～0.062	0.051	0.062
TBI-678	0.050～0.092	0.061	0.081
TBI-688	0.030～0.089	0.051	0.063
TBI-1002	0.051～0.110	0.062	0.102
TBI-1004	0.058～0.121	0.094	0.112
TBI-1010	0.015～0.054	0.015	0.027
CFM	0.108～0.240	0.219	0.221
INH	1.0～40	20	40
RIF	1.0～40	20	40
LVFX	0.05～10	1	10

MIC_{50} andMIC_{90} refer to MICs at which 50% and 90% of isolates are inhibited, respectively.

The MIC of each compound was evaluated for the following 10 clinical isolates with varying drug resistance profiles, including both MDR and XDR strains：

118 resistant to INH SM RIFRPT PTO OFX LVFX

040 resistant to INH RIFPTO RPT OFX LVFX

002 resistant to INH RIFSM

004 resistant to SM RIFRPT KM OFX

057 resistant to RIFRPT OFX LVFX

054 resistant to INH RIFRPT

109 resistant to INH EMB RIFKM CAP PTO OFX LVFX RPT

062 resistant to INH RIFRPT KM PTO OFX

1104 resistant to INH RIFOFX

1109 resistant to INH RIFOFX

INH = isoniazid; RIF = rifampin; EMB = ethambutol; SM = streptomycin; RPT = rifapentine; OFX = ofloxacin; PAS = p-aminosalicylic acid; CAP = capreomycin; KM = kanamycin sulfate; PTO = protionamid; LVFX = levofloxacin

Table 4. Activity of riminophenazine analogs against Intracellular *M. tuberculosis H37Rv*

	Inhibition (%)*		
Compounds	1 μg/ml	0.5 μg/ml	0.25 μg/ml
TBI-161	99.0 ± 2.1	81.8 ± 3.6	54.7 ± 1.7

TBI-166	93.6 ± 4.6	58.1 ± 0.5	10.6 ± 1.1
TBI-416	100.0 ± 1.6	98.0 ± 5.2	92.5 ± 3.8
TBI-443	99.9 ± 2.4	100.1 ± 4.1	92.7 ± 0.7
TBI-444	99.6 ± 3.1	101.4 ± 6.4	98.2 ± 0.8
TBI-449	99.5 ± 2.4	94.7 ± 6.2	58.7 ± 2.1
TBI-450	99.4±2.4	98±5.1	72.1±0.8
TBI-678	99.9 ± 1.1	99.2 ± 4.3	68.3 ± 4.7
TBI-688	99.7±3.0	65.1±2.1	61.5±2.7
TBI-1002	95.0 ± 5.8	96.2 ± 2.1	53.4 ± 0.4
TBI-1004	97.3 ± 5.2	77.2 ± 10.3	29.2 ± 3.1
TBI-1010	99.7 ± 3.4	100.2 ± 0.5	96.0 ± 1.2
CFM	99.5 ± 1.1	50.8 ± 5.3	7.3 ± 0.3
RIF	98.2 ± 3.3	98.8 ± 3.5	93.1 ± 3.9
INH	94.5 ± 2.7	89.5 ± 10.7	40.4 ± 1.4

*Variability in background fluorescence accounts for inhibition values >100%

Table 5. ***In vitro*** **activity of riminophenazine analogs against non-replicating** ***M. tuberculosis H37Rv***

Compound	MIC in LORA (µg/ml)

TBI-161	2.750
TBI-166	>25
TBI-416	7.440
TBI-443	>25
TBI-444	22.946
TBI-449	5.185
TBI-450	11.588
TBI-678	1.529
TBI-688	2.579
TBI-1002	>25
TBI-1004	6.950
TBI-1010	>25
CFM	1.209
RIF	0.980
INH	>25

Table 6. Pharmacokinetic parameters of clofazimine and riminophenazine analogs dosed orally in mice at 20 mg/kg

Compounds	T 1/2 (h)	Cmax (µg/ml)	AUC (ug.hr/ml)
TBI-161	5.54±1.31	1.55±0.61	20.72±4.53

TBI-166	41.25±10.23	1.30±0.77	24.06±8.48
TBI-416	8.90±4.28	0.94±0.55	13.50±5.29
TBI-443	6.62±3.26	2.01±1.16	33.09±10.81
TBI-444	7.00±2.44	3.01±0.97	43.86±14.31
TBI-449	9.56±1.91	2.30±0.86	32.67±6.45
TBI-450	16.08±8.53	1.56±0.73	22.09±9.18
TBI-678	12.97±7.51	0.66±0.47	11.17±5.86
TBI-688	9.23±5.23	1.33±0.28	18.44±10.27
TBI-1002	7.90±4.56	3.65±2.47	45.36±24.12
TBI-1004	17.5±8.45	3.02±1.57	52.42±14.28
TBI-1010	13.20±5.67	1.37±0.97	20.26±9.78
CFM	29.74±10.27	0.38±0.24	6.96±4.35

Table 7 Efficacy of CFM and new riminophenazines against *M. tuberculosis H37Rv*

20 days of treatment in BALB/c mice infected with *M. tuberculosis H37Rv.* (mean±SD)

Group/compounds	Dose(mg/kg)	logCFU/Lung
Untreated		8.53±0.32
TBI-166	20	4.66±0.19

TBI-416	20	3.83±0.27
TBI-443	20	5.13±0.20
TBI-444	20	4.71±0.56
TBI-449	20	3.87±0.33
TBI-450	20	3.64±0.28
TBI-1002	20	4.85±0.39
TBI-1004	20	4.28±0.37
TBI-1010	20	4.86±0.16
CFM	20	6.33±0.07
RIF	10	6.71±0.13

Note: On Day 3 there were 1.98 $\log_{10}$ CFU in the lungs of untreated mice. At the beginning of treatment, there were 3.17 ± 0.39 $\log_{10}$ CFU in the lungs of untreated mice.