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

Yu Lu,[1]* Meiqin Zheng,[1] Bin Wang,[1] Lei Fu,[1] Weijie Zhao,[1] Peng Li,[1] Jian Xu,[1] Hui Zhu,[1] Haixia Jin,[1] Dali Yin,[2] Haihong Huang,[2] Anna M. Upton,[3] and Zhenkun Ma[3]

Department of Pharmacology, Beijing Tuberculosis and Thoracic Tumor Research Institute, Beijing Chest Hospital, Capital Medical University, Beijing, China[1]; Department of Medicinal Chemistry, Institute of Materia Medica, Beijing, China[2]; and The Global Alliance for TB Drug Development, New York, New York[3]

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The global tuberculosis crisis urgently demands new, efficacious, orally available drugs with the potential to shorten and simplify the long and complex treatments for 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 leads to unwelcome skin discoloration. We recently conducted a systematic structure-activity study of more than 500 riminophenazine analogs for anti-*Mycobacterium tuberculosis* activity. We describe here the characteristics of 12 prioritized compounds in more detail. The new riminophenazine analogs demonstrated 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 of them were active against nonreplicating *M. tuberculosis*. Eleven of these more water-soluble riminophenazine analogs possess shorter half-lives than clofazimine when dosed orally to mice, suggesting that they may accumulate less. Most importantly, the nine compounds that progressed to efficacy testing demonstrated 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, along with their decreased potential for accumulation and therefore perhaps also for tissue discoloration, warrants further study.

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 multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains of *Mycobacterium 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 four-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.

* Corresponding author. Mailing address: Department of Pharmacology, Beijing Tuberculosis and Thoracic Tumor Research Institute, Beijing Chest Hospital, Capital Medical University, Machang 97, Tongzhou District, Beijing 101149, China. Phone: 8610 89509357. Fax: 8610 80505770. E-mail: luyu4876@hotmail.com.

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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 antimycobacterial compounds that may not have been fully exploited. One such compound is clofazimine (CFM), a riminophenazine and marketed antimycobacterial drug used since 1962 in the treatment of leprosy (16, 17) and, more recently, in the treatment of *Mycobacterium avium* complex infection (16) and MDR-TB (43). CFM, a redox active compound, with dye properties, was specifically designed as an antitubercular 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 drug-resistant strains (8, 29) and activity against *M. tuberculosis* replicating inside macrophages (44). Although activity against replicating and intracellular bacteria is desirable in an antitubercular drug, it is widely believed that subpopulations of nonreplicating 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* strains 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. tu-*

berculosis 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 rifampin (RIF) (10).

The efficacy of CFM was inconsistent in early *in vivo* models of TB, which is probably but not definitively due to interspecies variation in absorption (12); however, later studies have proven CFM and analogs to be as 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, has 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 (>70 days in humans) and the 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 antitubercular therapy, we systematically investigated the structure-activity relationship of more than 500 new CFM analogs for anti-*M. tuberculosis* activity (D. Yin and H. Huang, unpublished data). We report here the pharmacological properties of 12 prioritized compounds.

MATERIALS AND METHODS

Compounds. 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 (Yin and Huang, unpublished).

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

Determinations of solubility and logP. To determine the logP, water (2 ml) was added to 1 ml of a 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 high-pressure liquid chromatography (HPLC). The logP was calculated as follows: $\log P = \log ([\text{analyte}]_{\text{octan-1-ol}}/[\text{analyte}]_{\text{water}})$. To determine aqueous solubility, 10 mg of compound was dissolved in a pH 7.0 buffer solution and in a pH 1.0 buffer solution. Equilibration of test compound was achieved after 30 min by vigorous shaking for 30 s at a time over 5-min intervals at 20 ± 5°C.

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, respectively. 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 (catalog no. 271310) supplemented with 0.2% (vol/vol) glycerol, 0.05% Tween 80, and 10% (vol/vol) albumin-dextrose-catalase (BBL Middlebrook ADC Enrichment, catalog no. 212352) (7H9-ADC-TG). Cultures were centrifuged, washed twice, and then 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, NJ). *M. tuberculosis* (100 μl containing 2×10^5 CFU) was added, yielding a final testing volume of 200 μl. The plates were incubated at 37°C; on day 7 of incubation, 12.5 μl of 20% Tween 80 and 20 μl of Alamar blue were added to all wells. After incubation at 37°C for 16 to 24 h, the fluorescence was read at an excitation of 530 nm and an emission of 590 nm. The MIC was defined as the lowest concentration effecting a reduction in fluorescence of ≥90% relative to the mean of replicate bacterium-only controls. MICs against nonreplicating *M. tuberculosis* were determined using a low-oxygen-recovery assay (LORA) as described previously (10).

Cytotoxicity. Vero cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). The cells were incubated in a humidified atmosphere of 5% CO_2 at 37°C. Stocks of cells were cultured in 25-cm^2 tissue culture flasks and subcultured two to three times per week. Cytotoxicity testing was performed in a transparent 96-well microplate. Outer perimeter wells were filled with sterile water to prevent dehydration in experimental wells. The cells were incubated at 37°C under 5% CO_2 until confluent and then diluted with culture medium to 4×10^5 cells/ml. Threefold serial dilutions of the stock solutions resulted in final concentrations of 64 to 0.26 μg/ml in a final volume of 100 μl. After incubation at 37°C for 48 h, the medium was removed, and the monolayers were washed twice with 100 μl of warm Hanks balanced salt solution (HBSS). Warm medium (100 μl) and 10 μl of freshly made methyl-thiazolyldiphenyl-tetrazolium bromide (MTT) were added to each well, and then the plates were incubated for 4 h, after which the absorbance was determined at 492 nm.

Macrophage assay. J774A.1 cells were grown to confluence in 75-cm^2 cell culture flasks in Dulbecco modified Eagle medium (DMEM) containing 10% FBS. The cells were detached by using a cell scraper and centrifuged at 200 × *g* for 5 min at room temperature, and the pellet was resuspended to a final concentration of 3×10^5 to 6×10^5 cells/ml. Aliquots (1 ml) of cell suspension were distributed into 24-well plates (Corning/Costar), and the plates were incubated at 37°C in a 5% CO_2 incubator for 16 h. *M. tuberculosis* H37Rv frozen cultures were thawed, sonicated for 15 s, and diluted to a final concentration of 3×10^7 to 6×10^7 CFU/ml with DMEM, and 100 μl of the dilution was dispensed into each well to give multiplicity of infection of ~10 bacteria/cell. The plates were incubated at 37°C for 4 h to allow for phagocytosis. The supernatant was aspirated, and the cells were washed three times with DMEM without FBS to remove the extracellular mycobacteria. The contents of the wells were replaced with DMEM containing 1% FBS and different concentrations of the compounds (1.0, 0.5, and 0.25 μg/ml). Control wells received drug-free medium. The medium with or without drugs was replaced every day. After 3 days of incubation, monolayers were visually inspected under the microscope to ensure they remained intact, and then the medium was removed and the macrophages were lysed with 200 μl of 0.1% sodium dodecyl sulfate. After 10 min of incubation at 37°C, 800 μl of fresh medium was added. Portions (20 μl) of the lysates were inoculated into 96-well, black, clear-bottom microplates, and 7H9-ADC-TG was added, yielding a final testing volume of 200 μl. The plates were incubated at 37°C; on day 7 of incubation, 12.5 μl of 20% Tween 80 and 20 μl of Alamar blue were added to all wells. After incubation at 37°C for 16 to 24 h, the fluorescence of the wells was read at an excitation of 530 nm and an emission of 590 nm. Infected macrophages incubated in drug-free control wells were designated 100% for H37Rv viability, while 7H9 medium controls were used to determine background fluorescence. The intracellular mycobacterial growth inhibition activities of the compounds were calculated as the percent reduction in background-corrected fluorescence compared to the 100% H37Rv viability positive control fluorescence (Flu) as follows: $\%\ \text{inhibition} = [1 - (\text{Flu}_{\text{drug}} - \text{Flu}_{\text{background}})/(\text{Flu}_{\text{control}} - \text{Flu}_{\text{background}})] \times 100$.

Pharmacokinetics. Specific-pathogen-free (SPF) male BALB/c mice weighing 18 to 22 g were used with three mice per time point. Feed and water were freely given during acclimation with a 16-h 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 h after administration and centrifuged at 2,500 × *g* for 10 min to obtain serum which was then stored at −20°C. Portions (100 μl) of the serum was added to 200 μl of acetonitrile, and the resulting solution was vortex mixed for 2 min and then centrifuged at 2,500 × *g* for 10 min to remove protein. The supernatant was removed to an Eppendorf tube and centrifuged at 11,000 × *g* for 5 min. The supernatant was moved to a sample bottle, and 40 μl was injected for HPLC analysis (Waters 600 Controller, 2487 dual λ absorbance detector, 717 plus Autosampler and Waters Empower chromatogram worksta-

tion). The analytical column was a Waters SymmetryShield RP_{18} (3.9 by 150 mm, 5 μm). The mobile phase was prepared by mixing acetic sodium buffer solution and methanol in various proportions determined in an earlier experiment. The flow rate was 1 ml/min, with an λ_{max} of 495 nm. 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}), the elimination half-life, and the area under the concentration-time curve from 0 to 24 h (AUC_{0-24}). The PK data presented in Table 6 are reported as means ± standard deviations (SD).

Aerosol model of acute infection. SPF BALB/c male mice, weighing 18 to 20 g, were used in the present study. Mice were infected via aerosol with a suspension of 5×10^6 CFU of *M. tuberculosis* H37Rv/ml using a Glas-Col inhalation system to deposit 50 to 100 bacilli into the lungs of each animal. The course of infection was followed by plating homogenates of harvested organs (obtained from three animals) on 7H11 agar plates (7H11 plates containing 10% oleic acid-albumin-dextrose-catalase [OADC] enrichment and 50 μg of cycloheximide, 200 U of polymyxin B, 50 μg of carbenicillin, and 20 μg of trimethoprim/ml) and determining the CFU on days 3, 10, and 30 postinfection. The 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 days per week during the acute phase of infection from day 10 until day 30. Each treated group included five or six mice, while the control group, which received only CMC, was composed of seven to ten mice. Mice were sacrificed the day after the last day of treatment; the organ weights were determined, and the lungs were removed, homogenized, and serially diluted in 10-fold steps in HBSS. Portions (100 μl) were spread onto 7H11 agar in duplicate. The plates were incubated at 37°C for 2 to 3 weeks. The data are expressed as the $\log_{10}$ (and as the $\log_{10}$ 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. A Student *t* test was used to compare means between the test and control groups. A *P* value of ≤0.05 was considered significant.

RESULTS

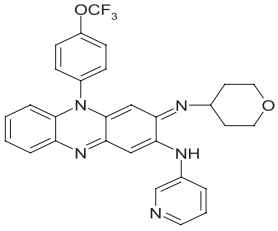
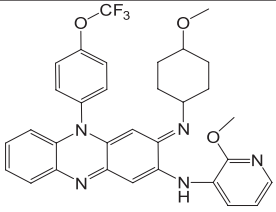
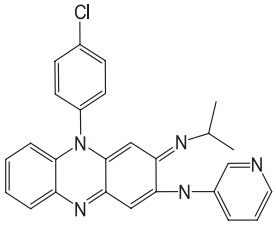
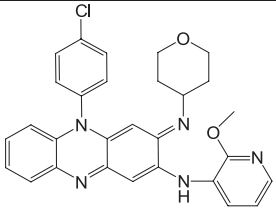
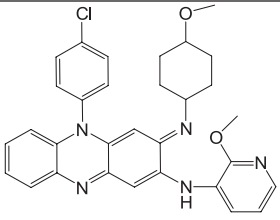
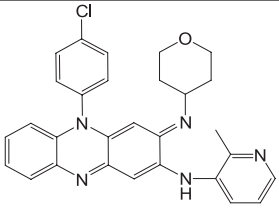
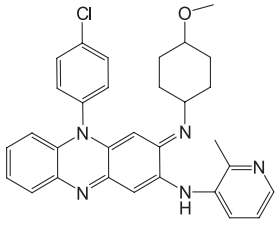
Riminophenazine analogs with enhanced potency compared to CFM against drug-sensitive and drug-resistant replicating *M. tuberculosis*. The 12 riminophenazine analogs possess MICs ranging from 0.011 to 0.038 μg/ml, with the most active exhibiting potency 10-fold better than CFM (MIC = 0.12 μg/ml). In addition, the compounds have acceptable cytotoxicity against Vero cells with selectivity indices ranging from 18 to >5,818 (Table 1). To further explore the *in vitro* activities of the compounds compared to CFM, MICs were determined using a 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 0.116 μg/ml, and most compounds are more potent than CFM (0.044 to 0.24 μg/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 to 0.24 μg/ml for CFM (Table 3). This result indicates the potential utility of these compounds against both MDR and XDR TB strains, including strains resistant to both fluoroquinolones and aminoglycosides. In addition, these data suggest a novel mode of action for this class compared to TB drugs in current use.

Riminophenazine analogs active against nonreplicating *M. tuberculosis* and intracellular *M. tuberculosis*. The activity of CFM against intracellular *M. tuberculosis* has been well documented (20, 37, 44). It is presumed to be related to the tendency of this antibacterial 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 by using a recovery assay. We compared the extracellular recovery of bacteria following 3 days of intracellular exposure to compounds at various concentrations. After 7 days of 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 ~1 $\log_{10}$ (6.07 ± 0.04 $\log_{10}$ CFU on day 0 and 7.16 ± 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 nonreplicating *M. tuberculosis* using the LORA assay. These bacteria may reflect a subpopulation 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.

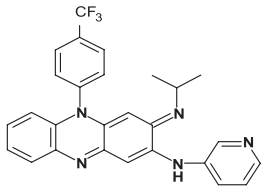
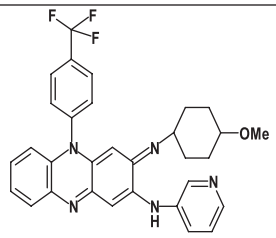
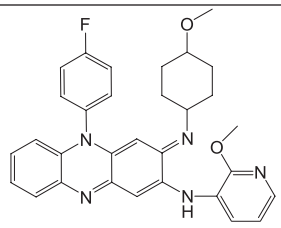
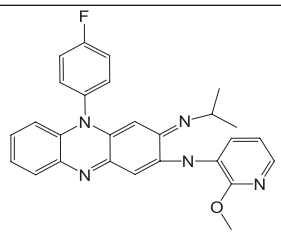
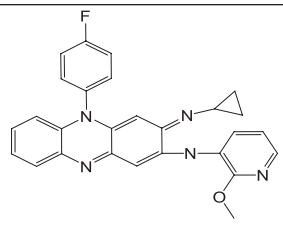
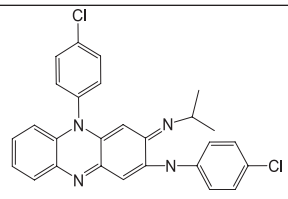
Less lipophilic riminophenazine analogs with decreased plasma half-lives compared to CFM. Riminophenazine analogs with lower lipophilicity and shorter half-lives compared to CFM can be expected to accumulate less in tissues, including those of the MPS. We hypothesize that a consequence of reduced tissue 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 the 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 to 4.82. No compound has aqueous solubility above the limit of detection (0.01 g/100 ml) at pH 7, but solubility at pH 1 is enhanced for the new compounds (ranging from 0.2 to 10.4 g/100 ml) 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 compared 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.

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

Compound	Structure	ClogP[a]	LogP[b]	Solubility (g/100 ml)[c]		MIC (μg/ml)	Vero cell IC_{50} (μg/ml)	SI[d]
				pH 1	pH 7			
TBI-161		5.50	4.30	1.18	I	0.03	>64	>2,133
TBI-166		6.85	4.52	1.09	I	0.016	>64	>4,000
TBI-416		5.88	3.51	10.4	I	0.03	0.54	18
TBI-443		5.47	4.15	1.00	I	0.016	>64	>4,000
TBI-444		6.50	3.74	1.80	I	0.016	>64	>4,000
TBI-449		5.17	4.81	1.10	I	0.03	9.73	324
TBI-450		6.20	4.11	1.50	I	0.016	50.88	3,180

Continued on following page

TABLE 1—*Continued*

Compound	Structure	ClogP[a]	LogP[b]	Solubility (g/100 ml)[c]		MIC (μg/ml)	Vero cell IC_{50} (μg/ml)	SI[d]
				pH 1	pH 7			
TBI-678		5.76	4.17	1.12	I	0.03	19.66	655
TBI-688		6.04	4.29	1.04	I	0.03	28.35	945
TBI-1002		5.93	4.82	0.20	I	0.016	>64	>4,000
TBI-1004		6.10	4.07	0.70	I	0.038	>64	>1,684
TBI-1010		5.85	4.37	1.00	I	0.011	>64	>5,818
CFM		7.7	5.34	I	I	0.12	68.62	572

[a] ClogP, calculated logP by computer.
[b] LogP, logarithm of the partition coefficient.
[c] I, insoluble (limit of detection = 0.01 g/100 ml).
[d] SI, selectivity index, calculated as the IC_{50}/MIC (for IC_{50} values of >64 μg/ml, a value of 64 μg/ml was used for the SI calculation).

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 three of the compounds (TBI-161, TBI-678, and TBI-688) have 50% lethal doses lower than 600 mg/kg. The remaining nine compounds were therefore prioritized for efficacy evaluation. The nine compounds demon-

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

Compound	MIC (μg/ml)[a]		
	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

[a] MIC_{50} and MIC_{90} refer to the MICs at which 50 and 90% of the isolates, respectively, are inhibited.

TABLE 4. Activity of riminophenazine analogs against intracellular *M. tuberculosis* H37Rv

Compound	Inhibition (%)[a] at:		
	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

[a] Variability in background fluorescence accounts for inhibition values of >100%.

strated 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 from 3.17 ± 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, the mean lung CFU counts for all riminophenazine-treated groups (including CFM) were 3 to 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 after 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 pretreatment 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). In the present study we observed discoloration of ears, internal organs, and fat as seen previously, following dosing of

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

Compound	MIC (μg/ml)[a]		
	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

[a] MIC_{50} and MIC_{90} refer to the MICs at which 50 and 90% of the isolates, respectively, are inhibited. The MIC of each compound was evaluated for the following 10 clinical isolates with varying drug resistance profiles, including both MDR and XDR strains: isolate 118, resistant to INH, SM, RIF, RPT, PTO, OFX, and LVFX; isolate 040, resistant to INH, RIF, PTO, RPT, OFX, and LVFX; isolate 002, resistant to INH, RIF, and SM; isolate 004, resistant to SM, RIF, RPT, KM, and OFX; isolate 057, resistant to RIF, RPT, OFX, and LVFX; isolate 054, resistant to INH, RIF, and RPT; isolate 109, resistant to INH, EMB, RIF, KM, CAP, PTO, OFX, LVFX, and RPT; isolate 062, resistant to INH, RIF, RPT, KM, PTO, and OFX; isolate 1104, resistant to INH, RIF, and OFX; and isolate 1109, resistant to INH, RIF, and OFX. Abbreviations: 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 5. *In vitro* activity of riminophenazine analogs against nonreplicating *M. tuberculosis* H37Rv

Compound	MIC (μg/ml)[a]
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

[a] As determined by a low-oxygen-recovery assay (LORA).

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

Compound	Mean ± SD		
	$t_{1/2}$ (h)	C_{max} (μg/ml)	AUC (μg · h/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

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

DISCUSSION

The riminophenazine CFM is one of very few compounds with demonstrated potential for use as an oral drug in the treatment of both drug-sensitive and drug-resistant TB. However, CFM, administered at 100 mg/day in the treatment of leprosy, induces well-documented and unwelcome discoloration of the patient's skin. This discoloration is evident within weeks of beginning treatment, fades months to years after the cessation of treatment, and occurs in a significant percentage of patients (16, 33, 42). CFM-induced skin discoloration has also been observed during treatment of *M. avium* complex and MDR TB infections, as well as during clinical trials to investigate the usefulness of CFM in inflammatory diseases, suggesting that this phenomenon is not leprosy specific (9, 16, 24, 43). The reports available suggest that the intensity, duration, and prevalence of skin discoloration are dependent on the dose and duration of treatment. Animal and clinical studies indicate that discoloration occurs in skin as well as in many other tissues, especially fat and organs of the MPS, via intracellular accumulation and crystallization of this bright red compound (15, 31, 32, 35, 41). CFM's 70-day half-life in humans and its lipophilic nature are the likely primary drivers of this side effect, as well as other dose- and duration-dependent side effects. Therefore, we have hypothesized that more water-soluble riminophenazine analogs with shorter half-lives may accumulate less and discolor tissues less. Further, more potent riminophenazine analogs that are as efficacious as CFM at a lower dose and/or possess rapidly sterilizing anti-*M. tuberculosis* efficacy may capture the potential efficacy benefit of CFM without the problematic discoloration side effect. Hence, we sought to discover riminophenazine analogs that have better *in vitro* potency than CFM, that demonstrate efficacy in a mouse model of TB, and that exhibit lower lipophilicity and shorter plasma half-lives compared to CFM.

TABLE 7. Efficacy of CFM and new riminophenazines against *M. tuberculosis* H37Rv[a]

Group or compound	Dose (mg/kg)	Log_{10} CFU/lung (mean ± SD)
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

[a] 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. Values were determined after 20 days of treatment in BALB/c mice infected with *M. tuberculosis* H37Rv.

Our *in vitro* activity studies reveal that all 12 compounds are more active than CFM against *M. tuberculosis* replicating in culture or inside macrophages, and most are active against low-oxygen-induced nonreplicating *M. tuberculosis*. However, there is a disconnect between the ranked activities of the new compounds and CFM against replicating and nonreplicating *M. tuberculosis in vitro*. This is particularly interesting in light of the apparently multifaceted antitubercular mode of action of CFM. The mode of action of CFM has been difficult to study due in part to the low frequency of resistance generation (38). Recent work suggests the bactericidal activity occurs via reactive oxygen species (ROS) production following the reduction of CFM by mycobacterial NADH quinone-oxidoreductase (46). This finding links CFM to both a pharmacologically validated *M. tuberculosis* target pathway, the electron transport chain (5), and the established mechanism of ROS-driven bactericidal action (22). The differences we noted between the rank order potencies of these analogs and CFM against extracellular replicating *M. tuberculosis* and low-oxygen-induced nonreplicating *M. tuberculosis* suggest a mode of action that emphasizes different mechanisms under different environmental conditions. Further complexity may be inherent in the activity of CFM against intracellular *M. tuberculosis*: interactions that may enhance macrophage-mediated, antimicrobial mechanisms have been observed for CFM (2, 3), suggesting that a combination of host-directed and antibacterial properties may affect growth inhibition of intracellular mycobacteria exposed to CFM. In addition, the clinically apparent anti-inflammatory properties of CFM, under investigation for the treatment of several autoimmune 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 nonreplicating bacteria have been preserved. Further 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 here confirms that the

nine compounds tested are active *in vivo*, at least against *M. tuberculosis* rapidly replicating within the lungs of mice. In the model presented, all nine new riminophenazine analogs demonstrate enhanced efficacy compared to CFM when administered for 20 days at 20 mg/kg in an acute murine TB model. However, simple inspection of the *in vivo* activities of the compounds compared to 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 the plasma half-lives of these compounds, from 5.54 to 41.25 h, may bias efficacy determinations following relatively short treatment periods toward those with shorter half-lives that reach steady state more quickly. Likewise, any correlation between tissue accumulation and efficacy for these riminophenazines would be difficult to detect over a 20-day course of treatment. Future studies evaluating the efficacy of these compounds over longer courses of treatment and at a range of doses are planned to permit clearer comparison of their efficacies and better understanding of the pharmacokinetic/pharmacodynamic drivers for this class.

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