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Antituberculosis Activities of Clofazimine and Its New Analogs B4154 and B4157

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In our efforts to develop new drugs for the treatment of tuberculosis, especially that caused by multidrug-resistant strains, we investigated clofazimine (CFM) and two of its analogs, B4154 and B4157, for their antituberculosis activities. Twenty *M. tuberculosis* strains were tested, including 16 drug-resistant strains (strains resistant to one or more antituberculosis drugs), for their susceptibilities to these three agents. All of the strains were found to be susceptible to B4154 and B4157, and one strain showed moderate resistance to CFM. The MICs of B4154, B4157, and CFM at which 90% of strains were inhibited were 0.25, 0.12, and ≤1.0 μg/ml, respectively. The intracellular activities of CFM and B4157 were superior to that of B4154. The chemotherapeutic activities of the three compounds were evaluated in C57BL/6 mice. At a dose of 20 mg/kg of body weight, the activity of CFM was slightly superior to that of B4157; however, both compounds prevented mortality and caused a significant reduction in the numbers of CFU in the lungs and spleens. The animals treated with B4157 showed less pigmentation than animals treated with CFM. The chemotherapeutic activity of CFM was comparable to those of rifampin and isoniazid. Complete susceptibility of multidrug-resistant strains to CFM and B4157 and the therapeutic efficacies of these compounds against mouse tuberculosis make these drugs attractive agents for the treatment of drug-resistant tuberculosis.

The increased incidence of tuberculosis caused by multidrug-resistant (MDR) strains throughout the world has created an urgent necessity for the discovery and development of new drugs for the treatment of such infections. In addition to searching for new agents, we are also investigating some drugs which were not fully exploited for various reasons. Clofazimine (CFM; also known as B663 or Lamprene) is one such example. It was developed initially as an antituberculosis drug (1–3). In the earlier experimental tuberculosis studies with hamster and mouse models, the drug was found to be effective; however, it showed less activity against tuberculosis in monkeys and guinea pigs. These inconsistencies were attributed to inadequate absorption of the drug in certain species of animals and the occurrence of low levels of the drug in the serum of animals in which the disease was considered extracellular (1, 4). Subsequently, on the basis of the fact that it concentrates extensively inside macrophages and on the basis of the logic of using an intracellular drug on an intracellular parasite, CFM has been used successfully for the treatment of leprosy. Earlier, in vitro and in vivo studies by us and others have demonstrated the activity of CFM and its analogs against *Mycobacterium avium* complex (MAC) strains (5, 6, 9, 12). Now CFM is being used for the treatment of MAC disease in AIDS patients.

Since tuberculosis and the more serious disease caused by MDR strains have recently emerged, we have sought to reestablish the chemotherapeutic roles of CFM and its analogs. Of the several riminophenazine compounds screened, only CFM, B746, B4100, B4101, B4154, and B4157 were found to show promising activity against tubercle bacilli. All five new analogs showed better in vitro activity than CFM; however, B4100 and B4101 were found to be less active in macrophage and animal models (10); consequently, they were not investigated further. We have previously described the chemotherapeutic activity of B746 against MAC and *Mycobacterium tuberculosis*, which was comparable to that of CFM (5, 7). In this report we describe the in vitro, intracellular, and in vivo activities of CFM and its two most active analogs, B4154 and B4157 (Fig. 1).

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MATERIALS AND METHODS

Mycobacteria. The *M. tuberculosis* strains used in the study consisted of susceptible strains as well as strains resistant to single or multiple drugs. Some of the strains were obtained from the Centers for Disease Control and Prevention, Atlanta, Ga., and the rest were from our hospital. The organisms were grown in 7H9 broth and were stored at −80°C in aliquots of 1.0 ml each. For each experiment, an aliquot was thawed and expanded in 7H9 broth, and organisms in the logarithmic phase of growth were used.

Drugs. CFM was obtained from CIBA-GEIGY, Basel, Switzerland, and its analogs were synthesized by J. F. O'Sullivan and colleagues at the Department of Chemistry, University of Dublin, Dublin, Ireland. For in vitro and intracellular studies, the compounds were dissolved in dimethyl sulfoxide and were stored in aliquots at −80°C; subsequent dilutions were made in distilled water. For in vivo studies, the compounds were emulsified in 0.5% methylcellulose–0.4% Tween 80. The drug suspensions were prepared every week and were stored at −10°C until use.

Macrophages and mice. The intracellular activities of the compounds were determined in the J774 A.1 mouse macrophage cell line. This cell line was obtained from the American Type Cell Collection and was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. Four- to 6-week-old C57BL/6 mice of either sex weighing 20 to 25 g were obtained from Jackson Laboratories, Bar Harbor, Maine. Mice were kept in microisolator cages, with five mice per cage.

Determination of MICs. MICs were determined by a radiometric (BACTEC) method as described elsewhere (6, 7). Briefly, the organisms were grown in 7H9 broth containing 10% oleic acid-albumin-dextrose (OADC) enrichment for 2 to 3 weeks, and the turbidity was adjusted to a no. 1 McFarland standard. Volumes of 0.1 ml of the different dilutions of compounds were placed into BACTEC 12B vials to reach final concentrations ranging from 0.06 to 4.0 μg/ml. All of the vials were inoculated with 0.1 ml of the *M. tuberculosis* suspension. In each test, two drug-free controls were included, one contained 0.1 ml of the bacterial suspension in the drug-containing vials and the other contained a 1/100 dilution of the same bacterial suspension. The vials were incubated at 37°C and were read in a BACTEC 460 reader (Becton Dickinson, Towson, Md.) daily until the growth index (GI) of the control vials diluted 1/100 reached ≥30 and there was a daily

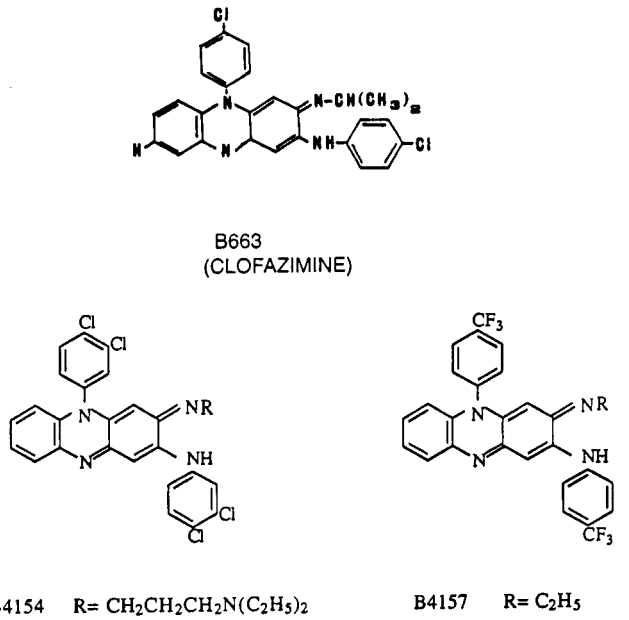

FIG. 1. Structures of CFM and its most promising analogs.

increase in the GI of ≥10 for 2 to 3 days. The MIC is defined as the lowest concentration of drug at which the increase in GI was equal to or less than that of the control diluted 1/100 (8).

Determination of intracellular activity. The mycobacterial suspension used to infect macrophages consisted of logarithmic-growth-phase *M. tuberculosis* H37Rv in 7H9 broth containing 10% OADC and 0.05% Tween 80. The growth was washed with saline three times by centrifuging at 5,000 × *g* (Sorvall RC-5B Centrige) for 10 min. After the final wash, the bacterial suspension in saline was allowed to stand at room temperature for 10 min so that the bacterial clumps could settle at the bottom. The smooth bacterial suspension was adjusted to a no. 1 McFarland standard, diluted 1/10 in DMEM containing 1% fetal calf serum, and used. Cytotoxicity was determined by exposing the macrophages to different concentrations (2.0 to 0.25 μg/ml) of the compounds. The intracellular activities of the drugs were studied as described earlier (7, 11). In brief, macrophages were scraped from culture plates and washed once, and viability was determined by trypan blue exclusion. Twenty-four-well tissue culture plates were seeded with 10^6 cells per well, and the plates were incubated for 2 h at 37°C in a CO_2 incubator. Nonadherent cells were washed off with warm Hanks' saline. The adherent monolayer of macrophages was infected with an *M. tuberculosis* H37Rv suspension in DMEM (about 10^7 CFU/ml) prepared as described above. After 2 h of incubation, the wells were washed three times with warm Hanks' saline and the contents of the wells were replaced with DMEM containing 1% fetal calf serum and different concentrations of the drugs (1.0, 0.5, and 0.25 μg of CFM and its analogs per ml and 0.1 μg of isoniazid per ml). Control wells received drug-free medium. The macrophage cultures were incubated at 37°C in a CO_2 incubator for 4 days. At days 0 and 4, the contents of triplicate wells in each group were lysed with 0.25% sodium dodecyl sulfate. Aliquots of the lysates were inoculated into BACTEC vials, and the results were read after 24 h (11).

Determination of in vivo activity. C57BL/6 mice were infected intravenously through a lateral caudal vein with mouse-passaged *M. tuberculosis* H37Rv in 0.2 ml containing approximately 10^6 CFU. On the next day, three mice were killed and the rest of the mice were divided into groups and dosed with different drugs for 8 to 12 weeks. CFM and its analogs were given at a dose of 20 mg/kg of body weight, isoniazid was given at a dose of 25 mg/kg, and rifampin was given at a dose of 25 mg/kg. Unless otherwise mentioned, the drugs were given 5 days a week and were not given on the day before the mice were killed. At 4, 8, and 12 weeks after treatment, three mice in each group were killed. Their lungs and spleens were collected aseptically and were homogenized in 10 ml of saline. The numbers of CFU in the homogenates were determined by plating 0.1 ml of three 10-fold dilutions onto 7H11 agar plates, and the numbers of CFU were counted after 3 weeks of incubation at 37°C and were expressed as the mean log number of CFU per gram of tissue (7).

Statistical analysis. The in vivo CFU data were statistically analyzed by using Student's *t* test.

RESULTS

In vitro activity. The MICs of CFM ranged from 0.06 to 2.0 μg/ml, whereas the MIC range of B4154 was ≤0.06 to 0.25 μg/ml and that of B4157 was ≤0.06 to 0.12 μg/ml (Table 1). The MICs of CFM at which 90 and 50% of the strains were inhibited (MIC_{90}s and MIC_{50}s) were ≤1.0 and ≤0.12 μg/ml, respectively; similarly, the MIC_{90} and MIC_{50} of B4154 were ≤0.25 and ≤0.12 μg/ml, respectively, and those of B4157 were ≤0.12 and ≤0.06 μg/ml, respectively, indicating the overall superior in vitro activities of the new analogs. However, the activity of B4154 was superior to that of CFM only against some strains tested. All of the drug-resistant strains were found to be susceptible (MICs, ≤1.0 μg/ml) to B4154 and B4157, and CFM had the highest level of activity against only one of the MDR strains, strain 2230 (MIC, 2.0 μg/ml).

TABLE 1. MICs of CFM and its analogs for *M. tuberculosis* strains[a]

M. tuberculosis strain	Susceptibility to antituberculosis drugs[b]	MIC (μg/ml)		
		CFM	B4154	B4157
H37Rv	Susceptible	0.12	0.12	≤0.06
14	Susceptible	0.12	0.12	≤0.06
2235	Susceptible	0.12	0.12	0.12
2237	Susceptible	0.12	0.25	≤0.06
2242	Susceptible	1.00	0.25	≤0.06
2257	Susceptible	0.25	0.25	≤0.06
1	MDR	0.12	0.12	≤0.06
2	RIF-R	0.25	0.12	0.06
9	RIF-R	0.50	0.25	≤0.06
11	EMB-R	1.00	0.12	≤0.06
29	MDR	0.25	≤0.06	≤0.06
33	RIF-R	0.12	0.06	≤0.06
35	INH-R	0.12	0.12	≤0.06
36	INH-R	0.12	0.12	≤0.06
37	RIF-R	0.12	0.25	≤0.06
2218	MDR	0.50	0.25	≤0.06
2219	MDR	0.12	0.25	≤0.06
2225	MDR	0.50	0.12	0.12
2227	MDR	0.06	0.25	0.12
2230	MDR	2.00	0.12	≤0.06

[a] MICs were determined by the radiometric (BACTEC) method.
[b] RIF, rifampin; INH, isoniazid; EMB, ethambutol; MDR, multidrug resistant; R, resistant.

Intracellular activity. All three compounds exerted good activity against *M. tuberculosis* in macrophages. Among the three compounds, B4154 was the least effective, and the activities of B4157 and CFM at 0.5 μg/ml were comparable to that of isoniazid at 0.1 μg/ml (Table 2).

TABLE 2. Intracellular activities of CFM analogs in J774A.1 macrophages

Compound	Concn (μg/ml)	GI (mean ± SD)
CFM	1.00	1.50 ± 0.70
	0.50	6.50 ± 2.10
	0.25	17.50 ± 2.10
B4154	1.00	6.00 ± 0.00
	0.50	18.00 ± 1.41
	0.25	33.00 ± 1.41
B4157	1.00	2.50 ± 0.7
	0.50	3.25 ± 1.41
	0.25	10.50 ± 0.70
Isoniazid	0.10	5.50 ± 0.70
Control		89.00 ± 1.41

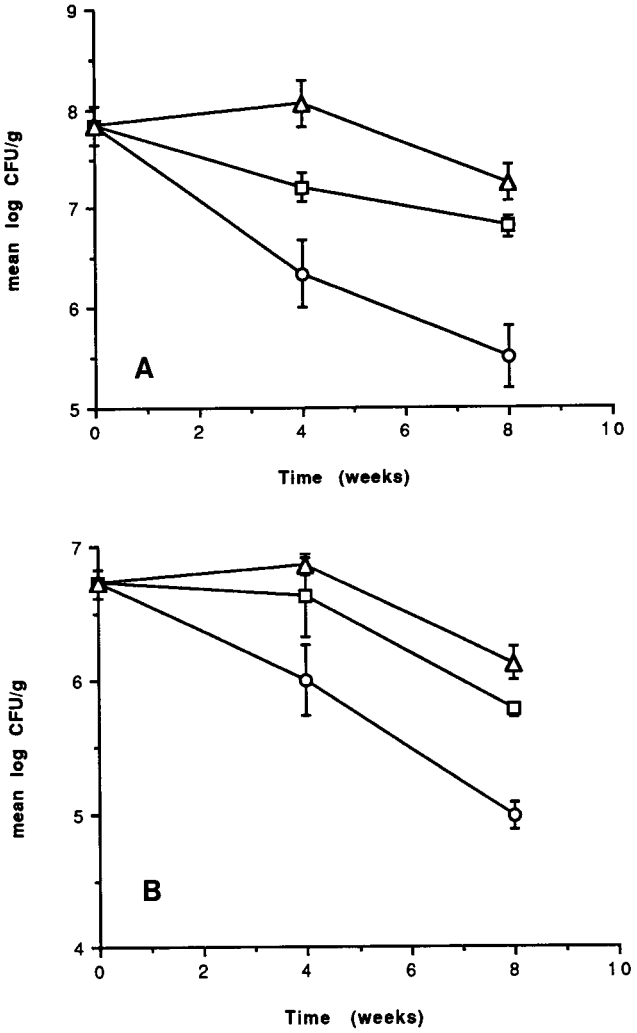

FIG. 2. In vivo activity of CFM in relation to frequency of treatment. Mice infected with *M. tuberculosis* H37Rv were treated with CFM at a dose of 20 mg/kg 5 days a week (△), twice a week (□), and once a week (○). The numbers of CFU in lungs (A) and spleens (B) were determined after 4 and 8 weeks of treatment.

In vivo activity. In the initial experiment, the chemotherapeutic activities of B4154 and B4157 were compared with that of CFM by using similar dosages (20 mg/kg, 5 days a week). All control mice died by the eighth week, and no mortality was observed in the drug-treated groups. Both CFM and B4157 caused a significant reduction in the numbers of CFU (more than 3 logs) in both lungs and spleens after 12 weeks of treatment (Table 3). The organisms were barely detectable at 12 weeks, the sensitivity of detection being about 3 log CFU/g of tissue. The activity of CFM seems to be slightly better than that of B4157 in lungs, with organisms being detectable in only one of three mice at 8 weeks and no organisms being detected at 12 weeks in all three mice. Despite having good in vitro activity, B4154 caused only a marginal reduction of 0.8 log CFU in the spleens, while there was an increase of 0.72 log CFU in the lungs. Because of its poor in vivo activity, the compound was not investigated further.

In the next experiment, CFM and B4157 were compared with isoniazid and rifampin (Table 4). All control mice died by the 12th week, and only one mouse was available for CFU determination at the 8th week. None of the mice in any of the treated groups died. All four drugs caused a significant reduction in the number of organisms in both lungs and spleens as early as 4 weeks following treatment. By 12 weeks the organisms were not detected in the lungs of mice treated with isoniazid or the spleens of mice treated with rifampin. In mice treated with CFM and B4157, the numbers of CFU were just above the threshold levels of detection in both organs; moreover, organisms were not detected in one or two mice in each group. Thus, the overall CFU data indicate that the in vivo activities of CFM and B4157 are comparable to those of isoniazid and rifampin. Even though the activities of CFM and B4157 were comparable, the internal organs of mice treated with B4157 showed less pigmentation than those treated with CFM.

The most outstanding feature of CFM is its long half-life (65 days) and its ability to concentrate in macrophages. We sought to determine whether these characters of the drug make it an ideal candidate for a regimen in intermittent chemotherapy. Groups of infected mice were treated with a 20-mg/kg dose of CFM 5 days a week, twice a week, and once a week. None of the mice in any of the groups died, including the mice treated once a week. In the lungs after 8 weeks, there was a 2.34-log reduction in CFUs with five doses a week, a 1.03-log reduction with two doses a week, and a 0.59-log reduction with one dose a week (Fig. 2). Similarly, in the spleens, there was a reduction of 1.73, 0.94, and 0.60 log with five, two, and one dose a week, respectively. Even though dosing for 5 days a week was optimal, dosing twice a week still brought about a 1-log reduction in the numbers of CFU in both organs.

DISCUSSION

Riminophenazine compounds, a group of compounds to which CFM belongs, were developed by Barry et al. (1–3) to be used specifically for the treatment of tuberculosis. Of the several compounds that they synthesized, B663 (CFM) was found to be the most active. Because of some early setbacks, the full potential of the drug was not exploited until its usefulness against leprosy and *M. avium* infections was recognized. The activities of CFM and its analogs against *M. avium* have been thoroughly studied in in vitro, macrophage, and animal models

TABLE 3. In vivo activities of CFM and its analogs against *M. tuberculosis* H37Rv in C57BL/6 mice

Drug	Organ	Log CFU/g of tissue (mean ± SD) at:			
		Day 1	Wk 1	Wk 8	Wk 12
CFM	Lungs		4.73 ± 0.63[a]	4.05[b]	0[c]
	Spleen		5.22 ± 0.02[c]	4.39 ± 0.18	3.63 ± 0.21
B4154	Lungs		7.49 ± 0.06	7.60 ± 0.15	7.27 ± 0.19
	Spleen		5.56 ± 0.87	6.00 ± 0.13	5.59 ± 0.63
B4157	Lungs		5.14 ± 0.88	4.25 ± 0.24	3.75 ± 0.41
	Spleen		4.51 ± 0.05[c]	3.75 ± 0.19	4.13[b]
Control	Lungs	6.55 ± 0.61	8.74 ± 0.95	—[e]	—
	Spleen	6.39 ± 0.11	6.87 ± 0.36	—	—

[a] $P < 0.05$. Statistical significance upon comparison with the numbers of CFU in controls on day 1.
[b] Data are for one mouse (organisms were not detected in the other two mice).
[c] 0 indicates that no CFU could be detected.
[d] $P < 0.001$ statistical significance upon comparison with the numbers of CFU in controls on day 1.
[e] —, all mice died.

TABLE 4. In vivo activities of CFM and B4157 in relation to those of rifampin and isoniazid against *M. tuberculosis* H37Rv in C57BL/6 mice

Drug (dose [mg])	Organ	Log CFU/g of tissue (mean ± SD) at:			
		Day 1	Wk 4	Wk 8	Wk 12
CFM (20)	Lungs		4.34 ± 0.20[a]	0[b]	3.49 ± 0.47[c]
	Spleen		5.09 ± 0.30[d]	4.07[e]	2.93 ± 0.18[c]
B4157 (20)	Lungs		5.67 ± 0.36[f]	4.38 ± 0.29	3.09 ± 0.14[c]
	Spleen		4.70 ± 0.28	4.06 ± 0.16[c]	2.97[e]
Rifampin (20)	Lungs		4.97 ± 0.03[a]	5.48 ± 0.01[c]	3.16[e]
	Spleen		3.65 ± 0.01[a,c]	0	0
Isoniazid (25)	Lungs		4.82 ± 0.10[a]	4.07 ± 0.45	0
	Spleen		4.91 ± 0.22[a]	5.11 ± 0.23	3.79 ± 0.15
Controls	Lungs	6.79 ± 0.11	8.35 ± 0.19	8.76[g]	—[h]
	Spleen	6.51 ± 0.12	7.05 ± 0.22	7.50[g]	—

[a] $P < 0.001$. Statistical significance upon comparison with the numbers of CFU in controls on day 1.
[b] 0 indicates that no CFU could be detected.
[c] Data are for two mice (organisms were not detected in the rest of the mice).
[d] $P < 0.01$. Statistical significance upon comparison with the numbers of CFU in controls on day 1.
[e] Data are for one mouse.
[f] $P < 0.05$. Statistical significance upon comparison with the numbers of CFU in controls on day 1.
[g] Data for one mouse (the rest of the mice died).
[h] All mice died.

by us and others (5, 6, 9, 12). However, the antituberculosis activities of these compounds in the context of the emergence of MDR strains has not been studied until recently. In addition to our attempts to rediscover the antituberculosis activity of CFM, we have been screening several new analogs and have identified some compounds with activity equal to or better than that of CFM and with reduced toxicity compared with that of CFM.

The most important feature with regard to the in vitro activity of CFM and its active analogs was that none of the *M. tuberculosis* strains including the MDR strains showed resistance to these drugs. For only one strain the MIC of CFM was 2.0 μg/ml. Even though the in vitro activities of B746 and B4157 were 4 and 8 times greater than that of CFM, respectively, their intracellular and chemotherapeutic activities in C57BL/6 mice were similar. Most importantly, the chemotherapeutic activities of CFM and B4157 were comparable to those of isoniazid and rifampin in the mouse model. Previously, we had shown that the chemotherapeutic activities of B746 and CFM were equal to that of isoniazid against virulent *M. tuberculosis* isolates (7). Thus repeated experiments have shown the promising activities of CFM, B746, and B4157.

One of the drawbacks of CFM is that it causes pigmentation of the skin. CFM accumulates in the reticuloendothelial organs and fatty tissues. In mice treated with B4157, pigmentation of internal organs was significantly less, as was the case with B746 (5, 7). Because of its unique nature of intracellular localization, the levels of CFM in serum are always low. For intracellular infections like tuberculosis, whether low levels in serum have any significance in the presence of high intracellular levels is not clear. However, in our pursuit of new riminophenazine compounds against *M. tuberculosis*, we are seeking to develop analogs with the least toxicity and increased activity and which produce increased levels in serum.

The fact that CFM is therapeutically effective against MAC infections, for which its MICs are 10-fold greater than that for *M. tuberculosis*, underscores its potential usefulness in the treatment of tuberculosis. Furthermore, the finding that all MDR strains are susceptible to CFM and its active analogs warrants examination of their usefulness for the treatment of tuberculosis caused by MDR strains as well. Moreover, the frequency of development of resistance to CFM is very low, and it has been demonstrated that CFM at sub-MICs prevented the emergence of isoniazid resistance in *M. tuberculosis* strains (3).

Treatment with CFM alone twice a week showed moderate activity; however, the activity of CFM or B4157 daily and intermittently in combination with other drugs should be evaluated against drug-susceptible and drug-resistant *M. tuberculosis* isolates. In the wake of the lack of new drugs against tuberculosis caused by MDR strains, we strongly feel that CFM, which is already being used extensively for the treatment of other mycobacterial infections, or its analogs (B746, B4157) should be explored in appropriate regimens for treating such disease.

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