Bactericidal Activity of the Nitroimidazopyran PA-824 in a Murine Model of Tuberculosis

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The nitroimidazopyran PA-824 has potent in vitro activity against *Mycobacterium tuberculosis*, a narrow spectrum of activity limited primarily to the *M. tuberculosis* complex, and no demonstrable cross-resistance to a variety of antituberculosis drugs. In a series of experiments, we sequentially characterized the activity of PA-824 in an experimental murine model of tuberculosis. The minimal effective dose was 12.5 mg/kg of body weight/day. The minimal bactericidal dose (MBD) was 100 mg/kg/day. When PA-824 was used as monotherapy at the MBD, it exhibited promising bactericidal activity during the initial intensive phase of therapy that was similar to that of the equipotent dose of isoniazid in humans. In combination with isoniazid, PA-824 prevented the selection of isoniazid-resistant mutants. Perhaps more importantly, PA-824 also demonstrated potent activity during the continuation phase of therapy, during which it targeted bacilli that had persisted through an initial 2-month intensive phase of treatment with rifampin, isoniazid, and pyrazinamide. Together, these data strongly support further evaluation of PA-824 in combination with first- or second-line antituberculosis drugs to determine its potential contribution to the treatment of drug-susceptible or multidrug-resistant tuberculosis, respectively.

A clear need exists for new antituberculosis drugs that have the potential to shorten the current duration of therapy for both active and latent tuberculosis (TB) and to improve the efficacies of regimens for the treatment of multidrug-resistant (MDR) TB (12). Yet, despite this clear need, no new class of drugs has been introduced into first-line usage since the rifamycins were introduced in the 1960s.

A series of nitroimidazofuran compounds with substantial antituberculosis activity was first described in 1993, but further development was halted over concerns that the compounds were mutagenic (1). Subsequent work gave rise to nitroimidazopyran derivatives that did not demonstrate genotoxicity but that retained potent antituberculosis activity that is highly specific for *Mycobacterium tuberculosis* via a novel mechanism of action (1, 13). The lead compound, PA-824, has an MIC of 0.015 to 0.25 $\mu\text{g/ml}$ against both susceptible and multidrug-resistant strains of *M. tuberculosis*. When PA-824 was compared to isoniazid at 25 mg/kg of body weight/day in a study with mice infected with a luciferase-producing mutant of *M. tuberculosis* H37Rv and by the use of relative light units from organ homogenates as a surrogate for determination of CFU counts, its activity was found to be similar to that of isoniazid when PA-824 was given at a dose of 25 mg/kg and greater than that of isoniazid when PA-824 was given at doses of 50 and 100 mg/kg. PA-824 was also shown to have activity against *M. tuberculosis* isolates that persisted under microaerophilic conditions in vitro, suggesting that it may have activity against latent or persisting *M. tuberculosis* isolates in vivo (13).

On the basis of these promising preliminary results, a series

of studies were undertaken with an experimental murine model to characterize the antituberculosis activity of PA-824. After measurement of its dose-response effect in mice, we compared the bactericidal activity of PA-824 to that of isoniazid during the 2-month initial phase of treatment using the standard methodology of quantitative organ CFU counts. We subsequently measured the activity of PA-824 during the continuation phase of treatment, in which tubercle bacilli persist in a state of limited metabolism following the 2-month initial phase of intensive combination chemotherapy. The bactericidal activity during this phase of therapy is often referred to as “sterilizing activity,” and it is this activity that ultimately determines the overall duration for which a treatment regimen must be given to successfully cure the patient.

MATERIALS AND METHODS

Antimicrobials. Moxifloxacin was provided by Bayer (Rolling Meadows, IL). Isoniazid and rifampin were purchased from Sigma (St. Louis, MO), and pyrazinamide was purchased from Fisher (Suwanee, GA). Stock solutions were prepared as described previously (14). PA-824 was provided as a pure powder by D. Rouse, Research Triangle Institute, Research Triangle Park, NC. For susceptibility testing and administration to mice, PA-824 was suspended in a cyclodextrin micelle (CM-2) formulation containing 10% hydroxypropyl- β -cyclodextrin (Sigma) and 10% lecithin (ICN Pharmaceuticals Inc., Aurora, OH). A 50-mg/ml suspension was prepared monthly. Dilutions in distilled water were made weekly to give the desired concentrations. Suspensions used for oral administration (gavage) were shaken between doses to ensure uniform dosing.

***M. tuberculosis* strain.** Strain H37Rv was passaged twice in mice to ensure its virulence, kept as frozen stocks, and then subcultured in Middlebrook 7H9 broth (Fisher) supplemented with 10% oleic acid albumin dextrose catalase (OADC) (Difco, Detroit, MI) and 0.05% Tween 80 (Sigma), and used for aerosol infection when the optical density at 600 nm was 0.8 to 1.0. The MICs for this strain are as follows: rifampin, 0.25 $\mu\text{g/ml}$; isoniazid, 0.05 $\mu\text{g/ml}$; and moxifloxacin, 0.5 $\mu\text{g/ml}$, on 7H10 medium; and pyrazinamide, 10 $\mu\text{g/ml}$, on Löwenstein-Jensen medium (pH 5.5).

Susceptibility testing. The susceptibility of *M. tuberculosis* to PA-824 and isoniazid was determined by the agar proportion method on Middlebrook 7H10 agar supplemented with 10% OADC and 0.5% glycerol (Difco).

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TABLE 1. Scheme of experiment used to assess the bactericidal activity of PA-824 during the 2-month initial and 4-month continuation phases of therapy

Regimen ^a	No. of mice killed by time point					Total
	Day -13	Day 0	2 mo	4 mo	6 mo	
Untreated	6	6				12
2 mo RHZ			6			6
2 mo H			6			6
2 mo Pa			6			6
2 mo HPa			6			6
2 mo RHZ + 4 mo RH				6	6	12
2 mo RHZ + 4 mo Pa				6	6	12
at 50 mg/kg						
2 mo RHZ + 4 mo Pa				6	6	12
at 100 mg/kg						
2 mo RHZ + 4 mo Pa				6	6	12
at 200 mg/kg						
2 mo RHZ + 4 mo H				6	6	12
2 mo RHZ + 4 mo M				6	6	12
Total	6	6	24	36	36	108

^a Drugs were given at the following dosages five times per week: isoniazid (H), 25 mg/kg; rifampin (R), 10 mg/kg; pyrazinamide (Z), 150 mg/kg; PA-824 (Pa), 50, 100, or 200 mg/kg.

Aerosol infection. Four- to 6-week-old female BALB/c mice (Charles River, Wilmington, MA) were infected via aerosol in a Middlebrook inhalation exposure system (Glas-col Inc., Terre Haute, IN).

Determination of MED and MBD. The minimal effective dose (MED) is defined as the lowest dose able to prevent the development of gross lung lesions and splenomegaly (4). The minimal bactericidal dose (MBD) is defined as the lowest dose able to reduce the lung CFU counts by 99% compared to the counts in the controls pretreatment. To determine MED and MBD, aerosol-infected mice were randomized into treatment groups (five to six mice/group). Treatment began the day after infection and consisted of daily doses of PA-824 ranging from 3.125 to 200 mg/kg. Untreated mice served as negative controls, while positive control mice received isoniazid at daily doses ranging from 3 to 25 mg/kg. Treatments were given once daily by gavage (in 0.2 ml) five times/week for 4 weeks. Body weight was assessed weekly. The following outcomes were assessed at the time that the mice were killed: (i) gross appearance of lung lesions, (ii) spleen weight, and (iii) CFU counts from lung and spleen homogenates for comparison with the values for untreated and pretreatment controls. Serial dilutions of lung and spleen homogenates were plated in duplicate on 7H10-OADC-glycerol agar. Agar plates for lung specimens were supplemented with cycloheximide (50 µg/ml), polymyxin B (200 U/ml), carbenicillin (50 µg/ml), and trimethoprim (20 µg/ml) to prevent contamination (adapted from Mitchison et al. [8]). CFU counts were determined after incubation for 4 weeks at 37°C with 5% CO₂.

Activity during the 2-month initial phase. Aerosol-infected mice were randomized to receive one of the following five treatments (six mice/group): no treatment; isoniazid (25 mg/kg) alone; PA-824 (100 mg/kg) alone; the combination of isoniazid (25 mg/kg) and PA-824 (100 mg/kg); or the combination of rifampin (10 mg/kg), isoniazid (25 mg/kg) and pyrazinamide (150 mg/kg). The last combination is abbreviated RHZ (Table 1). Treatment began 20 days after infection and was administered once daily, 5 days/week, for 8 weeks. Rifampin was administered at least 1 h apart from the time of administration of the other drugs to avoid drug interactions (2, 3, 5). At the end of treatment, the mice were killed to determine lung and spleen CFU counts and, for mice treated with isoniazid alone, PA-824 alone, or the combination isoniazid plus PA-824, the proportion of drug-resistant mutants in the lung homogenates. The latter assessment was made by plating similar dilutions on 7H10 agar containing either isoniazid (0.2 µg/ml) or PA-824 (2 µg/ml). The proportion of resistant mutants was calculated as the ratio of the CFU count obtained on antibiotic-containing medium over that obtained on antibiotic-free plates.

Activity during the 4-month continuation phase. Aerosol-infected mice were treated with RHZ for 8 weeks before they were randomized (12 mice/group) to receive one of the following six treatments: isoniazid (25 mg/kg), moxifloxacin (100 mg/kg), PA-824 (50, 100, or 200 mg/kg), or the combination of rifampin (10 mg/kg) plus isoniazid (25 mg/kg; the regimen is abbreviated RH) as a positive

TABLE 2. Spleen weights after 28 days of treatment

Treatment (dose [mg/kg])	Mean spleen wt ± SD (mg)	
	Day 1	Day 28
Untreated	85.8 ± 9.2	145.0 ± 20.7 ^a
PA-824 (3.125)		100.8 ± 14.3
PA-824 (6.25)		110.8 ± 16.9 ^a
PA-824 (12.5)		85.8 ± 5.0
PA-824 (25)		90.0 ± 11.0

^a Results significantly different from those for the control on day 1 ($P < 0.05$).

control. Treatment was administered daily, 5 days/week, for 16 weeks. The mice were killed after 8 and 16 weeks of treatment to determine lung and spleen CFU counts. The proportion of drug-resistant mutants was also determined in lung homogenates from mice treated with single-drug regimens.

Statistical analysis. Individual CFU counts were log transformed before analysis. Group mean spleen weights were compared with those for the pretreatment controls by one-way analysis of variance (ANOVA) with Dunnett's posttest. For CFU counts, multiple pairwise comparisons of group means were performed by one-way ANOVA with Bonferroni's posttest (GraphPad Prism, v.4; GraphPad Software, San Diego, CA).

RESULTS

Susceptibility of *M. tuberculosis* to PA-824. The MIC of PA-824 for *M. tuberculosis* H37Rv was determined to be 0.125 µg/ml.

MED and MBD of PA-824. Two consecutive experiments were performed. The first experiment used a PA-824 dose range of 12.5 to 200 mg/kg and established the MBD but could not confirm the MED. The second experiment used a lower PA-824 dose range of 3.125 to 25 mg/kg to confirm the MED. In both experiments, all groups of mice experienced weight gain over the course of the experiment. Overall, the cumulative weight gains were comparable for the isoniazid- and the PA-824-treated mice (data not shown). At the time that the mice were killed, untreated mice had enlarged lungs and many tubercles distributed over the lung surface. In the second experiment, mice treated with PA-824 at doses ≤6.25 mg/kg had visible lung lesions, while mice treated with isoniazid or PA-824 at doses ≥12.5 mg/kg did not. In addition, mice treated with PA-824 at doses as high as 6.25 mg/kg had enlarged spleens compared to the sizes of the spleens of the pretreatment controls ($P < 0.05$), while mice receiving ≥12.5 mg/kg did not (Table 2). Taken together, the data confirm that the MED of PA-824 is 12.5 mg/kg.

Lung CFU counts from the first experiment are presented in Fig. 1. At the MED, PA-824 had bacteriostatic activity, although a modest dissemination of the infection to the spleen (<10 CFU/spleen) was detected in 5 of 11 mice from the combined experiments (CFU range, 1 to 80 per spleen). No dissemination occurred at doses ≥25 mg/kg. The lowest dose required to reduce the lung CFU counts by 2 log₁₀ CFU (i.e., the MBD) was 100 mg/kg, eightfold higher than the MED. The 100-mg/kg dose was subsequently tested during the initial phase of therapy to confirm its bactericidal activity.

Activity of PA-824 during the initial phase. In the experiment performed to characterize the bactericidal activity of PA-824 during the initial phase of therapy, lung and spleen CFU counts at the initiation of treatment were 9.80 ± 0.14 and

FIG. 1. Log₁₀ CFU counts in lungs after 1 month of daily treatment with the indicated dose (in mg/kg) of PA-824 or isoniazid (INH). Arrows denote the MED and the MBD.

5.58 ± 0.44, respectively (Fig. 2). All untreated mice died within 2 months of infection.

Treatment with isoniazid or PA-824 alone for 8 weeks prevented death and reduced lung CFU counts by approximately 4 log units to 5.53 ± 0.17 or 6.02 ± 0.36 log₁₀, respectively, although isoniazid was slightly more active than PA-824 ($P < 0.05$). In mice treated with the combination isoniazid plus PA-824, the lung CFU counts were 5.83 ± 0.30 log₁₀, demonstrating that the combination has no additive or synergistic activity. The lung CFU counts were 3.94 ± 0.15 in mice treated with the combination RHZ, which was by far the most potent regimen ($P < 0.001$ versus the results for all other groups). As shown in Table 3, the spleen CFU counts in mice treated with isoniazid, PA-824, isoniazid plus PA-824, and RHZ for 2 months mirrored the CFU counts obtained in the lungs. No significant difference between mice treated with isoniazid or PA-824, alone or in combination, could be discerned. Treatment with RHZ, however, reduced the spleen CFU counts to a greater extent than the other regimens ($P < 0.001$).

Administration of either isoniazid or PA-824 alone in-

FIG. 2. Change in log₁₀ lung CFU counts after 2 months of treatment with isoniazid (H) and PA-824 (Pa), alone and in combination, compared to that following treatment with the standard initial-phase regimen of RHZ (HRZ).

TABLE 3. Spleen CFU counts and proportion of culture-positive spleens in infected mice after treatment with the indicated regimen

Regimen ^a	Log ₁₀ CFU count		No. of culture-positive spleens/total no. of spleens (CFU range)	
	0	2 mo	4 mo	6 mo
Untreated	5.58 ± 0.44			
2H		2.43 ± 0.24		
2Pa		2.97 ± 0.74		
2HPa		2.35 ± 0.24		
2RHZ and 4RH		1.43 ± 0.26	0/6	0/6
2RHZ and 4H			5/6 (1–5)	1/6 (1)
2RHZ and 4M			5/6 (1–10)	3/6 (1–600)
2RHZ and 4Pa			0/6	0/6
at 100 mg/kg				

^a H, isoniazid; Pa, PA-824; M, moxifloxacin; the numbers preceding the drug abbreviations indicate the number of months of treatment.

creased the proportion of drug-resistant mutants in the lungs (Table 4). When the drugs were given together, however, no significant selection occurred, further confirming the bactericidal activity of each drug.

Activity of PA-824 during the continuation phase. To determine the activity of PA-824 against the nonactively metabolizing bacilli that persist in the face of intensive chemotherapy, mice that had received RHZ for 2 months were allocated to receive up to 4 months of additional treatment with PA-824 (50, 100, or 200 mg/kg), isoniazid, moxifloxacin, or the combination RH (Table 1). Six mice per group were killed 2 and 4 months later to determine the extent to which each treatment was able to eradicate the persisting bacilli.

At the onset of the continuation phase, the mean log₁₀ CFU counts were 3.94 ± 0.15 and 1.43 ± 0.26 log₁₀ in the lungs and spleen, respectively. As shown in Fig. 3, the lung CFU counts for mice treated with moxifloxacin and isoniazid were 3.01 ± 0.17 and 2.50 ± 0.19 log₁₀, respectively, after 2 months and 2.48 ± 0.27 and 1.92 ± 0.21 log₁₀, respectively, after 4 months. Thus, although moxifloxacin and isoniazid alone had similar activities during the continuation phase, isoniazid was slightly more active ($P < 0.05$). PA-824 at a dose of 100 mg/kg exhibited activity that was significantly greater than that of isoniazid or moxifloxacin ($P < 0.01$), reducing the lung CFU counts to 1.37 ± 0.35 and 0.60 ± 0.36 log₁₀ after 2 and 4 months, respectively. The combination RH was more effective than moxifloxacin or isoniazid alone ($P < 0.001$), resulting in a reduction of the CFU count to 1.18 ± 0.35 log₁₀ after 2 months and complete culture-negative conversion after 4 months.

TABLE 4. Proportion of CFU resistant to isoniazid or PA-824 after 2 months of treatment with each drug alone or in combination

Regimen	Proportion of CFU resistant to:	
	Isoniazid (0.2 µg/ml)	PA-824 (2 µg/ml)
No treatment ^a	1.3 × 10 ⁻⁶	9.0 × 10 ⁻⁷
Isoniazid alone	2.5 × 10 ⁻⁴	
PA-824		3.8 × 10 ⁻³
Isoniazid + PA-824	<5.0 × 10 ⁻⁶	5.0 × 10 ⁻⁶

^a Data from Stover et al. (13).

FIG. 3. Change in lung $\log_{10}$ CFU counts after initial-phase treatment with RHZ for 2 months (2RHZ), followed by 4 months of treatment with isoniazid at 25 mg/kg (4H), moxifloxacin at 100 mg/kg (4M), PA-824 at 100 mg/kg (4Pa100), or rifampin plus isoniazid (4RH).

However, there was no statistically significant difference between RH activity and PA-824 activity after 2 or 4 months.

The same hierarchy of activity was evident in the cultures of spleen homogenates (Table 3). All six spleens from mice treated with RH or with PA-824 100 mg/kg were culture negative after both 2 and 4 months of continuation-phase therapy. Five of six spleens from the mice treated with either isoniazid or moxifloxacin remained culture positive after 2 months, whereas one and three of six spleens from isoniazid and moxifloxacin-treated mice, respectively, were culture positive after 4 months.

PA-824 exhibited dose-dependent activity during the continuation phase (Table 5). After 4 months of continuation-phase therapy (i.e., after a total of 6 months of therapy), the mean $\log_{10}$ lung CFU count from mice treated with PA-824 at 50 mg/kg was 2.36 ± 0.32 . At the same time, none of the six mice treated with PA-824 at 100 mg/kg had negative lung cultures, but the $\log_{10}$ CFU counts were 0.60 ± 0.36 , about 2 $\log_{10}$ units lower. Finally, five of six mice treated with PA-824 at 200

mg/kg had negative lung cultures. The results of the spleen CFU counts reinforced the findings for the lungs (Table 5). At the 50-mg/kg dose of PA-824, one of six spleens was still culture positive, with a single colony, while all mice treated with 100 or 200 mg/kg of PA-824 had negative spleen cultures.

DISCUSSION

In this series of experiments, we have demonstrated that the nitroimidazopyran compound PA-824 has substantial bactericidal activity during both the initial and the continuation phases of treatment in an experimental murine model of TB. The MED and MBD were 12.5 mg/kg and 100 mg/kg, respectively. At a dose of 100 mg/kg/day, the bactericidal activity of PA-824 approaches that of isoniazid at the equipotent dosage of 25 mg/kg/day for humans. By measuring light production from recombinant tubercle bacilli expressing firefly luciferase in mouse organ homogenates, Stover et al. (13) previously concluded that PA-824 was as potent as isoniazid at a dose of 25 mg/kg. The difference between the results of our two studies may be explained by the fact that assays based on ATP-dependent light production may fail to fully distinguish bactericidal activity from bacteriostatic activity (6). For instance, reversible alterations in light production may accompany antibiotic-associated changes in metabolism that are independent of cell death, and the impact of such alterations on light production relative to that on bactericidal activity may differ between antibiotic classes (7).

No additive or synergistic activity could be demonstrated when PA-824 was administered together with isoniazid during the 2-month initial phase of treatment, whereas the coadministration of rifampin and pyrazinamide with isoniazid clearly conferred additive activity compared to that of isoniazid alone. Whether PA-824 has additive or synergistic activity in combination with rifampin, isoniazid, pyrazinamide, and/or moxifloxacin remains to be demonstrated. The lack of an additive effect of the combination isoniazid and PA-824 during the initial phase of treatment does not preclude synergistic activity during the continuation phase in combination with other drugs, including rifampin or moxifloxacin, since *in vitro* data suggest that PA-824 has activity against latent or persisting *M. tuberculosis* in microaerophilic conditions (13). Similarly, PA-824 also has the potential to contribute significantly to the second-line regimens used to treat MDR TB, due to the apparent lack of cross-resistance with other antituberculosis drugs.

Perhaps the most important finding of our study is that PA-824 has substantial activity against tubercle bacilli that persist in a cultivable state, despite 2 months of daily treatment with RHZ. It is the activity against this bacillary population that ultimately determines the duration of therapy necessary for a given regimen to sterilize tuberculous lesions and effect a stable cure of the host (4). In this respect, it is remarkable that, at a dose of 100 mg/kg, the activity of PA-824 was significantly greater than that of isoniazid or moxifloxacin and approached that of the combination of rifampin and isoniazid. One may hope that PA-824, perhaps in combination with rifampin, might accelerate the killing of persisting *M. tuberculosis* and permit a reduction in the duration of TB therapy.

The choice of dose size is a critical aspect of experimental

TABLE 5. CFU counts and proportion of culture-positive lungs and spleens after treatment with increasing doses of PA-824 during the continuation phase of therapy

Dose (mg/kg)	Organ ^a	At 4 mo		At 6 mo	
		No. of samples culture positive	Mean $\log_{10}$ CFU count ^b	No. of samples culture positive	Mean $\log_{10}$ CFU count ^b
50	Lung	6/6	2.06 ± 0.24	6/6	2.36 ± 0.32
	Spleen	1/6	0.01 ^c	1/6	0.16 ^d
100	Lung	6/6	1.37 ± 0.35	6/6	0.60 ± 0.36
	Spleen	0/6		0/6	
200	Lung	6/6	2.28 ± 0.19	1/6	0.50 ± 1.22
	Spleen	0/6		0/6	

^a At the initiation of the continuation phase (at 2 months), the mean $\log_{10}$ CFU counts were 3.94 ± 0.15 in the lungs and 1.43 ± 0.26 in the spleens.

^b All isolates were susceptible to PA-824 (2 mg/liter).

^c One colony was isolated.

^d Eight colonies were isolated.

chemotherapy studies. Unfortunately, in the case of novel entities such as PA-824 there are no human pharmacokinetic or toxicity data on which to base the dose selection. At a dose of 100 mg/kg/day, PA-824 has substantial bactericidal activity during both the initial and the continuation phases of TB chemotherapy in the mouse model. Because this activity is dose dependent and 100 mg/kg was the lowest dose at which significant bactericidal activity could be demonstrated (i.e., the MBD), the equipotent dose in humans may be an important target dose for use for exploration of the activity of PA-824 in early clinical studies. The potential contribution of the compound to first-line chemotherapy regimens may be limited if a similar target exposure cannot be obtained in humans. That said, the promising activity of PA-824 demonstrated in this series of studies warrants further evaluation in the mouse model in order to (i) test regimens that combine PA-824 with first-line antituberculosis drugs and moxifloxacin to determine whether it has such a potential to further shorten the duration of therapy (10, 11), (ii) test regimens that combine PA-824 with other drugs active against MDR TB, and (iii) test PA-824 in combination with moxifloxacin in a paucibacillary model of latent MDR TB infection (9).

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