

# Sterilizing Activities of Novel Combinations Lacking First- and Second-Line Drugs in a Murine Model of Tuberculosis

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**Novel oral regimens composed of new drugs with potent activity against *Mycobacterium tuberculosis* and no cross-resistance with existing agents are needed to shorten and simplify treatment for both drug-susceptible and drug-resistant tuberculosis. As part of a continuing effort to evaluate novel drug combinations for treatment-shortening potential in a murine model, we performed two long-term, relapse-based experiments. In the first experiment, several 3- and 4-drug combinations containing new agents currently in phase 2/3 trials (TMC207 [bedaquiline], PA-824 and PNU-100480 [sut-ezolid], and/or clofazimine) proved superior to the first-line regimen of rifampin, pyrazinamide, and isoniazid. TMC207 plus PNU-100480 was the most effective drug pair. In the second experiment, in which 3- and 4-drug combinations composed of TMC207 and pyrazinamide plus rifapentine, clofazimine, PNU-100480, or both rifapentine and clofazimine were evaluated, the rank order of drugs improving the sterilizing activity of TMC207 and pyrazinamide was as follows: rifapentine plus clofazimine  $\geq$  clofazimine  $\geq$  rifapentine  $>$  PNU-100480. The results revealed potential new building blocks for universally active short-course regimens for drug-resistant tuberculosis. The inclusion of pyrazinamide against susceptible isolates may shorten the duration of treatment further.**

Novel oral regimens composed mostly or entirely of new drugs with potent activity against *Mycobacterium tuberculosis* and no cross-resistance with existing agents are needed to shorten and simplify treatment for tuberculosis (TB), including multidrug-resistant and extensively drug-resistant tuberculosis (DR-TB). Several new drugs in clinical development have demonstrated the potential to shorten TB treatment in animal models (16, 23, 26, 27, 32, 44) and even in clinical trials (6, 11, 34). We recently reported promising results in a murine model of TB with a regimen comprised of two new drugs in clinical development, TMC207 (TMC) and PA-824, plus moxifloxacin (MXF), and this regimen was at least as sterilizing as the first-line regimen of rifampin (RIF), isoniazid (INH), and pyrazinamide (PZA) (36). Combinations of TMC plus MXF or TMC plus PA-824 with PZA had even greater treatment-shortening abilities, suggesting that a 4-drug combination of TMC plus PZA plus MXF plus PA-824 may offer potential as a shorter (i.e.,  $\leq 4$ -month) regimen against PZA-susceptible isolates yet still constitute an effective short-course regimen (i.e.,  $\leq 6$  months) against isolates resistant to PZA (36). However, because PZA and MXF are unlikely to be active against extensively DR-TB strains and both PZA and fluoroquinolone resistance are on the rise in DR-TB patients (1, 11, 12, 25), replacement of one or both of these agents with one or more new drugs having novel mechanisms of action may lead to short-course regimens active against virtually all forms of DR-TB. Additionally, PA-824 has shown some potential in antagonizing the initial bacterial kill when added to TMC or TMC plus PZA, although the impact on the sterilizing activities of TMC-containing combinations remains unclear (36, 42). Therefore, it remains to be demonstrated whether replacing or removing this drug could lead to shorter durations of treatment.

PNU-100480 (PNU) is a new oxazolidinone with more potent antituberculosis activity than linezolid (LZD) *in vitro*, *in ex vivo*

whole blood cultures, and in a murine model (3, 41, 43, 45). In mice, the addition of PNU, but not LZD, increased the sterilizing activity of RIF plus PZA plus INH (44). In whole blood cultures, PNU showed additive activity with TMC and PZA (42). As it has now advanced to a phase 2 early bactericidal activity trial in pulmonary TB patients, PNU deserves further study as a component of novel TB regimens.

Interest in the riminophenazine derivative clofazimine (CFZ) was rekindled by a recent report that described successful treatment of DR-TB with a standardized 9-month regimen that included CFZ (39). CFZ has long been known to exhibit bactericidal activity against *M. tuberculosis* in mice, while results in other animal models have been less impressive (29). We recently found that addition of CFZ increased the initial bacterial kill with TMC plus PZA in mice, but the authors were unable to confirm that it also increased the sterilizing activity of this regimen (36).

Here, we report the results of two long-term, relapse-based chemotherapy studies conducted in mice to determine the potential efficacies of additional novel drug regimens. The objective of the first experiment was to compare the efficacies of 3- and 4-drug combinations of TMC, PA-824, PNU, and CFZ to that of RIF plus PZA plus INH. The objective of the second experiment was to determine whether PNU, CFZ, and/or rifapentine (RPT) in-

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TABLE 1 Scheme of experiment 1 for evaluation of completely novel oral regimens

Regimen type and group	Drug combination <sup>a</sup>	No. of mice sacrificed/group at <sup>b</sup> :					
		D-13	D0	M1	M2 (+3)	M3 (+3)	M4 (+3)
Control regimens							
Untreated		6	6				
A	RIF + PZA + INH <sup>c</sup>			5	4	(15)	(15)
Test regimens							
B	TMC + PNU + CFZ + PA-824			5	4 (15)	(15)	(15)
C	TMC + PNU + CFZ			5	4 (15)	(15)	(15)
D	TMC + PNU + PA-824			5	4 (15)	(15)	(15)
E	TMC + CFZ + PA-824			5	4 (15)	(15)	(15)
F	PNU + CFZ + PA-824			5	4 (15)	(15)	(15)
Total (n = 321)		6	6	30	24 (75)	(90)	(90)

<sup>a</sup> Drug doses (in mg/kg) were as follows: RIF (10), PZA (150), INH (10), TMC (25), PNU (50), CFZ (20), PA-824 (50).

<sup>b</sup> Time points are shown in days (e.g., D-13, day -13; D0, day 0) or months (e.g., M2, 2 months) of treatment. The notation "(+3)" indicates that the mice were held for 3 months after the completion of treatment at the indicated time point. When values are reported only in parentheses, this indicates that all mice in the group were sacrificed after the additional holding period posttreatment.

<sup>c</sup> For the RIF + PZA + INH regimen, PZA was given for the first 2 months only.

creased the sterilizing activity of the TMC plus PZA combination. The results reveal new building blocks for novel oral regimens with the potential to shorten the treatment duration of both drug-susceptible TB and DR-TB.

## MATERIALS AND METHODS

**Mycobacterial strain.** *M. tuberculosis* H37Rv was mouse passaged, frozen in aliquots, and subcultured in Middlebrook 7H9 broth with 10% oleic acid–albumin–dextrose–catalase (OADC; Fisher, Pittsburgh, PA) and 0.05% Tween 80 prior to infection.

**Antimicrobials.** INH, RIF, PZA, RPT, MXF, TMC, PA-824, PNU, and CFZ were obtained and formulated for oral administration as previously described (20, 32, 36, 38, 45), except that in experiment 1, CFZ was formulated in the same acidified 20% hydroxypropyl- $\beta$ -cyclodextrin solution as TMC.

**PK of CFZ.** The single-dose and multidose pharmacokinetics (PK) of CFZ were determined in uninfected 6-week-old female BALB/c mice. The single-dose group received a single oral dose of CFZ at 20 mg/kg of body weight in 5% dimethyl sulfoxide solution. Three mice per time point were anesthetized with isoflurane and exsanguinated by cardiac puncture at 2, 4, 8, 15, 18, 25, 36, and 48 h after CFZ administration. The multidose group received CFZ at 20 mg/kg once daily, 5 days per week, for 4 weeks. Three mice per time point were sampled at 0.25, 2, and 72 h after the last dose of CFZ. Serum was harvested and frozen at  $-80^{\circ}\text{C}$  before being shipped to the Peloquin laboratory, where concentrations of CFZ were determined using a validated high-performance liquid chromatography assay with UV detection. The standard curve covered the range from 4.0 to 0.05  $\mu\text{g/ml}$ . Overall validation precision (the coefficient of variation) of the standard curves ranged from 1.06% (at 1  $\mu\text{g/ml}$ ) to 7.49% (at 0.1  $\mu\text{g/ml}$ ). Overall validation precision of quality control samples ranged from 3.70% (at 1.5  $\mu\text{g/ml}$ ) to 5.59% (at 0.75  $\mu\text{g/ml}$ ). The within-sample precision was 3.57% (at 0.9  $\mu\text{g/ml}$ ). Serum drug concentration data were entered into a WinNonlin worksheet (version 4.0 [2002]; Pharsight, Mountain View, CA) and analyzed using standard noncompartmental techniques in order to determine the relevant pharmacokinetic parameters.

**Aerosol infection with *M. tuberculosis*.** All animal procedures were approved by the Institutional Animal Care and Use Committee. Aerosol infections were performed as previously described (26). Briefly, 5- to 6-week-old female BALB/c mice (Charles River, Wilmington, MA) were infected with *M. tuberculosis* H37Rv, using an inhalation exposure system (Glas-Col, Terre Haute, IN) and a fresh log-phase broth culture (optical

density at 600 nm of 0.8 to 1.0) with the goal of implanting between 3.5 and 4.0  $\log_{10}$  CFU in the lungs of each mouse. Two mice from each of 3 (experiment 1) or 2 (experiment 2) aerosol infection runs were humanely killed 1 day after infection and on the day of treatment initiation (D0) to determine the number of bacteria implanted in the lungs and at the start of treatment, respectively.

**Chemotherapy.** Mice were block randomized by run to experimental arms, and treatment was initiated 14 days (experiment 1) or 17 days (experiment 2) after infection. Treatment was administered 5 days per week, by gavage. Drug doses (in mg/kg) were as follows: INH (10), RIF (10), PZA (150), RPT (10), MXF (100), TMC (25), PNU (50), CFZ (20), and PA-824 (50) (33, 36, 45). In experiment 1 (Table 1), control mice received 2 months of RIF plus PZA plus INH, followed by up to 2 months of RIF plus INH (group A). Test regimens were the novel 4-drug combination of TMC plus PNU plus CFZ plus PA-824 (group B) and every 3-drug combination of those 4 drugs (groups C to F). The experimental scheme, including the duration of treatment for each regimen, is shown in Table 1. TMC and CFZ were formulated and administered together for groups receiving both drugs. PA-824 or a sham treatment with the CM-2 vehicle was administered immediately after the dose of TMC and/or CFZ. PNU was administered at least 4 h later.

In experiment 2 (Table 2), control mice received RPT plus PZA plus MXF or TMC plus PZA for up to 10 weeks. Test regimens included TMC plus PZA with RPT, CFZ, PNU, or RPT plus CFZ administered for up to 8 weeks. The experimental scheme, including the duration of treatment for each regimen, is shown in Table 2.

**Assessment of treatment efficacy.** Efficacy was assessed on the basis of lung CFU counts at selected time points during treatment (a measure of bactericidal activity) and the proportion of mice with culture-positive relapse after treatment completion (a measure of sterilizing activity). Quantitative cultures of lung homogenates were performed in parallel on 7H11 agar enriched with OADC (basic agar) and on basic agar supplemented with 0.4% activated charcoal to reduce drug carryover effects (36). Plates were incubated for up to 42 days at  $37^{\circ}\text{C}$  before final CFU counts were determined. The proportion of mice with culture-positive relapse was determined by holding cohorts of 15 mice for 3 additional months after completion of treatment and then humanely killing them to determine the proportion with positive lung cultures, as defined by  $\geq 1$  CFU of *M. tuberculosis* detected after plating the entire lung homogenate onto five 7H11 plates. Four of the five plates were supplemented with 0.4% activated charcoal. The remaining plate without charcoal was used to assess the risk of carryover at the time of relapse assessment.

TABLE 2 Scheme for experiment 2 to determine whether RPT, CFZ, or PNU increases the sterilizing activity of TMC plus PZA

Drug regimen <sup>a</sup>	No. of mice sacrificed/group at time point <sup>b</sup> :					
	D-16	D0	W4 (+12)	W6 (+12)	W8 (+12)	W10 (+12)
Control regimens						
Untreated	4	4				
RPT + PZA + MXF					(15)	(15)
TMC + PZA				(15)	(15)	(15)
Test regimens						
TMC + PZA + RPT				(15)	(15)	
TMC + PZA + CFZ				(15)	(15)	
TMC + PZA + PNU				(15)	(15)	
TMC + PZA + RPT + CFZ			(15)	(15)		
Total ( <i>n</i> = 203)	4	4	(15)	(75)	(75)	(30)

<sup>a</sup> Drug doses (in mg/kg) were as follows: RPT (10), PZA (150), MXF (100), TMC (25), CFZ (20), PNU (50).

<sup>b</sup> Time points are shown in days (D-16, day -16; D0, day 0) or weeks (e.g., W4, 4 weeks) of treatment. The notation "(+12)" indicates that the mice were held for 12 weeks after the completion of treatment at the indicated time point. When values are reported only in parentheses, this indicates that all mice in the group were sacrificed after the additional holding period posttreatment.

**Drug susceptibility testing.** Colonies isolated from several mice relapsing after 10 weeks of treatment with TMC plus PZA in experiment 2 were pooled directly from agar plates, suspended in phosphate-buffered saline, and homogenized with glass beads. After settling, the supernatant was plated in serial dilutions on drug-free 7H11 agar or 7H11 supplemented with TMC at 0.06 µg/ml.

**Statistical analysis.** The area under the serum drug concentration-time curve (AUC) was calculated using the trapezoidal method. CFU counts (*x*) were log transformed as (*x*+1) before analysis, and group means were compared by one-way analysis of variance with Dunnett's posttest to control for multiple comparisons. Group relapse proportions were compared using Fisher's exact test, adjusting for multiple comparisons. Comparisons of test regimens to TMC plus PNU plus CFZ plus PA-824 (experiment 1) and TMC plus PZA (experiment 2) were performed as one-sided tests, except when TMC plus PNU plus CFZ was compared to TMC plus PNU plus CFZ plus PA-824 (due to the potential for antagonism of PA-824 on TMC-containing regimens [36]). GraphPad Prism version 4 (GraphPad, San Diego, CA) was used for all analyses. Use of 15 mice per group for relapse assessment provided greater-than-80% power to detect 40% differences in the relapse rate, after setting  $\alpha$  at 0.01, to adjust for up to 5 simultaneous two-sided comparisons. Smaller differences may not be meaningful in terms of shortening the duration of treatment.

## RESULTS

**Single-dose and multidose PK of CFZ.** The mean maximal serum drug concentration ( $C_{max}$ ) of  $0.38 \pm 0.09$  µg/ml ( $\pm$  standard

deviation [SD]), half-life of  $29.11 \pm 4.66$  h, and  $AUC_{0-48\text{ h}}$  of  $8.52 \pm 0.69$  µg · h/ml observed after a single dose are consistent with prior results in mice (22). Significant accumulation occurred with multiple doses, resulting in a weekly mean  $C_{max}$  and trough concentrations of  $0.78 \pm 0.09$  and  $0.47 \pm 0.10$  µg/ml, respectively, during the fourth week of dosing, consistent with average serum drug concentrations of 0.7 µg/ml in patients receiving 100 mg daily (40). These results indicated that a 20-mg/kg daily dose in mice is equivalent to a 100-mg daily dose in humans.

**Treatment efficacy in experiment 1. (i) Lung CFU counts during treatment.** The day after aerosol infection, the mean lung  $\log_{10}$  CFU count was  $3.54 \pm 0.52$ . By D0, the mean CFU count had increased to  $7.27 \pm 0.44$ . The lung CFU counts observed after 1 and 2 months of treatment are presented in Table 3. One month of RIF plus PZA plus INH treatment (group A) reduced the CFU count by 2.54  $\log_{10}$ , to  $4.73 \pm 0.29$ . By comparison, 1 month of TMC plus PNU plus CFZ plus PA-824 (group B) reduced the CFU count by 3.79  $\log_{10}$ , to  $3.48 \pm 0.57$ . Only TMC plus PNU plus CFZ with or without PA-824 (groups B and C) produced statistically significant reductions in CFU counts compared to RIF plus PZA plus INH ( $P < 0.05$ ). The efficacy of the 4-drug combination (group B) was not significantly different from that of any of the novel 3-drug combinations in groups C to F ( $P = 0.0683$ ).

With 2 months of treatment, RIF plus PZA plus INH (group A)

TABLE 3 Lung CFU counts assessed during treatment and relapse, assessed 3 months after treatment completion in experiment 1

Group	Drug regimen	Mean ( $\pm$ SD) $\log_{10}$ CFU count at <sup>a</sup> :				Proportion (%) relapsing after treatment for:		
		D-13	D0	M1	M2	2 mos	3 mos	4 mos
Untreated		$3.54 \pm 0.52$	$7.27 \pm 0.44$	ND	ND	ND	ND	ND
A	RIF + PZA + INH <sup>b</sup>			$4.73 \pm 0.29$	$3.04 \pm 0.27$	ND	15/15 (100)	9/14 (64)
B	TMC + PNU + CFZ + PA-824			$3.48 \pm 0.57$	$0.37 \pm 0.75$	14/15 (93)	2/15 (13)	1/15 (7)
C	TMC + PNU + CFZ			$3.37 \pm 0.74$	0	13/15 (87)	4/15 (27)	1/14 (7)
D	TMC + PNU + PA-824			$3.99 \pm 0.89$	$0.97 \pm 1.18$	15/15 (100)	6/14 (43)	0/15 (0)
E	TMC + CFZ + PA-824			$4.39 \pm 0.51$	$1.55 \pm 1.14$	15/15 (100)	9/15 (60)	5/15 (33)
F	PNU + CFZ + PA-824			$4.47 \pm 0.39$	$0.82 \pm 1.64$	15/15 (100)	15/15 (100)	15/15 (100)

<sup>a</sup> Time points are shown in days (e.g., D-13, day -13; D0, day 0) or months (e.g., M1, 1 month) of treatment. ND, not done.

<sup>b</sup> For the RIF + PZA + INH regimen, PZA was given for the first 2 months only.

reduced the mean CFU count by 4.23 log<sub>10</sub>, to 3.04 ± 0.27. TMC plus PNU plus PA-824 (group D) and PNU plus CFZ plus PA-824 (group F) were significantly more active than RIF plus PZA plus INH ( $P < 0.05$ ). However, the best results were again observed with TMC plus PNU plus CFZ with or without PA-824 (groups B and C) ( $P < 0.01$  versus RIF plus PZA plus INH) which, collectively, rendered seven of eight mice culture negative. The efficacy of the 4-drug combination (group B) was not significantly different from that of any of the 3-drug combinations (groups C to F) ( $P = 0.3620$ ).

**(ii) Relapse after treatment completion.** The relapse results are displayed in Table 3. Three mice (1 each from the RIF plus PZA plus INH, TMC plus PNU plus PA-824, and TMC plus PNU plus CFZ groups) died of unknown causes during the follow-up period, well after treatment completion. As death due to *M. tuberculosis* infection so soon after 2 or more months of effective treatment is not expected and these mice did not appear ill prior to death, we censored them from further analyses. Relapse occurred in nearly all mice receiving only 2 months of treatment with the test regimens. Only 1 or 2 of the 15 mice receiving TMC plus PNU plus CFZ with or without PA-824 (groups B and C) did not relapse. After 3 months of treatment, all mice receiving RIF plus PZA plus INH (group A) relapsed. Compared to this control group, all TMC-containing test regimens produced significantly lower relapse rates ( $P < 0.0005$  for TMC plus PNU plus CFZ with or without PA-824 [groups B and C];  $P < 0.005$  for TMC plus PNU plus PA-824 [group D];  $P < 0.05$  for TMC plus CFZ plus PA-824 [group E]). Only 2 (13%) of 15 mice receiving the 4-drug TMC plus PNU plus CFZ plus PA-824 regimen (group B) relapsed. Removal of TMC from this combination, i.e., PNU plus CFZ plus PA-824 (group F), significantly increased the relapse rate ( $P < 0.0005$ ), as did removal of PNU, resulting in the regimen of TMC plus CFZ plus PA-824 (group E) ( $P < 0.05$ ). Removal of CFZ for group D or PA-824 for group C did not significantly affect the relapse rates.

After 4 months of treatment, 9 of 14 mice (64.3%) receiving RIF plus PZA plus INH (group A) relapsed. Compared to this control group, all TMC- plus PNU-containing test regimens (groups B to D) produced significantly lower relapse rates ( $P < 0.05$  for TMC plus PNU plus CFZ with or without PA-824;  $P = 0.001$  for TMC plus PNU plus PA-824), whereas PNU plus CFZ plus PA-824 (group F) was significantly worse before ( $P = 0.0169$ ), but not after, adjusting for multiple comparisons. Only 1 of 15 mice (7%) receiving the 4-drug regimen of TMC plus PNU plus CFZ plus PA-824 (group B) relapsed. Removal of TMC from this combination in group F significantly increased the relapse rate ( $P < 0.0005$ ). Removal of PNU in group E increased the relapse rate by 26%, but the difference was not statistically significant.

**Treatment efficacy in experiment 2.** The day after aerosol infection, the mean lung log<sub>10</sub> CFU count (± SD) was 4.41 ± 0.09. By D0, the mean CFU count had increased to 8.33 ± 0.26. The relapse results are displayed in Table 4. Treatment with RPT plus PZA plus MXF for 8 and 10 weeks resulted in relapse in 7 (47%) and 2 (13%) of 15 mice, respectively. These findings are consistent with our prior results (31, 32), considering that the initial burden of infection was higher in the current experiment. TMC plus PZA alone for 6, 8, and 10 weeks resulted in relapse in 14 (93%), 10 (67%), and 8 (53%) of 15 mice. Resistance to TMC was not observed in any of the relapsing mice sampled after 10 weeks of treatment and 12 weeks of follow-up. Addition of CFZ to the

TABLE 4 Relapse rates assessed 3 months after treatment completion in experiment 2

Drug regimen	Proportion (%) relapsing after treatment for:			
	4 wks	6 wks	8 wks	10 wks
RPT + PZA + MXF	ND <sup>a</sup>	ND	7/15 (47)	2/15 (13)
TMC + PZA	ND	14/15 (93)	10/15 (67)	8/15 (53)
TMC + PZA + RPT	ND	5/15 (33)	0/15 (0)	ND
TMC + PZA + CFZ	ND	1/15 (7)	0/15 (0)	ND
TMC + PZA + PNU	ND	8/15 (53)	6/15 (40)	ND
TMC + PZA + RPT + CFZ	4/15 (27)	0/15 (0)	ND	ND

<sup>a</sup> ND, not done.

regimen with TMC plus PZA reduced the number of mice relapsing after 6 and 8 weeks of treatment to 1 (7%) and 0 (0%), respectively ( $P < 0.001$  and 0.0001 versus TMC plus PZA). Addition of RPT also increased the sterilizing activity of TMC plus PZA, reducing the number of mice relapsing after 6 and 8 weeks of treatment to 5 (33%) and 0 (0%), respectively ( $P < 0.005$  and 0.0001). Remarkably, the 4-drug combination of TMC plus PZA plus RPT plus CFZ prevented relapse in all but 27% of mice after just 4 weeks of treatment, a result which typically requires 20 to 24 weeks of treatment with the first-line regimen to produce similar cure rates at this level of infection (30, 36). Addition of PNU resulted in a more modest increase in the sterilizing activity of TMC plus PZA, reducing the number of mice relapsing after 6 and 8 weeks of treatment to 8 (53%) and 6 (40%), respectively. The 40% reduction in relapse compared to TMC plus PZA alone after 6 weeks of treatment was significant before ( $P = 0.0176$ ), but not after, adjusting for multiple comparisons. Treatment with TMC plus PZA plus CFZ was superior to treatment with TMC plus PZA plus PNU after 6 weeks ( $P < 0.05$ ) and 8 weeks ( $P = 0.0507$ ) of treatment.

**Tolerability of the regimens.** Other than the expected discoloration of the fatty tissue and internal organs of mice receiving CFZ, no untoward effects of treatment were observed.

## DISCUSSION

The most novel finding of the present study was that 3- and 4-drug combinations comprised of 3 new drugs in clinical development with or without CFZ (i.e., lacking any current first- or second-line TB drug) had greater sterilizing activities than the standard first-line regimen of RIF plus PZA plus INH. This is the first evidence from an *in vivo* model that such novel combinations may provide the basis for universally active short-course regimens capable of treating all varieties of drug-susceptible and drug-resistant TB, thus reinforcing ongoing efforts to accelerate the clinical evaluation and eventual approval of novel drug combinations containing two or more unapproved agents (35).

The design of experiment 1 permitted analysis of the sterilizing contribution of each individual agent to the 4-drug combination of TMC plus PNU plus CFZ plus PA-824. The weaker performance of PNU plus CFZ plus PA-824 relative to the other experimental groups indicated that TMC contributes the greatest activity to the 4-drug combination and provided important confirmation of the significant sterilizing activity of TMC in the absence of the synergistic TMC plus PZA combination (47). PNU was the second most important agent in this 4-drug combination, as indicated by the better performance of TMC plus PNU plus CFZ plus PA-824 than of TMC plus CFZ plus PA-824. We previously showed that a higher dose of PNU

added to the sterilizing activity of the regimen with RIF plus PZA plus INH (44).

Although the initial bactericidal effects of TMC plus PNU plus CFZ with or without PA-824 tended to be greater than that of TMC plus PNU plus PA-824, the differences in CFU counts were not statistically significant. Perhaps more importantly, there were no discernible differences in the sterilizing activities of these three regimens. Although adding either CFZ to the TMC plus PNU plus PA-824 combination or PA-824 to the TMC plus PNU plus CFZ combination did not increase the sterilizing activity of the respective 3-drug combinations, it is important to recognize that the contribution of an individual agent to the sterilizing activity of a combination is a function of various factors, including the individual interactions with each companion drug in the combination and the dose of each agent. For example, these data cannot be used to gauge the contribution of CFZ or PA-824 to the TMC plus PNU combination itself. We previously reported the significant contributions of PA-824 to the sterilizing activities of other drug combinations, including PA-824 plus MXF plus PZA (14, 23) and RIF plus PA-824 plus PZA (37). Importantly, while PA-824 did not add significant sterilizing activity to the TMC plus PNU plus CFZ combination, there was also not any indication of the antagonistic effect we previously observed on the initial bacterial kill when PA-824 was added to TMC, TMC plus PZA, or TMC plus CFZ (36). The absence of an antagonistic effect on the sterilizing activity of the TMC plus PNU plus CFZ combination observed in this study is significant because TMC and another nitroimidazole derivative which is believed to act through the same mechanism as PA-824, OPC-67683, may soon be the first new drugs to be approved for treatment of TB in over 40 years. Although there are currently no phase 2b or phase 3 trials planned to evaluate novel regimens containing these 2 drug classes, a recent phase 2a trial also suggested indifference rather than antagonism when TMC and PA-824 were combined (24). Because any new approved drug or drugs will be heavily used to treat recalcitrant drug-resistant cases, thereby risking the selection of mutants resistant to the new agents, it is imperative that the potential for combinations containing these 2 drug classes be examined more thoroughly. Our results suggest that combining TMC and one of the nitroimidazole agents may be worthwhile, if for no other reason than to restrict the emergence of resistance.

The primary objective of experiment 2 was to confirm whether the addition of RPT, CFZ, or PNU would increase the sterilizing activity of the TMC plus PZA combination. The results showed that CFZ had significant sterilizing activity, even exceeding that of RPT when added to TMC plus PZA. Indeed, addition of CFZ may still increase the sterilizing activity of the TMC plus PZA plus RPT combination. This is the first demonstration of the sterilizing activity of CFZ based on relapse in mice as an end point and provides further support for inclusion of CFZ in regimens to treat DR-TB (39). The fact that regimens containing TMC plus PZA plus CFZ prevented virtually all relapses after 6 weeks of treatment is especially remarkable given the high burden of infection present in experiment 2. In previous experiments in this model with a similar bacterial burden, relapse rates were 100% and 25% after 16 and 24 weeks, respectively, of treatment with the first-line RIF plus PZA plus INH regimen (30, 36). The mechanism of the additive effects observed when combining TMC, PZA, and CFZ is open to speculation. All 3 drugs may have additive effects through inhibition of ATP synthesis (4, 21, 46). This may further increase susceptibility

to PZA by preventing efflux of pyrazinoic acid or energy-dependent mechanisms to remedy intracellular acidification. An additive effect of combining PZA and CFZ appears to play a role in the activity of TMC plus PZA plus CFZ, since PNU was not as effective as CFZ when added to the TMC plus PZA combination, despite PNU being the more important companion to TMC in experiment 1, in which PZA was not included. Still, PNU has shown additive effects with PZA in mice (data not shown) and whole blood cultures (41) and did appear to modestly increase the sterilizing activity of TMC plus PZA in experiment 2.

Because resistance to PZA may be found in 50 to 90% of MDR-TB isolates in some settings (11, 18, 25, 28), the ideal novel regimen would not rely on this sterilizing drug. Comparison of the results with TMC-containing regimens in experiment 1 with those of the combinations of TMC plus PZA plus CFZ and of TMC plus PZA plus PNU in experiment 2 revealed that neither CFZ nor PNU can replace PZA and its synergistic activity in TMC-containing regimens. We recently observed a similar inability to replace PZA with MXF, PA-824, or even RPT in TMC-containing combinations (36). Yet, despite lacking PZA, combinations of TMC plus PNU with PA-824 and/or CFZ produced fewer relapses after 3 to 4 months of treatment than did the same duration of treatment with RIF plus PZA plus INH. Such combinations may represent important new backbones for short-course DR-TB regimens. We recently reported that the 4-drug combination of TMC plus PZA plus MXF plus PA-824 may offer potential as a shorter (i.e.,  $\leq 4$ -month) regimen against PZA-susceptible isolates (based on the superior activity of TMC plus PZA plus either MXF or PA-824) yet still constitute an effective short-course regimen (i.e.,  $\leq 6$  months) against isolates resistant to PZA (based on the activity of TMC plus PA-824 plus MXF) (36). The current experiments suggest that PNU with or without CFZ may be able to replace MXF in the case of fluoroquinolone resistance to further enhance the universal value of such regimens.

An important limitation of this work is that it involved only one strain of mouse and one strain of *M. tuberculosis*. Efforts to confirm these promising results in a second animal model and against a second strain of *M. tuberculosis* are warranted (9). The second model could include another mouse strain (e.g., C3HeB/FeJ mice), guinea pigs, or larger species, which all exhibit more human-like pathology (2, 7, 15), or the intravenous inoculation mouse model, which is known to produce more conservative estimates of treatment durations needed to prevent relapse (8, 10). Use of a model with more human-like pathology may be preferable, because CFZ is concentrated intracellularly and the infecting bacilli in commonly used mouse strains (e.g., BALB/c) are virtually entirely intracellular. On the other hand, the tissue necrosis that occurs in C3HeB/FeJ mice and guinea pigs ensures that a majority of bacilli are extracellular (7, 15), as in human cavitary disease (13), where they may encounter lower CFZ concentrations than intracellular bacilli. The second *M. tuberculosis* strain could be a clinical isolate, such as CDC1551 or HN878.

Another limitation of the study was the risk of drug carryover caused by the significant accumulation and slow elimination of TMC and CFZ in the lungs of mice after repeated dosing (5, 17, 19). To reduce the risk of carryover when assessing lung CFU counts, we relied primarily on the addition of activated charcoal to the culture medium (36). However, there is no way to completely exclude carryover effects when few or no CFU are isolated, especially for samples containing high concentrations of CFZ. For this

reason, we relied upon relapse-based assessments of efficacy made at least 3 months after treatment discontinuation to allow time for serum and tissue drug concentrations to decrease and for regrowth of viable organisms. By comparing growth on culture plates with and without charcoal, we assessed the risk of drug carryover in lung homogenates obtained 3 months after treatment completion. Growth on all 4 charcoal-containing plates but not the single plain plate was observed for only 6 of the 130 relapses. In contrast, carryover effects were uniformly present when lung CFU counts were assessed at the completion of treatment for groups receiving TMC and/or CFZ. Therefore, although the risk of carryover still exists due to persistent tissue drug concentrations at the time of relapse assessment 3 months after treatment, the frequency and the magnitude of the observed effects are lower and are likely overcome with the use of charcoal-containing plates.

In conclusion, these experiments have demonstrated the potential for truly novel drug combinations containing TMC, PNU, PA-824, and CFZ to provide new foundations for universally active short-course regimens. For patients harboring isolates that remain susceptible to PZA, the inclusion of this bona fide sterilizing agent is expected to shorten treatment further. The promising results presented here should add impetus to ongoing efforts to accelerate the clinical evaluation and eventual approval of these and other potentially transformative regimens (35).

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