

# Activities of Drug Combinations against *Mycobacterium tuberculosis* Grown in Aerobic and Hypoxic Acidic Conditions

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*Mycobacterium tuberculosis* is exposed to hypoxia and acidity within granulomatous lesions. In this study, an acidic culture model of *M. tuberculosis* was used to test drug activity against aerobic 5-day-old (A5) and hypoxic 5-, 12-, and 19-day-old (H5, H12, and H19, respectively) bacilli after 7, 14, and 21 days of exposure. In A cultures, CFU and pH rapidly increased, while in H cultures growth stopped and pH increased slightly. Ten drugs were tested: rifampin (R), isoniazid (I), pyrazinamide (Z), ethambutol (E), moxifloxacin (MX), amikacin (AK), metronidazole (MZ), nitazoxanide (NZ), niclosamide (NC), and PA-824 (PA). Rifampin was the most active against A5, H5, H12, and H19 bacilli. Moxifloxacin and AK efficiently killed A5 and H5 cells, I was active mostly against A5 cells, Z was most active against H12 and H19 cells, and E showed low activity. Among nitrocompounds, NZ, NC, and PA were effective against A5, H5, H12, and H19 cells, while MZ was active against H12 and H19 cells. To kill all A and H cells, A5- and H5-active agents R, MX, and AK were used in combination with MZ, NZ, NC, or PA, in comparison with R-I-Z-E, currently used for human therapy. Mycobacterial viability was determined by CFU and a sensitive test in broth (day to positivity, MGIT 960 system). As shown by lack of regrowth in MGIT, the most potent combination was R-MX-AK-PA, which killed all A5, H5, H12, and H19 cells in 14 days. These observations demonstrate the sterilizing effect of drug combinations against cells of different *M. tuberculosis* stages grown in aerobic and hypoxic acidic conditions.

*Mycobacterium tuberculosis* is the causative agent of tuberculosis (TB), which kills about 2 million persons annually. Furthermore, 2 billion people are estimated to be latently infected with this organism, with 10% of them reactivating to an active lifetime disease.

Granulomatous lesions containing physiologic stages of *M. tuberculosis* ranging from actively replicating (AR) bacilli to dormant, nonreplicating (NR) bacilli coexist in the lungs of TB patients. Low oxygen pressure restricts the growth of aerobic to microaerophilic/anaerobic *M. tuberculosis* in the hypoxic core of solid and caseous granulomas, allowing bacilli to transit into a dormant state (1, 2). Current therapy for active TB, consisting of rifampin (R), isoniazid (I), pyrazinamide (Z), and ethambutol (E) for 2 months, followed by R-I for 4 months, results in killing of AR *M. tuberculosis* in few weeks, with moderate or no killing of NR bacilli. Therefore, a strategy to eliminate NR cells that survive current treatment needs to be developed to shorten therapy of active TB below 6 months and effectively reduce the reservoir of latently infected individuals (1, 3–5).

Several *in vitro* models to obtain NR *M. tuberculosis* have been developed over the years, based on reduced oxygen availability, nutrient starvation, standing cultures. One of the most popular methods is the Wayne model, in which dormant bacilli are obtained by gradual adaptation of stirred cultures of aerobic *M. tuberculosis* to anaerobiosis through the self-generated formation of an oxygen gradient (6, 7). By this and other models, it was shown that NR bacilli were insensitive to I, while being inhibited by R, Z, nitrocompounds (metronidazole [MZ], niclosamide [NC], nitazoxanide [NZ], and PA-824 [PA]), fluoroquinolones (e.g., moxifloxacin [MX]), aminoglycosides (e.g., amikacin [AK]), capreomycin (CP), and pyrazinamide (Z) (6, 8–12).

Few studies investigated *in vitro* activities of drug combinations against NR bacilli and the action of drugs against different growth stages of *M. tuberculosis* (12–14). Using the standard Wayne dormancy model at pH 6.6, we found that the combina-

tion of R with MX did not kill a small number of bacteria that had survived under aerobic and hypoxic conditions, while the addition to R-MX of a protein synthesis inhibitor (AK or CP) and a nitroimidazole active against anaerobes (MZ) killed all AR and NR bacilli (14). The recent observation that MZ prevents reactivation of latent *M. tuberculosis* infection in macaques (15) supports the need to find novel drugs or drug combinations targeting *M. tuberculosis* within hypoxic environments.

However, the Wayne model of hypoxia at pH 6.6 may not mimic the behavior of *M. tuberculosis* in active TB lesions, where due to inflammation, pH is estimated to be between 5.5 and 6 or lower (16, 17). Some support for this view comes from the knowledge that Z, a cornerstone drug active in the first 2 months of treatment, kills *M. tuberculosis* only at acidic pHs (16–19). Dormant bacilli may survive within granulomas in acidic and hypoxic microenvironments; thus, drug susceptibility tests of clinical isolates performed in neutral and aerobic media may not represent antibiotic activity against different populations within the lungs (20, 21). Examination of resistance levels of bacilli isolated from different lung sites is also difficult; therefore, measurement of the antibiotic sensitivity of AR and NR cells by *in vitro* models mimicking *in vivo* environments is an urgent need (1, 3, 5, 21).

The purpose of this study was to evaluate *in vitro* susceptibility of AR and NR bacilli to a panel of first- and second-line anti-TB drugs

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and nitrocompounds, alone and in combination, by using a modified Wayne model of hypoxia at pH 5.8 based on in vivo environments in which *M. tuberculosis* may reside. This approach allowed us to find combinations more bactericidal than the one currently used for human therapy (R-I-Z-E) against both AR and NR *M. tuberculosis*.

## MATERIALS AND METHODS

**Growth of aerobic and hypoxic cultures of *M. tuberculosis*.** *M. tuberculosis* strain H37Rv was grown in 20- by 125-mm screw-cap tubes containing Dubos Tween-albumin (DTA) broth prepared from Dubos broth base and Dubos medium albumin (Difco, Detroit, MI) and stirred with 8-mm magnetic bars, as described by Wayne and coworkers (6, 7), but in the present study, the medium was acidified to pH 5.8.

For preparation of AR aerobic (A) cells, mid-log-phase cultures were diluted in DTA broth to about  $1 \times 10^6$  CFU/ml and transferred to tubes in 12-ml volumes. Tubes were incubated at 37°C with loosened screw caps and fast stirring (about 250 rpm) to produce a small vortex at the top surface of the liquid. For preparation of NR hypoxic (H) cells, mid-log-phase cultures were diluted up to a cell density of about  $1 \times 10^6$  CFU/ml and transferred to tubes in 16-ml volumes, but in this case to obtain anaerobic conditions, the caps were tightly screwed and tight rubber caps were put under the caps. Tubes were incubated with slow stirring (about 120 rpm) at 37°C.

Mycobacterial growth was monitored for 40 days by measuring pH, optical density at 600 nm ( $OD_{600}$ ), and CFU/ml on Middlebrook 7H10 agar (Difco) plates incubated at 37°C under 5%  $CO_2$  for 3 weeks.

**Measurement of drug activity.** The activities of the first-line (R, I, Z, and E) and second-line (MX and AK) anti-TB drugs and of nitrocompounds (MZ, NZ, NC, PA) against A and H cultures were tested. R, I, Z, E, AK, MZ, NZ, and NC were purchased from Sigma Chemicals (St. Louis, MO); MX and PA were obtained from Bayer (Milan, Italy) and the Global Alliance for TB Drug Development (New York, NY), respectively. Rifampin was dissolved in ethanol; I, Z, E, MX, AK, and MZ were dissolved in distilled water; NZ, NC, and PA were dissolved in dimethyl sulfoxide.

To determine drug activity, 5-day-old A cultures (A5) and 5-, 12-, and 19-day-old H cultures (H5, H12, and H19, respectively) were incubated with single drugs or drug combinations for 0, 7, 14, and 21 days. Drugs (100  $\mu$ l) were added to A5 cultures by micropipette and to H5, H12, and H19 cultures by syringe (13, 14). R, I, E, MX, AK, MZ, NZ, NC, and PA were used at their maximum drug concentration in serum ( $C_{max}$ ) at 8, 2, 4, 4, 8, 8, 10, 0.3, and 2  $\mu$ g/ml, respectively (9, 22–26). Pyrazinamide was used at 100  $\mu$ g/ml (19).

After incubation, 1 ml of A or H cultures was washed and resuspended in 1 ml of DTA broth, and 0.2 ml was inoculated in Middlebrook 7H10 agar for CFU determination and in liquid medium (Bactec MGIT 960 system; Becton Dickinson, Sparks, MD) for determination of the number of days to reach a growth unit of  $\geq 75$  (days to positivity [DTP]). *M. tuberculosis* killing was defined as lack of regrowth in MGIT tubes after >100 days (DTP, >100 days) (14).

Statistical significance was assessed by a two-tailed Student's *t* test. A *P* value of <0.05 was considered significant.

## RESULTS

**Growth of *M. tuberculosis* under aerobic and hypoxic acidic conditions.** Figure 1 shows the growth of A and H bacilli monitored for 40 days. In A cultures, CFU increased rapidly up to day 18, remained stable up to day 35, and then decreased;  $OD_{600}$  showed a similar trend until day 18 and then stabilized. CFU of H cells increased for the first 8 days, and then a slow decrease up to day 24 was seen, followed by a drop to day 40. The turbidity of H cells was identical to that of A cells until day 6, and then turbidity increased slightly and stabilized. The pH values are shown in the inset to Fig. 1. In A cultures, pH remained stable at 5.8 from day 0 to 6, increased to 7.1 up to day 35, and then stabilized. In H cul-

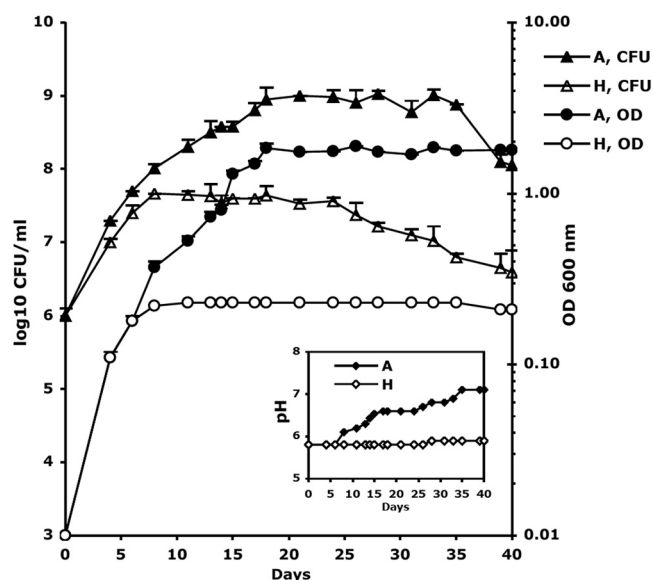


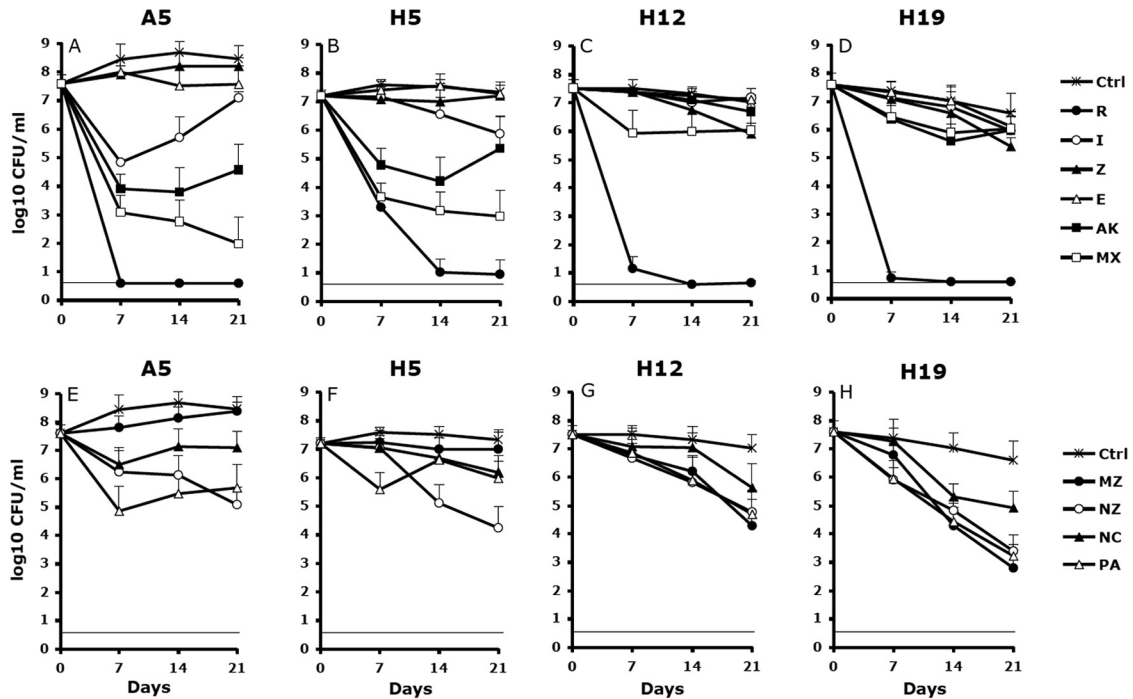
FIG 1 Growth of aerobic (A) and hypoxic (H) *M. tuberculosis* H37Rv in the acidic Wayne culture model at pH 5.8. Means and standard deviations of  $OD_{600}$  and  $\log_{10}$  CFU/ml from two experiments are shown. pH values are shown in the inset.

tures, pH did not change until day 26 and then increased to 5.9 from day 28 to 40.

**Activities of single drugs.** The activities of 10 drugs against A5, H5, H12, and H19 bacilli are shown in Fig. 2. Among first-line drugs, R was the most active against all four populations, with >7.8-, 4.3-, 6.4-, and 6.6- $\log_{10}$  decreases in A5, H5, H12 and H19 CFU, respectively, on day 7 and few or no CFU remaining on days 14 and 21 (Fig. 2A to D). Isoniazid was very active against A5 cells over the first 7 days (3.6- $\log_{10}$ -CFU reduction), and then bacteria regrew after  $\geq 14$  days; this drug had low activity but was significantly effective against H5 cells (1.5- $\log_{10}$ -CFU decrease on day 21,  $P = 0.015$ , in comparison with untreated control) and inactive against H12 and H19 cells. Pyrazinamide was inactive against A5 and H5 cells but showed a time-dependent activity against H12 and H19 cells (1- and 1.4- $\log_{10}$ -CFU reduction, respectively, on day 21;  $P = 0.034$  and 0.03, respectively). Ethambutol exhibited some activity only against A5 bacilli. Among second-line drugs, AK consistently killed A5 and H5 cells up to day 14 (4.9- and 3.3- $\log_{10}$ -CFU reduction, respectively), followed by regrowth, and showed a significant decrease of H19 cells on day 14 ( $P = 0.016$ ). MX efficiently killed A5 and H5 cells in a time-dependent manner (6.5- and 4.3- $\log_{10}$ -CFU reductions, respectively, on day 21), and exhibited some activity against H12 and H19 cells ( $P = 0.013$  and 0.06, respectively, on day 14).

As to the nitrocompounds (Fig. 2E to H), MZ was inactive against A5 and H5 cells, but its efficacy against H12 and H19 cells increased with hypoxia and exposure time (4- and 3.8- $\log_{10}$ -CFU decreases, respectively, on day 21). Unlike MZ, NZ showed a time-dependent activity against all four populations examined, with a reduction in the numbers of A5, H5, H12, and H19 cells of 3.4, 3.1, 2.2, and 3.2  $\log_{10}$  CFU, respectively, on day 21. Niclosamide killed all four populations to a lesser extent than NZ. PA-824 efficiently killed A5 and H5 cells up to day 7, followed by regrowth, and killed H12 and H19 cells to an extent similar to that of NZ and MZ.

To rapidly compare antibiotic activities shown in Fig. 2,  $\geq 2$ -



**FIG 2** Survival of A and H cultures of *M. tuberculosis* in the acidic Wayne model after 0, 7, 14, and 21 days of exposure to single drugs, as estimated by CFU counts. Five-day-old aerobic (A5) cultures and 5-, 12-, and 19-day-old hypoxic (H5, H12, and H19, respectively) cultures were incubated with drugs. Ctrl, control; R, rifampin; I, isoniazid; Z, pyrazinamide; E, ethambutol; AK, amikacin; MX, moxifloxacin; MZ, metronidazole; NZ, nitazoxanide; NC, niclosamide; PA, PA-824. The drug concentrations used were 8, 2, 100, 4, 8, 4, 8, 10, 0.3, and 2  $\mu\text{g/ml}$ , respectively. Dashed lines indicate the limit of detection (5 CFU/ml). Means and standard deviations from three experiments are shown.

$\log_{10}$ -CFU reductions are shown in Fig. 3 as black circles. Each cell population exhibited a different pattern of efficacy, with overall ranking of susceptibilities being A5 > H5 > H19 > H12. Major study results were as follows: (i) rifampin was the most active drug against A5, H5, H12, and H19 cells, (ii) AK and MX were very active against A5 cells and H5 cells, and (iii) NZ and PA were active against both A and H cells, albeit to a lesser extent than R.

**Activity of drug combinations.** On the basis of the single-drug results, we selected R, MX, and AK as potent anti-A5 and -H5 drugs to be used in combination with a fourth drug active against H12 and/or H19 cells, namely, MZ, NC, NZ, or PA.

Figure 4 shows the  $\log_{10}$  CFU/ml obtained after treatment of A5, H5, H12 and H19 cells with the combination R-I-Z-E, used for

human therapy, and with R-MX-AK, R-MX-AK-MZ, R-MX-AK-NZ, R-MX-AK-NC, or R-MX-AK-PA. In general, after 7 days of exposure, few or no colonies were found after treatment with all combinations; however, R-MX-AK-PA was the most active combination among those tested, with no A5, H5, H12, and H19 colonies being observed. No CFU were seen after  $\geq 14$  days of exposure to all combinations.

Since dormant *M. tuberculosis* may not form colonies on agar (13, 27), the samples for which results are shown in Fig. 4 were also inoculated in liquid medium (MGIT 960) in order to measure the DTP of surviving cells, a test providing a more sensitive measurement of viability than CFU (Table 1). After 7 days of exposure, no combination killed A5, H5, H12, and H19 bacilli (DTPs from 13 to 38 days); however, compared with the other combinations, R-MX-AK-PA was the most efficient at inhibiting them (DTPs of 38, 26, 20, and 24 mean days of regrowth, respectively). After 14 days of exposure, R-MX-AK-PA was the only combination killing all A5, H5, H12, and H19 cells inoculated in the MGIT tubes (DTP of > 100), while R-MX-AK-NZ killed H12 and H19 cells and R-MX-AK-MZ killed H19 cells only. Extending the exposure time to 21 days led all combinations, including R-MX-AK, to kill all A5, H5, H12, and H19 bacilli, with the exception of R-MX-AK-NC, which did not kill A5 cells and R-I-Z-E, which did not kill H5 cells.

**DISCUSSION**

The behavior of NR *M. tuberculosis* has been mostly studied in the Wayne model, which is based on the gradual oxygen depletion of the cultures. In addition to hypoxia, acidity is thought to be an important stress encountered by *M. tuberculosis* within granulomatous lesions

Drug	Growth stage and days of exposure											
	A5			H5			H12			H19		
	7	14	21	7	14	21	7	14	21	7	14	21
Rifampin	●	●	●	●	●	●	●	●	●	●	●	●
Isoniazid	●	●	○	○	○	○	○	○	○	○	○	○
Pyrazinamide	○	○	○	○	○	○	○	○	○	○	○	○
Ethambutol	○	○	○	○	○	○	○	○	○	○	○	○
Amikacin	●	●	●	●	●	●	○	○	○	○	○	○
Moxifloxacin	●	●	●	●	●	●	○	○	○	○	○	○
Metronidazole	○	○	○	○	○	○	○	○	●	○	●	●
Nitazoxanide	●	●	●	○	●	●	○	○	●	○	●	●
Niclosamide	○	○	○	○	○	○	○	○	○	○	○	○
PA-824	●	●	●	●	○	○	○	○	●	○	●	●

**FIG 3** Rapid comparison of antibiotic activities shown in Fig. 2, outlined as  $2\text{-}\log_{10}$ -CFU decreases. Symbols: ●,  $\geq 2\text{-}\log_{10}$ -CFU/ml decrease; ○,  $< 2\text{-}\log_{10}$ -CFU decrease.

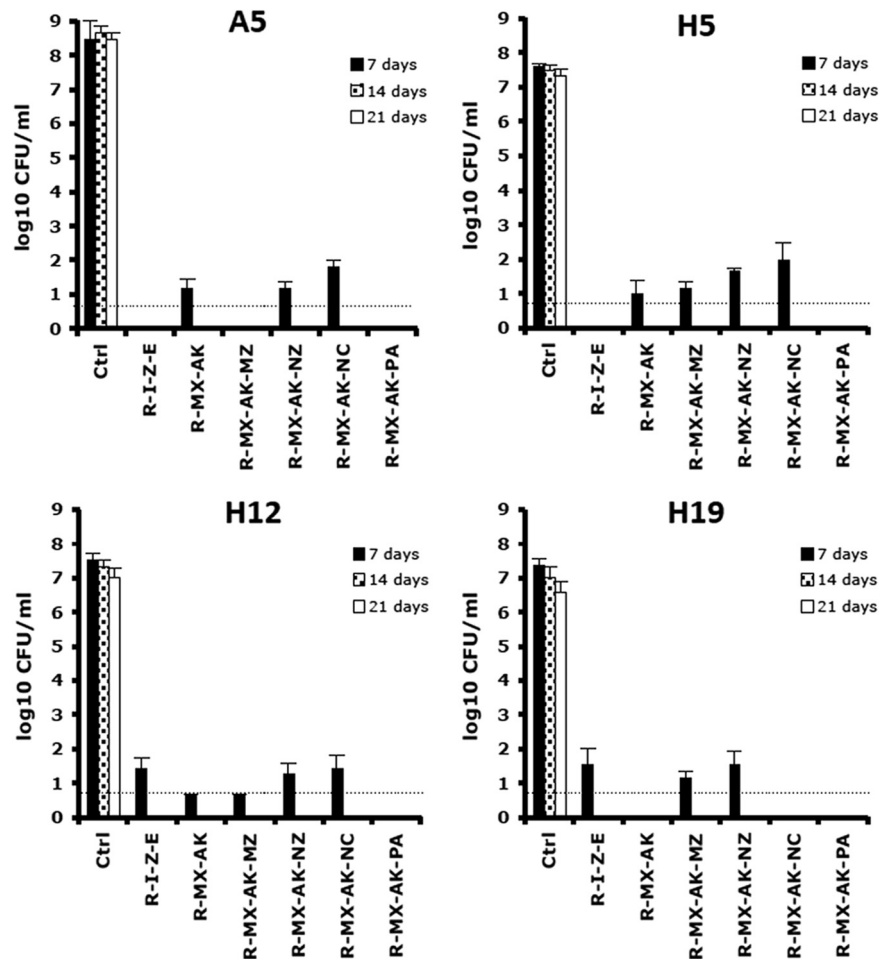


FIG 4 Survival of A and H cultures of *M. tuberculosis* in the acidic Wayne model after 7, 14, and 21 days of exposure to various drug combinations, shown by log<sub>10</sub> CFU/ml  $\pm$  standard deviation. Five-day-old aerobic (A5) cultures and 5-, 12-, and 19-day-old hypoxic (H5, H12, and H19, respectively) cultures were incubated with drugs. Ctrl, control; R, rifampin; I, isoniazid; Z, pyrazinamide; E, ethambutol; AK, amikacin; MX, moxifloxacin; MZ, metronidazole; NZ, nitazoxanide; NC, niclosamide; PA, PA-824. The drug concentrations used were 8, 2, 100, 4, 8, 4, 8, 10, 0.3, and 2  $\mu$ g/ml, respectively. Dashed lines indicate the limit of detection (5 CFU/ml).

and in the phagolysosomes of activated macrophages, while there is only limited information available regarding the pH of necrotic lesions and cavities (2, 16–18, 28). In this study, we measured the activity of single drugs and drug combinations in aerobic and hypoxic

acidic conditions to possibly mimic the environment of *M. tuberculosis* within granulomas and macrophages.

In aerobic conditions, *M. tuberculosis* efficiently multiplied and the pH rapidly increased, in keeping with the knowledge that

TABLE 1 Survival of A and H cultures of *M. tuberculosis* in the acidic Wayne model after 7, 14, and 21 days of exposure to various drug combinations, as estimated by regrowth in liquid medium by using the BACTEC MGIT 960 system<sup>a</sup>

Combination	DTP $\pm$ SD by days of exposure at growth stage shown <sup>b</sup> :											
	A5			H5			H12			H19		
	7	14	21	7	14	21	7	14	21	7	14	21
Control	2 $\pm$ 1	2 $\pm$ 1	2 $\pm$ 1	3 $\pm$ 1	3 $\pm$ 1	4 $\pm$ 1	3 $\pm$ 1	4 $\pm$ 2	5 $\pm$ 1	4 $\pm$ 1	5 $\pm$ 1	5 $\pm$ 1
R-I-Z-E	20 $\pm$ 4	27 $\pm$ 3	>100	19 $\pm$ 1	23 $\pm$ 5	24 $\pm$ 5	15 $\pm$ 1	26 $\pm$ 4	>100	18 $\pm$ 3	32 $\pm$ 10	>100
R-MX-AK	15 $\pm$ 2	24 $\pm$ 4	>100	16 $\pm$ 2	23 $\pm$ 1	>100	19 $\pm$ 2	51 $\pm$ 4	>100	19 $\pm$ 2	22 $\pm$ 1	>100
R-MX-AK-MZ	21 $\pm$ 7	18 $\pm$ 3	>100	17 $\pm$ 2	21 $\pm$ 7	>100	19 $\pm$ 5	28 $\pm$ 6	>100	17 $\pm$ 2	>100	>100
R-MX-AK-NZ	17 $\pm$ 3	24 $\pm$ 3	>100	15 $\pm$ 1	22 $\pm$ 2	>100	16 $\pm$ 2	>100	>100	18 $\pm$ 1	>100	>100
R-MX-AK-NC	16 $\pm$ 2	19 $\pm$ 2	20 $\pm$ 1	13 $\pm$ 2	18 $\pm$ 1	>100	15 $\pm$ 2	27 $\pm$ 3	>100	19 $\pm$ 1	27 $\pm$ 1	>100
R-MX-AK-PA	38 $\pm$ 8	>100	>100	26 $\pm$ 4	>100	>100	20 $\pm$ 2	>100	>100	24 $\pm$ 4	>100	>100

<sup>a</sup> Five-day-old aerobic (A5) cultures, and 5-, 12-, and 19-day-old hypoxic (H5, H12, and H19, respectively) cultures were incubated with various combinations of drugs for 7, 14, and 21 days. R, rifampin; I, isoniazid; Z, pyrazinamide; E, ethambutol; AK, amikacin; MX, moxifloxacin; MZ, metronidazole; NZ, nitazoxanide; NC, niclosamide; PA, PA-824. The drug concentrations used were 8, 2, 100, 4, 8, 4, 8, 10, 0.3, and 2  $\mu$ g/ml, respectively.

<sup>b</sup> Means  $\pm$  standard deviations from three experiments are shown.



optimal growth of tubercle bacilli in liquid media occurs between pH 5.8 and 6.7 (17). In contrast, in hypoxic conditions replication stopped after 8 days and the pH increased slightly, followed by a drop in the CFU count. A similar decrease was previously seen by us in hypoxic cultures in DTA broth at pH 6.6, in which we found that dormant bacilli older than 25 days grew in liquid but not on solid medium (13).

Among 10 antimicrobial agents tested, R, which inhibits RNA synthesis (5), was the most active against all the four cell stages examined and more effective than in our previous studies in DTA broth at pH 6.6 (14). The observation that R efficiently killed A and H cells in acidic conditions is important because R and Z, a drug active only at acidic pHs (17, 19), are together responsible for most of the sterilizing activity of the standard treatment of pulmonary TB (16, 19). Isoniazid, which acts on mycolic acid synthesis (5), was bactericidal against A5 cells, but showed limited activity against H5 cells and no activity against H12 and H19 cells, similarly to that reported by Wayne and coworkers in DTA broth at pH 6.6 (6). The exclusive activity of I against A5 and H5 cells and the growth curves of Fig. 1 indicated that in this acidic Wayne model, A5 cells were AR aerobic cells, while H5 cells were slowly replicating microaerophilic cells. Furthermore, inactivity of I against H12 and H19 cells indicated that these bacilli were NR anaerobic cells. Pyrazinamide, due to the pH increase, was inactive against A5 cells; the drug was inactive also against H5 cells but was effective late against H12 and H19 cells, in keeping with previous studies on aerobic, microaerophilic, and anaerobic *M. tuberculosis* (11, 19). Among second-line drugs, MX and AK, which inhibit DNA gyrase and protein synthesis, respectively (5), showed potent activities against A5 and H5 cells. Interestingly, MX was moderately effective also against H12 and H19 cells, and AK showed some activity against H19 cells. The latter observations extend the known activity of MX and/or AK against NR *M. tuberculosis* in the Wayne model at pH 6.6 to acidic conditions (8, 10). In line with these *in vitro* results, MX was shown to be an essential component of new building blocks for sterilizing regimens which have been shown in a murine model of TB (29).

As for the nitrocompounds, it is known that NZ, a drug highly effective against gastrointestinal protozoa, helminths, and anaerobic bacteria, kills both AR and NR *M. tuberculosis* at pH 5.5 under aerobic conditions (9). In this study, we extended this information to hypoxic, acidic *M. tuberculosis*, suggesting that this drug has a potential for treatment of diseases like TB, which are not primarily affecting the gastrointestinal tract. PA-824, a new anti-TB drug currently being tested in clinical trials (1, 3, 30), was more potent than NZ against A5 and H5 cells up to day 7 but not later; however, it killed H12 and H19 cells to the same extent as NZ and to a greater extent than NC, an anthelmintic nitrocompound for treatment of intestinal tapeworm infections. Metronidazole was effective only against NR H12 and H19 cells, in accordance with a recent report showing that it prevents reactivation of latent TB in macaques (15). Finally, it is important to point out that regrowth of A5 and/or H5 cells was seen after  $\geq 14$  days of treatment with PA, H, and AK but not MX and NZ. This was likely due to development of resistant mutants which are known to be generated at the concentrations used here for PA, H, and AK (31, 32), but not for MX and NZ (9, 33).

Overall, we analyzed the activity of several drugs against aerobic (A5), microaerophilic (H5), and anaerobic (H12 and H19) tubercle bacilli at pH 5.8 and found a variety of inhibitory effects.

This comparative analysis is important in designing combinations killing both AR and NR *M. tuberculosis*. In this view, the 3-drug combination R-MX-AK was selected as a building block for the high activities of R, MX, and AK against A5 and H5 cells and was potentiated by adding a fourth drug active against NR cells (i.e., MZ, NC, NZ, or PA). Combined drug activity was measured both by CFU and DTP, a method much more sensitive than colony counts (14). By CFU, it was found that all four stages of *M. tuberculosis* tested were apparently killed by the 4-drug combination R-MX-AK-PA in 7 days and by the other combinations in 14 days. In contrast, after growing the same inocula used for CFU counts in the MGIT tubes, it was found that no combination, including R-MX-AK-PA, had killed *M. tuberculosis* in 7 days, demonstrating that using plate counts can seriously underestimate mycobacterial killing after drug exposure. Since CFU are mostly used for determining the activity of new compounds against AR and NR *M. tuberculosis* (34), investigators should evaluate also the DTP assay to assess the *in vitro* sterilizing effect of anti-TB drugs and combinations. It is important to note that the 3-drug combination R-MX-AK, unlike R-I-Z-E, which is currently used for the human therapy, killed all four cell stages in 21 days, in accordance with good activity of AK and MX against aerobic and anaerobic *M. tuberculosis* strains reported by other investigators (8, 10). Again, among the other combinations, the most potent was R-MX-AK-PA, which killed A5, H5, H12, and H19 cells in 14 days and showed the highest DTP values after 7 days. The combination R-MX-AK-NZ was also potent because it killed H12 and H19 cells in 14 days but was as potent as R-MX-AK at killing A5 and H5 cells. R-MX-AK-NC was not bactericidal against A5 cells after 21 days, possibly due to antagonistic effects among drug components.

Overall, our study indicated that PA-824 efficiently enhanced R-MX-AK to kill aerobic, microaerophilic, and anaerobic cells in acidic conditions (i.e., in environments likely encountered by *M. tuberculosis* within activated macrophages and granulomatous lesions of patients with pulmonary TB). Strong support for the potential of PA as a combinatorial agent comes from the knowledge that PA-824 showed a high 14-day bactericidal activity when used in combination with MX and Z for treatment of pulmonary TB in a randomized trial (30). PA-824 kills NR *M. tuberculosis* by intracellular nitric oxide release (35). It is known that bactericidal antibiotics fluorquinolones and aminoglycosides (such as MX and AK, respectively) kill Gram-negative and Gram-positive bacteria by multiple mechanisms, including production of reactive radicals (36). This mode of action could have an important role also in the ability of R-MX-AK and R-MX-AK-PA to kill both AR and NR *M. tuberculosis*, including antibiotic-tolerant persisters, under acidic conditions; studies on the issue are in progress in our laboratory.

In conclusion, we systematically investigated the activity of 10 drugs alone and in combination against four different physiologic stages of *M. tuberculosis* that could live in TB granulomas and used a stringent method to demonstrate that tubercle bacilli were dead by using regrowth in broth as a test much more sensitive than CFU measurements. Overall, we think that this approach should be considered when studying the sterilizing activity of new drugs or drug combinations against *M. tuberculosis*, so as to facilitate further *in vivo* experimentation.

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