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Spectrum of Activity of Levofloxacin against Nontuberculous Mycobacteria and Its Activity against the *Mycobacterium avium* Complex in Combination with Ethambutol, Rifampin, Roxithromycin, Amikacin, and Clofazimine

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The spectrum of activity of levofloxacin was initially determined against 29 strains belonging to 16 species of atypical mycobacteria by measuring radiometric MICs. Levofloxacin MICs were 1 to 2 dilutions lower compared with those obtained for ofloxacin and 8 to 64 dilutions lower compared with those obtained for its D-isomer. Levofloxacin MICs were below its peak level in serum (5.5 μg/ml following administration of a single oral dose of 350 mg) for 25 of 29 isolates tested. It possessed MICs below its peak level in serum for *M. scrofulaceum*, *M. szulgai*, *M. malmoense*, *M. xenopi*, *M. marinum*, *M. kansasii*, *M. chelonei*, *M. abcessus*, *M. fortuitum*, and *M. peregrinum*. Regarding the *M. avium* complex, the MICs of levofloxacin for 11 clinical isolates (7 from human immunodeficiency virus-positive patients and 4 from human immunodeficiency virus-negative patients) were 1 to 2 dilutions lower than those of ofloxacin. Among 20 isolates belonging to 12 pathogenic mycobacterial species, the MBC/MIC ratios varied from 1 to 4 for levofloxacin and 2 to 4 for ofloxacin. When drug combinations were screened by using the radiometric *x/y* quotient methodology against five *M. avium* complex isolates, levofloxacin activity against all five isolates was enhanced by ethambutol and activity against three isolates was enhanced by clofazimine. Screening of three-drug combinations showed that the combination levofloxacin-ethambutol with a third potential anti-*M. avium* drug (rifampin, roxithromycin, amikacin, or clofazimine) resulted in enhanced activity for all 20 drug combinations screened.

The antimicrobial activity of levofloxacin (an optically active L-isomer of ofloxacin) against various microorganisms including *Mycobacterium tuberculosis* is 8 to 128 times greater than that of the corresponding D-isomer and about 2 times greater than that of ofloxacin, which consists of equal amounts of both the D- and L-isomers (7, 9, 15, 16, 18, 31, 33). Although both levofloxacin and ofloxacin are characterized by similar absorption rates (7, 17, 31, 33), the higher intracellular concentration/extracellular concentration ratios for levofloxacin compared with those for ofloxacin in cultured human cells (16, 19, 20) and the higher in vitro activity of levofloxacin against bacterial DNA gyrase (13) make it an interesting candidate drug to be screened against the full panel of atypical mycobacteria, particularly the AIDS-associated, multiple-drug-resistant opportunist pathogen *M. avium*. Although the activity of levofloxacin against *M. tuberculosis* (14–16, 30) and atypical mycobacteria including *M. avium* (5, 29) was recently evaluated, potential levofloxacin-containing drug combinations were not screened against these opportunistic pathogens. Because it is a usual practice to treat *M. avium* infections with regimens containing three to four drugs at a time (21–23), the present investigation was performed both to confirm the higher level of activity of levofloxacin compared with that of ofloxacin against a variety of atypical mycobacteria and to provide new data concerning the activity of levofloxacin in combination with other antituberculous drugs (ethambutol, rifampin, amikacin, clofazimine, and roxithromycin) against the *M. avium* complex (MAC).

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MATERIALS AND METHODS

Organisms. The various mycobacterial species used in the investigation (Tables 1 and 2) included both reference strains and human clinical isolates. All isolates except *M. malmoense*, *M. marinum*, and *M. chelonei* were grown on fresh Löwenstein-Jensen (LJ) slants at 37°C; *M. malmoense*, *M. marinum*, and *M. chelonei* were grown at 30°C. The clinical isolates either were from patients residing in Guadeloupe and Martinique and were isolated from clinical specimens at the Institut Pasteur of Guadeloupe or were various European isolates sent to the National Reference Center for Mycobacteria, Institut Pasteur, Paris, France. Strain identification was performed on the basis of biochemical and cultural characteristics including mycolic acid analysis. For drug activity studies, bacteria were scraped from the LJ slants, homogenized with glass beads (2 mm in diameter), grown in complete 7H9 broth (supplemented with Middlebrook albumin-dextrose-catalase [ADC] enrichment [Difco Laboratories, Detroit, Mich.]) containing 0.05% (vol/vol) Tween 80 to avoid clumping at 37°C, and harvested at their mid-logarithmic phase at an optical density of 0.15 (measured at 650 nm with a Coleman Junior II spectrophotometer), which corresponded to about 10^8 CFU/ml.

MIC and MBC determinations. Radiometric determination of the MICs was performed with the BACTEC 460-TB apparatus (Becton Dickinson, Towson, Md.), as reported earlier (24, 26, 28). Briefly, bacterial growth was measured in a confined atmosphere as a function of the ability of the bacteria to catabolize ^{14}C-labeled palmitic acid in 7H12 broth and by automatically measuring the amount of $^{14}CO_2$ released. The growth of bacteria was represented as a numerical value called the growth index (GI), which ranged from 1 to 999. Mycobacterial growth in this system is dependent on the standardization of the initial bacterial inoculum, and because of the more rapid growth of *M. avium* and some other rapid growers compared with the speed of growth of tubercle bacilli, in the BACTEC system, the initial bacterial inoculum added to the BACTEC vials depended on the species studied, as described previously (16).

After preculture of a strain in an initial BACTEC 12B vial to a GI of 500, the drug-containing vials were inoculated with 0.1 ml of the preculture, which was used directly in the case of *M. xenopi* and which was diluted 1:10 in the case of all other atypical mycobacteria except *M. avium*, *M. fortuitum*, and *M. chelonei*

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(which were inoculated with 0.1 ml of a 1:100-diluted preculture). The change in the daily GI (called the ΔGI) of the drug-containing vials described above was compared with the GI of a control vial inoculated with 100 times fewer bacteria in the absence of drug (referred to as the 1:100 control). Under these conditions, the MIC was interpreted once the GI in the 1:100 control reached a value of 30 or more. The MIC was defined as the minimal drug concentration resulting in a lower ΔGI in the drug-containing sample compared with that in the 1:100 control. All cultures except those of *M. malmoense*, *M. marinum*, and *M. chelonei* were incubated at 37°C; *M. malmoense*, *M. marinum*, and *M. chelonei* were incubated at 30°C.

The MBCs of levofloxacin and ofloxacin in the BACTEC system were compared for various isolates, as reported previously for *M. avium* (10, 11, 28). In the present study, the bacterial viability was determined by plating the bacterial suspensions from individual BACTEC vials at the beginning and at the end of the experiments onto 7H11 agar medium for viable count enumeration, and the results were expressed as the mean ± standard error viable count. The initial inoculum in the BACTEC vials was within a range of $1.42 \times 10^4 \pm 0.1 \times 10^4$ to $8.92 \times 10^4 \pm 1.0 \times 10^4$. The MBC was the lowest concentration of drug that was able to kill the initial inoculum by 2 logs or more.

Drug combination studies. All drugs were used at sub-MICs because at these concentrations, the drugs used alone were unable to significantly reduce the initial bacterial inoculum in the BACTEC vials (see Fig. 1). Consequently, any significant enhancement of drug activity obtained at these sub-MICs may indicate potential activity in *M. avium*-infected host cells, where these drugs are available at much higher concentrations. Drug combination studies were performed as reported earlier for *M. avium* by using the radiometric *x/y* quotients (25, 26, 28). Briefly, the combined drug action is equal to *x/y*, where *x* is the GI obtained with the combination of two or more drugs with the BACTEC system, and *y* is the lowest GI obtained at the same time with any of the drugs used alone. The various sub-MICs used and the *x/y* quotient interpretations are provided in footnotes *a* and *b* of Table 4. The radiometric data were also corroborated by plating the bacterial suspensions from individual vials at the beginning and end of the experiment onto 7H11 agar medium for viable count enumeration, and the results were expressed as mean ± standard error viable counts.

Drugs. Levofloxacin, ofloxacin, D-ofloxacin, roxithromycin (Hoechst-Marion-Roussel, Romainville, France), and clofazimine (CIBA-GEIGY, Basel, Switzerland) were kindly provided by their manufacturers, whereas all other drugs used in the investigation were purchased from Sigma Chemical Co., St. Louis, Mo.

RESULTS AND DISCUSSION

The results of the study are briefly summarized in Tables 1 to 4 and Fig. 1. The radiometric MICs of levofloxacin compared with those of ofloxacin and its D-isomer for 29 strains belonging to 16 species of atypical mycobacteria are summarized in Table 1. Levofloxacin MICs were 1 to 2 dilutions lower compared with those obtained for ofloxacin and 8 to 64 dilutions lower compared with those obtained for its D-isomer. These results corroborate recent findings obtained with 7H11 agar medium (29). Levofloxacin MICs were below its peak level in serum (5.5 μg/ml following administration of a single oral dose of 350 mg [8]) for 25 of 29 isolates belonging to species such as *M. scrofulaceum*, *M. szulgai*, *M. malmoense*, *M. xenopi*, *M. marinum*, *M. kansasii*, *M. chelonei*, *M. abcessus*, *M. fortuitum*, and *M. peregrinum*. Similarly, levofloxacin MICs for all clinical MAC isolates tested (seven from human immunodeficiency virus [HIV]-positive patients and 4 from HIV-negative patients) were 1 to 2 dilutions lower than those of ofloxacin (Table 2).

MBC/MIC ratios were determined for 20 isolates belonging to 12 pathogenic mycobacterial species (Table 3) and varied from 1 to 4 for levofloxacin and 2 to 4 for ofloxacin. If the absolute MBCs were considered in the context of the reported maximum concentration of the drug in serum (5.5 μg/ml [8]), it could be concluded that the drug was able to kill 2 logs or more of the bacterial inoculum of 9 of 12 species at clinically achievable concentrations (Table 3). However, the drug showed only marginal bactericidal activity against *M. intracellulare*, *M. avium*, and *M. simiae* (Table 3) when it was used alone. For these reasons, we decided to further investigate the anti-*M. avium* activity of levofloxacin in combination with other antituberculous drugs.

Apart from developing individual drugs for the treatment of MAC infections, one of the major issues today is the development of appropriate drug combination regimens (22, 23). In the present investigation, various drugs were combined at their sub-MICs and were screened by the radiometric *x/y* quotient methodology against five MAC isolates. The comparative radiometric MICs of levofloxacin, ofloxacin, and additional antituberculous drugs (ethambutol, rifampin, amikacin, clofazime, and roxithromycin) for five isolates that were selected for testing in the drug combination studies are presented in Table 2, whereas the sub-MICs selected for the latter experiments are provided in footnote *a* of Table 4. The sub-MICs exerted only marginal inhibitory activity when the drugs were used alone (data not shown); these corresponded to about a quarter of the respective MICs for each drug.

A radiometric comparison of the various two- and three-

TABLE 1. In vitro activities of levofloxacin, ofloxacin, and D-ofloxacin against a panel of 29 strains belonging to 17 species of atypical mycobacteria

Pathogen type and species	MIC (μg/ml)[a]		
	D-Ofloxacin	Ofloxacin	Levofloxacin
M. intracellulare			
ATCC 13950	>64.0	16.0	8.0
Clinical isolate 94-0070	>64.0	16.0	8.0
M. avium			
CIPT 140310002	32.0	1.0	0.5
Clinical isolate 89-0733	>64.0	16.0	8.0
M. scrofulaceum ATCC 19981	16.0	2.0	1.0
M. simiae			
ATCC 25275	>64.0	8.0	4.0
Isolate 91-0198 from AIDS patient	>64.0	16.0	8.0
M. szulgai NCTC 10831	64.0	2.0	1.0
M. malmoense ATCC 29571[b]	>64.0	2.0	1.0
M. xenopi ATCC 19970	64.0	2.0	1.0
M. marinum ATCC 927[b]	32.0	2.0	1.0
M. kansasii			
ATCC 12478	16.0	0.5	0.25
Clinical isolate 94-0069	16.0	0.5	0.25
M. gordonae ATCC 14470	8.0	0.25	0.125
M. terrae ATCC 15755	16.0	0.5	0.25
M. triviale ATCC 23292	≤4.0	≤0.25	≤0.125
M. gastri ATCC 15754	16.0	0.5	0.25
M. chelonae subsp. *chelonae*			
NCTC 946[b]	32.0	2.0	1.0
CIPT 140420005[b]	>64.0	8.0	4.0
Clinical isolate 81-0402[b]	4.0	0.25	0.125
Clinical isolate 92-0592[b]	16.0	2.0	1.0
M. chelonae subsp. *abcessus*			
Clinical isolate 92-0801	32.0	4.0	2.0
Clinical isolate 83-2319	32.0	4.0	2.0
M. fortuitum var. *fortuitum*			
ATCC 6841	≤4.0	≤0.25	≤0.125
CIPT 140410002	8.0	0.5	0.25
Clinical isolate 92-0542	≤4.0	≤0.25	≤0.125
M. peregrinum			
ATCC 14467	≤4.0	≤0.25	≤0.125
Clinical isolate 92-0469	16.0	1.0	0.5
Clinical isolate 92-0580	8.0	0.5	0.25

[a] All MICs were determined radiometrically. In the case of *M. xenopi*, the drug-containing vials were inoculated with 0.1 ml of a preculture grown to a BACTEC system GI of about 500, whereas in all other cases except for *M. avium*, *M. fortuitum* and *M. chelonei* the vials were inoculated with 0.1 ml of a 1:10-diluted culture (for *M. avium, M. fortuitum* and *M. chelonei* the vials were inoculated with a 1:100-diluted preculture). The values in each case were compared with the values for inoculum-containing control vial diluted 1:100.

[b] Incubated at 30°C.

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TABLE 2. Comparative radiometric MICs of levofloxacin and ofloxacin for 11 clinical MAC isolates and those of additional antituberculous drugs (ethambutol, rifampin, amikacin, clofazime, and roxithromycin) for five isolates selected for drug combination studies[a]

Drug	MIC (μg/ml) for the following clinical isolates[b]:										
	From HIV-positive patients							From HIV-negative patients			
	Av1	Av2	Av3	Av4	Av5	Av6	Av7	Av8	Av9	Av10	Av11
Ofloxacin	>32	>32	16	8	16	16	8	32	32	2	16
Levofloxacin	>16	>16	8	4	8	8	2	16	16	1	8
Ethambutol	ND[c]	ND	ND	ND	2	2	2	ND	ND	<2	2
Rifampin	ND	ND	ND	ND	2	8	8	ND	ND	<2	2
Amikacin	ND	ND	ND	ND	4	2	2	ND	ND	2	4
Clofazimine	ND	ND	ND	ND	0.12	0.25	0.25	ND	ND	0.12	0.5
Roxithromycin	ND	ND	ND	ND	2	8	8	ND	ND	8	8

[a] All MICs were determined by using 7H12 medium at a pH of 6.8 ± 0.2.
[b] Isolates Av1 to Av4 were isolated from Caribbean patients, whereas isolates Av5 to Av11 were from European patients.
[c] ND, not done, because these isolates were not used in combination studies with these drugs.

drug combinations screened is provided in Table 4. Among the two-drug combinations tested, levofloxacin-ethambutol showed considerable inhibition of the metabolism of all bacterial isolates tested; this was followed by levofloxacin-clofazimine, which inhibited three isolates. Further screening of three-drug combinations showed that the combination levofloxacin-ethambutol in combination with a third potential anti-*M. avium* drug (rifampin, roxithromycin, amikacin, or clofazimine) resulted in enhanced drug activity for all 20 drug combinations screened. The radiometric enhancement of various drug combinations, as evidenced by the *x/y* quotient calculations in Table 4, was further verified by bacterial viable count determinations in BACTEC vials at the beginning and at the end of the drug combination studies (Fig. 1). A satisfactory correlation between the BACTEC GI values, the *x/y* quotients, and the bacterial viable counts was observed.

It must be emphasized that the enhancement in the *x/y* method is intentionally performed by using sub-MICs of the drugs, which by itself is explanation for the fact that the overall effect observed from viable count data is not always highly bactericidal activity. Considering the published evidence about the refractory nature of *M. avium* to most of the antimicrobial agents used even at much higher concentrations (21–23), the 90 to 99% killing of the initial bacterial inoculum by levofloxacin-ethambutol in a two-drug combination or with a third drug which included rifampin, roxithromycin, amikacin, or clofazimine was noteworthy (Fig. 1).

The results presented above are in agreement with our previous observations that ethambutol is able to break the drug exclusion barrier located in the *M. avium* cell wall by inhibiting specific components (3, 26–28) and corroborate recent observations of Bermudez et al. (5) that levofloxacin in combination

TABLE 3. Comparative MBCs and MBC/MIC ratios of ofloxacin and levofloxacin for selected species of atypical mycobacteria

Pathogen type and species	Ofloxacin		Levofloxacin	
	MBC (μg/ml)[a]	MBC/MIC ratio	MBC (μg/ml)	MBC/MIC ratio
M. intracellulare ATCC 13950	32	2	16	2
M. avium complex				
Isolate Av3 from AIDS patient	32	2	16	2
Isolate Av4 from AIDS patient	16	2	8	2
Isolate Av5 from AIDS patient	64	4	32	4
Isolate Av6 from AIDS patient	ND[b]	ND	32	4
Isolate Av7 from AIDS patient	ND	ND	16	4
Clinical isolate Av8	ND	ND	32	4
Clinical isolate Av9	ND	ND	64	4
Clinical isolate Av11	ND	ND	8	1
M. scrofulaceum ATCC 19981	4	2	2	2
M. simiae				
ATCC 25275	32	4	8	2
Isolate 91-0198 from AIDS patient	32	2	16	2
M. szulgai NCTC 10831	4	2	2	2
M. malmoense ATCC 29571[c]	4	2	2	2
M. xenopi ATCC 19970	4	2	2	2
M. marinum ATCC 927[c]	2	4	1	4
M. kansasii 94-0069 (clinical isolate)	2	4	1	4
M. chelonae NCTC 946[c]	8	4	4	4
M. fortuitum CIPT 140410002	2	4	0.5	2
M. peregrinum 92-0469 (clinical isolate)	4	4	2	4

[a] The MBC was determined by plating suspensions from BACTEC vials at the time of drug addition and at the end of experiments onto 7H11 medium for assessment of the numbers of CFU. The initial inoculum in the BACTEC vials was within a range of $1.42 \times 10^4 \pm 0.1 \times 10^4$ to $8.92 \times 10^4 \pm 1.0 \times 10^4$. The MBC was the lowest concentration of drug that killed more than 2 logs (99%) of the initial inoculum.
[b] ND, not done.
[c] Incubated at 30°C.

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TABLE 4. In vitro enhancement of anti-*M. avium* activity of levofloxacin by selected drugs in two- and three-drug combinations

Drugs[a]	Enhancement of drug activity (*x/y* quotient) for indicated isolate[b]				
	From HIV-positive patients			From HIV-negative patients	
	Av5	Av6	Av7	Av10	Av11
Levo + Emb	0.14	0.078	0.26	0.11	0.005
Levo + Rif	—[c]	—	—	—	—
Levo + Rox	—	—	—	—	—
Levo + Amik	—	—	—	—	—
Levo + Clofa	0.19	0.44	—	—	0.36
Levo + Emb + Rif	0.04	0.049	0.025	0.04	0.006
Levo + Emb + Rox	0.02	0.078	0.016	0.066	0.074
Levo + Emb + Amik	0.01	0.005	0.026	0.02	0.016
Levo + Emb + Clofa	0.06	0.059	0.26	0.02	0.004

[a] All drugs were used at sub-MICs. The concentrations chosen were as follows: levofloxacin (Levo), 0.25 μg/ml for Av10, 0.5 μg/ml for Av7, and 2.0 μg/ml for the other strains; ethambutol (Emb), 1.0 μg/ml for each strain; rifampin (Rif), 0.25 μg/ml for all strains except 0.5 μg/ml for isolate Av7; roxithromycin (Rox), 0.5 μg/ml for isolate Av5, 1.0 μg/ml for strains Av6 and Av11, and 2 μg/ml for strains Av7 and Av10; amikacin (Amik), 0.5 μg/ml for isolate Av6 and 1.0 μg/ml for the other strains; clofazimine (Clofa), 0.05 μg/ml for all isolates except 0.1 μg/ml for isolate Av11. Refer to the text and Table 2 for further information.

[b] A radiometric *x/y* quotient of <0.5 (two-drug combinations) or <0.33 (three-drug combinations) indicated enhanced drug action.

[c] —, no enhancement of activity was observed.

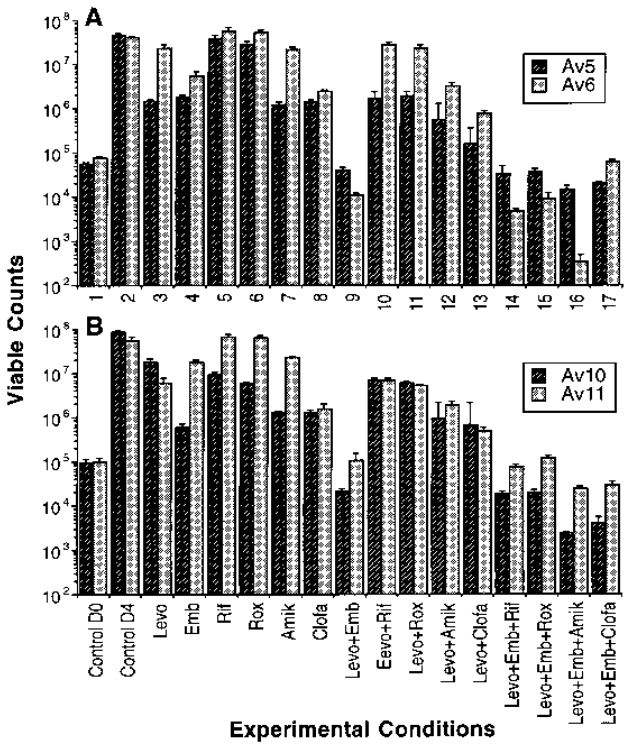

FIG. 1. Viable cell count data comparing the effects of various drugs used alone and in combination against four isolates of *M. avium*. The viable cell counts were determined by plating the cultures from experiments whose results are presented in Table 4 at the beginning and at the end of the experiments. All drugs were used at sub-MICs. Refer to footnote *a* of Table 4 for the sub-MICs. D0, day 0; D4, day 4; Levo, levofloxacin; Emb, ethambutol; Rif, rifampin; Rox, roxithromycin; Amik, amikacin; Clofa, clofazimine.

with ethambutol is more active than either drug alone in reducing the numbers of *M. avium* isolates in the blood, livers, and spleens, of experimentally infected beige mice. These data also support the practice of using combination drug therapy to prevent the emergence of resistant isolates in view of the large bacterial populations in patients with disseminated MAC infections and the resulting favorable response because of the synergistic activity of the drugs (1, 2, 6, 12). Indeed, according to the current notion, all patients with disseminated MAC infections not only should be treated but should also receive at least two or more antimycobacterial drugs to prevent and/or delay the emergence of resistance (22, 23); macrolides are a preferred first agent, with ethambutol being the suggested second drug. A third and a fourth drug are often added to this combination for severely ill, symptomatic patients and may include rifamycins, clofazimine, amikacin, and fluorinated quinolones (21–23). Similar drug combinations are also used to treat a variety of other atypical mycobacteria (23). In this context, the present investigation showed that levofloxacin is a potent drug against a variety of atypical mycobacteria and that its activity against MAC is successfully enhanced by other drugs like ethambutol, rifampin, roxithromycin, amikacin, and clofazimine.

The improved physicochemical and pharmacokinetic properties of levofloxacin compared with those of ofloxacin, e.g., lower MICs, similar absorption rates, higher levels of intracellular accumulation, and higher intracellular concentration/extracellular concentration ratios in human macrophages (4, 7, 9, 13, 15–17, 31–33), are interesting parameters suggesting that the improved in vitro activity of levofloxacin compared with that of ofloxacin may easily be reproducible in vivo. Furthermore, the pharmacokinetics and safety of levofloxacin were found to be unaltered in HIV-infected patients in a phase I, double-blind, randomized (1:1), placebo-controlled trial (8); the adsorption of levofloxacin was not rate limited by the gastrointestinal transit process and was almost immediate following oral administration, with a slow elimination process. In addition to the characteristics described above, levofloxacin also appeared to be equally safe even after the administration of multiple oral doses of 350 mg every 8 h to both healthy and HIV-infected patients (8), further suggesting its potential use in treating *M. avium*-infected AIDS patients. We therefore conclude that levofloxacin is a good candidate drug for treating atypical mycobacteria including *M. avium*. Its activity alone and in combination with other antituberculous drugs compared with the activity of ofloxacin should now be assessed in prospective, randomized, controlled clinical trials.

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