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Impact of Fluoroquinolone Resistance on Bactericidal and Sterilizing Activity of a Moxifloxacin-Containing Regimen in Murine Tuberculosis

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It has been shown previously that fluoroquinolone resistance (defined by resistance to at least 2 mg/liter ofloxacin) has a different impact on moxifloxacin monotherapy depending on the mutation in the sole fluoroquinolone target in *Mycobacterium tuberculosis*, i.e., DNA gyrase. Since tuberculosis treatment relies on multidrug therapy, we wished to determine the impact of fluoroquinolone resistance on the bactericidal and sterilizing activity of a second-line antituberculous regimen containing moxifloxacin. A total of 280 mice were inoculated with the wild-type *Mycobacterium tuberculosis* H37Rv strain or one of 3 isogenic fluoroquinolone-resistant mutant strains with increasing moxifloxacin resistance (the GyrB D500N, GyrA A90V, and GyrA D94G strains) and then treated for 6 months with a second-line regimen containing moxifloxacin, pyrazinamide, and ethionamide supplemented with amikacin during the first 2 months. Mice were sacrificed during treatment for measurement of bactericidal activity and 3 months after treatment completion for measurement of relapse rates (sterilizing activity). The CFU counts decreased faster in mice inoculated with the wild type than in mice inoculated with the mutant strains. The relapse rate after treatment completion was different among mice inoculated with mutant strains in relation to the drug resistance level: wild type, 0%; GyrB D500N strain, 33%; GyrA A90V strain, 50%; and GyrA D94G strain, 86%. The relapse rate observed with the GyrB D500N strain was the only one not statistically different from that observed with the wild-type strain. We demonstrated that the impact on sterilizing activity of the most active second-line drug regimen containing moxifloxacin depends on the MIC of moxifloxacin. We suggest that the precise level of moxifloxacin resistance be determined for all strains resistant to 2 mg/liter ofloxacin.

Drug resistance increases tuberculosis (TB) mortality from 5 to 10% for drug-susceptible TB to 13 to 24% for multidrug-resistant tuberculosis (MDR-TB; defined as resistance to both isoniazid and rifampin) and 18 to 50% for extensively drug-resistant tuberculosis (XDR-TB; defined as MDR-TB plus resistance to fluoroquinolones and an injectable second-line drug) (1–3). The incidence of XDR-TB is rising worldwide and has now been detected in 69 countries (4). The worsening prognosis of XDR-TB compared to that of MDR-TB is strongly related to fluoroquinolone resistance (5), which is defined by resistance to at least 2 mg/liter of ofloxacin (6).

Mycobacterium tuberculosis resistance to fluoroquinolones depends on the substitution in the GyrA and/or GyrB subunits of the DNA gyrase, a catalytically active complex ($GyrA_2GyrB_2$) (7, 8). The level of resistance of DNA gyrase mutants depends both on the mutation and the fluoroquinolone tested (9). Usually, the level of resistance is higher for ofloxacin than for moxifloxacin (2 to 16 mg/liter and 1 to 8 mg/liter, respectively) (10–12). Consequently, some strains categorized as fluoroquinolone resistant still have moxifloxacin MICs lower than the usual 4 mg/liter peak serum level (13). We previously demonstrated, in the murine model, that the impact of DNA gyrase mutations on moxifloxacin monotherapy activity was related to the level of resistance generated by each mutation (14). However, as tuberculosis treatment relies on drug combinations, this may not be true in the case of multidrug therapy. Therefore, our next aim was to evaluate whether the level of fluoroquinolone resistance also impacts the efficacy of moxifloxacin-containing multidrug regimens.

MATERIALS AND METHODS

Antimicrobials. Drugs were obtained and solutions prepared as previously described (14, 15).

***Mycobacterium tuberculosis* strains.** Four *M. tuberculosis* strains were used. Three isogenic mutant strains harboring DNA gyrase substitutions similar to those observed in clinical strains (12, 16), D94G and A90V in GyrA and D500N in GyrB, were obtained in a previous mouse study (15). The ofloxacin and moxifloxacin MICs were 0.5 and 0.25 mg/liter for the wild-type strain and were 4 and >8, >8 and 0.5, and 2 and 4 mg/liter for the strains harboring the GyrB D500N, GyrA A90V, and GyrA D94G substitutions, respectively (14). The wild-type reference H37Rv strain and the 3 mutant strains were grown from a mouse organ of a previous experiment as previously described (14, 15).

Intravenous infection. For each *M. tuberculosis* strain, 70 4-week-old female Swiss mice (Janvier Breeding Center, Le Genest Saint-Isle, France) were inoculated in the tail vein with 0.5 ml of a bacterial suspension prepared as previously described (17). The mean CFU counts were 6.8 for H37Rv, 6.4 log_{10} for the GyrA D94G strain, 7.1 for the GyrA A90V strain, and 6.5 for the GyrB D500N strain. After infection, mice were randomized

Received 12 March 2013 Returned for modification 29 April 2013
Accepted 29 June 2013
Published ahead of print 8 July 2013
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doi:10.1128/AAC.00506-13

TABLE 1 Scheme of experiment[a]

Strain	No. of mice untreated and kept under observation	No. of mice sacrificed				
		D−13	D0	M2	M6	M6+3
H37Rv	10	10	20	10	10	10
GyrB D500N strain	10	10	20	10	10	10
GyrA A90V strain	10	10	20	10	10	10
GyrA D94G strain	10	10	20	10	10	10

[a] Mice were inoculated at D−14, and treatment was given between D0 and M6.

by day of sacrifice (Table 1). We followed the animal experiment guidelines of the Faculté de Médecine Pitié-Salpêtrière.

Chemotherapy. Treatment began 14 days after infection (day 0 [D0]). Three drugs were administered by gavage 5 days per week for 6 months at the following dosages: moxifloxacin, 100 mg/kg of body weight twice daily; ethionamide, 50 mg/kg; and pyrazinamide, 150 mg/kg once daily. Amikacin, 150 mg/kg, was administered by subcutaneous injection 5 days per week for the first 2 months. Drug dosages used were similar to those in our previous experiments (15, 18). The moxifloxacin dosage allowed for an area under the curve (AUC) value close to that found in humans taking the recommended oral dose (400 mg/day), without excessively increasing the peak serum concentration (C_{max}) (14, 19, 20).

Assessment of treatment efficacy. We evaluated treatment efficacy by measuring lung CFU counts and the proportion of mice with culture-positive lung homogenates (i) during treatment and at the end of treatment, for assessing the bactericidal activity, and (ii) 3 months after treatment completion, for assessing sterilizing activity.

For each *M. tuberculosis* strain, 10 negative-control mice went untreated and were kept under observation until death. Ten and 20 untreated mice were sacrificed, respectively, the day after infection (D−13) and 14 days later, at the initiation of treatment (D0), to determine the initial CFU counts. Ten treated mice were killed after 2 months of treatment (M2), at the end of treatment (M6), and 3 months after treatment completion (M6+3) (Table 1).

Detection of second-step mutants after treatment completion. To check for *in vivo* selection of second-step mutants at M6+3, we used the direct proportion method. In addition to enumeration of the total number of CFU per organ, the undiluted organ suspensions were also plated onto Lowenstein-Jensen (LJ) medium containing ofloxacin at 16 mg/liter for the GyrB D500N mutant strain and 64 mg/liter for the GyrA A90V mutant strain, i.e., concentrations 4 times their MICs before treatment (14). The D94G mutant was not screened for second-step mutations, as we did show in a previous experiment that they do not occur when moxifloxacin is used alone, since the drug is inactive in this setting (14). Mutations leading to fluoroquinolone resistance were sought by sequencing the *gyrA* and *gyrB* genes of the bacilli isolated from the ofloxacin-containing medium by a method described elsewhere (21). We also sequenced colonies growing on LJ medium without ofloxacin when they could be tested individually, i.e., when there were less than 50 colonies on this medium and no growth on ofloxacin-containing medium.

Statistical analysis. CFU counts were log_{10} transformed before statistical analysis. Survival analyses were carried out using the Kaplan-Meier method. Quantitative data across two groups and across more than two groups were analyzed using the nonparametric Mann-Whitney-Wilcoxon and Kruskal-Wallis tests, respectively. The proportions of mice with positive cultures were compared using Fisher's exact test. Differences were considered to be statistically significant when the P value was <0.05. Statistical calculations were done using the website BiostaTGV (http://marne.u707.jussieu.fr/biostatgv/).

RESULTS

Survival rates. All 40 untreated mice died between 10 and 116 days after infection. Mortality was similar among untreated mice infected with the *M. tuberculosis* H37Rv wild-type strain and those infected with any of the three derivative mutant strains (data not shown). Thus, the mutant and the wild-type strains of *M. tuberculosis* exhibited equal virulence in the mouse.

Bactericidal activity of the moxifloxacin-containing regimen. The mean log_{10} CFU counts from lung homogenates and the proportions of mice with positive cultures are presented in Table 2.

The day after infection (D−13), mean log_{10} CFU counts in the lungs were 5.49 ± 0.18, 5.80 ± 0.18, 5.38 ± 1.0, and 5.46 ± 0.47 for mice infected with the H37Rv wild-type strain and the GyrB D500N, GyrA A90V, and GyrA D94G strains, respectively.

At D0, mean log_{10} CFU counts in the lungs increased to 6.61 ± 0.42, 7 ± 0.23, 7.59 ± 0.78, and 6.51 ± 0.50 for mice infected with the H37Rv wild-type strain and the GyrB D500N, GyrA A90V, and GyrA D94G strains, respectively. Thus, CFU counts before treatment were different across all four groups ($P = 3.6 \times 10^{-6}$). Consequently, comparison between the four groups was based on the measurement of the decline in CFU counts across D0 and M2 within each group.

Between D0 and M2, mean log_{10} CFU counts declined by 5.49, 4.52, 4.44, and 4.38 for mice infected with the H37Rv wild-type strain and the GyrB D500N, GyrA A90V, and GyrA D94G strains, respectively. These findings were mirrored in the proportion of mice with positive cultures. All mice infected with mutant strains (100%), but only three out of nine mice infected with the H37Rv wild-type strain (33%), had positive lung cultures, illustrating that initial bactericidal activity was significantly greater against the susceptible strain than the fluoroquinolone-resistant strains ($P =$

TABLE 2 Mean lung CFU counts and proportion of mice with culture-positive lung homogenates at the initiation of treatment, during treatment, and 3 months after treatment completion among mice infected with the wild-type *M. tuberculosis* strain H37Rv and those infected with the GyrB D500N, GyrA A90V, and GyrA D94G mutant strains

Strain	Lung CFU counts (log_{10}) (proportion of culture-positive mice)			
	Duration of treatment (mo)			Delay after treatment completion (+3 mo)
	0	2	6	
H37Rv	6.61 ± 0.42 (20/20)	1.12 ± 0.16 (3/9)[c]	0 ± 0 (0/9)[a]	0 ± 0 (0/9)[a]
GyrB D500N strain	7 ± 0.23 (20/20)	2.48 ± 0.52 (10/10)	0.21 ± 0.58 (1/9)[a]	1.24 ± 1.73 (3/9)[a]
GyrA A90V strain	7.59 ± 0.78 (20/20)	3.15 ± 0.66 (10/10)	0.06 ± 0.17 (1/8)[a,b]	1.94 ± 2.12 (4/8)[a,b]
GyrA D94G strain	6.51 ± 0.50 (20/20)	2.13 ± 0.51 (7/7)[a]	0 ± 0 (2/8)[a]	2.42 ± 1.43 (6/7)[a]

[a] Fourteen mice died of gavage accidents before the date of sacrifice.
[b] Two mice died from tuberculosis before the date of sacrifice (before the initiation of treatment).
[c] CFU counts were not available for one sacrificed mouse because of technical problems.

0.0031, $P = 0.0031$, and $P = 0.011$ for the GyrB D500N, GyrA A90V, and GyrA D94G strains, respectively).

Finally, the CFU counts in the lungs at the end of treatment (M6) were close to 0 in all groups: 0 CFU for H37Rv, 45 CFU in 1 mouse (11%) for the GyrB D500N strain, 3 CFU in 1 mouse (13%) for the GyrA A90V strain, and 1 CFU in 2 mice (25%) for the GyrA D94G strain. CFU counts ($P = 0.56$) and the proportion of mice with positive cultures ($P = 0.2$ to 1) were not significantly different among those infected with the respective four strains. Thus, bactericidal activity was not different against the susceptible strain and the fluoroquinolone-resistant strains after 6 months of the moxifloxacin-containing second-line regimen, despite an assessment at an earlier time point that had identified differences.

Relapse after treatment completion. Three months after treatment completion (M6+3), the relapse rates were related to the drug resistance level. Indeed, no mice infected with the *M. tuberculosis* H37Rv wild-type strain and three out of nine (33%), four out of eight (50%), and six out of seven (86%) mice infected with the GyrB D500N, GyrA A90V, and GyrA D94G strains, respectively, had culture-positive organs at this endpoint (Table 2). The relapse rate was significantly lower for the group infected with the H37Rv wild-type strain than for the groups infected with the GyrA A90V and GyrA D94G mutant strains ($P = 0.03$ and $P = 0.0009$, respectively), whereas it was not different from the group infected with the GyrB D500N mutant strain ($P = 0.2$). However, the difference in CFU counts between the latter group and the wild-type-infected mice approached statistical significance ($P = 0.077$). The difference between the group infected with the GyrB D500N mutant strain and the group infected with the GyrA D94G mutant strain closely approached significance ($P = 0.06$). The other differences between groups were not statistically significant (the GyrB D500N strain versus the GyrA A90V strain, $P = 0.64$; the GyrA A90V strain versus the GyrA D94G strain, $P = 0.28$).

Detection of second-step mutants after treatment completion. No colony grew on medium containing ofloxacin at 16 and 64 mg/liter for mice sacrificed at M6+3. Furthermore, no mutation in *gyrA* or *gyrB* was found in individual colonies growing on LJ medium without ofloxacin, demonstrating the absence of a second-step mutant selection in our experiment.

DISCUSSION

The consequence of detection of drug resistance has long been considered an on/off scenario in tuberculosis: diagnosing resistance was systematically leading to interruption of the drug. It is now well known that, for all antituberculous drugs, the level of resistance differs depending on the mutation generating resistance. Low-level resistance has been described for isoniazid and more recently for most antituberculous drugs (rifampin, ethambutol, aminoglycosides, fluoroquinolones, and ethionamide) (9, 16, 22, 23). However, the *in vivo* consequences of low-level resistance are still a matter of debate, as no clinical trial has assessed rigorously this question, and very few preclinical data exist (24).

In a previous murine study, we demonstrated that moxifloxacin, used alone, retains some activity *in vivo* against fluoroquinolone-resistant mutants and that this activity depends on the level of resistance (14). The present study aimed to find out whether the link between *in vivo* moxifloxacin activity and the level of fluoroquinolone resistance would still be apparent when combined with second-line antituberculous drugs.

We first demonstrated that fluoroquinolone resistance dramatically reduces the sterilizing activity of a second-line regimen, even when composed of the most active available second-line antituberculous drugs (25–27) (Table 2). This result is consistent with those of previous *in vivo* studies showing that withholding moxifloxacin from a second-line regimen against the wild-type H37Rv strain reduces both bactericidal and sterilizing activity (15, 18, 28) and with the numerous studies that have shown that fluoroquinolone resistance is a major prognostic factor for therapeutic failure in cases of multidrug-resistant tuberculosis (5, 29, 30).

Second, our data suggested that the sterilizing activity of the moxifloxacin-containing regimen decreases gradually against strains displaying levels of resistance increasing from low to intermediate to high. These results are consistent with data obtained for other bacterial species resistant to quinolones (31–35). Regarding the high-level resistant strain (the GyrA D94G strain), the detrimental impact of fluoroquinolone resistance was expected, as moxifloxacin alone is not active against this mutant in a murine model (14). For the strain displaying an intermediate level of resistance (the GyrA A90V strain), the relapse rate was significantly higher than that observed with the H37Rv wild-type strain but lower (although not statistically significant) than that seen with the high-level resistant strain (Table 2). The sterilizing activity of the moxifloxacin-containing regimen was not different for the low-level resistant (the GyrB D500N strain) and the wild-type strains, although it approached statistical significance for CFU counts (Table 2). Such low-level resistant strains account for up to 10% of fluoroquinolone-resistant strains (16, 36, 37). With the diagnosis of fluoroquinolone resistance being based mainly on susceptibility testing of ofloxacin (2 mg/liter), such strains may not be detected adequately. Therefore, our results suggest that, among strains resistant to 2 mg/liter ofloxacin, precise identification of the moxifloxacin level of resistance is required. This requires either genotypic testing including *gyrA* and *gyrB* sequencing or moxifloxacin susceptibility testing on low and high drug concentrations, which we suggest to be 2 mg/liter (9, 10, 38).

We demonstrated that although bactericidal activity against resistant mutants compared to H37Rv was reduced after 2 months, this reduction did not appear to depend on the level of fluoroquinolone resistance. This reinforces that in murine studies, bactericidal activity of drug combinations is not predictive of sterilizing activity (39, 40). However, here, the bactericidal activity was assessed only after 2 and 6 months. An intermediate time point may have been able to show a difference between the mutant strains. Also, when analyzing this and the apparently contradictory results regarding the sterilizing activity, one must bear in mind that, in our study, moxifloxacin was combined with the most active second-line drugs: amikacin, ethionamide, and pyrazinamide (25–27). Therefore, the fact that there was no difference in bactericidal activity against the three mutant strains after 2 and 6 months of treatment was probably due to the high activity of these three drugs. Since the second-line drugs still available to treat XDR-TB, such as cycloserine, *para*-aminosalicylic acid (PAS), or linezolid, are less active in clinical practice than those used in our study, the impact of fluoroquinolone resistance would probably be more important if moxifloxacin were combined with these drugs.

A caveat must be underlined when interpreting the results of this experiment. As the peak serum level of 100 mg/kg of moxifloxacin in mice is higher than that of 400 mg in humans, it could be possible that the activity against fluoroquinolone-resistant mu-

tants is exaggerated in mice compared to that in humans. However, we do not believe this is the case, since we demonstrated in a previous experiment that the main pharmacokinetic driver of moxifloxacin activity against fluoroquinolone-resistant mutants was the AUC and not the C_{max} (14).

Does this study support the WHO recommendation to include a later-generation fluoroquinolone, e.g., moxifloxacin, in a second-line TB regimen even in the case of fluoroquinolone-resistant tuberculosis (25, 41)? On one hand, there seems to be an impact of the level of resistance on the relapse rate, which supports the use of later-generation fluoroquinolone, at least against strains with low-level resistance (moxifloxacin MIC of less than 2 mg/liter). On the other hand, compared to the wild-type strain, the low-level resistance strain had a bacillary load higher at 2 months and almost significantly higher 3 months after the end of treatment, which means that even if a later-generation fluoroquinolone can improve treatment, it will not reverse fluoroquinolone resistance. Clearly, further studies need to be done, particularly to compare multidrug regimens with or without moxifloxacin to quantify more precisely the contribution of moxifloxacin. It would also be useful to compare multidrug regimens, including moxifloxacin at several dosages, to regimens without moxifloxacin in order to determine more precisely the impact of increasing moxifloxacin dosages on treatment efficacy. Indeed, since moxifloxacin activity depends on the AUC/MIC ratio (14, 42), we expect that doubling the dosage in humans (800 mg) could substantially improve the activity of a moxifloxacin-containing second-line drug regimen against strains with intermediate resistance. Such a dosage has already been used and seems safe (43).

Interestingly, our experiment could serve as a model for further design of studies aiming at determining the impact of different levels of resistance to other antituberculous drugs, which would help in implementing optimal treatments of patients with XDR-TB when the bacillus is resistant to all, or almost all, antituberculous drugs.

ACKNOWLEDGMENTS

This study was supported by the Fonds de Dotation Recherche en Santé Respiratoire, the Société de Pathologie Infectieuse de Langue Française, and a research grant from the European Society of Clinical Microbiology and Infectious Diseases. The animal facility was supported by a grant from Association Française Raoul Follereau.

We thank Maureen Beaudoin for technical assistance and Ekkehard Collatz for English editing.

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