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gyrA mutations and phenotypic susceptibility levels to ofloxacin and moxifloxacin in clinical isolates of *Mycobacterium tuberculosis*

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Objectives: To compare mutations in the quinolone resistance-determining region of the *gyrA* gene and flanking sequences with the MICs of ofloxacin and moxifloxacin for *Mycobacterium tuberculosis*.

Methods: The presence of mutations in 177 drug-resistant *M. tuberculosis* isolates was determined by DNA sequencing and the MICs quantified by MGIT 960.

Results: Single nucleotide polymorphisms were detected at codons 94 (n=30), 90 (n=12), 91 (n=3), 89 (n=1), 88 (n=1) and 80 (n=1). Four isolates with double mutations D94G plus A90V (n=2) and D94G plus D94N (n=2) reflect mixed populations. Agreement between genotypic and phenotypic susceptibility was high (≥97%) for both drugs. Mutant isolates had an MIC_{50} of 8.0 mg/L and an MIC_{90} of >10 mg/L for ofloxacin compared with an MIC_{50} and MIC_{90} of 2.0 mg/L for moxifloxacin. Codons 94 and 88 were linked to higher levels of fluoroquinolone resistance compared with codons 90, 91 and 89. The MIC distributions for the wild-type isolates ranged from ≤0.5 to 2.0 mg/L for ofloxacin and from ≤0.125 to 0.25 mg/L for moxifloxacin. However, 96% of the isolates with genetic alterations had MICs ≤2.0 mg/L for moxifloxacin, which is within its achievable serum levels.

Conclusions: This study provides quantitative evidence that the addition of moxifloxacin to extensively drug-resistant tuberculosis (XDR-TB) regimens based on a clinical breakpoint of 2.0 mg/L has merit. The use of moxifloxacin in the treatment of multidrug-resistant tuberculosis may prevent the acquisition of additional mutations and development of XDR-TB.

Keywords: fluoroquinolones, susceptibility testing, *in vitro* activity

Introduction

Fluoroquinolones are commonly used in the treatment of multidrug-resistant tuberculosis (MDR-TB), which is defined as resistant to both isoniazid and rifampicin.[1,2] Extensively drug-resistant tuberculosis (XDR-TB) arises when MDR strains acquire resistance to any fluoroquinolone in addition to at least one of the three injectable second-line drugs (SLDs), amikacin, kanamycin or capreomycin.[1,2] The design of individualized treatment regimens for XDR-TB should be based on reliable *in vitro* susceptibility results.[1,3] However, uncertainties about SLD susceptibility testing and the clinical interpretation of the test results need to be clarified in order to optimize the limited treatment options.[3]

Cross-resistance within the fluoroquinolone group is frequent.[1,4] However, it is unclear as to what extent this compromises the compound's antimycobacterial activity.[2] Ofloxacin belongs to the older-generation fluoroquinolones and has been widely used as a broad-spectrum antimicrobial agent against various infections, including MDR-TB.[1,5] New and more potent derivatives are now available, but ofloxacin is still being used because of its relatively low cost as opposed to the newer derivatives.[6] Moxifloxacin is one of the newer generation fluoroquinolones with enhanced activity against *Mycobacterium tuberculosis*.[4,7–12]

Moxifloxacin differs structurally from other fluoroquinolones, such as ofloxacin, in that it contains a methoxy group at the 8-position and a diazabicyclononyl ring moiety with an *S,S*-configuration at the 7-position.[7,8] The large hydrophobic moiety at C-7 reduces the ability of the bacterium to efflux the drug across its cell wall and this modification is important for

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preventing the emergence of resistance.[8] These structural changes lower the MIC of moxifloxacin (0.125–0.5 mg/L) compared with that of ofloxacin (0.5–2.0 mg/L).[4,12] The achievable peak serum concentrations of moxifloxacin and ofloxacin are 4.34 and 4.0 mg/L, respectively, after an oral dose of 400 mg.[4] The elimination half-life ranges from 10.7 to 13.3 h for moxifloxacin and is 4–5 h for ofloxacin.[4] Moxifloxacin has therefore been suggested for the therapy of ofloxacin-resistant TB.[2,4,6,10] However, it should only be used against strains with MICs that are below the maximum serum concentration and in combination with at least two other active antituberculosis agents.[4]

The new-generation fluoroquinolones have also been considered for use in a first-line regimen that has the ability to shorten the duration of TB treatment.[4] However, there are concerns that mutations conferring fluoroquinolone resistance may accumulate more rapidly[13] compared with other first-line antituberculosis drugs, thereby compromising the efficacy of the first-line treatment. An alternative view is to reserve the fluoroquinolones for the treatment of MDR- and XDR-TB. *M. tuberculosis* acquires resistance to the fluoroquinolones mainly through mutations in the quinolone resistance-determining regions (QRDRs) of the *gyrA* gene and, less frequently, in the *gyrB* gene.[9–12] The most frequent QRDR mutations are found at positions 90 (A90V), 91 (S91P) and 94 (D94G, D94A, D94Y, D94N and D94H).[4,9–11,14]

In this study, a large number of drug-resistant clinical *M. tuberculosis* isolates were used to: (i) assess the frequency of the different fluoroquinolone resistance mutations in *gyrA* in a high burden area with TB disease linked to coinfection with HIV;[15] and (ii) determine the association between nucleotide alterations in the A subunit of DNA gyrase and the level of phenotypic susceptibility to ofloxacin and moxifloxacin.

Materials and methods

Clinical isolates

M. tuberculosis isolates were collected from patients with drug-resistant TB and who were resident in the Western Cape, South Africa during the period 2007–09. These isolates have previously been subjected to routine drug susceptibility testing on Middlebrook 7H11 agar and had known IS*6110* restriction fragment length polymorphism[16] and spoligotype patterns.[17,18] One hundred and seventy-seven test isolates were selected from this collection, of which 43 were XDR, 25 pre-XDR (MDR with additional resistance to either a fluoroquinolone or an injectable), 54 MDR and 55 showed monoresistance to either isoniazid (n=52) or rifampicin (n=3). Sixty-five of the 177 isolates belonged to the typical Beijing family, 61 were atypical Beijing strains[19] and 42 were identified as members of the Low Copy Clade.[20] The remaining nine isolates were members of the Haarlem (n=4) and Latin American–Mediterranean (n=5) sublineages.[19] Each isolate represented a separate patient and their routinely determined susceptibility profiles were confirmed by critical concentration testing using MGIT 960 (Becton Dickinson Diagnostic Systems, Sparks, MD, USA).

DNA sequencing

A 345 bp region (codons 18–132) encompassing the QRDR of the *gyrA* gene (codons 74–113)[21] was PCR amplified with the QRDR primer set (forward 5′-TGACATCGAGCAGGAGATGC-3′ and reverse 5′-GGGCTTCGG TGTACCTCATC-3′) in combination with HotStarTaq DNA polymerase (Qiagen, Germany) under previously described conditions.[22] The primers were designed in-house using Primer3 software (v. 0.40).[23] Amplification products were sequenced with an ABI PRISM DNA sequencer (Applied Biosystems, Foster City, CA, USA) and the resulting chromatograms were analysed by use of Chromas software (Technelysium Pty Ltd).

Quantitative drug susceptibility testing and quality control

MICs were determined by quantitative drug susceptibility testing using an automated BACTEC MGIT 960 instrument (BD Bioscience, Sparks, MD, USA) equipped with TBeXiST and EpiCentre™ V5.75A software (BD Bioscience, Erembodegem, Belgium).[24] Stock solutions of ofloxacin (purchased from Sigma–Aldrich South Africa) and moxifloxacin (obtained from Bayer Pharma) were prepared by dissolving the respective drugs in 0.1 N NaOH before it was further diluted in distilled water. These were then filter-sterilized and stored at −80°C for up to 6 months. Ofloxacin was tested at 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 10 and 50.0 mg/L, and moxifloxacin at 0.125, 0.25, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0 and 10.0 mg/L. The κ coefficient was used to estimate the level of agreement between the phenotypic and genotypic results. Critical concentrations of 2.0 mg/L for ofloxacin and 0.25 mg/L for moxifloxacin, as per WHO criteria, were used to categorize isolates into susceptible and resistant strains.[1] For quality control purposes, a drug-susceptible *M. tuberculosis* reference strain H37Rv (ATCC 27294) was included as a control. Twenty-seven (15%) of the test isolates were also processed at the University of Zurich for genotypic and phenotypic susceptibility to monitor the accuracy and reproducibility of our results. The MIC results were based on at least duplicate findings.

Results

Mutations in the QRDR of gyrA

The genotypic and phenotypic findings of the 177 test isolates for ofloxacin and moxifloxacin are summarized in Table 1. Mutations in the QRDR were observed in 52 of the test isolates. The remaining 125 isolates lacked mutations and were considered wild-type for the sequenced region. The most prevalent mutations were observed at codon 94 (n=30), where aspartic acid was replaced with glycine (D94G; n=17), alanine (D94A; n=7) or asparagine (D94N; n=6). Twelve isolates harboured the A90V mutation and three the S91P mutation. Mutations G88C (n=1), D89G (n=1) and T80S (n=1) were detected in three separate isolates. Double point mutations were observed in four XDR isolates and their presence was confirmed by repeated sequencing. Both the D94G and A90V mutations were present in an atypical and a typical Beijing isolate, while D94G and D94N were detected in two atypical Beijing strains. The double mutants D94G/D94N can only be present as a result of infection with different strains (see the Discussion section).

Phenotypic resistance

The 55 selected monoresistant isolates were confirmed to be susceptible to both fluoroquinolones in MGIT 960. Fifty-four of these had a wild-type sequence in the fragment that was examined, while one isolate (a typical Beijing strain) carried a T80S mutation. All 54 MDR and 16 of the pre-XDR isolates were phenotypically and genotypically susceptible to ofloxacin and moxifloxacin. One atypical Beijing isolate was phenotypically classified as XDR, although no mutations were detected in the amplified *gyrA* fragment. The remaining pre-XDR (n=9) and

Table 1. MICs of ofloxacin and moxifloxacin for wild-type *M. tuberculosis* and mutated isolates with alterations in *gyrA* and flanking sequences

Codon mutation	Nucleotide change	*n*	MIC (mg/L)		Total number (%) with this mutation type	MIC range (mg/L)		MIC$_{50}$ (mg/L)		MIC$_{90}$ (mg/L)	
			OFX	MXF		OFX	MXF	OFX	MXF	OFX	MXF
T80S	ACC→TCC	1	≤0.5	≤0.125	1 (1.9)	≤0.5	≤0.125	≤0.5	≤0.125	≤0.5	≤0.125
G88C	GGC→TGC	1	10.0	4.0	1 (1.9)	10.0	2.0	10.0	2.0	10.0	2.0
D89G	GAC→GGC	1	2.0	1.0	1 (1.9)	2.0	1.0	2.0	1.0	2.0	1.0
A90V	GCG→GTG	1	2.0	0.25	12 (23.1)	2.0–6.0	0.25–1.0	4.0	1.0	6.0	1.0
		3	4.0	0.25							
		5	4.0	1.0							
		3	6.0	1.0							
S91P	TCG→CCG	1	4.0	0.5	3 (5.8)	4.0–6.0	0.5–1.0	4.0	1.0	4.0	1.0
		1	4.0	1.0							
		1	6.0	1.0							
D94A	GAC→GCG	3	4.0	1.0	7 (13.5)	4.0–10.0	1.0–2.0	6.0	1.0	10.0	2.0
		1	6.0	1.0							
		2	8.0	2.0							
		1	10.0	2.0							
D94G	GAC→GGC	1	6.0	1.0	17 (32.7)	6.0 to ≥10.0	1.0–2.0	≥10.0	2.0	≥10.0	2.0
		2	8.0	1.0							
		4	8.0	2.0							
		10	≥10.0	2.0							
D94N	GAC→AAC	1	4.0	0.5	6 (11.5)	4.0–10.0	0.5–4.0	10.0	2.0	10.0	4.0
		4	10.0	2.0							
		1	10.0	4.0							
A90V+D94G	GCG→GTG	1	4.0	1.0	2 (3.8)	4.0–8.0	1.0–2.0	4.0	1.0	8.0	2.0
	GAC→GGC	1	8.0	2.0							
D94N+D94G	GAC→AAC	1	8.0	2.0	2 (3.8)	8.0 to ≥10.0	2.0	8.0	2.0	10.0	2.0
	GAC→GGC	1	>10.0	2.0							
Mutants (*N*)		52						8.0	2.0	>10.0	2.0
Isolates with a wild-type *gyrA* sequence		1[a]	8.0	2.0		8.0	2.0	8.0	2.0	8.0	2.0
		106	≤0.5	≤0.125	none	≤0.5–2.0	≤0.125–0.25	≤0.5	≤0.125	1.0	≤0.125
		13	1.0	≤0.125							
		5	2.0	0.25							
Wild-type (*N*)		125									
Total number of isolates		177									
H37Rv[b]	control	6	≤0.5	≤0.125	none	≤0.5	≤0.125	≤0.5	≤0.125	≤0.5	≤0.125

OFX, ofloxacin; MXF, moxifloxacin.
[a]One isolate characterized as wild-type for *gyrA* had MICs of 8.0 and 2.0 mg/L for ofloxacin and moxifloxacin, respectively, but it may contain a mutation elsewhere.
[b]H37Rv (ATCC 27294) was included as a susceptible reference strain for *M. tuberculosis*.

XDR isolates (n=42) displayed decreased susceptibility to the two fluoroquinolones and all of them contained single nucleotide polymorphisms in a 21 kb region (codons 88–94) of the *gyrA* gene. Fluoroquinolone resistance occurred predominantly in two Beijing subgroups [atypical Beijing (33/61) and typical Beijing (17/65)], while only one (1/42) was from the Low Copy Clade. The overall agreement between genotypic and phenotypic susceptibility was 98% (174/177) for ofloxacin and 97% (172/177) for moxifloxacin. The κ values for agreement between the phenotypic and genotypic results were 0.945 (0.892–0.998) for

ofloxacin and 0.916 (0.851–0.982) for moxifloxacin. Minor susceptibility differences between the two fluoroquinolones were caused by borderline results rather than a lack of cross-resistance.

Quality control

Repeated genotypic and phenotypic testing of 27 isolates showed 100% agreement between the two laboratories. MIC variation between biological duplicates did not exceed one MIC dilution step, while the reference strain tested susceptible to both drugs in all repeats (Table 1).

Discussion

Our data demonstrate good correlation between nucleotide sequences in the A subunit of DNA gyrase and phenotypic susceptibility to ofloxacin and moxifloxacin in 177 *M. tuberculosis* isolates (see Table 1). Only one of the 125 isolates with a wild-type *gyrA* sequence showed phenotypic resistance to both drugs. This discrepancy is possibly caused by genetic changes in regions that we have not examined. A clear distinction between the susceptibility levels of the wild-type isolates compared with those harbouring fluoroquinolone-associated mutations was observed. However, a proportion of the isolates displayed decreased susceptibilities although the MICs were still within or marginally outside the normal wild-type distribution. Some of these isolates were identified with mutations associated with fluoroquinolone resistance, such as A90V and D89G, while others lacked nucleotide substitutions in the *gyrA* gene (see Table 1). Moderate increments in MICs may not result in therapeutic failure, but could possibly facilitate the accumulation of additional mutations in the QRDR and subsequently the selection of high-level resistant subpopulations. Mutations associated with fluoroquinolone resistance were found in 51 of the 177 isolates, which resulted from amino acid substitutions in codons 94, 90, 91, 89 and 88 and included four double mutations. One mutation in codon 80 (T80S) did not exhibit phenotypic resistance to either of the two fluoroquinolones. A previous study could also not associate a T80A mutation in GyrA of *M. tuberculosis* with fluoroquinolone resistance.[11] One isolate with a D89G substitution was resistant to moxifloxacin with an MIC (1.0 mg/L) 4-fold above the critical concentration (0.25 mg/L), although it remained susceptible to ofloxacin with an MIC equivalent to its critical concentration (2.0 mg/L). Based on our findings, this mutation, which was recently described,[25] confers borderline resistance to both drugs in *M. tuberculosis*. Twelve isolates harboured the A90V mutation. Eight of these had decreased susceptibilities to both fluoroquinolones and three to ofloxacin only, while one was susceptible to both drugs. A similar finding of an isolate susceptible to both ofloxacin and ciprofloxacin in the presence of the resistance-associated A90V mutation was previously reported.[25] Nine (9/12) isolates with an A90V mutation had MICs ranging from 2.0 to 4.0 mg/L for ofloxacin and from 0.25 to 1.0 mg/L for moxifloxacin. These MICs are scattered around the respective critical concentrations of the two drugs and implicate borderline susceptibility.[22] Conventional qualitative susceptibility testing is based on single critical concentrations, which are not suitable to distinguish between borderline (low-level), moderate-level and high-level resistance.[26] The MICs for four isolates with double mutations in codons 90 and 94 were not distinctively higher than those containing only single mutations (see Table 1). This is in contrast to previous reports that indicated a substantial decrease in susceptibility levels when multiple fluoroquinolone mutations are present in the same organism.[11,27,28] The presence of different populations either as variants of the same strain or as a mixture of two genetically independent strains was not established in this study. However, the double mutants D94G/D94N must be the result of mixed populations as two mutations cannot be present in the same codon, but the situation with the D94G/A90V combination is unclear.

Our data provide further evidence that decreased susceptibility to ofloxacin and moxifloxacin in *M. tuberculosis* isolates may occur from any one of several point mutations in the A subunit of DNA gyrase. Cross-resistance between ofloxacin and moxifloxacin was confirmed, although it was evident that the two drugs were not equally affected by mutations associated with fluoroquinolone resistance. The *gyrA* mutant isolates had an MIC_{50} of 8.0 mg/L and an MIC_{90} of >10 mg/L for ofloxacin, as opposed to an MIC_{50} and an MIC_{90} of 2.0 mg/L for moxifloxacin. Only two isolates (MIC 4.0 mg/L) had an MIC of >2.0 mg/L for moxifloxacin with a corresponding MIC of 10.0 mg/L for ofloxacin. The susceptibility levels for moxifloxacin in all of the isolates were below the peak serum concentration.[4] In contrast, 96% of the ofloxacin-resistant isolates had MICs equivalent to or above the achievable serum concentration.[4] In view of enhanced moxifloxacin activity, a clinical breakpoint of 2.0 mg/L is suggested for this drug to match the WHO's recommendation for ofloxacin.[1,9,10] A well-defined clinical breakpoint for moxifloxacin is important to guide MDR-TB treatment and should not be confused with an epidemiological cut-off (ECOFF) or critical concentration, which differentiates wild-type and non-wild-type strains.[12,26] The recommended ECOFF for moxifloxacin in MGIT 960 is 0.25 mg/L.[1] MDR-TB patients in South Africa are treated with ofloxacin prior to moxifloxacin and it is therefore likely that the test strains acquired fluoroquinolone resistance due to ofloxacin exposure. Based on our findings and the results of other *in vitro* studies,[9,10] we conclude that the use of moxifloxacin in the treatment of ofloxacin-resistant TB is justified, in particular as a component in combination with other drugs. A recent meta-analysis provided additional clinical evidence that the inclusion of new-generation fluoroquinolones in XDR-TB regimens significantly improved treatment outcomes.[2]

Judged by MIC_{50} and MIC_{90} values, mutations in codons 94 and 88 were linked to higher levels of resistance to both drugs compared with those in codons 90, 91 and 89, as shown in Table 1. This association is not absolute, since the MICs of fluoroquinolones for isolates with the same mutation may vary widely. Additional mechanisms other than *gyrA* mutations are likely to affect the level of fluoroquinolone resistance.[5]

Conclusions

Moxifloxacin is primarily administered against pre-XDR and XDR-TB, and its use is challenged by strains that have already acquired *gyrA* and/or *gyrB* mutations. The rationale of using moxifloxacin under these conditions is based on its enhanced antimycobacterial activity and favourable pharmacokinetic and pharmacodynamic characteristics.[4,7,8,12] Our findings demonstrated that moxifloxacin may still have clinical relevance in

the treatment of ofloxacin-resistant TB. However, a lack of effective companion drugs in a failing XDR regimen increases the risk of exposing TB infections to subinhibitory moxifloxacin concentrations.[13] Inappropriate use of this important drug class will radically worsen current attempts to control XDR-TB.

Moxifloxacin has the potential to eliminate both wild-type and first-step mutants more effectively than ofloxacin, which is important to prevent additional mutations and thus the development of XDR-TB. Bacteriological failure is generally associated with increased MICs[10] and for this reason we suggest that moxifloxacin is used as first-choice fluoroquinolone against MDR-TB. Sound scientific and clinical evidence should, however, be obtained and analysed to optimize treatment strategies.

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Transparency declarations

E. C. B. is a consultant for Becton Dickinson. All other authors: none to declare.

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