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ORIGINAL RESEARCH

Contribution of the R8 substituent to the in vitro antibacterial potency of besifloxacin and comparator ophthalmic fluoroquinolones

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Introduction: Previous work has shown that besifloxacin, an 8-chloro-fluoroquinolone, has more potent activity against gram-positive pathogens than moxifloxacin and gatifloxacin, which carry an 8-methoxy group. This study was conducted to determine the contribution of the R7 and R8 substituent to fluoroquinolone antibacterial activity.

Materials and methods: Besifloxacin, moxifloxacin, gatifloxacin, their R8 structural analogs, and ciprofloxacin were tested against representative isolates of various gram-positive and gram-negative species and previously characterized fluoroquinolone-resistant mutants of *Staphylococcus aureus*. Minimum inhibitory and minimum bactericidal concentrations were determined according to Clinical and Laboratory Standards Institute (CLSI) guidelines. Reserpine was used to determine the effect of efflux pumps on antibacterial activity.

Results: In general, exchanging the R8 residue in besifloxacin slightly reduced the molecule's potency, while introducing an 8-chloro group in moxifloxacin increased its potency. A similar change in gatifloxacin had little to no effect. Substituting the R8 residues did not increase the susceptibility to the efflux pump inhibitor reserpine or result in a loss of bactericidal activity. In contrast, the positive control, ciprofloxacin, was shown to be a substrate for reserpine and lost bactericidal activity against some fluoroquinolone-resistant isolates of *S. aureus*.

Conclusion: The data presented here show that, depending on the R7 substituent, replacing an 8-methoxy group with an 8-chloro substituent can improve potency or can have little-to-no effect. These findings highlight the importance of the interplay between the R7 and R8 substituents in determining antibacterial potency.

Keywords: moxifloxacin, besifloxacin, fluoroquinolone analogs, *Staphylococcus aureus*, resistance

Introduction

Fluoroquinolones have a broad spectrum of antibacterial activity, which makes them an ideal choice for the empiric treatment of infections of the surface of the eye. Ciprofloxacin, moxifloxacin, and gatifloxacin were approved many years ago for the systemic treatment of bacterial infections and more recently, for the treatment of bacterial conjunctivitis. However, resistance to commonly used fluoroquinolones is becoming more prevalent, even among ocular isolates.[1]

The potent antibacterial action of the fluoroquinolones is due to their ability to bind to the essential enzymes DNA gyrase and topoisomerase IV, which leads to double strand breaks in the DNA that ultimately results in cell death.[2] Bacterial resistance to this class of drugs primarily arises due to spontaneous mutations within the *gyrA*, *gyrB*, *parC* and *parE* genes that encode DNA gyrase and topoisomerase IV. In each of

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the four genes, most mutations that confer high-level fluoroquinolone resistance map within "hot spot" regions termed quinolone resistance-determining regions (QRDRs). Other resistance mechanisms, such as efflux pumps, can also play a contributing role in some instances.[3]

Besifloxacin is a novel chloro-fluoroquinolone that was approved in 2009 exclusively for the topical treatment of bacterial conjunctivitis.[4] Compared with the older fluoroquinolones, besifloxacin is unique due to the combination of a 7-azepinyl group and an 8-chloro substituent, making it the first chloro-fluoroquinolone in ophthalmic use (Figure 1). By comparison, the two fluoroquinolones most similar in structure and potency, moxifloxacin and gatifloxacin, both carry a methoxy group in the R8 position and a pyrrolol-pyridinyl or methyl-piperazinyl substituent, respectively, in the R7 position.[5,6]

Similar to many classes of antibacterial agents, the fluoroquinolones have undergone many rounds of chemical modifications to optimize their antibacterial, pharmacokinetic, and pharmacodynamic properties. One modification involves altering the substituent in the R8 position. Using a number of analogs to ciprofloxacin, gatifloxacin, and moxifloxacin, Lu et al[7] found that molecules with an 8-H atom were less potent than those with an 8-chloro or an 8-methoxy group. Interestingly, the differences in the analogs potency were more pronounced in the fluoroquinolone-resistant isolates of *Mycobacterium smegmatis* and *Staphylococcus aureus* when compared to the susceptible strains.

Besifloxacin was shown to be even more potent than moxifloxacin and gatifloxacin against gram-positive pathogens, while maintaining adequate activity against the gram-negatives.[8] A recent study by Sanfilippo et al[9] showed antibacterial activity to follow the order: besifloxacin > moxifloxacin > gatifloxacin > ciprofloxacin, when tested against 52 ocular clinical isolates of *S. aureus*.[9] Consistent with Lu's findings, the differences in antibacterial potency were more evident among the resistant isolates and generally increased proportionally to the number of mutations in the QRDRs of DNA gyrase and topoisomerase IV.

This improved activity of besifloxacin could be due to the unique 7-azepinyl substituent, the 8-chloro substituent that is lacking in the comparator drugs, or a combined effect of the R7 and R8 substituents. Since besifloxacin, moxifloxacin (aka BAY 12-8039), and gatifloxacin (aka AM1155, CG5501, or PD135432) differ only by their substituents in the R7 and R8 position, it was of interest to compare those molecules and their corresponding R8 structural analogs.[5,7] Therefore, the 8-methoxy structural analog of besifloxacin (BMO), the

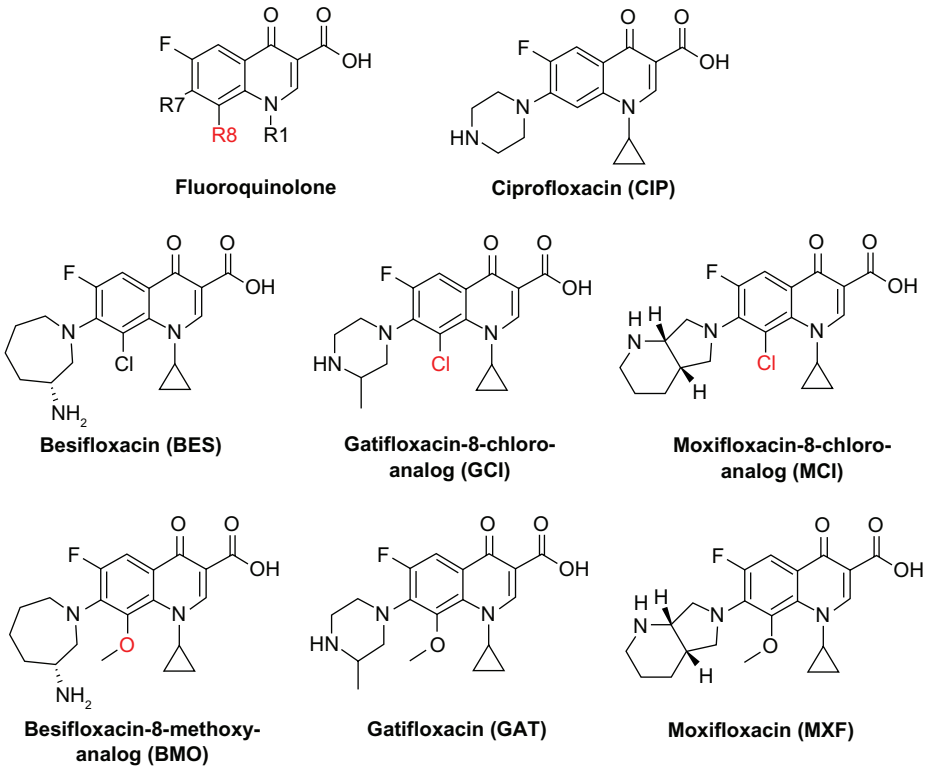

Figure 1 Chemical structure of the fluoroquinolones tested in this study.
Note: The R8 substituent that has been modified in the analogs is highlighted in red.

8-chloro analog of gatifloxacin (GCl) (aka PD138124), and the 8-chloro analog of moxifloxacin (MCl) (aka BAY y 3118) were obtained and compared with respect to their antibacterial potency (Figure 1).[6,7]

Materials and methods

Bacterial strains

The bacterial strains used in this study are listed in Table 1. The quality control strains *Enterococcus faecalis* ATCC 29212, *S. aureus* ATCC 29213, *Streptococcus pneumoniae* ATCC 49619, *Escherichia coli* ATCC 25922, *Haemophilus influenzae* ATCC 49247, and *Pseudomonas aeruginosa* ATCC 27853, as well as the wild-type clinical isolate *P. aeruginosa* PAO1 were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). The *P. aeruginosa* strains B1181 and D2133 were isolated at the University of Mississippi VA Medical Center (Jackson, MS, USA). The *H. influenzae* strain Hin1 was obtained from Eurofins Medinet Inc (Chantilly, VA, USA). The 52 *S. aureus* clinical isolates have been characterized and described previously.[9] The isolates were classified as ciprofloxacin-resistant, based on the Clinical and Laboratory Standards Institute (CLSI) interpretative criteria, and placed into different groups according to their QRDR mutations.[9,10]

Drugs and analogs

Besifloxacin was obtained from Bausch and Lomb, Inc (Rochester, NY, USA). Moxifloxacin, gatifloxacin, and ciprofloxacin were obtained from LKT Laboratories (St Paul, MN, USA). The 8-methoxy analog of besifloxacin, BMO, was synthesized by Dr Azhwarsamy Jeganathan at Bausch and Lomb, Inc (Rochester, NY, USA). Moxifloxacin and gatifloxacin and their 8-chloro analogs, MCl and GCl, respectively, were synthesized by Alembic Research Centre (Vadodara, India). All molecules made by Alembic were tested and confirmed by high-performance liquid chromatography (HPLC) and mass spectroscopy. The moxifloxacin and gatifloxacin produced by Alembic had the same antibacterial activity as the commercially available reagents. The efflux pump inhibitor reserpine was obtained from SPEX CertiPrep Group LLC (Metuchen, NJ, USA), and ethidium bromide was obtained from EMD Chemicals (Gibbstown, NJ, USA). All antimicrobial agents were solubilized and diluted as recommended by the manufacturers.

Antimicrobial susceptibility testing

All antimicrobial susceptibility tests were performed in triplicate; for each strain, modal or, when modal values could not be defined, central minimum inhibitory concentration

Table 1 Bacterial strains and groups of *Staphylococcus aureus* clinical isolates used in this study

Strain/group	Comments	Reference/source
Escherichia coli ATCC 25922	Quality control strain	ATCC
Pseudomonas aeruginosa ATCC 27853	Quality control strain	ATCC
Pseudomonas aeruginosa PAO1	Wild-type clinical laboratory isolate	ATCC
Pseudomonas aeruginosa B1181	Fluoroquinolone-resistant clinical isolate	University of Mississippi VA Medical Center
Pseudomonas aeruginosa D2133	Fluoroquinolone-resistant clinical isolate	University of Mississippi VA Medical Center
Haemophilus influenzae ATCC 49247	Quality control strain	ATCC
Haemophilus influenzae Hin1	Fluoroquinolone-resistant clinical isolate	Eurofins Medinet
Streptococcus pneumoniae ATCC 49619	Quality control strain	ATCC
Enterococcus faecalis ATCC 29212	Quality control strain	ATCC
Staphylococcus aureus ATCC 29213	Quality control strain	ATCC
Staphylococcus aureus group 1	13 ciprofloxacin-susceptible clinical isolates and strain ATCC 29213	Sanfilippo et al[9]
Staphylococcus aureus group 2	12 ciprofloxacin-resistant clinical isolates; mutations in GyrA (Ser84-Leu) and ParC (Ser80-Phe/Tyr); one strain had an additional ParE (Pro585-Ser) mutation that contributed to resistance	Sanfilippo et al[9]
Staphylococcus aureus group 3	6 ciprofloxacin-resistant clinical isolates; mutations in GyrA (Ser84-Leu) and ParC (Ser80-Tyr, Glu84-Gly)	Sanfilippo et al[9]
Staphylococcus aureus group 4	3 ciprofloxacin-resistant clinical isolates; mutations in GyrA (Ser84-Leu, Ser85-Pro), ParC (Ser80-Phe/Tyr), and ParE (Asp432-His/Asn)	Sanfilippo et al[9]
Staphylococcus aureus group 5	10 ciprofloxacin-resistant clinical isolates; mutations in GyrA (Ser84-Leu, Ser85-Pro) and ParC (Ser80-Tyr, Glu84-Gly)	Sanfilippo et al[9]
Staphylococcus aureus group 6	8 ciprofloxacin-resistant clinical isolates; mutations in GyrA (Ser84-Leu, Glu88-Ala/Lys) and ParC (Ser80-Tyr, Glu84-Gly); strains with a Glu88-Ala mutation had lower MICs than those with Glu88-Lys mutations	Sanfilippo et al[9]

(MIC) and minimum bactericidal concentration (MBC) values are reported here. MIC testing was performed by the broth microdilution method, in accordance with CLSI reference methods (CLSI M07-A8).[11] Briefly, 96-well panels containing serial twofold dilutions of antimicrobial agent were inoculated with ~5×10^4 colony forming units per well; the panels were incubated according to CLSI guidelines, and the MIC was reported as the lowest antimicrobial concentration that inhibited the visible growth of bacteria. To test for the contribution of the efflux pump NorA to the fluoroquinolone resistance of *S. aureus*, the MIC measurements were also performed in the presence of 20 μg/mL of the pump inhibitor reserpine as described elsewhere,[9] using ethidium bromide as a positive control.

Bactericidal activities were measured as follows: after overnight incubation to determine MIC values, MBC values were determined by spotting 10 μL from those wells that were at and above the recorded MIC values on drug-free agar medium, in accordance with CLSI reference methods (CLSI M26-A).[12] The number of surviving colony forming units after overnight incubation were counted and compared with the inocula. The MBC was defined as the drug concentration that resulted in a ≥3 log decrease in viable bacteria.

Results

Analogs against various species

In order to determine the contribution of the R7 and R8 substituents to the antimicrobial efficacy of the seven fluoroquinolones, we determined the MIC values against various gram-positive and gram-negative species, including fluoroquinolone-resistant isolates (Table 2).

The MIC values for besifloxacin were identical to or twofold lower than those of BMO, indicating that the R8 substituent did not influence antibacterial activity when the R7 substituent was an azepinyl moiety.

In contrast, the 8-chloro analog of moxifloxacin, MCl, was fourfold more potent than moxifloxacin itself against each of the three gram-positive species, while MCl was eight- to 16-fold more active than moxifloxacin against gram-negative strains. This indicates that a chloro substituent in the R8 position does improve potency if the R7 substituent is a pyrrolol-pyridinyl group.

The activity of the 8-chloro analog of gatifloxacin, GCl, was the same as that of gatifloxacin against *S. aureus* and was twofold lower against *E. faecalis* and *S. pneumoniae*. GCl was twofold more potent than gatifloxacin against *E. coli* and *P. aeruginosa*, while both drugs were equally potent against *H. influenzae*. In this instance, with a methyl-piperazinyl moiety in the R7 position, an 8-chloro group either had no effect on potency or decreased it by twofold.

While the fluoroquinolones besifloxacin and gatifloxacin, with an azepinyl or a methyl-piperazinyl group in the R7 position, respectively, were little affected by the 8-chloro or 8-methoxy substituent, the activity of the 7-pyrrolol-pyridinyl substituent-containing moxifloxacin and MCl were more strongly influenced by the nature of the R8 moiety. Overall, these data show that, depending on the bacterial species and the R7 substituent, replacing the 8-methoxy with an 8-chloro group can improve potency, have no effect, or reduce potency. This finding highlights the importance of the interplay between the R7 and R8 substituents in determining antibacterial potency.

Activity against 53 *S. aureus* isolates

Previous work with groups of ciprofloxacin-resistant *S. aureus* mutants has shown that fluoroquinolone MIC values generally increased with the number of mutations in the *gyrA*, *gyrB*, *parC*, and *parE* genes and that this increase affected older fluoroquinolones more drastically than newer ones.[9] This suggested that the R7, the R8, or a combination of the two moieties was able to reduce the impact of resistance-conferring mutations. To address this issue, we tested besifloxacin, moxifloxacin, gatifloxacin, their respective R8 analogs, and ciprofloxacin against six groups of *S. aureus* strains that differed in their levels of ciprofloxacin-resistance and that contained various QRDR mutations known to contribute to resistance (Figure 2).

The 14 isolates in group 1, which include 13 clinical isolates and 1 quality control strain, contained no resistance-conferring mutations and had correspondingly low MIC values. MIC_{50} values, the drug concentrations that inhibit the growth of 50% of isolates, increased in the order MCl (0.015 μg/mL) < besifloxacin (0.03 μg/mL) < BMO (0.06 μg/mL) = moxifloxacin (0.06 μg/mL) < gatifloxacin (0.12 μg/mL) = GCl (0.12 μg/mL) < ciprofloxacin (0.5 μg/mL). Exchanging the 8-chloro for an 8-methoxy group had different effects, depending on the R7 substituent: The MIC values for the besifloxacin analog BMO were either identical (in 64% of isolates) or twofold higher (36%) than the MIC values for besifloxacin. The MIC values for moxifloxacin were twofold (29%) or fourfold (71%) above that of the moxifloxacin analog MCl, while gatifloxacin had either identical (86%) or lower (14%) MIC values than the gatifloxacin analog GCl.

Against the 39 ciprofloxacin-resistant isolates in groups 2 through 6, the MIC values increased with the number and

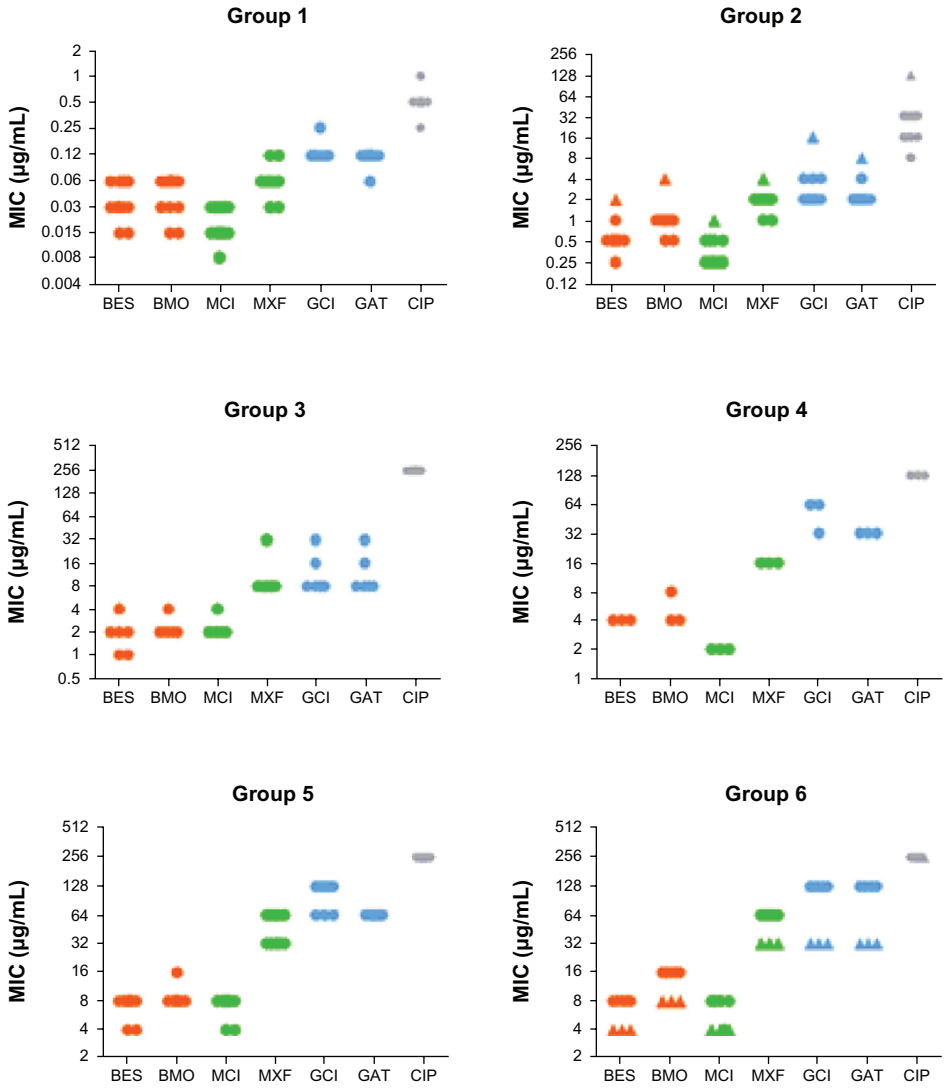

Figure 2 Minimum inhibitory concentrations of various clinical isolates of *Staphylococcus aureus* against the fluoroquinolones besifloxacin, besifloxacin-8-methoxy analog, moxifloxacin-8-chloro analog, moxifloxacin, gatifloxacin-8-chloro analog, gatifloxacin, and ciprofloxacin.

Notes: See Table 1 for the description of each group of isolates. Same colors indicate the identical C7 substituent on the fluoroquinolone. Triangles indicate strains with unique mutations that formed subgroups within groups 2 and 6.

Abbreviations: BES, besifloxacin; BMO, besifloxacin-8-methoxy analog; CIP, ciprofloxacin; MCI, moxifloxacin-8-chloro analog; MIC, minimum inhibitory concentrations; MXF, moxifloxacin; GAT, gatifloxacin; GCI, gatifloxacin-8-chloro analog.

nature of mutations, but the overall trends remained the same as in the ciprofloxacin-susceptible group: the MIC values for besifloxacin were identical (for 38% of isolates) or twofold (62%) lower than those for BMO, while GCl had identical (69%) or twofold higher (31%) MIC values than gatifloxacin. MCl was fourfold (38%), eightfold (59%), or 16-fold (3%) more potent than moxifloxacin.

These results show that, depending on the R7 moiety, exchanging the R8 substituent can have different effects on antibacterial potency against *S. aureus* isolates. Replacing the 8-chloro with an 8-methoxy group in besifloxacin either had no effect or resulted in a twofold decrease in potency. In the case of the gatifloxacin analog GCl, the same change either had no effect or resulted in a twofold increase in potency. The moxifloxacin analog MCl was always more potent than moxifloxacin; two- to fourfold against ciprofloxacin-susceptible isolates and four- to 16-fold against ciprofloxacin-resistant isolates.

The effect of reserpine

The staphylococcal efflux pump NorA has been shown to contribute to fluoroquinolone resistance in *S. aureus*.[3] While the pump has a wide spectrum of substrates, some molecules are more susceptible to the action of NorA than others. For example, ethidium bromide is rapidly exported, while the fluoroquinolones show various degrees of susceptibility.

In order to determine the impact of the fluoroquinolone's R7 and R8 substituents on NorA-mediated efflux, we determined the MIC values of the 53 *S. aureus* strains in the presence and absence of the plant alkaloid reserpine, which is an inhibitor of NorA (Table 3).

For 69.8% or more of *S. aureus* isolates, MIC values for moxifloxacin, gatifloxacin, and their 8-chloro analogs did not change in the presence of reserpine, suggesting that NorA has no impact on fluoroquinolone resistance in these strains. The remaining strains exhibited either a twofold increase or a twofold decrease in MIC values in the presence of reserpine, which could be attributed to experimental fluctuation. In the case of besifloxacin, reserpine did not change the besifloxacin MIC values for 64.2% of the isolates and resulted in twofold lower MIC values for 34.0% of isolates. For BMO, following reserpine treatment, no change in the MIC values was determined in 43.4% of isolates, and a twofold lower MIC was exhibited in 54.7% of the isolates. These data suggest that NorA-mediated export plays little to no role in the antistaphylococcal potency of besifloxacin, moxifloxacin, gatifloxacin, and their R8 analogs. In contrast, the ciprofloxacin MIC values decreased by twofold for 17.0% of isolates and by fourfold for 30.2% of isolates. Even more noticeably, MIC values for the ethidium bromide positive control increased by fourfold for 20.8% of isolates, by eightfold for 15.1% of isolates, and by 16-fold for 9.4% of isolates, confirming that ciprofloxacin and especially ethidium bromide are good substrates for NorA.

The contribution of the R8 substituent to bactericidal activity

Previous work has shown that ciprofloxacin loses its bactericidal activity against fluoroquinolone-resistant strains of *S. aureus*.[13] In order to determine whether the fluoroquinolone analogs maintain the same bactericidal activity as their commercially available counterparts, we measured the MBC for each strain listed in Table 2 and one representative isolate per *S. aureus* mutant group depicted in Figure 2. The MBC-to-MIC ratios for besifloxacin, BMO, moxifloxacin, MCl, gatifloxacin, GCl, and ciprofloxacin were ≤4:1 and usually 1:1 or 2:1 (data not shown). This indicates that all analogs maintained potent bactericidal activity, even against fluoroquinolone-resistant isolates. The only exception was a *S. aureus* isolate in mutant group 2, which had a MBC:MIC ratio of 16:1 for ciprofloxacin. An MBC:MIC ratio ≥8:1 is considered bacteriostatic, which was in good agreement with earlier time-kill experiments.[13] Overall, our results show that the presence of a chloro or a methoxy group in the R8 position does not notably alter bactericidal activity.

To test whether the loss of bactericidal action was linked to a specific resistance genotype, such as the one found in mutant group 2, we determined the ciprofloxacin MBC:MIC ratio for all of the 53 *S. aureus* isolates. Besifloxacin was used as a positive control. For most *S. aureus* isolates, the ciprofloxacin MBC:MIC ratios were 1:1 or 2:1 and did not exceed 4:1, indicating that these isolates were rapidly killed by the drug despite high levels of fluoroquinolone resistance (Figure 3). The exceptions to this were eight of the 12 isolates in mutant group 2, which had MBC:MIC ratios of 8:1 or 16:1, demonstrating that these isolates were no longer effectively killed by ciprofloxacin. In contrast, besifloxacin maintained its high bactericidal potency with MBC:MIC ratios of 1:1 or 2:1 against those strains. Further studies are required to determine why those particular isolates, and not others, lost their susceptibility to be killed by ciprofloxacin.

Discussion

The MIC data presented here for various species demonstrated that all of the seven fluoroquinolones had

Table 2 Minimum inhibitory concentrations of besifloxacin and comparators against various species and phenotypes

Species/strain	Phenotype	MIC (μg/mL)						
		BES	BMO	MCl	MXF	GCl	GAT	CIP
E. coli 25922		0.12	0.25	0.008	0.06	0.015	0.03	0.02
P. aeruginosa 27853		4	8	0.25	8	2	4	0.5
P. aeruginosa PAO1		1	1	0.25	1	0.5	0.5	0.02
P. aeruginosa B1181	FQR	8	8	2	8	8	4	2
P. aeruginosa D2133	FQR	4	8	8	64	32	32	16
H. influenzae 49247		0.03	0.06	0.008	0.06	0.03	0.03	0.02
H. influenzae Hin1	FQR	1	2	1	16	4	4	32
S. pneumoniae 49619		0.06	0.06	0.03	0.12	0.5	0.25	0.5
E. faecalis 29212		0.25	0.25	0.12	0.5	1	0.5	1
S. aureus 29213		0.03	0.03	0.015	0.06	0.12	0.12	0.5

Abbreviations: BES, besifloxacin; BMO, besifloxacin-8-methoxy analog; CIP, ciprofloxacin; FQR, fluoroquinolone-resistant; GAT, gatifloxacin; GCl, gatifloxacin-8-chloro analog; MCl, moxifloxacin-8-chloro analog; MIC, minimum inhibitory concentration; MXF, moxifloxacin.

Table 3 Contribution of reserpine-susceptible efflux pumps to fluoroquinolone MIC values in 52 clinical ophthalmic *Staphylococcus aureus* isolates and control strain ATCC29213

Drug	Number (percent) of isolates with x-fold change in MIC in response to reserpine					
	Increase	No change	Decrease			
	2x		2x	4x	8x	16x
BES	1 (1.9)	34 (64.2)	18 (34.0)			
BMO	1 (1.9)	23 (43.4)	29 (54.7)			
MXF	11 (20.8)	38 (71.7)	4 (7.5)			
MCl	7 (13.2)	37 (69.8)	9 (17.0)			
GAT	10 (18.9)	38 (71.7)	5 (9.4)			
GCl	5 (9.4)	43 (81.1)	5 (9.4)			
CIP		28 (52.8)	9 (17.0)	16 (30.2)		
EtBr		3 (5.7)	26 (49.1)	11 (20.8)	8 (15.1)	5 (9.4)

Notes: The MIC value of a strain grown in the presence of reserpine was divided by the MIC of the same strain grown in the absence of reserpine. The number of isolates that showed an increase or decrease in MIC values is shown.
Abbreviations: BES, besifloxacin; BMO, besifloxacin-8-methoxy analog; CIP, ciprofloxacin; EtBr, ethidium bromide; GAT, gatifloxacin; GCl, gatifloxacin-8-chloro analog; MCl, moxifloxacin-8-chloro analog; MIC, minimum inhibitory concentration; MXF, moxifloxacin.

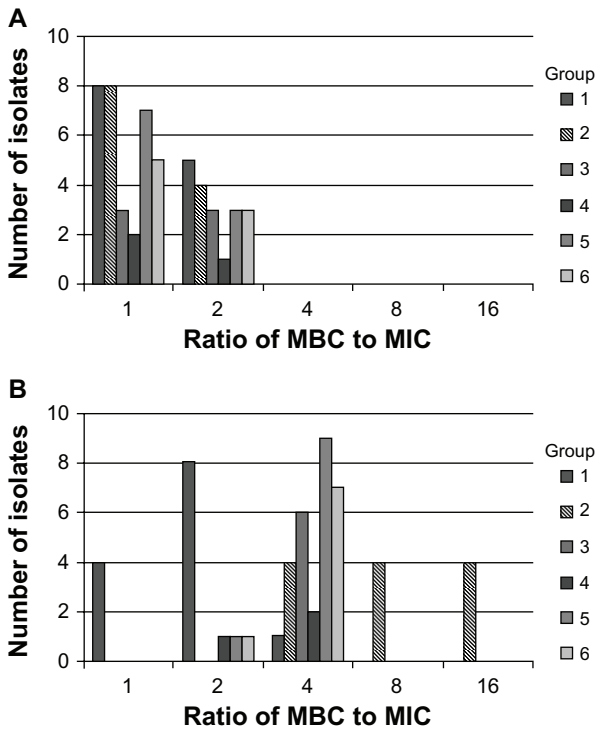

Figure 3 Ratio of minimum bactericidal concentrations to minimum inhibitory concentrations against 52 *Staphylococcus aureus* strains, including the fluoroquinolone-resistant isolates in groups 2–6, for (**A**) besifloxacin and (**B**) ciprofloxacin.
Notes: The number of isolates in each group was as follows: group 1: 13; group 2: 12; group 3: 6; group 4: 3; group 5: 10; and group 6: 8.
Abbreviations: MBC, minimum bactericidal concentrations; MIC, minimum inhibitory concentrations.

broad spectrum activity against various gram-positive and gram-negative species. Comparing besifloxacin, moxifloxacin, and gatifloxacin with their R8 analogs showed that it is the combination of the R7 and R8 substituents that determines the potency of the fluoroquinolone. For example, against the gram-positive pathogens, antibacterial potency followed the order: MCl > besifloxacin > GCl, when the R7 substituent was a chloro group; but it was: BMO > moxifloxacin > gatifloxacin when the R7 substituent was a methoxy group. When the R7 substituent was constant and the R8 moiety was changed from a chloro to a methoxy group, MIC values for the besifloxacin/BMO pair remained constant or increased by twofold, increased by four- to eightfold for the MCl/moxifloxacin pair, and remained constant or decreased by twofold for the GCl/gatifloxacin pair. Therefore, replacing the 8-chloro with an 8-methoxy group can have either no or little effect, as in the case of besifloxacin and the gatifloxacin analog GCl, or it can make a big difference in antibacterial potency, as exemplified by the moxifloxacin analog MCl.

MIC values for besifloxacin, moxifloxacin, gatifloxacin, and ciprofloxacin against *S. aureus*, *E. faecalis*, *E. coli*, and *P. aeruginosa* were within the quality control ranges suggested by CLSI.[10] The MIC data for the ATCC quality control strains of *E. faecalis*, *S. aureus*, *S. pneumoniae*, *E. coli*, and *P. aeruginosa* were similar to previously published MIC values for besifloxacin, moxifloxacin, MCl, and gatifloxacin.[5,8,14–18] Little has been published about the antibacterial potency of GCl, and no manuscripts that describe the antibacterial potency of BMO have been identified in the literature.[7]

For ciprofloxacin-resistant strains of *S. aureus*, the MIC values presented here were remarkably similar to those published by others, especially if one considers the differences in genetic background and testing methods. Fukuda et al[19] tested gatifloxacin, ciprofloxacin, and other fluoroquinolones against sequentially obtained quinolone-resistant mutants of *S. aureus* that were similar to mutant groups 2, 3, and 6 in our study.[19]

Lu et al[7] tested the activity of gatifloxacin, GCl, and comparator molecules that differed only in their R8 substituent against two strains of *S. aureus* that were fluoroquinolone-susceptible and -resistant, respectively. Similar to the data presented here, gatifloxacin and GCl had virtually identical activities against these two strains, while moxifloxacin was more potent than both drugs.[7] For the gatifloxacin analogs with various R8 substitutions, antistaphylococcal potency increased in the order: H < F < Br < Cl < methoxy (MO), for the fluoroquinolone-susceptible isolate, and H < F < Br < MO < Cl, for the fluoroquinolone-resistant isolate.[7] The enhancement in potency due to the Br, MO, or Cl R8 substituent was especially notable in the case of the

fluoroquinolone-resistant strain, which is consistent with the findings presented here.

The 53 strains of *S. aureus* were of interest because their susceptibility sheds light on the nature of the fluoroquinolone–target interaction. Strains were grouped based on their mutations in the QRDR of *gyrA* and *gyrB* (encoding DNA gyrase) and *parC* and *parE* (encoding topoisomerase IV), which confer high-level fluoroquinolone resistance.

Strains in group 1 contained no mutations, while isolates in groups 2 and 3 contained two or three mutations, respectively. All strains in groups 4–6 contained four mutations each, but the mutated amino acid was different in each group. It could be expected that, if a particular amino acid in the quinolone-binding site of the target protein was interacting with the R8 substituent of the fluoroquinolone, then a change in that amino acid or in the R8 substituent might be expected to change the MIC value. However, this does not seem to be the case based on the data presented here. Regardless of the group of *S. aureus* mutants, besifloxacin had the same potency or was twofold more potent than BMO, while GCl had the same potency or was twofold less potent than gatifloxacin. Similarly, MCl was more potent than moxifloxacin: two- to fourfold more potent against the ciprofloxacin-susceptible strains in group 1 and four- to eightfold (and in one case 16-fold) more potent against the ciprofloxacin-resistant strains. Moreover, there was some natural fluctuation in the MIC data, which was, at least to some degree, mitigated by taking MIC readings from three independent susceptibility tests. Despite this, MIC values were rather consistent within the mutant groups and rarely varied by more than a twofold dilution. In some instances, one strain in group 2 and three strains in group 6, this fluctuation seemed to be linked to specific mutations that caused those strains to be slightly different from the other strains in the group. The one strain in group 2 carries a Pro585-Ser mutation in *parE* that is absent in all the other strains, which might increase fluoroquinolone resistance.[9] The three strains in group 6 carry Glu88-Ala mutations in GyrA instead of the Glu88-Lys mutations found in the other strains in this group. Strains with the Glu88-Ala mutation showed consistently lower resistance levels than strains with a Glu88-Lys. Surprisingly, other mutations that were previously presumed to result in differences in fluoroquinolone resistance, such as the ParC-80 and ParE-432 mutations in the three strains of group 3, did not have the expected effect.[9]

These results show that a twofold difference in MIC is likely not to be biologically meaningful. Therefore, since the slight variations in MIC values within the pairs of R8 analogs is most likely due to natural variation and the overall trend remains the same from one mutant group to another, it is reasonable to assume that the R8 substituent does not interact with residues 84, 85, and 88 of GyrA, 80 and 84 of ParC, or 432 and 585 of ParE, at least not to the extent that it would alter the MIC value measurably. More sophisticated methods, such as X-ray crystallography, might shed a better light on these interactions.

Bax et al[20] investigated the three-dimensional structure of *S. aureus* DNA gyrase in a complex with DNA and ciprofloxacin.[20] Based on that model (NCBI [National Center for Biotechnology Information] Protein database code 2XCT), the R7 substituent of ciprofloxacin appears adjacent to Asn476, which is located at the end of an α-helix. The R8 moiety of the fluoroquinolone lies opposite of Arg458, which is located between a β-sheet and an α-helix. Both amino acids are part of the Toprim domain of the GyrB subunit. How Asn476 and Arg458 interact with the fluoroquinolones is currently unknown, and no *S. aureus* strain investigated in this study contained a mutation in these amino acids. However, Pan and Fisher,[21] using clinafloxacin selection in *S. pneumoniae*, obtained strains containing mutations in the corresponding amino acids, Glu474 and Pro454, respectively.[21] Both mutations resulted in minor increases in fluoroquinolone MIC values. Additional evidence for the importance of GyrB Arg458 in fluoroquinolone resistance comes from work done in *E. coli*, where Arg458 corresponds to Lys447. Strains with Lys447-Glu mutations in GyrB were found to be resistant to some quinolones, but hypersusceptible to others.[22] Unfortunately, the quinolones tested in this study were structurally very diverse, so no conclusions about possible interactions between the R8 substituent of the quinolone and the amino acids Lys447 or Glu447 of GyrB can be drawn. A better understanding of the interactions between Asn476 and Arg458 and the fluoroquinolones will have to await further mutational analysis.

Previous results by Shinabarger et al,[3] using genetically defined mutants and various pump inhibitors, have shown that moxifloxacin is not a substrate for NorA-mediated efflux. The data presented here confirm these results and further show that gatifloxacin, MCl, and GCl MIC values also remain virtually unchanged in the presence of the pump inhibitor reserpine. Therefore, the presence of a chloro or a methoxy group in the R8 position appears to have little impact on NorA-mediated efflux. The observation that some strains exhibited either a twofold increase or a twofold decrease in MIC values in the presence of reserpine might be due to natural variation in a biological system.

Shinabarger et al also showed that besifloxacin is a poor substrate for NorA, which was also confirmed in this study. Changing the 8-chloro to an 8-methoxy resulted in 11 (20.7%) additional strains that exhibited a reserpine-induced twofold decrease in MIC values, a change that could be due to natural fluctuations or due to an increased ability of NorA to export BMO when compared to besifloxacin. The latter hypothesis is consistent with work by Takenouchi et al, who proposed a correlation between the activity of efflux pumps and the bulkiness of the R7 substituent and the bulkiness and hydrophobicity of the R8 substituent.[23] However, even if replacement of the 8-chloro with an 8-methoxy group made BMO a better substrate for NorA, the effect is rather subtle and probably not biologically significant. In contrast, MIC values for ciprofloxacin and ethidium bromide changed more drastically in the presence of reserpine, confirming that they are good or very good substrates for NorA.

Work by Lu et al showed that the effect of the R8 substituent on the ability to kill cells was dependent on the R7 substituent, since changing the R8-H in ciprofloxacin to a 8-chloro group improved bactericidal activity, while the kill rates of moxifloxacin or gatifloxacin did not notably change when the 8-methoxy group was replaced with an 8-H or an 8-chloro group.[7] The results presented here show that, although their absolute potency varied, the ophthalmic fluoroquinolones besifloxacin, moxifloxacin, gatifloxacin, and their R8 analogs had potent bactericidal activity, even against ciprofloxacin-resistant isolates. Replacing the 8-chloro with an 8-methoxy substituent, or vice versa, did not alter the MBC:MIC ratios, suggesting that the two substituents contribute equally (or not at all) to the lethal activity of the agents tested.

In contrast, ciprofloxacin was bactericidal for some isolates of *S. aureus* but only bacteriostatic against others, particularly against strains in mutant group 2. These findings are consistent with previous time kill experiments that had shown that ciprofloxacin was unable to reduce the number of ciprofloxacin-resistant *S. aureus* and *Staphylococcus epidermidis* cells below the levels of the initial inocula within 2 hours.[13] Isolates in mutant group 2 all contain a Ser84-Leu mutation in GyrA and a Ser80-Tyr or -Phe mutation in ParC. However, there was no obvious correlation between the genotype of the mutants and the lack of bactericidal activity of ciprofloxacin, requiring further investigation. While ciprofloxacin had low potency and only bacteriostatic activity against ciprofloxacin-resistant *S. aureus* isolates, the more modern fluoroquinolones besifloxacin, moxifloxacin, and gatifloxacin that are in ophthalmic use today, have more potent activity and retain their bactericidal action.

The data presented here show that MCl is more potent than moxifloxacin, begging the question why moxifloxacin is commercially available while MCl is not. Development of MCl (aka BAY y 3118) has been discontinued because it is photochemically labile, producing radicals in the presence of ultraviolet A (UVA) light and oxygen.[6,24,25] While toxicity issues might have prevented the development of certain fluoroquinolones for systemic use, the ophthalmic fluoroquinolones available today for the treatment of ocular infections have been shown to be safe and effective.[26–29]

Previous work had shown that besifloxacin, an 8-chloro-fluoroquinolone, had more potent activity against gram-positive pathogens than moxifloxacin and gatifloxacin, which carry an 8-methoxy group. The data presented here show that, depending on the R7 substituent, replacing an 8-methoxy group with an 8-chloro substituent can improve potency or can have little-to-no effect. However, there was no difference between the 8-chloro and the 8-methoxy group with respect to NorA-mediated efflux or bactericidal activity. These findings highlight the importance of the interplay between and contributions from both the R7 and R8 substituents in determining antibacterial potency.

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Disclosure

All authors work for Bausch & Lomb Incorporated. The authors report no other conflicts of interest in this work.

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