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Activity of non-fluorinated quinolones (NFQs) against quinolone-resistant *Escherichia coli* and *Streptococcus pneumoniae*

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The newly developed 8-methoxy, non-fluorinated quinolones (NFQs) were studied to elucidate their enzyme inhibitory activity against wild-type and mutant GyrA (Ser-83→Trp) forms of *Escherichia coli* DNA gyrase. Using a DNA supercoiling inhibition assay, the NFQs were found to inhibit 50% (IC_{50}) of the *E. coli* DNA gyrase activity in the 1.6–3.2 mg/L concentration range and were comparable to ciprofloxacin. However, against the GyrA (Ser-83→Trp) mutant, the NFQs were ~16-fold more potent than ciprofloxacin. Antibacterial potency of the NFQs was investigated using clinical isolates of *E. coli* and penicillin-resistant *Streptococcus pneumoniae* (PRSP), including strains with reduced susceptibility to quinolones. Against 20 uncharacterized clinical isolates of *E. coli*, the MIC_{90}s of the NFQs were in the 0.125–0.25 mg/L range while those of ciprofloxacin, trovafloxacin, gatifloxacin and clinafloxacin were in the 0.016–0.125 mg/L range. Against clinical isolates with characterized mutations in *gyrA* and *parC*, PGE9262932, an NFQ, was two- to eight-fold more potent than ciprofloxacin. Against 23 clinical isolates of PRSP, the NFQs (MIC_{90} 0.031–0.125 mg/L) were more potent than ciprofloxacin, trovafloxacin, and gatifloxacin (MIC_{90} 0.25–2.0 mg/L), and at least as potent as clinafloxacin (MIC_{90} 0.125 mg/L). Against *S. pneumoniae* strains with *gyrA* and *parC* mutations, the NFQs (MIC 0.125–1.0 mg/L) were more potent than ciprofloxacin, trovafloxacin and gatifloxacin (MIC 4–32 mg/L), and comparable to clinafloxacin (MIC 0.5–1 mg/L).

Introduction

Point mutations in the conserved serine and aspartate/glutamate residues of the quinolone resistance determining regions (QRDR) of the target enzymes, DNA gyrase and topoisomerase IV, have been associated with quinolone resistance in many bacterial pathogens.[1] Apparently, the level of resistance to a given quinolone owing to these mutations depends on the effective potency toward the mutated target as well as the second target. While resistance to quinolones is widespread in certain bacterial species,[2] it is also observed in other clinically relevant pathogens including *Streptococcus pneumoniae*,[3] commonly encountered in respiratory tract infections, and *Escherichia coli*,[4] frequently associated with urinary tract infections. Over the past decade, resistance to penicillins and macrolides in *S. pneumoniae* has emerged as a significant problem.[5] The recent emergence of quinolone resistance in *S. pneumoniae* raises the potential for multidrug-resistant (resistant to quinolone, penicillin, macrolide) forms of this pathogen to pose a serious threat in the future. In the case of both *S. pneumoniae* and *E. coli*, point mutations in the QRDR are known to be a major factor in the development of resistance to quinolones in clinical isolates.[6–8] Thus, the effectiveness of quinolones as antibacterial therapy in the future will depend, in part, on their ability to overcome the effect of these point mutations.

Recently, a series of 8-methoxy, non-fluorinated quinolones (NFQs; Figure 1) has been developed and shown to have broad-spectrum antibacterial activity[9] with relatively high potency against quinolone-resistant staphylococci.[10] In this report, we describe: (i) the enzyme inhibitory activity of these NFQs against *E. coli* DNA gyrase; and (ii) the antibacterial activity of the NFQs against clinical isolates of *E. coli* and *S. pneumoniae*, including strains with specific point mutations in *gyrA* and *parC*.

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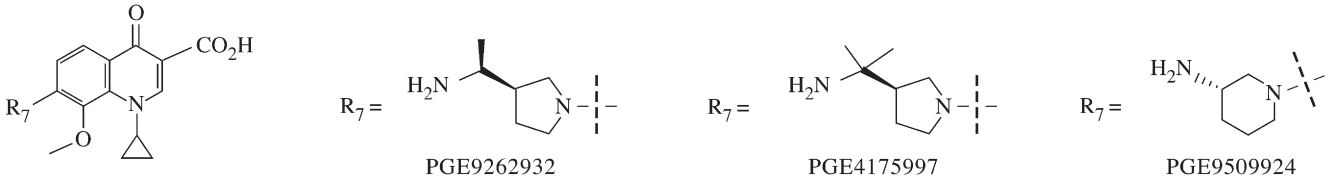

Figure 1. Chemical structures of the NFQs. The basic quinolone structure and different substitutions in the R7 positions of the three leads are shown.

Materials and methods

Materials and bacterial strains

Benchmark and test quinolones were synthesized in-house. For the MIC analysis, clinical isolates of *E. coli* and penicillin-resistant *S. pneumoniae* (PRSP) were obtained from Procter & Gamble Pharmaceuticals' internal culture collection and major hospitals in the greater Cincinnati area (Health Alliance Laboratories) during 1998–1999. These isolates were collected at random without regard for their susceptibility to specific quinolones. For studies with *gyrA* and *parC* mutants, *E. coli* strain WT, its *in vitro*-selected mutant MI, and the clinical isolate U12987 were obtained from Dr Peter Heisig.[11,12] Additional *E. coli* clinical isolates C-4, 1363, 1331 and 1334 were obtained from Dr Jordi Vila.[13] *S. pneumoniae* strains 02J1016, 02J1056 and their *in vitro*-selected mutants 1016-27, 1016-27-23, 1056-13 and 1056-13-18 were obtained from Dr Thomas D. Gootz.[14] Clinical isolates of *S. pneumoniae* with mutations in *parC* and *gyrA* were obtained from Dr Patrice Courvalin.[15,16] BM4203 and BM4204 were ciprofloxacin-susceptible strains isolated from patients before antibiotic therapy, while BM4203-R and BM4204-R were the corresponding ciprofloxacin-resistant strains isolated after therapy. In both cases, pre- and post-treatment isolates were indistinguishable based on pulsed-field gel electrophoresis patterns of genomic DNA digests, suggesting that the mutants were selected *in vivo* as a result of quinolone therapy.[15] BM4205 was a clinical isolate of *S. pneumoniae*, while BM4205-R3 was its *in vitro*-selected mutant.[15] Additional clinical isolates of ciprofloxacin-resistant *S. pneumoniae*, strains 1048, 1055 and 1056 were obtained from Dr Peter C. Appelbaum (Hershey Medical Center, PA, USA).

Experimental procedures

DNA gyrase was purified as subunits A and B from *E. coli* JM109 containing plasmids pPH3 and pAG111 to overexpress the *gyrA* and *gyrB* genes, respectively, from the *tac* promoter.[17] The mutated form of the A subunit (Ser-83→Trp) was purified from *E. coli* JM109 containing the overexpression plasmid pPH483.[18] The subunits were purified via anion-exchange column chromatography, following a previously described procedure with minor modifications.[17] The active, multimeric (A_2B_2) form of DNA gyrase was prepared by incubating equimolar amounts of the A and B subunits at room temperature in 50 mM Tris (pH 7.5)/100 mM KCl/1 mM EDTA/5 mM dithiothreitol/10% glycerol.[17] Inhibition of DNA gyrase supercoiling activity was ascertained by separating supercoiled and relaxed DNA via agarose gel electrophoresis, following an established procedure.[19] The IC_{50} was determined as the compound concentration at which 50% of the DNA gyrase supercoiling activity was inhibited.

The MIC of the test compounds was determined by incubating bacterial cultures (~5×10^5 cfu/mL) at 37°C overnight (18–24 h) in brain–heart infusion (BHI) broth in the presence of test compounds in a two-fold, broth microdilution series in duplicate.[20] *S. pneumoniae* strains were tested in the presence of 3% lysed horse blood in BHI broth and incubated in a 5% CO_2-enriched environment. In analysing multiple isolates of *E. coli* and penicillin-resistant *S. pneumoniae*, the MIC_{50} and MIC_{90} values were estimated as the minimum compound concentration at which growth of ⩾50% and ⩾90% of the strains were inhibited.

Results

DNA gyrase inhibition

The supercoiling activity of *E. coli* DNA gyrase was inhibited by the NFQs with IC_{50}s in the 1.6–3.2 mg/L range, while the IC_{50}s of ciprofloxacin, trovafloxacin, gatifloxacin and clinafloxacin were 1.6, 1.6, 0.8 and 0.2 mg/L, respectively (Figure 2). Using the same assay, IC_{50}s of additional benchmark compounds for the wild-type gyrase were as follows: nalidixic acid 50 mg/L; ofloxacin 1 mg/L; lomefloxacin 1.75 mg/L; sparfloxacin 1 mg/L and tosufloxacin 0.5 mg/L. When tested against the mutant gyrase [GyrA (Ser-83→Trp)], the IC_{50}s of the NFQs rose by factors of two to four, to 6.4 mg/L, while those of ciprofloxacin, trovafloxacin, gatifloxacin and clinafloxacin rose by factors of 64, 8, 4 and 5, respectively, to 102, 13, 3 and 1 mg/L (Figure 2).

Antibacterial activity against E. coli

The NFQs and benchmark quinolones were tested against 20 clinical isolates collected at random from patients with urinary tract infections for *in vitro* susceptibility. The MIC

data are presented in Table I. Based on the MIC_{90}s against these strains, two NFQs, PGE9262932 and PGE9509924, were as potent as trovafloxacin and eight-, four- and two-fold less potent than clinafloxacin, ciprofloxacin and gatifloxacin, respectively.

Antibacterial potency data on the NFQs and other quinolones against *E. coli* strains with point mutations, such as Ser-83→Leu and Asp-87→Asn in GyrA, Lys-447→Glu in GyrB, and Ser-80→Ile and Glu-84→Gly or Lys in ParC, are presented in Table II. When tested against *E. coli* strain WT (with wild-type residues in the QRDR[11]), the NFQs were four- to eight-fold less potent than ciprofloxacin. Against the laboratory-generated GyrA mutant strain MI (Ser-83→Leu), the NFQs were two- to four-fold less potent than ciprofoxacin. However, when tested against the clinical isolate U12987 with double mutations in GyrA (Ser-83→Leu, Asp-87→Asn) and ParC (Ser-80→Ile, Glu-84→Gly), the NFQs were four- to eight-fold more potent than ciprofloxacin. The NFQs were as potent as or two-fold less potent than ciprofloxacin, trovafloxacin and gatifloxacin against the clinical isolate C-4 [GyrA (Ser-83→Leu)]. Against clinical isolates 1363, 1331 and 1334, with mutations in GyrA, GyrB and ParC, PGE9262932 was two- to four-fold and four- to more than eight-fold more potent than ciprofloxacin and trovafloxacin, respectively. Strains, such as MIII and MIVb,[11] with deletions in *marR* (in addition to mutations in the serine/aspartate 'hot spots' of GyrA and ParC), were resistant to the NFQs as well as to several other quinolones (MICs in the range 64–>128 mg/L; data not shown).

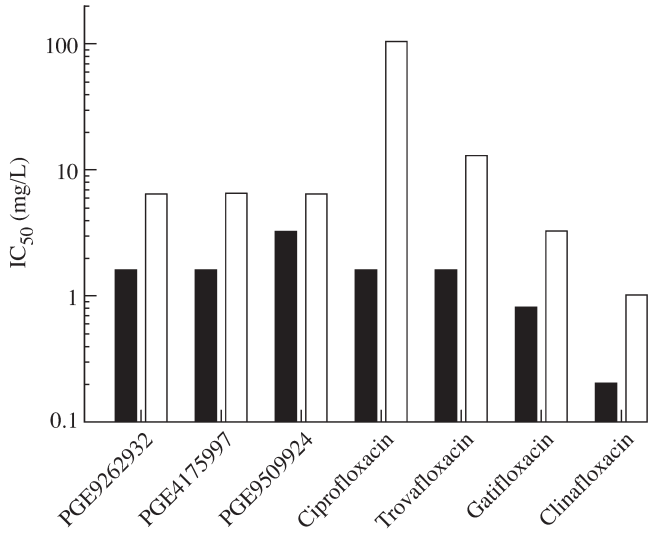

Figure 2. Inhibition of *E. coli* DNA gyrase by the NFQs and other quinolones. Inhibition potencies (IC_{50}) against the wild type (WT) (■) and the GyrA (Ser-83→Trp) mutant (□) are shown. PGE9509924 was used as a racemic mixture in this experiment.

Antibacterial activity against PRSP

In vitro antibacterial activity of the NFQs along with other quinolone and non-quinolone compounds was tested against 23 clinical isolates of PRSP. The results, summarized in Table III, show that the MICs of many classes of antibacterials including β-lactams (e.g. ampicillin) and macrolides (e.g. azithromycin) were relatively high (MIC_{90} 4–>16 mg/L) for these strains, although they appeared susceptible to vancomycin (MIC_{90} 1 mg/L). Based on the MIC_{90}s, the NFQs were more potent than other quinolones, such as ciprofloxacin, trovafloxacin and gatifloxacin, and at least as potent as clinafloxacin. Among the NFQs, PGE9262932 was 64-, 16-, eight- and four-fold more potent than ciprofloxacin, gatifloxacin, trovafloxacin and clinafloxacin, respectively, based on the MIC_{90} data. The cumulative susceptibility data, shown in Figure 3, also indicated that PGE9262932 was the most potent of all the quinolones tested in this study.

Antibacterial activity against GyrA and ParC mutants of S. pneumoniae

S. pneumoniae strains with mutations in the target genes (*parC* and *gyrA*) were tested against the NFQs and other

Table I. *In vitro* susceptibility of 20 clinical isolates of *E. coli* to the NFQs and other quinolones

Compound	MIC (mg/L)			Geometric mean (mg/L)
	range	MIC_{50}	MIC_{90}	
PGE9262932	0.063–0.125	0.125	0.125	0.113
PGE4175997	0.125–0.25	0.25	0.25	0.19
PGE9509924	0.063–0.25	0.125	0.125	0.14
Ciprofloxacin	0.016–0.063	0.031	0.031	0.027
Trovafloxacin	0.031–0.125	0.125	0.125	0.087
Gatifloxacin	0.031–0.125	0.063	0.063	0.058
Clinafloxacin	0.004–0.031	0.008	0.016	0.009

Table II. *In vitro* susceptibility of *E. coli* GyrA and ParC mutants to the NFQs and other quinolones

Strain	Properties	MIC (mg/L)						
		PGE9262932	PGE4175997	PGE9509924	ciprofloxacin	trovafloxacin	gatifloxacin	clinafloxacin
WT		0.25	0.5	0.25	0.063	0.125	0.25	0.125
MI	GyrA (S83L)	1	2	1	0.5	1	1	0.125
U12987	GyrA (S83L, D87N), ParC (S80I, E84G)	1	2	2	8	8	2	0.25
C-4	GyrA (S83L)	0.25	0.5	0.5	0.25	0.25	0.25	0.031
1363	GyrA (S83L), GyrB (K447E), ParC (E84K)	2	8	4	4	8	2	0.25
1331	GyrA (S83L, D87N), ParC (E84K)	2	8	4	8	16	4	0.5
1334	GyrA (S83L, D87N), ParC (S80I)	4	16	8	16	>32	8	1

quinolones in this study. The results from three sets of mutants are presented in Table IV. When tested against the laboratory-generated ParC mutants (Ser-79→Phe in strains 1016–27 and 1056–13), the MICs of the NFQs remained unchanged relative to those for the parent, while those of ciprofloxacin, trovafloxacin, gatifloxacin and clinafloxacin were elevated two- to four-fold (Table IV). When tested against the laboratory-generated, second-step mutant strains 1016-27-23 [ParC (Ser-79→Phe); GyrA (Ser-81→Phe)] and 1056-13-18 [ParC (Ser-79→Phe); GyrA (Glu-85→Lys)], the MICs of the NFQs were in the 0.125–1.0 mg/L range and were elevated two- to eight-fold (relative to the MICs for the parent), while the MICs of the other quinolones were in the range 0.5–32 mg/L and were elevated eight- to 32-fold (relative to the MICs for the parent). For a laboratory-generated GyrA mutant (Ser-81→Phe in strain BM4205-R3, Table IV), the NFQ MICs were elevated two- to four-fold, while those of the other quinolones were elevated four- to 16-fold (relative to the MICs for the parent).

The NFQs and other benchmark quinolones were also tested against clinical isolates of *S. pneumoniae* with elevated quinolone MICs. The results are shown in Table V. *In vivo*-selected mutations in ParC, in the Ser-79 and Asp-83 residues (strains BM4204-R and BM4203-R), did not reduce the potency of the NFQs significantly. Against clinical strains 1048 [ParC (Ser-79→Phe); GyrA (Ser-81→Cys)] and 1055 [ParC (Ser-79→Phe, Lys-137→Asn); GyrA (Glu-85→Lys)], the NFQs were 16- to 64-fold more potent than ciprofloxacin, trovafloxacin and gatifloxacin, and at least as potent as clinafloxacin.

Discussion

As judged by the potency profile against *E. coli* DNA gyrase, of the NFQs gatifloxacin and clinafloxacin appeared different from ciprofloxacin and trovafloxacin. This was indicated by our finding that the Ser-83→Trp mutation was less effective in reducing the potency of the former group of compounds, as judged by the smaller increase in the IC_{50}s against the mutant relative to the wild-type enzyme. The NFQs demonstrated a 16-fold higher potency against the mutant form of gyrase than did ciprofloxacin. In terms of antibacterial potency against clinical isolates of quinolone-sensitive *E. coli*, the NFQs were overall less potent than several quinolones. However, with MIC_{90}s in the 0.125–0.25 mg/L range, the NFQs were comparable to trovafloxacin against these clinical isolates of *E. coli*.

In *E. coli*, the GyrA subunit of DNA gyrase is believed to be a primary target for quinolones, while the ParC subunit of topoisomerase IV is considered a secondary target. This is based on the association of mutations in *gyrA* with low levels of ciprofloxacin resistance, and that of mutations in *gyrA* and *parC* with high levels of resistance to ciprofloxacin.[13] It appears that in *E. coli*, once GyrA is mutated,

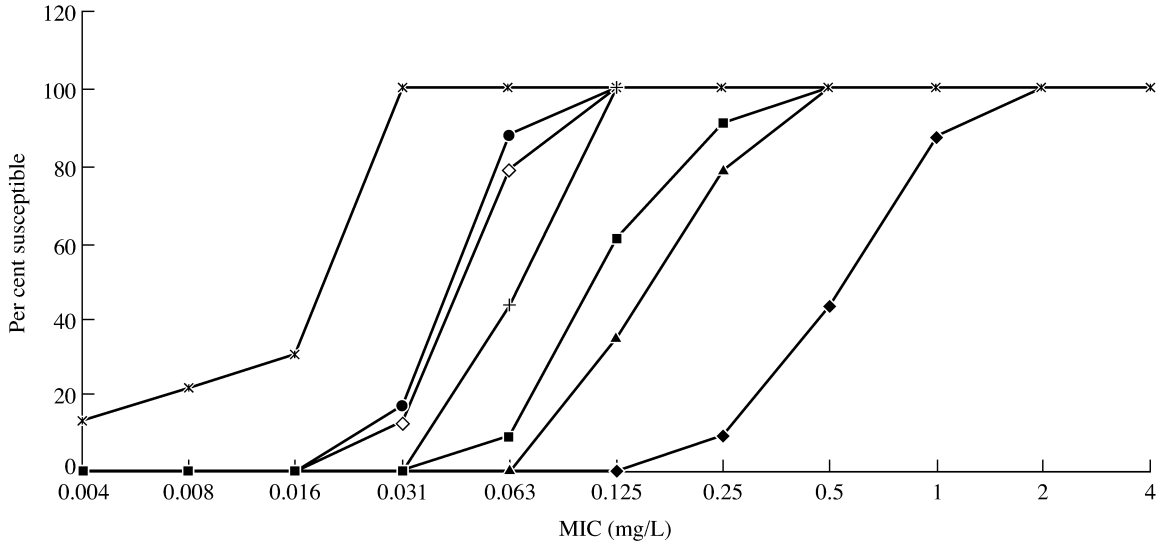

Figure 3. Cumulative susceptibility of 23 clinical isolates of PRSP to the NFQs and other quinolones. Symbols: ×, PGE9262932; ●, PGE4175997; +, PGE9509924; ◆, ciprofloxacin; ■, trovafloxacin; ▲, gatifloxacin; ◇, clinafloxacin.

Table III. *In vitro* susceptibility of 23 clinical isolates of PRSP to the NFQs and other quinolones

Compound	MIC (mg/L)			Geometric mean (mg/L)
	range	MIC_{50}	MIC_{90}	
PGE9262932	⩽0.004–0.031	0.031	0.031	
PGE4175997	0.031–0.125	0.063	0.125	0.062
PGE9509924	0.063–0.125	0.125	0.125	0.092
Ciprofloxacin	0.5–2.0	1	2	0.762
Trovafloxacin	0.0625–0.5	0.125	0.25	0.164
Gatifloxacin	0.125–0.5	0.25	0.5	0.228
Clinafloxacin	0.031–0.125	0.063	0.125	0.066
Ampicillin	0.25–4.0	2	4	2.47
Vancomycin	0.5–1.0	1	1	0.74
Azithromycin	0.5–>16	>16	>16	

quinolones such as ciprofloxacin inhibit ParC with higher potency and thereby exert their antibacterial activity via topoisomerase IV as the effective molecular target. However, since newer quinolones, such as the NFQs, have higher potency toward the Ser-83 mutant of GyrA, these compounds could potentially exert their antibacterial activity, at least in part, by inhibiting the mutated form of DNA gyrase, instead of utilizing the second target, topoisomerase IV, as the sole target. This scenario is consistent with our finding that the potency difference between the NFQs and ciprofloxacin was less against the GyrA (Ser-83→Leu) mutant MI than against the parent WT. A similar phenomenon was apparent against the clinical isolate C-4. While potency differences against various clinical isolates with similar mutations could be attributable to mechanisms other than mutations in *gyrA* and *parC*, the overall higher potency of the NFQs, relative to ciprofloxacin and trovafloxacin, against clinical isolates with *gyrA* and *parC* mutations, suggests that the NFQs retain higher potency against mutated forms of GyrA and/or ParC.

The *in vitro* potency data on the PRSP isolates indicated that the NFQs were extremely potent against this clinically important pathogen, which is widely resistant to penicillins and macrolides. PGE9262932 was exceptionally potent, with 100% of the isolates being susceptible at ⩽0.031 mg/L. The high antibacterial potency of the NFQs could be attributable to their high potency against DNA gyrase and/or topoisomerase IV in *S. pneumoniae*. Unlike *E. coli*,

Table IV. *In vitro* susceptibility of laboratory-generated ParC and GyrA mutants of *S. pneumoniae*

Strain	Properties	MIC (mg/L)						
		PGE9262932	PGE4175997	PGE9509924	ciprofloxacin	trovafloxacin	gatifloxacin	clinafloxacin
02J1016	parent	0.0625	0.125	0.125	1	0.25	0.5	0.0625
1016-27	ParC (S79F)	0.0625	0.125	0.125	4	0.5	1	0.125
1016-27-23	ParC (S79F), GyrA (S81F)	0.125	0.25	0.5	32	8	8	0.5
02J1056	parent	0.0625	0.125	0.125	1	0.125	0.25	0.0625
1056-13	ParC (S79F)	0.0625	0.125	0.125	4	0.5	1	0.125
1056-13-18	ParC (S79F), GyrA (E85K)	0.5	1	1	32	8	8	1
BM4205	parent	0.0625	0.0625–0.125	0.125–0.25	1	0.25	0.25–0.5	0.125
BM4205-R3	GyrA (S81F)	0.25	0.25	0.5–1.0	16	1	4.0–8.0	0.5–1.0

Table V. *In vitro* susceptibility of clinical isolates of ParC and GyrA mutants of *S. pneumoniae*

Strain	Properties	MIC (mg/L)						
		PGE9262932	PGE4175997	PGE9509924	ciprofloxacin	trovafloxacin	gatifloxacin	clinafloxacin
BM 4203	parent	0.0625	0.125	0.125	1	0.5	0.5	0.125
BM4203-R	ParC (D83H)	0.0625	0.125	0.25	4	0.5	1	0.25
BM4204	parent	0.0625	0.125	0.125	1	0.25	0.5	0.125
BM4204-R	ParC (S79F)	0.0625	0.125	0.125	2	0.5	0.5	0.125
1048	ParC (S79F), GyrA (S81C)	0.25	0.25	0.25	16	4	4	0.5
1055	ParC (K137N, S79F), GyrA (S81C)	0.25	0.5	0.5	16	8	8	0.5
1056	(K137N)	0.0625	0.125	0.125	2	0.5	1	0.25

previous studies showed that depending on the quinolone, the first-step mutations in *S. pneumoniae* could map to either *parC* or *gyrA*.[21–23] This suggests that in *S. pneumoniae*, certain quinolones, such as ciprofloxacin and trovafloxacin, have higher potency toward ParC (topoisomerase IV) than GyrA (DNA gyrase), while the reverse is true in the case of other quinolones, such as sparfloxacin and gatifloxacin.[21,22] As shown in Table IV, the MICs of the NFQs remained unchanged in the ParC mutants (Ser-79→Phe in strains 1016–27 and 1056–13), and were elevated two- to four-fold in a GyrA mutant (Ser-81→Phe in strain BM4205-R3) relative to the corresponding parents. These data suggest that in *S. pneumoniae*, wild-type GyrA is the target with higher effective potency for the NFQs.

Against *S. pneumoniae* strain 1016-27-23, with mutations in the serine 'hot spots' of both ParC and GyrA, the NFQ MICs increased two-fold (PGE9262932 and 4175997) or four-fold (PGE9509924) compared with eight- to 32-fold for the other quinolones. Against strain 1056-13-18, with mutations in the glutamate and serine 'hot spots' of GyrA and ParC, respectively, the NFQ MICs increased eight-fold, compared with 16- to 64-fold for the other quinolones. These data suggest that while the NFQs have higher potency against the Ser-81→Phe mutant form of GyrA than the Glu-85→Lys form, they are more potent than ciprofloxacin, trovafloxacin and gatifloxacin and at least as potent as clinafloxacin against the serine/glutamate 'hot spot' mutants of GyrA.

Data obtained with clinical isolates of *S. pneumoniae* with GyrA and ParC mutations were consistent with those obtained with laboratory-derived mutants. *In vivo*-selected mutations in either the Ser-79 or the Asp-83 residues of ParC (strains BM4203-R and BM4204-R) failed to show a consistent increase in the NFQ MICs relative to the parent strain, suggesting that in *S. pneumoniae* GyrA is the higher potency target for these compounds. In addition, the relatively high NFQ potency against clinical isolates with ParC and GyrA mutations (strains 1048 and 1055) suggest that these compounds are more potent than ciprofloxacin, trovafloxacin and gatifloxacin, and at least as potent as clinafloxacin against the mutated targets. Our data suggest that, like clinafloxacin, the NFQs are highly potent against PRSP, and are less affected *in vitro* by pre-existing and characterized target mutations that reduce quinolone potency against *S. pneumoniae*.

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