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Selection and Characterisation of Finafloxacin, Ciprofloxacin and Levofloxacin Resistant Mutants of *Escherichia coli*

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Revised Abstract

Background: Finafloxacin (FIN) is a novel fluoroquinolone (FQ) belonging to a new 8-cyano subclass. FIN exhibits optimal efficacy at slightly acidic pH (5.0 - 6.0), under which other FQs show decreased activity. Therefore, FIN is intended for therapeutic use against bacterial infections associated with an acidic environment. The *in vitro* emergence and genotypic mechanism of resistance to FIN, ciprofloxacin (CIP) and levofloxacin (LVX) was investigated in *E. coli* at pH 7.2 and pH 5.8.

Methods: Single-step mutants of *E. coli* ATCC 25922 were selected against FQ concentration gradients in Mueller-Hinton (MH) agar by plating 10^{10} CFU. MICs of stable mutants were determined by CLSI broth microdilution procedures at pH 7.2 and 5.8. Target mutation in gene segments of *gyrA* and *parC* of CIP, LVX and FIN resistant mutants were sequenced from PCR products. DNA sequences of mutants were aligned with parent.

Results: Resistance frequencies (8 x MIC) of first step mutants to FIN, CIP and LVX were 4.1 x 10^{-10}, 2.2 x10^{-9} and 1.3 x 10^{-9} respectively. First step mutants exhibited an 8 - 32-fold decrease in susceptibility over the parent and a relative decrease in susceptibility to the comparator FQs. All first step mutants (FIN, CIP & LVX) developed mutations within the quinolone resistance determination region (QRDR) of *gyrA*, the following substitutions were identified; G81D, S83L and D87N. No mutations in the QRDR of *parC* were detected.

Conclusion: FIN mutants arose at similar frequencies to the CIP and LVX mutants and exhibited similar decreases in susceptibility suggesting that FIN has the same low potential for resistance development. Mutations within the QRDR of *gyrA* were identified in FIN, CIP and LVX first step mutants of *E. coli* indicating this as a primary target.

Introduction

Finafloxacin (FIN, Figure 1) is a novel, broad spectrum fluoroquinolone (FQ) that belongs to a new 8-cyano subclass [1]. FIN contains a novel chiral base component which confers improved antibacterial activity at slightly acidic pH (pH 5.0 – 6.0) under which other marketed FQs exhibit significantly reduced activity [2].

FIN exhibited superior activity to comparator FQs against adherent bacteria *in vitro* [3] and in a wide range of rodent infection models [4,5]. Additionally, FIN displayed an excellent safety profile in a wide range of predictive, *in vitro,* toxicity assays [6] and was well tolerated in healthy human volunteers [7]. These attributes suggest that FIN warrants clinical investigation for bacterial infections that are associated with low pH such as urinary tract infection and *Helicobacter pylori* eradication.

The propensity for the development of resistance to FIN in *E. coli* was investigated in comparison to ciprofloxacin (CIP) and levofloxacin (LVX). Resultant FQ mutants were characterised to a molecular level.

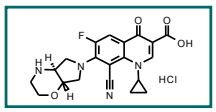

Figure 1. Finafloxacin hydrochloride.

Methods

Minimum inhibitory concentrations (MIC)

MICs were determined according to the CLSI procedure for broth microdilution [8] in cation-adjusted Mueller-Hinton broth (CAMHB) at pH 7.2 and pH 5.8.

Resistance frequencies

Single-step resistance frequencies of *E. coli* ATCC 25922 were determined on sodium phosphate buffered Mueller-Hinton agar (MHA) at pH 7.2 or pH 5.8, containing test drugs up to 32 x MIC. Stability of emergent resistant colonies was confirmed by MIC after three passages on drug free MHA. Mutation prevention concentration (MPC) was defined as the concentration of drug at which no resistant colonies were selected from an inoculum of 10^{10} CFU.

Serial passage

Resistant mutants were also selected following daily serial passage in the presence of subinhibitory FQ concentrations in CAMHB, pH 7.2 or pH 5.8.

Target gene sequencing

Genomic DNA was extracted from mutants and PCR performed according to the method of Lindgreen *et al.*, [9]. DNA sequencing of quinolone resistance determination regions (QRDRs) was performed using standard techniques.

Results and Discussion

The propensity for the development of resistance to FIN, CIP and LVX in *E. coli* 25922 was investigated through comparison of first-step, spontaneous resistance frequencies (Figure 3) and mutation prevention concentrations (Figure 2) at pH 7.2 and pH 5.8.

At pH 7.2, the MPCs for CIP (0.125 mg/L), LVX (0.25 mg/L) and FIN (1 mg/L) were each equivalent to 16 x $MIC_{[pH\ 7.2]}$. Below this threshold, at 8 x $MIC_{[pH\ 7.2]}$, resistance frequencies to each FQ were similar; FIN (4.1 x 10^{-10}), CIP (2.2 x 10^{-9}) and LVX (1.3 x 10^{-9}).

At pH 5.8, the MPCs of FIN (0.25 mg/L), CIP (1 mg/L) and LVX (2 mg/L) were between 16 – 32 x $MIC_{[pH\ 5.8]}$. As at pH 7.2, the FQ resistance frequencies at 8 x $MIC_{[pH\ 5.8]}$ were similar; FIN (1.1 x 10^{-9}), CIP (3.2 x 10^{-8}) and LVX (4.9 x 10^{-10}).

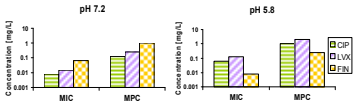

Figure 2. Minimum inhibitory concentration and mutation prevention concentration of FIN, CIP and LVX for *E. coli* 25922 at pH 7.2 (left) and pH 5.8 (right).

Results and Discussion

The potential for resistance development to FIN, CIP and LVX was also compared by serial passage of *E. coli* in the presence of subinhibitory FQ concentrations. The concentration of FQ at which the passaged culture could grow steadily increased over 22 passages, to 8 - 32 times greater than the starting concentration (Figure 4).

These findings, along with the single step resistance frequencies and MPCs (Figure 2 – 3) demonstrate that development of resistance to FIN in *E. coli* occurred at similar frequency to, and to a similar extent as CIP or LVX resistance.

Single-step, agar selected, FIN, CIP and LVX mutants are listed in Table 1. Mutants to each drug exhibited a similar susceptibility profile, comprising of a 8 – 32-fold increase in MIC to each of the FQs tested. Mutants to each drug harbored single point mutations conferring one of the following amino acid substitutions within the QRDR of *gyrA*; G81D, S83L or D87N. No mutations were detected in the QRDR of *parC*. This suggests that FIN shares a common target with CIP and LVX.

Susceptibilities of characterised FQ resistant mutants of common parentage, harboring different combinations of target mutations, to FIN, CIP and LVX were determined at pH 7.2 and 5.8 (Table 2).

The MIC increase exhibited by this panel was dependent on the nature of the target mutation(s). All mutants exhibited relative increases in MIC to FIN, CIP and LVX, when compared under the prevailing conditions of pH. In general, dual target mutants exhibited a 16 – 64 increase in MIC (to FIN, CIP and LVX) and a third mutation resulted in a further 4 – 16-fold increase in MIC.

In summary, the propensity for the development of FIN resistance in *E. coli* was very similar to that of CIP or LVX when compared at concentrations relative to the MIC under the prevailing conditions of pH. FIN exhibits much improved antibacterial activity at low pH. Subsequently, the limits of MPC and mutant selection window were lower at p 5.8 than at pH 7.2. Conversely, the negative effect of low pH on the activity of CIP and LVX is reflected in higher MPCs and concentrations at which mutants arise at pH 5.8.

This would imply that FIN may have an advantage in treating infections in acidic environments, such as in the gastrointestinal or urogenital tract, in abscesses, intra-abdominal infections, TB, CF and others.

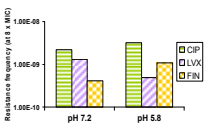

Figure 3. Spontaneous resistance frequencies of *E. coli* 25922 to 8 x MIC of FIN, CIP and LVX at pH 7.2 and pH 5.8.

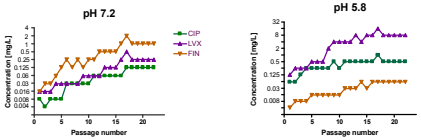

Figure 4. Selection of FQ resistance in *E. coli* 25922 by serial passage at pH 7.2 and pH 5.8.

Strain	Nt	*gyrA* mutation: Substitution	Amino acid	$MIC_{pH\ 7.2}$ [mg/L]: CIP	LVX	FIN	$MIC_{pH\ 5.8}$ [mg/L]: CIP	LVX	FIN
ATCC 25922			WT	0.008	0.016	0.063	0.06	0.125	0.008
CIP_14	242	GGT→GΔT	G81D	0.125	0.25	0.5	1	2	0.125
CIP_13	248	TCG→TΔG	S83L	0.25	0.5	0.5	2	2	0.125
CIP_35	259	GAC→ΔAC	D87N	0.125	0.25	0.5	2	4	0.06
LVX_17	242	GGT→GΔT	G81D	0.125	0.25	0.5	1	2	0.125
LVX_18	248	TCG→TΔG	S83L	0.25	0.25	0.5	2	2	0.125
LVX_19	259	GAC→ΔAC	D87N	0.06	0.125	0.25	1	1	0.06
FIN_21	242	GGT→GΔT	G81D	0.125	0.25	0.5	1	2	0.125
FIN_25	248	TCG→TΔG	S83L	0.25	0.25	0.5	2	4	0.125
FIN_27	259	GAC→ΔAC	D87N	0.125	0.25	0.5	1	2	0.125

Table 1. First-step FQ resistant mutants of *E. coli* ATCC 25922, selected against FIN, CIP or LVX. All mutants exhibited an 8 – 32-fold increase in MIC to both the selective and comparator FQs. QRDR sequencing of *gyrA* revealed the following substitutions; G81D, S83L and D87N in mutants selected by each of FIN, CIP and LVX.

gyrA allele	Second *gyrA* allele	*parC* allele	$MIC_{pH\ 7.2}$ [mg/L]: CIP	LVX	FIN	$MIC_{pH\ 5.8}$ [mg/L]: CIP	LVX	FIN
ATCC 25922			0.008	0.016	0.063	0.06	0.125	0.008
G81D		wt	0.125	0.25	0.5	1	2	0.125
G81D		S80R	8	16	64	64	128	16
G81D		S80I	1	4	8	8	32	2
G81D		S80I	2	8	32	16	64	4
G81D		G78D	2	16	16	16	128	4
D87N		wt	0.06	0.125	0.25	1	1	0.06
D87N	G81D	wt	2	4	8	16	16	2
S83L	D87N	wt	4	4	16	32	32	4
D87N	A84P	wt	8	8	64	64	64	8
D87N		S80R	2	4	8	16	16	1
D87N	A84P	E84K	128	32	64	>256	256	16
S83L		wt	0.25	0.5	0.5	2	2	0.125
S83L	D87N	S80R	64	32	128	>256	256	16

Table 2. Activity of FIN, CIP and LVX against a panel of double and triple target FQ resistant mutants of *E. coli* ATCC 25922. Mutants exhibited relative increases in MIC to each FQ.

Conclusions

- FIN exhibited improved antibacterial activity at low pH whereas CIP and LVX (along with other marketed FQs) lose activity. The mutant selection window for these FQs changed accordingly (and relative to potency) with pH.
- Resistance frequencies for FIN in *E. coli* were comparable to those for CIP and LVX at 8 x $MIC_{[pH\ 7.2]}$ and at 8 x $MIC_{[pH\ 5.8]}$.
- Single-step mutants to FIN, CIP and LVX exhibited relative decreases in susceptibility and common target allelic profiles, indicating *gyrA* as the primary target.
- These findings imply that FIN would have an advantage, in terms of its mutant selection window, under conditions of low pH, reflecting its unusual pH activation profile.

Literature

[1] Wohlert *et al.*, 48th ICAAC, Washington DC 2008, Poster No. F1-2036.
[2] Kresken *et al.*, 48th ICAAC, Washington DC 2008, Poster No. F1-2037.
[3] Goh *et al.*, 48th ICAAC, Washington DC 2008, Poster No. F1-2042.
[4] Endermann *et al.*, 48th ICAAC, Washington DC 2008, Poster No. F1- 2044.
[5] Endermann *et al.*, 48th ICAAC, Washington DC 2008, Poster No. F1-2045.
[6] Schmuck *et al.*, 48th ICAAC, Washington DC 2008, Poster No. F1-2047.
[7] Patel *et al.*, 48th ICAAC, Washington DC 2008, Poster No. F1-2048.
[8] Clinical Laboratory Standards Institute (CLSI). M7-A6. National Committee for Clinical Laboratory Standards, Wayne, Pa., 2003.
[9] Lindgreen *et al.*, (2003). *AAAC* **47(10):** 3222-3232.