Evaluation of the Potential for Delafloxacin Resistance Development in *Neisseria gonorrhoeae*

Poster C-1412a
54th ICAAC
Washington, DC
September 5-9, 2014

J. Remy, A. Marra, and E. Duffy
Melinta Therapeutics, Inc., New Haven, CT

Melinta Therapeutics, Inc.
Phone 203-624-5606
info@melinta.com

Abstract

Background
Neisseria gonorrhoeae (Ng) infections have limited treatment options of late due to the development of resistance to a number of antimicrobial agents. Mutations contributing to fluoroquinolone (FQ) resistance in Ng have been reported in *gyrA* and *parC* regions. We evaluated the propensity of Ng isolates to develop resistance to delafloxacin (DLX) over 30 passages, determined the frequency of resistance to DLX and ciprofloxacin (CIP) in Ng, and determined at which nucleotides, if any, mutations occurred.

Methods
Three clinical isolates (including one CIP-R) plus ATCC 49226 were included in this study. DLX, CIP, and rifampicin were evaluated for spontaneous resistance development. In addition, the four strains were passaged on DLX, CIP, ceftriaxone (CRO) and azithromycin. Susceptibility testing was performed on resistant isolates to determine cross-resistance to other agents. Using DNA from parent strains and resistant mutants PCR was performed to amplify *gyrA* and *parC*, and the PCR products were sequenced to identify any mutations in the quinolone-resistance determining region (QRDR).

Results
At 2X MIC, DLX resistance frequencies (RFs) were consistently lower than for CIP. At 4X MIC the DLX RF was lower than that of CIP for ATCC 49226 but the two FQs had similar RFs for the clinical isolates. Only one mutant demonstrated a newly-acquired mutation with a DLX MIC that was 4-fold higher than that of the parent, whereas the CIP MIC increased 16-fold. For all other clones, DLX and CIP MICs were within 2-fold of parent MICs.
Over 30 days passage, DLX MICs remained within 4-fold for the 4 isolates and did not revert to their pre-passage values after 10 days in drug-free medium. CIP and CRO MICs were also generally within 4-fold of parent MICs. Mutants isolated from ATCC 49226 acquired a novel mutation in *gyrA*. Cross-resistance to non-FQ compounds was not observed in either experiment.

Conclusion
DLX RFs were lower than that of CIP for 3 isolates at 2X MIC and for one isolate at 4X MIC.
New mutations were identified only in clones from the wild-type strain; no additional mutations were identified in isolates that already harbor one or more.
In those mutants with increased MICs to DLX, CIP MICs were also elevated. However, DLX MICs remained significantly lower than those for CIP for the mutants isolated from the clinical strains. No cross-resistance was seen for the other antibiotics.

Introduction

The available treatment options for *Neisseria gonorrhoeae* (Ng) infections have been severely limited in recent years due to the development of resistance to a number of antimicrobial agents, including fluoroquinolones[1]. New antimicrobial agents are desperately needed to combat these infections. Delafloxacin, a fluoroquinolone agent that has demonstrated *in vitro* potency against Ng including against ciprofloxacin-resistant isolates[2], is currently in development for the treatment of uncomplicated gonococcal infections and has received QIDP designation for this indication. Mutations in the Quinolone Resistance Determining Region (QRDR) have been reported for Ng, specifically in *gyrA* (including Ser91Phe, Ser91Tyr, Asp95Asn) and *parC* (Asp86Asn, Ser87Ile, Ser88Pro, and Glu91Gly)[3-4]. A low frequency of resistance has been reported for delafloxacin in *Staphylococcus aureus*[5], but until now Ng has not been assessed. We evaluated the propensity of Ng to become resistant to delafloxacin by determining the frequency of spontaneous resistance and by serial passage, and identifying the nucleotides, if any, at which genotypic alterations occurred.

Methods

GC broth medium was prepared as follows: 15 g Special Peptone (Oxoid), 1 g corn starch (Argo), 2 g NaCl (JT Baker), 4 g K2HPO4 (Alfa Aesar), and 1g KH2PO4 (BDH) were dissolved in 990 mL deionized water and autoclaved. After cooling, 10 mL of GCHI supplement (Remel) was added to the broth and mixed. GC agar medium was prepared in the same manner with the addition of 15 g/L Bacto Agar (BD) prior to autoclaving. The GCHI supplement was added once the medium had cooled to approximately 50˚ C.

Frozen isolates were subcultured on Chocolate II Agar (GC II Agar, with Hemoglobin and BD IsoVitaleX™) (CA; BD). Incubation conditions were 35˚ C in 5% CO_2 throughout this study. The Ng isolates included in this study were the quality control strain ATCC 49226 (purchased from ATCC, Manassas, VA) and three clinical isolates obtained from David Farrell (GR Micro, UK, currently at JMI Laboratories, North Liberty, IA). Minimum inhibitory concentrations (MICs) were determined by the agar dilution method[6] and are shown in Table 1.

Table 1: MICs (in µg/mL) for test compounds vs. *N. gonorrhoeae* isolates

Isolate	Delafloxacin	Ciprofloxacin	Ceftriaxone	Azithromycin	Rifampicin
ATCC 49226	0.001	0.002	0.015	0.125	0.125
255123	0.004	0.125	0.008	0.25	0.125
255124	0.015	0.25	0.008	0.25	>32
255126	0.125	8	0.03	0.25	0.125

For the spontaneous resistance experiment, agar plates containing antibiotics at 1X-8X MIC were prepared. A 0.5 McFarland equivalent was prepared for each isolate and diluted 1:200 into supplemented GC broth. The cultures were incubated overnight with shaking. Following incubation, cells were concentrated and spread onto plates containing the antibiotic concentrations of 1X-8X MIC for an inoculum of 10^9 cells/plate. Plates were incubated, and colonies were counted and recorded daily for 72 hrs. Clones were patched onto plates containing the selecting concentration of delafloxacin to confirm the resistant phenotype and for additional testing.

Methods

Broth microdilution serial passage was performed on the compounds listed in Table 1 as described[7]. Briefly, an initial inoculum was prepared to achieve ~1x10^6 CFU/well. Following incubation for 24 hours MICs were determined. A sample was removed from the ½ MIC well or from the next well showing adequate growth. Samples were diluted and inoculated into a fresh microtiter tray, maintaining an inoculum of ~1x10^6 CFU/well. Serial passage continued daily for 30 days, followed by 10 days of passage in drug-free medium to determine stability of any resistant phenotype. Each day, samples were removed from each drug/strain combination and frozen for additional testing.

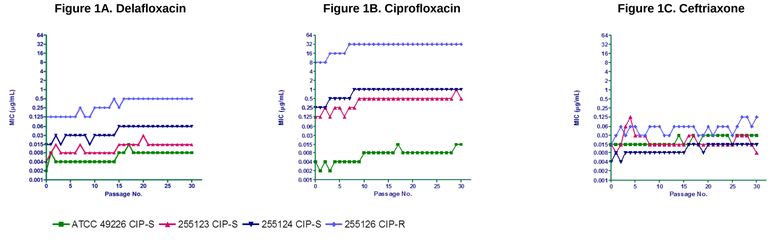
To determine cross-resistance to other antimicrobial agents, susceptibility testing was performed by the CLSI agar dilution method[6]. A 0.5 McFarland equivalent was prepared for each isolate, diluted 1:10 in PBS, and a 2 µL spot of each diluted sample was inoculated onto antibiotic plates. Plates without compound were included before and after each set of antibiotic plates as a growth control and to ensure no drug carryover occurred. Plate counts were performed to confirm an inoculum of ~10^4 per spot. Plates were incubated for 24 hr prior to reading. Plates were read against a dark background and the lowest concentration showing no growth was interpreted as the MIC.

To evaluate changes in the QRDR, DNA was isolated from the parent isolates and clones. Oligonucleotide primers were designed for amplification of *gyrA* and *parC* regions, and PCR and sequencing were performed using standard methods.

Results

Serial passage
Figure 1 shows a graphical representation of the MICs observed over the course of 30 days passaging in drug. Following 30 days of exposure to delafloxacin, the increase in delafloxacin MIC for the four isolates was ≤4-fold, as shown in Figure 1A. Ciprofloxacin and ceftriaxone MICs were also generally within 4-fold of parent MICs (Figures 1B and 1C). Delafloxacin MICs did not revert to their pre-passage values after 10 days of passaging in drug-free medium.

Figure 1: Daily passage MICs of Delafloxacin, Ciprofloxacin, and Ceftriaxone for *N. gonorrhoeae*



Spontaneous Resistance Selection
The resistance frequencies (RF) of delafloxacin, ciprofloxacin, and rifampicin against the four Ng strains were calculated at 48 and 72 hrs (no growth was observed at 24 hr). RFs for the 4 isolates are shown in Table 2. For ATCC 49226 at both 48 and 72 hours the RF for delafloxacin was lower than that for ciprofloxacin at 2X and 4X MIC. For Ng 255123, the RFs at 48 hours were the same for delafloxacin and ciprofloxacin, but colonies were observed on ciprofloxacin at 2X MIC at 72 hours. Colonies also were observed at both 48 and 72 hours on ciprofloxacin 2X MIC plates, but not delafloxacin plates, for Ng 255124. The RF could not be calculated for ciprofloxacin at 2X MIC, but at 4X MIC both antimicrobial agents showed identical RFs (<1.85E-10). Ng 255126, a ciprofloxacin-resistant isolate, also had a low RF for both delafloxacin at 48 and 72 hours, and no colonies of this strain were recovered on ciprofloxacin plates at 2, 4 or 8X MIC at either time point (starting MIC = 8 mg/mL). RFs for rifampicin were 2.74 – 7.78E-09 for ATCC 49226, 255123 and 255126 at 2X MIC, comparable to those against 255124 at 256 µg/mL.

Table 2. Resistance frequencies for Delafloxacin, Ciprofloxacin, and Rifampicin at 48 and 72 hours (MICs in µg/mL)

Isolate	ATCC 49226			255123			255124			255126	
Cell count	1.46E+09			1.80E+09			5.40E+09			9.00E+08	
Delafloxacin	48 hr	72 hr		48 hr	72 hr		48 hr	72 hr		48 hr	72 hr
MIC 0.001	TNTC	TNTC	MIC 0.004	<5.56E-10	<5.56E-10	MIC 0.015	<1.85E-10	5.67E-09	MIC 0.125	<1.11E-09	TNTC
2X-8X	<6.85E-10	<6.85E-10	2X-8X	<5.56E-10	<5.56E-10	2X-8X	<1.85E-10	<1.85E-10	2X-8X	<1.11E-09	<1.11E-09
Ciprofloxacin											
MIC 0.002	Lawn	Lawn	MIC 0.125	Lawn	Lawn	MIC 0.25	Lawn	Lawn	MIC 8	Lawn	Lawn
2X	1.37E-09	1.37E-09	2X	<5.56E-10	1.67E-09	2X	Lawn	Lawn	2X	<1.11E-09	<1.11E-09
4X	6.85E-10	6.85E-10	4X	<5.56E-10	<5.56E-10	4X	<1.85E-10	<1.85E-10	4X	<1.11E-09	<1.11E-09
8X	<6.85E-10	<6.85E-10	8X	<5.56E-10	<5.56E-10	8X	<1.85E-10	<1.85E-10	8X	<1.11E-09	<1.11E-09
Rifampicin											
MIC 0.125	Lawn	Lawn	MIC 0.125	1.72E-08	TNTC	(MIC >32 µg/mL) 64 µg/mL	Lawn	Lawn	MIC 0.125	Lawn	Lawn
2X	2.74E-09	2.74E-09	2X	3.33E-09	3.33E-09	128 µg/mL	Lawn	Lawn	2X	3.33E-09	7.78E-09
4X	<6.85E-10	<6.85E-10	4X	5.56E-10	5.56E-10	256 µg/mL	1.67E-09	5.56E-09	4X	1.11E-09	1.11E-09
8X	<6.85E-10	<6.85E-10	8X	<5.56E-10	<5.56E-10	-----	-----	-----	8X	1.11E-09	2.22E-09

TNTC, colonies too numerous to count; Lawn, confluent growth

Results

Determination of cross-resistance to other antimicrobial agents
Agar dilution MICs for delafloxacin and ciprofloxacin against the mutants selected in the spontaneous resistance experiment were within 2-fold of those of the parents with one exception: Mutant 226-S4, selected on 2X MIC of delafloxacin, had an increase of 4-fold for delafloxacin and 16-fold for ciprofloxacin. MICs for delafloxacin and ciprofloxacin against the clones selected in the serial passage experiment were 2- to 8-fold higher than those of the parent. No cross-resistance was seen with azithromycin, tetracycline, ceftriaxone, cefixime, penicillin, and spectinomycin for the other clones tested (data not shown).

Molecular Characterization

Serial passage
The four clones isolated from ATCC 49226 acquired a mutation in *gyrA* for which no prior mention has been found for Ng in a search of the literature: Glu161Gln. Compared to those of the ATCC 49226 parent, delafloxacin MICs for the clones were 8-fold higher, whereas those for ciprofloxacin were 2- to 4-fold higher. Delafloxacin MICs for 255123 mutants were also ~8-fold higher than those of the parent isolate; however, no additional mutations in *gyrA* or *parC* were identified. For the remaining two strains, additional mutations were not identified, and delafloxacin MICs for those mutants were within 2- to 4-fold of those of their parent isolates, as shown in Table 3.

Spontaneous Resistance
Following selection for spontaneous resistance, only one clone demonstrated a newly-acquired mutation: Ser91Tyr, a mutation that has been reported in the literature[8]. This clone had a delafloxacin MIC that was 4-fold higher than that of the parent, whereas the ciprofloxacin MIC increased 16-fold. For all other spontaneous mutants, delafloxacin and ciprofloxacin MICs were within 2-fold of parent MICs. Molecular characterization of the clones is shown in Table 4.

Table 3. Molecular characterization of clones isolated in the serial passage experiment

	Delafloxacin MIC (µg/mL)	Ciprofloxacin MIC (µg/mL)	*gyrA*	*parC*
ATCC 49226 Parent	0.001	0.002		-
4 clones	0.008	0.004-0.008	**Glu161Gln**	-
255123 Parent	0.001	0.125	Asp95Asn	-
6 clones	0.008-0.015	0.25-0.5	Asp95Asn	-
255124 Parent	0.015	0.5	Ser91Phe	-
4 clones	0.03-0.06	1-2	Ser91Phe	-
255126 Parent	0.125	8	Ser91Phe Asp95Asn	Asp86Asn
6 clones	0.25-0.5	16-32	Ser91Phe Asp95Asn	Asp86Asn

Table 4. Molecular characterization of clones isolated in the spontaneous resistance experiment

	Delafloxacin MIC (µg/mL)	Ciprofloxacin MIC (µg/mL)	*gyrA*	*parC*
ATCC 49226 Parent	0.001	0.004	-	-
3 clones	0.001	0.002	-	-
1 clone	0.004	0.06	**Ser91Tyr**	-
255123 Parent	0.002	0.125	Asp95Asn	-
2 clones	0.004-0.004	0.125-0.25	Asp95Asn	-
255124 Parent	0.015	1	Ser91Phe	-
13 clones	0.008-0.03	0.5-1	Ser91Phe	-
255126 parent	0.125	16	Ser91Phe Asp95Asn	Asp86Asn
5 clones	0.125-0.25	16-32	Ser91Phe Asp95Asn	Asp86Asn

Conclusions

- **The frequency of resistance for delafloxacin against *Neisseria gonorrhoeae* is low, and MICs remained within 4-fold of parent MICs following passage for 30 days.**
- **No cross-resistance was observed for non-fluoroquinolone antibiotics.**
- **Whereas new mutations were seen in the wild-type strain, no additional genotypic alterations in the QRDR were identified in isolates already harboring one or more mutations.**
- **Delafloxacin MICs, even when elevated, remained significantly lower than those of ciprofloxacin, and fall within the predicted range for probable PK/PD target attainment[9].**

References

1. Del Rio C., Hall G, K. Homes, O. Soge, E.W. Hook, R.D. Kirkcaldy, K.A. Workowski, S. Kidd, H.S. Weinstock, J.R. Papp, D. Trees, T.A. Peterman, and G. Bolan. Update to CDC's sexually transmitted diseases treatment guidelines, 2010: oral cephalosporins no longer a recommended treatment for gonococcal infections. MMWR Morb. Mortal. Wkly. Rep. 2012;61:590-594.
2. Roberts M.C., J.M. Remy, J.D. Longcor, A. Marra, E. Sun, E.M. Duffy. In vitro Activity of Delafloxacin against *Neisseria gonorrhoeae* Clinical Isolates. 2013. STI and AIDS World Congress. Poster P27.197. Vienna, Austria.
3. Deguchi, T., M. Yasuda, M. Nakano, S. Ozeki, T. Ezaki, I. Saito, and Y. Kawada. 1996. Quinolone-resistant *Neisseria gonorrhoeae*: correlation of alterations in the GyrA subunit of DNA gyrase and the ParC subunit of topoisomerase IV with antimicrobial susceptibility profiles. Antimicrob. Agents Chemother. 40: 1020-1023.
4. Giles, J.A., J. Falconio, J.D. Yuenger, J.M. Zenilman, M. Dan and M.C. Bash. 2004. Quinolone resistance-determining region mutations and *por* type of *Neisseria gonorrhoeae* isolates: resistance surveillance and typing by molecular methodologies. J. Infect. Dis. 189:2085-2093.
5. Remy J.M., C.A. Tow-Keogh, T.S. McConnell, J.M. Dalton, and J.A. DeVito. Activity of delafloxacin against methicillin-resistant *Staphylococcus aureus*: resistance selection and characterization. 2012. J Antimicrob. Chemother. 67:2814-2820.
6. Clinical and Laboratory Standards Institute. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically – Ninth Edition: Approved Standard M07-A9. CLSI, Wayne, PA. 2012.
7. Bogdanovich T., L.M. Ednie, S. Shapiro, and P.C. Appelbaum. 2005. Antistaphylococcal activity of ceftobiprole, a new broad-spectrum cephalosporin. Antimicrob. Agents Chemother. 49:4210-4219.
8. Kam, K.M., S.S. Kam, D.T. Cheung, V.W. Tung, W.F. Au, M.M. Cheung. 2003. Molecular characterization of quinolone-resistant *Neisseria gonorrhoeae* in Hong Kong. Antimicrob. Agents Chemother. 47:436-9.
9. Rubino C., S.M. Bhavnani, E. Burak, P.G. Ambrose. 2010. Pharmacokinetic-Pharmacodynamic Target Attainment Analyses Supporting Delafloxacin Phase 3 Dose Regimen Decisions. Abstr. 50th Intersci. Conf. Antimicrob. Agents Chemother., abstr A1-681.