Title: Determination of MIC Quality Control Ranges for the Novel Gyrase Inhibitor, Zoliflodacin

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Abstract: This report describes the results of two different, multi-laboratory QC studies that were used to establish QC ranges for the novel gyrase inhibitor, zoliflodacin, against the ATCC strains recommended by the Clinical and Laboratory Standards Institute (CLSI). Following the completion of an eight-laboratory, CLSI document M23-defined tier 2 study, the agar dilution MIC quality control (QC) range for zoliflodacin against the Neisseria gonorrhoeae QC strain ATCC 49226 was defined as 0.06 – 0.5 µg/mL and was approved by the CLSI Subcommittee on Antimicrobial Susceptibility Testing. This QC range will be used for in vitro susceptibility testing of zoliflodacin during phase 3 human clinical trials and surveillance studies, and eventually will be implemented in clinical labs. In a separate study, broth microdilution MIC quality control ranges for zoliflodacin against the following additional QC strains were determined to be: 0.12 – 0.5 µg/mL for Staphylococcus aureus ATCC 29213, 0.25 – 2 µg/mL for Enterococcus faecalis ATCC 29212, 1 - 4 µg/mL for Escherichia coli ATCC 25922, 0.12 – 0.5 µg/mL for Streptococcus pneumoniae ATCC 49619 and 0.12 – 1 µg/mL for Haemophilus influenzae ATCC 49247. These MIC QC ranges were also approved by CLSI for use in future in vitro susceptibility testing studies against organisms other than N. gonorrhoeae.

Key words: zoliflodacin, ETX0914, AZD0914, QC range
The US Centers for Disease Control and Prevention (CDC) has designated N. gonorrhoeae as an urgent public health threat that requires aggressive action due to emerging antibiotic resistance. In 2013, the CDC estimated an incidence of approximately 820,000 cases of gonorrhea in the United States alone, of which ~30% were resistant to existing antibiotics (1). Zoliflodacin (previously known as ETX0914 or AZD0914) is a novel, orally bioavailable spiropyrimidinetrione inhibitor of bacterial DNA gyrase (2) (Figure 1) currently in clinical development for the treatment of uncomplicated gonorrhea. Due to its unique mechanism of action, zoliflodacin is not cross-resistant to any other antibacterial agents, including other classes of gyrase inhibitors such as the fluoroquinolones, aminocoumarins or NBTIs (2). The compound, which has been granted qualified infectious disease product (QIDP) status and fast track designation by the U.S. Food and Drug Administration, has successfully completed both Phase I (3) and Phase 2 (4) clinical studies and will initiate Phase 3 testing in 2019. Zoliflodacin is rapidly bactericidal against clinical isolates of N. gonorrhoeae with low frequencies of resistance emergence (5). In recent surveillance studies, zoliflodacin was found to have potent antibacterial activity against contemporary clinical isolates from around the world (6, 7).

To ensure test performance and accuracy of results, a validated quality control (QC) method needs to be included in every study that involves in vitro susceptibility testing. QC methods rely on limits of variability which are established when testing an antibiotic against relevant QC strains and which are set according to the Clinical and Laboratory Standards Institute (CLSI) document M23 tier 2 studies. These include the evaluation of the reproducibility of antimicrobial susceptibility testing methods using different lots of reagents within a laboratory as well as among different laboratories over multiple days of testing and multiple replicates (8). The QC ranges established in this way are reported in applicable standard documents such as CLSI M100 (9).

In this report, we describe the results of two different, multi-laboratory QC studies that were used to establish QC ranges for zoliflodacin against the ATCC strains recommended by CLSI. The results of the agar dilution and broth microdilution MIC studies were approved at the June 2014 and January 2015 meetings, respectively, of the CLSI subcommittee on Antimicrobial Susceptibility Testing (https://clsi.org/meetings/ast-file-resources/) and will be used during Phase 3 clinical testing as well as future surveillance studies and eventually laboratories conducting testing on patient isolates.
Results

Agar dilution MIC results for zoliflodacin against \textit{N. gonorrhoeae} ATCC 49226 for the eight participating laboratories are presented in Table 1 and Figure 2. Based on guidance from the CLSI M23 document, the 57\% MIC shoulder present at 0.25 µg/mL indicated the need for a four-dilution quality control range \cite{8}. Therefore, 0.06 – 0.5 µg/mL was recommended for quality control testing, which was confirmed by the RangeFinder method analysis \cite{10}. This range included 100\% of all reported results. Significant lot-to-lot variability in base media was not observed with any of the three agar lots tested. All agar MIC results for ceftriaxone, the control agent, were within the CLSI-approved range on each day of testing \cite{data not shown}.

Because zoliflodacin has antibacterial activity against other bacterial species in addition to \textit{N. gonorrhoeae}, broth microdilution MIC results for zoliflodacin against \textit{S. aureus} ATCC 29213, \textit{E. faecalis} ATCC 29212, \textit{E. coli} ATCC 25922, \textit{S. pneumoniae} ATCC 49619 and \textit{H. influenzae} ATCC 49247 were performed by the eight participating laboratories and are shown in Tables 2 – 6 and Figure 3. Significant lot-to-lot variability in base media was not observed with any of the lots of broth tested. Quality control ranges were calculated using both the CLSI M23 criteria and the RangeFinder methods, which resulted in recommendations of three-dilution quality control ranges for all five strains \cite{10}. However, similar to what was observed with \textit{N. gonorrhoeae} ATCC 49226, both \textit{E. faecalis} ATCC 29212 (Table 3, Figure 3B) and \textit{H. influenzae} ATCC 49247 (Table 6, Figure 3E) demonstrated large MIC shoulders of 94.3\% and 82.7\%, respectively, suggesting bimodal distributions of the data divided between two 2-fold dilutions.

Therefore, four-dilution QC ranges were recommended for both \textit{E. faecalis} ATCC 29212 and \textit{H. influenzae} ATCC 49247. Zoliflodacin modal MICs for all lots of media were within the proposed dilution ranges. All values for the control antibiotic, levofloxacin, were within CLSI-approved ranges except for three replicates for \textit{H. influenzae} ATCC 49247 (data not shown). Removing the data associated with these three “out of control” replicates did not change the MIC range proposed for zoliflodacin.

Discussion

Establishing QC ranges for antibiotic susceptibility testing is an absolute necessity to ensure accurate and reproducible reporting of results. This is accomplished by the testing of well-established QC strains with known susceptibilities to the antimicrobial agent of interest \cite{8}. The goals of a QC program are to monitor the precision (reproducibility) and accuracy of susceptibility testing procedures, the performance of reagents used in routine tests, and the performance of laboratory personnel who...
execute the tests and report the results (8). The studies described here met CLSI document M23 requirements for tier 2 QC studies to establish MIC ranges for this novel antibacterial agent.

Table 7 summarizes the approved QC ranges for zoliflodacin MIC tests for the six organisms evaluated in these studies. Due to growth requirements, *N. gonorrhoeae* MIC tests had to be conducted using agar dilution methods in appropriate media (11). MIC tests for the remaining five bacterial species were conducted using the broth microdilution method (11). All (100%) of MIC values reported from the eight participating laboratories in this study were within the QC ranges shown in Table 7. The results from this multi-laboratory QC study for zoliflodacin were approved by the CLSI antimicrobial susceptibility testing subcommittee for future publication in document M100 (9).

**Materials and Methods**

Zoliflodacin (MW = 487.4 g/mol), provided by AstraZeneca Pharmaceuticals (Waltham, MA), was dissolved in DMSO and further diluted with water to a final concentration of 640 µg/mL. Levofloxacin (MW = 361.6 g/mol) purchased from Sigma-Aldrich (St. Louis, MO) (cat # 28266, lot # BCBK58) was dissolved with half volume water and 0.1N NaOH added dropwise until dissolved and further diluted with water to a final concentration of 10 µg/mL. Ceftriaxone (MW = 554.6 g/mol) purchased from Sigma-Aldrich (cat # C5793, lot #SLBH9174V) was dissolved and diluted with water to a final concentration of 2.5 µg/mL.

Eight laboratories participated in these CLSI document M23-defined tier 2 studies (8). They were: the Clinical Microbiology Institute (CMI), Wilsonville, OR (M. Traczewski); Laboratory Specialists, Inc., Westlake, OH (L. Koeth); the UCLA Medical Center, Los Angeles, CA (J. Hindler); Thermo Fisher Scientific, Oakwood Village, OH (C. Knapp); the University of Rochester Medical Center, Rochester, NY (D. Hardy); International Health Management Associates, Inc. (IHMA), Schaumburg, IL (M. A. Hackel); Tufts New England Medical Center, Boston, MA (L. McDermott) and University of Alberta Hospital, Edmonton, AB (R. Rennie). Each of the participating laboratories is an experienced microbiology facility, and each laboratory followed CLSI procedures for broth microdilution testing exactly as written (11). Supplies, including frozen broth microdilution panels, agar plates, and disks, were distributed by CMI to the other seven sites. The QC strain tested in agar dilution MIC studies was *N. gonorrhoeae* ATCC 49226. Five ATCC QC strains were tested in the broth microdilution studies: *S. aureus* ATCC 29213, *E. coli* ATCC 25922, *E. faecalis* ATCC 29212, *S. pneumoniae* ATCC 49619 and *H.s influenzae* ATCC 49247. Control
agents used were levofloxacin for the broth microdilution studies and ceftriaxone for the agar dilution studies (9).

The agar dilution MIC method specified in CLSI standard M07 (11) for dilution testing of *N. gonorrhoeae* antibiotic susceptibility was used at all laboratories. Three lots of GC agar media from three manufacturers were prepared by CMI and dispensed into sterile tubes to a final volume of 18 mL of agar. Lots included were obtained from Difco (Becton Dickinson, Franklin Lakes, NJ) (Lot # 9266070), Criterion (Hardy Diagnostics, Albany, NY) (Lot # 13248) and Remel (Thermo Fisher Scientific, Bedford, MA) (Lot # 403989). Defined growth supplement was obtained from Remel (GCHI Enrichment, Lot # 393311). Agar deeps, antibiotic powders, and defined growth supplement were shipped from CMI to the other seven testing laboratories. Zoliflodacin was tested on all three lots of media and ceftriaxone was tested on the Difco lot. Each laboratory tested 5 replicates of the organism each day for two days to obtain 30 zoliflodacin agar MIC values and 10 ceftriaxone agar MIC values.

The broth microdilution method specified in the CLSI standards M07 and M100 (9, 11) was used at all laboratories. Three lots of Mueller Hinton broth media from three manufacturers were prepared by CMI and used to prepare broth microdilution MIC panels containing serial two-fold dilutions of zoliflodacin. Lot one was used for serial two-fold dilutions of the control drug levofloxacin. Lots included were Difco (Product #275730, Lot #2319300), BBL (Becton Dickinson, Franklin Lakes, NJ) (Product #212322, Lot #4044343) and Oxoid (Thermo Fisher Scientific, Bedford, MA) (Product #CN0405, Lot #970220). Three types of broth microdilution panels were prepared. The first contained cation adjusted Mueller Hinton broth. The second and third panel types were prepared using the same three lots of Mueller Hinton broth supplemented with either 3% lysed horse blood (LHB) for testing of *S. pneumoniae* or made up as HTM broth for testing of *H. influenzae*. MIC panels were frozen at -70°C and shipped to each of the labs frozen. Each laboratory tested 10 replicates of each QC organism on a minimum of 3 days in order to obtain 30 zoliflodacin MIC values and 10 levofloxacin MIC values from each participating laboratory.

All data for both studies was sent to CMI for analysis. Colony counts of prepared inocula were performed by each laboratory on each day of testing.
Proposed QC ranges for zoliflodacin for all ATCC strains for both broth and agar dilution testing were generated using document M23 criteria (8) and the method by Turnidge et al. (10) (see CLSI RangeFinder tool http://clsi.org/standards/micro/rangefinder/).

Acknowledgements

We would like to thank Sara Patey and Linda Otterson for their preliminary QC range finding studies for zoliflodacin at AstraZeneca. A.A.M and J.P.M are employees of, and P.A.B is a consultant for, Entasis Therapeutics, who is the sponsor of zoliflodacin. Neither M.M.T. nor M.D.H. have a personal financial interest in Entasis Therapeutics. All authors provided analysis input and have read and approved the final manuscript.

References


Table and Figure Legends

Table 1. Medium lot and interlaboratory comparisons for zoliflodacin agar MIC test results against *N. gonorrhoeae* ATCC 49226

Table 2. Medium lot and interlaboratory comparisons for zoliflodacin broth microdilution MIC test results against *S. aureus* ATCC 29213

Table 3. Medium lot and interlaboratory comparisons for zoliflodacin broth microdilution MIC test results against *E. faecalis* ATCC 29212

Table 4. Medium lot and interlaboratory comparisons for zoliflodacin broth microdilution MIC test results against *E. coli* ATCC 25922

Table 5. Medium lot and interlaboratory comparisons for zoliflodacin broth microdilution MIC test results against *S. pneumoniae* ATCC 49619

Table 6. Medium lot and interlaboratory comparisons for zoliflodacin broth microdilution MIC test results against *H. influenzae* ATCC 49247

Table 7. Summary of Accepted QC Ranges

Figure 1. Chemical structure of zoliflodacin.

Figure 2. Distribution of zoliflodacin agar MIC test results against *N. gonorrhoeae* ATCC 49226

Figure 3. Distribution of zoliflodacin broth MIC test results against five QC strains
Table 1. Medium lot and interlaboratory comparisons for zoliflodacin agar MIC test results against \textit{N. gonorrhoeae} ATCC 49226$^a$

<table>
<thead>
<tr>
<th>Agar MIC (µg/mL)</th>
<th>No. of occurrences for medium lot:</th>
<th>No. of occurrences for laboratory:</th>
<th>Total no. of occurrences</th>
</tr>
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<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
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<tr>
<td></td>
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<tr>
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<tr>
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<td>80</td>
</tr>
</tbody>
</table>

Geomean$^b$ 0.149 0.134 0.188

Range 2 2 3 2 2 3 2 2 3 3

$^a$ dashed box corresponds to approved QC range; $^b$ geometric mean of observed values

Table 2. Medium lot and interlaboratory comparisons for zoliflodacin broth microdilution MIC test results against \textit{S. aureus} ATCC 29213$^a$

<table>
<thead>
<tr>
<th>Broth MIC (µg/mL)</th>
<th>No. of occurrences for medium lot:</th>
<th>No. of occurrences for laboratory:</th>
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<tr>
<td></td>
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Geomean$^b$ 0.165 0.241 0.259

Range 2 2 3 2 2 2 2 3 2 3 2 3

$^a$ dashed box corresponds to approved QC range; $^b$ geometric mean of observed values
Table 3. Medium lot and interlaboratory comparisons for zoliflodacin broth microdilution MIC test results against *E. faecalis* ATCC 29212<sup>a</sup>

<table>
<thead>
<tr>
<th>Broth MIC (µg/mL)</th>
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<th>No. of occurrences for laboratory:</th>
<th>Total no. of occurrences</th>
</tr>
</thead>
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<tr>
<td></td>
<td>A</td>
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</tr>
<tr>
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<tr>
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<td>3</td>
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</table>

<sup>a</sup>dashed box corresponds to approved QC range; <sup>b</sup>geometric mean of observed values

Table 4. Medium lot and interlaboratory comparisons for zoliflodacin broth microdilution MIC test results against *E. coli* ATCC 25922<sup>a</sup>

<table>
<thead>
<tr>
<th>Broth MIC (µg/mL)</th>
<th>No. of occurrences for medium lot:</th>
<th>No. of occurrences for laboratory:</th>
<th>Total no. of occurrences</th>
</tr>
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<tr>
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<tr>
<td>Geomean&lt;sup&gt;b&lt;/sup&gt;</td>
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</tbody>
</table>

<sup>a</sup>dashed box corresponds to approved QC range; <sup>b</sup>geometric mean of observed values
Table 5. Medium lot and interlaboratory comparisons for zoliflodacin broth microdilution MIC test results against *S. pneumoniae* ATCC 49619

<table>
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<th>No. of occurrences for medium lot:</th>
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</thead>
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Geomean\(^b\) 0.249 0.252 0.239

Range 3 3 3 1 1 3 2 1 3 3 1 3

*\(^a\) dashed box corresponds to approved QC range; \(^b\) geometric mean of observed values

Table 6. Medium lot and interlaboratory comparisons for zoliflodacin broth microdilution MIC test results against *H. influenzae* ATCC 49247

<table>
<thead>
<tr>
<th>Broth MIC (µg/mL)</th>
<th>No. of occurrences for medium lot:</th>
<th>No. of occurrences for laboratory:</th>
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<tr>
<td></td>
<td>A</td>
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Geomean\(^b\) 0.352 0.346 0.405

Range 2 2 2 2 2 1 1 2 2 2 3 2 3 3

*\(^a\) dashed box corresponds to approved QC range; \(^b\) geometric mean of observed values*
<table>
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<th>Organism</th>
<th>MIC range (µg/mL)</th>
<th>% of occurrences in range</th>
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<td><em>N. gonorrhoeae</em></td>
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</tr>
<tr>
<td><em>S. aureus</em> ATCC 29213</td>
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<td>100</td>
</tr>
<tr>
<td><em>E. faecalis</em> ATCC 29212</td>
<td>0.25 - 2</td>
<td>100</td>
</tr>
<tr>
<td><em>E. coli</em> ATCC 25922</td>
<td>1 - 4</td>
<td>100</td>
</tr>
<tr>
<td><em>S. pneumoniae</em> ATCC 49619</td>
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<td>100</td>
</tr>
<tr>
<td><em>H. influenzae</em> ATCC 49247</td>
<td>0.12 - 1</td>
<td>100</td>
</tr>
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</table>