

In vitro antimicrobial combination testing of and evolution of resistance to the first-in-class spiropyrimidinetrione zoliflodacin combined with six therapeutically relevant antimicrobials for *Neisseria gonorrhoeae*

Foerster, Sunniva; Drusano, George; Golparian, Daniel; Neely, Michael; Piddock, Laura; Alirol, Emilie; Unemo, Magnus

DOI:

[10.1093/jac/dkz376](https://doi.org/10.1093/jac/dkz376)

License:

Other (please specify with Rights Statement)

Document Version

Peer reviewed version

Citation for published version (Harvard):

Foerster, S, Drusano, G, Golparian, D, Neely, M, Piddock, L, Alirol, E & Unemo, M 2019, 'In vitro antimicrobial combination testing of and evolution of resistance to the first-in-class spiropyrimidinetrione zoliflodacin combined with six therapeutically relevant antimicrobials for *Neisseria gonorrhoeae*' *Journal of Antimicrobial Chemotherapy*, vol. 74, no. 12, pp. 3521–3529. <https://doi.org/10.1093/jac/dkz376>

[Link to publication on Research at Birmingham portal](#)

Publisher Rights Statement:

This is a pre-copyedited, author-produced version of an article accepted for publication in *Journal of Antimicrobial Chemotherapy* following peer review. The version of record Sunniva Foerster, George Drusano, Daniel Golparian, Michael Neely, Laura J V Piddock, Emilie Alirol, Magnus Unemo, In vitro antimicrobial combination testing of and evolution of resistance to the first-in-class spiropyrimidinetrione zoliflodacin combined with six therapeutically relevant antimicrobials for *Neisseria gonorrhoeae*, *Journal of Antimicrobial Chemotherapy*, Volume 74, Issue 12, December 2019, Pages 3521–3529, <https://doi.org/10.1093/jac/dkz376> is available online at: <https://academic.oup.com/jac/article/74/12/3521/5561459>

General rights

Unless a licence is specified above, all rights (including copyright and moral rights) in this document are retained by the authors and/or the copyright holders. The express permission of the copyright holder must be obtained for any use of this material other than for purposes permitted by law.

- Users may freely distribute the URL that is used to identify this publication.
- Users may download and/or print one copy of the publication from the University of Birmingham research portal for the purpose of private study or non-commercial research.
- User may use extracts from the document in line with the concept of 'fair dealing' under the Copyright, Designs and Patents Act 1988 (?)
- Users may not further distribute the material nor use it for the purposes of commercial gain.

Where a licence is displayed above, please note the terms and conditions of the licence govern your use of this document.

When citing, please reference the published version.

Take down policy

While the University of Birmingham exercises care and attention in making items available there are rare occasions when an item has been uploaded in error or has been deemed to be commercially or otherwise sensitive.

If you believe that this is the case for this document, please contact UBIRA@lists.bham.ac.uk providing details and we will remove access to the work immediately and investigate.

1 ***In vitro* antimicrobial combination testing and evolution of resistance to the first-**
2 **in-class spiropyrimidinetrione zoliflodacin combined with six therapeutically**
3 **relevant antimicrobials for *Neisseria gonorrhoeae***

4
5 **Sunniva FOERSTER^{1,*}, George DRUSANO², Daniel GOLPARIAN¹, Michael**
6 **NEELY³, Laura JV PIDDOCK⁴, Emilie ALIROL⁴ and Magnus UNEMO^{1,**}**

7
8 *¹WHO Collaborating Centre for Gonorrhoea and other STIs, Swedish Reference*
9 *Laboratory for STIs, Faculty of Medicine and Health, Örebro University, Örebro,*
10 *Sweden; ²Institute for Therapeutic Innovation, Department of Medicine, College of*
11 *Medicine, University of Florida, Orlando, USA; ³Children's Hospital of Los Angeles,*
12 *Department of Pediatrics, Division of Infectious Diseases, University of Southern*
13 *California, USA; ⁴Global Antibiotic Research & Development Partnership (GARDP),*
14 *Geneva, Switzerland*

15
16 *Current address. Sahlistrasse 17, 3012 Bern, Switzerland

17 **Corresponding author. Department of Laboratory Medicine, Clinical Microbiology,
18 Örebro University Hospital, SE-701 85 Örebro, Sweden. Tel: +46-19-6022038; Fax:
19 +46-19-127416; E-mail: magnus.unemo@regionorebrolan.se

20 **Running title:** Zoliflodacin in combination with six therapeutic antimicrobials

21
22
23
24
25 **Word count:** Text: 3644 words, Abstract: 248 words

26 **Objectives:** Resistance in *Neisseria gonorrhoeae* to all gonorrhoea therapeutic
27 antimicrobials has emerged. Novel therapeutic antimicrobials are imperative and the
28 first-in-class spiropyrimidinetrione zoliflodacin appears promising. Zoliflodacin could
29 be introduced in dual antimicrobial therapies to prevent the emergence and/or spread of
30 resistance. We investigated the *in vitro* activity and selection of resistance to
31 zoliflodacin alone and in combination with six gonorrhoea therapeutic antimicrobials
32 against *N. gonorrhoeae*.

33 **Methods:** The international gonococcal reference strains WHO F (wild-type), and
34 WHO O, WHO V, and WHO X (strains with different AMR profiles) were examined.
35 Zoliflodacin was evaluated alone or combined with ceftriaxone, cefixime,
36 spectinomycin, gentamicin, tetracycline, cethromycin, and sitafloxacin in checkerboard
37 assays, time-kill curve analysis, and selection of resistance studies.

38 **Results:** Zoliflodacin alone or in combination with all six antimicrobials showed a rapid
39 growth inhibition against all examined strains. The time-kill curve analysis indicated
40 that tetracycline or cethromycin combined with zoliflodacin can significantly decrease
41 the zoliflodacin kill rate *in vitro*. The frequency of selected zoliflodacin resistance
42 mutations was low when evaluated as a single agent and further reduced for all
43 antimicrobial combinations. All resistant mutants contained the GyrB mutations
44 D429N, K450T or K450N, resulting in zoliflodacin MICs of 0.5-4 mg/L.

45 **Conclusions:** Zoliflodacin, alone or in combination with STI therapeutic antimicrobials,
46 rapidly kills gonococci with infrequent resistance emergence. Zoliflodacin remains
47 promising for gonorrhoea oral monotherapy and as part of dual antimicrobial therapy
48 with low resistance emergence potential. A phase III trial evaluating efficacy and safety
49 of zoliflodacin for uncomplicated gonorrhoea treatment is planned in 2019.

50

51 **Introduction**

52 Compromised treatment of gonorrhoea due to antimicrobial resistance (AMR) in
53 *Neisseria gonorrhoeae* is a global public health concern.¹⁻⁴ AMR to all previously or
54 currently used therapeutic drugs has developed in *N. gonorrhoeae*; this facilitates the
55 transmission of gonorrhoea and the emergence of severe sequelae.^{2,3} *In vitro* resistance
56 to ceftriaxone, the last option for empiric first-line monotherapy, has been documented
57 in many countries.²⁻²⁰ Sporadic failures to cure pharyngeal gonorrhoea with ceftriaxone
58 have also been verified in many countries.^{5,12,13,17,19,21} Dual antimicrobial therapy
59 (mainly ceftriaxone plus azithromycin) was introduced for empirical first-line
60 gonorrhoea therapy in many countries worldwide.²²⁻²⁷ However, in 2016 the first global
61 failure of treating pharyngeal gonorrhoea with the recommended dual therapy was
62 reported in England.²⁸ International spread of ceftriaxone-resistant gonococcal strains
63 has also been documented in recent years.¹⁴⁻¹⁸ Finally, it is a grave concern that the first
64 global gonococcal strain with combined ceftriaxone resistance and high-level
65 azithromycin resistance was reported in 2018 in England¹⁹ and Australia.²⁰ To forestall
66 gonorrhoea becoming exceedingly-difficult-to-treat or even untreatable with any
67 feasible first-line antimicrobial regimen, novel, ideally oral, antimicrobials with new
68 mechanism(s) of action for treatment of gonorrhoea are essential.

69 The first-in-class spiropyrimidinetrione zoliflodacin targets the GyrB subunit of the
70 DNA gyrase, and has no cross-resistance to any previously developed antimicrobial.²⁹
71 Zoliflodacin was shown to have potent *in vitro* activity against geographically,
72 temporally, and genetically diverse wild-type, MDR and XDR *N. gonorrhoeae* strains.³⁰
73 Follow-up investigations of contemporary, consecutive and/or selected clinical isolates
74 in Europe, USA, and China further verified the potent activity and lack of resistance to
75 zoliflodacin.³¹⁻³³ A phase II randomised controlled clinical trial (RCT) evaluating single

76 oral doses of zoliflodacin (2 g or 3 g) for the treatment of uncomplicated gonorrhoea
77 was recently completed.³⁴ The cure rates for urogenital gonorrhoea were 98% (48/49)
78 and 100% (47/47), respectively. The cure rates for the low number of rectal infections
79 were 100% (5/5) and 100% (7/7), and for pharyngeal infections, 50% (4/8) and 82%
80 (9/11), respectively. Zoliflodacin was well-tolerated with transient gastrointestinal upset
81 being the most commonly reported adverse effect.³⁴ Consequently, zoliflodacin appears
82 promising for the future treatment of gonorrhoea and a phase III RCT is planned in
83 2019. Once introduced, zoliflodacin could be used in dual antimicrobial regimens, to
84 mitigate potential emergence and/or spread of resistance.

85 We firstly investigated the *in vitro* activity of zoliflodacin alone and in combination
86 with six therapeutic antimicrobials (novel, currently, or previously used) against *N.*
87 *gonorrhoeae* using checkerboard assays. Second, time-kill curve analysis and the *in*
88 *vitro* selection of resistance mutations in *N. gonorrhoeae* due to zoliflodacin exposure
89 alone or in combination with these antimicrobials were performed.

90

91 **Material and methods**

92 *Neisseria gonorrhoeae* reference strains, culture, and zoliflodacin susceptibility testing

93 The reference strains examined were WHO F (susceptible to all gonorrhoea therapeutic
94 antimicrobials), and WHO O, WHO V, and WHO X with different phenotypic AMR
95 and AMR determinants (Supplementary Table 1).^{35,36} These gonococcal reference
96 strains were used to investigate zoliflodacin alone and in combination with ceftriaxone,
97 cefixime, spectinomycin, gentamicin, tetracycline, cethromycin, and sitafloxacin in
98 checkerboard assays, time-kill curve analysis, and selection of resistance studies. All
99 strains were initially cultured on GCAGP agar plates³⁷ for 18-20 h at 37°C in a humid

100 5% CO₂-enriched atmosphere. The MICs (mg/L) of zoliflodacin (Entasis Therapeutics)
101 were determined by recommended agar dilution technique (www.clsi.org; M07-A10).

102

103 *Checkerboard analysis*

104 Checkerboard assays for the evaluation of zoliflodacin in combination with seven
105 therapeutic antimicrobials separately (ceftriaxone [Sigma_Aldrich], cefixime
106 [Sigma_Aldrich], spectinomycin [Sigma_Aldrich], gentamicin [Sigma_Aldrich],
107 doxycycline to represent tetracyclines [Sigma_Aldrich], cethromycin [Advanced Life
108 Sciences], and sitafloxacin [Daiichi Sankyo] were performed in Graver-Wade (GW)
109 medium as described,³⁸⁻⁴⁰ with minor modifications e.g. OD_{450nm} was used to measure
110 growth inhibition after 18 h of incubation. All experiments were performed in
111 triplicates.

112

113 *Time-kill curve analysis*

114 Time-kill curve analyses were performed as described.^{39,41} Zoliflodacin alone and in
115 combination with ceftriaxone, spectinomycin, cethromycin, tetracycline, gentamicin, or
116 sitafloxacin were examined. Cefixime was not evaluated due to the identical mechanism
117 of action and similar checkerboard results as ceftriaxone. Zoliflodacin alone and all the
118 antimicrobial combinations were examined against the antimicrobial susceptible WHO
119 F reference strain. Additionally, WHO X (high-level ceftriaxone resistant, tetracycline
120 resistant) was tested for zoliflodacin alone and in combination with ceftriaxone,
121 tetracycline, and gentamicin. WHO O (high-level spectinomycin resistant) and WHO V
122 (high-level cethromycin resistant) were tested for zoliflodacin alone and in combination
123 with spectinomycin and cethromycin, respectively, due to their resistance profiles.

124

125 *Fractional inhibitory concentration index (FICI) analysis*

126 The fractional inhibitory combination index (FICI) was calculated using the
127 checkerboard data to indicate synergy, additive or indifferent effect, or antagonism, as
128 described.⁴² As cut-off defining growth, an OD_{450nm} of ≤ 0.5 was defined. The cut-off for
129 potential synergy, indifferent and antagonism was ≤ 0.5 , $>0.5-4$, and >4 , respectively, as
130 described.⁴³

131

132 *Time-kill mathematical modeling⁴⁴*

133 For each isolate, all colony counts for all fractions/multiples of the MIC were modeled
134 simultaneously. The Non-Parametric Adaptive Grid (NPAG) algorithm within the
135 Pmetrics package (v1.5) for R (v3.5) was employed for the modeling process.^{45,46} This
136 algorithm is known to be mathematically consistent. The fractions/multiples of the MIC
137 were assumed to be stable (zoflupredacin has been shown to be heat stable over 24 h) and
138 were modeled by a very rapid loading infusion followed by a continuous infusion to
139 attain the desired exposure.

140 Weighting was by the inverse of the observation variance to approximate the
141 homoscedastic assumption. As there were multiple observations for each concentration,
142 the adaptive γ function was employed to optimize the weights. The Mean Weighted
143 Error was the measure of Bias and the Bias-Adjusted Mean Weighted Squared Error
144 was the measure of Precision. Both Pre-Bayesian (Population) and Bayesian
145 (Individual) regressions were performed in a Predicted-Observed plot.

146

147 *Population pharmacokinetic/pharmacodynamic mathematical model*

148 Because zoliflodacin concentration was constant in the system, we modeled one system
149 output, total bacterial burden, for the analysis of colony count data with the following
150 equations:

$$151 \quad \frac{dN}{dt} = K_g \times N \times E - K_{kmax} \times M \times N \quad (1)$$

$$152 \quad E = 1 - [N/POP_{MAX}] \quad (2)$$

$$153 \quad M = (\text{conc})^H / [(\text{conc})^H + EC_{50}^H] \quad (3)$$

154 Equation 1 describes the rates of change of the bacterial burden (N) over time. The
155 model equations for describing the rate of change of the numbers of microorganisms
156 were developed based on the *in vitro* observation that bacteria in the system are in
157 logarithmic growth phase in the absence of drug and exhibit an exponential density-
158 limited growth rate (equation 2). First-order growth was assumed, up to a density limit.
159 As bacterial population approaches maximal density, they approach stationary phase.
160 This is accomplished by multiplying the first-order growth terms by *E* (equation 2; a
161 logistic growth term). The maximal bacterial density (POP_{MAX}) is identified as part of
162 the estimation process. Most of the information for identifying this parameter is derived
163 from the bacterial growth in the control group. Equation 1 allows the antibacterial
164 effects of the different drug exposures administered to be modeled. There is a maximal
165 kill rate that the drug can induce (K_{kmax}). The killing effect of the drug was modeled as a
166 saturable kinetic event *M* [equation 3] that relates the kill rate to drug concentration,
167 where *H* is the slope or Hill's constant and EC₅₀ (mg/L) is the drug concentration at
168 which the bacterial kill rate is half-maximal. Thus, the drug effect observed on the
169 population is the difference between intrinsic growth rate and the kill rate observed at
170 the drug concentrations achieved.

171

172 *Construction of 95% credible intervals*

173 To summarize population parameter values, we used a bootstrapping procedure to
174 calculate median values and 95% credibility intervals. Briefly, using all four of the
175 support points which each contain a vector of values for every parameter in the model
176 and an associated probability of that parameter set, we generated 1000 sets of 4 random
177 weighted samples (with replacement) for any parameter, e.g. $K_{\text{kill-max}}$. From these 1000
178 sets, we calculated the median, 2.5th percentile, and 97.5th percentile.

179

180 *Selection of zoliflodacin-resistant mutants*

181 Selection of zoliflodacin-resistant mutants was performed for WHO F, WHO O, WHO
182 V, and WHO X (Supplementary Table 1) as described,³⁹ with minor modifications.
183 Briefly, GCVIT plates (3.6% Difco GC Medium Base agar [BD, Diagnostics]
184 supplemented with 1% IsoVitalex [BD, Diagnostics]) were prepared to contain 4×MIC,
185 2×MIC and 1×MIC of ceftriaxone, spectinomycin, cethromycin, doxycycline,
186 gentamicin, and sitafloxacin alone or in combination with zoliflodacin at the same
187 concentrations. The WHO strains were initially cultured on GCAGP plates³⁷ for 18–20
188 h at 37°C in a humid 5% CO₂-enriched atmosphere. Fresh cultures (18 h) from 10
189 GCAGP agar plates were pooled and suspended in 2 mL of sterile PBS. A dilution
190 series of the strain suspensions in PBS was plated on antimicrobial-free GCVIT plates.
191 Undiluted 100 µL aliquots were plated on antimicrobial-containing GCVIT plates and
192 grown for 48 h at 37°C in a humid 5% CO₂-enriched atmosphere. For each tested
193 antimicrobial combination and strain, zoliflodacin alone was tested in parallel. All
194 zoliflodacin-resistant mutants inhibited by ≥16 times the zoliflodacin MIC of the wild
195 type strain, a significant MIC increase, were genome sequenced as described.⁴⁷

196

197 **Results**

198 *Checkerboard analysis*

199 The results from the checkerboard analyses are summarised in Table 1. Except for one
200 strain, the mean FICIs for all evaluable strains ranged between 0.97-2.50 (standard
201 deviations (SDs): 0.04-1.14), indicating an indifferent effect. There were no significant
202 interactions between zoliflodacin and ceftriaxone, cefixime, spectinomycin,
203 cethromycin, tetracycline, gentamicin, or sitafloxacin. The only significant interaction
204 (*in vitro* antagonism) observed was for WHO F for zoliflodacin in combination with
205 cethromycin, with a mean FICI of 7.44, although the mean SD for the FICI was also
206 large (6.73) (Table 1).

207

208 *Time-kill curve analysis*

209 In general, zoliflodacin alone and in combination with the six antimicrobials showed
210 rapid growth inhibition against all tested strains. For zoliflodacin alone, similar time-kill
211 curve profiles were observed for all the four WHO reference strains (Supplemental
212 Figure 1). The rates of killing of the strains were dose-dependent with a rapid reduction
213 in observed cfus at 16×MIC and 8×MIC, and slower rates of kill at 4×MIC and 2×MIC
214 of zoliflodacin. For WHO X and particularly WHO F, the growth was typically
215 inhibited also at 1×MIC, and in several experiments by lower zoliflodacin
216 concentrations. For the highest zoliflodacin concentrations, the growth rates decreased
217 quickest in the first hour of exposure and then leveled off. Qualitative evaluations of the
218 time-kill curves indicated that tetracycline, cethromycin, ceftriaxone or gentamicin
219 combined with zoliflodacin affected the zoliflodacin growth inhibition *in vitro*. For
220 mathematical modeling of these interactions, see below. The combinations of
221 zoliflodacin plus spectinomycin or sitafloxacin showed an indifferent effect compared
222 to zoliflodacin alone (Supplementary Figure 1).

223

224 *Mathematical modeling of zoliflodacin for isolates with different antimicrobial*
225 *resistance mechanisms*

226 The mean, median and SD for the parameter estimates for WHO F, O, V, and X are
227 displayed in Table 2. For all the isolates, the ratio of the maximal kill rate constant
228 (K_{kmax}) to the growth rate constant (K_g) was in excess of unity and ranged from a ratio
229 of two to a ratio of eight. This indicates that zoliflodacin was able to induce substantial
230 kill in all four strains, even though three of the four strains had multiple AMR
231 determinants for other antimicrobials. The isolates all grew well, with turnover half-
232 time estimates that ranged from 0.44 h (WHO O) to 1.18 h (WHO V). The strains
233 differed substantially regarding the EC_{50} , with the antimicrobial wild-type WHO F
234 strain having an EC_{50} of 0.123 mg/L, while the strains isolates had EC_{50} values that were
235 6-fold to greater than 20-fold higher. This was reflected in the kill curves, where a
236 substantial proportion of the WHO F population was killed after 2-3 h exposure to
237 relatively low concentrations compared to the other strains, where killing required
238 concentrations at or above the MIC value. Note that the differences were not reflected in
239 the MIC, as there is only a 2-fold difference between the wild-type WHO F and the
240 other three strains (0.032 mg/L versus 0.064 mg/L).

241

242 *Model fit to the data*

243 The fit of the model to the data is displayed in Supplementary Table 2. Observed-
244 Predicted plots for both the Pre-Bayesian (Population) analyses and the Bayesian
245 (Individual) analyses were good. The measures of Bias and Precision demonstrate that
246 the analyses were reasonably precise and unbiased.

247

248 *Interaction between zoliflodacin and either cethromycin, tetracycline, ceftriaxone or*
249 *gentamicin in a time-kill assay*

250 In WHO F, the fit of the model to the data is shown in Supplementary Table 2. A
251 bootstrapping approach was employed to develop 95% credible intervals around the
252 point estimates of the system parameter values. In Table 3, we show the estimates of the
253 credible intervals for model parameters for the activities of zoliflodacin monotherapy
254 against WHO F. As we sought to ascertain the interaction between zoliflodacin and
255 either cethromycin, tetracycline, ceftriaxone or gentamicin in combination, we also
256 show the point estimates of the parameter values, but concentrate upon the rate of
257 bacterial cell kill (K_{kmax}) and the drug concentration of zoliflodacin at which the kill rate
258 is half maximal (EC_{50}), which is potency. The concentration shown is for zoliflodacin
259 alone, ignoring the concentration of cethromycin, tetracycline, ceftriaxone, or
260 gentamicin. As can be seen in Table 3, the estimates of K_{kmax} for zoliflodacin when
261 WHO F is also exposed to either cethromycin or tetracycline are significantly lower
262 than seen with zoliflodacin alone and fall outside the 95% credible interval; likewise,
263 the estimates of EC_{50} for zoliflodacin with either cethromycin or tetracycline are both
264 significantly higher than with zoliflodacin alone and fall outside the 95% credible
265 interval. These findings indicate a statistically significant *in vitro* decrease in bacterial
266 killing (i.e. potential *in vitro* antagonism) with the combinations of zoliflodacin plus
267 cethromycin or zoliflodacin plus tetracycline. The estimates of K_{kmax} and EC_{50} for
268 zoliflodacin with either ceftriaxone or gentamicin were also lower and higher,
269 respectively, than seen with zoliflodacin alone and fell outside the 95% credible
270 intervals. However, the EC_{50} remained relatively low, the inhibition of zoliflodacin kill
271 rates of these antimicrobials was substantially more limited, and the gonococcal
272 population was still relatively rapidly and effectively killed (Supplemental Figure 1).

273

274 *Selection of zoliflodacin-resistant mutants*

275 When exposed to zoliflodacin alone, zoliflodacin-resistant mutants were selected at very
276 low frequencies from the reference strains WHO F, WHO O, WHO V, and WHO X
277 (Table 4). No zoliflodacin-resistant mutants with a ≥ 16 fold increase of the wild-type
278 MIC, were selected when the four WHO strains were exposed to zoliflodacin in
279 combination with ceftriaxone, spectinomycin, cethromycin, doxycycline, gentamicin, or
280 sitafloxacin. All selected zoliflodacin-resistant mutants contained a single amino acid
281 alteration (D429N, K450N or K450T) in GyrB, which resulted in zoliflodacin MICs of
282 0.5-4 mg/L (up to 125 times increases in zoliflodacin MICs). The selected *gyrB*
283 zoliflodacin-resistant mutations did not affect the MICs of the two other bacterial
284 topoisomerase II inhibitors ciprofloxacin and sitafloxacin (targetting GyrA), or the
285 MICs of ceftriaxone, cefixime, spectinomycin, cethromycin, azithromycin, tetracycline,
286 gentamicin, or tetracycline (data not shown).

287

288 **Discussion**

289 The increasing prevalence of gonorrhoea in many settings and AMR in *N. gonorrhoeae*
290 is a major global public health concern.¹⁻⁴ Internationally, MDR *N. gonorrhoeae* strains
291 are spreading, significantly compromising the effectiveness of gonorrhoea treatment,
292 including the last remaining option, ceftriaxone plus azithromycin dual therapy.²²⁻²⁷
293 Novel antimicrobials for effective treatment of urogenital and extragenital gonorrhoea
294 are essential. The first-in-class spiropyrimidinetrione zoliflodacin, with a novel mode of
295 action, appears promising for the future treatment of gonorrhoea based on *in vitro*
296 activity against wild type, MDR and XDR *N. gonorrhoeae* strains, phase I and II
297 RCTs,²⁹⁻³⁴ and a multi-continental phase III RCT is planned in 2019. In the phase II

298 RCT,³⁴ the cure rate for the low number of pharyngeal gonococcal infections was lower
299 than the one for anogenital infections, which is the case for most antimicrobials.
300 Accordingly, it is essential to include sufficient number of pharyngeal gonococcal
301 infections in the phase III RCT as well as enhance our understanding of
302 pharmacokinetic/pharmacodynamic properties of zoliflodacin and other antimicrobials
303 in especially pharyngeal gonorrhoea. Once introduced, zoliflodacin could be used in a
304 dual antimicrobial regimen to mitigate emergence and/or spread of resistance and
305 potentially extend the life span of a new treatment modality.

306 We investigated the *in vitro* activity of zoliflodacin alone and in combination with
307 six therapeutic antimicrobials against *N. gonorrhoeae* using checkerboard analysis and
308 time-kill curve analysis, and selection of resistance mutations in *N. gonorrhoeae* when
309 exposed to zoliflodacin alone and zoliflodacin in combination with the additional
310 antimicrobials. The differences between the results in the checkerboard analyses and
311 time-kill curve analyses for several antimicrobials were likely due to the different times
312 for measuring growth inhibition (18 h versus 6 h), antimicrobial concentration ratios
313 (1:1 versus 64 different ratios) and experimental setup (direct inoculation versus 4 h
314 pre-incubation without antimicrobials). Longer time-kill experiments are not feasible
315 due to autolysis reducing the viable cell count (cfu/mL) of many strains. The OD_{450nm}
316 can be measured at later time-points because the turbidity accumulates and is not strictly
317 dependent on viable bacteria. Accordingly, the time-kill curve analysis supplemented
318 the checkerboard analyses, by measuring the early activity of different 1:1 combinations
319 of the antimicrobials. In general, zoliflodacin had a kill rate constant that resulted in a
320 rapid decline of bacterial counts for *N. gonorrhoeae* alone and in combination with all
321 the six antimicrobials. As previously reported,³⁹ zoliflodacin alone showed a
322 bactericidal profile similar to ciprofloxacin⁴¹ for all examined strains, In the

323 checkerboard analyses, the only strong interaction (potential *in vitro* antagonism)
324 identified was for WHO F and zoliflodacin in combination with cethromycin. However,
325 qualitative and quantitative evaluations of the time-kill curves indicated that
326 zoliflodacin combined with tetracycline, cethromycin, ceftriaxone, or gentamicin may
327 affect the kill rate *in vitro* compared to zoliflodacin alone. Mathematical modeling
328 subsequently verified statistically significant loss of potency *in vitro* (potential *in vitro*
329 antagonism) with the combinations of zoliflodacin plus cethromycin or tetracycline.
330 Some *in vitro* growth inhibition was also verified with the combinations of zoliflodacin
331 plus ceftriaxone or gentamicin. However, this inhibition was substantially more limited
332 and the gonococcal population remained relatively rapidly and effectively killed
333 (Supplemental Figure 1) with a low resistance emergence (Table 4). The combinations
334 of zoliflodacin plus spectinomycin and zoliflodacin plus sitafloxacin showed an
335 indifferent effect compared to zoliflodacin alone. It is important to stress that these *in*
336 *vitro* static results should be interpreted with caution. Optimising combination (or
337 single) therapies to achieve both a rapid growth inhibition and a suppression of AMR
338 emergence is very challenging, since these represent different goals of therapy.
339 Additionally, a static *in vitro* experiment might not completely reflect a dynamic *in vivo*
340 infection where antimicrobial concentrations and bacterial population numbers vary
341 over time. In order to design ideal dual therapies, two different antimicrobial
342 concentration-time profiles at all anatomical sites need to be monitored, while
343 additionally monitoring the impact of both antimicrobials on the susceptible bacterial
344 populations and subpopulations that have *a priori* AMR. To enhance our understanding
345 of the dynamic activity and selection of resistance mutations of zoliflodacin alone and
346 in combination with additional antimicrobials, a Hollow Fiber Bioreactor (HFB) for *N.*
347 *gonorrhoeae* would be ideal. This would remove the assay time restriction due to

348 autolysis, limited nutrients, and accumulation of metabolites. A HFB would additionally
349 address the dynamic rate of bacterial killing, post-antibiotic effect, drug exposure
350 parameters influencing efficacy, pharmacodynamic targets for optimal drug dosing, and
351 in combination with pharmacokinetic data dosage profiles that prevent or facilitate
352 resistance selection for any antimicrobial monotherapy or combination therapy.

353 When exposed to zoliflodacin alone, zoliflodacin-resistant mutants were selected at
354 very low frequencies from all four examined WHO reference strains and no
355 zoliflodacin-resistant mutants (with ≥ 16 -fold increased MIC) were selected when the
356 strains were exposed to zoliflodacin in combination with ceftriaxone, spectinomycin,
357 cethromycin, doxycycline, gentamicin, or sitafloxacin. The agar plate-based method
358 used for selection of zoliflodacin-resistant mutants in the present study, as all currently
359 available similar methods for *N. gonorrhoeae*, has inherent limitations, particularly for
360 antimicrobials such as zoliflodacin where resistance mutations are selected at very low
361 frequencies. This is likely part of the reason that zoliflodacin-resistance mutations have
362 been selected in different frequencies in diverse *N. gonorrhoeae* strains and on different
363 culture media, from $< 2 \times 10^{-14}$ to 1×10^{-8} , in previous studies.^{39,48} Accordingly, the
364 reported mutation frequencies need to be interpreted with caution. In the present study,
365 the parallel comparisons between resistance frequencies when exposed to zoliflodacin
366 alone and in combination with other antimicrobials show qualitatively that the
367 combination resulted in lower frequencies than expected in an additive model.
368 Experiments with *Escherichia coli* have previously demonstrated that the evolution of
369 resistance in response to a drug pair is independent from synergistic or antagonistic drug
370 interactions.⁴⁹ Theory shows that synergistic drug pairs, preferred for their immediate
371 efficacy, could even favor the evolution of resistance due to increased selective
372 pressure.⁵⁰ In the present study, all selected zoliflodacin-resistant mutants contained a

373 single amino acid alteration (D429N or, less frequently, K450T or K450N) in the
374 zoliflodacin target GyrB, which resulted in zoliflodacin MICs of 0.5-4 mg/L. Notably,
375 the *in vitro* selected zoliflodacin-resistant mutants with the GyrB D429N mutation
376 appear to have a reduced growth rate *in vitro*,³⁹ which make it difficult to predict the
377 emergence and spread of zoliflodacin-resistant mutants *in vivo*. The less frequently
378 selected GyrB D429A zoliflodacin-resistance mutation has also been reported
379 previously, as well as that an over-expression of the MtrCDE efflux pump might
380 slightly increase zoliflodacin MICs.^{39,48}

381 In conclusion, zoliflodacin, alone and in combination with other STI therapeutic
382 antimicrobials, had a rapid and high efficacy against gonococci. Zoliflodacin resistance
383 mutations were selected *in vitro* at very low frequencies, which were even lower when
384 zoliflodacin was combined with an additional antimicrobial. Tetracycline and
385 cethromycin significantly reduced the bactericidal activity of zoliflodacin *in vitro*: these
386 and additional interactions need to be further investigated. To enhance our
387 understanding of the dynamic activity and selection of resistance mutations of
388 zoliflodacin alone and in combination with additional antimicrobials, as well as fitness
389 of zoliflodacin-resistant selected mutants, a future optimized and quality-assured HFB
390 for *N. gonorrhoeae* would be ideal. Our findings suggest several potentially new
391 candidate zoliflodacin combinations. Zoliflodacin remains a promising novel, oral
392 therapy for treatment of gonorrhoea and our data support that appropriate dual
393 antimicrobial therapy can be highly effective as well as suppress selection of
394 zoliflodacin resistance mutations *in vitro* and therefore might extend the life span of a
395 potentially new oral treatment modality.

396

397 **SUPPLEMENTARY MATERIAL**

399

400 **Acknowledgements**

401 We are grateful to Advanced Life Sciences Inc, Woodridge, Illinois, USA and Entasis
402 Therapeutics for providing cethromycin and zoliflodacin, respectively. We thank our
403 Entasis colleagues, John Mueller, Alita Miller and John O'Donnell, for critical review
404 of the manuscript.

405

406 **Funding**

407 The present work was supported by the GARDP, the Örebro County Council Research
408 Committee, Örebro, Sweden, and the Foundation for Medical Research at Örebro
409 University Hospital, Örebro, Sweden.

410

411 **Transparency declarations**

412 None to declare.

413

414 **References**

- 415 1. Newman L, Rowley J, Vander Hoorn S *et al*. Global estimates of the prevalence and
416 incidence of four curable sexually transmitted infections in 2012 based on systematic
417 review and global reporting. *PLoS One* 2015; **10**: e0143304.
- 418 2. Unemo M, Shafer WM. Antimicrobial resistance in *Neisseria gonorrhoeae* in the
419 21st Century: past, evolution, and future. *Clin Microbiol Rev* 2014; **27**: 587-613.
- 420 3. Wi T, Lahra MM, Ndowa F *et al*. Antimicrobial resistance in *Neisseria gonorrhoeae*:
421 Global surveillance and a call for international collaborative action. *PLoS Med* 2017;
422 **14**: e1002344.

- 423 4. Cole MJ, Spiteri G, Jacobsson S *et al.* Overall low extended-spectrum cephalosporin
424 resistance but high azithromycin resistance in *Neisseria gonorrhoeae* in 24 European
425 countries, 2015. *BMC Infect Dis* 2017; **17**: 617.
- 426 5. Ohnishi M, Golparian D, Shimuta K *et al.* Is *Neisseria gonorrhoeae* initiating a
427 future era of untreatable gonorrhoea? Detailed characterization of the first strain with
428 high-level resistance to ceftriaxone. *Antimicrob Agents Chemother* 2011; **55**: 3538-45.
- 429 6. Cámara J, Serra J, Ayats J *et al.* Molecular characterization of two high-level
430 ceftriaxone-resistant *Neisseria gonorrhoeae* isolates detected in Catalonia, Spain. *J*
431 *Antimicrob Chemother* 2012; **67**: 1858-60.
- 432 7. Unemo M, Golparian D, Nicholas R *et al.* High-level cefixime- and ceftriaxone-
433 resistant *N. gonorrhoeae* in France: novel *penA* mosaic allele in a successful
434 international clone causes treatment failure. *Antimicrob Agents Chemother* 2012; **56**:
435 1273-80.
- 436 8. Gianecini R, Oviedo C, Stafforini G *et al.* *Neisseria gonorrhoeae* resistant to
437 ceftriaxone and cefixime, Argentina. *Emerg Infect Dis* 2016; **22**: 1139-41.
- 438 9. Lahra MM, Ryder N, While DM. A new multidrug-resistant strain of *Neisseria*
439 *gonorrhoeae* in Australia. *N Engl J Med* 2014; **371**: 1850-1.
- 440 10. Deguchi T, Yasuda M, Hatazaki K *et al.* New clinical strain of *Neisseria*
441 *gonorrhoeae* with decreased susceptibility to ceftriaxone in Japan. *Emerg Infect Dis*
442 2016; **22**: 142-4.
- 443 11. Nakayama S-I, Shimuta K, Furubayashi K-I *et al.* New ceftriaxone and multidrug-
444 resistant *Neisseria gonorrhoeae* strain with a novel mosaic *penA* gene isolated in Japan.
445 *Antimicrob Agents Chemother* 2016; **60**: 4339-41.

- 446 12. Golparian D, Ohlsson A, Janson H *et al.* Four treatment failures of pharyngeal
447 gonorrhoea with ceftriaxone (500 mg) or cefotaxime (500 mg), Sweden, 2013 and 2014.
448 *Euro Surveill* 2014; 19(30). pii: 20862.
- 449 13. Unemo M, Golparian D, Potonik M *et al.* Treatment failure of pharyngeal
450 gonorrhoea with internationally recommended first-line ceftriaxone verified in Slovenia,
451 September 2011. *Euro Surveill* 2012; 17(25). pii: 20200.
- 452 14. Lahra MM, Martin I, Demczuk W *et al.* Cooperative recognition of internationally
453 disseminated ceftriaxone-resistant *Neisseria gonorrhoeae* strain. *Emerg Infect Dis*
454 2018; **24**(4).
- 455 15. Lefebvre B, Martin I, Demczuk W *et al.* Ceftriaxone-resistant *Neisseria*
456 *gonorrhoeae*, Canada, 2017. *Emerging Infect Dis* 2018; **24**: 381-3.
- 457 16. Terkelsen D, Tolstrup J, Johnsen CH *et al.* Multidrug-resistant *Neisseria*
458 *gonorrhoeae* infection with ceftriaxone resistance and intermediate resistance to
459 azithromycin, Denmark, 2017. *Euro Surveill* 2017; 22(42).
- 460 17. Poncin T, Fouere S, Braille A *et al.* Multidrug-resistant *Neisseria gonorrhoeae*
461 failing treatment with ceftriaxone and doxycycline in France, November 2017. *Euro*
462 *Surveill* 2018; 23(21).
- 463 18. Golparian D, Rose L, Lynam A *et al.* Multidrug-resistant *Neisseria gonorrhoeae*
464 isolate, belonging to the internationally spreading Japanese FC428 clone, with
465 ceftriaxone resistance and intermediate resistance to azithromycin in Ireland, August
466 2018. *Euro Surveill* 2018; 23(47).
- 467 19. Eyre DW, Sanderson ND, Lord E *et al.* Gonorrhoea treatment failure caused by a
468 *Neisseria gonorrhoeae* strain with combined ceftriaxone and high-level azithromycin
469 resistance, England, February 2018. *Euro Surveill* 2018; 23(27).
- 470 20. Whiley DM, Jennison A, Pearson J *et al.* Genetic characterization of *Neisseria*

471 *gonorrhoeae* resistant to both ceftriaxone and azithromycin. *Lancet Infect Dis* 2018; **18**:
472 717-8.

473 21. Read PJ, Limnios EA, McNulty A *et al.* One confirmed and one suspected case of
474 pharyngeal gonorrhoea treatment failure following 500 mg ceftriaxone in Sydney,
475 Australia. *Sex Health* 2013; **10**: 460-2.

476 22. Bignell C, Unemo M. 2012 European guideline on the diagnosis and treatment of
477 gonorrhoea in adults. *Int J STD AIDS* 2013; **24**: 85-92.

478 23. Bignell C, Fitzgerald M. UK national guideline for the management of gonorrhoea
479 in adults, 2011. *Int J STD AIDS* 2011; **22**: 541-7.

480 24. Workowski KA, Bolan GA. Sexually transmitted diseases treatment guidelines,
481 2015. *MMWR Recomm Rep* 2015; **64**(RR-03): 1-137.

482 25. World Health Organization (WHO), Department of Reproductive Health and
483 Research. WHO guidelines for the treatment of *Neisseria gonorrhoeae*. Geneva: WHO,
484 2016: p. 1-64. [https://apps.who.int/iris/bitstream/handle/10665/246114/9789241549691-](https://apps.who.int/iris/bitstream/handle/10665/246114/9789241549691-eng.pdf;jsessionid=090680F3DF4BD97479F394BC9CB837EB?sequence=1)
485 [eng.pdf;jsessionid=090680F3DF4BD97479F394BC9CB837EB?sequence=1](https://apps.who.int/iris/bitstream/handle/10665/246114/9789241549691-eng.pdf;jsessionid=090680F3DF4BD97479F394BC9CB837EB?sequence=1)

486 26. Public Health Agency of Canada. Canadian Guidelines on Sexually Transmitted
487 Infections. Gonococcal Infections Chapter, 2013. [www.phac-aspc.gc.ca/std-mts/sti-](http://www.phac-aspc.gc.ca/std-mts/sti-its/cgsti-ldcits/assets/pdf/section-5-6-eng.pdf)
488 [its/cgsti-ldcits/assets/pdf/section-5-6-eng.pdf](http://www.phac-aspc.gc.ca/std-mts/sti-its/cgsti-ldcits/assets/pdf/section-5-6-eng.pdf)

489 27. Australasian Sexual Health Alliance (ASHA). Australian STI Management
490 Guidelines for Use in Primary Care. [www.sti.guidelines.org.au/sexually-transmissible-](http://www.sti.guidelines.org.au/sexually-transmissible-infections/gonorrhoea#management)
491 [infections/gonorrhoea#management](http://www.sti.guidelines.org.au/sexually-transmissible-infections/gonorrhoea#management)

492 28. Fifer H, Natarajan U, Jones L *et al.* Failure of dual antimicrobial therapy in
493 treatment of gonorrhoea. *N Engl J Med* 2016; **374**: 2504-6.

494 29. Basarab GS, Kern GH, McNulty J *et al.* Responding to the challenge of untreatable
495 gonorrhea: ETX0914, a first-in-class agent with a distinct mechanism-of-action against
496 bacterial type II topoisomerases. *Sci Rep* 2015; **5**: 11827.

497 30. Jacobsson,S, Golparian D, Alm RA *et al.* High in vitro activity of the novel
498 spiropyrimidinetrione AZD0914, a DNA gyrase inhibitor, against multidrug-resistant
499 *Neisseria gonorrhoeae* isolates suggests a new effective option for oral treatment of
500 gonorrhea. *Antimicrob Agents Chemother* 2014; **58**: 5585-8.

501 31. Unemo M, Ringlander J, Golparian D *et al.* High in vitro susceptibility to the novel
502 spiropyrimidinetrione ETX0914 (AZD0914) among 873 contemporary clinical
503 *Neisseria gonorrhoeae* isolates from 21 european countries from 2012 to 2014.
504 *Antimicrob Agents Chemother* 2015; **59**: 5220-5.

505 32. Papp JR, Lawrence K, Sharpe S *et al.* In vitro growth of multidrug-resistant
506 *Neisseria gonorrhoeae* isolates is inhibited by ETX0914, a novel spiropyrimidinetrione.
507 *Int J Antimicrob Agents* 2016; **48**: 328-30.

508 33. Su XH, Wang BX, Le WJ *et al.* Multidrug-resistant *Neisseria gonorrhoeae* isolates
509 from Nanjing, China, are sensitive to killing by a novel DNA Gyrase inhibitor,
510 ETX0914 (AZD0914). *Antimicrob Agents Chemother* 2015; **60**: 621-3.

511 34. Taylor SN, Marrazzo J, Batteiger BE *et al.* Single-dose zoliflodacin (ETX0914) for
512 treatment of urogenital gonorrhea. *N Engl J Med* 2018; **379**: 1835-45.

513 35. Unemo M, Fasth O, Fredlund H *et al.* Phenotypic and genetic characterization of the
514 2008 WHO *Neisseria gonorrhoeae* reference strain panel intended for global quality
515 assurance and quality control of gonococcal antimicrobial resistance surveillance for
516 public health purposes. *J Antimicrob Chemother* 2009; **63**: 1142-51.

517 36. Unemo M, Golparian D, Sánchez-Busó L *et al.* The novel 2016 WHO *Neisseria*
518 *gonorrhoeae* reference strains for global quality assurance of laboratory investigations:

519 phenotypic, genetic and reference genome characterization. *J Antimicrob Chemother*
520 2016; **71**: 3096-108.

521 37. Jönsson A, Foerster S, Golparian D *et al.* In vitro activity and time-kill curve
522 analysis of sitafloxacin against a global panel of antimicrobial-resistant and multidrug-
523 resistant *Neisseria gonorrhoeae* isolates. *APMIS* 2018; **126**: 29-37.

524 38. Meletiadiis J, Pournaras S, Roilides E *et al.* Defining fractional inhibitory
525 concentration index cutoffs for additive interactions based on self-drug additive
526 combinations, Monte Carlo simulation analysis, and in vitro-in vivo correlation data for
527 antifungal drug combinations against *Aspergillus fumigatus*. *Antimicrob Agents*
528 *Chemother* 2010; **54**: 602-9.

529 39. Foerster S, Golparian D, Jacobsson S *et al.* Genetic resistance determinants, in vitro
530 time-kill curve analysis and pharmacodynamic functions for the novel topoisomerase II
531 inhibitor ETX0914 (AZD0914) in *Neisseria gonorrhoeae*. *Front Microbiol* 2015; **6**:
532 1377.

533 40. Wade JJ, Graver MA. A fully defined, clear and protein-free liquid medium
534 permitting dense growth of *Neisseria gonorrhoeae* from very low inocula. *FEMS*
535 *Microbiol Lett* 2007; **273**: 35-7.

536 41. Foerster S, Unemo M, Hathaway LJ *et al.* Time-kill curve analysis and
537 pharmacodynamic functions for *in vitro* evaluation of antimicrobials against *Neisseria*
538 *gonorrhoeae*. *BMC Microbiol* 2016; **16**: 216.

539 42. White RL, Burgess DS, Manduru M *et al.* Comparison of three different in vitro
540 methods of detecting synergy: time-kill, checkerboard, and E test. *Antimicrob Agents*
541 *Chemother* 1996; **40**: 1914-8.

542 43. Odds FC. Synergy, antagonism, and what the chequerboard puts between them. *J*
543 *Antimicrob Chemother* 2003; **52(1)**: 1.

- 544 44. Drusano GL. Antimicrobial pharmacodynamics: critical interactions of “bug and
545 drug”. *Nat Rev Microbiol* 2004; **2**: 289-300.
- 546 45. Leary R, Jelliffe R, Schumitzky A *et al.* An adaptive grid non-parametric approach
547 to pharmacokinetic and dynamic (PK/PD) models. In "Proceedings of the 14th IEEE
548 Symposium on Computer-Based Medical Systems". IEEE Computer Society, Bethesda,
549 MD 2001: 389-94.
- 550 46. Neely MN, van Guilder MG, Yamada WM *et al.* Accurate detection of outliers and
551 subpopulations with Pmetrics, a nonparametric and parametric pharmacometric
552 modeling and simulation package for R. *Ther Drug Monit* 2012; **34**: 467-76.
- 553 47. Jacobsson S, Golparian D, Cole M *et al.* WGS analysis and molecular resistance
554 mechanisms of azithromycin-resistant (MIC >2 mg/L) *Neisseria gonorrhoeae* isolates
555 in Europe from 2009 to 2014. *J Antimicrob Chemother* 2016; **71**: 3109-16.
- 556 48. Alm RA, Lahiri SD, Kutschke A *et al.* Characterization of the novel DNA gyrase
557 inhibitor AZD0914: low resistance potential and lack of cross-resistance in *Neisseria*
558 *gonorrhoeae*. *Antimicrob Agents Chemother* 2015; **59**: 1478-86.
- 559 49. Munck C, Gumpert HK, Wallin AI *et al.* Prediction of resistance development
560 against drug combinations by collateral responses to component drugs. *Sci Transl Med*
561 2014; **6**: 262ra156.
- 562 50. Michel JB, Yeh PJ, Chait R *et al.* Drug interactions modulate the potential for
563 evolution of resistance. *Proc Natl Acad Sci U S A* 2008; **105**: 14918-23.

564

565

566

567
568
569
570
571
572
573
574
575
576
577
578
579
580
581
582
583
584
585
586

Table 1 Fractional inhibitory concentration index (FICI) from the checkerboard assay

Strain	Antimicrobial combination	Checkerboard FICI (SD)^a
WHO F	Zoliflodacin+ceftriaxone	1.80 (0.59)
WHO O	Zoliflodacin+ceftriaxone	1.11 (0.04)
WHO V	Zoliflodacin+ceftriaxone	1.24 (0.14)
WHO X	Zoliflodacin+ceftriaxone	1.04 (0.05)
WHO F	Zoliflodacin+cefixime	2.50 (1.14)
WHO O	Zoliflodacin+cefixime	1.23 (0.08)
WHO V	Zoliflodacin+cefixime	1.21 (0.16)
WHO X	Zoliflodacin+cefixime	1.15 (0.40)
WHO F	Zoliflodacin+spectinomycin	1.27 (0.59)
WHO O	Zoliflodacin+spectinomycin	NA
WHO V	Zoliflodacin+spectinomycin	1.00 (0.16)
WHO X	Zoliflodacin+spectinomycin	1.43 (0.42)
WHO F	Zoliflodacin+cethromycin	7.44 (6.73)
WHO O	Zoliflodacin+cethromycin	1.35 (0.18)

WHO V	Zoliflodacin+cethromycin	NA
WHO X	Zoliflodacin+cethromycin	0.97 (0.16)
WHO F	Zoliflodacin+doxycycline	1.96 (0.07)
WHO O	Zoliflodacin+doxycycline	1.24 (0.09)
WHO V	Zoliflodacin+doxycycline	1.47 (0.42)
WHO X	Zoliflodacin+doxycycline	1.09 (0.09)
WHO F	Zoliflodacin+gentamicin	1.21 (0.34)
WHO O	Zoliflodacin+gentamicin	1.09 (0.04)
WHO V	Zoliflodacin+gentamicin	1.49 (0.68)
WHO X	Zoliflodacin+gentamicin	1.13 (0.37)
WHO F	Zoliflodacin+sitafloracin	1.08 (0.22)
WHO O	Zoliflodacin+sitafloracin	1.01 (0.07)
WHO V	Zoliflodacin+sitafloracin	1.06 (0.05)
WHO X	Zoliflodacin+sitafloracin	1.03 (0.33)

587 NA, not applicable (due to high-level resistance to spectinomycin (WHO O) or
588 cethromycin (WHO V))

589 ^aMean values from three experiments. The cut-off for potential synergy, indifferent and
590 antagonism was ≤ 0.5 , $>0.5-4$, and >4 , respectively, as previously described.⁴³

591

592

593

594

595

596

597

598

599

600

601

602

603

604

605

Table 2 Parameter estimates for zoliflodacin kill of four WHO *Neisseria gonorrhoeae* reference strains

Parameter	K_g	K_{kmax}	EC_{50}	H	POPMAX	IC
Units	hr^{-1}	hr^{-1}	mg/L	unitless	cfu/mL	cfu/mL
<i>WHO F</i>						
Mean	1.10	4.91	0.123	9.94	4.78×10^8	6.27×10^5
Median	0.790	4.42	0.0187	9.14	4.18×10^8	6.23×10^5
SD	0.726	2.20	0.175	2.46	2.13×10^8	6.64×10^4
<i>WHO O</i>						
Mean	1.59	6.88	2.65	0.872	6.87×10^7	2.18×10^6
Median	1.55	6.88	2.89	0.846	7.65×10^7	2.52×10^6
SD	0.0470	0.0642	0.312	0.113	2.91×10^6	4.91×10^5

<i>WHO V</i>						
Mean	0.586	4.91	1.01	9.61	7.02×10^8	2.50×10^6
Median	0.390	4.68	0.801	2.61	7.95×10^8	2.90×10^6
SD	0.479	0.813	0.406	8.66	2.60×10^6	4.96×10^5
<i>WHO X</i>						
Mean	0.771	1.67	0.789	2.25	9.78×10^8	9.55×10^5
Median	0.754	1.11	0.947	0.675	9.63×10^8	1.06×10^6
SD	0.0318	0.655	0.180	1.87	1.96×10^7	1.23×10^5

606 K_g = Growth rate constant; K_{kmax} = maximal kill rate constant; EC_{50} = Zoliflodacin
607 concentration at which the kill rate is 50% of maximal; H = Hill's constant; POPMAX =
608 Maximal population size in stationary phase; IC = Initial Condition, the number of
609 Colony Forming Units at baseline.

610

611

612

613

614

615

616

617

618

619

620

621 **Table 3 Determination of the interaction of zoliflodacin with cethromycin,**
622 **tetracycline, ceftriaxone or gentamicin as a function of whether the maximal**
623 **bacterial kill rate (K_{kmax}) and the concentration of zoliflodacin in combination with**
624 **the second drug fall outside the 95% credible interval around the point estimates**
625 **of the parameters for zoliflodacin alone.** The highlighted numbers from the
626 combination analyses should be compared to the 95% credible intervals for zoliflodacin
627 alone.

628 **Zoliflodacin alone (WHO F)**

	Mean	SD	CV%	Median	2.50 Pctle	97.5 Pctle
K_g	1.56	0.8	51.16	1.59	0.68	2.4
K_{kmax}	9.35	2.96	31.62	10.38	5.15	12.05
EC_{50}	0.07	0.03	36.26	0.08	0.04	0.1
H_k	9.48	7.61	80.27	7.43	2.85	19.9

POPMAX	4.61E+08	1.37E+08	29.74	4.43E+08	3.54E+08	6.31E+08
IC	7.66E+06	7.65E+06	99.95	8.27E+06	1.02E+05	1.64E+07

629

630 **Zoliflodacin plus cethromycin (WHO F)**

	Mean	SD	CV%	Median
K _g	0.817	0.110	13.5	0.741
K _{kmax}	4.13	0.384	9.30	4.28
EC ₅₀	0.559	0.308	55.1	0.729
H _k	5.10	5.67	111	1.34
POPMAX	6.62E+09	4.36E+09	65.8	9.93E+09
IC	1.66E+06	7.48E+05	44.9	2.22E+06

631

632 **Zoliflodacin plus tetracycline (WHO F)**

	Mean	SD	CV%	Median
K _g	0.900	0.0261	2.90	0.919
K _{kmax}	2.65	0.241	9.09	2.84
EC ₅₀	3.02	0.301	9.96	2.82
H _k	1.11	0.335	0.335	1.10
POPMAX	1.01E+09	6.15E+06	0.610	1.04E+09
IC	1.61E+06	5.82E+05	3.61	1.56E+06

633

634 **Zoliflodacin plus ceftriaxone (WHO F)**

	Mean	SD	CV%	Median
K _g	1.24	0.0685	5.54	1.21
K _{kmax}	3.63	0.417	11.5	3.46
EC ₅₀	0.333	0.108	32.4	0.353
H _k	1.73	0.936	54.1	1.133
POPMAX	5.67E+08	2.52E+08	44.4	4.55E+08
IC	1.23E+06	4.38E+05	35.5	1.17E+06

635

636 **Zoliflodacin plus gentamicin (WHO F)**

	Mean	SD	CV%	Median
K_g	1.14	0.206	18.0	1.26
K_{kmax}	6.80	2.67	39.3	8.23
EC_{50}	0.763	0.340	44.5	0.898
H_k	1.85	1.85	88.4	1.12
POPMAX	8.10E+08	3.49E+08	43.1	9.94E+08
IC	1.22E+06	9.64E+05	78.8	7.15E+05

637 K_g = Growth rate constant; K_{kmax} = maximal kill rate constant; EC_{50} = Zoliflodacin
638 concentration at which the kill rate is 50% of maximal; H = Hill's constant; POPMAX =
639 Maximal population size in stationary phase; IC = Initial Condition, the number of
640 Colony Forming Units at baseline.

641

642

643

644

645

646

647

648

649

650

651

652

653

654

655 **Table 4 Frequency of selected zoliflodacin resistance mutants, when *Neisseria***
656 ***gonorrhoeae* strains were exposed to zoliflodacin alone and zoliflodacin in**
657 **combination with additional antimicrobials, and selected GyrB resistance**
658 **mutations**

Isolate ^a	Frequency – zoliflodacin ^b	Additional drug	Frequency – Additional drug	Frequency – combination ^b	Expectation (additive model)
WHO F	3.1×10^{-12}	Ceftriaxone	1.25×10^{-13}	-	lower
WHO O	2.0×10^{-12}	Ceftriaxone	3×10^{-12}	2.0×10^{-12}	lower
WHO V	2.0×10^{-11}	Ceftriaxone	ND ^c	-	lower

WHO X	2.5×10^{-12}	Ceftriaxone	2.5×10^{-12}	8.3×10^{-13}	lower
WHO F	3.1×10^{-12}	Spectinomycin	ND ^c	-	lower
WHO O	2.0×10^{-12}	Spectinomycin	ND ^c	-	lower
WHO V	2.0×10^{-11}	Spectinomycin	ND ^c	-	lower
WHO X	2.5×10^{-12}	Spectinomycin	ND ^c	-	lower
WHO F	1.1×10^{-12}	Cethromycin	2.2×10^{-11}	-	lower
WHO O	1.9×10^{-11}	Cethromycin	ND ^c	-	lower
WHO V	9.4×10^{-12}	Cethromycin	ND ^c	7.2×10^{-12}	lower
WHO X	-	Cethromycin	ND ^c	-	NA
WHO F	3.1×10^{-12}	Doxycycline	-	-	lower
WHO O	3.0×10^{-10}	Doxycycline	ND ^c	-	lower
WHO V	3.3×10^{-11}	Doxycycline	ND ^c	-	lower
WHO X	-	Doxycycline	ND ^c	-	lower
WHO F	1.0×10^{-13}	Gentamicin	7.0×10^{-13}	-	lower
WHO O	1.3×10^{-11}	Gentamicin	ND ^c	-	lower
WHO V	5.5×10^{-11}	Gentamicin	-	-	lower
WHO X	2.5×10^{-13}	Gentamicin	1.0×10^{-11}	-	lower
WHO F	ND ^c	Sitafloxacin	ND ^c	ND ^c	NA
WHO O	1.4×10^{-11}	Sitafloxacin	2×10^{-12}	2.0×10^{-12}	lower
WHO V	ND ^c	Sitafloxacin	1.7×10^{-12}	2.0×10^{-11}	lower
WHO X	2.5×10^{-13}	Sitafloxacin	ND ^c	2.5×10^{-13}	lower

659 ^aFor each tested combination of zoliflodacin plus one additional antimicrobial,
660 zoliflodacin alone was tested in parallel for the same strain.

661 ^bFrequency of zoliflodacin resistance mutations (cfu/mL) when exposed to zoliflodacin
662 alone or zoliflodacin in combination with additional antimicrobial. -, no mutants
663 detected.

664 ^cNot determined as outside the experimental range.

665