

# 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*

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

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22  
23  
24  
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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.<sup>1-4</sup> 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.<sup>2,3</sup> *In vitro* resistance  
56 to ceftriaxone, the last option for empiric first-line monotherapy, has been documented  
57 in many countries.<sup>2-20</sup> Sporadic failures to cure pharyngeal gonorrhoea with ceftriaxone  
58 have also been verified in many countries.<sup>5,12,13,17,19,21</sup> Dual antimicrobial therapy  
59 (mainly ceftriaxone plus azithromycin) was introduced for empirical first-line  
60 gonorrhoea therapy in many countries worldwide.<sup>22-27</sup> However, in 2016 the first global  
61 failure of treating pharyngeal gonorrhoea with the recommended dual therapy was  
62 reported in England.<sup>28</sup> International spread of ceftriaxone-resistant gonococcal strains  
63 has also been documented in recent years.<sup>14-18</sup> 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 England<sup>19</sup> and Australia.<sup>20</sup> 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.<sup>29</sup>  
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.<sup>30</sup>  
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.<sup>31-33</sup> 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.<sup>34</sup> 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.<sup>34</sup> 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).<sup>35,36</sup> 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 plates<sup>37</sup> for 18-20 h at 37°C in a humid

100 5% CO<sub>2</sub>-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,<sup>38-40</sup> with minor modifications e.g. OD<sub>450nm</sub> 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.<sup>39,41</sup> 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.<sup>42</sup> As cut-off defining growth, an OD<sub>450nm</sub> of  $\leq 0.5$  was defined. The cut-off for  
129 potential synergy, indifferent and antagonism was  $\leq 0.5$ ,  $>0.5-4$ , and  $>4$ , respectively, as  
130 described.<sup>43</sup>

131

132 *Time-kill mathematical modeling<sup>44</sup>*

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.<sup>45,46</sup> 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  $\gamma$  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<sub>MAX</sub>) 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 EC<sub>50</sub> (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, 2.5<sup>th</sup> percentile, and 97.5<sup>th</sup> 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,<sup>39</sup> 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 plates<sup>37</sup> for 18–20  
188 h at 37°C in a humid 5% CO<sub>2</sub>-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% CO<sub>2</sub>-enriched atmosphere. For each tested  
193 antimicrobial combination and strain, zoliflodacin alone was tested in parallel. All  
194 zoliflodacin-resistant mutants inhibited by  $\geq 16$  times the zoliflodacin MIC of the wild  
195 type strain, a significant MIC increase, were genome sequenced as described.<sup>47</sup>

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  $\geq 16$  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.<sup>1-4</sup> 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.<sup>22-27</sup>  
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,<sup>29-34</sup> and a multi-continental phase III RCT is planned in 2019. In the phase II

298 RCT,<sup>34</sup> 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<sub>450nm</sub>  
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,<sup>39</sup> zoliflodacin alone showed a  
322 bactericidal profile similar to ciprofloxacin<sup>41</sup> 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  $\geq 16$ -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  $1 \times 10^{-8}$ , in previous studies.<sup>39,48</sup> 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.<sup>49</sup> 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.<sup>50</sup> 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*,<sup>39</sup> 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.<sup>39,48</sup>

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

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#### 411 **Transparency declarations**

412 None to declare.

413

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

<b>Strain</b>	<b>Antimicrobial combination</b>	<b>Checkerboard FICI (SD)<sup>a</sup></b>
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+sitafloxacin	1.08 (0.22)
WHO O	Zoliflodacin+sitafloxacin	1.01 (0.07)
WHO V	Zoliflodacin+sitafloxacin	1.06 (0.05)
WHO X	Zoliflodacin+sitafloxacin	1.03 (0.33)

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

589 <sup>a</sup>Mean values from three experiments. The cut-off for potential synergy, indifferent and  
590 antagonism was  $\leq 0.5$ ,  $>0.5-4$ , and  $>4$ , respectively, as previously described.<sup>43</sup>  
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604 **Table 2 Parameter estimates for zoliflodacin kill of four WHO *Neisseria***  
605 ***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	$4.78 \times 10^8$	$6.27 \times 10^5$
Median	0.790	4.42	0.0187	9.14	$4.18 \times 10^8$	$6.23 \times 10^5$
SD	0.726	2.20	0.175	2.46	$2.13 \times 10^8$	$6.64 \times 10^4$
<i>WHO O</i>						
Mean	1.59	6.88	2.65	0.872	$6.87 \times 10^7$	$2.18 \times 10^6$
Median	1.55	6.88	2.89	0.846	$7.65 \times 10^7$	$2.52 \times 10^6$
SD	0.0470	0.0642	0.312	0.113	$2.91 \times 10^6$	$4.91 \times 10^5$

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

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.

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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
$K_{kmax}$	<b>9.35</b>	2.96	31.62	10.38	<b>5.15</b>	<b>12.05</b>
$EC_{50}$	<b>0.07</b>	0.03	36.26	0.08	<b>0.04</b>	<b>0.1</b>
$H_k$	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

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

	Mean	SD	CV%	Median
K <sub>g</sub>	0.817	0.110	13.5	0.741
K <sub>kmax</sub>	<b>4.13</b>	0.384	9.30	<b>4.28</b>
EC <sub>50</sub>	<b>0.559</b>	0.308	55.1	<b>0.729</b>
H <sub>k</sub>	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

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

	Mean	SD	CV%	Median
K <sub>g</sub>	0.900	0.0261	2.90	0.919
K <sub>kmax</sub>	<b>2.65</b>	0.241	9.09	<b>2.84</b>
EC <sub>50</sub>	<b>3.02</b>	0.301	9.96	<b>2.82</b>
H <sub>k</sub>	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

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

	Mean	SD	CV%	Median
K <sub>g</sub>	1.24	0.0685	5.54	1.21
K <sub>kmax</sub>	<b>3.63</b>	0.417	11.5	<b>3.46</b>
EC <sub>50</sub>	<b>0.333</b>	0.108	32.4	<b>0.353</b>
H <sub>k</sub>	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

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

	Mean	SD	CV%	Median
$K_g$	1.14	0.206	18.0	1.26
$K_{kmax}$	<b>6.80</b>	2.67	39.3	<b>8.23</b>
$EC_{50}$	<b>0.763</b>	0.340	44.5	<b>0.898</b>
$H_k$	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.

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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 <sup>a</sup>	Frequency – zoliflodacin <sup>b</sup>	Additional drug	Frequency – Additional drug	Frequency – combination <sup>b</sup>	Expectation (additive model)
<b>WHO F</b>	$3.1 \times 10^{-12}$	Ceftriaxone	$1.25 \times 10^{-13}$	-	lower
<b>WHO O</b>	$2.0 \times 10^{-12}$	Ceftriaxone	$3 \times 10^{-12}$	$2.0 \times 10^{-12}$	lower
<b>WHO V</b>	$2.0 \times 10^{-11}$	Ceftriaxone	ND <sup>c</sup>	-	lower

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

659 <sup>a</sup>For each tested combination of zoliflodacin plus one additional antimicrobial,  
660 zoliflodacin alone was tested in parallel for the same strain.

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

664 <sup>c</sup>Not determined as outside the experimental range.

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