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DOI:
10.1093/jac/dkz376

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

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Running title: Zoliflodacin in combination with six therapeutic antimicrobials

Word count: Text: 3644 words, Abstract: 248 words

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

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

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

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

Introduction

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

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

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

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

Material and methods

Neisseria gonorrhoeae reference strains, culture, and zoliflodacin susceptibility testing

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

5% CO_2-enriched atmosphere. The MICs (mg/L) of zoliflodacin (Entasis Therapeutics) were determined by recommended agar dilution technique (www.clsi.org; M07-A10).

Checkerboard analysis

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

Time-kill curve analysis

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

Fractional inhibitory concentration index (FICI) analysis

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

Time-kill mathematical modeling[44]

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

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

Population pharmacokinetic/pharmacodynamic mathematical model

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

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

$$E = 1 - [N/POPMAX] \quad (2)$$

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

Equation 1 describes the rates of change of the bacterial burden (N) over time. The model equations for describing the rate of change of the numbers of microorganisms were developed based on the *in vitro* observation that bacteria in the system are in logarithmic growth phase in the absence of drug and exhibit an exponential density-limited growth rate (equation 2). First-order growth was assumed, up to a density limit. As bacterial population approaches maximal density, they approach stationary phase. This is accomplished by multiplying the first-order growth terms by *E* (equation 2; a logistic growth term). The maximal bacterial density (POPMAX) is identified as part of the estimation process. Most of the information for identifying this parameter is derived from the bacterial growth in the control group. Equation 1 allows the antibacterial effects of the different drug exposures administered to be modeled. There is a maximal kill rate that the drug can induce (K_{kmax}). The killing effect of the drug was modeled as a saturable kinetic event *M* [equation 3] that relates the kill rate to drug concentration, where *H* is the slope or Hill's constant and EC_{50} (mg/L) is the drug concentration at which the bacterial kill rate is half-maximal. Thus, the drug effect observed on the population is the difference between intrinsic growth rate and the kill rate observed at the drug concentrations achieved.

Construction of 95% credible intervals

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

Selection of zoliflodacin-resistant mutants

Selection of zoliflodacin-resistant mutants was performed for WHO F, WHO O, WHO V, and WHO X (Supplementary Table 1) as described,[39] with minor modifications. Briefly, GCVIT plates (3.6% Difco GC Medium Base agar [BD, Diagnostics] supplemented with 1% IsoVitalex [BD, Diagnostics]) were prepared to contain 4×MIC, 2×MIC and 1×MIC of ceftriaxone, spectinomycin, cethromycin, doxycycline, gentamicin, and sitafloxacin alone or in combination with zoliflodacin at the same concentrations. The WHO strains were initially cultured on GCAGP plates[37] for 18–20 h at 37°C in a humid 5% CO_2-enriched atmosphere. Fresh cultures (18 h) from 10 GCAGP agar plates were pooled and suspended in 2 mL of sterile PBS. A dilution series of the strain suspensions in PBS was plated on antimicrobial-free GCVIT plates. Undiluted 100 µL aliquots were plated on antimicrobial-containing GCVIT plates and grown for 48 h at 37°C in a humid 5% CO_2-enriched atmosphere. For each tested antimicrobial combination and strain, zoliflodacin alone was tested in parallel. All zoliflodacin-resistant mutants inhibited by ≥16 times the zoliflodacin MIC of the wild type strain, a significant MIC increase, were genome sequenced as described.[47]

Results

Checkerboard analysis

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

Time-kill curve analysis

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

Mathematical modeling of zoliflodacin for isolates with different antimicrobial resistance mechanisms

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

Model fit to the data

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

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

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

Selection of zoliflodacin-resistant mutants

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

Discussion

The increasing prevalence of gonorrhoea in many settings and AMR in *N. gonorrhoeae* is a major global public health concern.[1-4] Internationally, MDR *N. gonorrhoeae* strains are spreading, significantly compromising the effectiveness of gonorrhoea treatment, including the last remaining option, ceftriaxone plus azithromycin dual therapy.[22-27] Novel antimicrobials for effective treatment of urogenital and extragenital gonorrhoea are essential. The first-in-class spiropyrimidinetrione zoliflodacin, with a novel mode of action, appears promising for the future treatment of gonorrhoea based on *in vitro* activity against wild type, MDR and XDR *N. gonorrhoeae* strains, phase I and II 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 ciprofloxacin[41] for all examined strains, In the

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

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

When exposed to zoliflodacin alone, zoliflodacin-resistant mutants were selected at very low frequencies from all four examined WHO reference strains and no zoliflodacin-resistant mutants (with ≥16-fold increased MIC) were selected when the strains were exposed to zoliflodacin in combination with ceftriaxone, spectinomycin, cethromycin, doxycycline, gentamicin, or sitafloxacin. The agar plate-based method used for selection of zoliflodacin-resistant mutants in the present study, as all currently available similar methods for *N. gonorrhoeae*, has inherent limitations, particularly for antimicrobials such as zoliflodacin where resistance mutations are selected at very low frequencies. This is likely part of the reason that zoliflodacin-resistance mutations have been selected in different frequencies in diverse *N. gonorrhoeae* strains and on different culture media, from $<2\times10^{-14}$ to 1×10^{-8}, in previous studies.[39,48] Accordingly, the reported mutation frequencies need to be interpreted with caution. In the present study, the parallel comparisons between resistance frequencies when exposed to zoliflodacin alone and in combination with other antimicrobials show qualitatively that the combination resulted in lower frequencies than expected in an additive model. Experiments with *Escherichia coli* have previously demonstrated that the evolution of resistance in response to a drug pair is independent from synergistic or antagonistic drug interactions.[49] Theory shows that synergistic drug pairs, preferred for their immediate efficacy, could even favor the evolution of resistance due to increased selective pressure.[50] In the present study, all selected zoliflodacin-resistant mutants contained a

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

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

SUPPLEMENTARY MATERIAL

Foerster et al_Supplementary Figure 1, Table 1 and Table 2.pdf

Acknowledgements

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

Funding

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

Transparency declarations

None to declare.

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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+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)

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

[a]Mean values from three experiments. The cut-off for potential synergy, indifferent and antagonism was ≤0.5, >0.5-4, and >4, respectively, as previously described.[43]

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**
WHO F						
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
WHO O						
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

WHO V						
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
WHO X						
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

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

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

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

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

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

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

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

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

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

[a]For each tested combination of zoliflodacin plus one additional antimicrobial, zoliflodacin alone was tested in parallel for the same strain.

[b]Frequency of zoliflodacin resistance mutations (cfu/mL) when exposed to zoliflodacin alone or zoliflodacin in combination with additional antimicrobial. -, no mutants detected.

[c]Not determined as outside the experimental range.