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In vitro activity of EDTA and TOL-463 against *Neisseria gonorrhoeae*

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ABSTRACT

Neisseria gonorrhoeae quickly develops drug resistance. Time-kill curves revealed that EDTA and TOL-463 inhibit growth similar to penicillin, ciprofloxacin, and azithromycin. Furthermore, synergistic and additive antimicrobial interactions occurred when EDTA and TOL-463 were combined with penicillin or azithromycin, respectively, suggesting that further investigations into these unconventional antimicrobials may be advantageous.

Neisseria gonorrhoeae, a common sexually transmitted bacterial pathogen, has progressively developed resistance to antibiotics recommended for gonorrhea treatment (reviewed by Unemo and Nicholas, 2012). In an effort to impede resistance to the last few remaining antimicrobial agents, the Centers for Disease Control and Prevention recommend a combination therapy consisting of ceftriaxone as the primary antibiotic and azithromycin as a secondary therapy (Workowski and Bolan, 2015). Despite efforts to slow the spread of antibiotic resistance, *N. gonorrhoeae* isolates with reduced susceptibility to 1 or both agents have been reported (CDC, 2017; Fifer et al., 2016; Papp et al., 2017).

TOL-463 is a boric acid (BA)– and EDTA-based anti-infective agent in clinical development for vaginitis (ClinicalTrials.gov Identifier NCT02866227). BA is a weak inorganic acid used clinically to treat vulvovaginal candidiasis and bacterial vaginosis (Workowski and Bolan, 2015), with antimicrobial effects caused by oxidative metabolism inhibition (De Seta et al., 2009). EDTA also exhibits antimicrobial activity via microbial membrane disruption (Finnegan and Percival, 2015) and can interact synergistically with antibiotics to inhibit multidrug-resistant bacteria (Buckley et al., 2013; Hamoud et al., 2014). Since TOL-463 is safe and effective against lower genital tract infections, i.e., vulvovaginal candidiasis and bacterial vaginosis (Marrazzo et al., 2018), the objective of this study was to investigate the in vitro activity of TOL-463 against *N. gonorrhoeae* and characterize drug interactions when combined with antibiotics known for reduced efficacy due to elevated rates of resistance (CDC, 2017).

For this proof-of-concept study, we tested 2 *N. gonorrhoeae* reference strains: F-18 (ATCC 49226), displaying intermediate resistance to penicillin and susceptible to ciprofloxacin and azithromycin, and SPL-4, which contains chromosomally mediated resistance to penicillin and tetracycline, high-level resistance to ciprofloxacin, and decreased susceptibility to cefixime (CDC, 2005). To analyze the antimicrobial effects of TOL-463 on these isolates, a standardized in vitro time-kill curve assay (Foerster et al., 2015) using Graver–Wade medium (Wade and Graver, 2007) was implemented with minor assay modifications. Briefly, overnight *N. gonorrhoeae* colonies from GC II base medium supplemented with 1% IsoVitaleX were used to prepare a 0.5 McFarland standard in Muller–Hinton broth. Inoculum was diluted 1:500 in prewarmed (37 °C) Graver–Wade medium. Ninety microliters of inoculum was transferred to a 96-well microtiter plate and incubated for 4 h, shaking at 150 RPM at 37 °C under 5% CO_2 to reach *N. gonorrhoeae* log-phase growth. BA and EDTA were dissolved in sterile water, and 10 μl of each agent alone or combined at a 1:1 ratio was added to *N. gonorrhoeae* culture. PBS served as a growth control, and all groups were run in triplicate. At 0, 3, 6, and 24 h postexposure, the total volume was serially diluted, spotted (10 μl) onto gonococcal resistance agar plates (3.6% Difco GC Medium Base agar supplemented with 1% hemoglobin and 1% IsoVitalex) (Foerster et al., 2015), and incubated for 24 h at 37 °C under 5% CO_2. Colonies were counted and numbers extrapolated to log colony-forming unit (CFU). Preliminary experiments identified 1 mg/mL BA +1 mg/mL EDTA as the minimum inhibitory concentration (MIC) of TOL-463 against both *N. gonorrhoeae* strains after 24 h of co-incubation (data not shown). Furthermore, 1 mg/mL EDTA alone completely inhibited both strains. BA alone, at concentrations ranging from 1 mg/mL to 10 mg/mL, was tested with no impact on growth.

Abbreviations: BA, boric acid; FICI, fractional inhibitory concentration index.

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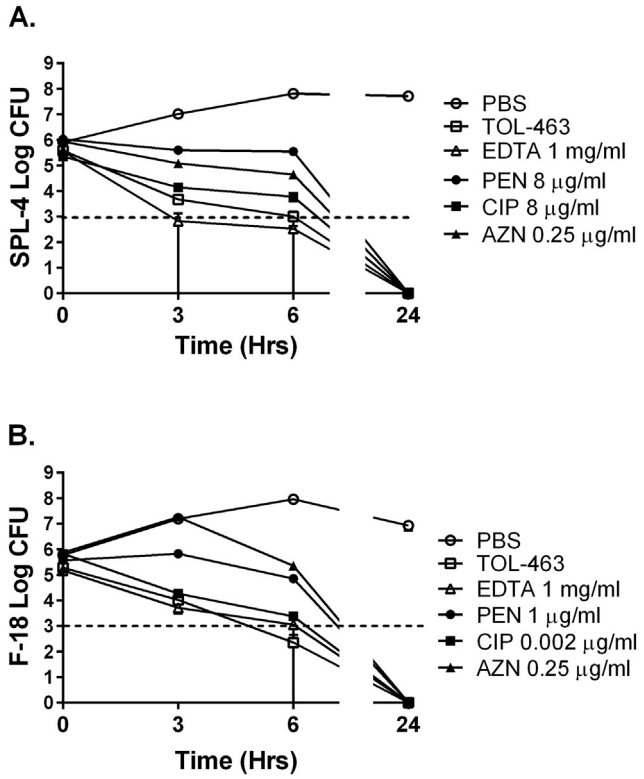

Fig. 1. Time-kill curves of 2 *N. gonorrhoeae* strains comparing antimicrobial effects of TOL-463 or EDTA to 3 antibiotics. *N. gonorrhoeae* strains SPL-4 (A) and F-18 (B) were exposed to TOL-463 (1 mg/mL BA +1 mg/mL EDTA), EDTA, penicillin (PEN), ciprofloxacin (CIP), or azithromycin (AZN) at MIC concentrations. PBS served as the growth control. Experiments were run in triplicate, and results are expressed as mean CFU ± standard error of the means (SEM); some error bars are too small to visualize. Dashed line represents limit of detection for this assay. CFUs at log 0 represent no growth.

We continued to further characterize TOL-463 antimicrobial effects and included 1 mg/mL EDTA as an additional experimental group.

N. gonorrhoeae time-kill curves were generated to compare TOL-463 and EDTA to penicillin, ciprofloxacin, and azithromycin at MIC concentrations. The MICs of penicillin, ciprofloxacin, and azithromycin were determined by agar dilution technique in accordance with the Clinical and Laboratory Standards Institute guidelines (Clinical and Laboratory Standards Institute, 2017) for both strains. Control growth curves were similar for both strains, and all antimicrobial agents required 24 h to fully inhibit *N. gonorrhoeae* below the limit of detection (10^3 CFUs). Interestingly, TOL-463 and EDTA alone inhibit SPL-4 (Fig. 1A) and F-18 (Fig. 1B), at early time points, to the same extent or greater than the 3 antibiotics tested.

BA's and EDTA's mechanisms of action are not shared by antibiotics (Soares et al., 2012); therefore, we hypothesized that TOL-463 and/or EDTA could act as antibiotic adjuvants, enhancing the antimicrobial properties of penicillin, ciprofloxacin, or azithromycin when combined. These antibiotics were selected based on the high percentage of *N. gonorrhoeae* isolates resistant to penicillin and ciprofloxacin, and the steadily increasing number of isolates with reduced susceptibility to azithromycin (CDC, 2017). We characterized these antimicrobial combinations as synergistic, additive, no interaction, or antagonistic using a checkerboard assay followed by fractional inhibitory concentration index (FICI) analysis (Banerjee et al., 2014). For checkerboard assays, serial dilutions were performed for TOL-463, EDTA, and all 3 antibiotics starting at 4× MIC down to 0.03× MIC. Serial dilutions for TOL-463 retained the 1:1 ratio of EDTA and BA, and arbitrary units (U) were assigned to TOL-463 dilutions, where 1 mg/mL EDTA +1 mg/mL BA (1X MIC) is equivalent to 1 U TOL-463. EDTA or TOL-463 was combined with each antibiotic in 1:1 ratios, resulting in 10 μL of drug mixture added to 90 μL of log-phase culture. MICs were determined 24 h postexposure by plating all 100 μl onto gonococcal resistance agar plates and visualizing colony growth 18–24 h later. Using the MICs derived from the checkerboard assay, i.e., MICs of each antimicrobial alone and the MICs of antimicrobial combinations, the FICI was calculated as follows: $FICI = FIC_A + FIC_B = MIC_A^{Comb}/MIC_A^{alone} + MIC_B^{Comb}/MIC_B^{alone}$. The FICI was used to assess synergy (FICI ≤0.5), additive interaction (FICI >0.5 to ≤1), no interaction (FICI >1 to ≤4), or antagonism (FICI >4) with antimicrobial combinations (Meletiadis et al., 2010; Sopirala et al., 2010). Table 1 shows the FICI median, range, and interpretation of 3 independent experiments. The range of MIC values for penicillin, ciprofloxacin, and azithromycin was reproducible and did not span more than 3 dilutions for both isolates. The azithromycin MIC for SPL-4 occasionally fell 1 dilution below the expected reference range (0.125–0.5 μg/mL) previously determined by agar dilution testing (CDC, 2005). Differences in media, growth conditions, and antimicrobial susceptibility methods may contribute to this MIC discrepancy. Interestingly, both TOL-463 and EDTA interact synergistically with penicillin against both strains. Similarly, TOL-463 and EDTA exhibit additive effects with azithromycin against F-18.

Table 1
MICs and FICIs of TOL-463-antibiotic or EDTA-antibiotic combinations against *N. gonorrhoeae*.

			Median (range)					
	Strain	Antibiotic	MIC_{AB}[a,b]	$MIC_{AB(with\ TOL\text{-}463)}$[b]	$MIC_{TOL\text{-}463}$[c]	$MIC_{TOL\text{-}463(with\ AB)}$[c]	FICI	Interpretation[e]
TOL-463	SPL-4	Penicillin	4 (4)	1 (1)	1 (0.5–1)	0.125 (0.125–0.25)	0.5 (0.375–0.5)	Synergistic
		Ciprofloxacin	8 (8–16)	8 (4–8)	1 (1)	0.03 (0.03–0.5)	1.03 (0.75–1.03)	No interaction
		Azithromycin	0.06 (0.06–0.5)	0.06 (0.015–0.25)	1 (0.5–1)	0.03 (0.03–0.5)	0.75 (0.53–1.06)	Additive
	F-18	Penicillin	0.5 (0.5–1)	0.125 (0.125–0.25)	1 (0.5–1)	0.25 (0.06–0.25)	0.5 (0.31–0.75)	Synergistic
		Ciprofloxacin	0.004 (0.004)	0.002 (0.001–0.002)	1 (1)	0.5 (0.5)	1 (0.75–1)	No interaction
		Azithromycin	0.25 (0.25–0.5)	0.03 (0.015–0.125)	1 (1)	0.5 (0.25–0.5)	0.56 (0.5–0.62)	Additive
	Strain	Antibiotic	MIC_{AB}[a,b]	$MIC_{AB(with\ EDTA)}$[b]	MIC_{EDTA}[d]	$MIC_{EDTA(with\ AB)}$[d]	FICI	Interpretation[e]
EDTA	SPL-4	Penicillin	4 (4)	1 (1–2)	1 (0.5–1)	0.25 (0.06–0.25)	0.5 (0.5–.62)	Synergistic
		Ciprofloxacin	8 (8–16)	8 (8)	1 (0.5–1)	0.06 (0.06–0.5)	1.03 (1–1.12)	No interaction
		Azithromycin	0.06 (0.06–0.5)	0.06 (0.06–0.25)	1 (1)	0.06 (0.06–0.5)	1.06 (1–1.06)	No interaction
	F-18	Penicillin	1 (0.5–2)	0.25 (0.25)	1 (0.5–1)	0.25 (0.06–0.5)	0.5 (0.185–0.62)	Synergistic
		Ciprofloxacin	0.004 (0.004)	0.002 (0.001–0.004)	1 (0.5–1)	0.25 (0.06–0.5)	1 (0.75–1.06)	Additive
		Azithromycin	0.5 (0.25–0.5)	0.125 (0.125–.25)	1 (0.5–1)	0.06 (0.06–0.5)	0.62 (0.56–0.75)	Additive

[a] AB = antibiotic.
[b] MIC_{AB} in μg/mL.
[c] $MIC_{TOL\text{-}463}$ in arbitrary units (U) consisting of 1:1 *(w/v)* ratio of EDTA and BA, where 1 mg/mL EDTA +1 mg/mL BA is equivalent to 1 U TOL-463.
[d] MIC_{EDTA} in mg/mL.
[e] The FICI was interpreted as drug synergy (FICI ≤0.5), additive (FICI >0.5 to ≤1), no interaction (FICI >1 to ≤4), or drug antagonism (> 4).

These results suggest that TOL-463 and 1 mg/mL EDTA can inhibit *N. gonorrhoeae* independent of antibiotics and, in some cases, enhance the inhibitory capacity of antibiotics *in vitro*. The synergistic interactions of TOL-463-penicillin and EDTA-penicillin observed against SPL-4 are especially interesting since this isolate has high-level penicillin resistance conferred through mutations in *ponA* and *penA* (Johnson et al., 2014). However, these combinations failed to reduce the MIC of SPL-4 to that of a fully susceptible strain, defined as an MIC of ≤0.06 μg/mL (Clinical and Laboratory Standards Institute, 2017). Furthermore, the synergistic effects with penicillin were observed in 2 strains lacking β-lactamase. Future studies will determine whether this interaction is sustained in the presence of penicillin-inactivating enzymes and is repeatable with extended-spectrum cephalosporins.

Development of TOL-463 as a topical treatment for vaginitis is safe and effective (Marrazzo et al., 2018), but this formulation limits its clinical utility against urethral and rectal gonorrhea infections. EDTA, the active component of TOL-463, is FDA-approved to treat lead poisoning and lead encephalopathy through intravenous administration (Arnold and Morgan, 2015; Ogawa et al., 2008), suggesting that EDTA could potentially be delivered systemically to treat extragenital and urethral gonococcal infections. However, bioavailability studies at these anatomical sites are needed. Nevertheless, the antimicrobial effects of EDTA and TOL-463 observed in this study warrant further investigation of their therapeutic potential.

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Declaration of interest

The authors declare no conflicts of interest.

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