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In vitro activity of the novel triazaacenaphthylene gepotidacin (GSK2140944) against MDR *Neisseria gonorrhoeae*

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Objectives: Increased antimicrobial resistance surveillance and new effective antimicrobials are crucial to maintain treatable gonorrhoea. We examined the *in vitro* activity of gepotidacin, a novel triazaacenaphthylene, and the effect of efflux pump inactivation on clinical *Neisseria gonorrhoeae* isolates and international reference strains ($n = 252$) and compared gepotidacin with antimicrobials currently or previously recommended for gonorrhoea treatment.

Methods: MICs (mg/L) were determined by agar dilution (gepotidacin) or by Etest (seven other antimicrobials). The *gyrA* and *parC* genes were sequenced and the impact of inactivation of the MtrCDE, MacAB and NorM efflux pumps on gepotidacin MICs was examined.

Results: Gepotidacin showed potent *in vitro* activity against all gonococcal isolates ($n = 252$; MIC $\leq$4 mg/L). The modal MIC, MIC_{50}, MIC_{90} and MIC range of gepotidacin were 0.5, 0.5, 1 and 0.032–4 mg/L, respectively. Inactivation of the MtrCDE efflux pump, but not MacAB or NorM, decreased the gepotidacin MICs for most strains. No significant cross-resistance between gepotidacin and any other antimicrobials, including the fluoroquinolone ciprofloxacin, was identified. However, the ParC D86N mutation (possibly together with additional antimicrobial resistance mutation), which is associated with fluoroquinolone resistance, was associated with increased gepotidacin MICs.

Conclusions: Gepotidacin demonstrated high *in vitro* activity against gonococcal strains, indicating that gepotidacin could potentially be an effective option for gonorrhoea treatment, particularly in a dual antimicrobial therapy regimen and for patients with resistance or allergy to extended-spectrum cephalosporins. Nevertheless, elucidating *in vitro* and *in vivo* resistance emergence and mechanisms in detail, together with further gonorrhoea clinical studies, ideally also including chlamydia and *Mycoplasma genitalium* are essential.

Introduction

Neisseria gonorrhoeae, the aetiological agent of the sexually transmitted infection gonorrhoea, remains a significant global public health concern. The WHO estimated in 2012 there were ~78 million new cases of gonococcal infections among adult women and men globally.[1] According to the 2013 global burden of disease study, gonorrhoea results in 225 400 years lived with disability per year and 313 900 disability-adjusted life years.[2,3] Undetected and/or untreated gonorrhoea imposes significant human and socio-economic costs and consequences worldwide. These consequences disproportionally affect women and include pelvic inflammatory disease potentially resulting in ectopic pregnancy and infertility, and increased risk of acquisition and transmission of HIV.[4–6]

In the absence of a gonococcal vaccine, effective prevention, diagnostics, surveillance and particularly antimicrobial treatment are the mainstays in the management of gonorrhoea. However, *N. gonorrhoeae* has since the beginning of the antimicrobial era developed antimicrobial resistance (AMR) to all antimicrobials introduced for gonorrhoea treatment and this resistance has spread internationally within 10–20 years.[6,7] The third-generation extended-spectrum cephalosporins (ESCs), which represent the last available antimicrobial class that is effective as empirical monotherapy, are also threatened by emerging AMR.[7–11] To combat ESC resistance development, current gonorrhoea treatment guidelines in many more-resourced settings have introduced a dual antimicrobial therapy, mainly ceftriaxone at 250–500 mg plus azithromycin at 1–2 g, as empirical first-line treatment of all

gonorrhoea cases.[12–16] Subsequently, ESC resistance has slightly decreased in many settings worldwide; however, azithromycin resistance has increased or stabilized at a relatively high level in many settings.[10,11] Most worryingly, in 2016 the first global treatment failure with recommended dual antimicrobial therapy was reported from the UK.[17] Accordingly, continuing AMR surveillance and new therapeutic antimicrobials are crucial to maintain gonorrhoea as a treatable infection.[5,7,8,18–20]

Gepotidacin (GSK2140944) is a novel, first-in-class triazaacenaphthylene antibacterial (bacterial type II topoisomerase inhibitor). Structural data have shown that gepotidacin inhibits bacterial DNA gyrase and topoisomerase IV by a novel mode of action and has a binding site close to but distinct from that of quinolones.[21] Gepotidacin in earlier studies has shown *in vitro* activity against a small collection of *N. gonorrhoeae* isolates[22] and a broad spectrum of *in vitro* activity against other bacterial species, including MRSA and other primary causative pathogens of acute bacterial skin and skin structure infections.[23] A Phase II randomized controlled clinical trial (RCT) evaluating gepotidacin 1.5 and 3 g single oral dose, respectively, for treatment of uncomplicated gonorrhoea was recently performed.[24] Microbiological success in the treatment of urogenital gonorrhoea was achieved by 97% (29 of 30) and 95% (37 of 39) of subjects, respectively. The most frequent adverse effects associated with gepotidacin treatment were gastrointestinal with the majority being mild or moderate in intensity.[24] All isolates from the urogenital treatment failures ($n = 3$) were resistant to ciprofloxacin with a pre-existing D86N amino acid substitution in ParC.[25] Post-treatment isolates from the two treatment failures with the gepotidacin 3 g dose demonstrated resistance emergence to gepotidacin (gepotidacin MIC increased ≥32-fold to ≥32 mg/L) and had an additional A92T mutation in GyrA.[25]

The aims of the present study were to examine the *in vitro* activity of gepotidacin and the effect of inactivation of efflux pumps (MtrCDE, MacAB and NorM) on a large collection of clinical *N. gonorrhoeae* isolates and international reference strains ($n = 252$). The collection included all described types of high-level *in vitro* and clinical resistance to antimicrobials currently or previously recommended for treatment of gonorrhoea, as well as numerous MDR[26] and XDR[26] gonococcal isolates. Additionally, the QRDRs of the *gyrA* gene, encoding the GyrA subunit of DNA gyrase, and the *parC* gene, encoding the ParC subunit of topoisomerase IV, were sequenced, i.e. to investigate further the potential cross-resistance between gepotidacin and fluoroquinolones.

Materials and methods

N. gonorrhoeae isolates

The examined strains represented a large geographically (mainly global representativeness), temporally (obtained from 1991 to 2016), phenotypically and genetically diverse collection. They comprised 35 international gonococcal reference strains, including the 2016 WHO reference strains,[27] 100 consecutive clinical Swedish gonococcal isolates obtained in 2016 and 117 isolates selected for their resistance phenotype including XDR gonococcal isolates,[17,28–30] isolates with *in vitro* or clinical resistance to ESCs, as well as other high-level *in vitro* and clinical resistance and MDR to other antimicrobials previously used for treatment of gonorrhoea.

Antimicrobial susceptibility testing

The MICs (mg/L) of gepotidacin (GlaxoSmithKline, London, UK) were determined by agar dilution, according to current CLSI guidelines (www.clsi.org). The MICs (mg/L) of ceftriaxone, cefixime, azithromycin, spectinomycin, ciprofloxacin, ampicillin and tetracycline were determined by Etest (AB bioMérieux, Marcy-l'Étoile, France), according to the manufacturer's instructions. With the exception of gepotidacin, for which no breakpoints exist, all MICs were interpreted for susceptibility, intermediate susceptibility and resistance according to EUCAST breakpoints (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_7.0_Breakpoint_Tables.pdf). Only whole MIC dilutions are reported in this article.

Sequencing of the gyrA, parC and mtrR genes

The QRDRs of the *gyrA* gene and the *parC* gene were sequenced in all isolates as previously described,[31,32] to investigate further the potential cross-resistance between gepotidacin and fluoroquinolones. The *mtrR* (promoter and coding sequence) was sequenced in selected isolates, as earlier reported.[32]

Inactivation of efflux pumps

The *mtrD*, *macA* and *norM* genes, coding for subcomponents of the MtrCDE, MacAB and NorM efflux pumps, were inactivated in five strains, as previously described.[33] These five strains consisted of the 2016 WHO reference strains WHO F, WHO O, WHO P and WHO X[27] and one clinical strain with high-level azithromycin resistance (azithromycin MIC ≥256 mg/L).

Results

Gepotidacin demonstrated *in vitro* activity against all the tested *N. gonorrhoeae* isolates ($n = 252$). The susceptibility results for gepotidacin and seven antimicrobials currently or previously recommended for gonorrhoea treatment are summarized in Table 1. Antimicrobial susceptibility results were divided into different subgroups, i.e. all isolates, consecutive isolates, selected isolates, international reference strains, ciprofloxacin-resistant isolates and high-level ciprofloxacin-resistant isolates (MIC ≥32 mg/L) (Table 1). Briefly, the modal MIC, MIC_{50}, MIC_{90} and MIC range of gepotidacin were 0.5, 0.5, 1 and 0.032–4 mg/L, respectively.

In general, no significant cross-resistance between gepotidacin and the previously recommended fluoroquinolone ciprofloxacin or any other tested antimicrobial was observed, with exception of the isolates with a gepotidacin MIC of 4 mg/L ($n = 3$), which all had a ciprofloxacin MIC of ≥32 mg/L. There were no substantial differences in the *in vitro* activity of gepotidacin against ciprofloxacin-resistant isolates compared with ciprofloxacin-susceptible isolates. A total of 152 (60%) ciprofloxacin-resistant isolates, including 75 (30%) with a ciprofloxacin MIC of ≥32 mg/L, were compared with 100 (40%) ciprofloxacin-susceptible isolates and the modal MIC, MIC_{50}, MIC_{90} and MIC range of gepotidacin for these groups were 0.5, 0.5, 2 and 0.032–4 mg/L, and 0.25, 0.25, 0.5 and 0.032–2 mg/L, respectively (Table 1). The MIC distributions for gepotidacin and ciprofloxacin and a comparison of the MIC values of gepotidacin and ciprofloxacin are shown in Figures S1 and S2 (available as Supplementary data at *JAC* Online), respectively.

Notably, all four XDR isolates[28–30] had a gepotidacin MIC of only 0.25–0.5 mg/L and four isolates with high-level resistance to

Table 1. MIC range, MIC_{50}, MIC_{90} and modal MIC of gepotidacin and additional antimicrobials for all *N. gonorrhoeae* isolates ($n = 252$), as well as different groups of isolates (in the case of gepotidacin), and proportion of all isolates ($n = 252$) categorized as susceptible, intermediately susceptible and resistant to antimicrobials currently or previously recommended for treatment of gonorrhoea

Antimicrobial, isolate group (no.)	MIC range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)	Modal MIC (mg/L)	S/I/R[a] (%)
Gepotidacin					
all isolates (252)	0.032–4	0.5	1	0.5	ND[b]
consecutive isolates (100)	0.032–2	0.25	1	0.25	ND[b]
selected isolates (117)	0.032–4	0.5	2	0.5	ND[b]
reference strains (35)	0.125–4	0.5	2	0.5	ND[b]
ciprofloxacin-resistant isolates (152)	0.032–4	0.5	2	0.5	ND[b]
high-level ciprofloxacin-resistant isolates (75)[c]	0.032–4	0.5	1	0.5	ND[b]
Ceftriaxone (252)	<0.002–4	0.016	0.125	0.004	96.8/ND[b]/3.2
Cefixime (252)	<0.016–8	<0.016	0.25	<0.016	88.9/ND[b]/11.1
Azithromycin (252)	0.016 to >256	0.5	2	1	44.0/13.9/42.1
Spectinomycin (252)	4 to >1024	16	16	16	98.0/ND[c]/2.0
Ciprofloxacin (252)	<0.002 to >32	2	>32	>32	39.7/0.0/60.3
Ampicillin (252)	<0.016 to >256	0.5	4	1	27.4/59.1/13.5
Tetracycline (252)	0.125–256	2	16	4	22.2/17.5/60.3

MICs were determined by agar dilution for gepotidacin and Etest for the additional antimicrobials.
[a]S, susceptible; I, intermediately susceptible; R, resistant. EUCAST breakpoints (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_7.0_Breakpoint_Tables.pdf) were applied for all antimicrobials.
[b]Not determined due to lack of interpretative criteria.
[c]Ciprofloxacin MICs of ≥32 mg/L.

azithromycin (MIC ≥256 mg/L) showed a gepotidacin MIC of 0.25–0.5 mg/L.

Most identified non-synonymous mutations in the QRDR of GyrA, associated with fluoroquinolone resistance, or in the QRDR of ParC were not in any obvious manner associated with increased MICs of gepotidacin. However, the fluoroquinolone resistance-associated ParC D86N mutation, which is also a critical amino acid residue in gepotidacin binding, appeared to be associated with increased gepotidacin MICs. In total, 21 isolates contained the ParC D86N mutation and the gepotidacin MIC_{50} for these isolates was 2 mg/L (range 0.5–4 mg/L; Table 2). However, 6 of these 21 isolates containing the ParC D86N mutation had lower gepotidacin MICs, i.e. 0.5 mg/L ($n = 2$) and 1 mg/L ($n = 4$).

Furthermore, of the three isolates with a gepotidacin MIC of 4 mg/L, two had the ParC D86N mutation in addition to *gyrA* QRDR mutations (Tables 2 and 3). In Table 3, the MICs of gepotidacin and ciprofloxacin in all isolates with the ParC D86N mutation, in combination with many other GyrA and ParC QRDR mutations, have been summarized.

The ParC D86N mutation was not found in any isolates lacking GyrA QRDR S91 and D95 mutations. Notably, the third isolate with a gepotidacin MIC of 4 mg/L contained the GyrA QRDR S91F and D95Y mutations together with a ParC QRDR E91K mutation (Table 2). Surprisingly, the GyrA S91F mutation solely [together with a WT ParC QRDR ($n = 8$)] also appeared to be associated with increased gepotidacin MICs (gepotidacin MIC_{50} 2 mg/L) (Table 2).

Inactivation of the MtrCDE efflux pump decreased the MICs of gepotidacin by 2–3-fold, in all strains except WHO F. Inactivation of the MacAB and NorM efflux pumps had no obvious impact on the gepotidacin MICs (Table S1). Notably, the *mtrR* (promoter and coding sequence) was sequenced in 31 selected isolates. These included all 21 isolates with a ParC D86N mutation (gepotidacin MIC = 0.5–4 mg/L) and 10 additional isolates that were lacking the ParC D86N mutation but showed increased gepotidacin MICs (2–4 mg/L). Twenty-four (77.4%) of these 31 isolates contained *mtrR* mutations [A-deletion in the repeated sequence of the promoter plus MtrR G45D ($n = 14$), only the A-deletion in the promoter ($n = 7$), only MtrR G45D ($n = 2$) and mtr_{120} ($n = 1$)] resulting in an overexpression of the MtrCDE efflux pump. The seven isolates lacking *mtrR* mutations had gepotidacin MICs of 0.5–2 mg/L.

Discussion

This is the first comprehensive *in vitro* evaluation of gepotidacin, a novel topoisomerase II inhibitor belonging to the new class of triazaacenaphthylene antimicrobials, as a treatment option for gonorrhoea. The *in vitro* activity of gepotidacin against a large geographically, temporally and genetically diverse collection of clinical *N. gonorrhoeae* isolates and international reference strains, including various types of high-level AMR, MDR and XDR isolates, was high. Gepotidacin inhibited all *N. gonorrhoeae* isolates at MIC ≤4 mg/L with MIC_{50} and MIC_{90} of 0.5 and 1 mg/L, respectively.

In general, no significant cross-resistance between gepotidacin and any other antimicrobials, including the fluoroquinolone ciprofloxacin [Spearman's rank correlation coefficient of 0.30 for the gepotidacin and ciprofloxacin MICs (Figure S2)], was identified. However, the ParC D86N mutation, which is associated with fluoroquinolone resistance, appeared to be associated also with increased gepotidacin MICs. This confirms the findings of the recently performed gepotidacin Phase II RCT for uncomplicated gonorrhoea, in which all three gepotidacin treatment failures of urogenital gonorrhoea were caused by gonococcal isolates

containing this pre-existing mutation.[25] Nevertheless, in the present study, the gepotidacin Phase II RCT[24,25] or large European gonococcal strain material (5% of 1054 isolates from 2013 had ParC D86N),[34] the ParC D86N mutation has only been found together with additional fluoroquinolone resistance-associated GyrA and/or ParC QRDR mutations and it remains unknown if and how the ParC D86N mutation alone affects gepotidacin MICs. Surprisingly, the presence of the GyrA S91F mutation alone also appeared to be associated with increased gepotidacin MICs, which shows that additional gepotidacin resistance determinants and/or GyrA and ParC mutations outside the QRDRs can increase gepotidacin MICs. Finally, the GyrA A92T mutation induced during treatment (gepotidacin 3 g dose) of two subjects in the gepotidacin Phase II RCT of treatment of uncomplicated gonorrhoea, which resulted in ≥32-fold increased MICs of gepotidacin,[25] was not found in any isolates in the present study or in a large sample of European gonococcal strain material from 2013.[34] Accordingly, this mutation in GyrA amino acid residue A92, which is located in the gepotidacin binding pocket, is likely induced by gepotidacin but not fluoroquinolones. The frequency of spontaneous single-step resistance mutations, when *N. gonorrhoeae* strains (none had any pre-existing ParC D86N mutation) were exposed to 4× MIC and 8× MIC of gepotidacin, has been shown to be low ($<1.25 \times 10^{-9}$; no gepotidacin-resistant mutants were obtained).[22] However, subsequent studies ($n = 2$) of induction of resistance mutations to gepotidacin have examined selected ciprofloxacin-resistant clinical isolates ($n = 5$) with mutations in GyrA (S91F and D95A/G) and ParC (D86N), that is, the genotype observed in isolates from three microbiological failures in the gepotidacin Phase II trial.[25] In both of these studies, at 4× MIC and 10× MIC of gepotidacin, the frequency of resistance mutations to gepotidacin was low (≤2.9 to $<9.1\times10^{9}$). However, in both studies gepotidacin-resistant mutants ($n = 3$) were isolated from one of the five strains at 4× MIC of gepotidacin. All three isolated gepotidacin-resistant mutants contained an induced GyrA A92T mutation in addition to the pre-existing GyrA S91F, D95A and ParC D86N mutations. The gepotidacin MIC increased 16-fold compared with the parent strain (N. Scangarella-Oman and Sharon Min, unpublished data). This indicates that given the dual-targeting mechanism of action of gepotidacin, mutations in QRDRs of both ParC and GyrA are required for resistance and resistance is induced at a higher frequency if pre-existing mutations are present in one of the targets, e.g. the ParC D86N mutation. The impact on gepotidacin MICs by inactivation of the MtrCDE, MacAB and NorM efflux pumps was relatively minor. Only inactivation of the MtrCDE efflux pump significantly influenced the gepotidacin susceptibility by decreasing the MICs 2–3-fold in 4 of the 5 examined strains, and 23 (92%) of the 25 isolates with the highest gepotidacin MICs (2–4 mg/L) had mutations resulting in an overexpression of the MtrCDE efflux

Table 2. MIC range and MIC_{50} of gepotidacin and ciprofloxacin for 252 clinical *N. gonorrhoeae* isolates and international reference strains divided into the different mutation patterns observed in the QRDRs of the *gyrA* and *parC* genes

	Gepotidacin		Ciprofloxacin	
Mutation	MIC range	MIC_{50}	MIC range	MIC_{50}
gyrA[a]				
WT ($n = 101$)	0.032–2	0.25	<0.002–0.125	0.004
S91F, D95G ($n = 107$)	0.032–4[b]	0.5	0.5 to >32	>32
S91F, D95A ($n = 21$)	0.125–2	0.5	0.5 to >32	2
S91F, D95N ($n = 13$)	0.064–4[c]	0.5	1 to >32	>32
S91F ($n = 8$)	0.25–2	2	0.125–1	0.25
S91F, D95Y ($n = 1$)	4[d]	–	>32	–
S91Y ($n = 1$)	1	–	0.25	–
parC				
WT ($n = 118$)	0.032–2	0.5	<0.002–4	0.004
S87R ($n = 75$)	0.032–2	0.5	2 to >32	>32
E91G ($n = 21$)	0.125–1	0.5	1 to >32	4
D86N ($n = 20$)	0.5–4	2	1 to >32	8
S87R, S88P ($n = 6$)	0.064–0.5	0.5	8 to >32	>32
S87N, E91Q ($n = 3$)	0.125–0.25	–	2–8	–
S87N, E91K ($n = 2$)	0.5	–	4–8	
S87N ($n = 2$)	0.5, 2	–	1 to >32	–
S87I ($n = 1$)	0.5	–	4	–
S87W ($n = 1$)	0.25	–	0.002	–
E91K ($n = 1$)	4	–	>32	–
E91Q ($n = 1$)	0.5	–	1	–
D86N, S88P ($n = 1$)	4	–	>32	–

MICs (mg/L) were determined by agar dilution for gepotidacin and Etest for ciprofloxacin.
[a]Of the 151 isolates with any *gyrA* QRDR mutation, 133 (88.1%) also had a *parC* QRDR mutation.
[b]Includes one isolate with a gepotidacin MIC of 4 mg/L, which also had the ParC D86N mutation.
[c]Includes one isolate with a gepotidacin MIC of 4 mg/L, which also had the ParC D86N and S88P mutations.
[d]Isolate also had the ParC E91K mutation.

Table 3. MIC range and MIC_{50} of gepotidacin and ciprofloxacin for *N. gonorrhoeae* isolates ($n = 21$) with a D86N mutation in the QRDR of ParC combined with other GyrA and ParC QRDR mutations

	Gepotidacin		Ciprofloxacin	
Mutation pattern (no.)	MIC range	MIC_{50}	MIC range	MIC_{50}
ParC D86N, GyrA S91F + D95G (14)	1–4	2	4 to >32	8
ParC D86N, GyrA S91F + D95A (5)	0.5–1	1	1–8	2
ParC D86N + S88P, GyrA S91F + D95N (1)	4	not applicable	>32	not applicable
ParC D86N, GyrA S91F + D95N (1)	2	not applicable	8	not applicable

MICs (mg/L) were determined by agar dilution for gepotidacin and Etest for ciprofloxacin.

pump. Further clinical and laboratory studies are needed to elucidate gepotidacin resistance determinants in detail in *N. gonorrhoeae* and to be able to predict future emergence of resistance to gepotidacin in *N. gonorrhoeae*.

The recently performed Phase II RCT evaluating gepotidacin 1.5 and 3 g single oral dose, respectively, for treatment of uncomplicated gonorrhoea achieved microbiological success in the treatment of urogenital gonorrhoea by 97% and 95% of subjects, respectively.[24] To improve the cure rate, gepotidacin might have to be considered for use in a combination therapy or, if possible, the gepotidacin dose and/or dose frequency increased (in single or likely multiple doses) or the formulation of gepotidacin improved, e.g. in regard to pharmacodynamic/pharmacokinetic parameters. In fact, to mitigate resistance development, gepotidacin and any other new treatment option for gonorrhoea has to be considered to be included in a combination therapy in public health guidelines. In a previously performed *in vitro* chequerboard analysis of gepotidacin in combination with other antimicrobials from multiple antimicrobial classes in *N. gonorrhoeae*, *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Escherichia coli* no antagonism occurred and only one instance of synergy (with moxifloxacin) was seen in *N. gonorrhoeae*.[22,35] Gepotidacin is available in both oral and intravenous formulations and a multicentre, Phase II RCT has also been performed for acute bacterial skin and skin structure infections.[36] This study demonstrated that both the oral and intravenous administration were efficacious. The incidence of adverse events was for similar between the three treatment groups included in the study, with nausea (20%) and diarrhoea (13%) as the most frequently reported.[36]

In conclusion, gepotidacin demonstrated high *in vitro* activity against a large collection of gonococcal strains, including many MDR and XDR strains, and could potentially be a promising future treatment for gonorrhoea. However, the pre-existing ParC D86N mutation, associated with fluoroquinolone resistance, appeared to also increase the MICs of gepotidacin (possibly in association with additional fluoroquinolone resistance-associated GyrA and ParC QRDR mutations). Additional studies, such as of *in vitro* induction/selection and *in vivo* resistance emergence and mechanisms of resistance are needed. Finally, additional appropriate RCTs including patients with both urogenital and extragenital, in particularl pharyngeal, gonorrhoea, are crucial. Ideally, these studies should also examine concomitant STIs such as *Chlamydia trachomatis* and *Mycoplasma genitalium* infections.

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Transparency declarations

N. S.-O. is employed by GlaxoSmithKline. All other authors: none to declare.

Supplementary data

Figures S1 and S2 and Table S1 are available as Supplementary data at *JAC* Online.

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