Antimicrobial activity of enacyloxin IIa and gladiolin against the urogenital pathogens Neisseria gonorrhoeae and Ureaplasma spp

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Keywords
Enacyloxin IIa, gladiolin, Neisseria gonorrhoeae, novel antimicrobials, Ureaplasma parvum, Ureaplasma urealyticum.

Abstract
Aims: To determine the antimicrobial activity of enacyloxin IIa and gladiolin against Neisseria gonorrhoeae and Ureaplasma spp.
Methods and Results: The Burkholderia polyketide antibiotics enacyloxin IIa and gladiolin were tested against 14 N. gonorrhoeae and 10 Ureaplasma spp. isolates including multidrug-resistant N. gonorrhoeae isolates WHO V, WHO X and WHO Z as well as macrolide, tetracycline and ciprofloxacin-resistant ureaplasmas. Susceptibility testing of N. gonorrhoeae was carried out by agar dilution, whereas broth micro-dilution and growth kinetic assays were used for Ureaplasma spp. The MIC range for enacyloxin IIa and gladiolin against N. gonorrhoeae was 0.015–0.06 mg l⁻¹ and 1–2 mg l⁻¹ respectively. The presence of resistance to front line antibiotics had no effect on MIC values. The MIC range for enacyloxin IIa against Ureaplasma spp. was 4–32 mg l⁻¹ with a clear dose-dependent effect when observed using a growth kinetic assay. Gladiolin had no antimicrobial activity on Ureaplasma spp. at 32 mg l⁻¹ and limited impact on growth kinetics.
Conclusions: Enacyloxin IIa and gladiolin antibiotics have antimicrobial activity against a range of antibiotic susceptible and resistant N. gonorrhoeae and Ureaplasma isolates.
Significance and Impact of the Study: This study highlights the potential for a new class of antimicrobial against pathogens in which limited antibiotics are available. Development of these compounds warrants further investigation in the face of emerging extensively drug-resistant strains.

Introduction
Antimicrobial resistance (AMR) is of growing concern among sexually transmitted pathogens. Neisseria gonorrhoeae diagnoses are increasing yearly which is ultimately putting a pressure on increased prescribing and maybe driving development of AMR (PHE 2019b). In many countries resistance to ciprofloxacin persists, high-level azithromycin resistance is present and resistance to third-generation cephalosporins such as ceftriaxone has begun to emerge (Unemo et al. 2019). In 2018, the first description of extensively drug-resistant (XDR) N. gonorrhoeae was identified in the United Kingdom and subsequently in Australia, with all cases linked to recent travel to South East Asia (Eyre et al. 2018; Jennison et al. 2019). Further reports of XDR cases with ceftriaxone resistance and intermediate azithromycin resistance were reported in the UK from two women with recent travel to Ibiza, Spain, in which genomically identical strains belonging to the characterized FC428 were isolated (Eyre et al. 2019).
Ureaplasma spp. are unique genus of bacteria which have an essential requirement for urea in energy production and also have one of the smallest genomes of any free living organism (Glass et al. 2000). These bacteria are...
linked to non-gonococcal urethritis in men (Beeton et al. 2019) strongly associated with chorioamnionitis and subsequent preterm birth, (Sweeney et al. 2017) development of bronchopulmonary dysplasia, necrotizing enterocolitis and intraventricular haemorrhaging among preterm neonates (Viscardi 2014) and identified as a cause of infectious hyperammonemia among immunocompromised patients (Bharat et al. 2015). AMR is a significant challenge among these organisms due to substantial levels of intrinsic resistance owing to the absence of a cell wall and lack of metabolic pathways for de novo folic acid synthesis. Current treatment relies on macrolide, fluoroquinolone and tetracycline antibiotics, although acquired resistance is present to all antibiotics and prevalence varies depending on geographical location (Beeton and Spiller 2017). The lack of a cell wall affords Ureaplasma spp. intrinsic resistance to all beta lactam and glycopeptide antibiotics, but also provides an excellent model included with multidrug-resistant N. gonorrhoeae (WHO X) and NCTC 13822 (WHO Z) (Unemo et al. 2016). Neisseria gonorrhoeae were propagated on Brain Heart Infusion (BHI) agar (Sigma, Dorset, UK) supplemented with 5% lysed horse blood (TCS Biosciences, Buckingham, UK). All cultures were incubated at 37°C in the presence of CO2.

A total of 10 Ureaplasma spp. were tested. These included Ureaplasma parvum serovar 1 (ATCC 27813), U. parvum serovar 3 (ATCC 700970), U. parvum serovar 6 (ATCC 27818), U. parvum serovar 14 (ATCC 33697), U. parvum HPA5, erythromycin-resistant U. parvum UHWO10 (Beeton et al. 2009), ciprofloxacin-resistant U. parvum U6 (Beeton et al. 2016), Ureaplasma urealyticum serovar 2 (ATCC 27814), U. urealyticum serovar 4 (ATCC 27816) and tetracycline-resistant U. urealyticum serovar 9 (ATCC 33175). Ureaplasma were grown in ureaplasma selective media (Mycoplasma Experience, Bletchingley, UK) within microtitre plates and sealed with adhesive film. Plates were incubated at 37°C under normal atmospheric conditions. Antibiotics were purchased from Sigma-Aldrich (Dorset, UK).

Preparation of enacyloxin IIA and gladiolin

Enacyloxin IIA and gladiolin were prepared from Burkholderia gladioli strains BCC1701 and BCC0238 respectively. Both antibiotics were induced by growing Burkholderia on solidified basal salts medium with 4 g l−1 glycerol (BSM-G) for 72 h at 30°C and extracted using dichloromethane (Mullins et al. 2019). Extracts were concentrated using a Buchi Rotavapor R-3 system, eluted in 60% (v/v) acetonitrile and purified by preparative HPLC (Waters Autopurification HPLC system fitted with a XSelect CSH C18, 5 μm OBD, 19×100× mm column). Fractions were collected at 360 and 230 nm for enacyloxin IIA and gladiolin respectively pooled and dried to a powder by a combination of vacuum centrifugation and freeze drying. Powdered aliquots of each antibiotic were stored at −80°C prior to dissolving in dimethyl sulfoxide to create working stocks for antimicrobial susceptibility testing.

Materials and methods

Neisseria gonorrhoeae and Ureaplasma spp. strain collection

A total of 14 N. gonorrhoeae were examined in this study. Seven isolates (Gwent 1-7) were recent clinical isolates from South Wales, UK. The remaining seven comprised of characterized susceptible and multidrug-resistant isolates NCTC 8375, NCTC 13798, NCTC 13799, NCTC 13477 (WHO F), NCTC 13818 (WHO V), NCTC 13820 (WHO X) and NCTC 13822 (WHO Z) (Unemo et al. 2016). Neisseria gonorrhoeae were propagated on Brain Heart Infusion (BHI) agar (Sigma, Dorset, UK) supplemented with 5% lysed horse blood (TCS Biosciences, Buckingham, UK). All cultures were incubated at 37°C in the presence of CO2.

In this study, we examined the antimicrobial activity of enacyloxin IIA and gladiolin against a panel of susceptible and multidrug-resistant N. gonorrhoeae isolates from South Wales and characterized collections. Additionally, we sought to determine the activity of these compounds against the cell wall-free Ureaplasma spp. as a representative of the Mollicutes class.

Journal of Applied Microbiology 130, 1546–1551 © 2020 The Authors. Journal of Applied Microbiology published by John Wiley & Sons Ltd on behalf of Society for Applied Microbiology

N.L. Heath et al. Novel antimicrobials for STIs
Antimicrobial susceptibility testing of *Ureaplasma* spp

The susceptibility of *Ureaplasma* spp. to antimicrobials was determined by broth microdilution following the Clinical and Laboratory Standards Institute approved guidelines for testing human mycoplasmas (CLSI 2011). In brief, doubling dilutions of the test antimicrobial were prepared in USM within microtitre plates. *Ureaplasma* were added to each well giving a final concentration of $10^4$–$10^5$ colour changing units per ml. Plates were sealed and subsequently incubated at 37°C under ambient air. MIC values were determined as the lowest concentration of antimicrobial to inhibit colour change at the point at which the positive control well gave a colour change. Growth curve assays were set up in an identical way with the modification of static incubation within a Tecan Infinite M200 spectrophotometer. Plates were sealed and incubated at 37°C for 20 h with readings taken at 550 nm were taken every 20 min.

Antimicrobial susceptibility testing of *N. gonorrhoeae*

Agar dilution was used to determine the susceptibility of *N. gonorrhoeae* to the test antimicrobials. Briefly, doubling dilutions of antimicrobials were prepared in agar plates containing BHI supplemented with 5% defibrinated horse blood giving a final concentration range from 32 to 0.015 mg l$^{-1}$. *Neisseria gonorrhoeae* colonies were suspended in saline to a 0.15 McFarland Standard. A multi-point inoculator was used to inoculate plates with 1 µl of bacterial suspension in duplicate. Plates were incubated for 30 h at 37°C in the presence of CO$_2$. For quality assurance purposes, MIC values for the WHO strains were confirmed using the method described.

Results

Activity of enacyloxin IIa and gladiolin against *N. gonorrhoeae*

The activity of enacyloxin IIa and gladiolin against *N. gonorrhoeae* was determined using an agar dilution assay. The MIC range for enacyloxin IIa against *N. gonorrhoeae* was lower than that of gladiolin (0.015–0.06 mg l$^{-1}$ versus 1–2 mg l$^{-1}$ respectively) (Table 1). The presence of defined high-level azithromycin resistance (WHO V) or ceftriaxone resistance (WHO X) in a background of ciprofloxacin and tetracycline resistance had no impact on the MIC compared with fully susceptible strains.

Activity of enacyloxin IIa and gladiolin against *Ureaplasma* spp

Using broth microdilution the MIC range of enacyloxin IIa against *Ureaplasma* spp. was determined to be...
between 4 and 32 mg l\(^{-1}\) whereas the MIC for gladiolin was greater than 32 mg l\(^{-1}\) (Table 2). The effect of enacyloxin IIa on the growth kinetics of the macrolide resistant \(U.\) \(parvum\) strain UHWO10 was determined by spectrophotometry (Fig. 1). There was a clear dose-dependent inhibition of growth over time with enacyloxin IIa, which was not seen with gladiolin or erythromycin.

### Table 2 MIC values for \(Ureaplasma\) spp

|          | U. \(p\) ATCC 27813 (SV1) | U. \(p\) ATCC 700970 (SV3) | U. \(p\) ATCC 27818 (SV6) | U. \(p\) ATCC 33697 (SV14) | U. \(u\) ATCC 27814 (SV2) | U. \(u\) ATCC 27816 (SV4) | U. \(u\) ATCC 33175 (SV9) | MIC range (mg l\(^{-1}\)) |
|----------|-----------------------------|-----------------------------|---------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Enacyloxin IIa | 32 32 4 8 16 4 8 16 4 32 10 4-32 |
| Gladiolin   | >32 >32 >32 >32 >32 >32 >32 >32 >32 >32 >32 >32 |
| Erythromycin| 0.5 0.5 1 1 0.5 1 0.5 1 0.5 1 0.5 1 |
| Ciprofloxacin | 2 2 32 2 2 1 2 2 2 2 8 10 1-32 |
| Tetracycline | 0.25 0.125 0.125 0.25 0.125 0.125 0.25 0.125 0.125 0.125 0.125 0.125 |

\(U.u = U.\) \(urealyticum\), \(U.p = U.\) \(parvum\), \(SV = serovar.\)

### Discussion

Antibiotic resistance among sexually transmitted pathogens is of growing concern. In 2018, the first reports of XDR \(N.\) \(gonorrhoeae\) with ceftriaxone and high-level azithromycin resistance were noted in England and shortly after in Australia (Jennison \textit{et al.} 2019). Although numbers of XDR cases are limited, recent data from the
Gonococcal Resistance to Antimicrobials Surveillance Programme (GRASP) identified a drift in all antimicrobials tested away from susceptible ranges (PHE 2019a). In light of these data there is a need to invest and develop novel antimicrobial compounds. Enacyloxin represents a novel class of antibiotic for future development (Mahenthiralingam et al. 2011). The MIC values for enacyloxin IIa against *N. gonorrhoeae* were lower than for front line agents azithromycin and ceftriaxone. The MIC values were substantially lower than those previously reported for *A. baumannii* (3 mg l$^{-1}$) and *Pseudomonas aeruginosa* (100 mg l$^{-1}$; Mahenthiralingam et al. 2011). Of particular interest was the activity of this compound against the multidrug-resistant strains with documented resistance to azithromycin, ceftriaxone, tetracycline and ciprofloxacin. By inhibiting protein synthesis via interactions with EF-TU, enacyloxin can bypass current mechanisms of resistance. In addition, the recent finding that the chain release mechanism required enacyloxin biosynthesis can be manipulated to produce novel analogues, opens up multiple possibilities for the clinical development of this antibiotic (Masschelein et al. 2019). Although the activity of gladiolin was not as pronounced as enacyloxin IIa, it was again comparable to that of azithromycin and ceftriaxone.

*Ureaplasma* spp. are recognized pathogens associated with in utero infection, infection among immunocompromised patients and mounting evidence as pathogens in sexual health (Bharat et al. 2015; Sweeney et al. 2017; Beeton et al. 2019). Intrinsic resistance to many antimicrobials and emergence of acquired resistance makes treatment complicated, especially in the context of neonatal infection in which tetracyclines and fluoroquinolones are contraindications. Endpoint MIC readings showed that MIC values ranged from 1 to 16 mg l$^{-1}$ for enacyloxin IIa and ≥32 for gladiolin. These values were comparable to those previously seen for *A. baumannii* (Mahenthiralingam et al. 2011). Due to the lack of quantifiable turbidity when growing *ureaplasma* in broth culture, a colorimetric and kinetic based method was used to observe any dose-dependent impact of antimicrobials. For enacyloxin IIa there was a clear dose dependent inhibition of growth which was absent in the presence of gladiolin as well erythromycin to which the UHWO10 is resistant to. Enacyloxin is known to bind serum proteins and therefore the presence of 20% serum in the *ureaplasma* media may have chelated out active drug explaining the higher MIC values. Reasons for the lack of gladiolin activity is unknown, but may be linked to the lack of antimicrobial activity against *ureaplasmas* seen for other RNA polymerase inhibiting antibiotics such as rifampin (Waites et al. 2005).

In conclusion, this study demonstrates the potential for enacyloxin IIa and gladiolin as future therapeutics against *N. gonorrhoeae*, with particular reference to multidrug-resistant strains. These data suggest further investigation and development of this compound as a potential for treating XDR *N. gonorrhoeae* and mycoplasmas.

**Acknowledgements**

The authors thank Aneurin Bevan University Health Board, Royal Gwent Hospital, Department of Microbiology for their help with this project.

**Funding**

This project was supported by an internal Research and Innovation Services fund at Cardiff Metropolitan University. E.M. and G.W. acknowledge funding from Biotechnology and Biological Sciences Research Council (BBSRC; grants BB/L021692/1 and BB/S007652/1), and the Welsh Government Life Sciences Bridging Fund (Grant LSBF R2-004) which fund *Burkholderia* antibiotic discovery and exploitation research.

**Author contributions**

N.J.H, R.S.R, G.W, E.M and M.L.B conceived and designed the study. N.J.H, R.S.R, G.W and M.L.B undertook experimental procedures. N.J.H, R.S.R and MLB undertook data analysis. M.L.B drafted the manuscript. All authors approved the final manuscript.

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