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ORIGINAL ARTICLE

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

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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 for ruling out cellular targets of novel antimicrobials (Hillitt *et al.* 2017).

With the emergence of XDR *N. gonorrhoeae* and presence of macrolide resistance among ureaplasmas, which may complicate treatment of neonatal infections, there is a growing need to develop antibiotics with novel cellular targets to overcome current mechanisms of resistance. Enacyloxin IIa (Mahenthiralingam *et al.* 2011) and gladiolin (Song *et al.* 2017) are novel polyketide antibiotics isolated from *Burkholderia* bacteria. These antimicrobial compounds are of interest due to either their activity against novel bacterial targets not targeted by currently approved antibiotics, or ability to overcome current resistance as follows. Enacyloxin IIa inhibits elongation factor-Tu (EF-Tu) which has not yet been clinically exploited as an antibiotic target (Parmeggiani and Nissen 2006). The novel macrolide gladiolin inhibits the bacterial RNA polymerase, but is able to overcome resistance to other antibiotics such as rifampicin which has the same target (Song *et al.* 2017). The antibacterial capacity of these compounds have been examined against a number of bacteria including *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Enterococcus faecium* and *Acinetobacter baumannii* (Mahenthiralingam *et al.* 2011; Song *et al.* 2017), but to date have yet to be examined against either *N. gonorrhoeae* or any mollicute.

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.

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 CO_2.

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 sulphoxide to create working stocks for antimicrobial susceptibility testing.

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% defibrinized horse blood giving a final concentration range from 32 to 0·015 mg l^{-1}. *Neisseria gonorrhoeae* colonies were suspended in saline to a 0·5 McFarland Standard. A multipoint 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

Table 1 MIC values for *Neisseria gonorrhoeae* isolates

	NCTC 13477 (WHO F)	NCTC 13818 (WHO V)	NCTC 13820 (WHO X)	NCTC 13822 (WHO Z)	NCTC 8375	NCTC 13798	NCTC 13799	Gwent 2	Gwent 3	Gwent 4	Gwent 5	Gwent 6	Gwent 7	Gwent 8	Gwent 9	*n*	MIC range (mg l^{-1})
Enacyloxin IIa	<0·015	0·03	0·06	0·06	<0·015	<0·015	<0·015	N/D	0·03	0·03	0·03	0·03	0·03	0·03	0·03	14	<0·015 to 0·06
Gladiolin	1	1	1	2	2	1	1	2	1	1	1	1	N/D	1	N/D	13	1–2
Azithromycin	0·125	>32	0·5	1	0·25	N/D	N/D	N/D	0·25	0·25	N/D	N/D	0·125	N/D	0·25	9	0·125 to >32
Ceftriaxone	<0·015	0·06	2	0·5	0·06	0·06	0·06	0·06	0·125	0·06	0·06	0·06	N/D	0·06	0·06	14	0·015 to 2

N/D = not determined.

Table 2 MIC values for *Ureaplasma* spp

	U.p HPA5	U.p UHWO10	U.p U6	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)	*n*	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	10	>32
Erythromycin	0·5	>32	0·5	1	1	0·5	1	1	0·5	2	10	0·5 to >32
Ciprofloxacin	2	2	32	2	2	1	2	2	2	8	10	1–32
Tetracycline	0·25	0·125	0·125	0·125	0·25	0·125	0·125	0·25	0·125	>32	10	0·125 to >32

U.u = *U. urealyticum*, U.p = *U. parvum*, SV = serovar.

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.

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 *et al.* 2019). Although numbers of XDR cases are limited, recent data from the

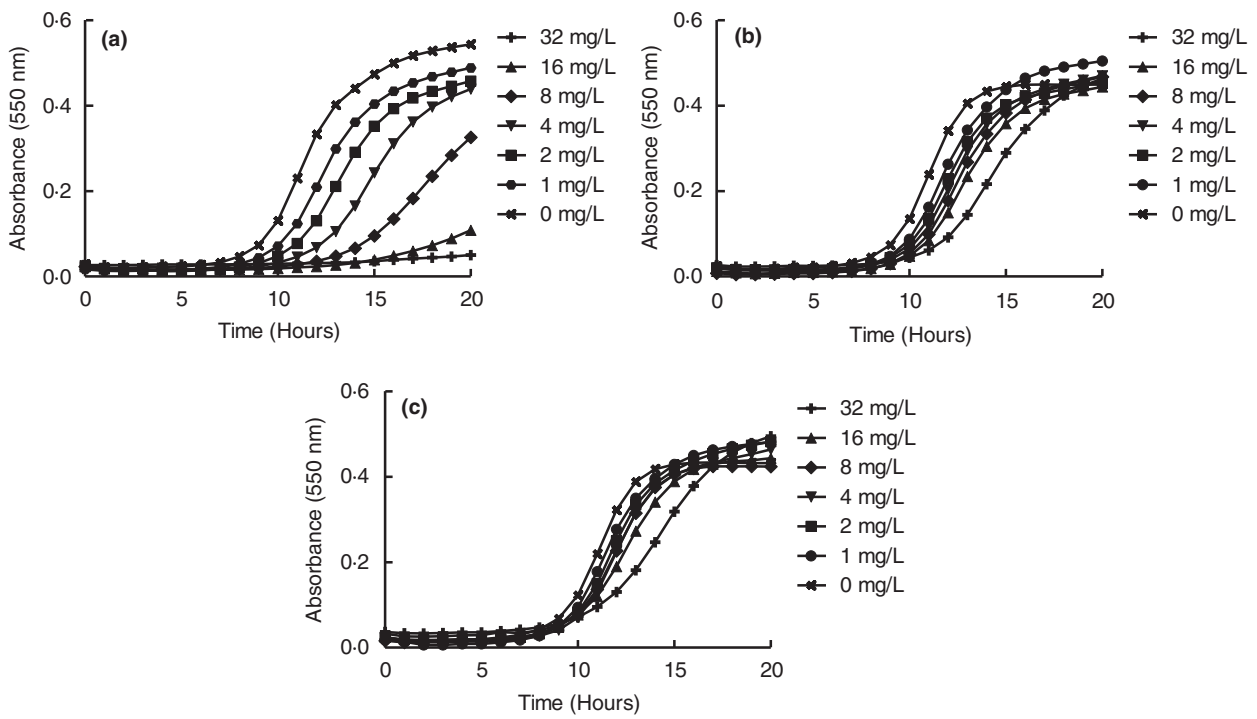

Figure 1 Impact of antimicrobials on the growth dynamics of erythromycin-resistant *Ureaplasma parvum* isolate UHWO10. A colour change in the culture media was recorded over time indicating growth of *U. parvum* while in the presence of varying concentration of (a) enacyloxin IIa, (b) gladiolin and (c) erythromycin.

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 4 to 32 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. Enaycloxin 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.

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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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