***In vitro* activity of solithromycin and metabolites, CEM-214 and N-Acetyl-CEM-101, against 100 clinical *Ureaplasma* spp. isolates in comparison to azithromycin**

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Abstract

There is a strong association between vaginal and/or amniotic fluid *Ureaplasma* spp. colonisation and risk of preterm birth (PTB). The novel fluoroketolide antibiotic, solithromycin (CEM-101), is active against *Ureaplasma* spp. *in vitro*. Evidence from *ex vivo* and *in vivo* models suggests that, unlike most macrolide antibiotics, it readily crosses the placenta. Solithromycin metabolism varies according to species; in pregnant sheep, the bioactive metabolites CEM-214 and N-Acetyl-CEM-101 (NAC-CEM-101) have been shown to accumulate in the amniotic cavity following maternal solithromycin administration, potentially contributing to its antimicrobial effects. To determine the antimicrobial activity of these metabolites against *Ureaplasma* spp., the effects of solithromycin, CEM-214, NAC-CEM-101 and (for comparison) azithromycin were tested on a collection of 100 clinical *Ureaplasma* spp. isolates from the United Kingdom and Australia using a modified 96-well broth microdilution method. The MIC₉₀ values (mg/L) observed for the combined cohort were: solithromycin, 0.125; CEM-214, 0.5; NAC-CEM-101, 0.5 and azithromycin, 2. Solithromycin showed 34-fold greater activity against *Ureaplasma* spp. isolates than azithromycin, while CEM-214 and NAC-CEM-101 possessed approximately 22% and 17% activity of solithromycin, respectively, significantly greater than that of azithromycin. One bacterial isolate showed resistance to azithromycin (MIC 16 mg/L) but had much lower MIC values for solithromycin (MIC 0.25 mg/L). We conclude that, the metabolites of solithromycin had reduced but still potent activity against 100 clinical *Ureaplasma* spp. isolates *in vitro*. This may be important in some instances such as pregnancy, however, studies to determine levels of the metabolites in these settings are required.

Key words: *Ureaplasma*, antibiotic, solithromycin, azithromycin, minimum inhibitory concentration, preterm birth.

1. INTRODUCTION

Preterm birth (PTB) accounts for 11.1% of all live births worldwide and is the leading cause of neonatal morbidity and mortality in the developed world [1]. Infection has been established as an important aetiological factor, contributing to approximately 40% of PTB cases, in particular births before 32 weeks' gestation [2]. *Ureaplasma* spp. are the most common organisms isolated from preterm gestational tissues and amniotic fluid [3] and are a major cause of early preterm birth. They also readily colonise the vagina in 40-80% of pregnant women, and this is believed to be the reservoir for intraamniotic infection in the vast majority of cases [4]. Lung colonisation in neonates has also been established as a cause of the chronic pulmonary disease, bronchopulmonary dysplasia (BPD), another major cause of neonatal morbidity and mortality [5].

Ureaplasma spp. are part of the Class Mollicutes, a group of organisms that includes other human pathogens such as *Mycoplasma hominis* and *Mycoplasma pneumoniae*. Organisms within this class are unique in that they lack a cell wall and possess limited biosynthetic pathways as a result of their greatly reduced genome size [6]. Such attributes create a complex problem when attempting to formulate effective antibiotic treatment regimens, especially during pregnancy. The most effective antimicrobial agents against *Ureaplasma* spp. are the quinolones, tetracyclines and macrolides; however, during pregnancy both the quinolones and tetracyclines are contra-indicated due to their teratogenic effects [7], and some macrolides such as clarithromycin are also contraindicated [8]. As a result, macrolides such as erythromycin and azithromycin are the preferred antibiotics for treatment of *Ureaplasma* spp. in pregnancy. However, their efficacy is limited by their poor placental transfer characteristics (<5%) [9], which impairs their ability to efficiently treat intraamniotic infection. There are also additional safety concerns surrounding some clinical applications of these antibiotics in pregnancy [10, 11]. Finally, the hyper-mutable phenotype of these organisms, combined with the long residence time of some macrolides (e.g. azithromycin), may contribute to the generation of antibiotic resistance [12]. The emergence of macrolide-resistant strains of *Ureaplasma* spp. has the potential to jeopardise their efficacy for widespread clinical use, including during pregnancy [13, 14]. The development of new antimicrobial agents, therefore, is of substantial importance when considering antenatal treatment of *Ureaplasma* spp. infections.

The fluoroketolide antibiotic, solithromycin, is one such candidate. Acting by inhibiting protein synthesis, this compound binds to a total of three sites on the bacterial 50S ribosome (compared to one binding site of the current macrolides) [15]. The compound has been shown to be highly potent against Gram-positive and negative bacteria, as well as *Mycoplasma* and *Ureaplasma* spp. [16-18]. Currently in phase III clinical trials, solithromycin has also shown a good safety profile, is well-

tolerated in oral formulations, and exhibits excellent placental transfer as demonstrated both in an ovine model (34%) and in a human *ex vivo* placental perfusion model [19-22]. Following its metabolism, solithromycin can produce at least two bioactive polar metabolites: CEM-214 and NAc-CEM-101. These are minor species in adult human studies, but can be found in significant amounts in sheep and other animals. In the pregnant sheep model, these metabolites have been demonstrated to accumulate in the amniotic fluid following both intravenous (IV) and intra-amniotic (IA) administration, exhibiting delayed clearance and a prolonged IA half-life [21]; levels of metabolites in the human amniotic cavity are unknown, although the extent of metabolism by the human placenta is low [22]. The bioactivity of CEM-214 and NAc-CEM-101 has been evaluated against respiratory pathogens and *Escherichia coli*, but not against *Ureaplasma* spp. This could be of significance with respect to solithromycin's use in treating pregnancy infections, as (depending on the extent of metabolism) the accumulation of bioactive solithromycin metabolites within the amniotic cavity could extend its antimicrobial activity *in utero*, enhancing its ability to treat *Ureaplasma* spp. infections at reduced doses and improving pregnancy outcomes accordingly.

The aims of this study, therefore, were to assess the activity of solithromycin, CEM-214 and NAc-CEM-101 against a range of clinical *Ureaplasma* spp. isolates sourced from the United Kingdom and Australia. For comparison, the second generation macrolide azithromycin was also included in the studies.

2. MATERIALS AND METHODS

2.1. Specimen collection

A total of 100 clinical *Ureaplasma* spp. isolates were sourced from a range of clinical samples (Table 1). Specimens were sourced from two geographically separate populations, 50 isolates from the United Kingdom (UK) and the remaining 50 isolates from Australia (AUS).

2.2. Purification

All specimens were purified through a process of triple cloning. This involved inoculation of a 10 µL loop of exponential-phase *Ureaplasma* spp. broth culture onto A8 agar (Melbourne University Media Preparation Unit, Melbourne, Australia) and incubated at 37°C, 5% CO₂/2% O₂ for 24 h. *Ureaplasma* spp. colonies were visualised under a stereomicroscope (Nikon, Tokyo, Japan) and a single, well-isolated colony was selected by puncturing the surface of the agar with a sterile P1000 pipette tip, engulfing the colony. The colony was expelled into 2 mL of 10B broth (Melbourne University Media Preparation Unit) and incubated for a further 24 h as previously described, until growth was observed in the form of a pH-associated colour change (*Ureaplasma* spp. metabolise urea and

subsequently produce ammonia, resulting in a pH shift within the media). This process was repeated a total of three times to ensure isolation of a purified *Ureaplasma* spp. isolate.

2.3. Antimicrobial susceptibility testing

Stock solutions of each antibiotic were prepared and frozen in single-use aliquots. Solithromycin (5 g/L), CEM-214 (5 g/L) and NAc-CEM-101 (5 g/L) (all supplied by Cempira Inc, Chapel Hill, USA) were re-suspended from powder form in a mannitol-tartrate-thioglycerol perfusion buffer (pH 4.2), whilst azithromycin (5 g/L) (Aspen Pharmacare Pty Ltd, St Leonards, NSW, Australia) was re-suspended from powder form in saline solution. Working stock solutions were diluted in 10B broth to concentrations of 4, 8 and 16 mg/L for solithromycin, CEM-214 and NAc-CEM-101, and azithromycin, respectively. A modified 96-well broth microdilution method, previously described by Beeton *et al.* [23], was subsequently employed to ascertain the minimum inhibitory concentration (MIC) of each antibiotic compound required to inhibit the growth of *Ureaplasma* spp. below 10^4 colour changing units (CCU). CLSI requirements for antibiotic and organism handling as well determination of 10^4 - 10^5 CCU input organisms were adhered to in performing MIC determinations [24], with the exception of incubation conditions. Briefly, 90 μ L of 10B broth (Melbourne University Media Preparation Unit) were added to each well in a 96-well microtitre plate (Axygen[®], Corning, Massachusetts, USA) with the exception of rows 1 and 12. Row 12 received 180 μ L of one of the four working antibiotic stocks and using this, a 2-fold dilution series was formed up the plate from rows 11-2. Row 1 acted as a positive growth control, and received 90 μ L of 10B broth diluted with the corresponding antibiotic vehicle (in the same ratio as the working stock). 10 μ L of exponential phase *Ureaplasma* spp. broth culture was added to each well of column 1 and serially diluted 1:10 across the plate. The plate was sealed with adhesive plate film (Axygen[®]) and incubated for 48 h as described above.

2.4. Species differentiation

Payne *et al.* [25] had already established species-level identification of UK triple-cloned isolates. For the Australian isolates, to determine whether the triple-cloned *Ureaplasma* spp. were either *U. parvum* or *U. urealyticum*, a real-time PCR assay targeting the urease gene of these species as described by Yi *et al.* [29], adapted for use on a ViiA7 real-time PCR thermocycler (Life Technologies, Carlsbad, California, USA), was used. Reaction mixtures (final volume or concentration) consisted of: 1X Taqman FAST Advanced Master Mix (Life Technologies), 0.9 μ M primers UU1613F and UU1524R (Life Technologies), 0.25 μ M probes UU-parvo (FAM) and UU-T960 (VIC) (Life Technologies), 5 μ L of template DNA and nuclease-free water (Ambion) to a final volume of 20 μ L. PCR cycling conditions

consisted of an initial denaturation/Taq activation at 95°C for 20 s, followed by 40 quantification cycles of 95°C for 1 s and 60°C for 20 s (data acquiring).

2.5. Statistical analyses

MIC results were recorded as geometric mean, MIC₅₀, MIC₉₀ and range. Statistical comparisons between antibiotic agents, Australian and UK sample cohorts and delivery outcomes were conducted using non-parametric analyses including Friedman, Mann-Whitney and Wilcoxon tests to assess significance ($p < 0.05$) (IBM SPSS Statistics Version 22).

3. RESULTS

3.1. Overall MIC values

In all of our experiments we used microaerophilic incubation conditions, which are optimal for the growth of *Ureaplasma* spp. and reflect the clinical environment that it grows in. As these are not currently endorsed under CLSI guidelines, we compared aerobic vs microaerophilic incubation conditions for two type strains, *U. parvum* ATCC 27815 and *U. urealyticum* ATCC 27618. MIC titrations were conducted against solithromycin and azithromycin, 10 replicates of each drug/incubation environment. Quality control MIC ranges for both ATCC strains showed no significant difference in MIC endpoint between aerobic and microaerophilic incubation over a 96h period (Table S1).

Of the 100 isolates tested 99 showed susceptibility to all four antibiotic compounds, while only one exhibited azithromycin resistance. The geometric mean and MIC₉₀ values were calculated with the exclusion of this value. In order of activity, solithromycin possessed the lowest geometric mean and MIC₉₀ against the 99 *Ureaplasma* spp. isolates tested, followed by CEM-214, NAc-CEM-101 and azithromycin (Table 2). Based on the geometric mean MIC values, relative potency was $1 < 4.5 < 5.8 < 31.2$, respectively.

3.2. Comparison of Australian and UK isolates

Significantly lower median MIC values were observed against solithromycin ($P=0.007$), CEM-214 ($P=0.011$) and NAc-CEM-101 ($P=0.011$) within the UK strains compared to the Australian isolates (Table 3), although the differences were not pharmacologically significant. Comparison of the median MIC of azithromycin between the two populations yielded no significant differences.

3.3. Antibiotic-resistant isolates

As no MIC breakpoints have currently been established for solithromycin or its metabolites, it is not possible to classify an isolate as resistant or susceptible. No azithromycin-resistant isolates were found within the Australian cohort, whilst one was detected in the UK cohort. This isolate, UHWO10, showed an MIC value of 16 mg/L for azithromycin, compared with the median MIC of 2 mg/L ($P<0.001$). In addition, this isolate also showed significantly higher MIC values for the remaining antibiotic compounds tested ($P<0.001$), compared to the median values observed (Figure 1). For solithromycin, CEM-214 and NAc-CEM-101, the MIC values increased 4, 14 and 3-fold respectively, although the isolate's growth was still inhibited at much lower concentrations compared to Azithromycin.

3.4. Comparison between *Ureaplasma* spp. isolates associated with preterm and term pregnancies

Full pregnancy outcome data were available for *Ureaplasma* spp. isolates obtained from the Australian cohort. No significant differences in median MIC values were observed between *Ureaplasma* spp. isolates from preterm and term pregnancies.

3.5. Species comparison between *Ureaplasma urealyticum* and *Ureaplasma parvum* isolates

The Australian cohort of samples contained both *U. urealyticum* and *U. parvum* isolates, whilst the UK contained only *U. parvum*. Collectively, the *U. urealyticum* clinical isolates exhibited significantly greater median MIC values against antibiotics solithromycin ($P<0.001$), CEM-214 ($P<0.001$) and NAc-CEM-101 ($P=0.004$) in comparison to *U. parvum*. Median MIC values between the two species against azithromycin were not statistically significant (Figure 2).

4. DISCUSSION

The present findings expand upon a previous smaller study and confirm that the 4th generation macrolide solithromycin has potent activity against *Ureaplasma* spp. [22]. These findings, in conjunction with our animal studies [26] showing its ability to achieve therapeutic concentrations in the amniotic fluid from a single maternal dose [27], further highlight the potential clinical benefits of solithromycin for the treatment of reproductive tract infections in pregnancy. The observation that the two solithromycin metabolites CEM-214 and NAc-CEM-101 maintain considerable bioactivity against *Ureaplasma* spp. may have additional implications for its use in pregnancy, in light of data from the sheep showing significant accumulation and extended residence time of these metabolites within the amniotic cavity. However, the relevance of this phenomenon to human pregnancy remains to be confirmed.

Solithromycin metabolites showed reduced bioactivity against *Ureaplasma* spp., although their potency against was still very high: 4 times more potent than azithromycin, a macrolide antibiotic that is efficacious and widely used during pregnancy [28]. The only other study that has assessed the metabolite activity of solithromycin, to our knowledge, was completed by Pereira *et al.* [29]. They determined the activity of both metabolites against several Gram positive organisms including: *Streptococcus pneumoniae*, *Streptococcus pyogenes* and *Staphylococcus aureus*, plus the Gram negative organism *E. coli*. For respiratory pathogens, they found CEM-214 and NAc-CEM-101 possessed 25% and 50% of solithromycin's activity, respectively. Our study, however, found no significant difference between the median MICs of the two metabolites against *Ureaplasma* spp. Pereira *et al.* 2010 [29] found that NAc-CEM-101 was produced in greater amounts than CEM-214 in rats, whereas in pregnant sheep, we found the opposite was true [21], similar to findings in monkeys [29]. The accumulation of the metabolites in the amniotic cavity in sheep raises the possibility that either the fetus or placenta is capable of significant metabolism. However, our studies using the placental perfusion model suggest that in humans the placenta is not a major source of either metabolite [23], although it remains to be determined to what extent the fetus contributes to levels in amniotic fluid in human pregnancy.

Solithromycin was 29-34 fold more potent than azithromycin. This supports the findings of Waites *et al.* [27], who were the first to outline this compound's high potency against *Ureaplasma* spp. Their reported MIC range (0.002-0.063 mg/L), MIC₅₀ (0.008 mg/L) and MIC₉₀ (0.016-0.031 mg/L) values, however, are lower than those observed in this study. These differences may be explained by their much smaller sample set of 20 isolates, compared with our more comprehensive sample of 100. Methodological differences may also have contributed to the disparities observed, however both studies adhered to the CLSI methods, with the exception of incubation conditions, a factor that we have demonstrated do not significantly affect MIC endpoints.

There are two species of *Ureaplasma* that are known to colonise humans, *Ureaplasma parvum* and *Ureaplasma urealyticum*. Using real-time PCR, we previously confirmed that all of the UK isolates used in this study were *U. parvum* [25]. Subsequent analysis of the Australian isolates in the current study showed that 20% were *U. urealyticum*. We found that the geometric mean MIC, MIC₅₀ and MIC₉₀ values for the *U. urealyticum* isolates were up to 2 times higher than for *U. parvum* isolates. This finding is similar to that of Waites *et al.*, who previously reported that *U. urealyticum* commonly exhibits greater MIC values compared to *U. parvum* [27]. Whether or not this means that *U. urealyticum* infections are harder to eradicate with macrolides than *U. parvum* remains to be

determined. However, it is worth noting that clinically *U. urealyticum* is much less common than *U. parvum* [30].

Our study also compared MIC values for isolates from two geographically distinct regions, UK and Australia. We hypothesised that a similar level of activity would be observed across all isolates in response to solithromycin, CEM-214 and NAc-CEM-101. In contrast, azithromycin is a widely used antibiotic and we expected fluctuating susceptibility results reflecting recurrent exposures to this compound. Although minor differences were observed, all isolates showed inhibited growth when exposed to solithromycin and its metabolites at levels consistently lower than those demonstrated with azithromycin.

We detected one apparently azithromycin-resistant isolate, UHWO10. This strain was previously documented to possess a 6 bp in-frame deletion within the L4 protein gene which resulted in erythromycin resistance (MIC >64 mg/L), although the MIC range for azithromycin (0.5–4 mg/L) was within susceptible limits [23]. However, those results were obtained for a non-clonal clinical isolate, whereas in our study it was subjected to triple clone purification before being assessed.

Perhaps of greater interest was the relative efficacy of solithromycin, CEM-214 and NAc-CEM-101 against this strain. All three compounds were highly active against the isolate, further supporting the clinical potential of solithromycin treatment.

With full pregnancy outcome data available for the 50 isolates analysed from Australia, we were able to conduct, to our knowledge, the first antibiotic susceptibility comparison of vaginal *Ureaplasma* spp. isolates originating from preterm (10 isolates) and term (40 isolates) births. No significant difference in antibiotic susceptibility was observed between the isolates from the two outcomes. This is a similar result to that described by Martinez *et al.* [31], where no association was found between the susceptibility of organisms to erythromycin and tetracycline in amniotic fluid samples collected from pregnancies with or without ruptured or intact membranes and preterm labour [31].

5. CONCLUSION

Solithromycin and its two metabolites, CEM-214 and NAc-CEM-101, show potent antimicrobial activity against clinical *Ureaplasma* spp. isolates *in vitro*. The effectiveness of this drug and its metabolites against a macrolide-resistant *Ureaplasma* spp. isolate at low MIC levels is a notable characteristic in a time of emerging resistance. The collective findings to date indicate that solithromycin represents an exciting antimicrobial option for the treatment of reproductive tract infections in pregnancy, particularly in those pregnancies complicated by *Ureaplasma* spp. infection. Future studies are now required to assess the extent of metabolite formation and accumulation in

261 human pregnancy, the efficacy and safety of the drug during pregnancy, and to identify which
262 women will benefit most from antenatal treatment.

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269 **7. AUTHOR INFORMATION**

270 MSP and JAK are co-senior authors.

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279 **9. CONFLICT OF INTEREST**

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281 Competing interests: None declared

282 Ethical approval: Not required

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10. FIGURE LEGENDS

Figure 1. Comparisons between the median MIC values of the 99 *Ureaplasma* spp. isolates and the azithromycin-resistant isolate (UHW010) against solithromycin (SOLI), CEM-214, NAc-CEM-101 (NAC) and azithromycin (AZI). Data labels above each column distinguish the exact value and (*) denotes a significant difference between the two data series as determined by non-parametric one-sample Wilcoxon analysis with *P* value <0.05.

Figure 2. Median MIC comparisons[#] for solithromycin (SOLI), CEM-214, NAc-CEM-101 (NAC) and azithromycin (AZI) against *U. urealyticum* and *U. parvum* isolates. The symbol (*) denotes a significant difference between median MIC values as determined by Mann-Whitney analysis with *P* value <0.05.

[#]Values were calculated excluding isolate UHW010, which was resistant to azithromycin and has known molecular mechanisms of macrolide resistance.

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