

1 *In vitro* susceptibility to closthioamide among clinical and reference strains of *Neisseria*

2 *gonorrhoeae*

3 *Original article*

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11 Runing Head: Closthioamide activity against *N. gonorrhoeae*

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14

15 **Abstract**

16 *Neisseria gonorrhoeae* is one of the leading antimicrobial resistance threats worldwide and there  
17 is a need for the development and evaluation of new antimicrobials. The aims of this study were  
18 to determine the *in vitro* susceptibility to the novel antimicrobial closthioamide (CTA) of clinical  
19 *Neisseria gonorrhoeae* strains, reference *N. gonorrhoeae* strains and related commensal  
20 *Neisseria* species. Minimum inhibitory concentration (MIC) to CTA and six antibiotics were  
21 determined using agar dilution for 149 clinical *N. gonorrhoeae*, eight World Health Organisation  
22 reference *N. gonorrhoeae* and four commensal *Neisseria* species. The correlation between CTA  
23 MICs and ciprofloxacin, penicillin, cefixime, ceftriaxone, azithromycin and tetracycline were also  
24 determined using Spearman's Rank correlation test. CTA MIC for the clinical and reference  
25 gonococcal strains were 0.008-0.25 mg/L and 0.063-0.5 mg/L respectively. The MIC range for  
26 commensal species was 0.063-1 mg/L. The MIC<sub>50</sub> and MIC<sub>90</sub> of the clinical gonococcal strains  
27 were 0.063 mg/L and 0.125 mg/L respectively. The MICs of CTA did not correlate with the MICs  
28 of the other antibiotics tested. Closthioamide has high *in vitro* activity against *N. gonorrhoeae* and  
29 cross-resistance due to existing antimicrobial resistance was not detected, indicating that CTA  
30 could be used to treat drug-resistant infections. However, further research on the mechanism of  
31 action, toxicity, pharmacokinetics and pharmacodynamics of CTA need to be conducted to  
32 evaluate the clinical suitability of this antimicrobial.

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## 36 **Introduction**

37 *Neisseria gonorrhoeae* is one of the most important antimicrobial resistance (AMR) threats  
38 worldwide(1). The discovery of penicillin in the 1940s revolutionised the treatment of *N.*  
39 *gonorrhoeae* however resistance has developed to every therapeutic antimicrobial agent  
40 used(2). In the past 15 years empirical therapy in the UK has had to be changed three times due  
41 to increasing rates of resistance, on average every five years(3).

42 Dual therapy for gonorrhoea, with ceftriaxone and azithromycin, was introduced in 2011(4) as a  
43 strategy to delay AMR. These antibiotics represent the last reliable classes of antibiotics  
44 recommended for empirical treatment of *N. gonorrhoeae* infection(5) and worryingly, the  
45 minimum inhibitory concentration (MIC) to both antibiotics are increasing annually(6). This is  
46 complicated further by reports of treatment failure due to extended spectrum cephalosporin  
47 (ESC) resistance occurring worldwide(7–16). In 2016, treatment failure occurred after dual  
48 therapy with ceftriaxone and azithromycin in a UK patient with urethral and pharyngeal  
49 infection(17). Phenotypic and molecular AMR testing indicated that the gonococcal isolate was  
50 resistant to both agents, providing challenging prospects for the future treatment of gonorrhoea.  
51 In 2012, the World Health Organisation (WHO) published an action plan to combat the spread  
52 and impact of *N. gonorrhoeae*(1). Given that there is no effective vaccine against gonorrhoea  
53 and antimicrobial therapy is still one the most important means of gonorrhoea control, the WHO  
54 advocates research into new antimicrobials(1).

55 Closthioamide, discovered in 2010, was isolated from the anaerobic bacterium *Clostridium*  
56 *cellulolyticum*(18). It represents a new class of natural polythioamide antibiotics and has been  
57 shown to have high *in vitro* activity against AMR microorganisms such as methicillin resistant  
58 *Staphylococcus aureus* (MRSA) and vancomycin resistant Enterococci (VRE)(19). Its mode of  
59 action is not yet well understood but there is evidence it may impair DNA replication and inhibit  
60 DNA gyrase(19). Cross-resistance to quinolone antibiotics has not been observed to-date,  
61 suggesting a different mechanism of action(19). Given its high potency with multi-drug resistant  
62 (MDR) bacteria, closthioamide is a candidate antibiotic to test against *N. gonorrhoeae*.

63 The aim of this study is to determine the *in vitro* activity of closthioamide against clinical and  
64 laboratory reference strains of *N. gonorrhoeae*, as well as commensal *Neisseria* species.

65

## 66 **Results**

### 67 **Bacterial Isolates**

68 A total of 149 clinical *N. gonorrhoeae* isolates were examined in this study; 97 isolates were  
69 obtained from Barts Health NHS Trust, 50 from St George's University Hospitals NHS  
70 Foundation Trust and one each from Royal Free NHS Foundation Trust and Tunbridge Wells  
71 NHS. The gonococcal isolates were cultured from pharyngeal (n=11, 7.4%), urethral (n=19,  
72 12.7%), cervical (n=3, 2%) or rectal (n=19, 12.7%) infection and 65% (n=97) had an unknown  
73 site.

74

### 75 **CTA susceptibility**

76 The MICs for the novel antibiotic closthioamide were determined for 149 clinical gonococcal  
77 isolates, eight reference gonococcal isolates and four commensal *Neisseria* species. Of the 149  
78 clinical strains, 131 had previously determined MICs to penicillin, ceftriaxone, azithromycin,  
79 ciprofloxacin, tetracycline and spectinomycin and 127 had known MICs to cefixime.

80 The CTA MIC range of the 149 clinical strains was between 0.008 mg/L – 0.25 µmg/L. The  
81 number of isolates with MICs of 0.008 mg/L, 0.015 µmg/L, 0.031 µmg/L, 0.063 mg/L, 0.125 mg/L  
82 and 0.25 mg/L were one (1%), six (4%), 14 (9%), 53 (36%), 72 (48%) and three (2%)  
83 respectively (Figure 1). The MIC<sub>50</sub> and MIC<sub>90</sub> were 0.063 mg/L and 0.125 mg/L respectively.

84 The CTA MICs of *N. lactamica* and *N. perflava* were 0.063 mg/L and 0.5 mg/L respectively and  
85 both *N. flavescens* strains had an MIC of >1 mg/L. The CTA MICs of the WHO gonococcal  
86 control strains were higher than the MIC<sub>50</sub> of the clinical strains and the MIC of WHO strain K  
87 was 0.5 mg/L, higher than any of the clinical strains (Table 1).

88

### 89 **Cross-resistance to CTA**

90 The MICs for seven antibiotics were compared to CTA MICs to identify any cross-resistance.  
91 Resistance rates, using WHO breakpoints (Table 1)(20), for the clinical gonococcal strains for  
92 penicillin, cefixime, ceftriaxone, azithromycin, ciprofloxacin, tetracycline and spectinomycin were

93 7.6% (10/131), 2.4%(3/127), 0.8%(1/131), 0.8% (1/131), 23.7%(31/131), 15.3%(20/131) and 0%  
94 (0/131) respectively. No significant correlation was identified between the tested antibiotics;  
95 ciprofloxacin, a fluoroquinolone, had a correlation coefficient of 0.07 (Figure 2, Table 2), the  
96 highest correlation was 0.48 with azithromycin.

97

## 98 Discussion

99 The imminent threat of untreatable gonorrhoea is a global problem that urgently requires the  
100 development of new antimicrobial agents. In this study, the novel antimicrobial closthioamide was  
101 evaluated against 149 clinical and eight reference strains of *N. gonorrhoeae* and four commensal  
102 *Neisseria* species. CTA was effective *in vitro* against 146/149 (98%) of clinical gonococcal strains  
103 at  $\leq 0.125$ mg/L, suggesting a low therapeutic dose concentration which would reduce any  
104 potential toxicity. Importantly isolates resistant to ciprofloxacin and the first line therapeutic  
105 agents ceftriaxone and azithromycin are as susceptible to CTA as strains sensitive to these  
106 antibiotics, suggesting CTA could be effective clinically against MDR *N. gonorrhoeae* strains.  
107 Closthioamide activity against *N. gonorrhoeae* is comparable to its activity against other AMR  
108 organisms; *N. gonorrhoeae* MIC<sub>90</sub> (0.125mg/L) was higher than for MRSA (0.027mg/L) but lower  
109 than for VRE (0.44mg/L)(19). This is noteworthy, as previous studies have shown CTA to be  
110 more effective *in vitro* against Gram-positive organisms than Gram-negative organisms such as  
111 *E. coli* where the MIC ranges between 0.31 mg/L. and 3.75 mg/L(19).

112 CTA mode of action has been linked to gyrase and DNA replication which is also a target for  
113 fluoroquinolones suggesting a potential cross-resistance with antibiotics such as ciprofloxacin.  
114 Analysis of the WHO reference strains demonstrated that although Strain K had the highest CTA  
115 MIC (0.5mg/L) and resistance to ciprofloxacin (>32 mg/L) due to gyrase mutations(21); Strain L,  
116 with equal ciprofloxacin resistance, was still sensitive to CTA. Analysis of the clinical isolates  
117 demonstrated no correlation between the two antibiotics. This is also supported by a study by  
118 Chiriac *et al* who found no cross-resistances between the two antimicrobials, although they did  
119 not examine *N. gonorrhoeae*(19). These data suggests that CTA susceptibility is not linked to  
120 fluoroquinolone resistance, as mutations in the *gyrA* region which confer resistance to

121 fluoroquinolone do not seem to influence CTA MICs, indicating that the active site for CTA may  
122 be elsewhere in the quinolone resistance determining region (QRDR).  
123 Interestingly, the two *N. perflava* strains had the highest CTA MIC (>1 mg/L) and the basis of this  
124 relative resistance requires further research to understanding the specific resistance  
125 mechanisms.  
126 Closthioamide has been shown to have low toxicity in tissue culture(19), making it a good  
127 candidate for clinical use, however further work should be carried out in terms of its toxicity,  
128 pharmacokinetics and pharmacodynamics(22). Successful treatment of pharyngeal infection is  
129 critical to the gonorrhoea control efforts(23–25) meaning that penetration of any new  
130 antimicrobial into the pharyngeal mucosa is of particular importance. Clinical trials investigating  
131 the efficacy of existing antimicrobials such as gentamicin are currently being carried out(26),  
132 however these agents have poor pharyngeal penetration and even if successful will not offer a  
133 long term solution, as development of resistance to aminoglycosides occurs readily(27).  
134 In conclusion, CTA has high anti-gonococcal activity *in vitro*, even for multidrug resistant isolates,  
135 but further studies to evaluate the clinical potential of this antimicrobial are urgently required in  
136 light of the public health threat that gonorrhoea poses.

137

## 138 **Materials & Methods**

### 139 **Bacterial Isolates**

140 Clinical, anonymised *Neisseria gonorrhoeae* isolates cultured from patients at Barts Health NHS  
141 Trust, St George's University Hospitals NHS Foundation Trust, Royal Free NHS Foundation  
142 Trust and Tunbridge Wells NHS Trust hospital laboratories during the period 2013-2014 were  
143 examined in this study. Eight fully characterised WHO gonococcal reference strains, F, G, K, L,  
144 M, N, O and P were provided by the Sexually Transmitted Bacteria Reference Unit (STBRL),  
145 Public Health England (PHE), UK. Commensal *Neisseria lactamica* (n=1), *Neisseria perflava*  
146 (n=1) and *Neisseria flavescens* (n=2) were provided by the London School of Hygiene & Tropical  
147 Medicine (LSHTM) and the Royal Free NHS Foundation Trust Microbiology Laboratory. Isolates  
148 were preserved in 20% glycerol Brain Heart Infusion (BHI) broth at -80°C. Prior to MIC testing,

149 isolates were cultured on Columbia agar supplemented with chocolated horse blood (Oxoid,  
150 Basingstoke, UK) at 37°C, in 5% CO<sub>2</sub> for 24 hours.

151

## 152 Antimicrobial Susceptibility Testing

153 The MICs for CTA, cefixime, ceftriaxone, spectinomycin, tetracycline and azithromycin were  
154 determined by the agar dilution method, as previously described(20). A multi-point inoculator  
155 (Denley, Colchester, UK) was used to inoculate 1µl of each suspension onto each plate in the  
156 respective antimicrobial agar dilution series. The CTA MIC range tested was 0.002 mg/L - 1  
157 mg/L. The MICs for penicillin and ciprofloxacin were determined via gradient strip (Launch  
158 Diagnostics, Kent, UK and Biomerieux, Crappone, France respectively) as previously  
159 described(20).

160

## 161 Synthesis of CTA

162 Closthioamide was synthesized according to the route of Hertweck and coworkers(28, 29).  
163 Closthioamide stock solution was prepared at 100 mg/L in 100% ethanol. The core of CTA was  
164 synthesized by two consecutive peptide couplings and deprotections onto a 1,3-diaminopropane  
165 core with an N-protected beta-alanine, followed by a third peptide coupling to install the aromatic  
166 benzoic acid end caps. Thionation (oxygen to sulphur conversion) with Lawesson's reagent and  
167 deprotection under highly acidic conditions yielded CTA in five longest linear steps. It was noted  
168 during purification of CTA that ethanol present in the chloroform solvent as a stabilizer was  
169 retained.

170 All reagents involved in the synthesis of intermediates, peptide coupling, protection/deprotection  
171 and synthesis of CTA were obtained from Sigma-Aldrich, with the exception of 1-ethyl-3-(3-  
172 dimethylaminopropyl)carbodiimide (EDCI) from Manchester Organics. All reaction solvents used  
173 in synthesis were anhydrous and of the highest grade from Sigma-Aldrich. All routine solvents for  
174 workup and purification were obtained from VWR. In all cases, reagents and solvents were used  
175 as received.

176

177 **Statistical analysis**

178 Data were analysed in Microsoft Excel. MIC<sub>50</sub> and MIC<sub>90</sub> were calculated with MIC data from  
179 clinical gonococcal strains only. The correlation between CTA MICs and those for other  
180 antibiotics was determined with a Spearman's Rank correlation test, using STATA 14.2. The  
181 correlation coefficient was calculated using MIC data from clinical and reference gonococcal  
182 strains.

183

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191

192 **Transparency declaration**

193 None to declare

194

195 **Ethical considerations**

196 None

197

198 **Supplementary data**

199 Full MIC data for all antibiotics tested as well as graphs showing MIC distributions are provided

200 as supplementary data with this manuscript.

201

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- 281

282 TABLES AND FIGURES LEGENDS

283

284 **Figure 1. Susceptibility of gonococcal isolates to closthioamide (CTA).**

285 CTA was tested on 149 clinical gonococcal strains (range tested was 0.002 – 1 mg/L). MIC =

286 Minimum Inhibitory Concentration.

287

288

289 **Table 1. MICs of WHO gonococcal reference strains.** Minimum inhibition concentration (MICs)

290 (mg/L) determined by World Health Organisation (WHO) for penicillin (PEN), cefixime (CFX),

291 ceftriaxone (CRO), azithromycin (AZI), ciprofloxacin (CIP), tetracycline (TET), spectinomycin

292 (SPE). Closthioamide (CTA) MIC was determined by agar dilution in this study.

293

294

295 **Figure 2. Correlation between CTA and ciprofloxacin MICs.** MIC data for CTA and

296 ciprofloxacin from 139 clinical strains was used to calculate a correlation coefficient ( $R^2$ ) of 0.07.

297

298

299 **Table 2. Raw data showing number of clinical isolates with given combination of CTA and**

300 **Ciprofloxacin MICs.**

301

302