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

Original article

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Runing Head: Closthioamide activity against *N. gonorrhoeae*

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Abstract

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

Introduction

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

Dual therapy for gonorrhoea, with ceftriaxone and azithromycin, was introduced in 2011(4) as a strategy to delay AMR. These antibiotics represent the last reliable classes of antibiotics recommended for empirical treatment of *N. gonorrhoeae* infection(5) and worryingly, the minimum inhibitory concentration (MIC) to both antibiotics are increasing annually(6). This is complicated further by reports of treatment failure due to extended spectrum cephalosporin (ESC) resistance occurring worldwide(7–16). In 2016, treatment failure occurred after dual therapy with ceftriaxone and azithromycin in a UK patient with urethral and pharyngeal infection(17). Phenotypic and molecular AMR testing indicated that the gonococcal isolate was resistant to both agents, providing challenging prospects for the future treatment of gonorrhoea.

In 2012, the World Health Organisation (WHO) published an action plan to combat the spread and impact of *N. gonorrhoeae*(1). Given that there is no effective vaccine against gonorrhoea and antimicrobial therapy is still one the most important means of gonorrhoea control, the WHO advocates research into new antimicrobials(1).

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

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

Results

Bacterial Isolates

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

CTA susceptibility

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

The CTA MIC range of the 149 clinical strains was between 0.008 mg/L – 0.25 µmg/L. The 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 and 0.25 mg/L were one (1%), six (4%), 14 (9%), 53 (36%), 72 (48%) and three (2%) respectively (Figure 1). The MIC_{50} and MIC_{90} were 0.063 mg/L and 0.125 mg/L respectively. The CTA MICs of *N. lactamica* and *N. perflava* were 0.063 mg/L and 0.5 mg/L respectively and both *N. flavescens* strains had an MIC of >1 mg/L. The CTA MICs of the WHO gonococcal control strains were higher than the MIC_{50} of the clinical strains and the MIC of WHO strain K was 0.5 mg/L, higher than any of the clinical strains (Table 1).

Cross-resistance to CTA

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

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% (0/131) respectively. No significant correlation was identified between the tested antibiotics; ciprofloxacin, a fluoroquinolone, had a correlation coefficient of 0.07 (Figure 2, Table 2), the highest correlation was 0.48 with azithromycin.

Discussion

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

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

fluoroquinolone do not seem to influence CTA MICs, indicating that the active site for CTA may be elsewhere in the quinolone resistance determining region (QRDR).

Interestingly, the two *N. perflava* strains had the highest CTA MIC (>1 mg/L) and the basis of this relative resistance requires further research to understanding the specific resistance mechanisms.

Closthioamide has been shown to have low toxicity in tissue culture(19), making it a good candidate for clinical use, however further work should be carried out in terms of its toxicity, pharmacokinetics and pharmacodynamics(22). Successful treatment of pharyngeal infection is critical to the gonorrhoea control efforts(23–25) meaning that penetration of any new antimicrobial into the pharyngeal mucosa is of particular importance. Clinical trials investigating the efficacy of existing antimicrobials such as gentamicin are currently being carried out(26), however these agents have poor pharyngeal penetration and even if successful will not offer a long term solution, as development of resistance to aminoglycosides occurs readily(27).

In conclusion, CTA has high anti-gonococcal activity *in vitro*, even for multidrug resistant isolates, but further studies to evaluate the clinical potential of this antimicrobial are urgently required in light of the public health threat that gonorrhoea poses.

Materials & Methods

Bacterial Isolates

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

isolates were cultured on Columbia agar supplemented with chocolated horse blood (Oxoid, Basingstoke, UK) at 37^{o}C, in 5% CO_2 for 24 hours.

Antimicrobial Susceptibility Testing

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

Synthesis of CTA

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

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

Statistical analysis

Data were analysed in Microsoft Excel. MIC_{50} and MIC_{90} were calculated with MIC data from clinical gonococcal strains only. The correlation between CTA MICs and those for other antibiotics was determined with a Spearman's Rank correlation test, using STATA 14.2. The correlation coefficient was calculated using MIC data from clinical and reference gonococcal strains.

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Transparency declaration

None to declare

Ethical considerations

None

Supplementary data

Full MIC data for all antibiotics tested as well as graphs showing MIC distributions are provided as supplementary data with this manuscript.

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TABLES AND FIGURES LEGENDS

Figure 1. Susceptibility of gonococcal isolates to closthioamide (CTA).
CTA was tested on 149 clinical gonococcal strains (range tested was 0.002 – 1 mg/L). MIC = Minimum Inhibitory Concentration.

Table 1. MICs of WHO gonococcal reference strains. Minimum inhibition concentration (MICs) (mg/L) determined by World Health Organisation (WHO) for penicillin (PEN), cefixime (CFX), ceftriaxone (CRO), azithromycin (AZI), ciprofloxacin (CIP), tetracycline (TET), spectinomycin (SPE). Closthioamide (CTA) MIC was determined by agar dilution in this study.

Figure 2. Correlation between CTA and ciprofloxacin MICs. MIC data for CTA and ciprofloxacin from 139 clinical strains was used to calculate a correlation coefficient (R^2) of 0.07.

Table 2. Raw data showing number of clinical isolates with given combination of CTA and Ciprofloxacin MICs.