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Methenamine as an Alternative Treatment of *Neisseria gonorrhoeae* Urethritis? An In Vitro and In Vivo Study in *Galleria mellonella*

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Abstract

Background: There is an urgent need for novel treatment options for *Neisseria gonorrhoeae*. Methenamine is an interesting urinary antiseptic with a very low propensity to induce antimicrobial resistance. **Methods:** We assessed the MICs of methenamine-hippurate for 18 *N. gonorrhoeae* isolates. We then assessed the in vivo efficacy of methenamine-hippurate against *N. gonorrhoeae* using the *Galleria mellonella* infection model. **Results**: We found that all the gonococcal isolates had a methenamine-hippurate MIC of 300 mg/L. This MIC was not higher in isolates with higher ceftriaxone MICs. No toxicity of methenamine at the doses tested was found, and doses as low as 200 mg/kg were effective in the *G. mellonella* model. **Conclusions:** Further studies in mice and humans are required to assess if methenamine-hippurate could be used to treat gonococcal urethritis alone or in combination with other agents such as ceftriaxone.

Keywords: methenamine; *N. gonorrhoeae*; AMR; treatment; gonorrhoea

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1. Introduction

Neisseria gonorrhoeae is a sexually transmitted infection whose main clinical manifestations are urethritis, cervicitis and proctitis [1]. Current therapy guidelines for gonorrhoea typically recommend ceftriaxone with or without azithromycin [2,3]. *N. gonorrhoeae* has developed antimicrobial resistance to all the classes of antimicrobials used to treat it [4]. This means that novel therapeutics are urgently required [5]. It would be particularly useful if these compounds would target novel targets that could be taken orally, are safe and cheap, and have a low probability of generating antimicrobial resistance [4,6]. One such compound is methenamine-hippurate which has been used as an oral urinary antiseptic to prevent urinary tract infections for over 100 years [7].

Methenamine is a urinary antiseptic first discovered in 1859 and first used as a urinary antiseptic in 1895 [7]. Methenamine is rapidly absorbed after oral administration [7]. More than 80% of methenamine is recovered in the urine of normal volunteers, which indicates excellent oral bioavailability [8]. Methenamine-hippurate undergoes hydrolysis in the acidic urine where it is converted into formaldehyde. Formaldehyde has a number of antimicrobial effects (Figure 1) [7,8]: (1) It reacts with the amino groups in proteins and

thereby forms crosslinks that denature the proteins. (2) It forms covalent bonds between nucleotides or between nucleotides and proteins, thus inhibiting DNA replication, transcription, and repair. (3) It can compromise the integrity of cell membranes by crosslinking membrane proteins and lipids, increasing permeability and causing leakage of cellular contents. A dose of 1 g of methenamine-hippurate every 12 h results in a urine concentration of >500 µg/mL of methenamine, which exceeds the MICs of almost all urinary pathogens [8,9]. The elimination half-life is approximately 4.3 h, the volume of distribution is 0.56 L/kg, the renal clearance is about 1.46, and the total clearance is 1.58 mL/min/kg [9]. Between 70% and 90% of a 1 g dose of oral methenamine is excreted in the urine within 24 h [10]. The urinary antibacterial effects begin within 30 min of ingestion [10].

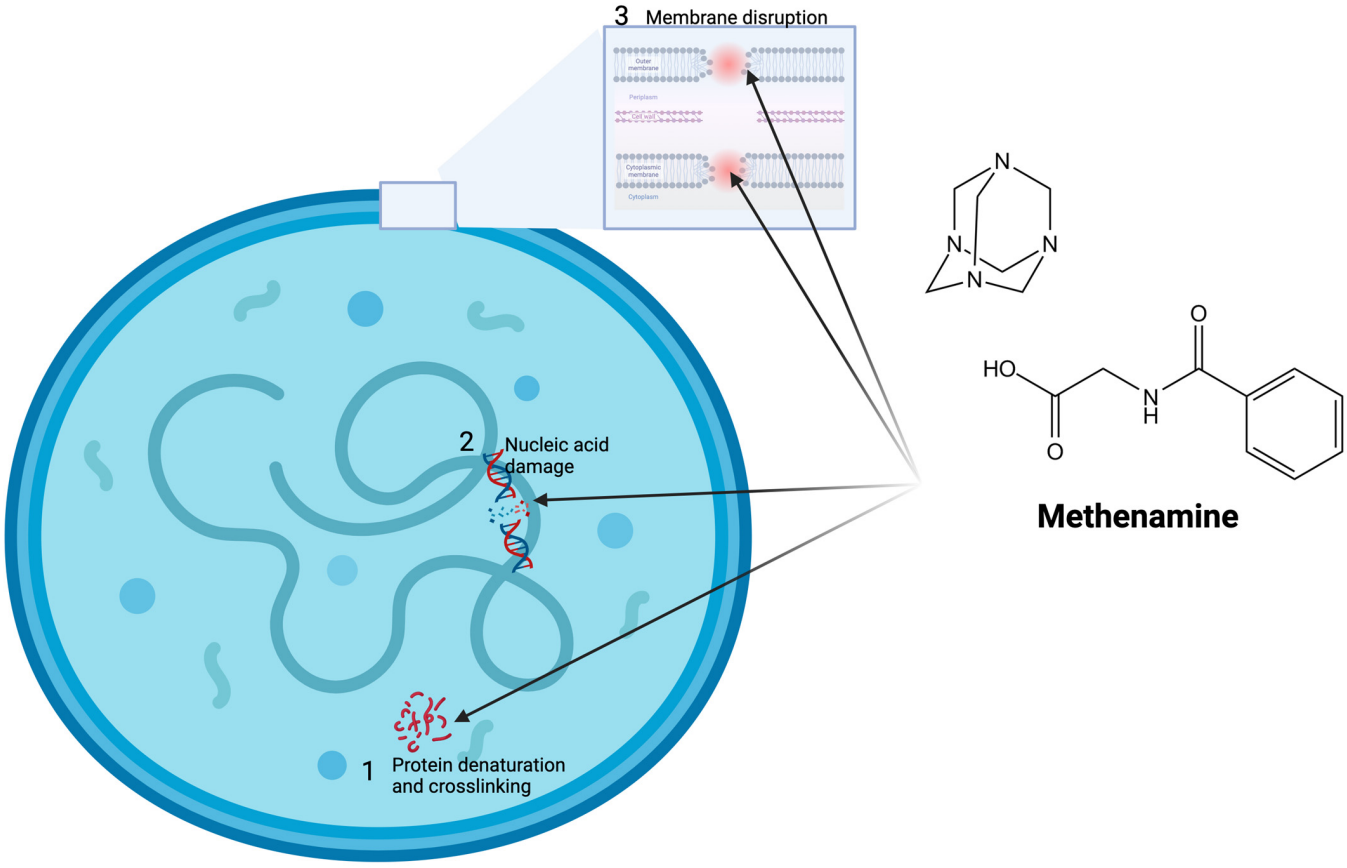


Figure 1. Mechanism of action of methenamine-hippurate. Methenamine is broken down in the acid urine into formaldehyde, which has numerous antimicrobial actions including (1) protein denaturation and crosslinking, (2) nucleic acid damage, and (3) membrane disruption via crosslinking membrane proteins and lipids. Figure produced with BioRender.com (version 1.1).

Further advantages of methenamine-hippurate are its low cost and few contraindications or side effects. The most common side effects are nausea, vomiting, dyspepsia and diarrhea, which are generally mild [10,11]. Very rarely, hypersensitivity reactions have been reported [11]. We could not find any reports of treatment emergent resistance [7–9]. Two recent randomized controlled trials (RCTs) found methenamine to be as efficacious as long-term antibiotics at preventing recurrent urinary tract infections (rUTIs) but without the elevated risk of AMR found with antibiotic prophylaxis [12]. An obvious disadvantage of methenamine-hippurate is that because of poor penetration to other body sites, it could only be used to treat gonococcal infection of the urethra [4]. Adverse reactions occur in less than 4% of patients [11].

We have recently successfully introduced a methenamine-based regimen to treat recalcitrant, multidrug-resistant *Mycoplasma genitalium* urethritis [13]. A search in PubMed and Google Scholar could not find any evidence that methenamine has been evaluated for the treatment of *N. gonorrhoeae*. This provided the motivation for the current study, where we evaluated the in vitro and in vivo activity of methenamine against *N. gonorrhoeae*.

2. Materials and Methods

2.1. Neisseria gonorrhoeae Isolates

To assess the effects of methenamine-hippurate on *Neisseria gonorrhoeae*, a panel of 3 WHO reference strains (WHO X, Y, and Z), ATCC 49226, and 15 clinical isolates were chosen. All clinical isolates were from symptomatic clinical infections obtained from individuals attending the STI clinic at the Institute of Tropical Medicine, Antwerp, between 2023 and 2025. These isolates were chosen to incorporate both low and high ceftriaxone susceptible isolates. Eleven of these isolates had high ceftriaxone MICs and four isolates had low ceftriaxone MICs. Further information pertaining to the bacterial strains used in this study is provided in Table 1.

Table 1. Methenamine-hippurate, ceftriaxone, ciprofloxacin and azithromycin Minimum Inhibitory Concentrations (MICs—µg/mL) of the *Neisseria gonorrhoeae* panel used in this study [10].

Strain ID	Ceftriaxone	Ciprofloxacin	Azithromycin	Methenamine
15.441	0.25	16	0.25	300
17.360	0.25	16	0.5	300
18.227	0.19	32	1	300
18.243	0.19	16	0.25	300
18.293	0.19	8	0.25	300
19.598	0.5	>32	0.38	300
20.090	0.19	32	0.25	300
20.442	0.19	32	0.75	300
21.140	0.25	32	0.75	300
21.272	0.19	32	1	300
23.509	0.19	6	0.25	300
24.758	<0.002	<0.002	0.032	300
24.772	0.004	3	1.5	300
24.773	0.003	0.002	0.003	300
24.774	<0.002	0.002	0.5	300
ATCC 49226	0.008	0.002	0.25	300
WHO X	2	>32	0.5	300
WHO Y	1	>32	1	300
WHO Z	0.5	>32	1	300

2.2. Antimicrobial Susceptibility Testing

A stock solution of 5 mg/mL methenamine hippurate (Mylan, NV, Canonsburg, PA, USA) was prepared by crushing two 1 g tablets and dissolving them in 5 mL distilled water (pH adjusted to 5.05), followed by gentle heating and preparations of the required dilutions.

The methenamine MICs were ascertained via agar dilution testing. This was performed using gonococcal (GC) agar base medium (BD Difco™, Becton Dickinson, Temse, Belgium) supplemented with 1% IsoVitaleX (BD, Temse, Belgium). This was performed according to the Clinical and Laboratory Standards Institute (CLSI) agar dilution method [14,15]. First, the *N. gonorrhoeae* strains were grown on Columbia blood agar with 5% sheep blood and incubated at 36 °C in 5% CO_2 for 16 h. Second, a 0.5 McFarland suspension of each strain was prepared in a tube with 2 mL of Mueller Hinton Broth (BD Difco™, BD, Temse,

Belgium). Third, approximately 10^4 CFU of a dilution of the suspension per spot was inoculated within 15 min of preparation onto the agar surface with a multipoint inoculator. Fourth, the plates were incubated at 36 °C in 5% CO_2 for 20 to 24 h. The MICs were then interpreted by the standard method of reading growth inhibition.

2.3. Preparation of Live Microbial Inocula for G. mellonella Infection

N. gonorrhoeae 19.598 was used for these experiments. This isolate was chosen on the basis of it having the highest ceftriaxone MIC of all the clinical isolates in our study. It was cultured from frozen stocks onto blood agar (BA) plate for $\leq$16 h at 36 °C with 5% CO_2. Single colonies were then taken from these cultures and plated onto fresh agar plates. These were incubated at 36 °C with 5% CO_2 for 6 h. The *N. gonorrhoeae* were then injected into the larvae-hemocoel (30 µL of GC broth containing 10^8 CFU/larva). This dose of *N. gonorrhoeae* was similar to that used in previous analogous experiments [16,17].

2.4. Injection of G. mellonella Larvae

We used the last larval stage of *Galleria mellonella* (Terramania, Arnhem, The Netherlands) to assess the in vivo efficacy of methenamine. Healthy, non-discolored larvae weighing 250 to 450 mg were selected and placed into individual sterile Petri dishes in groups of 10 per Petri dish. The larvae were then kept in an incubator at 36 °C with a 5% CO_2 atmosphere for the length of the experiments. Each experimental group consisted of at least 30 larvae.

The larvae were injected in the last right pro-leg with 30 µL of bacterial suspension, followed 5–20 min later by 10 µL of various doses of methenamine-hippurate (2 g/g and 4 g/g; Sanbio, Uden, The Netherlands), using 0.3 mL U-100 insulin syringes (BD Micro-Fine, Temse, Belgium). For each group of 10 larvae, one syringe and needle was used.

The larvae were then assessed at prespecified time points (16, 24, 32, 48, 64, 72 and 88 h) post inoculation for mortality (defined as a lack of movement in response to tactile stimulus [18]) and melanization [18].

2.5. Concentration of Methenamine Injected

We tested two doses of methenamine-hippurate: 2 g/g and 4 g/g. These doses were based on the MIC result for *N. gonorrhoeae*, 19.598, and a median larvae liquid volume of 192 µL (based on a median weight of 300 mg) [19]. Based on the formula,

$Conc_{in\ vivo} = (Conc_{compound} \times 10\ \mu L)/Vol_{larvae}$ [20,21], this dose should translate into a hemolymph concentration of 104 µg/mL and 208 µg/mL for the 2 g/g and 4 g/g doses, respectively.

One positive control group was included that received the same protocol which consisted of bacterial inoculation (30 µL/larva) followed by 10 µL/larva of ceftriaxone (20 mg/kg; Sigma–Aldrich, St. Louis, MO, USA). One negative control group was included that received the same protocol: bacterial inoculation (30 µL/larva) followed by 10 µL/larva of phosphate-buffered saline (PBS). This dose of ceftriaxone was the same as that used in a previous study that found that this dose of ceftriaxone was effective against *N. gonorrhoeae* WHO P in *G. mellonella* [7]. One PBS control group was included. This control group received two injections of PBS: first 30 µL/larva followed by 10 µL/larva.

The surviving and dead *G. mellonella*, were kept at −80 °C overnight to sedate and kill them. They were then autoclaved at 121 °C for 15 min and disposed of according to local regulations.

2.6. Data Analysis

Statistical analyses and data visualization were conducted using GraphPad PrismV.10® with ANOVA or Mann–Whitney tests to compare groups. A Mantel–Cox test was performed to compare the degree of melanization and mortality rates between groups.

3. Results

3.1. Minimum Inhibitory Concentrations

3.1.1. In Vitro

The MIC of all the isolates was 300 μg/mL (Table 1).

3.1.2. Toxicity

At the doses tested (4, 8 and 12 g/g), methenamine-hippurate did not result in an increase in mortality or melanization of the larvae compared to PBS in the 4 days this was assessed (Figure 2).

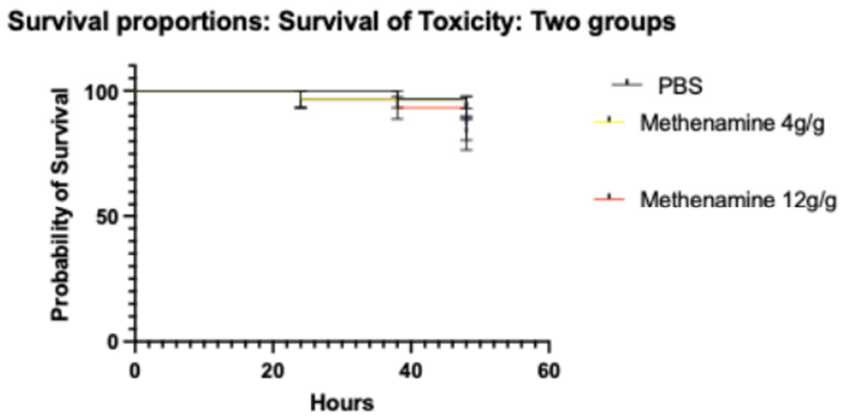


Figure 2. Survival curves of *Galleria mellonella* treated with varying doses of methenamine-hippurate (4 to 12 g/g) or PBS (Phosphate Buffered Saline; symbols in each panel represent the mean survival from 3 independent experiments and the error bars represent the standard deviation of the mean).

3.1.3. Mortality

The ceftriaxone group as well as the 2 g/g and 4 g/g methenamine-hippurate groups had a statistically significant increase in survival compared to the no-treatment group (all $p \leq 0.01$; Figure 3).

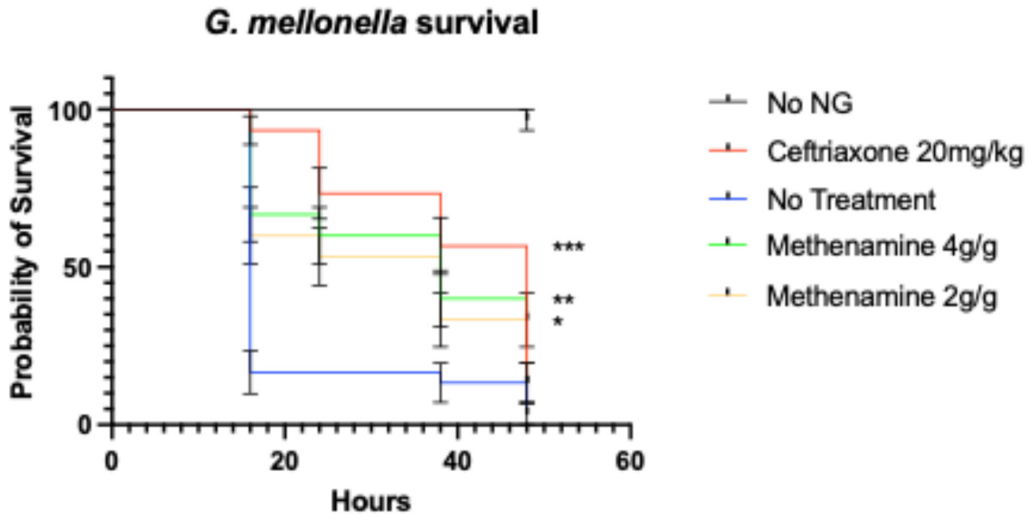


Figure 3. Survival curves of *Galleria mellonella* infected with *N. gonorrhoeae* (NG) and treated with varying doses of methenamine-hippurate, ceftriaxone (20 mg/kg) or PBS (*** $p < 0.0001$, ** $p < 0.001$, * $p < 0.01$; symbols in each panel represent the mean survival from 3 independent experiments and the error bars represent the standard deviation of the mean).

4. Discussion

We found that all the gonococcal isolates had a methenamine-hippurate MIC of 300 mg/L. This MIC was not higher in isolates with higher ceftriaxone MICs. No toxicity of methenamine at the doses tested was found, and doses as low as 2 g/g were effective in the *G. mellonella* model. While these findings are promising, the efficacy observed in an invertebrate model should be interpreted cautiously. Further validation in mammalian infection models is necessary to assess its clinical relevance.

A single oral dose of 1 g methenamine-hippurate results in a mean urinary concentration of methenamine-hippurate of 792 mg/L within 2 h, increases to 1000 mg/L at 4 h, and then steadily declines to 230 mg/L. Dosing 1 g every 12 h results in a steady state above 500 mg/L [9]. These concentrations are higher than the methenamine MIC and the doses of methenamine assessed in our in vivo model.

These findings provide preliminary evidence that methenamine-hippurate could be used for the treatment of *N. gonorrhoeae* urethritis. We also found that methenamine is safe. More importantly, the safety of methenamine in humans has been well established in humans in other studies [11]. There are however a number of limitations to our study and this conclusion. As noted above, methenamine could only be used to treat urethritis, which is only one of multiple presentations of *N. gonorrhoeae*. We only evaluated the effect of methenamine on a relatively limited panel of *N. gonorrhoeae* isolates and in a single strain in the in vivo model. We did not assess the probability of resistance emerging to methenamine. Interactions with other antimicrobials were also not assessed [22]. We only investigated the in vivo efficacy of methenamine in a *G. mellonella* model of infection. There are a number of differences between gonococcal infections in this model and humans. These include the fact that the larvae do not have urethras, they have different immune systems, and the pharmacokinetics and pharmacodynamics likely vary [19,20]. The pH of urine varies according to a number of factors such as diet and co-medications [7,11]. Since methenamine requires an acidic pH for conversion to formaldehyde, this can affect its efficacy. Studies of methenamine for other conditions have not found that this has made much of an impact on its clinical efficacy but this would need to be investigated in future studies using methenamine for gonococcal urethritis [8,10].

There is an important need to search for companion antimicrobials to prevent the emergence of gonococcal resistance to ceftriaxone [5]. Methenamine may be useful in this regard. However, in vitro and in vivo interaction assessments should first be performed [23].

5. Conclusions

Methenamine is an interesting urinary antiseptic with a very low propensity to induce AMR. As such, it could be useful to treat gonococcal urethritis alone or in combination with other agents such as ceftriaxone [5]. It is crucial to acknowledge that our results represent early preclinical results. As such, further assessments in mammalian models including humans would be required before methenamine could be considered for clinical use.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript

MIC	Minimum Inhibitory Concentration
RCT	Randomized Controlled Trial

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