In vitro and in vivo activity of combination antimicrobial agents on Haemophilus ducreyi

Josée E. Roy-Leon1, Wallace D. Lauzon1, Baldwin Toye2,3, Neera Singhal4 and D. William Cameron1,4*

1Faculty of Medicine, Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, Canada; 2Department of Pathology and Laboratory Medicine, University of Ottawa, Ottawa, Canada; 3Department of Pathology and Laboratory Medicine, The Ottawa Hospital, Ottawa, Canada; 4Clinical Epidemiology Program, Ottawa Health Research Institute at The Ottawa Hospital, Ottawa, Canada

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Objectives: Development of single dose antibiotic treatments for chancroid has been followed by drug-resistant Haemophilus ducreyi in endemic areas. We examined the activity and interactions of antimicrobial agents and combinations against H. ducreyi.

Methods: We evaluated the in vitro susceptibility of three virulent strains of H. ducreyi to ceftriaxone, azithromycin, rifabutin and streptomycin, and each two-drug combination by the agar dilution method. We then tested each two-antibiotic combination for activity by the checkerboard method. Lastly, we chose the antibiotic combination with the lowest fractional inhibitory concentration index (FICI) and tested combined sub-therapeutic doses of ceftriaxone (0.05 mg/kg) and streptomycin (10 mg/kg) in five treated animals versus three untreated controls, compared with controls, for in vivo interaction in the temperature-dependent rabbit model of H. ducreyi infection.

Results: Each H. ducreyi strain was susceptible in vitro to each antibiotic and two-antibiotic combination, and combined ceftriaxone and streptomycin had the lowest FICI at 0.63. In five treated animals versus three untreated controls, combined sub-therapeutic doses of ceftriaxone (0.05 mg/kg) and streptomycin (10 mg/kg) reduced mean (SD) duration of culture positivity from 7.3 (1.1) to 2.6 (1.7) days (P < 0.001), time to 50% reduction in lesion size from 9.7 (1.5) to 5.8 (0.8) days (P < 0.005), and time to resolution of ulcer from 11.7 (2.3) to 6.6 (1.7) days (P < 0.05).

Conclusions: Ceftriaxone and streptomycin have in vivo synergic interaction against H. ducreyi lesions in the temperature-dependent rabbit model of infection. Antibiotic combinations may be evaluated clinically as single-dose therapy for chancroid.

Keywords: antimicrobial interactions, chancroid, H. ducreyi, synergy

Introduction

The Gram-negative bacterium Haemophilus ducreyi is the causative agent of the sexually transmitted disease (STD) chancroid. Chancroid is a common form of genital ulcer disease in the developing world, and also occurs in marginalized groups with limited access to healthcare services. Chancroid has received considerable interest in recent years because it has epidemiological synergy with HIV infection, and each may facilitate transmission of the other. Chancroid can be treated with relative ease, and even established outbreaks can be controlled. In addition to HIV infection, chancroid outbreaks are associated with prostitution and crack-cocaine use. Studies of improved STD treatment in endemic regions demonstrate a 40% reduction in HIV transmission in Mwanza, Tanzania, and no effect on HIV incidence in Rakai, Uganda, where there was a low incidence of treatable STD and a high prevalence of HIV.

In a 1988 survey of H. ducreyi strains isolated in South Africa, most were found to be resistant to penicillin, tetracycline, sulfamethoxazole, and trimethoprim. H. ducreyi, once susceptible to sulfonamides, penicillins, tetracyclines, and trimethoprim, has developed plasmid or chromosomally encoded resistance to...
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Each 16-18 discrete susceptibility profiles in different regions are also reported, often reflecting differential drug use.19 Several single-dose regimens have been shown to be effective for the treatment of chancroid, including ceftriaxone, ciprofloxacin, azithromycin, and sulframethoxazole/trimethoprim.20 Despite documented *H. ducreyi* resistance to it,21 streptomycin is still an alternative treatment.22 With the growing prevalence of HIV co-infection, however, single-dose therapies of ceftriaxone, azithromycin, fleroxacin, ciprofloxacin, erythromycin and trimethoprim/sulframethoxazole are associated with greater than 20% chancroid treatment failure rates.23-26 Also, there is regional variation of chancroid response to azithromycin,27 and chancroid treatment failure attributable to herpes simplex virus (HSV) co-infection28 or drug resistance. As *H. ducreyi* is adept at acquiring new gene products through horizontal mechanisms, both in its genome, e.g. cytolethal distending toxin,29 and on stably transmitted plasmids,18,30 it may acquire resistance genes through horizontal transmission as well as through de novo mutation. Although clinical evidence of this complex problem is limited to simple observational study methods, theoretical and mathematical models show that effective combination therapy can limit the emergence of antibiotic resistance at the population level.31 Efficacious single-dose treatment regimens may improve compliance-related effectiveness of treatment programmes and reduce the emergence of antibiotic resistance,20 and are desirable as chancroid therapy. Combination antibiotic therapy has improved therapeutic efficacy in other bacterial infectious conditions and in the immunosuppressed patient.32

We aimed to demonstrate the principle, and propose that antibiotic combinations may be developed as single-dose, effective and sustained treatment of chancroid, suitable for treatment-based chancroid control programmes. We screened antibiotics of unrelated class and each of their two-antibiotic combinations for *in vitro* antimicrobial activity against susceptible *H. ducreyi* strains, and then evaluated the most promising antibiotic combination for *in vivo* treatment efficacy in the temperature-dependent rabbit model of chancroid.33

Materials and methods

**Bacterial strains and culture conditions**

Type strain 35000 of *H. ducreyi* was originally isolated during an outbreak in Winnipeg, Manitoba, in 1975,34 while strains RO 40 and RO 34 are clinical isolates from Nairobi, Kenya, 1987. Strains 35000 and RO 34 are virulent in the temperature-dependent rabbit model of chancroid,33,35 and possess distinct outer membrane profiles.35 The strain RO 40 has a distinct outer membrane profile and is also virulent in the temperature-dependent rabbit model of chancroid (D. W. Cameron and J. E. Roy-Leon, unpublished data). Reference strains *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were obtained from the American Type Culture Collection, Rockville, MD, USA.

*H. ducreyi* strains were grown on chocolate agar (CA) plates consisting of 3.6% gonococcal (GC) agar base (B-D Microbiology Systems, Cockeysville, MD, USA) +1% bovine haemoglobin (B-D Microbiology Systems) supplemented with 1% IsoVitalX (B-D Microbiology Systems) and 5% fetal bovine serum (FBS; Gibco BRL, Burlington, ON, Canada); and in broth consisting of a 1:1 (v/v) mixture of Mueller–Hinton broth (B-D Microbiology Systems) and α-minimal essential medium (Gibco BRL) supplemented with 17% FBS. Plates were incubated at 33°C for 48 h with 5% CO₂ and high humidity, while broth cultures were incubated for 12 h at 33°C in an environmental shaker at 175 rpm. Reference strains of *E. coli* and *S. aureus* were grown in CA or Mueller–Hinton broth supplemented with 1% IsoVitalX. Plates were incubated for 24 h at 37°C in 5% CO₂ and broth cultures were incubated for 2 h at 37°C in an environmental shaker (Lab-line, Melrose Park, IL, USA).

**Animals**

Animals were treated in accordance with the Animal Care and Veterinary Services procedures. The Animal Care Committee at the University of Ottawa approved all experiments. Male New Zealand White Rabbits (2.5–3 kg) (Charles River Laboratories, Montreal, PQ, Canada) were housed in an 11.7 m² room at the Animal Care Facility at the University of Ottawa Health Science Centre. The temperature in the room was maintained at 14°C with a Thermo Air plus air conditioning unit. The backs of the rabbits were shaved prior to infection and when necessary. All rabbits were kept under identical conditions for the duration of the experiments.

**Antibiotics**

Ceftriaxone (Sigma Chemical Co., St Louis, MO, USA), streptomycin (Sigma), azithromycin (Pfizer, Pointe-Claire-Dorval, PQ, Canada), and rifabutin (Farmitaliz Carlo Erba, Nerviano, Italy) were used in the experiments.

**In vitro antimicrobial susceptibility testing**

MIC and fractional inhibitory concentration index (FICI) determinations were measured by agar dilution on CA plates supplemented with 1% gonococcal supplement B (Difco Laboratories, Detroit, MI, USA). Plates were incubated for 48 h at 33°C in 5% CO₂ in a humid chamber. All strains were tested for their susceptibility to ceftriaxone, streptomycin, azithromycin and rifabutin. All stock solutions were prepared in double distilled water. Azithromycin and rifabutin were initially dissolved in methanol (<2% of the final volume) because of their lack of solubility in water.

**MIC determination.** MIC was determined using the agar dilution method of the NCCLS with modifications.36,37 Ceftriaxone, streptomycin, azithromycin and rifabutin were diluted in water and incorporated into CA at 50°C to yield the appropriate 2-fold dilution series. Final concentration ranges tested were 0.001–8 mg/L for ceftriaxone, 0.063–512 mg/L for streptomycin, 0.00005–4 mg/L for azithromycin, and 0.002–2 mg/L for rifabutin. The agar was poured into 100 × 15 mm Petri dishes (Fisher Scientific, Nepean, ON, Canada), to a depth of 3–4 mm. Plates were used the same day or were stored at 4°C for use the following day. *H. ducreyi* strains were grown on CA plates for 48 h, and inoculated on new plates that were incubated for 24 h. The growth was suspended in Mueller–Hinton broth with 1% IsoVitalX and allowed to sediment for at least 15 min at room temperature. The optical density of each supernatant was adjusted to that of a 0.5 McFarland barium sulphate standard. A few colonies of the reference strains were selected from plates grown for 24 h at 37°C and were inoculated into 4 mL of Mueller–Hinton broth supplemented with 1% IsoVitalX. This suspension was incubated at 37°C for 2 h. All suspensions were diluted 1:10 and 2 µL (~10⁴ cfu) was inoculated onto the plates with a pipette. MIC was defined as the lowest concentration of an antibiotic that completely inhibits growth (disregarding a single colony) after 48 h at 33°C in 5% CO₂ and high humidity. Assays were performed in duplicate.

**FICI determination.** FICI for *H. ducreyi* strains 35000, RO 40 and RO 34 exposed to varying concentrations of all two-drug combinations of ceftriaxone, azithromycin, rifabutin and streptomycin were determined
using the chequerboard agar dilution method. Plates and inocula were prepared and incubated for MIC determination as described above. The assays were performed in duplicate in three separate experiments.

In vivo antimicrobial susceptibility testing

Experimental induction of infection. Experimental infections were induced and assayed as previously described. Briefly, broth-grown *H. ducreyi* strains 35000 and RO 40 were harvested at the late mid-log phase, defined by time of incubation, i.e. 4–6 h, by centrifugation at 3000 g for 10 min. Pellets were washed once with PBS (Gibco BRL), pH 7.2, and resuspended in Mueller–Hinton broth. Serial 10-fold dilutions of *H. ducreyi* broth, from 10⁶ to 10⁷ cfu/mL were prepared, and injected intraepithelial in triplicate in 100 μL doses, for a total of 15 injections, into the shaved backs of the animals, to give five final inocula doses of 10⁸ to 10⁵ cfu. The actual inoculum size was determined by colony count after diluting and plating 100 μL on CA plates and incubating at 33°C with 5% CO₂ and high humidity.

Determination of lesion severity. Two of three lesions were measured and scored daily, for a period of 21 days for each inoculum size on each rabbit (0 = nil, 1 = erythema, 2 = induration, 3 = suppuration, 4 = ulceration). The third lesion was cultured for the presence of *H. ducreyi* by lateral injection of 0.1 mL of PBS at pH 7.2, manipulation, and aspiration. The aspirate was cultured on CA plates for 48 h at 33°C and examined for evidence of typical *H. ducreyi* colony morphology by the push test, Gram stain, and microscopic examination. Culture was discontinued after four consecutive days of negative culture results.

Determination of sub-therapeutic antibiotic dose. Preliminary experiments using single-dose, single-drug treatment with 0.05–5 mg/kg of ceftriaxone and 2.5–15 mg/kg of streptomycin established that 0.05 mg/kg of ceftriaxone and 10 mg/kg of streptomycin were the highest doses which had no effect on lesion healing relative to controls without antibiotic treatment in the temperature-dependent rabbit model of chancroid.

Efficacy of antimicrobial treatment. *H. ducreyi* 35000 was used to examine in vivo antibiotic efficacy. Inocula of 10⁷ cfu were injected into the shaved backs of the rabbits in a 15-location grid pattern. Rabbits were treated with antibiotics upon ulceration of the lesions on day 4 post-infection. Single sub-therapeutic doses of 0.05 mg/kg of ceftriaxone (n = 4), 10 mg/kg of streptomycin (n = 4), and a combination of both antibiotics (n = 5) were administered intramuscularly. Control rabbits (n = 3) received no treatment. Odd numbered lesions (8/rabbit) were measured and scored daily for 17 days post-treatment. Even numbered lesions were cultured until negative for four consecutive days.

Table 1. MIC of antimicrobial agents against *Haemophilus ducreyi* and reference bacterial strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>n</th>
<th>MIC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ceftriaxone</td>
</tr>
<tr>
<td><em>H. ducreyi</em> 35000</td>
<td>10</td>
<td>0.002</td>
</tr>
<tr>
<td><em>H. ducreyi</em> RO 40</td>
<td>10</td>
<td>0.002</td>
</tr>
<tr>
<td><em>H. ducreyi</em> RO 34</td>
<td>10</td>
<td>0.002</td>
</tr>
<tr>
<td><em>E. coli</em> ATCC 25922</td>
<td>3</td>
<td>0.063</td>
</tr>
<tr>
<td><em>S. aureus</em> ATCC 29213</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

ND, not done. MIC was determined by the agar dilution technique for each antibiotic against each bacterial strain.

Statistical analysis

Analysis of variance (ANOVA) was used to compare the FICI between strains and the Student–Newman–Keuls method was used where ANOVA showed a significant difference. The Kruskal–Wallis ANOVA on ranks was used in instances where normality failed. Comparative analysis of in vivo efficacy of treatment on ulcerative lesions was done on data from treatment day 0 (on the fourth day from infection) to day 17. Serial lesion sizes were expressed as a percentage of pre-treatment lesion size, so that all lesions were 100% on day 0. For each rabbit, for each of 17 treatment days, the median lesion size percentage and median lesion score were calculated from eight of the 15 inoculation sites. The mean time in days for a 50% decrease in median lesion size, and the mean time in days to reach a median score of 2 were compared between groups. For each rabbit, the median duration of lesion culture positivity was calculated. Mean time in days to culture positivity was compared between groups. For each rabbit, the median inoculum size was calculated, and the means of the groups were compared. Comparisons were carried out by ANOVA, followed by the Tukey post-hoc test where appropriate.

Results

In vitro antimicrobial susceptibility testing

MIC determination. MICs for *H. ducreyi* strains 35000, RO 40 and RO 34 show that they were susceptible to the four antibiotics tested, and the MICs for the reference strains *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 were all within one dilution of the accepted ranges (Table 1).

FICI determination. FICIs for each two-antibiotic combination tested in vitro against each *H. ducreyi* strain are shown in Table 2. All three *H. ducreyi* strains had an FICI of 0.63 for the combination of ceftriaxone and streptomycin. FICI was determined to be 1.5 for ceftriaxone + azithromycin and streptomycin + azithromycin for all three strains tested, indicating that these antibiotics do not interact in either a synergic or antagonistic manner. Drug combinations including rifabutin were much more variable in their effects in all strains than combinations without this drug.

In vivo antimicrobial susceptibility testing

Experimental induction of infection. The lowest inoculum size of *H. ducreyi* strain 35000 to consistently produce ulcerative lesions in this model is 10⁴ cfu. Rabbits were inoculated with 10⁵ cfu of *H. ducreyi* strain 35000 (100 μL of 10⁵ cfu/mL) in a pattern of...
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Table 2. FICI for all two-drug combinations of antimicrobial agents against *Haemophilus ducreyi*

<table>
<thead>
<tr>
<th>Strain</th>
<th>FICI, mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ceftriaxone + streptomycin</td>
</tr>
<tr>
<td><em>H. ducreyi</em> 35000 (n = 3)</td>
<td>0.63 (0)</td>
</tr>
<tr>
<td><em>H. ducreyi</em> RO 40 (n = 3)</td>
<td>0.63 (0)</td>
</tr>
<tr>
<td><em>H. ducreyi</em> RO 34 (n = 3)</td>
<td>0.63 (0)</td>
</tr>
</tbody>
</table>

FICI, fractional inhibitory concentration index. Values shown are means (SD).

*Comparison of strains by ANOVA (P = 0.05). Student–Newman–Keuls method showed no difference between groups.

*Comparison of strains by Kruskal–Wallis ANOVA on ranks (P = 0.14).

*Comparison of strains by ANOVA (P = 0.22).

Table 3. Effect of treatment with individual and combined sub-therapeutica doses of ceftriaxone and streptomycin on experimental chancroid lesions in rabbits

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>n</th>
<th>Inoculum size (cfu × 104)</th>
<th>Days culture positiveb</th>
<th>Days to 50% reduction in lesion size</th>
<th>Days to reduction in lesion scorec from 4 to 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls, untreated</td>
<td>3</td>
<td>3.99 (2.48)</td>
<td>7.3 (1.1)</td>
<td>9.7 (1.5)</td>
<td>11.7 (2.3)</td>
</tr>
<tr>
<td>Ceftriaxone, 0.05 mg/kg</td>
<td>4</td>
<td>3.78 (2.11)</td>
<td>7.0 (2.7)</td>
<td>8.2 (1.7)</td>
<td>9.7 (2.7)</td>
</tr>
<tr>
<td>Streptomycin, 10 mg/kg</td>
<td>4</td>
<td>6.04 (0.74)</td>
<td>7.5 (2.1)</td>
<td>8.5 (0.6)</td>
<td>9.4 (1.7)</td>
</tr>
<tr>
<td>Ceftriaxone + streptomycin</td>
<td>5</td>
<td>5.13 (2.47)</td>
<td>2.6 (1.7)d</td>
<td>5.8 (0.8)e</td>
<td>6.6 (1.7)f</td>
</tr>
</tbody>
</table>

Values shown are means (SD). After intraepithelial injection of *H. ducreyi* 35000 in the temperature-dependent rabbit model, lesions were allowed to ulcerate for 4 days, and then rabbits received intramuscular injections of antimicrobials or no treatment.

aThe highest dose which had no effect alone on lesion healing compared with controls.

bThe last day of culture positivity, after which at least two sequential cultures were negative.

cLesion score: 0 = nil, 1 = erythema, 2 = induration, 3 = suppuration, 4 = ulceration.

dANNOVA, P < 0.05, and Tukey all pairwise comparison between ceftriaxone + streptomycin treatment and each of the other groups, P < 0.05.

eANNOVA, P < 0.05, and Tukey all pairwise comparison between ceftriaxone + streptomycin treatment and controls and ceftriaxone + streptomycin and streptomycin treatment, P < 0.05.

Discussion

Our findings indicate an in vivo synergic interaction between ceftriaxone and streptomycin. As there is no generally accepted definition of in vivo synergy, we used the definition of Cleeland and Squires. In vitro, each virulent strain of *H. ducreyi* was...
Rabbits were inoculated with $10^4$ cfu intraepithelial $H. ducreyi$ and treated with sub-therapeutic doses of antibiotics compared with untreated controls. Rabbits were inoculated with $10^4$ cfu intraepithelial $H. ducreyi$ strain 35000 and lesions allowed to ulcerate for 4 days before being treated (day 0). (a) Residual lesion size as a percentage of pre-treatment size. (b) Lesion score (0 = nil, 1 = erythema, 2 = induration, 3 = suppuration, 4 = ulceration), and the last culture positive day that was followed by at least two culture negative days. Controls, white bars and open circles; streptomycin 10 mg/kg, diagonally hatched bars and filled squares; ceftriaxone 0.05 mg/kg, vertically hatched bars and filled circles; combined streptomycin and ceftriaxone, horizontally hatched bars and open diamonds. Error bars indicate standard errors of the mean.

Figure 1. Course of experimental Haemophilus ducreyi lesions in rabbits treated with sub-therapeutic doses of antibiotics compared with untreated controls. Rabbits were inoculated with $10^4$ cfu intraepithelial $H. ducreyi$ strain 35000 and lesions allowed to ulcerate for 4 days before being treated (day 0). (a) Residual lesion size as a percentage of pre-treatment size. (b) Lesion score (0 = nil, 1 = erythema, 2 = induration, 3 = suppuration, 4 = ulceration), and the last culture positive day that was followed by at least two culture negative days. Controls, white bars and open circles; streptomycin 10 mg/kg, diagonally hatched bars and filled squares; ceftriaxone 0.05 mg/kg, vertically hatched bars and filled circles; combined streptomycin and ceftriaxone, horizontally hatched bars and open diamonds. Error bars indicate standard errors of the mean.

with statistically significant reduction in time of culture positivity, size, and resolution of lesions.

This is the first time positive antibiotic interactions against $H. ducreyi$ have been reported. This in vivo synergy is not unexpected, as there is evidence of broad synergic interactions with combination β-lactam and aminoglycoside antibiotics, both in vitro and in vivo. The theoretical basis for this interaction is the increased uptake of the aminoglycoside in the presence of the cell-wall-active β-lactam. Streptomycin and ceftriaxone have been used separately in the treatment of chancroid and are affordable. Ceftriaxone is well tolerated and safe, with occasional allergic reactions. Streptomycin is associated with ototoxicity and nephrotoxicity when used at high doses for extended periods of time.

Although commonly used to determine in vitro antibiotic interactions, the chequerboard method has several limitations, such as reliance on a single time point, assumption of a linear dose–response for all antimicrobials, and an all-or-nothing read out, and has been described as inherently unstable as a predictor of synergy because of its reliance on 2-fold dilutions. Time–kill methods can address some of the shortcomings of the chequerboard method of susceptibility testing. It is recognized in the field of antibiotic testing that special measures are needed in dealing with fastidious organisms, but no standardized culture procedure exists for this fastidious organism, and there was no precedent for the evaluation of antimicrobial activity on $H. ducreyi$ in liquid media. We opted for the chequerboard agar dilution technique, which has been used with $H. ducreyi$ in MIC determination in the past.

We chose streptomycin, an aminoglycoside, ceftriaxone, a cephalosporin, and azithromycin, an azalide, as they have all been used to treat chancroid. We chose rifabutin, a semi-synthetic ansamycin, as it may be appropriate for single-dose therapy because of high in vitro susceptibility of $H. ducreyi$, high tissue binding, and long elimination half-life. By evaluating sub-therapeutic doses of antibiotics, we were able to properly evaluate synergic, indifferent, or additive effects, and not antagonistic effects, of the selected antibiotic combinations. We did not test potentially additive or synergic rifabutin antibiotic combinations in vivo, as they lacked consistent in vitro activity. As with other organisms and antibiotics, however, in vitro activity may not always reflect in vivo efficacy, especially when the chequerboard agar dilution method is used to determine FICI, and other two-drug combinations including fluoroquinolones may well deserve consideration.

It may be reasonable to predict similar interactions of other aminoglycosides and cephalosporins against $H. ducreyi$, however, several authors have cautioned that interaction between antibiotics is dependent upon the antibiotics used, the organism being tested, and, to some extent, the medium or the host. This study demonstrates the in vivo therapeutic synergy of streptomycin and ceftriaxone against $H. ducreyi$ in an animal model of infection and disease. Antibiotic combinations need clinical evaluation as chancroid treatment, especially where consistently efficacious treatment is lacking or improved single-dose observed therapy is desirable.

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