Efficacy of surotomycin in an in vitro gut model of Clostridium difficile infection

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Objectives: We investigated the efficacy of the cyclic lipopeptide surotomycin in treating clindamycin-induced Clostridium difficile infection (CDI) using an in vitro gut model.

Methods: Two three-stage chemostat gut models were inoculated with human faeces, spiked with C. difficile spores (~10^7 cfu/mL, PCR ribotype 027 or 001). Clindamycin (33.9 mg/L, four times daily for 7 days) was dosed to induce CDI. Following high-level toxin production, surotomycin (250 mg/L, twice daily for 7 days) was instilled. Microflora populations, C. difficile vegetative cells and spores, cytotoxin titres and antimicrobial levels (LC–MS/MS and bioassay) were determined. The emergence of C. difficile and enterococci with reduced susceptibility to surotomycin was monitored on breakpoint agar (4×MIC).

Results: Counts of viable C. difficile were reduced to near the limit of detection on Days 1 and 3 of surotomycin instillation, and cytotoxin was undetectable on Days 3 and 4 of surotomycin instillation in the 027 and 001 models, respectively. Recurrence of vegetative growth and toxin production occurred 11 days (001 model) and 15 days (027 model) after surotomycin instillation had ceased, and remained for the duration of the experiment. Surotomycin instillation decreased populations of bifidobacteria, clostridia, enterococci and lactobacilli, but was sparing of Bacteroides fragilis group populations. All enumerated organisms had recovered to steady-state levels by 3 weeks post-surotomycin instillation. No evidence of the emergence of reduced susceptibility to surotomycin was observed.

Conclusions: Surotomycin successfully reduced C. difficile vegetative cell counts and toxin levels in the gut model and was sparing of B. fragilis group populations. There was no evidence of decreased susceptibility to surotomycin during exposure or post-exposure.

Keywords: chemostat, ribotype 027, recurrence

Introduction

Clostridium difficile infection (CDI) is a leading cause of antibiotic-associated diarrhoea among the hospitalized elderly and is a major burden on healthcare facilities worldwide, with associated incremental costs ranging from £4577 to £8843 across Europe,1 and from US$4846 to US$8570 in the USA.2 Treatment strategies for CDI have changed little over the past two decades, with conventional antimicrobial treatment limited to either metronidazole or vancomycin. Reports of reduced metronidazole efficacy, notably in severe CDI cases,3 and reduced metronidazole susceptibility among epidemic C. difficile ribotypes3 has led to the increased evaluation of new antimicrobial agents. However, fidaxomicin remains the only novel treatment agent to be approved in the past two decades. One of the major problems facing clinicians is the management of disease recurrence, which is observed in approximately 20%–30% of patients.3 Fidaxomicin is associated with significantly reduced rates of recurrence versus vancomycin (regardless of strain type);6 however, additional therapies are still required to address recurrent CDI.

Surotomycin is an orally available cyclic lipopeptide antibiotic with selective antimicrobial activity against C. difficile and other Gram-positive bacteria. Surotomycin successfully completed a Phase 2 clinical trial and was demonstrated to be safe and well tolerated in patients with CDI.7 In addition, better sustained cure rates were achieved with surotomycin than with vancomycin. Importantly, a reduction and delay in the recurrence of CDI...
episodes were recorded with surotomycin compared with vancomycin. The median time to recurrence for surotomycin (250 mg twice daily) versus vancomycin (125 mg four times daily) was 17.5 versus 6 days.7 Surotomycin is now undergoing Phase 3 clinical trials.

The gut model used here has been validated against the intestinal contents of sudden death victims and provides a very close simulation of bacterial activities and compositions in different areas of the hindgut.8 Observations in this gut model reflect clinical observations, in terms of both the induction and treatment of CDI. Antibiotics with a known clinical propensity to cause CDI, such as cephalosporins and clindamycin, promote C. difficile germination and toxin production in the gut model,9–11 whereas antibiotics with low clinical risk of CDI induction, such as tigecycline and piperacillin/tazobactam, do not.12,13 Vancomycin, ramoplanin, oritavancin and fidaxomicin have demonstrated efficacy in resolving simulated CDI in the gut model, reflective of good efficacy in animal models and clinical studies.10,14,15 Conversely, metronidazole and tolvaptam have demonstrated poor efficacy in the gut model against epidemic C. difficile strains, again reflective of clinical observations.16,17

Methods

C. difficile strains

The PCR ribotype 027 strain used in this experiment (027 210) was isolated during an outbreak of CDI at the Maine Medical Centre (Portland, ME, USA) and was kindly supplied by Dr Rob Owens. The PCR ribotype 001 strain (P24) was isolated in 1996 from a symptomatic patient at Leeds General Infirmary, UK.

The in vitro gut model

We have previously reported the use of this model to study the interplay of gut microbiota and C. difficile following antimicrobial exposure. The model consists of three chemostat vessels, pH controlled (pH 5.5 ± 0.2, Vessel 1; pH 6.2 ± 0.2, Vessel 2; pH 6.8 ± 0.2, Vessel 3) and arranged in a weir cascade system. Vessel 1 is top-fed with a complex growth medium (containing 10 mg/L CaCl2), and all vessels are sparged with nitrogen to maintain an anaerobic atmosphere. The model is inoculated with a pooled faecal slurry (10% in pre-reduced PBS). Faeces are from volunteers (n=3–5) from the age bracket most at risk of CDI (>60 years), with no history of antimicrobial therapy in the last 3 months, and are screened for the presence of C. difficile before use. Gut microflora populations, total counts of viable C. difficile and C. difficile spore counts are enumerated on selective agars. Colonies are identified to genus level on the basis of colony morphology, Gram reaction and microscopic appearance on selective and non-selective agars (Table 1). The presence of C. difficile cytotoxin is determined by Vero cell cytotoxicity assay.9

Experimental design

Two models were run in parallel (Figure 1). Following inoculation with faecal slurry, the models were left to equilibrate to allow the bacterial populations to stabilize before inoculation with C. difficile spores (~107 cfu/mL). Model 1 was inoculated with PCR ribotype 001 spores and Model 2 with PCR ribotype 027 spores. Both models were left for a further 7 days prior to the instillation of clindamycin (33.9 mg/L, four times daily for 7 days) at levels reflecting observed biliary/faecal levels to induce simulated CDI. When evidence of germination and high toxin titre was observed [toxin titre ≥3 relative units (RU) for 3 days], both models were inoculated with 70 mg of surotomycin (to achieve 250 mg/L surotomycin in Vessel 1) twice daily for 7 days. Surotomycin was instilled to reflect the faecal concentrations measured in subjects during Phase 1 clinical trials.7 Both models were then left for 3 weeks with no further interventions. The total viable counts of indigenous gut microflora were monitored daily (post-steady-state), along with the total viable counts, spore counts and cytotoxin titres for C. difficile.

Determination of antimicrobial concentrations

Bioassay

Antimicrobial concentrations were determined using an in-house large plate bioassay. Samples (1 mL) from all the vessels of each gut model were centrifuged (16 000 g) and the supernatants sterilized by filtration through 0.22 μm syringe filters before being stored at −20 °C prior to bioassay. Bioassay agar (100 mL) was sterilized by autoclaving, cooled to 50°C and inoculated with 1 mL of indicator organisms. The indicator organisms were grown on blood agar for 24 h and then suspended in sterile saline at a turbidity equivalent to that of a 0.5 McFarland standard. Inoculated agars were mixed by inversion, transferred aseptically into 245×245 mm agar plates (Fisher Scientific, Loughborough, UK) and allowed to set. The inoculated agars were dried (37°C) for 10 min and 25 wells (9 mm in diameter) were removed from the agar using a number 5 cork borer. Antibiotic calibrator or filter-sterilized samples (30 μL) from the gut model were randomly assigned to bioassay wells in triplicate. The bioassay plates remained at ambient temperature for 4 h prior to overnight aerobic incubation at 37°C. The zone diameters were measured using calipers accurate to 0.1 mm. Calibration lines were plotted from squared zone diameters and unknown concentrations from culture supernatants were determined. All assays were performed in duplicate.

To determine the clindamycin concentrations, Wilkins-Chalgren agar was inoculated with Kocuria rhizophila (ATCC 9341) and a calibration series ranging from 4 mg/L to 256 mg/L was used. The limit of detection of this bioassay is ~2 mg/L.

To determine the surotomycin concentrations, calcium (50 mg/L)-controlled Antibiotic Medium No. 1 was inoculated with K. rhizophila indicator organism (ATCC 9341). Again a calibration series ranging from 4 mg/L to 256 mg/L was used; the assay limit of detection was ~2 mg/L.

Liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS)

Faecal pellets were homogenized with water in a 1:19 ratio. The homogenized faecal samples were cleaned by solid phase extraction and analysed by LC–MS/MS. An API 4000 was operated in the selected reaction monitoring mode under optimized conditions for detection of surotomycin positive ions formed by electrospray ionization.

The method used for the quantification of surotomycin in human faeces (5% faecal homogenate in water) was calibrated over a concentration range of 50 to 10 000 ng/mL. The precision and accuracy of the method were set at ≤20% (for %bias and %coefficient of variation). Samples were analysed by Tandem Labs (Salt Lake City, UT, USA).

Monitoring for emergence of reduced susceptibility to surotomycin

The emergence of enterococci and C. difficile populations showing reduced susceptibility to surotomycin, daptomycin and vancomycin was monitored twice weekly on antimicrobial-containing breakpoint agars. The MICs of each antimicrobial for both C. difficile strains and Enterococcus faecalis (ATCC 29212) were determined by agar incorporation, and antimicrobial concentrations of 4×MIC were used in breakpoint agars. C. difficile shifts in
surotomycin susceptibility (surotomycin MIC for both strains = 0.25 mg/L) were monitored on Brazier’s CCEYL Agar containing 2 mg/L surotomycin in addition to the usual supplements (outlined in Table 1). C. difficile shifts in daptomycin susceptibility (daptomycin MIC for both strains = 1 mg/L) were monitored on Braizer’s CCEYL Agar containing 4 mg/L daptomycin in addition to the usual supplements (outlined in Table 1). Antimicrobial-resistant enterococci populations were monitored on Kanamycin Aesculin Azide Agar containing 2 mg/L surotomycin, 8 mg/L daptomycin or 4 mg/L vancomycin in addition to the usual supplements (outlined in Table 1). For E. faecalis, surotomycin MIC = 0.25, daptomycin MIC = 2 mg/L and vancomycin MIC = 1 mg/L. All agars containing surotomycin or daptomycin were controlled to contain 50 mg/L calcium.

Table 1. Selective and non-selective media used to enumerate gut microflora populations

<table>
<thead>
<tr>
<th>Target group</th>
<th>Agar</th>
<th>Supplements</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total anaerobes</td>
<td>Fastidious Anaerobe Agar</td>
<td>5% horse blood</td>
</tr>
<tr>
<td>Total clostridia</td>
<td>Fastidious Anaerobe Agar</td>
<td>5% horse blood</td>
</tr>
<tr>
<td>Bifidobacteria</td>
<td>Beerens’ Agar (42.5 g/L Columbia Agar + 5 g/L Agar Technical)</td>
<td>0.5 g/L cysteine HCl, 5 g/L glucose</td>
</tr>
<tr>
<td>Bacteroides fragilis</td>
<td>Bacteroides Bile Aesculin Agar</td>
<td>5 mg/L haemin, 10 µg/L vitamin K, 7.5 mg/L vancomycin, 1 mg/L penicillin, 75 mg/L kanamycin, 10 mg/L colistin</td>
</tr>
<tr>
<td>Lactobacilli</td>
<td>LAMVAB Agar (20 g/L Agar Technical, 52.2 g/L MRS broth)</td>
<td>0.5 g/L cysteine HCl, 20 mg/L vancomycin</td>
</tr>
<tr>
<td>Total facultative anaerobes</td>
<td>Nutrient Agar</td>
<td></td>
</tr>
<tr>
<td>Lactose-fermenting Enterobacteriaceae</td>
<td>MacConkey Agar</td>
<td></td>
</tr>
<tr>
<td>Enterococci</td>
<td>Kanamycin Aesculin Azide Agar + 5 g/L Agar Technical</td>
<td>10 mg/L nalidixic acid, 10 mg/L aztreonam, 20 mg/L kanamycin</td>
</tr>
<tr>
<td>C. difficile spores</td>
<td>Brazier’s CCEYL Agar</td>
<td>2% lysed horse blood, 5 mg/L lyszyme, 250 mg/L cycloserine, 8 mg/L cefoxitin</td>
</tr>
<tr>
<td>C. difficile total counts</td>
<td>Brazier’s CCEYL Agar</td>
<td>2% lysed horse blood, 5 mg/L lyszyme, 250 mg/L cycloserine, 8 mg/L cefoxitin, 2 mg/L moxifloxacin</td>
</tr>
</tbody>
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Figure 1. Schematic to show experimental design. CD, C. difficile spores; CLI, clindamycin; QID, four times daily; SUR, surotomycin; BID, twice daily.

Results

C. difficile total viable counts, spore counts and cytotoxin

C. difficile populations of both PCR ribotypes remained as spores before (Period B) and during (Period C) clindamycin instillation (Figure 2). An increase in total viable counts over spore counts (germination) was observed 6 days (ribotype 001, Figure 2a) and 5 days (ribotype 027, Figure 2b) after the end of clindamycin instillation. Corresponding toxin production was observed, reaching a titre of 3 and 5 RU in the PCR ribotype 001 and 027 gut models, respectively (Figure 2). Surotomycin instillation rapidly
reduced the total counts to the level of the spore counts, which continued to wash out of the model and were around the limit of detection (1.52 log_{10} cfu/mL) by the end of Period E. Cytotoxin was no longer detectable 4 and 3 days into surotomycin instillation in the PCR ribotype 001 and 027 models, respectively. *C. difficile* continued to be detected as spores (both models) into Period F, albeit at reduced levels compared with the pre-surotomycin period, until an increase in total viable counts over spores (recurrence) was observed 11 days (PCR ribotype 001) and 15 days (PCR ribotype 027) after the end of surotomycin instillation (Figure 2). Cytotoxin was detected 14 days after the cessation of surotomycin instillation in the 001 model, and reached a titre of 5 RU by the end of the experiment. Cytotoxin was detected 16 days after the cessation of surotomycin instillation in the 027 model and reached a titre of 2 RU.

**Gut microflora viable counts**

Changes in gut microflora populations following clindamycin instillation were similar to those previously reported\[10,14,15\] and consisted mainly of minor decreases in the *Bacteroides fragilis* group and increases in lactose-fermenting *Enterobacteriaceae* and enterococci populations (Figures 3 and 4). Clindamycin caused a decrease in bifidobacteria populations in the 001 model (~3 log_{10} cfu/mL) but not the 027 model (Figure 3). In most cases, populations recovered to steady-state levels during Period D, although enterococci populations in both models continued to fluctuate (Figure 4).

The effects of surotomycin instillation were similar in both models. Counts of lactose-fermenting *Enterobacteriaceae* increased by 2–3 log_{10} cfu/mL (Figure 4). There were decreases in enterococci, *clostridia*, bifidobacteria and lactobacilli populations. Enterococci and *clostridia* decreased to below, or around, the limit of detection.
in both models. Bifidobacteria decreased by \( \approx 8 \log_{10} \text{cfu/mL} \) to below the limit of detection in the 001 model, and by \( \approx 5 \log_{10} \text{cfu/mL} \) in the 027 model (Figure 3). Lactobacilli decreased by \( \approx 6 \log_{10} \text{cfu/mL} \) to below the limit of detection in the 001 model and by \( \approx 4 \log_{10} \text{cfu/mL} \) in the 027 model (Figure 4). \textit{B. fragilis} group populations in both models were unaffected by surotomycin instillation. In both models, all populations recovered to steady-state levels following the end of surotomycin instillation.

**Emergence of reduced susceptibility**

No evidence of reduced susceptibility to surotomycin, daptomycin or vancomycin, as indicated by growth on breakpoint agar (CCEYL Agar, 2 mg/L surotomycin or 4 mg/L daptomycin; Kanamycin Aesculin Azide Agar, 2 mg/L surotomycin, 8 mg/L daptomycin or 4 mg/L vancomycin), was observed throughout the experiments (data not shown).

**Antimicrobial concentrations**

In both models, clindamycin concentrations in all three vessels peaked at around 60 mg/L, and levels were undetectable 4 days after cessation of instillation (Figure 2). Germination occurred approximately 3 days after clindamycin washout. Surotomycin levels, as measured by bioassay, peaked at \( \approx 250 \text{mg/L} \) and \( \approx 110 \text{mg/L} \) in Vessel 3 of the 001 and 027 models, respectively (Figure 2). Surotomycin levels as measured by LC–MS/MS peaked at 401 mg/L and 123 mg/L in Vessel 3 of the 001 and 027 models, respectively (data not shown).

**Discussion**

Vancomycin has been the treatment of choice for severe CDI and has previously been investigated in the human gut model.\textsuperscript{10,14,15} In the present experiments, surotomycin successfully and rapidly reduced vegetative cell counts and toxin production of \textit{C. difficile} in the human gut model of CDI, indicating that surotomycin is comparable to vancomycin in the resolution of simulated CDI in an in \textit{vitro} model. The effects of surotomycin were similar on the two ribotype strains examined.

In Phase 2 clinical trials, surotomycin was not inferior to vancomycin in the initial resolution of CDI, but a higher sustained cure rate and a delay in time to recurrence following surotomycin compared with vancomycin was reported.\textsuperscript{7} Following the initial
successful reduction of *C. difficile* by surotomycin in the gut model, the recurrence of vegetative *C. difficile* growth and subsequent toxin production was observed 11 and 15 days, respectively, after the cessation of instillation. Following vancomycin instillation, recurrence has sometimes been observed,14,18 and sometimes not.10 However, in these cases, the models were only monitored for 14 days post-vancomycin instillation, which may not be sufficient time for recurrence to occur; for example, if the observation period in the current experiments had been limited to 14 days, we would have detected CDI recurrence in one but not both models. It is therefore difficult to determine whether surotomycin instillation delays recurrence in comparison with vancomycin in the gut model. Phase 3 clinical trials should provide useful additional data on this point and may help in validating this *in vitro* gut model as an appropriate tool to explore and assess the recurrence rate after a successful antibiotic exposure.

Surotomycin is a cyclic lipopeptide, which is dependent on calcium for its activity. We have therefore measured calcium levels in the gut model and found the calcium concentration to be $\sim$15 mg/L. This is far below the optimal 50 mg/L for surotomycin activity (the activity of surotomycin at 25 mg/L calcium has been shown to be reduced 4-fold compared with 50 mg/L calcium19). However, we have demonstrated that these low calcium concentrations do not prevent activity of surotomycin against *C. difficile* and other gut microflora components. Given the amount of drug in the system, this reduced activity may have no impact on the initial killing of the *C. difficile* load during the treatment window. However, it may potentially have an impact when the level of surotomycin gets closer to the MIC level for the isolate used in the system (several days after the end of instillation). In a system maintained at 50 mg/L, we might have seen faster killing or further delayed recurrence. The calcium concentration of the gut model is much lower than that of pooled faecal slurry (86 mg/L), due to dilution of the faecal slurry with gut model growth media. Concentrations in the colon are likely to be more reflective of faecal slurry than the gut model fluid.

Levels of surotomycin detected in both models were similar as detected by bioassay and LC–MS/MS (110–250 mg/L versus 123–401 mg/L) and were equivalent to those measured in stool samples of healthy volunteers given 1000 mg twice daily in a Phase 1 clinical trial in healthy volunteers (Cubist Pharmaceuticals Inc., data on file). During a Phase 2 clinical trial, the faecal surotomycin

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**Figure 4.** Mean facultative anaerobic gut microbiota populations (log$_{10}$ cfu/mL) in Vessel 3 of (a) the PCR ribotype 001 model and (b) the PCR ribotype 027 model.
levels of patients receiving 250 mg twice daily were 2- to 5-fold lower than measured in the gut model but were of a similar order of magnitude (Cubist Pharmaceuticals Inc., data on file).

Surotomycin had a greater deleterious effect on some gut microbiota populations (clostridia and lactobacilli) than vancomycin, and a similarly devastating effect on bifidobacteria and enterococci populations to that previously reported following vancomycin instillation. 

Notably, however, surotomycin was sparing of B. fragilis group populations, whereas vancomycin was not. Other authors have reported an averageweight of Enterobacteriaceae following surotomycin exposure, but this was not observed in either model here. Importantly, no evidence of reduced susceptibility to either surotomycin or doptomycins was observed for either C. difficile or enterococci throughout the experiment. There was no evidence of selection for vancomycin-resistant enterococci.

In conclusion, surotomycin shows comparable efficacy to vancomycin in the resolution of simulated CDI in an in vitro gut model but, unlike vancomycin, is sparing of Bacteroides spp. populations. Further investigation of surotomycin as a treatment for CDI is warranted.

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