Comparison of the efficacy of ramoplanin and vancomycin in both in vitro and in vivo models of clindamycin-induced Clostridium difficile infection

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Received 11 April 2005; returned 15 June 2005; revised 22 June 2005; accepted 15 August 2005

Objectives: Treatment of Clostridium difficile infection (CDI) is limited primarily to either metronidazole or vancomycin. We compared vancomycin and a novel glycolipodepsipeptide, ramoplanin, in both hamster and in vitro gut models of clindamycin-induced CDI.

Methods: We used an in vitro triple-stage chemostat model that simulates the human gut, and an in vivo hamster model, both primed with clindamycin.

Results: Clindamycin exposure elicited symptomatic disease in the hamster model, and promoted C. difficile germination and toxin production in the gut model. C. difficile germination and toxin production were not associated with depletion of gut microflora in the gut model, but were temporarily associated with subinhibitory concentrations of clindamycin. Both ramoplanin and vancomycin were associated with rapid symptom resolution in the hamster model, and rapid toxin titre decrease in the in vitro gut model. In both models of CDI, vancomycin was associated with greater persistence of C. difficile spores. C. difficile spores were recovered significantly more often from the caecal contents of vancomycin-treated (n = 19/23) compared with ramoplanin-treated (n = 6/23) hamsters (P < 0.05).

Conclusions: Results from the in vitro gut and hamster models were concordant. Ramoplanin and vancomycin were similarly effective at reducing cytotoxin production in the gut CDI model and in resolving symptoms in the hamster model. Ramoplanin may be more effective than vancomycin at killing spores and preventing spore recrudescence. These findings suggest a potential therapeutic role for ramoplanin in CDI that requires further clinical investigation.

Keywords: antibiotic-associated diarrhoea, chemostat, hamster

Introduction

Clostridium difficile infection (CDI) is a major cause of morbidity, notably among the hospitalized elderly. C. difficile is the aetiological agent of pseudomembranous colitis, its toxin being demonstrable in 95% of cases, and is responsible for ~30% of cases of antibiotic-associated diarrhoea.¹ ² CDI occurs almost exclusively as a result of antimicrobial chemotherapy and is thought to occur following antibiotic-mediated depletion of the normal gut microflora.³ ⁴ Clindamycin, third generation cephalosporins and aminopenicillins are associated with the greatest risk of CDI, whereas ureidopenicillins are uncommonly associated with the disease.⁶

Treatment of CDI is mainly limited to oral metronidazole or vancomycin, both given for 7–10 days, following withdrawal of the precipitating antibiotic whenever possible.⁷ Both treatments are highly effective against C. difficile and are equivalent in terms of overall response, although vancomycin therapy has been associated with a shorter mean duration of symptoms (mean 1.6 days shorter).⁸ Symptomatic recurrences are common following treatment with metronidazole or vancomycin, but there is no statistically significant difference in recurrence rates between these two agents.⁹ Most authorities now recommend metronidazole as the first-line agent because it is considerably less expensive than vancomycin, and in order to reduce the selective pressure for vancomycin-resistant enterococci (VRE).

Ramoplanin is a glycolipodepsipeptide antibiotic that is undergoing clinical trials for the prevention of VRE bloodstream infections. It also shows good activity against C. difficile¹⁰–¹² and is currently being evaluated as treatment for CDI. Activity
against both C. difficile and enterococci may provide a further therapeutic option in CDI, without the potential promotion of VRE colonization associated with vancomycin treatment. We have investigated the effects of ramoplanin or vancomycin treatment on C. difficile in the hamster model of CDI. In addition, we used an in vitro gut model of CDI to investigate the effects of ramoplanin and vancomycin exposure on C. difficile and human gut microflora following priming with clindamycin.

Methods

Hamster model of CDI

Bacterial strain. C. difficile strain 421 was used to challenge hamsters orogastrically. This strain was isolated from the faeces of a hospitalized patient, and was susceptible to both vancomycin (2 mg/L) and ramoplanin (0.03 mg/L). The strain was kindly provided by G. Privitera (Pollicinico Ospedale Maggiore, Milan, Italy) and was stored in small aliquots at −80°C in nutrient no. 2 broth (Oxoid S.p.A., Garbagnate, Italy) with 20% (v/v) glycerol (Baker Malinkrodt B. V., Denvertier, The Netherlands). A suspension of C. difficile 421 for oral challenge was prepared by picking 4–5 colonies from a 48 h sheep blood agar plate and resuspending in tryptic soy broth supplemented with 1% oxynaprox (Oxyrase, Mansfield, USA). The suspension was adjusted to an A625 of 1.0 and further diluted 1:1000 before challenge. The inoculum was 10⁸ viable bacteria in 1 mL.

Antimicrobial agents. Ramoplanin was produced by Vicuron Pharmaceuticals (formerly Biosearch Italia S.p.A.). Clindamycin (CL, Dalacin C phosphate; Pharmacia & Upjohn) was supplied as 4 mL ampoules of sterile solution for clinical use, and vancomycin (VA, Vancocin; Eli Lilly) was purchased as clinical lyophilized vials. All supplements were supplied by Sigma–Aldrich Co., Poole, UK.

Animals. Male Golden Syrian hamsters (Harlan Italy, S. Pietro al Natisone, Italy) weighing 60–80 g were used in all experiments. For the quarantine period, hamsters were housed in plastic cages with raised floor grids (to prevent coprophagia), in an air-conditioned animal room and were provided food and water ad libitum. Procedures were approved by the Animal Care and Use Committee (Vicuron, Italy) in accordance with local regulations.

Procedure for collection of caecal samples. Fifty-six hamsters were given 10⁴ C. difficile strain 421 organisms by gavage, and were then randomized into two groups and housed in a cabinet ventilated with HEPA-filtered air (Day −1). Twenty-four hours later, the hamsters were given a single subcutaneous injection of clindamycin (100 mg/kg body weight) to induce colitis (Day 0). After a further 24 h, animals received oral vancomycin (n = 23) (Group A) or ramoplanin (n = 23) (Group B) at 50 mg/kg/day in sterile saline for 5 days (Days 1–5). Another group of animals (n = 8) (Group C) remained untreated and acted as controls. Serial sacrifices (n = 2–4) for microbiological evaluation of caecal contents were performed on Days −1 (baseline), 1, 2, 4, 6, 8, and 9 and after challenge with clindamycin (Figure 1). Hamsters were euthanized by asphyxiation with CO₂, and caeca were removed and immediately transferred into a sterile anaerobic chamber. The caeca were incised and 0.5–1 mL of content was placed into a sterile biofreeze vial (Costar, Corning Costar Corporation). Vials were stored at −80°C until they were transported on dry ice for microbiological analysis in Leeds, UK.

Microbiological analysis of caecal contents. All manipulations and analyses of hamster caecal contents were performed in an anaerobic cabinet (Don Whitley Scientific Ltd, Shipley, UK). Caecal contents were emulsified 1:4 in sterile pre-reduced PBS to give a 25% (w/v) caecal slurry. Two hundred microlitres of each sample of caecal contents were serially diluted 10-fold to 10⁻⁸ in pre-reduced peptone water (Sigma–Aldrich Co., Poole, UK). Brazier’s Cycloserine Cefoxitin Egg Yolk agar plates (Bioconnections, Leeds, UK), supplemented with 5 mg/L of lysozyme and 2% lysed horse blood (CCEYL) were inoculated in triplicate, and 20 µL of each dilution were spread per quarter plate. Plates were incubated anaerobically for 48 h, and then single colonies were counted.

For the enumeration of C. difficile spores, 200 µL of each hamster caecal sample were treated with an equal volume of 96% (v/v) ethanol (VWR International, Lutterworth, UK) for 1 h. Alcohol-shocked suspensions were then serially diluted 10-fold to 10⁻⁸ in pre-reduced peptone water. CCEYL plates were processed as described above.

In vitro gut model of CDI

The gut model was developed by MacFarlane et al., and validated against the caecal contents of sudden death victims. We have described its modification for use as a model of CDI. Briefly, the model consists of three vessels operating in a weir cascade system in an oxygen-free nitrogen atmosphere. The system is top-fed with growth medium at a controlled rate, and each vessel operates at a controlled pH to reflect the increasing alkalinity of the gut. Thus, Vessel 1 (280 mL) operates at a low pH (5.5) and high substrate availability, whereas Vessels 2 and 3 (300 mL) operate at more neutral pH (6.2 and 6.8, respectively) and lower substrate availability. The model is primed with pooled emulsified faecal samples, and allowed to equilibrate in respect of bacterial populations for 2 weeks.

Preparation of the gut model. Human faeces for inoculation of the model were collected from five healthy elderly volunteers (>65 years) with no history of antimicrobial use for 2 months prior to donation, and screened for the presence of C. difficile. The gut model was primed with a 10% (w/v) faecal slurry and media as previously described. The system was then allowed to equilibrate for 2 weeks. The model was then sampled daily thereafter for C. difficile total and spore counts, toxin titres and for enumeration of gut bacterial populations.

Gut model growth medium. Gut model growth media were prepared as previously described. Media were pre-reduced by sparging with oxygen-free nitrogen overnight before use.

Enumeration of faecal bacteria. Samples were removed from each vessel and serially diluted (from 10-fold to 10⁻⁸) under anaerobic conditions in pre-reduced peptone water. The following selective agars were inoculated in triplicate with 20 µL of each appropriate dilution (all agars were supplied by Oxoid, Basingstoke, UK, and all supplements were supplied by Sigma–Aldrich Co., Poole, UK, unless otherwise stated): Brazier’s CCEY agar supplemented with cycloserine and cefsulodin (Bioconnections, Leeds, UK), 5 mg/L of lysozyme and 2% lysed horse blood (total C. difficile); Brazier’s CCEY agar with 5 mg/L of lysozyme and 2% lysed horse blood, (C. difficile spores); Fastidious Anaerobe agar (FAA) (Bioconnections, Leeds, UK) supplemented with 5% whole horse blood (total anaerobes); Modified Neomycin Blood agar: FAA medium supplemented with 100 mg/L of neomycin, 5 mg/L of lysozyme, 5% lysed horse blood (total clostridia); Tween agar: FAA medium supplemented with 1 mL/L of Tween 80 and 5% whole horse blood (Gram-positive cocci); Brucella medium base supplemented with 5% laked horse blood, 5 mg/L of haemin, 10 µL/L of vitamin K₃, 75 mg/L of kanamycin and 7.5 mg/L of vancomycin, (total bacteroides); Beersen agar: 42.5 g/L of Columbia agar base supplemented with 5 g/L of Agar Technical no. 3, 0.5 g/L of cysteine hydrochloride, 5 g/L of glucose, 5 mL/L of propionic acid, pH 5 (bifidobacteria); LAMVAB agar: 500 mL/L of MRS broth, 20 g/L of Agar Technical no. 3, 20 mg/L of vancomycin (lactobacilli); Kanamycin Aesculin Azide agar
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(KAA) supplemented with 20 mg/L of kanamycin (enterococci); Kanamycin Vancomycin Aesculin Azide agar (KVAA) supplemented with 20 mg/L of kanamycin and 6 mg/L of vancomycin (VRE); MacConkey Agar no. 3 (lactose fermenters); Nutrient agar (total facultative bacteria). All selective agars excepting KAA, KVAA, nutrient and MacConkey agars were pre-reduced in an anaerobic cabinet for 24 h prior to inoculation. All plates were incubated anaerobically for 48 h, except KAA, KVAA, nutrient and MacConkey agars, which were incubated aerobically at 37°C for 24 h. After incubation, colonies were counted and identified on the basis of colony morphology, colony fluorescence, Gram’s stain, biochemical reactivity and genus-specific PCR where appropriate. *C. difficile* spores were enumerated as described above.

*C. difficile* cytotoxin quantification. *C. difficile* cytotoxin titres were determined using a VERO cell cytotoxicity/neutralization assay with *C. sordellii* antitoxin (Prolab Diagnostics, Neston, UK) as described previously.14,15 *C. difficile* cytotoxin titres were expressed in relative units (RU).

**Figure 1.** (a) *C. difficile* total viable counts in caecal contents of hamsters in Group A (vancomycin treatment) (dark grey bar); Group B (ramoplanin treatment) (open bar); or Group C (no treatment) (light grey bar); following induction of CDI with clindamycin. (b) *C. difficile* spore counts in caecal contents of hamsters in Group A (vancomycin treatment) (dark grey bar); Group B (ramoplanin treatment) (open bar); or Group C (no treatment) (light grey bar); following induction of CDI with clindamycin.
C. difficile gut model inoculum. The gut model inoculum comprised C. difficile spores of strain P24 (UK epidemic strain, PCR ribotype 1). These were prepared using alcohol shock in sterile saline as previously described, adjusted to ~10⁷ cfu/mL and stored at 4°C until required.

Experimental design

Time periods for the three gut model experiments are shown in Figure 2. Following establishment of steady-state (Period A) for bacterial populations in the gut model, ~10⁷ cfu C. difficile spores were added to Vessel 1. C. difficile total counts, spore counts, cytotoxin titres and faecal bacterial counts were monitored daily for 7 days (Period B). A further 10⁷ cfu C. difficile spores were added to Vessel 1 and clindamycin instillation commenced for a period of 7 days (Period C). Clindamycin was instilled into Vessel 1 at 33.9 mg/L, 6 hourly, to maintain the biliary/faecal levels observed following a single 600 mg dose. C. difficile total counts, spore counts, cytotoxin titres and faecal bacterial counts were monitored daily. Thereafter, three experimental approaches were taken.

Clindamycin control. No further interventions were made, monitoring of C. difficile, C. difficile spores, cytotoxin and faecal bacterial populations continued for 14 days (Period D).

Ramoplanin instillation. Following the attainment of a high cytotoxin titre (4 RU or above) for two consecutive days (Period D), ramoplanin was instilled into Vessel 1 at 1 g/L, 12 hourly, to reflect the human faecal levels observed during a 400 mg twice daily dosing regimen (Period E). (D. Jabes, personal communication). C. difficile, C. difficile spores, cytotoxin and faecal bacterial populations were monitored throughout ramoplanin instillation (Period E) and continued for 14 days post-treatment (Period F).

Vancomycin instillation. Following the attainment of a high cytotoxin titre (4 RU or above) for two consecutive days (Period D), vancomycin was instilled into Vessel 1 at 125 mg/L, 6 hourly, to reflect the human faecal levels observed during a 125 mg four times daily dosing regimen (Period E). (D. Jabes, personal communication). C. difficile, C. difficile spores, cytotoxin and faecal bacterial populations were monitored throughout vancomycin instillation (Period E) and continued for 14 days post-treatment (Period F).

Antimicrobial assays. Samples were removed daily from each vessel for the determination of antibiotic concentrations. C. difficile levels were determined using a large plate bioassay with Staphylococcus aureus NCTC 6571. Ramoplanin levels were not determined in this investigation.

Results

Effects of ramoplanin or vancomycin in the hamster model of CDI

There was no evidence of C. difficile in caecal contents collected from C. difficile inoculated hamsters before challenge with a single 100 mg/kg dose of clindamycin (Day –1), and none of these animals developed signs of CDI. Evidence of CDI (wet tail, watery or haemorrhagic caecal contents) was seen only at 48 h (Day 2) after challenge with clindamycin. On day 2, C. difficile total counts were quantifiable in 66% of Group A (ramoplanin-treated) hamsters compared with 33% of Group B (vancomycin-treated) hamsters, with C. difficile spores detectable in 100% of Group A hamsters compared with 30% of Group B hamsters. CDI symptoms (watery faeces) were present in 33% of both Groups A and B animals. In comparison Group C (control) hamsters were uniformly asymptomatic (wet tail, watery or haemorrhagic caecal contents) and caecal contents had high counts of C. difficile (total counts range 6.04–7.79 log₁₀ cfu/g caecal contents) and (predominantly) C. difficile spores (range 7.09–7.67 log₁₀ cfu/g caecal contents) (Figure 1a and b). On Day 4, C. difficile total counts and spores were quantifiable in 75 and 100% of Group A hamsters, respectively, but C. difficile was undetectable in all Group B hamsters (Figure 1a and b). All animals in both treatment groups were asymptomatic at Day 4 and thereafter.

During the post-treatment period (Days 6, 8, 9 and 10) C. difficile total counts were unmeasurable in both Groups A and B hamsters, with the exception of one Group B animal which had quantifiable C. difficile total counts on Day 6. During the post-treatment period, C. difficile spores were detected in 50–100% of Group A hamsters on Days 6–10, but were undetectable in Group B hamster caecal contents until Days 9 and 10 (50 and 75%, respectively) (Figure 1a and b). Overall, C. difficile spores were recovered significantly more often from the caecal contents of vancomycin-treated (n = 19/23) compared with ramoplanin-treated (n = 6/23) hamsters (χ² analysis with Yates’ correction, P < 0.05).

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Figure 2. Schematic representation of gut model experiments.
Effects of ramoplanin versus vancomycin treatment upon CDI in the gut model

Steady-state bacterial populations were achieved by Day 14 in each of the three experiments. Addition of *C. difficile* spores had little effect upon faecal bacterial populations (Figure 3a–c, time period B). *C. difficile* was present as spores and numbers decreased in accordance with the dilution rate in all vessels. *C. difficile* cytotoxin was not detected in this time period (Figure 4a–c, time period B). Following commencement of clindamycin instillation, increases in counts of total facultative anaerobes, lactose fermenters, enterococci and Gram-positive cocci were observed in all three experiments. Total anaerobe counts remained constant throughout the clindamycin dosing period, although marked decreases (≥6 log) in numbers of bifidobacteria and less dramatic decreases in *Bacteroides* spp. populations were observed.

Following further addition of *C. difficile* spores and commencement of clindamycin instillation, *C. difficile* remained largely as spores, and numbers decreased steadily in all vessels, with no evidence of germination. *C. difficile* cytotoxin was either not detectable or intermittently present at a titre of only 1 RU during clindamycin instillation (Figure 4a–c, time period C). Cessation of clindamycin prompted a swift recovery of the majority of faecal bacterial populations to levels similar to those seen before antibiotic instillation. Bifidobacterial populations were generally slower to recover and were not detectable at all in one experiment (Figure 3a–c, time period D). *C. difficile* total counts remained constant for ≥5 days after cessation of clindamycin instillation. Germination then occurred, seen as marked divergence of total viable and spore *C. difficile* counts. Subsequently, cytotoxin was detected in Vessels 2 and 3, 6–8 days after cessation of clindamycin instillation (Figure 4a–c, time period D).

Effects of no interventions: control gut model

Faecal bacterial populations, which had been re-established to pre-clindamycin instillation levels, remained constant until the end of the experiment (Figure 3a, Period E). *C. difficile* cytotoxin was

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Effects of ramoplanin versus vancomycin treatment upon CDI in the gut model

Figure 3. (a) Effect of clindamycin instillation followed by no further interventions upon gut bacterial populations in Vessel 3 of the gut model. (b) Effect of clindamycin instillation followed by ramoplanin instillation upon gut bacterial populations in Vessel 3 of the gut model. (c) Effect of clindamycin instillation followed by vancomycin instillation upon gut bacterial populations in Vessel 3 of the gut model.

Figure 4. (a) Effect of clindamycin instillation followed by no further interventions upon *C. difficile* total viable counts, spore counts and cytotoxin titres in Vessel 3 of the gut model. (b) Effect of clindamycin instillation followed by ramoplanin instillation upon *C. difficile* total viable counts, spore counts and cytotoxin titres in Vessel 3 of the gut model. (c) Effect of clindamycin instillation followed by vancomycin instillation upon *C. difficile* total viable counts, spore counts and cytotoxin titres in Vessel 3 of the gut model.
detected 8 days after clindamycin instillation ceased (Day 37) and achieved a maximum titre of 6 RU, 2 days later. This level of cytotoxin was maintained for a further 4 days (Day 43), and then titres decreased slowly to 1 RU at the end of the experiment. Cytotoxin was detectable for a total of 12 days. C. difficile total and spore counts remained divergent until Day 43, when total counts decreased to the same numbers as spores. This coincided with the decrease in toxin titre. C. difficile total and spore counts then decreased steadily until the end of the experiment (Figure 4a, Period E). No VRE were detected.

Effects of ramoplanin in the gut model
Ramoplanin instillation commenced when cytotoxin reached and sustained a titre of at least 4 RU for two consecutive days. Cytotoxin titres of 5 and 5.5 RU were achieved (Day 33) and maintained for 2 days in Vessels 2 and 3, respectively, before ramoplanin instillation (Figure 4b, Period D/E). Bacteroides spp. bifidobacteria, lactobacilli, clostridial and enterococcal counts all decreased markedly to below the limits of detection 3–5 days after ramoplanin instillation commenced. Only lactose fermenters remained at a steady level during the instillation period (Figure 3b, Period E). After cessation of ramoplanin, all faecal bacteria that had decreased in numbers remained undetectable, except for enterococci, which increased in counts to around the limits of detection by the end of the experiment (Figure 3b, Period F). C. difficile total and spore counts decreased by 7 and 3 logs respectively, to below the limit of detection by the second day of ramoplanin instillation. Cytotoxin titres began to decrease on the second day of ramoplanin instillation, and were undetectable by the fifth day (Figure 4b, Period E). Neither C. difficile spores nor cytotoxin were detectable from this point until the end of the experiment (the remainder of ramoplanin instillation and a 14 day recovery period). C. difficile total counts sporadically increased during the recovery period to around the limit of detection (Figure 4b, Period F). No VRE were detected.

Effects of vancomycin in the gut model
Vancomycin instillation commenced when cytotoxin reached and sustained a titre of at least 4 RU for two consecutive days. A titre of 5 RU was achieved (Day 33) and maintained for 2 days in Vessels 2 and 3 before vancomycin instillation (Figure 4c, Period D/E). Bacteroides spp. bifidobacteria, lactobacilli, clostridial and enterococcal counts all decreased steadily until the end of the vancomycin instillation period (Figure 3c, Period E). Lactose fermenters and lactobacilli increased steadily throughout the instillation period and up to the end of the experiment. After vancomycin cessation all faecal bacteria, which had decreased in counts, remained undetectable except enterococci, which were present sporadically around the limit of detection up to the end of the experiment (Figure 3c, Period F). C. difficile total counts decreased to the same level as those for spores (4.5 log cfu/mL) by the third day of vancomycin instillation, and this level was maintained throughout the instillation period. Cytotoxin began to decrease steadily on the second day of vancomycin instillation, and was undetectable by the final day of instillation (Figure 4c, Period E). C. difficile spore and total viable counts were similar and declined at a steady rate following cessation of vancomycin. Cytotoxin was not detectable up to the end of the experiment (Figure 4c, Period F). No VRE were detected.

Antimicrobial assays
In all experiments clindamycin concentrations peaked in Vessel 1 on Day 2 of instillation control experiment, 34 mg/L; ramoplanin experiment, 38 mg/L; vancomycin experiment, 33 mg/L, on Days 3 or 4 in Vessel 2 (41, 42 and 27 mg/L, respectively) and on Days 5, 4 and 3 in Vessel 3 (50, 45 and 27 mg/L, respectively). Antibiotic levels remained approximately constant until clindamycin instillation ceased, and were undetectable after 3 and 5 days.

Vancomycin peaked in both Vessels 1 and 2 at 431 mg/L, and at 274 mg/L in Vessel 3 on Day 4 of instillation. Concentrations decreased thereafter and on the final day of instillation were measured at 137, 244 and 203 mg/L, respectively. Concentrations then decreased steadily to around the limit of detection (1 mg/L) 6 days after cessation of instillation.

Discussion
We have previously reported use of the gut model to investigate the effects of cefotaxime and its active metabolite desacetylcefotaxime and piperacillin–tazobactam upon C. difficile and gut flora. We showed that cefotaxime, an agent frequently associated with CDI,22–25 elicited rapid and sustained C. difficile germination and high-level toxin production, while piperacillin–tazobactam, which uncommonly pre-disposes to CDI,26,27 did not. The gut model may therefore be used to investigate infection pathogenesis, and specifically antibiotic-mediated responses and potential therapeutic interventions in CDI. In the present study, we verified the clinical observation of a high risk of CDI associated with clindamycin.3–6 Clindamycin instillation to achieve levels equivalent to those measured in bile consistently promoted C. difficile germination and subsequently toxin production (before sporation ensued). Interestingly, germination and toxin production were not observed during clindamycin instillation, but instead up to 8 days after cessation of antibiotic. This contrasts with our observations in experiments with cefotaxime and its active desacetyl metabolite, where effects were seen during antibiotic instillation.15 These observations can probably be explained according to the gut model concentration of antibiotic in relation to the MIC of clindamycin (1 mg/L) for the C. difficile UK epidemic strain under investigation.28 Thus, germination and toxin production occurred in the present experiments only when the gut model concentrations of clindamycin decreased below the MIC. Conversely, as the MIC of cefotaxime and desacetylcefotaxime for the C. difficile strain is much higher (256 mg/L) than that for clindamycin, concentrations of the former antibiotics in the gut model did not reach an inhibitory level before C. difficile proliferation and toxin production.

We have previously shown that in the absence of antibiotic C. difficile remains in its quiescent spore state, and is eluted from the gut model.15,16 We postulate that two conditions must be met in order for C. difficile germination and toxin production to occur in the gut model: firstly, a CDI-precipitating antibiotic must be present and secondly, the antibiotic concentration must be sub-inhibitory to growth before germination and toxin production can occur. Previous simplistic in vitro models of CDI, which relied on test tube or batch culture, failed to demonstrate reproducible differences between antibiotics and/or controls in terms of effects on C. difficile toxin production.30,31 However, such studies did not take into account gut levels of antimicrobials and their fluctuation. In addition, toxin production as a result of nutrient limitation may
have masked any antibiotic-mediated toxin production. By contrast, the gut model is a well-controlled in vitro system. Minor inter-experiment variation in antimicrobial concentrations and numbers of *C. difficile* present in any vessel at a particular time may occur. Such variability, possibly related to difference in faeces pools used to prime the gut model, may account for slight differences between experiments in the timing of *C. difficile* germination and toxin production.

Colonization resistance by gut flora is considered to be a key determinant in the prevention of CDI.12–14 CDI is thought to occur following antibiotic-mediated impairment of the gut flora, allowing *C. difficile* to proliferate and produce toxin. Colonization resistance theory suggests that *C. difficile* germination and toxin production, as a prelude to subsequent sporulation, might be expected to occur at the point of maximum disruption to the gut flora. However, the present study and previous experiments have shown that this is not necessarily true. Cefotaxime and desacetylceftaxime had a limited suppressive effect upon gut microflora, whereas profoundly deleterious changes were observed during piperacillin–tazobactam exposure.16 In the present study, while bifidobacteria and *Bacteroides* spp. were markedly depleted by clindamycin, other components of the gut microflora (total facultative anaerobes, lactose fermenters, enterococci and Gram-positive cocci) increased in numbers. Indeed, baseline levels of most gut bacteria were swiftly restored after cessation of clindamycin, and indeed before *C. difficile* germination and toxin production was observed. These results demonstrate that the relationship between gut flora, pre-disposing antibiotics and *C. difficile* is more complex than previously believed. Development of CDI may be more dependent upon gut levels of antimicrobials and the antibiotic susceptibility of the infecting *C. difficile* strain than on colonization resistance of gut microflora per se.

Choice of antimicrobial therapy for the management of CDI is generally confined to metronidazole or vancomycin. While both of these agents are successful in terms of overall response rates, such limited options, in addition to the continuing problem of high recurrence rates and threat of resistance emergence, have stimulated a search for alternative therapeutic agents for CDI. Ramoplanin is a glycolipodepsipeptide that has good activity against *C. difficile*.10–12 It was not absorbed from the gastrointestinal tracts of patients suffering from pseudomembranous colitis.35 In the present study, vancomycin and ramoplanin reduced *C. difficile* numbers in both hamster and gut models of CDI, accompanied by symptomatic resolution in the former. The rates of decline of *C. difficile* cytotoxin titres in the gut model were similar following vancomycin and ramoplanin exposure. Cytotoxin was detectable at low titre sporadically after cessation of ramoplanin but not after vancomycin. In both animal and in vitro models vancomycin was associated with the persistence of *C. difficile* spores. In the gut model during vancomycin instillation, total *C. difficile* counts reduced, whereas numbers of spores were unchanged. By contrast, ramoplanin had a profound effect upon both total *C. difficile* counts and spore counts, with neither vegetative cells nor spores being detectable 2 days after commencement of antibiotic. Spores were recovered only sporadically after cessation of ramoplanin. Ramoplanin and vancomycin achieve high concentrations in the gut lumen in vivo and these were simulated in the gut model (D. Jabes, personal communication).14 In order to minimize antibiotic carry-over during faecal culture, samples were centrifuged and resuspended in sterile, pre-reduced PBS immediately before dilution and inoculation of plates.

The hamster model has been widely accepted as the most predictive in vivo model of CDI, and has been an important tool in the study of *C. difficile* pathogenesis. However, the ethical and practical problems associated with animal models means that it is available at relatively few centres. In the present study, the results in animal and in vitro models of CDI were similar, particularly with respect to the persistence of *C. difficile* spores during vancomycin treatment/exposure. These observations provide further evidence that the gut model is a practicable alternative to animal models. Although the gut model is limited by the lengthy experimental cycle, and also by limited capacity to test multiple *C. difficile* strain-antibiotic combinations, it circumvents the ethical and practical drawbacks associated with animal testing.

The in vitro activity of ramoplanin against *C. difficile* isolates is at least equivalent to that of vancomycin and metronidazole.10–12 Ramoplanin is functionally related to lipid II binding antibiotics such as nisin,36 which has been shown to inhibit spore germination and outgrowth of *Bacillus* and *Clostridium* spp.37 Our results suggest that ramoplanin may exert a direct effect upon *C. difficile* spores in the gut lumen. Spore suppression following ramoplanin treatment may reduce the likelihood of CDI relapse in comparison with vancomycin therapy. Symptomatic recurrences following treatment of CDI are common (up to 37% of cases) and subsequent treatment poses a therapeutic dilemma.38 However, the role of residual spores in the recurrence of symptoms in CDI is controversial. Most studies have shown that the majority of patients with recurrent CDI experience re-infection with distinct strains.39,40 Thus, the potential of ramoplanin to reduce symptom recurrence in CDI, possibly via enhanced activity against spores, requires further clinical investigation. We were unable to detect VRE following either vancomycin or ramoplanin instillation in the present study, probably reflecting the absence of these bacteria in donor faecal samples used to prime the model. Several studies report an association between VRE and *C. difficile*.41–43 Vancomycin treatment (intravenous or oral) is a well-documented risk factor for colonization or infection by VRE, but metronidazole has also been implicated in some studies.44 Conversely, ramoplanin is active against VRE and administration can temporarily suppress gastrointestinal VRE carriage.45–47

In conclusion, ramoplanin and vancomycin were similarly effective at reducing cytotoxin production in the gut CDI model, and in resolving symptoms in hamsters. Results from the in vitro gut and hamster models were similar, and notably showed that ramoplanin may be more effective than vancomycin at killing spores and preventing spore recrudescence. These findings suggest a potential therapeutic role for ramoplanin in CDI that requires further clinical investigation.

Acknowledgements

Clindamycin was supplied as a gift by Pfizer, for which we are grateful. This work was supported in part by a grant from The British Society for Antimicrobial Chemotherapy. Experiments with ramoplanin were supported by a grant from Vicuron.

References


Vancomycin/ramoplanin in *C. difficile* infection models


