In vitro activity of cadazolid against clinically relevant Clostridium difficile isolates and in an in vitro gut model of C. difficile infection

C. H. Chilton1, G. S. Crowther1, S. D. Baines2, S. L. Todhunter1, J. Freeman3, H. H. Locher4, A. Athanasiou4 and M. H. Wilcox1,3*

1Leeds Institute for Molecular Medicine, University of Leeds, Leeds, UK; 2Department of Human and Environmental Sciences, University of Hertfordshire, Hatfield, UK; 3Department of Microbiology, Leeds Teaching Hospitals NHS Trust, The General Infirmary, Old Medical School, Leeds, UK; 4Actelion Pharmaceuticals Ltd, Allschwil, Switzerland

*Corresponding author. Microbiology, The General Infirmary, Old Medical School, Leeds LS1 3EX, UK. Tel: +44-113-3926818; Fax: +44-113-3922696; E-mail: mark.wilcox@leedsth.nhs.uk

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Objectives: We investigated the in vitro activity of cadazolid against 100 Clostridium difficile isolates and its efficacy in a simulated human gut model of C. difficile infection (CDI).

Methods: MICs of cadazolid, metronidazole, vancomycin, moxifloxacin and linezolid were determined using agar incorporation for 100 C. difficile isolates, including 30 epidemic strains (ribotypes 027, 106 and 001) with reduced metronidazole susceptibility, 2 linezolid-resistant isolates and 2 moxifloxacin-resistant isolates. We evaluated the efficacy of two cadazolid dosing regimens (250 versus 750 mg/L twice daily for 7 days) to treat simulated CDI. Microflora populations, C. difficile total viable counts and spores, cytotoxin titres, possible emergence of cadazolid, linezolid or quinolone resistance, and antimicrobial concentrations were monitored throughout.

Results: Cadazolid was active against all (including linezolid- and moxifloxacin-resistant) C. difficile strains (MIC90 0.125, range 0.03–0.25 mg/L). The cadazolid geometric mean MIC was 152-fold, 16-fold, 9-fold and 7-fold lower than those of moxifloxacin, linezolid, metronidazole and vancomycin, respectively. Both cadazolid dosing regimens rapidly reduced C. difficile viable counts and cytotoxin with no evidence of recurrence. Cadazolid levels persisted at 50–100-fold supra-MIC for 14 days post-dosing. Cadazolid inhibition of enumerated gut microflora was limited, with the exception of bifidobacteria; Bacteroides fragilis group and Lactobacillus spp. counts were unaffected. There was no evidence for selection of strains resistant to cadazolid, quinolones or linezolid.

Conclusions: Cadazolid activity was greater than other tested antimicrobials against 100 C. difficile strains. Cadazolid effectively treated simulated CDI in a gut model, with limited impact on the enumerated gut microflora and no signs of recurrence or emergence of resistance within the experimental timeframe.

Keywords: MICs, antimicrobial persistence, chemostat

Introduction

Clostridium difficile is a leading cause of antibiotic-associated diarrhoea in hospitals and long-term care facilities around the world, 1,2 and a significant burden on healthcare systems. Recent systematic reviews have estimated that the incremental costs associated with C. difficile infection (CDI) range from £4577 to £8843 across Europe,3 and US$4846 to US$8570 in the USA.4 Conventional treatment for CDI has been limited to oral vancomycin or metronidazole, with vancomycin thought to be more effective in severe cases,5 although still associated with high rates of recurrent disease (~20%).6 The approval of fidaxomicin in 2011 for use in the treatment of CDI has provided an additional therapeutic option and has been associated with a reduction of ~50% in the rates of recurrence of non-PCR ribotype 027 infections. However, rates of recurrence of PCR ribotype 027 infections following fidaxomicin treatment are comparable to those observed following vancomycin treatment.7,8

Clinical and in vitro reports have questioned the efficacy of metronidazole treatment for CDI because of a greater observation of recurrent disease and also the emergence of C. difficile with reduced susceptibility to metronidazole.9,10 Additionally, the continued prevalence of PCR ribotype 027 strains11 and associated poorer response to metronidazole in these infections is suggestive that additional treatment options for CDI are required.
Cadazolid (formerly ACT-179811) is a novel oxazolidinone antibiotic with a fluoroquinolone side chain, shown to have potent activity against *C. difficile*, and currently in clinical development for CDI.\textsuperscript{12-13} In this study we evaluated the in vitro activity of cadazolid against a panel of clinical *C. difficile* isolates and determined the efficacy of two dosing regimens in treating simulated NAP1/027 CDI in a human gut model.

### Methods

#### Susceptibility testing of *C. difficile* strains and control organisms

The susceptibilities of 100 *C. difficile* strains (outlined in Table S1, available as Supplementary data at JAC Online) to cadazolid, metronidazole, vancomycin, moxifloxacin and linezolid were evaluated in this study using a previously described agar incorporation method.\textsuperscript{15} This method was used instead of the CLSI method, as for many antimicrobials we have found it to be more reliable when looking at MICs for clinical *C. difficile* isolates. Other authors have reported the same observation, particularly with respect to metronidazole.\textsuperscript{16,17} Four control organisms were used in the susceptibility studies: *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *Bacteroides fragilis* ATCC 25285 and *C. difficile* PCR ribotype 010 identified with intermediate susceptibility to metronidazole (MIC 8–16 mg/L).\textsuperscript{18}

Antimicrobial stock solutions were prepared in either DMSO (cadazolid) or deionized water (metronidazole, vancomycin, moxifloxacin and linezolid). Antimicrobial stock solutions (1600 – 3200 mg/L) were sterilized by filtration through 0.22 μm syringe filters. Dilutions of cadazolid were prepared in DMSO due to poor solubility of the drug in vitro, while comparator antimicrobials were diluted in deionized water. Preliminary experiments demonstrated that the presence of 10% DMSO in agar was inhibitory to the growth of *C. difficile* and *B. fragilis*, but not *S. aureus* or *E. faecalis*. Cadazolid was therefore prepared 100-fold concentrated and diluted in agar such that the final concentration of DMSO was 1%. Cadazolid solutions could not be sterilized by filtration due to filter degradation by DMSO. Antimicrobial solutions of comparator antimicrobials were prepared 10-fold concentrated and diluted in Wilkins-Chalgren agar to give the appropriate concentration. MIC ranges were 0.06 – 16 mg/L for metronidazole, vancomycin and linezolid, 0.06 – 256 mg/L for moxifloxacin and 0.07 – 16 mg/L for cadazolid.

Agar incorporation MICs were determined using previously published methods.\textsuperscript{16} *C. difficile* were initially cultured on Brazier’s CCYE agar supplemented with 20% (v/v) defibrinated horse blood and 5 mg/L lysisyme for 48 h at 37°C in an anaerobic cabinet. Control organisms were cultured on Columbia blood agar for 48 h at 37°C in an anaerobic environment. All organisms were subsequently inoculated in pre-reduced Schaedler’s anaerobic broth (5 mL) to a turbidity equivalent to that of a 0.5 McFarland standard and incubated anaerobically at 37°C for 24 h.

Wilkins-Chalgren agars were dried for 20 min at 37°C prior to inoculation. Overnight cultures of *C. difficile* were diluted 1:5 in sterile pre-reduced 0.85% (w/v) saline to achieve 10<sup>4</sup> cfu/spot (BSAC recommended inocula). *S. aureus* and *E. faecalis* were diluted 1:100 in sterile pre-reduced saline due to more profuse growth in overnight cultures. *B. fragilis* cultures were not diluted prior to inoculation onto Wilkins-Chalgren agar. Non-antimicrobial-containing control agar and agar containing 1% (v/v) DMSO were inoculated at the start and end of the inoculation procedure to ensure organism viability. Inoculated agars were incubated for 48 h at 37°C in an anaerobic environment. MIC endpoints were read as the lowest concentration of antimicrobial agent where there was no apparent growth, disregarding a visible haze of growth or a single colony.

### In vitro human gut model

#### C. difficile strain

The *C. difficile* PCR ribotype 027 strain (CD 210) evaluated in the in vitro human gut model was isolated during an outbreak of CDI at the Maine Medical Centre (Portland, MA, USA) in 2005 and was supplied courtesy of Dr Rob Owens. The MIC of clindamycin for this strain is 0.5 mg/L.

#### Gut model

We have described previously the use of a triple-stage chemostat human gut model to study the interplay between antimicrobial agents, the indigenous gut microflora and *C. difficile*.\textsuperscript{16-17} The gut model was validated against physico-chemical and microbiological measurements from the intestinal contents of sudden death victims,\textsuperscript{24} but is limited by its inability to simulate immunological and secretory events that occur in the human colon in vivo. The gut model comprises three pH-maintained fermentation vessels (pH 5.5 ± 0.2, vessel 1; pH 6.2 ± 0.2, vessel 2; pH 6.8 ± 0.2, vessel 3), top-fed with a growth medium\textsuperscript{18} at a controlled rate (D = 0.015 h<sup>−1</sup>). The gut model is inoculated with a pooled faecal emulsion (~10% w/v in pre-reduced PBS) prepared from *C. difficile*-negative faeces of five healthy elderly (>60 years) volunteers. The faecal donors were in good health and received no antimicrobial therapy for at least 3 months prior to commencement of this study. Gut microflora populations were monitored throughout the experimental duration and enumerated on selective and non-selective agars as previously described.\textsuperscript{16}

Briefly, colonies were identified to genus level on the basis of colony morphology, Gram reaction and microscopic appearance on selective and non-selective agas as follows: fastidious anaerobe agar supplemented with 5% horse blood (total anaerobes and total clastidia); Beeners agar—4.2 g/L Columbia agar, 5 g/L agar technical, 0.5 g/L cysteine HCl and 5 g/L glucose ( bifidobacteria); Bacteroides bile aesculin agar supplemented with 5 mg/L haemin, 10 μL/L vitamin K, 7.5 mg/L vancomycin, 1 mg/L penicillin, 75 mg/L kanamycin and 10 mg/L calistin (*B. fragilis* group); LAMVAB agar—20 g/L agar technical, 52.2 g/L MRS broth, 0.5 g/L cysteine HCl and 20 mg/L vancomycin ( lactobacilli); nutrient agar (total facultative anaerobes); MacConkey agar (lactose-fermenting Enterobacteriaceae (LFE)); KAA supplemented with 10 mg/L nalidixic acid, 10 mg/L aztreonam and 20 mg/L kanamycin (enterococci); Brazier’s CCEY agar supplemented with 2% lysed horse blood, 5 mg/L lysisyme, 250 mg/L cycloserine and 8 mg/L cefoxitin (*C. difficile* spores); and Brazier’s CCEY agar as above and supplemented with 2 mg/L moxifloxacin (*C. difficile* total viable counts). All agar bases were supplied by Oxoild and made according to the manufacturer’s instructions, except where indicated. The presence of *C. difficile* cytotoxin was determined using the Vero cell cytotoxicity assay.\textsuperscript{18}

#### Experimental design

In this study, two gut models were run in parallel. Following inoculation of the faecal slurry, the models were left for a control period of 2 weeks (Figure 1, period A) to allow equilibration of the bacterial populations. *C. difficile* cultures were not diluted prior to inoculation onto Wilkins-Chalgren agar. Non-antimicrobial-containing control agar and agar containing 1% (v/v) DMSO were inoculated at the start and end of the inoculation procedure to ensure organism viability. Inoculated agars were incubated for 48 h at 37°C in an anaerobic environment. MIC endpoints were read as the lowest concentration of antimicrobial agent where there was no apparent growth, disregarding a visible haze of growth or a single colony.
C. difficile cytotoxin titres were monitored for a further 2 weeks with no further interventions (Figure 1, period F).

Determination of antimicrobial concentrations

Antimicrobial concentrations achieved in each of the vessels of the gut model were determined using a microbiological bioassay. Aliquots of gut model fluid (1 mL) were removed daily from each vessel during the experiments so that retrospective analyses could be performed. Samples for bioassay were centrifuged (15 min, 16000 × g) and the supernatants sterilized by filtration through 0.22 μm filters and stored at − 20 °C.

Both clindamycin and cadazolid bioassays used Kocuria rhizophila ATCC 9341 as an indicator organism. Briefly, K. rhizophila was inoculated onto nutrient agar and incubated overnight at 37 °C. One millilitre of a standard suspension (turbidity equivalent to that of a 0.5 McFarland standard, 1.7 × 10^7 cfu) in sterile saline was inoculated into 100 mL of molten Mueller–Hinton agar and mixed gently by inversion. Inoculated agars were poured into sterile polystyrene bioassay dishes (245 mm², Nunc) and allowed to set at room temperature for 1 h. Agars were dried at 37 °C for 10 min; following which 25 wells (9 mm diameter) were cut from the agar using a sterilized number 5 cork borer. Wells were inoculated with either antimicrobial calibrators of known concentration (1–64 μg/mL clindamycin and 0.5–512 μg/mL cadazolid) or culture fluid from the gut models.

Additional samples were also assayed for cadazolid concentrations using an HPLC method. To measure the total concentration of cadazolid, 25 μL of gut model fluid was mixed with two volumes of methanol and centrifuged for 5 min at 13 000 rpm. Then 25 μL of the supernatant was mixed 1:1 with 1% (w/v) formic acid before measurement by HPLC (as described later). This method resulted in nearly 100% extraction of the compound from spiked samples (as compared with standard samples dissolved in DMSO). The free soluble concentrations were measured by centrifugation of the samples for 5 min at 13 000 rpm, adding 25 μL of the supernatant to two volumes of methanol, mixing, centrifuging and treating further as for total concentration.

The calibration curve was obtained by spiking blank gut model fluid with different concentrations of cadazolid followed by sample preparation as already detailed, resulting in a linear curve from 0 to 250 mg/L.

HPLC was carried out on an Agilent 1200 system using a Zorbax Eclipse XDB-C18 column (4.6 × 50 mm, 1.8 μm particle size) (Agilent Technologies). Cadazolid was eluted with an acetonitrile/0.05% formic acid gradient (0%–100%). The free soluble concentrations were measured by centrifugation of the samples for 5 min at 13 000 rpm, adding 25 μL of the supernatant to two volumes of methanol, mixing, centrifuging and treating further as for total concentration.

The calibration curve was obtained by spiking blank gut model fluid with different concentrations of cadazolid followed by sample preparation as already detailed, resulting in a linear curve from 0 to 250 mg/L.

Results

Susceptibility testing

The MICs of the antimicrobial agents evaluated in this study are shown in Table 1. Cadazolid was active against all C. difficile strains tested, with an MIC₉₀ of 0.125 mg/L (range 0.03–0.25 mg/L). Cadazolid geometric mean (GM) MICs for the entire C. difficile panel were 152–fold, 16–fold, 9–fold and 7–fold lower than the corresponding MICs for moxifloxacin, linezolid, metronidazole and vancomycin, respectively. Cadazolid demonstrated good anti-microbial activity against all C. difficile isolates with reduced susceptibility to metronidazole. The GM MIC of cadazolid for C. difficile PCR ribotype 001 isolates with reduced susceptibility to metronidazole was 0.07 mg/L (range 0.06–0.125 mg/L) versus 5.28 mg/L for metronidazole. Cadazolid MICs for C. difficile PCR ribotypes 027 and 106 isolates with reduced susceptibility to metronidazole were 0.06–0.125 mg/L, although metronidazole MICs for some of these isolates were lower than initially demonstrated (range 2–4 mg/L) raising questions as to whether these strains are truly showing reduced susceptibility. In addition, cadazolid demonstrated potent activity (MICs 0.06 and 0.03 mg/L) against two C. difficile isolates with mutations in gyrA/gyrB, and against the two linezolid-resistant isolates (MICs 0.25 and 0.06 mg/L).

In vitro human gut model

Equilibration period and internal C. difficile control period

Viable counts of gut microflora populations were similar in vessels 2 and 3 of both models. As conditions in vessel 3 most resemble the in vivo conditions of the distal colon, only vessel 3 data are presented here (Figures 2 and 3). In both models, viable counts were dominated by obligate anaerobes, particularly Bifidobacterium spp. and B. fragilis group, with lower viable counts of facultative anaerobe bacterial groups. C. difficile was not detected during the equilibration period (period A), and remained as spores during the internal control.
Effect of clindamycin instillation on gut microflora populations

The results following clindamycin instillation were similar in both models and reflected previous observations (Figures 2 and 3). The most marked effect was on bifidobacteria populations, with viable counts decreasing in both models by $\approx 2\log_{10}$ cfu/mL to below the limit of detection by the end of clindamycin instillation (period C). Populations of other organisms remained relatively stable during clindamycin instillation, with small reductions in lactobacilli ($\approx 2\log_{10}$ cfu/mL in the 250 mg/L twice daily model and $\approx 1\log_{10}$ cfu/mL in the 750 mg/L twice daily model). Substantial decreases in B. fragilis group viable counts ($\approx 4\log_{10}$ cfu/mL in the 250 mg/L twice daily model and $\approx 2\log_{10}$ cfu/mL in the 750 mg/L twice daily model) were observed following cessation of clindamycin instillation, while increases in facultative anaerobe populations occurred in the same period, most notably for enterococci ($\approx 4\log_{10}$ cfu/mL in both models). Lactose fermenters also increased ($\approx 3\log_{10}$ cfu/mL in both models). Bacterial groups deleteriously affected by clindamycin instillation largely recovered by the end of period D (Figures 2 and 3).

Effect of clindamycin instillation on C. difficile total viable counts, spore counts and cytotoxin production

C. difficile remained quiescent during clindamycin instillation in both gut models; populations were comprised principally of spores, and cytotoxin was undetectable. Following the cessation of clindamycin instillation, C. difficile spore germination, proliferation of vegetative cells and cytotoxin production was observed, reflecting prior studies performed in the gut model.\textsuperscript{20,22,23,27} In the 250 mg/L twice daily gut model, C. difficile viable counts remained dominated by spores until 10 days after cessation of clindamycin instillation (day 37; Figure 4a). C. difficile total viable counts then increased over spore counts, indicating that germination of C. difficile spores had occurred (Figure 4). In the 750 mg/L twice daily gut model, C. difficile viable counts remained dominated by spores until 13 days after cessation of clindamycin instillation (day 40; Figure 4b), when increased C. difficile total viable counts over spore counts were observed. C. difficile cytotoxin was detected $\approx 2$ days after spore germination (Figure 4) of both gut models, and titres rapidly increased to 4 RU. Instillation of cadazolod commenced on days 43 and 45 in the 250 mg/L twice daily and 750 mg/L twice daily regimens, respectively. Vessel 2 counts reflected vessel 3 counts in both models. Some evidence of C. difficile spore germination and subsequent proliferation was observed in vessel 1 of both gut models at the end of period D, but cytotoxin was not detected (data not shown). This is consistent with previous observations,\textsuperscript{22,28} although low-level toxin production has occasionally been detected in vessel 1.\textsuperscript{18}

Clindamycin concentrations detected in the gut models

Clindamycin was detected in both models at levels within the range of those previously observed; peak concentrations were 35.1 and 62.3 mg/L in vessel 3 of the 250 mg/L twice daily and 750 mg/L twice daily models, respectively. In vessel 3, clindamycin was undetectable 5 and 6 days, respectively, after the end of the period (period B). All bacterial groups increased during the steady-state period, compared with those observed on the first day of the experiment, but stabilized before the end of period B.

<table>
<thead>
<tr>
<th>Number of isolates</th>
<th>CDZ</th>
<th>MXF</th>
<th>LZD</th>
<th>MTZ</th>
<th>VAN</th>
</tr>
</thead>
<tbody>
<tr>
<td>All CD strains</td>
<td>100</td>
<td>0.1, 0.125, 0.125</td>
<td>15.24, 32, 32</td>
<td>1.66, 2, 2</td>
<td>0.93, 1, 4</td>
</tr>
<tr>
<td>Genotypically distinct</td>
<td>32</td>
<td>0.11, 0.125, 0.125</td>
<td>3.59, 2, 32</td>
<td>1.96, 2, 2</td>
<td>0.27, 025, 1</td>
</tr>
<tr>
<td>All MTZ RS</td>
<td>30</td>
<td>0.08, 0.06, 0.125</td>
<td>32, 32, 32</td>
<td>1.55, 2, 2</td>
<td>3.32, 4, 8</td>
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<tr>
<td>MTZ RS 001</td>
<td>10</td>
<td>0.07, 0.06, 0.125</td>
<td>32, 32, 32</td>
<td>1.41, 1, 2</td>
<td>5.28, 4, 8</td>
</tr>
<tr>
<td>MTZ RS 027</td>
<td>10</td>
<td>0.08, 0.06, 0.125</td>
<td>32, 32, 32</td>
<td>1.41, 2, 2</td>
<td>2.46, 2, 4</td>
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<tr>
<td>MTZ RS 106</td>
<td>10</td>
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<td>32, 32, 32</td>
<td>1.87, 2, 2</td>
<td>2.83, 2, 4</td>
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<tr>
<td>All MTZ S</td>
<td>33</td>
<td>0.11, 0.125, 0.125</td>
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<td>1.59, 2, 2</td>
<td>0.96, 1, 1</td>
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<td>0.12, 0.125, 0.125</td>
<td>32, 32, 32</td>
<td>1.66, 2, 2</td>
<td>1.07, 1, 1</td>
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<tr>
<td>MTZ RS CD ribotype 010</td>
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<td>0.06–0.125</td>
<td>32</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>B. fragilis ATCC 25285</td>
<td>1</td>
<td>2</td>
<td>0.25</td>
<td>1–2</td>
<td>0.25</td>
</tr>
<tr>
<td>S. aureus ATCC 29213</td>
<td>1</td>
<td>0.125</td>
<td>0.125</td>
<td>2</td>
<td>NR</td>
</tr>
<tr>
<td>E. faecalis ATCC 29212</td>
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<td>0.125</td>
<td>0.25</td>
<td>2</td>
<td>NR</td>
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<tr>
<td>MXF-resistant CD</td>
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<td>0.12</td>
<td>2</td>
<td>16</td>
<td>0.25</td>
</tr>
<tr>
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<td>1.07, 1, 1</td>
</tr>
<tr>
<td>MTZ RS CD ribotype 010</td>
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<td>0.06–0.125</td>
<td>32</td>
<td>1</td>
<td>8</td>
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<tr>
<td>B. fragilis ATCC 25285</td>
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<td>2</td>
<td>0.25</td>
<td>1–2</td>
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<tr>
<td>S. aureus ATCC 29213</td>
<td>1</td>
<td>0.125</td>
<td>0.125</td>
<td>2</td>
<td>NR</td>
</tr>
<tr>
<td>E. faecalis ATCC 29212</td>
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<td>0.125</td>
<td>0.25</td>
<td>2</td>
<td>NR</td>
</tr>
<tr>
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<td>64</td>
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<td>0.5</td>
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<td>1</td>
<td>2</td>
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<tr>
<td>Gut model 027 UK</td>
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<td>0.03</td>
<td>32</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>Gut model 106 UK</td>
<td>1</td>
<td>0.13</td>
<td>32</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 1. GM MIC, MIC$50$ and MIC$90$ values of cadazolid (CDZ), moxifloxacin (MXF), linezolid (LZD), metronidazole (MTZ) and vancomycin (VAN)
instillation period in the 250 mg/L twice daily and 750 mg/L twice daily models (data not shown).

Effect of cadazolid instillation on gut microflora populations

The overall inhibitory effect of both cadazolid dosing regimens against the enumerated indigenous gut microflora was limited, although some bacterial groups were more markedly affected (Figures 2 and 3). Modest decreases in the viable counts of enterococci were observed for both dosing regimens, while only the higher cadazolid instillation dose of 750 mg/L twice daily reduced viable counts of LFE and lactobacilli. Interestingly, total Clostridium spp. initially appeared more deleteriously affected by 250 mg/L twice daily than 750 mg/L twice daily, but viable counts in both models had decreased by ~2 log₁₀ cfu/mL by the end of the experiments (period F). Bifidobacteria were adversely affected by the instillation of both dosing regimens of cadazolid, declining to below the limits of detection by the end of the dosing period (period E) and not recovering for the remainder of the experiment. Neither dosing regimen was inhibitory to B. fragilis group populations.

Effect of cadazolid instillation on C. difficile total viable counts, spore counts and cytotoxin production

Vegetative forms of C. difficile were markedly inhibited by the instillation of both dosing regimens of cadazolid (Figure 4), with a
decrease of $\approx 5 \log_{10} \text{cfu/mL}$ in viable count observed in both models by the end of the cadazolid instillation period (period E). *C. difficile* cytotoxin titres also declined rapidly, with low titres ($\leq 1 \text{RU}$) observed by the end of the dosing period. Following the cessation of cadazolid instillation, *C. difficile* was sporadically isolated at the limits of detection, with no evidence of recurrent spore germination, proliferation or cytotoxin production.

**Cadazolid concentrations detected in the gut models**

Active concentrations of cadazolid measured by bioassay were similar in both of the gut models, despite the 3-fold higher concentration of antimicrobial agent instilled into the 750 mg/L twice daily gut model. Mean concentrations (± SEM) in vessel 3 were 4.10 mg/L (0.87) in the 250 mg/L model and 3.68 mg/L (0.88) in the 750 mg/L model and peaked at $\approx 10.25$ and $\approx 21.5$ mg/L, respectively. In both gut models, markedly lower concentrations of cadazolid were detected in vessel 1 compared with vessels 2 and 3. Cadazolid persisted in vessels 2 and 3 of both gut models until the end of the experiments, and was still detectable 14 days after cessation of its instillation at supra-MIC levels (cadazolid MIC = 0.06 mg/L).

Results obtained by HPLC analysis of clear supernatants of the samples were generally similar to those obtained by the bioassay method (data not shown), whereas total concentrations as measured by HPLC were much higher (10-fold to 100-fold). Interestingly, the higher dose did not result in a higher cadazolid concentration.
in the supernatants, but the higher dose resulted in generally higher total concentrations of cadazolid (up to 120 mg/L) compared with the lower dose (up to 80 mg/L), and the concentrations declined in the post-treatment phase.

**Isolation of bacteria on breakpoint agars**

No *C. difficile* or enterococci were isolated from breakpoint agars containing cadazolid or linezolid, respectively, for the duration of the experiment. LFE and non-LFE were isolated from both ciprofloxacin and nalidixic acid breakpoint agars throughout the experiment, even prior to instillation of cadazolid (data not shown). No evidence of increased resistance to ciprofloxacin, nalidixic acid or cadazolid was detected in LFE or non-LFE recovered during or following the cadazolid instillation period (data not shown).

**Discussion**

Cadazolid has potent activity against *C. difficile*, indicating its potential as a novel CDI treatment agent.\textsuperscript{12,13,15} The MIC data presented here indicate that against a panel of 100 clinically relevant *C. difficile* isolates, cadazolid has 7-fold to 152-fold superior activity compared with other antimicrobials, including metronidazole (9-fold higher activity) and vancomycin (7-fold higher activity). In addition, cadazolid retained potent activity (MIC $\leq$ 0.125 mg/L) against the 30 strains showing reduced susceptibility to metronidazole.

In the *in vitro* gut model, cadazolid instillation of both dosing regimens rapidly reduced vegetative cell counts and cytotoxin titres, which declined to the limit of detection by the end of the 7 day dosing period, and showed no sign of CDI recurrence. There
was no difference in the efficacy of 250 mg/L twice daily or 750 mg/L twice daily dosing regimens in resolving simulated CDI, both of which were comparable to vancomycin treatment as determined in previous gut model experiments.20,22,27,29 Metronidazole has previously been demonstrated to be ineffective in treating simulated CDI in the gut model.21 It is tempting to speculate that the lack of recurrent vegetative growth and toxin production observed following cadazolid treatment may indicate a reduced risk of recurrent CDI compared with conventional treatment agents. However, as the models were only monitored for 2 weeks following cadazolid instillation these observations should be interpreted with caution. Previous observations of recurrent vegetative growth and toxin production following vancomycin instillation have occurred both before22 and after23 this 14 day cut-off point, or not at all.29 Cadazolid concentrations remained at supra-MIC (>0.06 mg/L) levels for the 14 day post-instillation period, which would have prevented the outgrowth of any spores during this period, thereby preventing the recurrence of simulated CDI.

The approximate 10–100-fold difference in solubile and total cadazolid concentrations measured by HPLC suggests that a large proportion of the drug within the model remains insoluble. The higher dosing regimen resulted in a higher total, but not soluble, concentration of cadazolid, suggesting that ‘drug saturation’ may occur. The total cadazolid concentration decreased following the cessation of instillation, whereas the soluble concentration did not. Slow release of cadazolid from insoluble to soluble phases after cessation of dosing may explain the persistence of antibiotic activity as measured by the bioassay. These theoretical explanations require further study, but do point to a potential advantageous pharmacodynamic effect of cadazolid.

Cadazolid instillation was sparing of the enumerated gut microflora with the exception of bifidobacteria. Slight decreases in total clostridia and enterococci populations were also observed, although the higher cadazolid concentration regimen did not appear to have a greater inhibitory effect on microflora populations than the 250 mg/L twice daily regimen. This may be due to the drug saturation discussed earlier. If drug saturation was achieved by both dosing regimens, and only the soluble cadazolid was active, the effects following cadazolid instillation would be expected to be similar in both models. The persistence of detectable cadazolid activity did not appear to increase the adverse effect on gut microbrial populations. Enterococci populations appeared unaffected by cadazolid, despite the mean active concentration of ~4 mg/L being higher than the MIC for the control E. faecalis strain (Table 1), indicating that the majority of enterococci within the model were less susceptible to cadazolid than this control strain. Cadazolid instillation had little effect on B. fragilis group populations, whereas vancomycin instillation has been shown to be highly deleterious to B. fragilis in previous gut model experiments,22,27 and by other research groups.30 The significance of this is not clear, but the limited inhibitory effects of cadazolid on the gut microflora population may help to protect against recurrent infection due to colonization resistance.

Cadazolid is a novel oxazolidinone-type antibiotic with a quinoline side chain. We have previously investigated the effects of fluoroquinolones26 and linezolid (an oxazolidinone)23 in the in vitro gut model. Fluoroquinolones (ciprofloxacin, levofloxacin and moxifloxacin) induced CDI and had a marked deleterious effect on the gut flora, particularly bifidobacteria, Bacteroides spp., enterococci and lactobacilli. Unlike for cadazolid, detected levels of active fluoroquinolone (measured by bioassay) were similar to levels instilled and fell below the limit of detection 6–10 days after the end of instillation. Linezolid, given after induction of CDI, elicited fewer changes in gut microflora populations than the fluoroquinolones, but had a greater effect than cadazolid. The reasons for the reduced activity of the two antimicrobial moieties against the normal gut flora when combined are unclear and warrant further investigation. It is plausible that coupling two antibiotic moieties will not result in an entirely additive spectrum for the new molecule; e.g. steric hindrance at active sites or pharmacodynamic issues could account for the difference in activity of parent versus constituent molecules. Interestingly, active linezolid concentrations detected by bioassay were approximately half the expected levels, but levels detected by HPLC were similar to those expected. Notably, the persistence of active antimicrobial following cadazolid instillation was not observed for linezolid. The results of previous gut model studies, evaluating both the propensity of antimicrobials to induce CDI19,31,32 and the efficacy of various CDI treatment regimens,20,22,23,33 have correlated well with clinical observations in vivo, and so the present results suggest that cadazolid has potential as a CDI treatment agent. Recently, the clinical efficacy of cadazolid was shown in Phase 2 trials of CDI.14

The data presented here indicate that cadazolid has potent activity against clinically relevant C. difficile strains and is effective in treating clindamycin-induced CDI caused by a C. difficile PCR ribotype 027 strain in an in vitro gut model, while having modest effects on gut microflora populations. Such observations, especially taken alongside the observed persistence of cadazolid after the cessation of dosing, may be beneficial in terms of the likelihood of CDI recurrence. The two dosing regimens evaluated were equally successful in resolving simulated CDI, and neither regimen appeared to select for resistant organisms. Further clinical investigations to determine the potential of this antimicrobial to treat CDI are warranted.

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Supplementary data
Table S1 is available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).
References


