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Impact on toxin production and cell morphology in *Clostridium difficile* by ridinilazole (SMT19969), a novel treatment for *C. difficile* infection

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Objectives: Ridinilazole (SMT19969) is a narrow-spectrum, non-absorbable antimicrobial with activity against *Clostridium difficile* undergoing clinical trials. The purpose of this study was to assess the pharmacological activity of ridinilazole and assess the effects on cell morphology.

Methods: Antibiotic killing curves were performed using the epidemic *C. difficile* ribotype 027 strain, R20291, using supra-MIC (4× and 40×) and sub-MIC (0.125×, 0.25× and 0.5×) concentrations of ridinilazole. Following exposure, *C. difficile* cells were collected for cfu counts, toxin A and B production, and morphological changes using scanning electron and fluorescence microscopy. Human intestinal cells (Caco-2) were co-incubated with ridinilazole-treated *C. difficile* growth medium to determine the effects on host inflammatory response (IL-8).

Results: Treatment at supra-MIC concentrations (4× and 40× MIC) of ridinilazole resulted in a significant reduction in vegetative cells over 72 h (4 log difference, $P<0.01$) compared with controls without inducing spore formation. These results correlated with a 75% decrease in toxin A production ($P<0.05$) and a 96% decrease in toxin B production ($P<0.05$). At sub-MIC levels (0.5× MIC), toxin A production was reduced by 91% ($P<0.01$) and toxin B production was reduced by 100% ($P<0.001$), which resulted in a 74% reduction in IL-8 release compared with controls ($P<0.05$). Sub-MIC (0.5×)-treated cells formed filamentous structures ~10-fold longer than control cells. Following fluorescence labelling, the cell septum was not forming in sub-MIC-treated cells, yet the DNA was dividing.

Conclusions: Ridinilazole had robust killing effects on *C. difficile* that significantly reduced toxin production and attenuated the inflammatory response. Ridinilazole also elicited significant cell division effects suggesting a potential mechanism of action.

Introduction

According to the US CDC, the annual incidence of *Clostridium difficile* infection (CDI) exceeds 500 000 hospitalized cases with 29 000 deaths.[1,2] Treatment options for CDI are limited to metronidazole, fidaxomicin and oral vancomycin.[3,4] Morbidity and mortality, including the recurrence of CDI, continues to be a major concern with recurrence rates up to 25% after discontinuation of treatment in patients given metronidazole or vancomycin.[5,6] Moreover, patients with at least one recurrence have a higher probability of experiencing further recurrences.[7] In addition to the significant morbidity and mortality, CDI increases healthcare costs due to extended hospitalizations and re-hospitalizations.[8,9] The pathogenesis of CDI involves secretion of active toxins (A and B) by *C. difficile* with host inflammatory response to these toxins playing a vital role in disease progression. IL-8 and other cytokines directly contribute to the proinflammatory response.[10–12] Although *C. difficile* is largely confined to the colon, CDI is typically associated with a systemic inflammatory reaction marked by leucocytosis that is, in part, thought to be mediated by toxins A and B disseminating systemically from the gastrointestinal tract.[13,14]

Ridinilazole is a novel, non-absorbable narrow-spectrum antibiotic that is currently in clinical development for the treatment of CDI.[15–17] Ridinilazole has been shown to have potent activity against multiple strains of *C. difficile* and has demonstrated efficacy in both *in vitro* gut and *in vivo* hamster models.[18–21] Although it has been shown to be highly potent against *C. difficile*, the mechanism of action of ridinilazole has not been fully elucidated. This includes the effect of ridinilazole on toxins A and B

and associated changes in the inflammatory response. The purpose of this study was to assess the pharmacological activity of ridinilazole and determine the morphological effects of ridinilazole on *C. difficile*.

Materials and methods

Compounds, bacterial strains and growth media

Ridinilazole was provided by Summit Therapeutics and re-suspended in DMSO (Sigma–Aldrich). *C. difficile* strain R20291 (BI/027/NAP1) was used for all the experiments. All cultures were grown in a vinyl anaerobic chamber (Coy Lab Products) at 37°C. Liquid microbiological cultures were grown in brain–heart infusion (BHI) medium (Hardy Diagnostics) supplemented with 0.1% sodium taurocholate (Alfa Aesar) during time–kill experiments. For colony recovery and counts, bacteria were grown on blood agar plates (Hardy Diagnostics). Metronidazole and vancomycin were purchased from commercial suppliers (Sigma–Aldrich). Caco-2 human gut epithelial cells were grown in Eagle's minimal essential medium (EMEM; ATCC 302003) for measurements of IL-8 release.

Kill kinetics

MICs of ridinilazole, metronidazole and vancomycin were determined for strain R20291 by broth microdilution in 0.1% sodium taurocholate BHI medium. Supra-MICs were used to mimic physiological concentrations and sub-MICs to assess the array of efficiency and efficacy of the drug. For experiments, cultures of *C. difficile* strain R20291 were prepared by inoculating one isolated colony on blood agar plate into BHI medium. After 24 h incubation at 37°C in an anaerobic chamber, cultures were diluted 1:100 to ~10^6 cfu/mL in fresh BHI medium supplemented with 0.1% sodium taurocholate and the appropriate concentration of antibiotic (0 h). Total viable counts were determined at 0, 24, 48 and 72 h of antibiotic treatment. One mL of culture was supplemented with 100 μL of killed yeast solution and centrifuged for 1 min at 10000 rpm.[22] The supernatant was removed and the pellet resuspended in fresh BHI medium. Aliquots (100 μL) of sample serial dilutions were spread onto blood agar plates in duplicate and incubated at 37°C in an anaerobic chamber for 48 h prior to colony counting. Spore viability was determined at 0 and 24 h. Total samples were centrifuged at 10000 rpm for 1 min and re-suspended in 100 μL of isopropanol to kill vegetative cells. Spore samples were then diluted and 100 μL was spread onto blood agar plates in duplicate and incubated for 48 h at 37°C in an anaerobic chamber prior to colony counting. The limit of detection for these assays was 500 cfu/mL.

Sample preparation for microscopy

Five mL of each sample from time–kill studies was centrifuged for 5 min at 5000 rpm. The pellets were resuspended in 200 μL of 4% paraformaldehyde and incubated for 1 h at room temperature. The samples were centrifuged again for 1 min at 10000 rpm, resuspended in 50 μL of H_2O and stored at 4°C prior to microscopy experiments.

Scanning electron microscopy

Samples were incubated for 30 min on poly-L-lysine-coated coverslips and allowed to dry. Twenty nanometres of gold was applied to the sample for coating using a Denton Desk II Sputtering System (Denton Vacuum). Coverslips were transferred to a FEI XL-30FEG scanning electron microscope (FEI). Working distance was set to 5 mm and high-resolution mode was used to image the cells. Images were taken at 15.0 kV at designated magnifications. After acquiring the images, the image files were opened in FIJI (http://fiji.sc/Welcome). Scale bars were calibrated and cell length was determined using the multi-measure function.

Confocal microscopy

The cells were stained for 1 h with 500 μg/mL FM4-64 (Life Technologies) in DMSO. Cells were washed twice with PBS and then re-diluted in PBS before being mounted on slides in mounting medium containing DAPI (Vector Labs). The slides were then transferred to a laser-scanning confocal microscope (LEICA TCS SP8) and images were taken using 405 nm and 560 nm lasers and a ×63 objective lens. Images files were opened in FIJI (http://fiji.sc/Welcome) and analysed using the cell counter function.

Toxin A and B concentration

Toxin A and B levels were measured by a commercially available ELISA with the *C. difficile* Toxin A or B quanti kit (tgcBIOMICS), according to the manufacturer's instructions. Limit of detection for toxin A or B levels was 0.31 ng/mL.

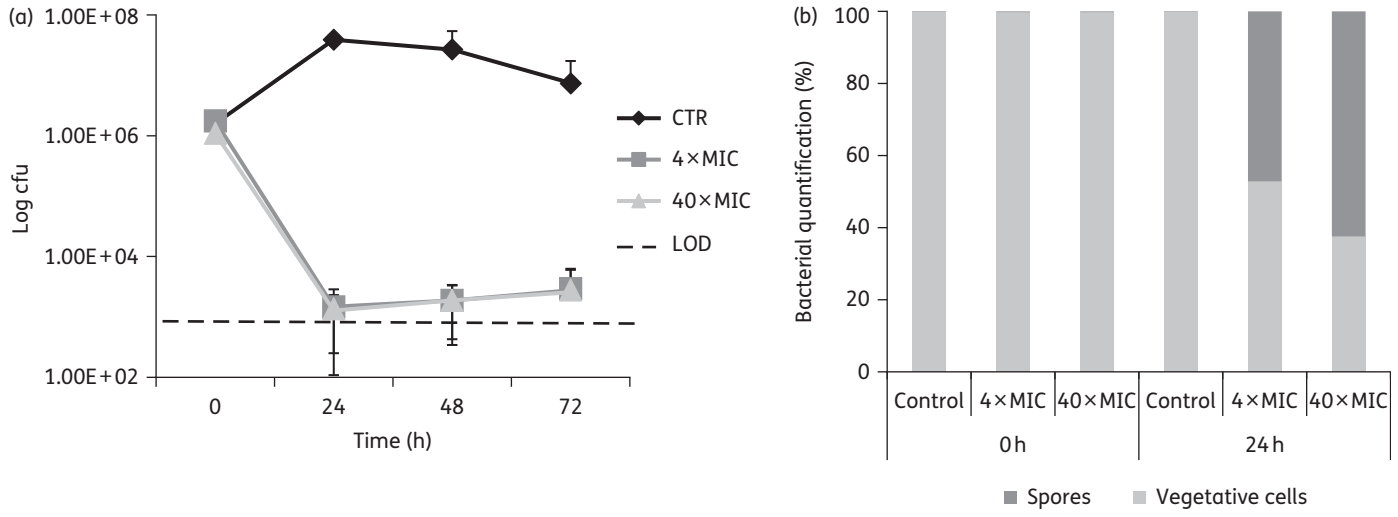

Figure 1. (a) Time–kill kinetics of ridinilazole against *C. difficile* strain 20291 at 4× and 40× MIC (mean values of two experiments ±SEM). CTR, control or non-treated bacterial experiments; LOD, limit of detection. (b) Spores/vegetative cells ratio at 0 and 24 h (mean values of two experiments). Error bars represent standard errors.

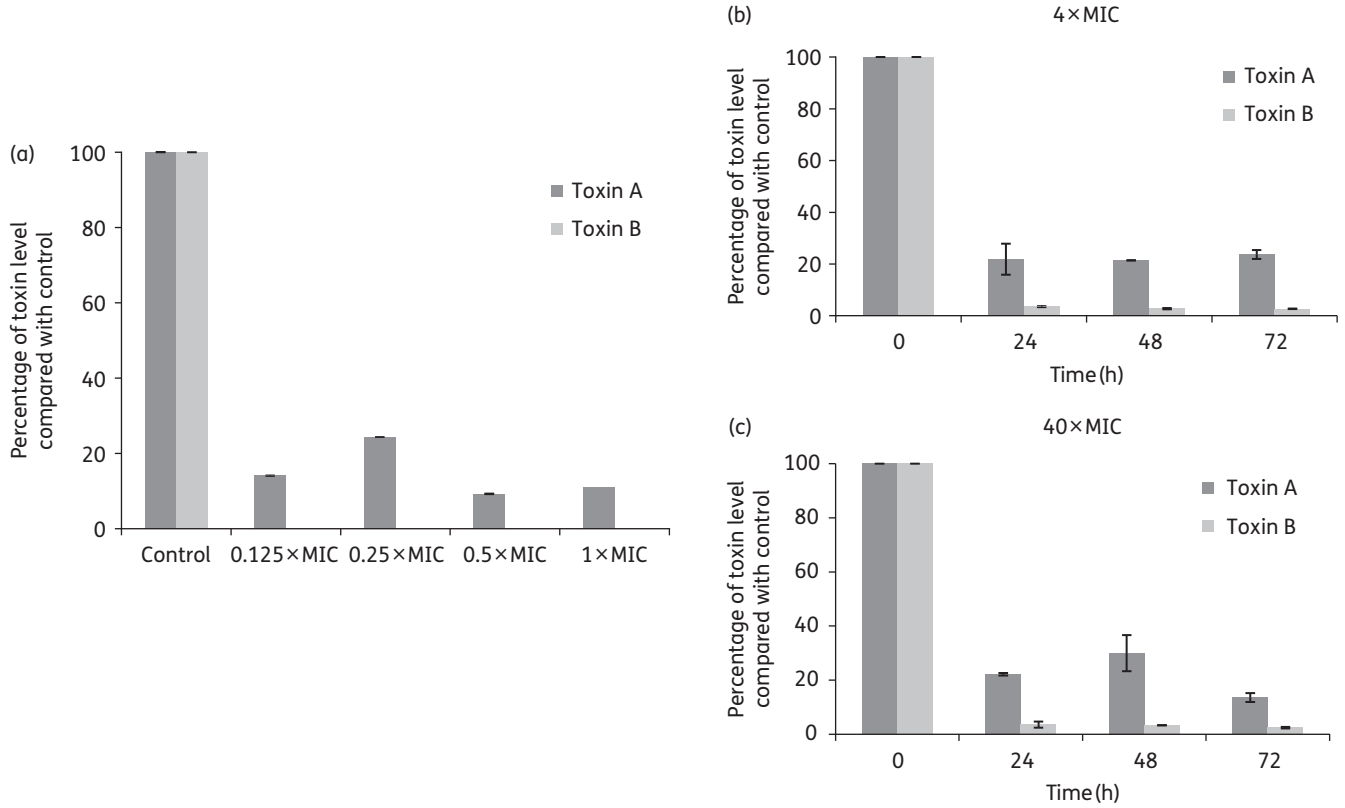

Figure 2. Measurements of toxin A and B levels at sub-MIC (0.125×, 0.25× and 0.5× MIC) and MIC concentrations (a) and supra-MIC (4× and 40× MIC) concentrations (b and c, respectively) after 24 h of exposure to ridinilazole against *C. difficile* strain R20291. Error bars represent standard errors.

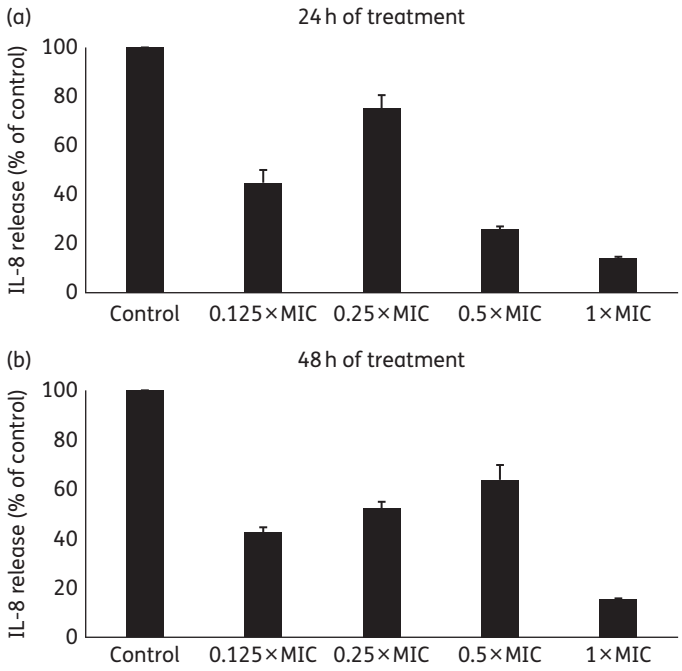

Figure 3. IL-8 concentration from Caco-2 cells treated with supernatants of ridinilazole-treated time–kill samples at 24 h (a) and 48 h (b). Error bars represent standard errors.

Eukaryotic cell culture

Caco-2 cells (ATCC) were maintained and propagated in EMEM with 10% FBS (Hyclone), 100 U/mL penicillin G and 100 μg of streptomycin (Life Technologies) at 37°C and in 5% CO_2, according to the cell line supplier's instruction manual. All assays were carried out between cell passage numbers 5 and 15. In a 24-well plate, the cells were seeded and allowed to grow for 2 weeks to reach 95% confluence in the presence of complete medium to become two-dimensionally polarized before adding toxin. Medium was changed every other day with 20% FBS complete medium during the two-dimensional cell polarization event.

Inflammatory response determination

From the time–kill experiments, supernatants were collected and filtered at the indicated timepoints. Caco-2 cells were washed twice with PBS and 600 μL of EMEM was used as medium before adding 300 μL of filtered supernatant in duplicate. After a 24 h co-incubation, the supernatants from the Caco-2 cells were harvested and IL-8 release was quantified via a commercially available IL-8 ELISA kit (ThermoFisher Scientific), according to the manufacturer's instructions. The limit of detection for IL-8 levels was 5 pg/mL.

Experimental set-up and statistical analyses

For all sub-MIC experiments, the study drug was added at the same time as the initial inoculation of the one isolated colony on blood agar plate into BHI medium. The antibiotic and strain were then co-incubated for the next 24 h during an overnight culture prior to initial cfu counts and other experiments. For all supra-MIC experiments, the study drug was added after an

overnight culture was grown in the absence of antibiotics. Statistical analysis was performed using SPSS statistics software, version 22 (IBM). Data are expressed as the mean ± SEM. Data were assessed for significance using a Student's *t*-test or one-way ANOVA as appropriate. $P<0.05$ was considered significant.

Results

Killing kinetics

For the *C. difficile* strain R20291 the MIC of ridinilazole was 0.06 mg/L, the MIC of metronidazole was 0.48 mg/L and the MIC of vancomycin was 0.96 mg/L. Supra-MIC killing kinetics showed that ridinilazole had a bactericidal effect at 4× and 40× MIC with a $\geq$3 $\log_{10}$ reduction in cfu compared with control by 24 h (Figure 1a). No recovery of growth was observed at 48 and 72 h post-inoculation. Spore recovery was also quantified at 0 and 24 h post-infection at supra-MIC concentrations and were found present in <1% of the total population in all conditions at the start of the experiment (0 h) with no changes observed in control experiments at 24 h. Although the proportion of spores increased (Figure 1b), the absolute number of spores remained constant (data not shown) indicating that ridinilazole did not induce sporulation of *C. difficile*.

Toxin production and IL-8 release

The effect of ridinilazole on the production of toxins A and B after 24 h of exposure is shown in Figure 2. Toxins A and B were both present in all control experiments. Toxin B was not detected at any concentration of ridinilazole, including sub-MIC concentrations (0.125×−0.5× MIC) during the first 24 h of growth (−Δ100%; $P<0.001$). Toxin A concentrations were reduced 80%–90% ($P<0.01$) compared with control at all sub-MIC concentrations (Figure 2a). In supra-MIC experiments, adding ridinilazole at 4× or 40× MIC after 24 h of growth reduced toxin A and B by 75% to >90% at 24, 48 and 72 h compared with control ($P<0.05$ for each; Figure 2b and c).

Caco-2 cells were exposed to *C. difficile* culture supernatants following sub-MIC exposure to ridinilazole for 24 h. Following 24 h of exposure to ridinilazole at 0.5× MIC, a 74% reduction in IL-8 release compared with drug-free controls was observed ($P<0.05$). At 0.125× MIC and 0.25× MIC IL-8 reductions of 55% and 25% were recorded, respectively (Figure 3a and b).

Comparison of results with metronidazole and vancomycin

The effect of exposure to ridinilazole, vancomycin and metronidazole at 0.5× MIC on cfu change (Figure 4a), toxin A and B concentrations (Figure 4b) and IL-8 concentrations (Figure 4c) is shown. Metronidazole and vancomycin were shown to have a minimal

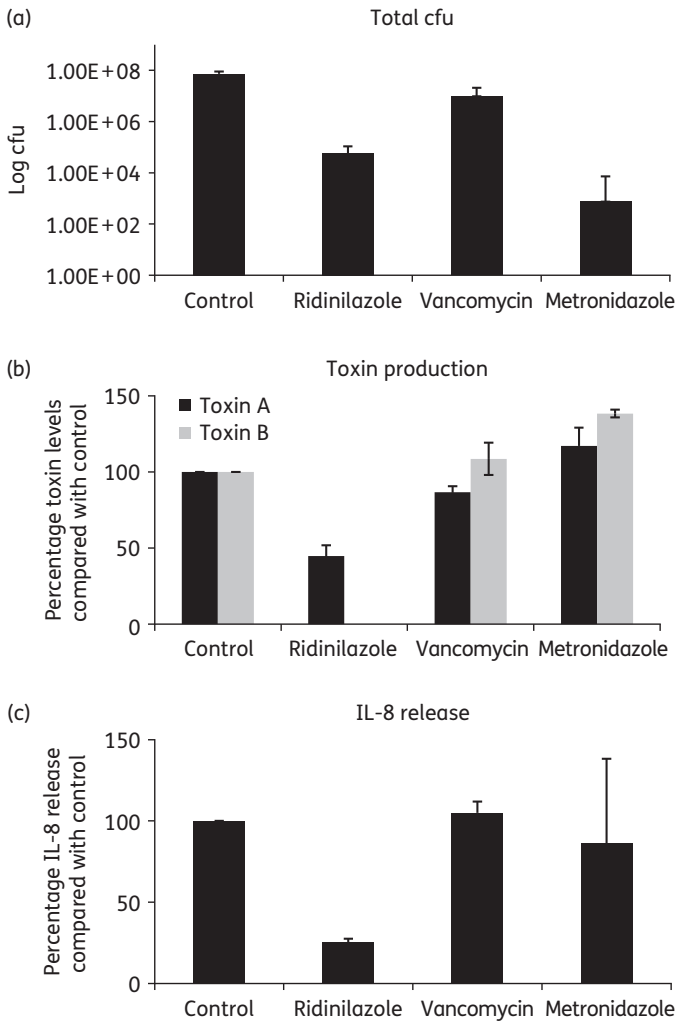

Figure 4. cfu (a), toxins A and B (b) and IL-8 concentrations from Caco-2 exposed cells (c) after 24 h of exposure to 0.5× MIC of ridinilazole, vancomycin or metronidazole. Error bars represent standard errors.

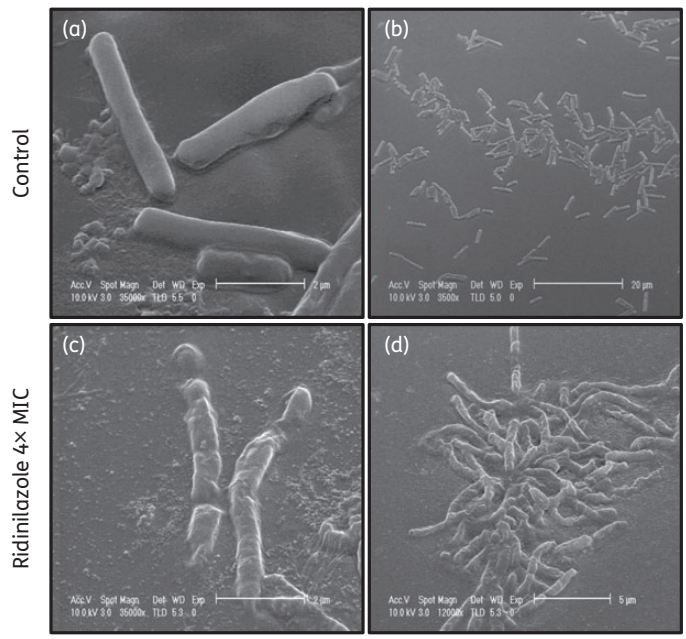

Figure 5. Scanning electron microscopy pictures of control cells (upper panels) at ×35000 (a) and ×3500 (b) magnifications. Scanning electron microscopy pictures of cells treated with 4× MIC (lower panels) at ×35000 (c) and ×12000 (d) magnifications. All pictures were taken 6 h after the start of the time–kill kinetics.

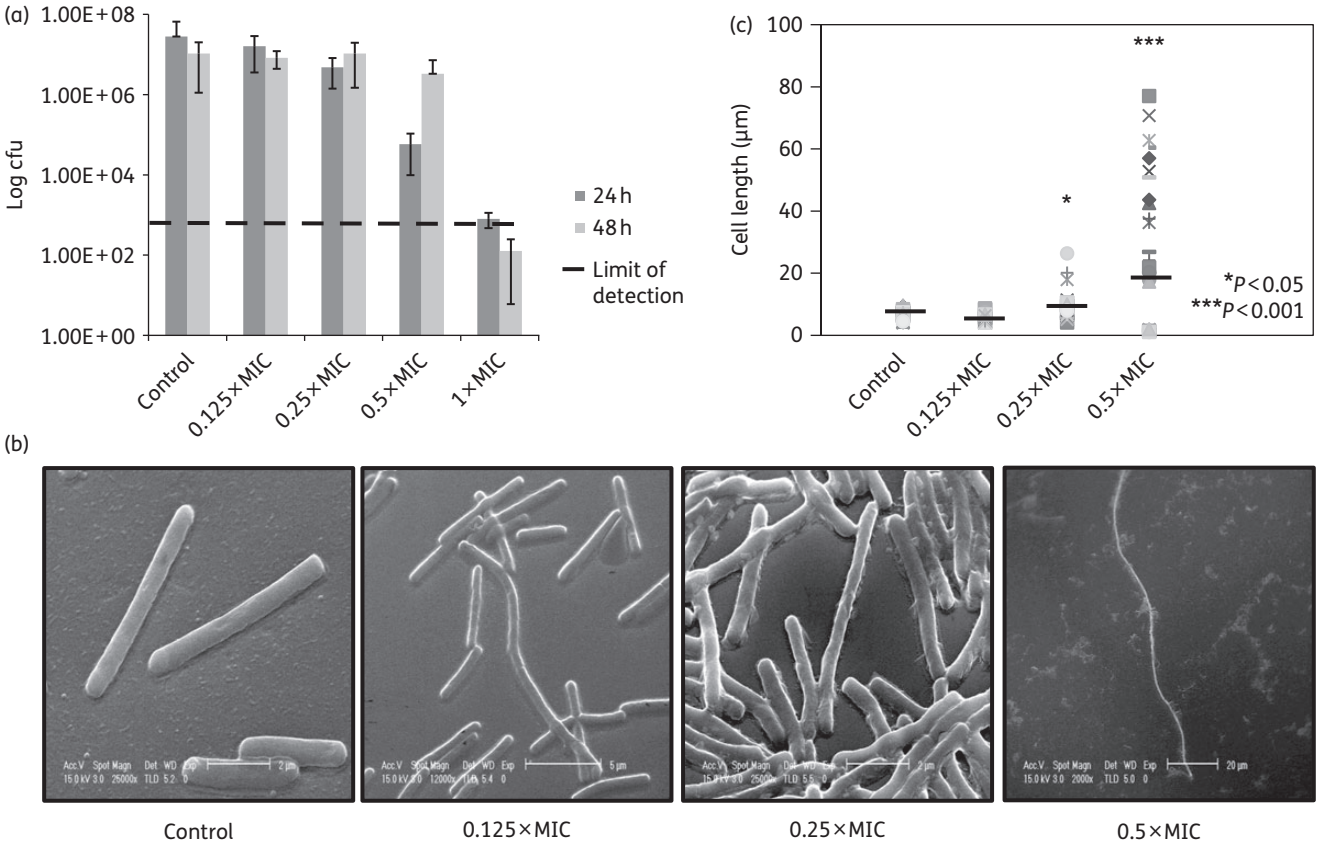

Figure 6. (a) 48 h time–kill kinetics of ridinilazole against *C. difficile* strain R20291 at 0.125×, 0.25×, 0.5× and 1× MIC (mean values of two experiments ±SEM). (b) Scanning electron microscopy pictures of control cells and cells treated with sub-MIC concentrations from time–kill kinetic experiments. Pictures were taken at the 24 h timepoint; magnification ×35 000 for control, ×25 000 for 0.125× MIC, ×12 000 for 0.25× MIC and ×2000 for 0.5× MIC. (c) Quantification of cell lengthening upon treatment with ridinilazole. Error bars represent standard errors.

effect on toxin A or B concentrations, whereas ridinilazole demonstrated significant efficacy (Figure 4b). In addition, IL-8 concentrations from Caco-2 cells exposed to vancomycin or metronidazole were similar to that of control (Figure 4c).

Cell morphology

At supra-MIC concentrations, the cell morphology was severely perturbed after 24 h of treatment (Figure 5). Most of the cells were destroyed (Figure 5c) or forming aggregates (Figure 5d) compared with control cells (Figure 5a and b). At sub-MIC concentrations, a dose-dependent effect on cell length occurred at 24 h of treatment from 0.25× MIC and higher (Figure 6a). Scanning electron microscopy images demonstrate an effect on cell length from 0.125× MIC that was even more dramatic at 0.25× and 0.5× MIC (Figure 6b). Cell length ranged from 4 μm in control (left-hand panel of Figure 6b) to ∼100 μm at 0.5× MIC (right-hand panel of Figure 6b). Cell lengthening was quantified and shown in Figure 6(c). A significant increase in cell length was observed from 0.25× MIC ($P<0.05$), from 7 μm on average for control to 9 μm at 0.25× MIC and 20 μm at 0.5× MIC ($P<0.001$). These results were statistically significant despite the heterogeneity in size (from 1 to 100 μm at 0.5× MIC). This could be due to the presence of cell debris from killed cells at 0.5× MIC. To characterize this cell-lengthening phenotype further, bacterial DNA and the cell wall were stained and visualized on a confocal microscope (Figure 7a). While control cells displayed DNA division and septum formation (upper panels), DNA from treated cells was dividing, yet there was no septum formation (lower panels). This absence was observed starting at 0.125× MIC with a greater effect noted at 0.5× MIC (Figure 7b). Cumulatively, these results suggest that ridinilazole may alter *C. difficile* cell division or proliferation.

Discussion

C. difficile is a spore-forming, Gram-positive, anaerobic organism that is transmitted from person-to-person via the oral–faecal route.[23] Progression to CDI most commonly involves release of toxins A and B by the vegetative cells of *C. difficile* eliciting a host inflammatory response that damages the colonic epithelial lining leading to diarrhoea, formation of pseudomembranes and systemic complications.[24] Colitis induced by *C. difficile* is characterized by a massive influx of neutrophils into the colonic mucosa. The movement of these neutrophils into the site of injury is believed to be a crucial event during the inflammatory process mediated primarily by IL-8 release by the colonocytes.[25,26] In this study, we investigated the pharmacological properties of a novel, narrow-spectrum agent targeting *C. difficile*, ridinilazole.

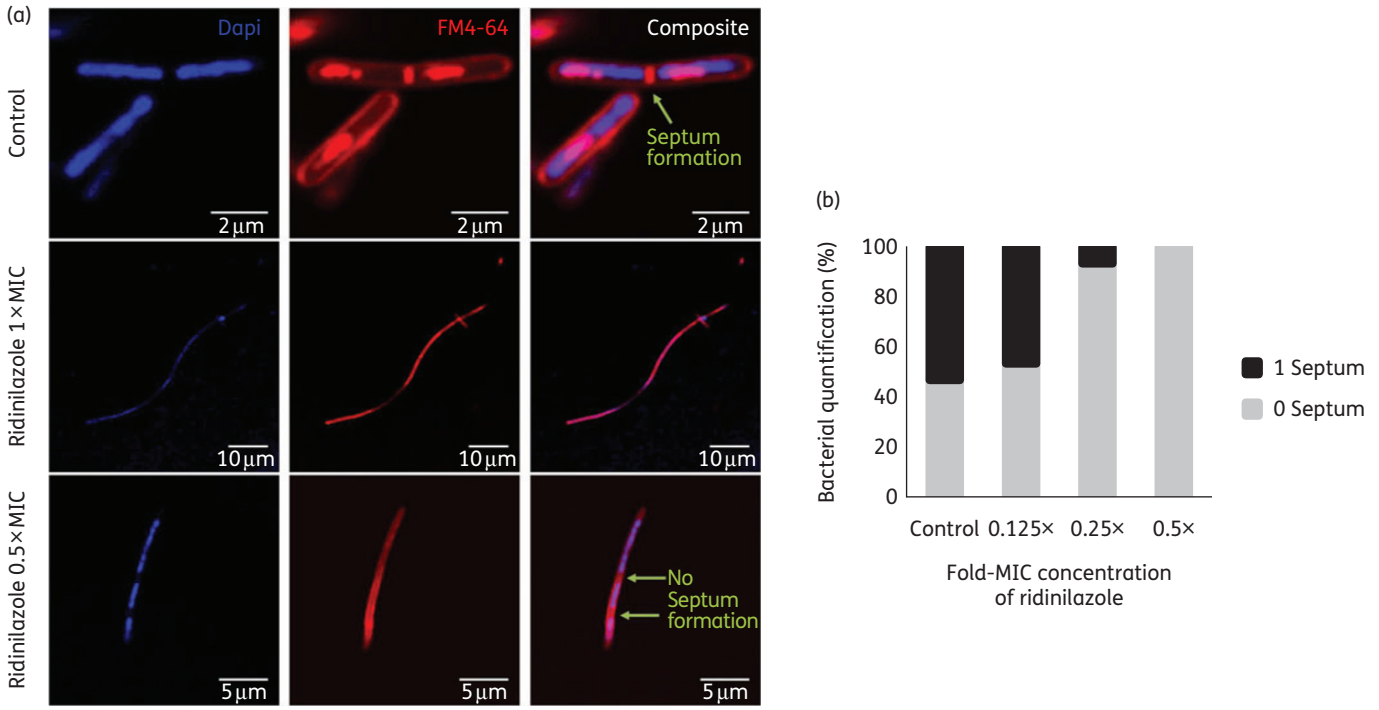

Figure 7. (a) Confocal pictures; DNA (blue, DAPI) and cell membrane (red, FM4-64) at 0.5× and 0.25× MIC for *C. difficile* strain R20291 after 24 h of treatment with ridinilazole. (b) Quantification of septum presence after 24 h of treatment with ridinilazole at sub-MIC concentrations (0.125×, 0.25× and 0.5× MIC). This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Novel findings from this study include the discovery of a marked decrease in toxins A and B production in *C. difficile* isolates exposed to sub-MIC concentrations of ridinilazole leading to decreased IL-8 production in cell lines exposed to ridinilazole-treated *C. difficile* isolates. The other novel finding on assessment of cell morphology following exposure to ridinilazole was that cell division was halted and an absence of septum formation, which may be a direct effect of, or a downstream response to, ridinilazole. Lastly, the potent bactericidal effect of this drug was demonstrated and confirmed results from previous studies.[18]

In this study, toxins A and B concentrations were significantly decreased in *C. difficile* strains grown in the presence of sub-MIC concentrations of ridinilazole. Toxin concentrations also decreased significantly in *C. difficile* strains exposed to supra-MIC concentrations during 24 h. As toxins trigger the inflammatory response (mainly IL-8),[27] ridinilazole-exposed isolates were less bioactive as demonstrated by decreased IL-8 concentrations on exposed cell lines. Collectively, these data provide evidence that ridinilazole has bactericidal properties that reduces toxin levels and decreases the host inflammatory response during *C. difficile* infection. Previous studies have shown that fidaxomicin also inhibits toxin production at sub-MIC concentrations and a comparison of these two agents in the future will be necessary.[28]

To the best of our knowledge, this is the first paper demonstrating morphological changes upon exposure of ridinilazole to *C. difficile* isolates. Using scanning electron microscopy and confocal microscopy, we showed significant cell lengthening in ridinilazole-exposed isolates that was associated with decreased septum formation. A study by Ransom *et al.*[29] identified and characterized midcell localizing division proteins or MLD proteins as essential for *C. difficile* cell division. Using mldABC and mldAB overexpressed mutants, *C. difficile* cells were shown to divide improperly and demonstrated a phenotype similar to what was observed in our ridinilazole-exposed isolates. Whether ridinilazole affects these particular cell division proteins or other proteins involved in the cell division pathway will require further study.

It is unclear the exact mechanisms for the toxin and anti-inflammatory effects observed in this study; to determine if this is a direct or indirect effect mediated by membrane modification or cell division defects will also require further analysis. Likewise, we used a current epidemic 027 strain for these studies. Confirmation of these effects in other strains will be required. Finally, clinical applications taking advantage of these beneficial pharmacological effects will require more thorough investigation.

In conclusion, this study confirmed the bactericidal activities of ridinilazole and discovered decreased toxin A and B concentrations in *C. difficile* strains exposed to ridinilazole, associated with decreased bioactivity and IL-8 concentrations. This anti-inflammatory effect may be important to resolve the main symptoms of CDI. While the mechanism of action of ridinilazole remains to be fully determined, it is apparent that this antibiotic has a promising future for the treatment of CDI.

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Transparency declarations

R. J. V. is an employee of Summit Therapeutics and holds share options. K. W. G. has received research support from Summit Therapeutics and Merck & Co. All other authors: none to declare.

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