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Killing kinetics of fidaxomicin and its major metabolite, OP-1118, against *Clostridium difficile*

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The kinetics of bacterial killing for fidaxomicin and its major metabolite, OP-1118, were investigated against *Clostridium difficile* strains, including two clinical strains belonging to the restriction endonuclease analysis group BI (ORG 1687 and 1698), the ATCC 43255 strain and two laboratory-derived mutant strains with decreased susceptibility to fidaxomicin (ORG 919 and 1620). Both fidaxomicin and OP-1118 demonstrated time-dependent killing of *C. difficile* strains. Fidaxomicin (at 4× MIC) reduced bacterial counts of the ATCC 43255 strain, clinical strain ORG 1687 and the two laboratory-generated mutant strains by $\geqslant$3 logs within 48 h of exposure. The other BI strain, ORG 1698, was tested at 2× MIC fidaxomicin with bacterial counts decreasing 1 log in 48 h. Exposure to OP-1118 (at 4× MIC) also resulted in a $\geqslant$3 log drop in c.f.u. counts for the ATCC 43255 strain, the clinical BI strain ORG 1687 and the mutant strain ORG 919. Higher concentrations of OP-1118 (32× MIC) were required for a 3 log reduction in c.f.u. counts for the other BI strain, ORG 1698. In summary, the results indicate that both fidaxomicin and its major metabolite, OP-1118, are bactericidal against *C. difficile* strains, including the hypervirulent restriction endonuclease analysis group BI strains, at concentrations that are many fold below the detected faecal concentrations of these compounds after oral administration of fidaxomicin.

INTRODUCTION

Clostridium difficile is the leading cause of nosocomial infections in community hospitals, surpassing meticillin-resistant *Staphylococcus aureus* infections in prevalence (Miller *et al.*, 2010). Following several major outbreaks of *C. difficile* infection by hypervirulent strains in North America and Europe, the disease continues to be a major cause of diarrhoea in hospitalized patients and among the elderly in long-term care facilities. The mortality and morbidity associated with *C. difficile* infections is high, resulting in significant economic burden (Dubberke & Wertheimer, 2009). Regardless of the impact, there has been little improvement in *C. difficile* treatment in the last 30 years. Current standard therapies are inadequate, the response rate to metronidazole has declined and there is a high rate of recurrence of infection following treatment with either metronidazole or vancomycin (Miller, 2010; Rupnik *et al.*, 2009).

Fidaxomicin (formerly OPT-80 and PAR-101) is a novel, narrow-spectrum antibiotic in development for the treatment of *C. difficile* infection (Ackermann *et al.*, 2004; Credito & Appelbaum, 2004; Finegold *et al.*, 2004). The putative target for fidaxomicin is the RNA polymerase (Talpaert *et al.*, 1975). Although this target is shared by the rifamycin class of compounds, the site of action for fidaxomicin, which is structurally related to lipiarmycin, is thought to be distinct and there is no cross-resistance between rifampicin and fidaxomicin in *C. difficile* (Babakhani *et al.*, 2004). Furthermore, lipiarmycin is thought to act at the early stages of transcription and has been shown to inhibit the holoenzyme better than the core enzyme (Sonenshein & Alexander, 1979).

Oral administration of both vancomycin and fidaxomicin results in a high faecal concentration of the drugs [$>$1 mg (g faeces)$^{-1}$] with minimal systemic absorption (Johnson *et al.*, 1992; Shue *et al.*, 2008). Following oral administration (400 mg per day), fidaxomicin is excreted mainly in the faeces along with high concentrations of its major metabolite, OP-1118, which is derived by hydrolysis of the isobutyryl ester located at the 4′ position of fidaxomicin. Similar to the parental compound, OP-1118 demonstrates a narrow-spectrum activity against *C. difficile*, albeit with a 32-fold higher MIC_{90} (Babakhani *et al.*, 2007). In recent phase 3 clinical trials in patients with *C. difficile* infection, fidaxomicin not only was well tolerated and achieved the primary end point of clinical cure but also demonstrated a significantly lower rate of recurrence of infection compared with vancomycin (Crook *et al.*, 2010; Louie *et al.*, 2009b; Miller *et al.*, 2009). Analysis of stool samples from subjects during a phase 2 dose-ranging study demonstrated that fidaxomicin not only lacks activity against Gram-negative bacteria but is also sparing of Gram-positive commensals (Louie *et al.*, 2009a; Tannock *et al.*, 2010).

The narrow antimicrobial spectrum of activity of fidaxomicin has been well established by several studies;

however, there are no data on the killing kinetics of the compound and its major metabolite (Ackermann *et al.*, 2004; Credito & Appelbaum, 2004; Finegold *et al.*, 2004). This study investigated the killing rate of both fidaxomicin and OP-1118 in comparison with vancomycin in wild-type hypervirulent strains, as well as in laboratory-derived strains with reduced susceptibility to fidaxomicin.

METHODS

Bacterial strains. Clinical *C. difficile* strains ORG 1687 and 1698 belonging to the restriction endonuclease analysis group BI were collected from subjects in fidaxomicin phase 3 trials in Canada. *C. difficile* strains 9689 and 43255 were obtained from the ATCC. Laboratory-generated *C. difficile* strains with reduced susceptibility to fidaxomicin were derived either from wild-type ATCC 43255 strain through multiple passages in the presence of increasing concentrations of fidaxomicin (ORG 919) or from wild-type ATCC 9689 strain following a single exposure of the bacteria to high concentrations of fidaxomicin (ORG 1620). All strains were stored at −80 °C and subcultured on 5 % blood agar plates under anaerobic conditions (80 % nitrogen, 10 % carbon dioxide and 10 % hydrogen) prior to testing.

Antimicrobial agents. Vancomycin was prepared as a 10 mg ml^{-1} stock in water. Similar stock concentrations of fidaxomicin and OP-1118 were prepared by dissolving the compounds in DMSO. All drugs were further diluted to an appropriate concentration in growth medium (*Brucella* broth supplemented with vitamin K and haemin) before testing.

MIC determination and media. The Clinical and Laboratory Standards Institute microbroth (rather than agar) dilution method (recommended only for *Bacteroides fragilis*) was used with slight modifications for MIC determination because it more closely represents the conditions of the killing studies (CLSI, 2007). Lysed blood, which obscures the colour of the redox indicator resazurin in media, was omitted from the culture medium without affecting the growth of *C. difficile*. Briefly, microtitre plates containing tenfold serially diluted drugs were equilibrated inside an anaerobic glove box for a minimum of 3 h. *C. difficile* (10^5 c.f.u.) was added to each well and, after 48 h incubation at 35 °C, the plates were examined for growth of *C. difficile* colonies. The MIC was determined as the drug concentration where no growth or the most significant reduction in growth was observed. MIC determinations were performed in duplicate. However, as MIC values can vary slightly between runs, duplicate MIC determination plates were also included in each killing kinetics experiment to confirm the MIC for that experiment.

Time–kill curve studies. All killing kinetics experiments were performed inside an anaerobic chamber. Colonies from blood agar plates were inoculated into *Brucella* broth supplemented with vitamin K and haemin and allowed to grow at 35 °C until they reached a growth turbidity (OD_{600}) of 0.4, which, based on our experience, corresponds to ~1×10^8 cells ml^{-1}. A portion of the cells (~1×10^6 cells) was inoculated into 5 ml supplemented *Brucella* broth containing fidaxomicin, OP-1118 or vancomycin at concentrations that were multiples of the MIC. A no-drug (control) tube was also included in each run. At different time intervals over a 48 h incubation period, aliquots from each treatment were removed and serially (tenfold) diluted, and 100 µl of the dilution was plated onto duplicate blood agar plates. The number of c.f.u. was determined after incubation of the plates at 35 °C for 48 h. With this method, the threshold of detection was 100 c.f.u. ml^{-1}, or a single colony when 100 µl of a tenfold-diluted culture was plated. Killing kinetics experiments for the most part were performed once for each strain and drug combination (at multiples of the MIC) except as specified in Results (in duplicate for OP-1118 against ORG 1698 or triplicate for fidaxomicin against ORG 919).

The problem of residual drug carryover was addressed by dilution of cultures at least tenfold prior to plating, i.e. once plated, the drug would diffuse and be diluted a further 250-fold, based on a 100 µl inoculum and a 25 ml plate volume. To confirm the lack of effect of residual drug on growth, a broth culture of a *C. difficile* ATCC strain was grown overnight and 50 µl was removed at different time points, serially diluted and mixed with an equal volume of the drug (final concentration 4–8 × MIC) immediately prior to plating. C.f.u. counts with and without exposure to the drug were compared to verify that dilution steps during plating led to negligible drug concentrations that did not affect the growth of the bacteria. Assessment of the impact of drug carryover was performed once with both the ATCC 43255 and 9689 strains.

RESULTS AND DISCUSSION

MIC values

The MIC values for each strain, determined by the broth microdilution method, are summarized in Table 1. Both clinical strains had fidaxomicin MICs of 0.25 mg l^{-1} and OP-1118 MIC values of 2–4 mg l^{-1}. The ATCC strains 43255 and 9689 demonstrated fidaxomicin MICs of 0.016–0.25 and 0.016 mg l^{-1}, respectively, and OP-1118 MICs of 4 and 0.25 mg l^{-1}, respectively. The laboratory strains generated from the ATCC strains demonstrated reduced susceptibility to both fidaxomicin (2–4 mg l^{-1} for ORG 919 and 1 mg l^{-1} for ORG 1620) and OP-1118 (128 mg l^{-1} for ORG 919). The vancomycin MIC for all strains, with the exception of ORG 919, was 1 mg l^{-1}. The MIC for ORG 919 was fourfold higher than that for the parental ATCC strain 43255 at 4 mg l^{-1}.

Killing kinetics

The killing kinetics of fidaxomicin and OP-1118 for each strain are described below and shown separately in Figs 1–5.

To demonstrate lack of effect of drug carryover on growth, bacterial counts from growing broth cultures of *C. difficile*

Table 1. MIC values (mg l^{-1}) for fidaxomicin, OP-1118 and vancomycin

ND, Not done.

Antibiotic	ATCC strains		Clinical strains		Laboratory strains	
	43255	9689	ORG 1687	ORG 1698	ORG 919	ORG 1620
Fidaxomicin	0.25, 0.06	0.016	0.25	0.25	2, 4	1
OP-1118	4	0.25	2	2, 4	128	ND
Vancomycin	1	1	1, 2	1	4	1

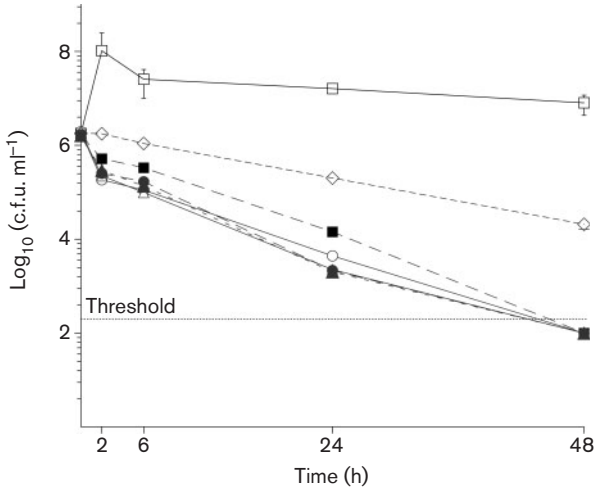

Fig. 1. Killing kinetics of fidaxomicin (○, 4× MIC; △, 32× MIC), OP-1118 (▲, 2× MIC; ●, 4× MIC; ■, 16× MIC) and vancomycin (◇, 4× MIC) against *C. difficile* ATCC 43255. A no-drug control (□) was included with each run. Samples of <100 c.f.u. ml^{-1} were below the limit of detection.

ATCC 43255 or 9689 were determined at various time points by serial dilution followed by mixing with equal volume of 8× or 16× concentrations or no drug immediately prior to plating. Exposure of cells to 4–8× MIC concentrations of drugs during serial dilution and plating did not affect the viability of cells. The numbers of c.f.u. were similar at each time point whether the cells were mixed with high concentrations of the drugs or not.

The killing kinetics against *C. difficile* ATCC 43255 are depicted in Fig. 1. Both fidaxomicin and its major metabolite, OP-1118, at 4× MIC exerted bactericidal activity against this strain; bacterial counts dropped below the detection limit (100 c.f.u. ml^{-1}) by 48 h for both

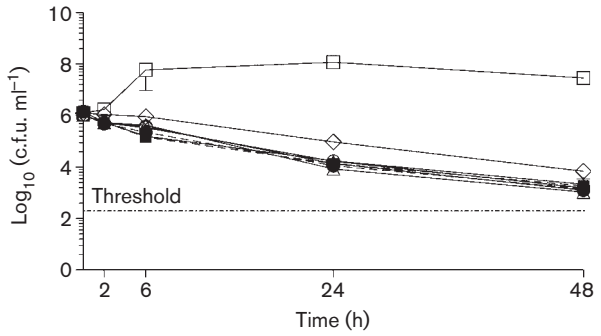

Fig. 2. Killing kinetics of fidaxomicin (△, 2× MIC; ○, 4× MIC; ⊗, 8× MIC), OP-1118 (▲, 4× MIC; ●, 8× MIC; ■, 16× MIC) and vancomycin (◇, 2–4× MIC) against *C. difficile* clinical BI strain ORG 1687. A no-drug control (□) was included with each run. Samples of <100 c.f.u. ml^{-1} were below the limit of detection.

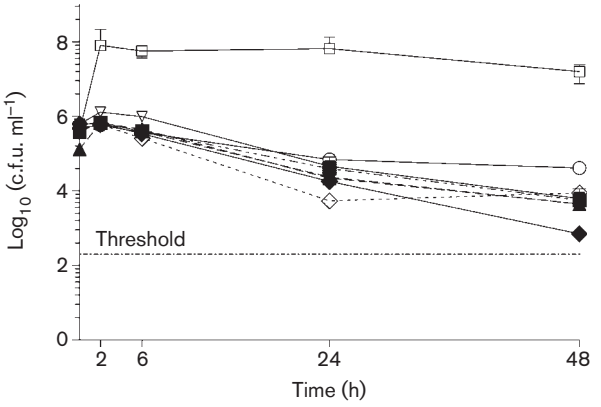

Fig. 3. Killing kinetics of fidaxomicin (○, 2× MIC), OP-1118 (▲, 4× MIC; ●, 8× MIC; ■, 16× MIC; ◆, 32× MIC) and vancomycin (◇, 2× MIC) against *C. difficile* clinical BI strain ORG 1698. A no-drug control (□) was included with each run. Samples of <100 c.f.u. ml^{-1} were below the limit of detection.

compounds. OP-1118 was also bactericidal at 2× MIC. In contrast, cells exposed to vancomycin at 4× MIC were, as expected, killed more slowly and the bacterial count dropped by only 2 logs within 48 h.

Similarly, both fidaxomicin (even at 2× MIC) and OP-1118 demonstrated time-dependent bactericidal activity against the clinical BI strain ORG 1687 (Fig. 2). The number of c.f.u. dropped 3 logs by 48 h in the presence of fidaxomicin or OP-1118. In contrast, killing activity by vancomycin at 4 μg ml^{-1} was slower, dropping only 1 log in 24 h and slightly over 2 logs in 48 h.

The killing activity of fidaxomicin against the other clinical BI strain, ORG 1698, was tested once at 2× MIC, with bacterial counts dropping slightly more than 1 log by 48 h

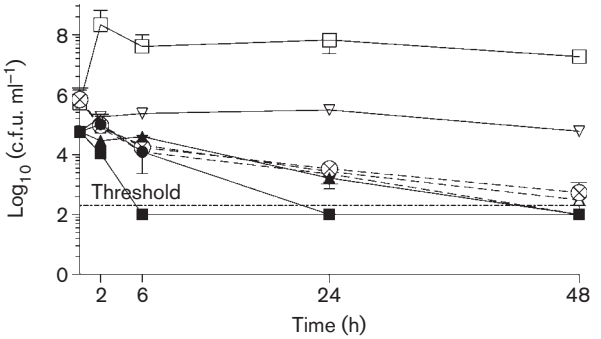

Fig. 4. Killing kinetics of fidaxomicin (○, 4–8× MIC; △, 16× MIC; ⊗, 32× MIC), OP-1118 (▲, 4× MIC; ●, 8× MIC; ■, 16× MIC) and vancomycin (▽, 4× MIC) against laboratory-generated mutant *C. difficile* 919 with reduced susceptibility to fidaxomicin. A no-drug control (□) was included with each run. Samples of <100 c.f.u. ml^{-1} were below the limit of detection.

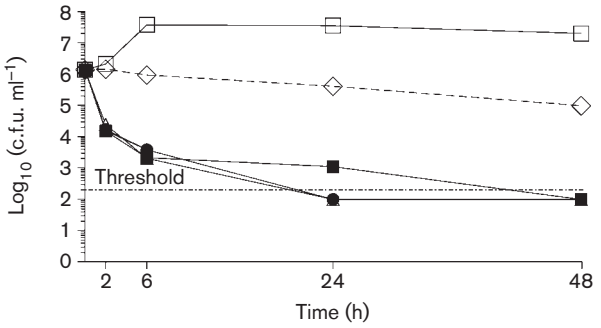

Fig. 5. Killing kinetics of fidaxomicin (○, 2× MIC; △, 4× MIC; ●, 8× MIC; ■, 16× MIC) and vancomycin (◇, 4× MIC) against laboratory-generated mutant *C. difficile* 1620 with reduced susceptibility to fidaxomicin. A no-drug control (□) was included with each run. Samples of <100 c.f.u. ml^{-1} were below the limit of detection.

(Fig. 3). In the same experiment, however, c.f.u. counts for 2× MIC vancomycin-treated cells initially dropped by ~2 logs within 24 h and then began to recover, and by 48 h the c.f.u. count was <2 logs lower than the initial inoculum, indicating a lack of efficient killing. OP-1118 bactericidal activity against *C. difficile* ORG 1698 could not be determined reliably; inocula in duplicate experiments were insufficient such that the final counts were below the limit of detection. Nevertheless, bacterial counts dropped by at least 2 logs at 4–16× MIC and by more than 2 logs at 32× MIC.

Similar to the wild-type parental strain ATCC 43255, the mutant strain ORG 919 was killed by fidaxomicin and OP-1118, with c.f.u. counts dropping below the detection threshold by 48 h (Fig. 4). A 3 log drop in c.f.u. counts could not be determined reliably for this organism, as inoculum densities were lower than anticipated during the three separate trials. Vancomycin at 4× MIC inhibited c.f.u. counts by 1 log at 48 h.

Fidaxomicin demonstrated a >3 log drop in c.f.u. at concentrations as low as 2× MIC against the mutant strain ORG 1620 (Fig. 5). In contrast, vancomycin killing at 4× MIC was slow and the c.f.u. counts dropped only slightly more than 1 log in 48 h. The bactericidal activity of OP-1118 against this mutant strain was not investigated.

The results of this investigation demonstrated that both fidaxomicin and its metabolite OP-1118 have bactericidal activity against *C. difficile* strains *in vitro*. Both ATCC and clinical strains were killed by fidaxomicin, with c.f.u. counts dropping significantly for ATCC strain 43255 (>3 log drop) and the clinical BI strain ORG 1687 (3 log drop). The bactericidal activity of fidaxomicin against the other clinical BI strain, ORG 1698, could not be established in this report as it was not tested above 2× MIC concentrations; at 1× and 2× MIC concentrations, however, the bacterial count for this strain dropped by 1 log in 24 h and declined only slightly thereafter.

This report further demonstrates that fidaxomicin's major metabolite, OP-1118, is also bactericidal in a time-dependent manner. Both ATCC strain 43255 and the clinical BI strain ORG 1687 were killed in the presence of a 4× MIC concentration of OP-1118. Similar to the parental compound, the bactericidal activity against the ATCC strain was more pronounced (>3 logs) than against clinical BI strains ORG 1687 (3 logs) and ORG 1698 (2 logs). For the latter strain, a higher concentration of metabolite (32× MIC) was required to obtain a 3 log reduction in c.f.u. counts.

Bactericidal activity of fidaxomicin was also observed against the laboratory-generated mutant strains with increased fidaxomicin MIC values. ORG 919 (MIC 2–4 μg ml^{-1}) was derived in our laboratory from *C. difficile* ATCC 43255 after multiple passages in the presence of increasing concentrations of fidaxomicin and had two mutations in the *rpoC* region of the RNA polymerase (Gln^{781}→Arg and Asp^{1127}→Glu) (J. Seddon, Optimer, personal communication). ORG 1620 with increased fidaxomicin MIC (1 mg l^{-1}) was generated through a single-step exposure of ATCC 9689 to a high concentration of fidaxomicin and had a single mutation in the *rpoB* region of the RNA polymerase (Gln^{1074}→Lys). The latter amino acid mutation has been identified at homologous positions in other species with a low susceptibility to lipiarmycin, a related macrocyclic compound (Kurabachew *et al.*, 2008; Mekler *et al.*, 2004). Despite a several-fold higher MIC compared with the parental strains, both strains with mutations in the RNA polymerase were rapidly killed by fidaxomicin at 4× MIC concentrations, indicating that mutants that may arise during fidaxomicin therapy will probably be eliminated by the high levels of drug achieved in the gut.

In summary, the results of this study confirmed that fidaxomicin and its major metabolite, OP-1118, are bactericidal against *C. difficile*, including hypervirulent strains, whereas the other leading drug used, vancomycin, is bacteriostatic against *C. difficile* (Odenholt *et al.*, 2007). It is expected that with high concentrations of both compounds, reproducibly obtained in the gut, even mutant strains with increased fidaxomicin MIC values are likely to be killed effectively during therapy.

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