Tigecycline Exhibits Inhibitory Activity against *Clostridium difficile* in the Colon of Mice and Does Not Promote Growth or Toxin Production[▽]

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Tigecycline is a broad-spectrum glycylcycline antibiotic with potent *in vitro* activity against *Clostridium difficile*. We used a mouse model to test the hypothesis that tigecycline has a low propensity to promote colonization and toxin production by *C. difficile* due to inhibitory activity in the colon. Mice (5 to 8 per group) received subcutaneous injections of tigecycline (low and high doses) alone or in combination with clindamycin for 6 days. Growth of and toxin production by 3 strains of *C. difficile* (tigecycline MICs ≤ 0.012 μg/ml) were measured in cecal contents collected 6 h or 3 days after the final antibiotic dose. Antibiotic concentrations were measured using a bioassay, and concentrations of total anaerobes and *Bacteroides* spp. were measured. The effects of tigecycline on rendering mice susceptible to colonization with and reducing the burden of *C. difficile* were also examined. In comparison to saline controls, clindamycin promoted the growth of *C. difficile* ($P < 0.001$) in cecal contents, whereas tigecycline did not. Tigecycline did not suppress total anaerobes or *Bacteroides* spp. in comparison to saline controls. Concurrent administration of tigecycline prevented clindamycin-induced promotion of *C. difficile* in cecal contents collected 6 h or 3 days (high dose only) after the final antibiotic dose. Tigecycline did not promote the establishment of colonization in mice, yet it did not reduce concentrations of *C. difficile* in animals with established colonization. In summary, tigecycline did not promote the growth of or toxin production by *C. difficile*, probably due to inhibitory activity against *C. difficile* and relative sparing of indigenous anaerobic microflora.

Antimicrobial therapy plays a central role in the pathogenesis of *Clostridium difficile* infection (CDI). The presumed mechanism by which antibiotics induce CDI is through disruption of the indigenous microflora of the colon, thereby allowing *C. difficile* to grow to high concentrations with production of toxin (13). Although nearly all classes of antibiotics have been associated with CDI, clindamycin, broad-spectrum cephalosporins, and penicillins have traditionally been considered the agents that pose the greatest risk (13). With the emergence of the North American pulsed-field gel electrophoresis type 1 (NAP1) epidemic strain of *C. difficile* that exhibits increased resistance to fluoroquinolone antibiotics, fluoroquinolones have also been associated with CDI in multiple studies (1, 9, 13). However, there remains some uncertainty regarding the relative importance of fluoroquinolones as a risk factor for CDI because these agents cause only minor disruption of intestinal anaerobes (1, 13). Some recent studies suggest that antibiotics with inhibitory activity against *C. difficile* may be less likely to promote CDI (1–3, 7, 13–15, 17–19). For example, piperacillin-tazobactam, a beta-lactam/beta-lactamase inhibitor combination, not only disrupts the indigenous intestinal microflora but also has potent activity against *C. difficile* and has been infrequently associated with CDI in clinical studies (15, 19). In mice, piperacillin-tazobactam inhibited growth of *C. difficile* in the colon during treatment but facilitated growth and toxin production when exposure occurred after treatment

during the period of recovery of the indigenous microflora (14).

Tigecycline, a broad-spectrum glycylcycline antibiotic, also has potent activity against *C. difficile* and is excreted in significant concentrations in bile (median fecal concentration in human volunteers, 5.6 mg/kg of body weight on day 8 of administration) (12). In a chemostat model of the human intestinal microflora, tigecycline markedly decreased concentrations of bacteroides and bifidobacteria but did not induce proliferation or toxin production by *C. difficile* (3). Wilcox (17) has noted that tigecycline has infrequently been associated with CDI in clinical studies. Moreover, Herpers et al. recently reported successful use of intravenous tigecycline as salvage therapy for 4 patients with refractory CDI (7). These data suggest that tigecycline may achieve sufficient concentrations in the intestinal tract to inhibit the growth of *C. difficile*. In this study, we used a mouse model to examine the effect of tigecycline on the growth of and toxin production by *C. difficile*. We hypothesized that the effect of tigecycline on *C. difficile* in the colon is similar to the effect of piperacillin-tazobactam: inhibition of growth and toxin production during tigecycline treatment but promotion of growth when exposure occurs after treatment during the period of recovery of the indigenous microflora.

MATERIALS AND METHODS

***C. difficile* strains.** Three strains of *C. difficile* were studied. ATCC 43593 is a nontoxigenic strain from the American Type Culture Collection (ATCC). The other strains were cultured from patients with CDI in Cleveland, OH. VA 17 is an epidemic North American pulsed-field gel electrophoresis type 1 (NAP1) strain. VA 11 is a restriction endonuclease analysis (REA) J-type strain. For all three strains, the MICs of tigecycline, piperacillin-tazobactam, and clindamycin

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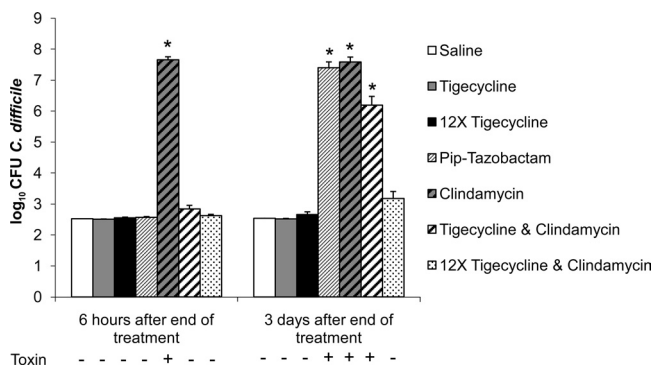
FIG. 1. Mouse model of *in vitro* colonization resistance to *C. difficile*. Mice ($n = 8$ per group) received daily subcutaneous antibiotics for 4 days. Six hours or 3 days after the final antibiotic dose, cecal contents were collected, and aliquots were inoculated with 10^4 CFU/ml of *C. difficile*. Samples were incubated anaerobically for 24 h and then plated onto selective media to quantify *C. difficile* (lower limit of detection, 2.5 $\log_{10}$CFU/ml). Data for three strains of *C. difficile* were pooled. Error bars represent standard errors of the means. Pip = piperacillin. Detection of *C. difficile* toxin by *C. difficile* Tox A/B II (Wampole Laboratories) is indicated below the *x* axis. ∗, $P < 0.001$ versus saline.
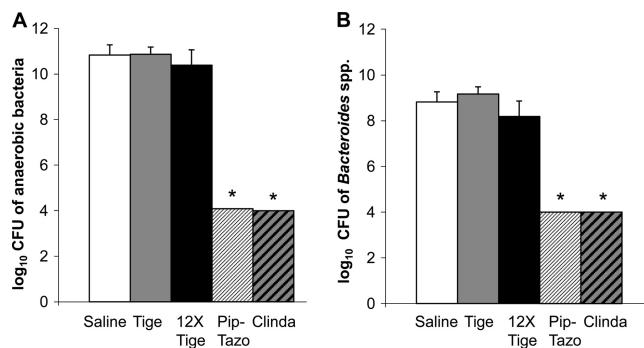

FIG. 2. Effect of antibiotic treatment on concentrations of total anaerobes and *Bacteroides* spp. Mice ($n = 6$) received daily subcutaneous antibiotic treatment for 4 days. Six hours after the final dose, cecal contents were collected and plated anaerobically onto prereduced brucella agar to quantify total anaerobes (A) and onto *Bacteroides* bile-esculin agar to quantify *Bacteroides* spp. (B). The lower limit of detection was 4 logs. Error bars represent standard errors of the means. Tige = tigecycline; Pip-Tazo = piperacillin-tazobactam; Clinda = clindamycin. ∗, $P < 0.001$ versus saline.

were ≤0.012 μg/ml, ≤1 μg/ml, and ≥256 μg/ml, respectively, as determined by broth dilution (10).

Bioassay for tigecycline concentrations. The concentration of tigecycline in stool samples on day 5 of treatment was determined using an agar diffusion assay, as described previously by Nord et al. (12), but *Escherichia coli*, rather than *Bacillus cereus*, was used as the indicator strain. The limit of detection was 1 μg/gm of feces.

Mouse model of *in vitro* colonization resistance to *C. difficile*. The *in vitro* mouse model was adapted from the hamster model of colonization resistance to CDI, developed by Borriello et al. (5). These investigators demonstrated that antibiotics that promoted *in vitro* growth of and toxin production by *C. difficile* in cecal emulsions of hamsters also caused CDI in hamsters, whereas antibiotics that did not promote *in vitro* growth and toxin production did not cause disease (5). We have previously found that this model yields similar results in mice (1, 13). The Animal Care Committee of the Cleveland Veterans Affairs Medical Center approved the experimental protocol.

Female CF-1 mice weighing 25 to 30 g (Harlan Sprague-Dawley, Indianapolis, IN) were housed in individual cages. Mice received daily subcutaneous injections (total volume, 0.2 ml) of saline, tigecycline (0.05 mg/day), clindamycin (1.4 mg/day), or piperacillin-tazobactam (8 mg/day) for 4 days. In some experiments, mice received daily injections of tigecycline and clindamycin. The antibiotic doses were equal to the usual human doses administered over a 24-hour period (milligrams of antibiotic per gram of body weight). Because tigecycline did not promote overgrowth of *C. difficile*, the effect of a higher daily dose of 12 times the initial dosage was also measured.

Mice (8 per group) were killed by CO_2 asphyxiation 6 h or 3 days after the final antibiotic dose. The ceca were removed and opened longitudinally, and the contents were collected and transferred to an anaerobic chamber (Coy Laboratories, Grass Lake, MI) within 5 min. The cecal contents were diluted 3-fold (volume/volume) with sterile prereduced phosphate-buffered saline (PBS). A final concentration of 10^4 CFU/ml of each *C. difficile* strain was added to separate aliquots of cecal contents from individual mice. The *C. difficile* strains were prepared for inoculation by serially diluting 72-hour broth cultures in sterile, prereduced PBS. To quantify the *C. difficile*, after incubation for 24 h, samples were diluted in sterile PBS and plated on prereduced cycloserine-cefoxitin-brucella agar containing 0.1% taurocholic acid and 5 mg/ml lysozyme (*C. difficile* brucella agar [CDBA]) (11). To determine toxin production in cecal contents, the *C. difficile* Tox A/B II (Wampole Laboratories, Princeton, NJ) test kit was used, according to the manufacturer's instructions.

Effect of antibiotic treatment on concentrations of total anaerobes and *Bacteroides* spp. Six hours after completion of the final dose of 4 days of subcutaneous antibiotic treatment, fresh cecal contents were collected and transferred to the anaerobic chamber. The contents were serially diluted in prereduced PBS and plated onto brucella agar and *Bacteroides* bile-esculin agar (Becton Dickin-

son) to measure the concentrations of total anaerobes and *Bacteroides* spp., respectively. The lower limit of detection was ~4 $\log_{10}$CFU/g of stool.

Mouse model of *in vivo* *C. difficile* colonization. To assess the effect of tigecycline on the establishment of *in vivo* colonization with *C. difficile*, mice (6 per group) were given subcutaneous saline, tigecycline, clindamycin, or both antibiotics for 5 days. On day 2 of antibiotic treatment, mice were administered 10^4 CFU of *C. difficile* spores in 0.5 ml of PBS by oral gavage. To quantify the burden of *C. difficile*, fresh stool samples were collected on days 1, 3, and 7 after administration of *C. difficile*. Stool samples were emulsified with 5-fold (weight/volume) prereduced PBS, serially diluted, and inoculated onto CDBA plates.

To assess whether tigecycline treatment results in suppression of *C. difficile* after colonization has been established, mice (5 per group) received subcutaneous injections of clindamycin for 2 days, followed by administration of 4 $\log_{10}$CFU of *C. difficile* spores. Two days after administration of spores, the mice received daily subcutaneous saline, subcutaneous tigecycline (0.6 mg/day), subcutaneous piperacillin-tazobactam (8 mg/day), or oral vancomycin (0.25 mg/day) for 5 days. Piperacillin-tazobactam and oral vancomycin were included for comparison because these agents also have potent inhibitory activity against *C. difficile*; the dose of oral vancomycin was equivalent (in mg/kg) to the human dose used for treatment of CDI.

Statistical analysis. Data analyses were performed using R software (version 2.10.1, 14 December 2009). A one-way analysis of variance (ANOVA) (Fig. 1 and 2) or multivariate ANOVA (MANOVA) (Fig. 3 and 4) was performed to compare the groups, with *P* values adjusted for multiple comparisons using Tukey's honestly significant difference test.

RESULTS

Mouse model of *in vitro* colonization resistance to *C. difficile*. Figure 1 shows the effects of antibiotic treatment on *in vitro* growth of *C. difficile* in cecal contents collected 6 h or 3 days after the final antibiotic dose. In comparison to saline controls, clindamycin promoted overgrowth of *C. difficile* at both time points ($P < 0.001$), whereas piperacillin-tazobactam treatment prevented overgrowth in contents collected at 6 h but promoted overgrowth in samples collected 3 days after the final antibiotic dose ($P < 0.001$). Tigecycline treatment at both doses did not promote overgrowth of *C. difficile* in cecal contents collected at either time point. When administered in combination with clindamycin, tigecycline prevented clindamycin-induced overgrowth of *C. difficile* in cecal contents collected 6 h (both doses of tigecycline) and 3 days (high-dose tigecycline only) after antibiotics were withdrawn. By bioassay,
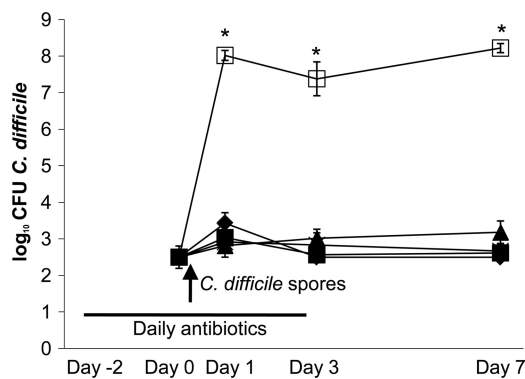
FIG. 3. Mouse model of *in vivo* *C. difficile* colonization. Mice ($n =$ 6) received subcutaneous antibiotic treatment daily for 5 days. On day 0, 10^4 CFU of *C. difficile* spores were administered by oral gavage (arrow). Stool was collected, and dilutions were plated onto selective media to quantify *C. difficile*. The lower limit of detection was ~3 $\log_{10}$CFU/g. Saline, ◆; tigecycline, ■; 12× tigecycline, ▲; clindamycin, □; tigecycline and clindamycin, ●. *, $P < 0.001$ versus saline.
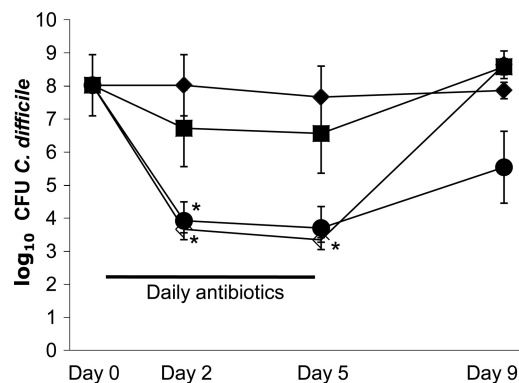

FIG. 4. Impact of antibiotic treatment on *C. difficile* levels in colonized mice. Mice ($n = 5$) were colonized with *C. difficile* and then administered saline, piperacillin-tazobactam, or tigecycline by subcutaneous injection or vancomycin by oral gavage for 5 days. Stool was collected, and dilutions were plated onto selective media to quantify *C. difficile* spores. The lower limit of detection was ~4 logs. Saline, ◆; tigecycline, ■; piperacillin-tazobactam, □; oral vancomycin, ●. *, $P < 0.05$ versus saline.

levels of tigecycline in cecal contents collected 6 h after the final dose on day 4 were 2.8 ± 0.4 and 9.0 ± 3.4 μg/ml ($\pm$ standard errors of the means [SEM]) at the human-equivalent (mg/kg) and the 12-times-higher doses, respectively. Tigecycline was not detected in the cecal contents of mice receiving either dose when collected 3 days after the final dose. *C. difficile* toxin was detected in all cecal content samples obtained from clindamycin-treated mice, whereas none of the mice in the other groups had detectable levels of toxin in cecal contents.

Figure 2 shows the effects of antibiotic treatment on concentrations of total anaerobes and *Bacteroides* spp. in cecal contents. Tigecycline did not suppress the levels of total anaerobes or *Bacteroides* spp., whereas piperacillin-tazobactam and clindamycin did ($P < 0.001$).

Mouse model of *in vivo* *C. difficile* colonization. Figure 3 shows the effect of antibiotic treatment on the establishment of colonization with the nontoxigenic *C. difficile* strain ATCC 43593. In comparison to saline controls, clindamycin-treated mice developed high concentrations of *C. difficile* in stool ($P < 0.001$), whereas mice receiving either the human-equivalent dose or the 12-times-higher dose of tigecycline did not.

Figure 4 shows the assessment of whether antibiotic treatment results in suppression of *C. difficile* levels after colonization has been established. Subcutaneous injection of piperacillin-tazobactam resulted in a significant decrease in *C. difficile* concentrations in stool during treatment (P of <0.05 on days 2 and 5). Oral vancomycin also caused a decrease in stool concentrations of *C. difficile* that was significant on day 2 ($P <$ 0.05) and approached significance on day 5 ($P = 0.058$). After treatment discontinuation, *C. difficile* levels in the groups of mice rapidly rebounded to pretreatment levels. Tigecycline treatment did not reduce *C. difficile* concentrations in stool in comparison to saline controls.

DISCUSSION

We found that tigecycline did not promote *in vitro* growth of or toxin production by *C. difficile* in cecal contents collected

from mice during or 3 days after completion of treatment. As in previous studies, piperacillin-tazobactam inhibited growth of *C. difficile* during treatment but promoted overgrowth in cecal contents collected 3 days after completion of treatment (14). Clindamycin promoted overgrowth of clindamycin-resistant *C. difficile* in cecal contents during and after treatment. In previous studies, clindamycin inhibited the growth of clindamycin-susceptible strains during, but not after, treatment (1). Piperacillin-tazobactam and clindamycin treatment reduced concentrations of total anaerobes and *Bacteroides* spp. in cecal contents, whereas tigecycline treatment did not. For each of the antibiotics studied, the levels detected in mouse stool or cecal contents are comparable to concentrations detected in stool samples of healthy human volunteers or patients receiving the same antibiotics (6, 8, 11, 18). Our findings suggest that tigecycline might have a relatively low propensity to promote CDI due to inhibitory activity against *C. difficile* and relative sparing of indigenous anaerobic microflora.

Our results are consistent with some findings of previous studies in human volunteers and using a chemostat model of human intestinal microflora (3, 12). As in mice, tigecycline did not inhibit *Bacteroides* spp. in healthy human adults (12). Bifidobacteria were significantly reduced in human volunteers (12), but levels of bifidobacteria were not measured in the current study. In the chemostat model, instillation of tigecycline resulted in a 3- to 4-log decrease in total anaerobes and marked suppression of bacteroides, which failed to recover during the 14-day postantibiotic period (3). Despite the reduction in anaerobic microflora in the chemostat model, instillation of tigecycline was not associated with germination or outgrowth of *C. difficile* spores, suggesting that growth was inhibited (3).

Tigecycline treatment did not promote *C. difficile* colonization in mice, yet it did not reduce *C. difficile* levels in animals with preestablished colonization. In contrast, piperacillin-tazobactam and oral vancomycin suppressed levels of *C. difficile* in mice with preestablished colonization. The concentration of the antibiotics in the intestinal tract may explain the

differing effects of the 3 agents. Compared to piperacillin-tazobactam and vancomycin levels determined in previous mouse model studies (piperacillin-tazobactam, 31.2 ± 9.7 μg and 2.0 ± 0.8 μg/ml of cecal contents at 6 h and 3 days after discontinuation of treatment, respectively; vancomycin, >500 μg/ml of cecal contents at both time points) (13; our unpublished data), the peak fecal concentration of tigecycline was much lower (2.8 ± 0.4 μg/gm), and no drug was detectable 3 days after discontinuation of treatment. Although tigecycline achieves peak concentrations in stool several times above the typical MICs of *C. difficile* isolates, factors such as rapid clearance from the intestinal tract, an inoculum effect (i.e., intestinal concentrations above the 10^5 inoculum used for MIC testing), or binding to fecal matter could potentially reduce its *in vivo* effects in the intestinal tract (13).

In conclusion, tigecycline did not promote growth of or toxin production by *C. difficile*, probably due to inhibitory activity against *C. difficile* and relative sparing of indigenous anaerobic microflora.

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