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ORIGINAL ARTICLE

In vitro and *in vivo* antibacterial activity of tigecycline against *Vibrio vulnificus*

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Abstract *Background/purpose:* The aim of this study is to investigate the role of tigecycline in *Vibrio vulnificus* infection.
Methods: Eight randomly selected clinical *V. vulnificus* isolates were studied to obtain the minimal inhibitory concentrations (MICs) of minocycline, cefotaxime, and tigecycline, and the time–kill curves of tigecycline alone or in combination with other drugs. A peritonitis mouse model was used for the evaluation of the therapeutic efficacy of tigecycline alone or cefotaxime in combination with minocycline or tigecycline.
Results: The MIC of minocycline, cefotaxime, and tigecycline for eight clinical *V. vulnificus* isolates was 0.06–0.12 μg/mL, 0.03–0.06 μg/mL, and 0.03–0.06 μg/mL, respectively. In time–killing studies, at the concentration of 1 × MIC, the inhibitory effect of tigecycline persisted for 24 hours in five of eight isolates. With 2 × MIC and trough level, the inhibitory effect was noted in all isolates for 24 hours. With the combination of minocycline plus cefotaxime and tigecycline plus cefotaxime at 1/2 × MIC, the bactericidal effect was noted in 25% and 62.5% of eight isolates and synergism in 50% and 75% of isolates. With a low (1.25×10^5 CFU/mL) inoculum, all infected mice survived with tigecycline alone, tigecycline plus cefotaxime, or minocycline plus cefotaxime on the 14th day. At the inoculum of 1.25×10^6 CFU, the survival rate

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was 33.3% on the 14th day in the tigecycline plus cefotaxime-treated group, but none of the mice treated by tigecycline alone or minocycline plus cefotaxime survived (33.3% vs. 0%, $p = 0.01$ by Fisher's exact test).
Conclusion: Our *in vitro* combination and animal studies indicate that tigecycline could be an option for the treatment of invasive *V. vulnificus* infections.

Introduction

Vibrio vulnificus is primarily associated with severe, distinctive septicemia and soft tissue infection, including necrotizing fasciitis, or both, especially in patients with malignancy, adrenal insufficiency, liver cirrhosis, or diabetes.[1–5] The clinical courses of septicemic patients with *V. vulnificus* are often rapidly progressing, and more than 50% of such patients die within 48 hours of hospitalization.[6] Our previous study showed that cefotaxime combined with minocycline was *in vitro* active against *V. vulnificus*, and was effective in a mouse model.[7] Clinical experiences also support the use of minocycline plus cefotaxime for severe *V. vulnificus* infections.[6,8]

According to previous *in vitro* studies, tigecycline, a member of a new class of glycylcycline, exhibits good tissue penetration and has been reported to be active against *Vibrio* species, making it a potential choice for invasive human *Vibrio* infections.[9,10] Successful tigecycline salvage therapy for *V. vulnificus* necrotizing fasciitis in a child was reported recently.[11] Because of the lack of parenteral preparation of minocycline in Taiwan, we decided to study the role of tigecycline in the treatment of *V. vulnificus* infection. Moreover, as severe *V. vulnificus* infections often occurred in immunocompromised patients, it is important to initiate broad spectrum antibiotics, such as cefotaxime plus tigecycline, to cover many potential pathogens for severe infectious diseases. Therefore, we examined *in vitro* killing effect of tigecycline and initiated *in vivo* survival studies to evaluate the efficacy of tigecycline alone or in combination with cefotaxime in treatment of murine *V. vulnificus* infections.

Methods

Bacterial isolates

Eight clinical *V. vulnificus* isolates were randomly selected from Chi Mei Medical Center in southern Taiwan. The isolates were stored at −80°C in Protect Bacterial Preservers (Technical Service Consultants Ltd., Heywood, UK) prior to use. Species confirmation was performed using standard biochemical methods, via a VITEK 2 automated system (bioMérieux, Marcy l'Etoile, France).

Antibiotics and minimal inhibitory concentrations

The minimal inhibitory concentrations (MICs) of ampicillin, cefazolin, cefotaxime, ceftriaxone, ciprofloxacin, gentamicin, minocycline (Sigma, St. Louis, MO, USA), tigecycline (Pfizer, New York, NY, USA), and imipenem (U.S. Pharmacopeia, Rockville, MD, USA), were determined by agar dilution on Mueller–Hinton agar (Oxoid, Basingstoke, UK), according to the Clinical and Laboratory Standards Institute (CLSI) recommendations. Interpretation criteria for susceptibility data were based on CLSI and Food and Drug Administration guidelines.[12,13] Inocula were prepared by suspending growth from overnight cultures in saline to a turbidity of a 0.5 McFarland standard. Inoculated plates were then incubated in ambient air at 37°C for 24 hours. *Escherichia coli* ATCC 25922 was included as the control strain in each run. Tigecycline MICs were measured by broth microdilution as recommended.[12,14] The Mueller–Hinton broth with calcium (25 μg/mL) and magnesium (12.5 μg/mL) (CAMHB) was freshly prepared. Appropriate 5×10^5 colony-forming units (CFU)/mL was mixed with serial drug dilutions and incubated in ambient air at 35°C for 18–24 hours.

Time–kill studies of tigecycline

Time–kill studies for *V. vulnificus* isolates were performed according to the CLSI-defined methodology.[15] In brief, bacterial suspensions were diluted to 5.0×10^5 CFU/mL in 25 mL fresh Mueller–Hinton broth. Drug concentrations of tigecycline in the time–kill studies were adjusted to 1/4 × MIC, 1/2 × MIC, 2 × MIC, and serum trough level (0.13 μg/mL). Bacterial counts were measured at 2 hours, 4 hours, 8 hours, 24 hours, and 48 hours by enumerating the colonies in 10-fold serially diluted specimens of 100-μL aliquots plated on the nutrient agar (Difco Laboratories, Sparks, MD, USA) at 37°C.

In vitro antibacterial activity of antibiotic combinations

The *in vitro* antibacterial activity of three antimicrobial agents alone or in combination (tigecycline plus cefotaxime, and minocycline plus cefotaxime) was tested. Approximately 1×10^6 CFU/mL *V. vulnificus* was used for the combination test. Drug concentrations were adjusted to 1/2-fold of MICs. Bacterial counts were measured at 24 hours on nutrient agar (Difco Laboratories) at 37°C.

Definitions

Synergy and antagonism were defined as ≥ 2 $\log_{10}$ greater and lesser kills between the combination and the most active constituent after 24 hours. Bacteriostatic activities

were defined as $\geq$2 $\log_{10}$, but <3 $\log_{10}$ and bactericidal activities were defined as $\geq$3 $\log_{10}$ reductions in CFU/mL at 24 hours, respectively, relative to the starting inoculum.[16] All experiments were performed in duplicate.

In vivo mouse study

Female inbred BALB/c mice (Animal Center, National Science Council, Taipei, Taiwan) weighing 18—20 g (6—8 weeks old) were used in this study. Vv14-3 was randomly selected and incubated in Mueller—Hinton broth overnight and subcultured. After 3 hours of incubation in sterile broth, the pellet obtained after centrifugation was diluted to the anticipated turbidity for mouse experiments. The dosage of cefotaxime for mice is 150 mg/kg every 6 hours and minocycline 20 mg/kg every 12 hours intraperitoneally administered,[17] and tigecycline 6.25 mg/kg every 12 hours subcutaneously administered as described previously.[18] Antibiotics were initiated 2 hours after intraperitoneal bacterial inoculation and administered for 48 hours. The number of surviving mice was recorded at 8-hour intervals for 120 hours.

Pharmacokinetic studies

The dose of tigecycline, subcutaneous injection of 6.25 mg/kg, was selected based on published pharmacokinetic data, which indicated that this dose in mice can achieve a serum maximum concentration (C_{max}) of 1.17 μg/mL, similar to the C_{max} of 0.93 μg/mL achieved at the dose of 100 mg every 12 hours in humans.[9,19] At multiple time points of 0.25 hours, 0.5 hours, 1 hour, 2 hours, 3 hours, 5 hours, 7 hours, 9 hours, and 12 hours, blood and thigh muscle samples were collected from six mice. Tigecycline concentrations were estimated using the paper-disk diffusion method with a control strain, *Bacillus cereus* BCRC10446. All samples were assayed in triplicate. The lower limit of detection for tigecycline is 0.06 μg/mL.

Statistical analysis

Data analysis was performed using SPSS 10.0 for Windows (SPSS Inc., Chicago, IL, USA). To compare the effects between different treatment groups, two-way and one-way within-repeated subjects analysis of variance tests were applied. Fisher's exact test was applied to compare the survival rates between groups. A p value < 0.05 was considered statistically significant.

Results

We tested the biotyping of these *V. vulnificus* isolates in our study and found that all tested isolates are Biotype 1 (data not shown). The MIC_{50} of tigecycline, cefotaxime, and minocycline for eight randomly selected *V. vulnificus* isolates was 0.03 μg/mL, 0.06 μg/mL, and 0.12 μg/mL, respectively.

Time—kill studies of tigecycline

The time—kill studies of eight *V. vulnificus* isolates cocultured with tigecycline at the concentrations of 1/2 × MIC, 1 × MIC, 2 × MIC, and 0.13 μg/mL (serum trough level) are shown in Figure 1. When *V. vulnificus* isolates at an inoculum of 5×10^5 CFU/mL were incubated with tigecycline at the concentration of 1/2 × MIC (0.03 μg/mL), the bacterial load increased to 10^8 CFU/mL at 24 hours. At the tigecycline concentration of 1 × MIC (0.06 μg/mL), *V. vulnificus* was temporarily inhibited at 8 hours, but regrew later (Figure 1). At a higher concentration of 2 × MIC (0.12 μg/mL), bacterial growth was inhibited until 48 hours, and bactericidal activity was evident at 24 hours. At the concentration of 0.13 μg/mL, the result can be expected to be similar to that of 0.12 μg/mL.

Among the *V. vulnificus* isolates tested, the isolate numbers with a decrease of at least 1 $\log_{10}$ CFU/mL, 2 $\log_{10}$ CFU/mL, or 3 $\log_{10}$ CFU/mL at different incubation times and tigecycline concentrations are shown in Table 1. Of note, tigecycline at the concentration of 2 × MIC and serum trough level, can exhibit bactericidal activity at 24 hours.

In vitro antibacterial activity of antibiotic combinations

With the combination of minocycline plus cefotaxime, both at the concentration of 1/2 × MIC, the colony count decreased from 0 $\log_{10}$ to 4.26 $\log_{10}$ compared with the starting inoculum, and such a combination was bactericidal to two (25%) of eight isolates (Figure 2). With the same concentration combination, the colony count decreased from 0.98 $\log_{10}$ to 4.78 $\log_{10}$ compared with the most active drug. Such a combination regimen was shown to be synergistic against four (50%) isolates. By contrast, the combination of tigecycline plus cefotaxime can decrease the bacterial load from 2.2 $\log_{10}$ to 3.9 $\log_{10}$ compared with starting inoculum, and be bactericidal to five (62.5%) isolates and synergistic against six (75%) isolates.

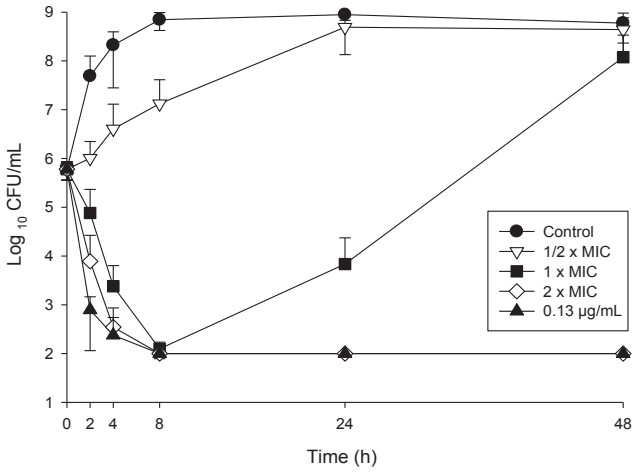

Figure 1. Time—kill curves of eight clinical *Vibrio vulnificus* isolates incubated with different concentrations of tigecycline. MIC = minimal inhibitory concentration.

Table 1 Isolate numbers of eight clinical *Vibrio vulnificus* strains with a decline of ≥1, 2, or 3 $\log_{10}$ CFU/mL at different incubation time, with tigecycline at the concentrations of 1/2 × MIC, 1 × MIC, 2 × MIC, and 0.13 μg/mL (the serum trough level, if standard doses of tigecycline are intravenously given).

Drug level	No. of isolates with a specific decline of bacterial load											
	2 h			4 h			8 h			24 h		
	−1 $\log_{10}$	−2 $\log_{10}$	−3 $\log_{10}$	−1 $\log_{10}$	−2 $\log_{10}$	−3 $\log_{10}$	−1 $\log_{10}$	−2 $\log_{10}$	−3 $\log_{10}$	−1 $\log_{10}$	−2 $\log_{10}$	−3 $\log_{10}$
1/2 × MIC	1	0	0	2	0	0	0	0	0	0	0	0
1 × MIC	3	2	1	1	4	3	0	0	8	2	1	5
2 × MIC	1	3	4	0	1	7	0	0	8	0	0	8
0.13 μg/mL	0	4	4	0	1	7	0	0	8	0	0	8

MIC = minimal inhibitory concentration.

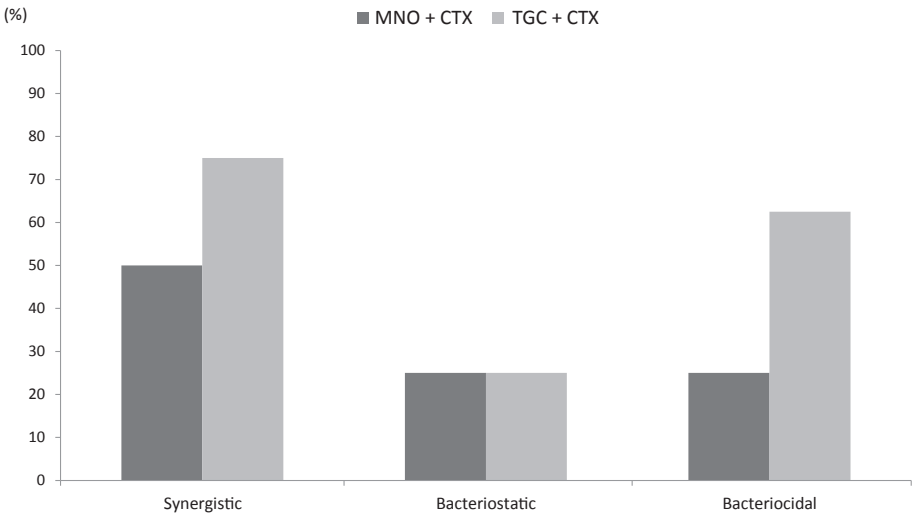

Figure 2. *In vitro* combination effect of 1/2 × MIC of minocycline (MNO) or tigecycline (TGC) and 1/2 × MIC of cefotaxime (CTX) for eight clinical *Vibrio vulnificus* isolates after 24 hours of incubation. MIC = minimal inhibitory concentration.

Bioassays and pharmacodynamic parameters

After blood and muscle of thigh samples were collected from six mice, the serum C_{max} of tigecycline was 0.98 μg/mL in average, and thigh tissue C_{max} was 1.65 μg/mL.

Survival rates of mice with *V. vulnificus* peritonitis

The survival rates of mice infected by Vv14-3 with a low (1.25×10^5 CFU/mL) and high inoculum (1.25×10^6 CFU/mL) and treated by tigecycline alone, or tigecycline or minocycline plus cefotaxime are shown in Table 2. In the low inoculum group, all mice treated by either tigecycline alone, tigecycline plus cefotaxime, or minocycline plus cefotaxime survived for 14 days. However, with a high inoculum, all mice died, except the mice in the tigecycline plus cefotaxime group, which had a survival rate of 33.3%. Such an outcome was significant between the former group and two other treatment groups (i.e., mice treated by tigecycline alone or minocycline plus cefotaxime) (33.3% vs. 0%; $p = 0.01$ by Fisher's exact test).

Table 2 Survival rates of mice infected by a clinical isolate of *Vibrio vulnificus*, Vv14-3, at a low or high inoculum and treated by tigecycline (TGC) alone, tigecycline plus cefotaxime (CTX), or minocycline (MNO) plus cefotaxime.

Antibiotic regimens	Survival rate (%)				
	Day 0	Day 1	Day 2	Days 3—5	Day 14
Low inoculum (1.25×10^5 CFU)					
Control, $n = 10$	100	0	0	0	0
TGC, $n = 10$	100	100	100	100	100
TGC + CTX, $n = 10$	100	100	100	100	100
MNO + CTX, $n = 10$	100	100	100	100	100
High inoculum (1.25×10^6 CFU)					
Control, $n = 12$	100	0	0	0	0
TGC, $n = 12$	100	0	0	0	0
TGC + CTX, $n = 12$	100	33.3	33.3	33.3	33.3
MNO + CTX, $n = 12$	100	0	0	0	0

Discussion

In the present study, the potent antibacterial activity and rapid bactericidal effect of tigecycline against *V. vulnificus* were observed, because the bacterial load can be decreased at least 3 $\log_{10}$ at 2 hours in 50% of eight clinical isolates. The MIC of *V. vulnificus* to tigecycline was 0.03 μg/mL or 0.06 μg/mL, and the tigecycline concentration we tested was 1 × MIC or 2 × MIC, which is close to the serum trough level, 0.13 μg/mL.[9,18,19] This indicates that tigecycline could be bactericidal for *V. vulnificus*.

By contrast, in time–kill assays tigecycline alone and in combination with other antibiotics often showed bacteriostatic effect against enterococci, Gram-negative bacilli including carbapenem-susceptible and carbapenem-resistant *Acinetobacter baumannii*, extended-spectrum beta-lactamase (ESBL)-producing *E. coli*, or *Klebsiella pneumoniae* carbapenemase-producing Enterobacteriaceae strains.[20–24] However, the bactericidal effect of tigecycline against penicillin-susceptible and penicillin-resistant pneumococci and some ESBL-producing Enterobacteriaceae was reported by some studies.[25,26] Our data indicate that tigecycline alone can pose a rapid bactericidal effect for clinical *V. vulnificus* isolates.

Severe *V. vulnificus* infection, especially in immunocompromised patients, can manifest as bacteremia with or without necrotizing fasciitis. In mice, the tigecycline C_{max} measured by bioassays was 0.98 μg/mL in serum and 1.65 μg/mL in uninfected thigh tissue, and both drug levels are higher than the MIC_{90} for *V. vulnificus*. Accordingly, tigecycline therapy could be reasonable for *V. vulnificus* skin and soft-tissue infection, as reported in the literature.[9,10] According to our study, the concentration of 0.12 μg/mL or 0.13 μg/mL is rapidly bactericidal for *V. vulnificus*. However, tigecycline at currently recommended dosages may be theoretically effective for septicemia due to *V. vulnificus*, which is highly susceptible to tigecycline. More clinical reports or investigations are warranted prior to such a clinical practice.

Our *in vivo* data demonstrate that tigecycline monotherapy poses a similar killing effect as the combination of minocycline and cefotaxime for experimental murine infection with low inoculum of *V. vulnificus*. Furthermore, the antibacterial effect of tigecycline was enhanced, if combined with cefotaxime, for the treatment of high-inoculum *V. vulnificus* infection. Tigecycline may be a potential alternative to treat human *V. vulnificus* infection in areas with limited access to old antibiotics. However, in light of the species differences between mice and humans, the extrapolation of animal data to clinical medicine should be done with caution. More clinical trials involving tigecycline monotherapy or combination regimens for invasive *V. vulnificus* infections are warranted.

In our *in vivo* study, we use two different inoculums 1.25×10^5 and 1.25×10^6, which are higher than the LD_{50} (lethal dose, 50%) of this strain (LD_{50} approx. 10 CFU/mouse) with around 10^4 and10^5 times. Therefore, even in the deficiency of the immunocompromised mice to imitate the immunocompromised status, the infection status seems to be more severe than general condition. Such *in vivo* results demonstrate the combination of tigecycline and cefotaxime can be used to treat severe *V. vulnificus* infection in immunocompromised populations.

Previous reports have suggested that, in addition to primary surgery, fluoroquinolones or third-generation cephalosporins plus minocycline are the best option for antibiotic treatment of necrotizing fasciitis caused by *V. vulnificus*.[8,27,28] Our major purpose was to investigate the role of tigecycline instead of minocycline. Perhaps we can perform another study to compare the effect of such an agent with fluoroquinolones in the future.

In conclusion, the *in vitro* and animal studies indicate that tigecycline alone or in combination with cefotaxime might be as effective as the traditional combination of minocycline and cefotaxime against *V. vulnificus*, and could be an option for the treatment of invasive *V. vulnificus* infections in areas without access to minocycline (for injection).

Conflicts of interest

The authors declare no competing interest.

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