

In vitro time-kill activities of ciprofloxacin alone and in combination with the iron chelator deferasirox against *Vibrio vulnificus*

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Abstract Iron plays a major role in the growth and virulence of ferrophilic organisms like *Vibrio vulnificus*. People who reside in the coastal areas with raw fish eating habits have a high risk of *Vibrio* infection and aggressive therapy can only reduce their mortality. We investigated the in vitro efficacy of ciprofloxacin, a bactericidal drug used in *V. vulnificus* patients, and the orally active iron chelator deferasirox against *V. vulnificus* infection. We performed in vitro time-kill studies on two ATCC strains and one clinical isolate of *V. vulnificus* collected from a patient admitted to Chosun University Hospital with either ciprofloxacin or iron chelator deferasirox alone and the two drugs in combination. The combination of an iron chelator plus an antibiotic creates a novel form of synergism at 24 h. The antimicrobial effect of deferasirox may be ascribed to its ability to deplete iron that would otherwise be used for bacterial growth. Combination therapy with ciprofloxacin plus deferasirox has potential clinical application by lowering the iron availability against a ferrophilic organism like *V. vulnificus* infections.

Introduction

Vibrio vulnificus, an opportunistic and emerging pathogen of humans, is a halophilic gram-negative, motile, curved

marine bacterium that is endemic in warm coastal waters. *V. vulnificus* infection is extremely invasive and rapidly fatal [1], and the prognosis is directly linked to the speed and accuracy of the diagnostic and treatment measures [2]. Mortality rates increase by 33–53% if treatment is delayed by 24 h, and approach 100% in patients not treated within 72 h [2, 3]. Treatment involves the administration of highly active antimicrobial agents and, when necessary, surgical debridement. *V. vulnificus* infection is associated with a variety of clinical syndromes: primary septicemia with high fever and chills; wound infection caused by direct inoculation; and gastrointestinal, characterized by vomiting, diarrhea, and abdominal pain [4]. Persons who are immunocompromised or have elevated serum iron levels, typically due to chronic liver damage, long-term alcohol abuse, or hemochromatosis, are highly vulnerable to infection by *V. vulnificus* [4, 5].

Iron plays an essential and complex role in the growth and virulence of virtually all microbial pathogens [6]. The level of freely available iron in the body is too low to sustain the growth of bacteria [7]. However, some pathological conditions in the body increase the availability of iron as a result of the release of intracellular iron due to cell death, or a rise in transferrin iron saturation level; as a consequence, host susceptibility to a variety of ferrophilic pathogens, including *V. vulnificus*, is augmented [8, 9].

No time-kill study has been performed on *V. vulnificus* infections using a combination of an antimicrobial agent and the iron chelator deferasirox. Deferasirox (Exjade, DFS) is a tridentate iron chelator belonging to a new class of oral chelator available for routine use in the United States, Europe, and many parts of the world against transfusional iron overload in pediatric and adult patients. We have investigated the in vitro efficacy of ciprofloxacin, a bactericidal drug used in *V. vulnificus* patients, and the

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orally active iron chelator deferasirox. It appeared to be a matter of considerable interest to establish whether the potential antimicrobial action of deferasirox augmented the antimicrobial efficacy of antibiotics.

Materials and methods

Bacterial strains

V. vulnificus ATCC 27562 was obtained from the American Type Culture Collection, ATCC 33815 was purchased from the Korean Collection for Type Cultures (KCTC) Center, and 14 clinical isolates of *V. vulnificus* for the susceptibility test were selected randomly from the samples collected from the patients admitted to Chosun University Hospital, Gwangju, South Korea, in the period 2006 to 2008. The two ATCC strains and one clinical isolate CUH 42-14 were used in the time-kill studies.

Reagents

The media used in this study were Mueller–Hinton broth and Mueller–Hinton agar (Difco Laboratories, Detroit, MI, USA). Standard ciprofloxacin powder was obtained from Bayer HealthCare, Korea, and deferasirox from Novartis Pharma, Basel, Switzerland. Both reagents were prepared as 2.048 mg/mL stock solutions, and the deferasirox was dissolved in dimethyl sulfoxide (DMSO; Amresco, Solon, OH, USA).

MICs

The minimum inhibitory concentrations (MICs) of ciprofloxacin and deferasirox for all 14 clinical isolates and two ATCC strains were determined based on the Clinical and Laboratory Standards Institute (CLSI) guidelines for the micro-broth dilution procedure [10]. The MICs of ciprofloxacin readings were interpreted using the CLSI break-point criteria. The MIC was defined as the lowest concentration of a drug that completely inhibited visible growth of the organism.

Time-kill studies

Time-kill studies were performed to evaluate synergy, as previously described [11, 12]. Bacteria were diluted to a standard cell suspension of about 5.0×10^5 CFU/mL in 50 mL fresh Mueller–Hinton broth (MHB) containing 2% NaCl in a 250-mL conical flask and exposed to the drug. To evaluate the in vitro activities of the drugs, the 3/4 MICs of ciprofloxacin and 1.5 MIC of deferasirox were compared individually and in combination. Control growth was

assessed in an additional conical flask without drugs. The flasks were incubated at 35°C in a shaking incubator at 200 rpm. Then, 2-, 4-, 8-, 12-, and 24-h aliquots of 100 µl were taken, diluted 10-fold in freshly prepared MHB containing 2% NaCl, and cultured overnight at 37°C on duplicate Muller–Hinton Agar (MHA) plates also containing 2% NaCl. Synergy is defined as a decrease of ≥ 2 log₁₀ (100-fold drop) in CFU per milliliter in the drug combination compared to the most active single constituent [13]. Indifference and antagonism were defined at 24 h as a ± 1 log₁₀ kill to < 2 compared to the most efficient agent alone and > 1 log₁₀ growth compared with the less active single agent, respectively. All of the experiments were performed at least in duplicate.

Results

MICs

The MICs for ciprofloxacin and deferasirox against all 14 clinical isolates and two ATCC strains of *V. vulnificus* are shown in Table 1.

Time-kill studies

The time-kill study of ATCC 27562 revealed growth inhibition by 0.0225 µg/mL ciprofloxacin (3/4 MIC) and 12 µg/mL deferasirox (1.5 MIC) until 8 h and 12 h, respectively; the bacteria then began to regrow and increased to an extended level. In the combination regimen, ciprofloxacin (3/4 MIC) plus deferasirox (1.5 MIC) had an inhibitory effect up to 24 h and the mean reduction in bacterial counts was 3.79 ± 0.27 CFU/mL, and showed synergistic effect (Fig. 1a).

Similarly, with ATCC 33815, ciprofloxacin inhibited the growth of bacteria at 0.0469 µg/mL (3/4 MIC), and deferasirox at 12 µg/mL (1.5 MIC), up to 12 h and 2 h, respectively. A combination regimen of ciprofloxacin (3/4 MIC) plus

Table 1 Susceptibilities of two ATCC strains and 14 Chosun University Hospital (CUH) isolates of *Vibrio vulnificus* to three antimicrobial agents and the iron chelator deferasirox

Antimicrobial agents/iron chelator	MICs (mg/L)		
	CUH isolates	ATCC strains	
		Range	27562
Ciprofloxacin	0.0156–0.0625	0.0301	0.0625
Cefotaxime	0.0156–0.0625	0.0156	0.125
Doxycycline	0.0156–0.25	0.0625	0.0625
Deferasirox	8.0	8.0	8.0

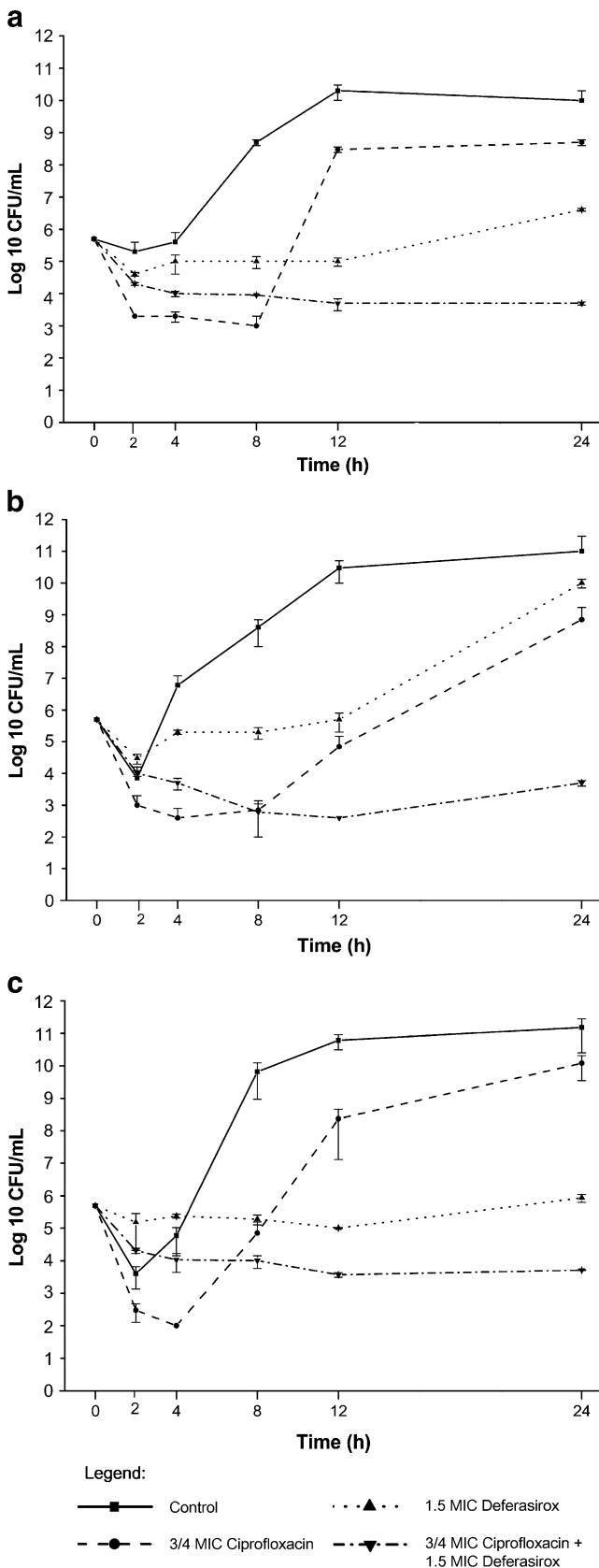


Fig. 1 Time-kill kinetics of *Vibrio vulnificus* (a) ATCC 27562, (b) ATCC 33815, and (c) CUH 42-14 during incubation with 3/4 MIC ciprofloxacin, 1.5 MIC deferasirox, and the combination of 3/4 MIC ciprofloxacin and 1.5 MIC deferasirox. The initial inoculum of *V. vulnificus* was 5×10^5 CFU/mL

deferasirox (1.5 MIC) also showed growth inhibition up to 12 h, with less killing of bacteria than ciprofloxacin monotherapy. Although bacteria in all three regimens started to proliferate after 12 h, the mean reduction of the bacterial counts in combination therapy was 5.15 ± 0.61 log CFU/mL as compared to a single active agent of ciprofloxacin at 24 h and indicating clear synergistic effect (Fig. 1b).

The time-kill study of clinical isolate CUH 42-14 revealed a growth inhibitory effect of ciprofloxacin at $0.0225 \mu\text{g/mL}$ (3/4 MIC) up to 8 h and of deferasirox at $12 \mu\text{g/mL}$ (1.5 MIC) up to 12 h. However, in a combination regimen of ciprofloxacin (3/4 MIC) and deferasirox (1.5 MIC), the growth inhibitory effect continues until 24 h and the mean reduction of bacterial counts was 2.17 ± 0.32 log CFU/mL as compared to the most active single agent of deferasirox and showed synergistic effect (Fig. 1c).

Discussion

V. vulnificus is a halophilic gram-negative bacillus that causes life-threatening skin and soft tissue infections. Chuang et al. [11] reported synergism between cefotaxime and minocycline by inhibiting *V. vulnificus* in vitro, and fluoroquinolones alone have been shown to be as effective as the combination of cefotaxime plus minocycline in vitro and in vivo [14]. Many combinations of antimicrobial agents have been studied for treating *V. vulnificus* infections [1, 4, 11, 14], but few effective combinations to reduce mortality effectively have been identified.

Elevated serum iron levels may play an important role in the pathogenesis of *V. vulnificus* infections [9]. The standard iron chelating agent, deferoxamine, a hydroxamate siderophore derived from streptomyces species, can accelerate the in vitro growth of many ferrophilic organisms, such as *V. vulnificus*, *Yersinia enterocolitica*, and Mucorales. These organisms bind specifically to deferoxamine iron complexes and strip the iron from the chelator via an energy-dependent reductive process, so facilitating iron uptake [15]. *V. vulnificus* is known to bind deferoxamine (Desferal) via the specific receptor *DesA* [16]. Hence, in some patients treated with iron chelator such as deferoxamine in whom yersiniosis or mucormycosis was promoted, the drug may have enhanced the growth of a microbial or neoplastic cell invader by acting as a siderophore instead of assisting the host [17]. Indeed, Neupane and Kim showed recently that the growth of *V. vulnificus* was stimulated by

deferoxamine, whereas two other oral chelators, deferasirox and deferiprone, had an inhibitory effect on growth [18]. Hence, deferasirox and deferiprone are not only useful for the treatment of iron overload, but also function as antimicrobial agents with the potential to act synergistically with various antibiotics [19].

Kim et al. suggested that iron chelation therapy may be an effective means for preventing the growth of *V. vulnificus* in susceptible patients [9]. Therefore, we investigated the in vitro efficacy of the combination of ciprofloxacin plus deferasirox compared to monotherapy with ciprofloxacin against *V. vulnificus*. When ciprofloxacin at 3/4 MICs was combined with deferasirox at 1.5 MICs, the inhibition of bacterial growth persisted in vitro in all strains for at least 24 h, and the combination was superior to ciprofloxacin alone. The antimicrobial effect of deferasirox may be ascribed to its ability to deplete iron that would otherwise be used for bacterial growth. The combination of an iron chelator plus an antibiotic creates a novel form of synergism. It is possible that the quinolone antibiotic interferes with DNA gyrase and that the microbe, therefore, becomes more sensitive to iron deprivation [19]. Based on our time-kill studies, it would appear that ciprofloxacin plus deferasirox is more active combination than ciprofloxacin alone against *V. vulnificus*. Even though ciprofloxacin plus deferasirox had a synergistic effect against *V. vulnificus*, it should be noted that, at early times, ciprofloxacin alone appeared to be more active than the combination treatment. In other experiments, we have found that the higher the ciprofloxacin concentration, the more effective it was at inhibiting the proliferation of micro-organisms (data not shown). Therefore, a high dose of ciprofloxacin may be required to overcome the suboptimal effect of ciprofloxacin plus deferasirox at early times. It will be important to test for the in vivo synergism of this combination to establish the clinical relevance of our findings.

In conclusion, our in vitro time-kill results show that ciprofloxacin plus deferasirox is superior to ciprofloxacin alone. Iron chelators such as deferasirox may, therefore, have clinical utility in combination with antibiotics such as ciprofloxacin on account of their ability to exploit the ferrophilic characteristics of *V. vulnificus*.

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