Studies on the antibacterial activity of a novel fluoroquinolone, OPS-2071, against enteropathogenic bacteria

（腸管感染症起因菌に対する新規フルオロキノロン抗菌剤 OPS-2071 の抗菌活性に関する研究）

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ABBREVIATIONS

95% CI	95% Confidence interval
AAD	Antibiotic-associated diarrhea
AUC	The area under the curve
CDAD	*Clostridioides difficile*-associated diarrhea
CDC	The centers for disease control and prevention
CDI	*Clostridioides difficile* infection
CDT	*Clostridioides difficile* transferase toxin
CFU	Colony forming unit
C_{max}	Maximum concentration
CLSI	The Clinical and Laboratory Standards Institute
D90N	Amino acid substitution from aspartic acid at position 90 to asparagine
D90Y	Amino acid substitution from aspartic acid at position 90 to tyrosine
DNA	Deoxyribonucleic acid
EAEC	Enteroaggregative *Escherichia coli*
ED_{50}	50% effective doses
ETEC	Enterotoxigenic *Escherichia coli*
ESBL	Extended spectrum beta-lactamase
EUCAST	European committee on antimicrobial susceptibility testing
FDA	The United States food and drug administration
FMT	Fecal microbiota transplantation
GyrA	DNA gyrase subunit A
GyrB	DNA gyrase subunit B
IBD	Inflammatory bowel disease
IC_{50}	50% inhibitory concentration
LC-ESI-MS/MS	Liquid chromatography electrospray ionization tandem mass spectrometry
MBC	Minimum bactericidal concentration

MBC_{90}	Minimum bactericidal concentration required to achieve killing of 90% of organisms
MIC	Minimum inhibitory concentration
MIC_{50}	Minimum inhibitory concentration required to inhibit the growth of 50% of organisms
MIC_{90}	Minimum inhibitory concentration required to inhibit the growth of 90% of organisms
MPC	Mutant prevention concentration
MRSA	Methicillin resistant *Staphylococcus aureus*
MSSA	Methicillin susceptible *Staphylococcus aureus*
MSW	Mutant selection window
PAE	Post antibiotic effect
PCR	Polymerase chain reaction
PD	Pharmacodynamic
PK	Pharmacokinetic
QRDR	Quinolone resistance determination region
S83L	Amino acid substitution from serin at position 83 to leucine
S83W	Amino acid substitution from serin at position 83 to tryptophan
S84L	Amino acid substitution from serin at position 84 to leucine
T86A	Amino acid substitution from threonine at position 86 to alanine
T86I	Amino acid substitution from threonine at position 86 to isoleucine
T86K	Amino acid substitution from threonine at position 86 to lysin
WHO	World Health Organization

PREFACE

Intestinal infections are diseases caused by the growth of pathogens in the intestines, causing diarrhea and other symptoms and most frequently resulting from consuming contaminated food or water. Although bacteria, viruses, and protozoa are the causative pathogens, this thesis focuses only on bacteria as the target pathogens. Intestinal infections are common in developing countries where inadequate sanitation is widespread. It is one of the most serious diseases, with 1.7 million deaths reported annually worldwide, especially in children and the elderly over 70 years of age [70)]. The number of patients who have died from intestinal infections is still too high, even though economic development has significantly reduced it over the past decades by improving access to treatment (Figure 1) [2)].

Drug-resistant bacteria are becoming a therapeutic threat in intestinal infections. Drug-resistant bacteria, such as typhoidal/non-typhoidal *Salmonella*, *Shigella* spp, *Campylobacter* spp, *Vibrio* spp, and diarrheal *Escherichia coli*, have been reported as a problem in their treatment [40, 62, 70)]. The spread of *Salmonella* with ESBL (extended spectrum beta-lactamase), azithromycin-resistant *Shigella*, and ciprofloxacin resistance among a wide range of enteric infectious bacteria has reduced the effectiveness of antimicrobial agents, resulting in a depletion of therapeutic agents [70)]. Antibiotic resistance is associated with the widespread use and misuse of antibiotics in humans and agriculture. In developing countries, in addition to the widespread of infectious diseases, the abuse and misuse of antibiotics through the purchase without prescriptions, and the lack of restrictions on antibiotic use in agriculture further contribute to the emergence of resistant bacteria[13, 49)].

Enteric infections are also an important public health problem in developed countries; the Centers for Disease Control and Prevention (CDC) reported on the threat of antibiotic resistance in the United States in 2019, listing 18 antimicrobial-resistant bacteria and fungi including intestinal infectious bacteria, such as *Clostridioides difficile*, *Campylobacter*, typhoidal/non-typhoidal *Salmonella*, and *Shigella* [9)]. Resistance to therapeutic agents against these organisms is rapidly increasing, and if this trend continues, treatment options may be lost for patients who need treatment. For instance, *Campylobacter* spp, one of the most common intestinal infections in the developed world, is estimated at 1.5 million cases per year in the U.S. and an annual medical cost of $2.7 million. Ciprofloxacin and azithromycin are used for treatment, but their

drug resistance is increasing every year. Ciprofloxacin-resistant strains have increased to 28% as of 2017 [9].

C. difficile is also one of the important pathogens that cause infections in the intestinal tract. *C. difficile* is a Gram-positive anaerobic rod that is resistant to many clinically used antimicrobial agents and is known to cause antimicrobial-associated diarrhea caused by disruption of the intestinal flora due to antimicrobial treatment, which leads to microbial substitution, followed by an increase in *C. difficile* (Figure 2) [12]. *C. difficile* is not becoming increasingly resistant to therapeutic agents, but in addition to being inherently resistant to almost all clinically used antimicrobials, it has an estimated 12,800 deaths and 2,230,900 hospitalizations in the U.S. in 2017 and $1 billion in annual health care costs, making it a major problem [9]. The risk factors include antimicrobial therapy, prolonged hospitalization, use of immunosuppressants or proton pump inhibitors, and being over 65 years of age; *C. difficile* is known as one of the most important nosocomial organisms. Vancomycin, metronidazole, and fidaxomicin are therapeutic drugs for *C. difficile* Infection (CDI), metronidazole, however, is considered less effective than the other two drugs. The therapeutic challenge of CDI is its frequent recurrence after treatment. Although treatment with antimicrobial agents is reported to be highly effective, exceeding 80%, recurrence occurs in 10-25% of patients after treatment, and further recurrence is reported in up to 65% of patients with recurrent disease [21]. *C. difficile* is a spore-forming bacterium and is thought to recur when bacteria remaining in the intestine as spores re-grow after treatment. Spores are resistant to heat, acid, and antibiotics. CDI is caused by inoculation with the spores, and the growth of *C. difficile* is normally inhibited by the intestinal flora. When the flora is disrupted by the administration of antimicrobial agents, *C. difficile* begins to proliferate, causing damage to the intestinal tract by two toxins called enterotoxin A and cytotoxin B, the pathogenic agents of CDI (Figure 2) [12]. The *C. difficile* BI/NAP1/027 strain is reported as a hypervirulent strain producing *C. difficile* transferase toxin (CDT; or binary toxin), with strong sporulation ability and increased production of enterotoxin A and cytotoxin B, which are said to be involved in the severe disease. Epidemics of this highly virulent strain have been reported in North America and are considered a new threat to *C. difficile*. Fidaxomicin has been available as a therapeutic agent since 2011 and showed lower results than vancomycin for relapse (25% vancomycin, 15% fidaxomicin), but this inhibition was not confirmed for the BI/NAP/027 strain. Another treatment option, Bezlotoxumab (a

monoclonal antibody that binds to *C. difficile* cytotoxin B), was approved by the FDA in 2016. It has shown efficacy in reducing relapse, but its use has been limited by its high cost and potential side effects. Fecal microbiota transplantation (FMT) is also being investigated as a new treatment option for CDI. Since disruption of intestinal flora is responsible for the development of CDI, FMT, which transplants normal intestinal flora, is a promising treatment for CDI [36]. In fact, the therapeutic effect in combination with conventional antimicrobial agents has shown the lowest recurrence compared to any other therapy and has been reported as a promising treatment, but the therapeutic process has not yet been established, and more time is needed before it can be widely offered as a treatment. These facts have increased the need for further therapeutic agents for intestinal infections.

An important aspect in the development of a therapeutic drug is not only its therapeutic efficacy but also considerations regarding the risk of the emergence of bacterial resistance. In the long history of antibiotic use, the emergence of resistant bacteria seems inevitable. However, the emergence of resistant bacteria must be prevented as much as possible by their proper use. Pharmacokinetic/pharmacodynamic (PK/PD) modeling is an important concept for its proper use (Figure 3) [4]. The PD parameter is sometimes the minimum inhibitory concentration (MIC), an indicator of antimicrobial activity, particularly MIC_{90}, which is the MIC value widely evaluated in clinical isolates, or breakpoint, which is considered the clinically treatable MIC. However, these are only concentrations at which antimicrobial activity is observed, and they do not take into account whether they prevent the emergence of resistant bacteria. In contrast, mutant prevention concentration (MPC) is a parameter for inhibiting the emergence of resistant bacteria and is defined as the concentration that prevents the emergence of resistant bacteria. On the other hand, concentrations below the MPC but above the MIC are defined as a mutant selection window (MSW) because they inhibit the growth of susceptible bacteria but not of resistant bacteria, and thus have a high risk of selectively increasing the number of resistant bacteria, and treatment should avoid this MSW as much as possible [8]. Unfortunately, MPC is not currently widely used as an indicator of PD, as standardized test methods such as the Clinical and Laboratory Standards Institute (CLSI) method, like MIC, have not yet been defined and have not been widely evaluated through clinical isolates. The variability among strains has not been adequately studied. Nevertheless, the emergence of resistant bacteria is a major challenge

common to the entire world, and this concept will become even more important in the future for the long use of antimicrobial agents.

OPS-2071 is a novel quinolone compound synthesized by Otsuka Pharmaceutical Co., Ltd. that targets intestinal infections (Figure 5) [54]. Quinolone antibiotic is one of the most important classes of antibiotics due to their wide spectrum and potent antimicrobial activity, and their favorable pharmacokinetics [20]. In particular, ciprofloxacin, a second-generation quinolone antibacterial agent, was launched in the late 1980s and has still been widely used to date including for intestinal infections. Despite reports of bacterial resistance, it remains a therapeutically important antimicrobial agent, listed by the WHO as an essential drug and an extremely important antibiotic, and one of the most commonly prescribed drugs in the world [79]. Although ciprofloxacin is also recommended as a treatment for intestinal infections, its long-standing use has led to increasing reports of quinolone-resistant strains, particularly in developing countries.

Quinolones target DNA gyrase and topoisomerase, which are essential for bacteria, and are known to work bactericidal by inhibiting these enzymes. There are two major mechanisms of quinolone resistance: one is direct mutations of the target molecule that reduces the binding affinity of the quinolone antimicrobial agent, and the other is mutations to induce inhibition of the uptake of quinolone antimicrobial agents into the bacteria or an accelerated efflux of the drug out of the bacteria. The combination of these two mechanisms is known to induce highly resistant bacteria, but the resistance mechanisms that induce inhibition of drug uptake or promotion of drug efflux do not induce highly resistant bacteria and do not lead to clinically significant resistance [20, 38]. In contrast, direct mutations to target molecules are known to induce high levels of resistance on their own. The region where resistance mutations are introduced is called the quinolone resistance determination region (QRDR), and similar mutations have been reported in many strains of different species [20]. Evaluation against these resistance mutations is useful in terms of predicting antibacterial activity against resistant strains and differentiating them from existing quinolone antimicrobials.

In my study, the potential use of OPS-2071 for various intestinal infections as a therapeutic drug was evaluated. Various aspects of the drug need to be tested in order to evaluate its potential as a therapeutic agent. *In vitro* antibacterial activity, *in vivo* pharmacokinetics, and *in*

vivo therapeutic efficacy are essential for predicting therapeutic efficacy. In addition to estimating therapeutic efficacy, it is also important to consider the risk of the emergence of resistant strains, since the problem is that antimicrobial therapy may become ineffective due to the acquisition of resistance after an initial strong therapeutic effect. In addition, the evaluation of inhibitory activity against the target molecules is useful information for characterizing the drug.

This thesis is composed of two chapters and a conclusion. In CHAPTER I, to evaluate its potential as an antimicrobial agent against *C. difficile* infection, the *in vitro* activity, *in vivo* PK profile, and *in vivo* efficacy were examined. In addition, the frequency of spontaneous resistance and mutant prevention concentration was evaluated as the evaluation of the risk of the emergence of drug resistance. CHAPTER II, to see if OPS-2071 is effective against a wide range of intestinal infections, we evaluated the *in vitro* antibacterial activity and mechanism of action of OPS-2071 against a wide range of enteric infection-causing bacteria, excluding *C. difficile*, as well as the risk of emergence of drug-resistant strains.

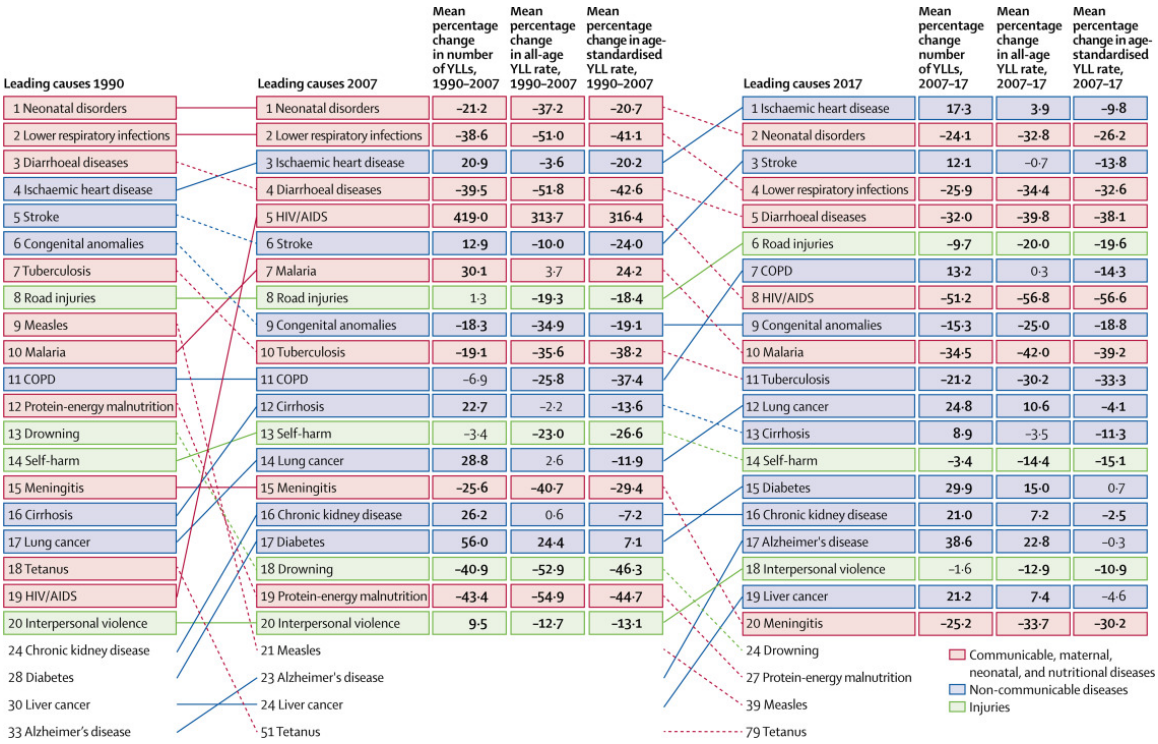

Figure 1. Leading 20 causes of global years of life lost (YLLs) for 1990, 2007, and 2017 with percentage change in number of YLLs, in all-age and age-standardized rates for both sexes combined [2)]

COPD: chronic obstructive pulmonary disease. YLLs: a measure of premature death calculated as the sum of each death multiplied by the standard life expectancy at each age.

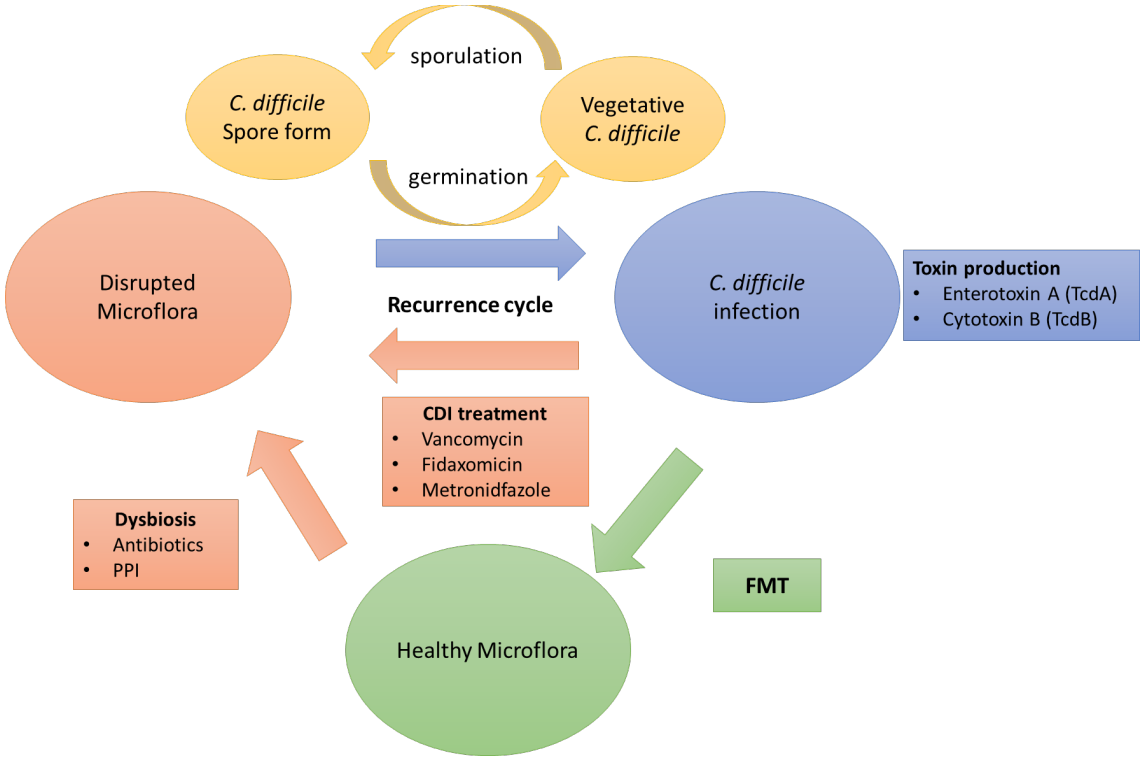

Figure 2. Schematic representation of *C. difficile* infection cycle (based on Chilton CH et al. 2018)[12)]

The disease occurs when *C. difficile* spore proliferates in the intestinal tract and produces toxins due to disruption of the intestinal flora. Recurrence is repeated as sporulated *C. difficile* proliferates again after drug treatment. It can be treated by restoring the normal intestinal microflora with FMT.

(FMT: fecal microflora transplantation, PPI: proton pump inhibitor)

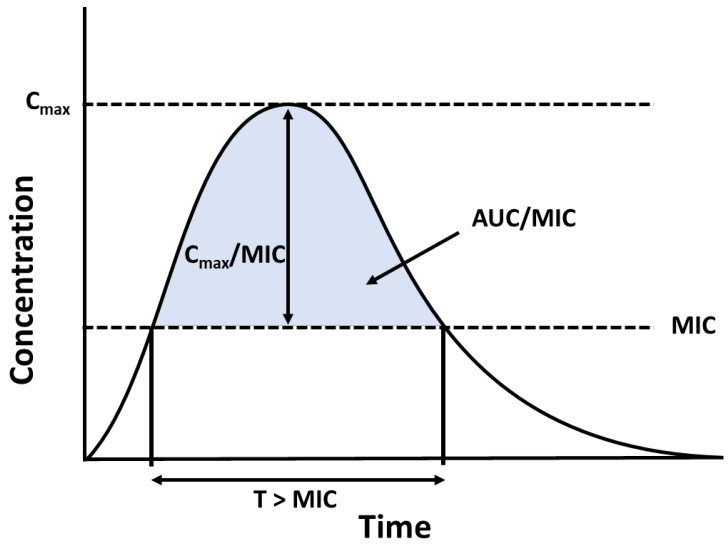

Figure 3. Pharmacokinetic/Pharmacodynamic (PK/PD) indices [4)]

There are three types of antibiotics: concentration-dependent and time-dependent antimicrobial activity. Concentration-dependent drugs increase their activity with increasing concentration. For such drugs, drug administration is optimized by the parameters AUC/MIC (the ratio of the 24-h area under the concentration-time curve over the MIC) and C_{max}/MIC (the peak concentration and MIC ratio). On the other hand, for time-dependent drugs, the effect does not change with increasing concentration, so drug administration is optimized with the parameter T>MIC (the time during which the concentration of the drug was over the MIC) in order to maintain a concentration above the MIC for an extended period of time. Another time-dependent drug shows time-dependent killing and no or very short persistent effects, so drug administration is optimized by the duration of time that active antibiotic concentrations exceeded the MIC. It is usually expressed as the percentage of the dosing interval and only the fraction of the drug not bound to proteins is considered.

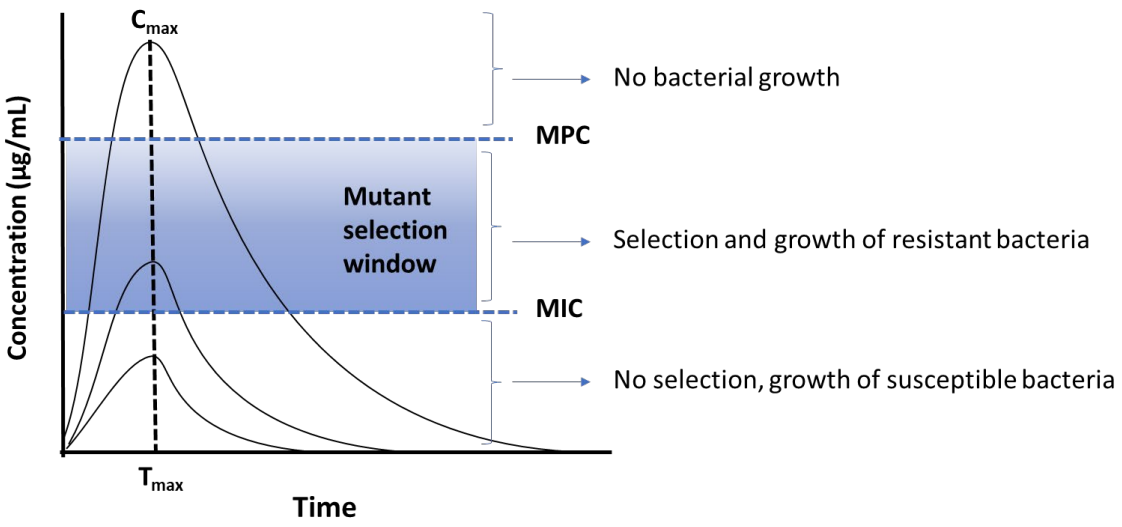

Figure 4. Schematic representation of the relation of MIC, MPC, and MSW (based on Cantón R et al. 2011) [8)]

The figure illustrates three dosing patterns: the low dose does not reach MIC concentration and does not inhibit the growth of the susceptible bacteria and no therapeutic effect is expected. The middle dose exceeds the MIC, which inhibits the growth of susceptible bacteria but does not inhibit resistant bacteria, resulting in selective increase in resistant bacteria. The high dose exceeds the MPC, which also inhibits the growth of resistant bacteria.

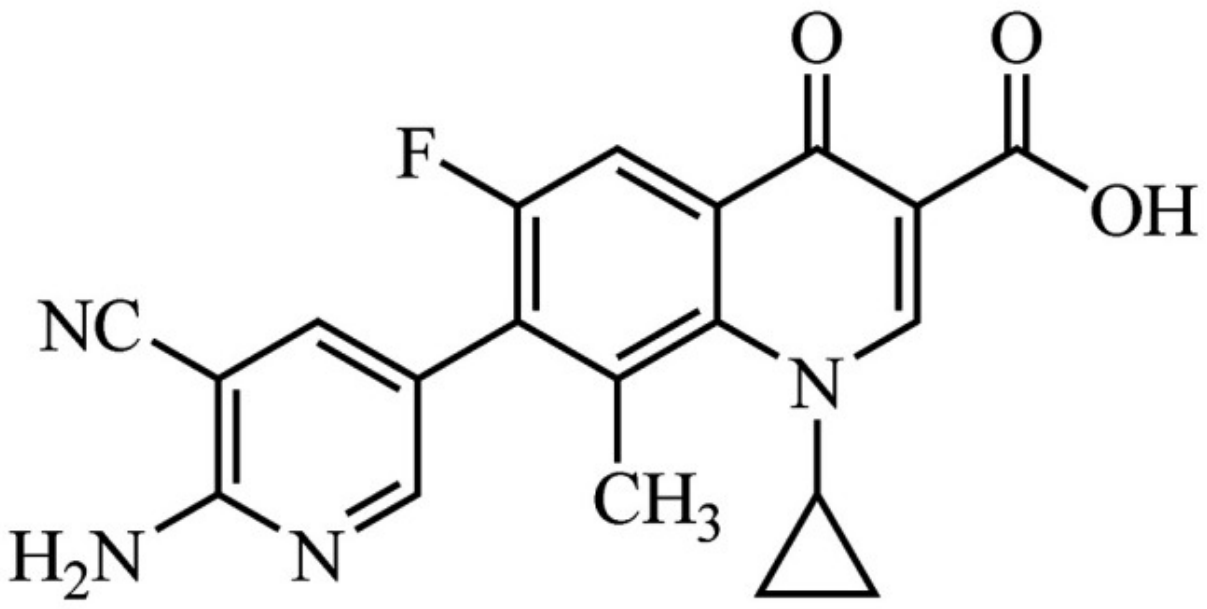

Figure 5. Chemical structure of OPS-2071

CHAPTER I:

In Vitro and *In Vivo* Antibacterial Activities of OPS-2071 against *Clostridioides difficile*

Introduction

Many commercially available antibiotics have the potential to cause diarrhea during treatment of infectious diseases [43)], and they often disrupt the normal balance of intestinal flora. This can lead to an increase in pathogenic bacteria that may eventually induce so-called antibiotic-associated diarrhea (AAD). One of the most important causes of AAD is expansion of *C. difficile*, as it is resistant to many currently available antibiotics. It is specifically designated *C. difficile* infection (CDI) or *C. difficile*-associated diseases (CDAD), which are implicated in 10 to 25% of AAD and the most prevalent infective cause of AAD [3, 44)]. In 2011, the estimated incidence of CDI in the United States was 453,000 and the estimated annual mortality was 295,000 [34)].

C. difficile is capable of producing three toxins, toxin A (TcdA), toxin B (TcdB), and *C. difficile* transferase toxin (CDT). These toxins are closely associated with certain clinical symptoms [29)]. In the early 2000s, hypervirulent strains such as the PCR ribotype 027, referred to as BI/NAP1/027, emerged and spread rapidly [5, 77)]. The BI/NAP1/027 strain produces all three of the toxins referred to above. They damage the gut barrier, leading to severe enterotoxicity in humans, and the BI/NAP1/027 strain is characterized by high-level fluoroquinolone resistance [6, 28)].

Since the toxins of *C. difficile* damage the intestinal epithelial barrier and promote mucosal inflammation, CDI affects gut-related diseases. There are many reports describing how CDI dramatically increases in patients with inflammatory bowel disease (IBD). Consequently, morbidity, mortality, the need for surgery, and health care costs have been increasing due to CDI in IBD patients compared with IBD patients who are not infected [22, 39, 61, 64, 75)]. Therefore, there is general agreement that CDI is the most common gastrointestinal infection in patients with IBD. Treatment of CDI is a critical subject for gut-related diseases such as IBD.

Given the current range of treatment options, vancomycin and fidaxomicin are the preferred first-line therapeutic agents for the initial episode of CDI, but their use in cases of

multiple recurrences is not well established. Metronidazole is also used to treat CDI but is only recommended for treatment of the initial episode in nonsevere cases [51]. Over the preceding decade, new CDI treatments have been developed, such as fecal microbiota transplantation (FMT). FMT restores the diversity of the gut microbiota and has achieved cure rates exceeding 85%. However, there is a risk that the donor stool may cause infection in immunocompromised patients [35]. While FMT is a promising therapy, it has not been widely accepted for broad clinical use. Thus, there is a pressing need for the development of a new therapeutic agent for CDI that can successfully address these issues.

OPS-2071, 7-(6-amino-5-cyanopyridin-3-yl)-1-cyclopropyl-6-fluoro-8-methyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid, is a novel quinolone antibacterial agent (Figure 5) developed at Otsuka Pharmaceutical Co., Ltd., targeting intestinal infection pathogens, including *C. difficile*. It has decreased potential for absorption from the intestine, which in turn reduces the adverse events commonly associated with the fluoroquinolone class of antibiotics. In order to assess the potential utility of OPS-2071 against CDI, the evaluation of *in vitro* and *in vivo* antibacterial activity, spontaneous resistance, and pharmacokinetics was performed and compared to reference compounds.

Materials and methods

Antibiotics

OPS-2071 was synthesized by Otsuka Pharmaceuticals. Co., Ltd. (Tokushima, Japan). ^{14}C-OPS-2071 was synthesized by Curachem, Inc. (Chungcheongbuk-do, South Korea). Ciprofloxacin and levofloxacin were purchased from Sigma-Aldrich (St. Louis, MO). Vancomycin was purchased from Fujifilm Wako Pure Chemical Corp. (Osaka, Japan). Fidaxomicin was purchased from Optimer Pharmaceuticals, Inc. (Jersey City, NJ) and extracted and purified at Otsuka Pharmaceuticals. Co., Ltd.

Microorganisms

Clinically isolated strains were obtained with 105 strains in total. We obtained 4 strains from Aino Hospital (Osaka, Japan) and 39 strains from Miroku Medical Laboratory (Nagano, Japan) as shown in Table 1. A total of 17 hypervirulent and 18 non-hypervirulent strains were obtained from the University of Western Australia (Perth, Australia), and 3 hypervirulent and 24 non-hypervirulent strains were obtained from Rakuno Gakuen University (Hokkaido, Japan) as shown in Table 2. ATCC 700057 and ATCC 43255 were purchased from the American Type Culture Collection (ATCC; Manassas, VA).

Animals

All studies were carried out in adherence to the Guidelines for Animal Care and Use [68)], which were approved by the Animal Care and Use Committee of Otsuka Pharmaceutical Co., Ltd.

Four to five-week-old, specific-pathogen-free, male golden Syrian hamsters were purchased from Japan SLC, Inc. (Shizuoka, Japan). Five- to six-week-old, specific-pathogen-free, male Sprague-Dawley (SD) rats were purchased from Charles River Laboratories Japan, Inc. Hamsters were housed in mouse Hi-PSF cages (Clea Japan, Inc.) during the infection study and Econ TPX cages (Clea Japan, Inc.) during the PK study. SD rats were housed in stainless bracket cages (Nihon Cage Co, Ltd.) and Econ TPX cages (Clea Japan, Inc.) Animals were fed with a certified diet (CRF-1; Oriental Yeast Co., Ltd.) ad libitum. Water, food, and bedding were autoclaved prior to use. Animals were allowed to acclimate in the animal facility, in which environmental

controls were set to the following conditions: a temperature of 23 ± 2°C, humidity of 60% ± 10%, and a 12 hours light-dark cycle (light period, 7 a.m. to 7 p.m.) for more than 1 week. Ten randomly selected hamsters per group were used for the experimental infection model. Five hamsters per group and three rats per group, randomly selected, were used for the pharmacokinetic study.

Determination of antibacterial activity

MICs obtained using the agar dilution method were determined visually as described for the Clinical and Laboratory Standards Institute (CLSI) method [16, 19]. MICs obtained using the broth dilution method were determined visually based on the CLSI method except for using Gifu anaerobic medium (GAM) broth instead of supplemented Brucella broth [16, 19].

Minimum bactericidal concentration testing

From among 43 strains, the 8 *C. difficile* strains sensitive to the antibiotics being tested were selected for the minimum bactericidal concentration (MBC) test. Using the agar dilution method, these sensitivities were determined based on the MIC for OPS-2071, vancomycin, fidaxomicin, and metronidazole as shown in Table 1. In this test, MIC testing was performed using the broth dilution method as recommended in the CLSI [16]. After the MICs were determined, 10 µl of the bacterial suspension from the MIC test tube was inoculated onto agar plates and incubated at 37°C. The bacterial colonies were then counted, and bacterial numbers were calculated. The MBC was considered to be the lowest concentration of the antibiotics that prevented growth and reduced the inoculum by >99.9% within 48 hours, irrespective of counts of survivors at higher antibiotic concentrations.

Killing kinetics

The bacterial suspensions of *C. difficile* ATCC 700057 in GAM broth were precultured for 2 hours, and the test agents were added to the test tubes at concentrations of 0.5-, 1-, 2-, and 4-fold MIC. The test tubes were incubated for 48 hours. The colony forming unit (CFU)/ml values of the bacterial suspensions were calculated at 0, 2, 4, 6, 24, and 48 h by culturing 0.1-ml samples of serial 10-fold dilutions on GAM agar plates at 37°C under anaerobic conditions and then counting the number of growing colonies. The CFU/ml value at each time point is from one sample of datum.

Inhibition of DNA gyrase activity

The subunits A and B of DNA gyrase of *C. difficile* were purchased from Inspiralis Ltd. (Norwich, UK). The supercoiling activity of DNA gyrase was determined as described previously [30]. The inhibitory effect of compounds was assessed by determining the concentration required to inhibit 50% of the enzyme (IC_{50}). The gyrase supercoiling reaction mixtures (30 µl), which contained gyrase (1 unit) and relaxed pBR322 DNA (500 ng), with or without drug solution, were incubated at 37°C for 30 min. The assay reaction mixture was analyzed by electrophoresis. The gels were stained with ethidium bromide, and the density of the supercoiled plasmid was obtained using a UV illuminator. The image of the supercoiled plasmid was analyzed using image processing software (ImageJ 1.50i; National Institutes of Health, USA). The IC_{50} was defined as the concentration that caused 50% inhibition of the supercoiled plasmid.

Frequency of spontaneous resistance

The frequency of spontaneous *C. difficile* ATCC 700057 resistance to OPS-2071, vancomycin, metronidazole, and fidaxomicin was measured by inoculating the bacterial suspension onto a supplemented Brucella agar (Brucella agar with 5 µg/ml of hemin, 1 µg/ml of vitamin K1, and 5% laked horse blood) plate containing antibiotics at 4-, 16-, and 64-fold the agar dilution MIC. The frequency of spontaneous resistance to each compound was calculated as the number of resistant colonies formed per the number of inoculated bacteria.

Mutant prevention concentration

The mutant prevention concentration (MPC) was measured by plating approximately 10^{10} CFU of *C. difficile* ATCC 700057 onto a GAM agar plate containing the test agent. Inoculated plates were incubated for 3 days, and the MIC for each antibiotic that prevented the growth of colonies was determined using the agar dilution method as described by the CLSI [16]. The MIC values of grown colonies were compared to determine which bacteria were resistant. The MPC was defined as the lowest concentration that prevented the growth of resistant bacteria.

Determination of post antibiotic effect

C. difficile ATCC 700057 was cultured in GAM agar and adjusted in GAM broth to approximately 10^6 CFU/ml. The bacterial suspensions were preincubated at 37°C for 1 hour. Before and after preincubation, the CFU/ml was determined using serial cultures of 10-fold dilutions on GAM agar at 37°C. After preincubation, test and reference solutions were added to each bacterial suspension in a test tube, and the suspensions were cultured for 1 hour. After exposure to the test compounds, bacterial suspensions were washed with GAM broth and cultured for 6 hours. In order to calculate the CFU/ml of these suspensions every hour, serial 10-fold dilutions were cultured on agar plates. The post antibiotic effect (PAE) was defined according to Craig and Gudmundsson as PAE = T − C, where T is the time required for the viable counts of the exposed bacteria to increase by 1 log10 above the counts observed immediately after washing, and C is the corresponding time for the antibiotic unexposed controls [76)]. The PAEs of OPS-2071 were compared with those of other compounds at 4× and 8× the MIC.

Cytotoxicity assay

The BALB/c mouse fibroblast cell line, BALB/3T3 clone A31 cell (JCRB9005), was obtained from the Health Science Research Resources Bank (Osaka, Japan) and was cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% heat-inactivated newborn calf serum (Gibco), 100 IU/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich). The neutral red uptake assay was adapted to determine cytotoxicity as follows [7)]. The cells were seeded into a 96-well plate at 1×10^4 cells/well in 100 µl of culture medium and incubated for 25 hours at 37°C in 5% CO_2. After the culture medium was removed, cells were treated with negative control (dimethyl sulfoxide) and OPS-2071 (1.77 to 100 µg/ml, six wells/dose) in Earl's balanced salt solution (EBSS; Sigma-Aldrich) supplemented with 10 mmol/liter N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES; Sigma-Aldrich) for 1 h and 50 min, followed by incubation with culture medium for 21 hours. The culture medium was replaced with 50 µg/ml neutral red (Sigma-Aldrich) containing culture medium and incubated for 3 hours. The cells were washed with Dulbecco's phosphate-buffered saline (Gibco), and neutral red was extracted with an extraction solution (50% ethanol, 49% water, and 1% acetic acid). Absorbance

at 540 nm was measured using a Molecular Devices Emax plate reader (Molecular Devices, San Jose, CA), and cell viability was calculated.

Pharmacokinetics in the hamster

OPS-2071 was orally administered at 2 mg/kg to male Syrian hamsters, and the cecal contents were collected 0.5, 1, 2, 4, 8, 24, 48, and 72 hours postdose (n = 5 for each time point). After homogenizing with saline and treatment using acetonitrile-formic acid (100:1 [vol/vol]), the OPS-2071 concentration in cecal contents was determined using a validated liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) method. The mass spectrometer 4000 QTRAP (AB Sciex Pte. Ltd.) and high-performance liquid chromatography Prominence UFLC system (Shimadzu Corp.) were used. The pharmacokinetic (PK) parameter, the maximum concentration (C_{max}) was determined with WinNonlin professional software version 6.3, (Pharsight Corp.).

Pharmacokinetics in the rat

OPS-2071 was orally or intravenously administered at 1 mg/kg to male SD rats, and plasma was collected 0.5, 1, 2, 4, 6, 8, 12, 24, 48, and 72 hours postdose (oral, n = 3 for each time point) or 0.083, 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 48, and 72 hours postdose (intravenous, n = 3 for each time point). After solid-phase extraction, the OPS-2071 concentration in plasma was determined by a validated LC-ESI-MS/MS method, and bioavailability was calculated. The mass spectrometer QTRAP 5500 (AB Sciex Pte. Ltd.) and high-performance liquid chromatography Prominence UFLCXR system (Shimadzu Corp.) were used. The PK parameter, the area under the curve at time t (AUC_t), was determined with the same WinNonlin professional software, and the bioavailability was calculated from $AUC_{t,oral}$ and $AUC_{t,intravenous}$.

^{14}C-OPS-2071 was orally administered at 3 mg/kg to male SD rats. Blood was collected 1, 2, 4, 6, 8, 12, 24, 72, and 168 hours postdose (n = 3 for each time point), the stomach, small intestine, and large intestine were collected 1, 4, 8, 24, 72, and 168 hours postdose (n = 3 for each time point), and feces were collected up until 168 hours postdose (n = 3). The collected blood, stomach, small intestine, large intestine, and feces were dissolved in a tissue solubilizer, and radioactivity was measured using a liquid scintillation counter LSC-6101 (Aloka Co., Ltd.) to

calculate the concentration. Cumulative excretion in feces was calculated from fecal radioactivity concentrations.

In vivo hamster model of *C. difficile* infection

C. difficile ATCC 43255 was cultured on GAM agar, and a bacterial suspension was prepared in saline [50]. The MIC values of OPS-2071, vancomycin, and fidaxomicin against *C. difficile* ATCC 43255 are 0.016, 2, and 0.06, respectively. Hamsters were infected using oral gavage with 0.5 ml of suspension containing approximately 10^6 CFU of *C. difficile* (day −1). The next day, the animals were administered clindamycin phosphate (0.2 mg/ml in water) by oral gavage at a dose of 5 ml/kg of body weight, for a final dose of 1 mg/kg (day 0). The animals were allocated to the test groups using a stratified randomization method based on the body weight of each infected animal. The test compounds were administered by oral gavage once daily for 5 days (OPS-2071, 0.008 to 1 mg/kg; fidaxomicin, 0.04 mg/kg to 5 mg/kg; vancomycin, 0.04 to 5 mg/kg [5-fold dilution]). Infected control animals were administered 5% (wt/vol) gum arabic solution by oral gavage. Animals were observed at least once daily for mortality and the presence or absence of diarrhea, and mortality was recorded once daily. From the perspective of animal protection, any significantly debilitated hamsters were euthanized for humane reasons. The cecal contents of dead animals were tested for toxin A and toxin B using C.Diff Quik Chek Complete (Abbott Diagnostics Medical Co., Ltd., Tokyo, Japan) to confirm that the death of the animal was due to CDI.

Statistical analysis

Statistical analyses were conducted using SAS software release 9.3 (SAS Institute Japan). The significance level of the test was set at 5%. Differences between hypervirulent and non-hypervirulent strains were statistically analyzed using two-tailed Wilcoxon rank sum tests. The statistical significance of OPS-2071, fidaxomicin, and vancomycin treatment was analyzed at all doses against the vehicle. Survival curves were estimated for each group using the Kaplan-Meier method. Differences in survival homogeneity in each group were evaluated with a log-rank test with Dunnett-type (two-sided) comparison. The effective dose at 50% (ED_{50}) of the dose-response was generated for OPS-2071, vancomycin, and fidaxomicin on day 20, and its 95% confidence interval was determined using a probit method.

Results

Antibacterial activity of OPS-2071 against 43 clinically isolated strains of *C. difficile*

The *in vitro* antibacterial activities of OPS-2071 and other relevant compounds against 43 clinically isolated *C. difficile* strains obtained from Aino Hospital (Osaka, Japan) and Miroku Laboratory (Nagano, Japan) are shown in Table 1. As expected, commonly used quinolone antibiotics such as ciprofloxacin and levofloxacin are inactive against *C. difficile* isolates. Compared with conventional therapeutic agents (vancomycin, metronidazole, and fidaxomicin), the minimum inhibitory concentration required to inhibit the growth of 50% and 90% of organisms (MIC_{50} and MIC_{90}) of OPS-2071 were, respectively, 16-fold and 8-fold lower than that of vancomycin, 16-fold and 4-fold lower than that of metronidazole, and 2-fold and 8-fold higher than that of fidaxomicin. Using the method described by CLSI, the MICs of OPS-2071, vancomycin, and fidaxomicin against a quality control strain, *C. difficile* ATCC 700057, were 0.031, 2, and 0.063 μg/ml, respectively [19)]. These results indicate that OPS-2071 possesses comparable activity to that of fidaxomicin and was more active than both vancomycin and metronidazole against *C. difficile* on a concentration basis.

Antibacterial activity comparison between 20 hypervirulent and 42 non-hypervirulent strains of *C. difficile*

In order to compare the antibacterial activity of OPS-2071 between hypervirulent and non-hypervirulent strains, 20 hypervirulent and 42 non-hypervirulent strains were obtained from the University of Western Australia (Perth, Australia) and Rakuno Gakuen University (Hokkaido, Japan). *C. difficile* can be characterized by PCR ribotyping [28)]. Several ribotypes are known to be epidemiologically hypervirulent, high-toxin-producing strains. In addition, these strains were reported to be highly resistant to existing fluoroquinolones [43, 47, 52, 57)]. We tested the *in vitro* antibacterial activity against 62 strains of *C. difficile*, which were genetically characterized and different from the 54 strains in Table 1, including 20 epidemiologically hypervirulent strains (PCR ribotype 018, 2 strains; 023, 2 strains; 027, 4 strains; 056, 2 strains; 078, 7 strains; and 244, 3 strains) [5, 23, 46, 77)]. There was no significant difference in the susceptibility to OPS-2071 and vancomycin between hypervirulent and non-hypervirulent strains

(Table 2); however, significant differences in susceptibility to fidaxomicin ($P < 0.01$) and metronidazole ($P < 0.05$) were found (Wilcoxon rank sum test).

Effect of pH and serum on *in vitro* activity

The MICs for OPS-2071 and reference compounds against *C. difficile* (ATCC 700057) were assessed under different pH conditions (Table 3). Little or no difference in MIC of OPS-2071 was observed at pH 6, 7, or 8, with a MIC of 0.016, 0.016, and 0.03 µg/ml, respectively. To investigate the effect of human serum on antibacterial activity, the MICs of OPS-2071, vancomycin, and fidaxomicin against *C. difficile* ATCC700057 were determined in the presence of 10%, 30%, and 50% human serum. The MIC of OPS-2071 was increased by only two to four-fold in the presence of 30% and 50% human serum, respectively; while the MIC of fidaxomicin increased eight to thirty-two fold by human serum (Table 4). These data indicate that OPS-2071 and vancomycin are less influenced by human serum than fidaxomicin.

Bactericidal activity

The inhibitory and bactericidal activities of OPS-2071 and other drugs against 8 of the 43 clinical isolates are summarized in Table 5. These 8 clinical isolates were selected from 35 of 43 clinical isolates, described in Table 1, based on the inclusion criterion of a smaller MIC_{90} to OPS-2071, vancomycin, metronidazole, and fidaxomicin. The minimum bactericidal concentration of OPS-2071 required to eradicate 90% of organisms (MBC_{90}) was the same as the MIC_{90}. In contrast, the MBC_{90} of the other drugs was 1- to 2-fold higher than the corresponding MIC_{90} values. These data indicate that OPS-2071 has bactericidal activity against clinically isolated strains of *C. difficile* at a concentration equivalent to the MIC.

Inhibition of DNA gyrase activity

DNA gyrase and DNA topoisomerase IV are, respectively, the primary and secondary targets of fluoroquinolones. In *C. difficile*, evidence exists that the secondary target is absent [26]. To evaluate the mechanism of action, the ability of OPS-2071 to inhibit DNA gyrase was evaluated and compared with that of existing quinolones in *C. difficile*. As expected, the 50% inhibitory concentration (IC_{50} [µg/ml]) of OPS-2071 was much lower than those of ciprofloxacin and levofloxacin (OPS-2071 IC_{50}, 0.23 [range, 0.17–0.32]; ciprofloxacin IC_{50}, 8.59 [4.23–22.29]; levofloxacin IC_{50}, 11.49 [6.08–46.29]).

Killing kinetics

Time-kill curves for OPS-2071, vancomycin, metronidazole, and fidaxomicin were determined using *C. difficile* ATCC 700057 (Figure 6). In order to do this assay, the MICs of OPS-2071, vancomycin, metronidazole, and fidaxomicin against the strain were retested for this assay, and the MICs determined by a broth dilution method were 0.03, 4, 0.5, and 0.03 μg/ml, respectively. After the addition of OPS-2071 at concentrations higher than the MIC, the numbers of viable bacteria decreased rapidly, with a 2-log reduction in the first 2 hours and a continued decrease to an undetectable level within 24 hours. After 48 hours of incubation, regrowth of the bacteria did not occur. The killing kinetics of fidaxomicin were close to those of OPS-2071. As for vancomycin and metronidazole, these drugs needed over 2-fold MIC for an undetectable level to be reached. The killing kinetics of OPS-2071 showed that the drug is effective against *C. difficile*.

Frequency of spontaneous resistance

To test the frequency of spontaneous resistance, the MICs were determined for this assay. The MICs of OPS-2071, vancomycin, and fidaxomicin against *C. difficile* ATCC 700057 were 0.03, 2, and 0.06 μg/ml, respectively. The frequency of spontaneous resistance to OPS-2071 and other compounds are shown in Table 6. No mutants were observed at concentrations of 4-, 16-, and 64-fold MIC of OPS-2071 with a frequency of less than 9.17×10^{-9}. In contrast, resistant mutants were observed at 4-fold MIC of fidaxomicin. *C. difficile* strain ATCC 700057 did not show a propensity to develop spontaneous resistance in response to OPS-2071.

Mutant prevention concentration

The MPC against *C. difficile* ATCC 700057 is defined as the minimal concentration at which there is no emergence of resistant bacteria [48)]. The MPC of OPS-2071, vancomycin and fidaxomicin are 0.5, 8, and 8 μg/ml, respectively. The MPC of OPS-2071 was the lowest among the tested compounds. This study suggests that the risk of emergence of OPS-2071-resistant *C. difficile* strains is considerably lower than that for vancomycin and fidaxomicin.

Post antibiotic effect of OPS-2071

PAE is defined as the delayed regrowth or the persistent growth suppression of bacteria after short antimicrobial exposure. The PAEs of OPS-2071 against *C. difficile* were determined and compared with those of other drugs (Table 7). A broth method was used to determine the MICs of OPS-2071, vancomycin, and fidaxomicin against *C. difficile* ATCC 700057. The MICs were 0.06, 4, and 0.06 μg/ml, respectively, and the PAEs of OPS-2071 at 4- and 8-fold the MIC against *C. difficile* (3.93 to 4.04 hours) were longer than those of vancomycin (1.03 to 1.25 hours) and fidaxomicin (2.86 to 2.77 hours). As OPS-2071 showed a longer PAE against *C. difficile* than either vancomycin or fidaxomicin, the duration of OPS-2071's antibacterial efficacy against *C. difficile* can be expected to be longer than that of the other two agents.

Cytotoxicity assay

The cytotoxicity of OPS-2071 was determined with the neutral red uptake assay in BALB/3T3 clone A31 cells. OPS-2071 did not show cytotoxicity up to 100 μg/ml (IC_{50}, >100 μg/ml).

Pharmacokinetics in the hamster

OPS-2071 was orally administered at 2 mg/kg. The maximum concentration (C_{max}) in the cecal contents reached 42.95 μg/g at 4 hours postdose and then decreased to 1.84 μg/g at 24 hours postdose (Figure 7). This concentration was many times higher than the MIC_{90} and MBC_{90} of OPS-2071 for *C. difficile*.

Pharmacokinetics in the rat

OPS-2071 showed low systemic exposure, with values for the area under the concentration-time curve calculated to the last observable concentration at time t (AUC_t) after oral and intravenous administration of OPS-2071 at 1 mg/kg of 19.48 and 671.7 ng · h/ml, respectively (Figure 8). Therefore, the calculated oral bioavailability was 2.9%. In addition, orally administered ^{14}C-OPS-2071 derived radioactivity was distributed to the large intestine at a high concentration (Figure 9), and more than 95% of dosed radioactivity was excreted in feces. These results indicate that the low absorption of OPS-2071 makes it suitable for treating CDI.

In vivo activity in the hamster

The effectiveness of OPS-2071 and other drugs was assessed in a hamster model of CDI. This model is the current gold standard used to assess potential efficacy in the treatment of CDI [50, 60, 66, 74]. The survival curve for each drug tested in this study is shown in Figure 10. After infection, all hamsters treated with vehicle died within 5 days. Compared with the vehicle control, significant efficacy was observed for OPS-2071 at dosages of 0.04 mg/kg and above. In contrast, significant efficacy was observed for vancomycin and fidaxomicin only at dosages of 1 mg/kg and above. The ED_{50} of OPS-2071, vancomycin, and fidaxomicin were 0.0313 mg/kg/day (95% confidence interval [CI], 0.0131 to 0.0686 mg/kg/day), 1.22 mg/kg/day (95% CI, 0.597 to 2.39 mg/kg/day), and 1.63 mg/kg/day (95% CI, 0.836 to 3.20 mg/kg/day), respectively. OPS-2071 also had an ED_{50} 39.0-fold lower than that of vancomycin and 52.1-fold lower than that of fidaxomicin.

Table 1. MICs for 43 clinically isolated strains of *C. difficile*

	MIC (µg/ml)		
	MIC_{50}	MIC_{90}	MIC range
OPS-2071	0.125	0.5	0.016 - 1
Ciprofloxacin	64	128	16 - 128
Levofloxacin	128	>128	4 - >128
Vancomycin	2	4	1 - 8
Metronidazole	2	2	1 - 4
Fidaxomicin	0.063	0.063	0.016 - 0.125

Table 2. MIC distribution of each drug against hypervirulent and non-hypervirulent strains

MIC (μg/ml)	Cumulative percentage of 62 strains							
	OPS-2071		Vancomycin		Fidaxomicin		Metronidazole	
	hyp	non-hyp	hyp	non-hyp	hyp	non-hyp	hyp	non-hyp
Strain No.	20	42	20	42	20	42	20	42
<0.004						0		
0.008						5		
0.016	0	0				5		
0.031	65	79				10		
0.063	75	88			0	52		
0.125	80	90			40	86	0	0
0.25	100	100		0	90	98	45	74
0.5			0	10	100	100	85	95
1			95	81			95	100
2			100	98			95	
4				100			95	
8							95	
>16							100	
MIC range (μg/ml)	0.03 - 0.25	0.03 - 0.25	1 - 2	0.5 - 4	0.125 - 0.5	0.008 - 0.5	0.25 - 16	0.25 - 1
MIC_{50} (μg/ml)	0.03	0.03	1	1	0.25	0.06	0.5	0.25
MIC_{90} (μg/ml)	0.25	0.125	1	2	0.25	0.25	1	0.5

hyp: hypervirulent strain, non-hyp: non-hypervirulent strain

Table 3. Effect of pH on antibacterial activity against *C. difficile* ATCC 700057

		MIC (μg/ml)		
		OPS-2071	Vancomycin	Fidaxomicin
	6	0.016	2	0.03
pH	7	0.016	2	0.06
	8	0.03	2	0.06

MICs for *C. difficile* ATCC 700057 were determined visually by the agar dilution method. In order to examine the effect of pH, Supplemented Brucella Broth with 5 μg/ml hemin, 1 μg/ml vitamin K1 was adjusted using 1N NaOH or 1N HCl and added agar powder (final 1.5%) to produce pH values of 6, 7, or 8. The differences in the MICs of OPS-2071 over a range of pH were comparable to those of vancomycin and fidaxomicin.

Table 4. Effect of human serum on antibacterial activity against *C. difficile* ATCC 700057

		MIC (μg/ml)		
		OPS-2071	Vancomycin	Fidaxomicin
	None	0.06	4	0.06
Human	10%	0.125	4	0.5
serum	30%	0.125	4	1
	50%	0.25	8	2

MICs for *C. difficile* ATCC 700057 were determined visually by the agar dilution method. In order to test the concentration of human serum, heat-inactivated (for 30 minutes at 56 °C), human serum was added to GAM broth at concentrations of 0, 10, 30, and 50%. OPS-2071 showed a lower MIC than that of vancomycin and fidaxomicin even in 50% human serum.

Table 5. Comparison of MICs and MBCs of OPS-2071 and other compounds against *C. difficile*

	MIC (µg/ml)		MBC (µg/ml)		MBC_{90}/MIC_{90}
	range	90%	range	90%	
OPS-2071	0.03 - 0.5	0.5	0.03 - 0.5	0.5	1
Vancomycin	2 - 4	4	4 - 8	8	2
Metronidazole	1 - 4	4	1 - 4	4	1
Fidaxomicin	0.06 - 0.25	0.25	0.06 - 0.25	0.25	1

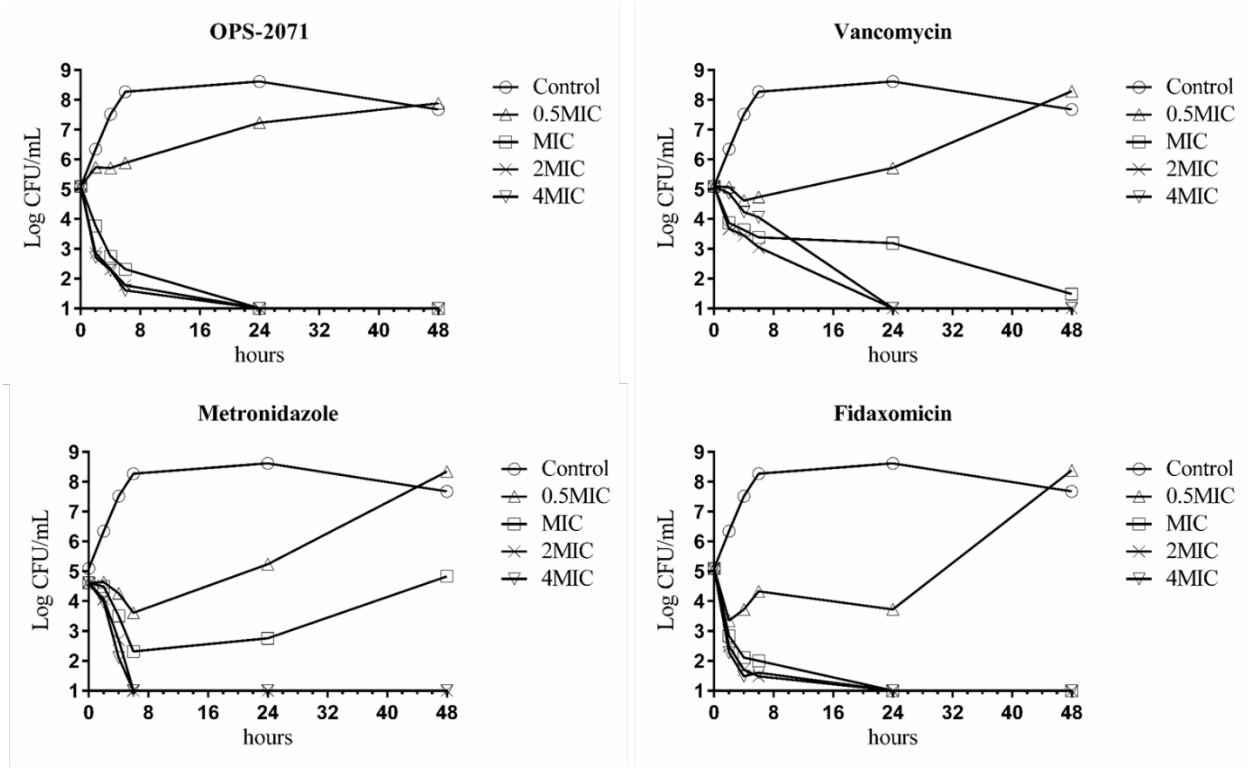

Figure 6. ***In vitro*** **time-killing curve against** ***C. difficile***

Table 6. Frequency of spontaneous resistance against *C. difficile*

	Dose	Test concentration (μg/ml)	Frequency
OPS-2071	4 × MIC	0.125	$<9.17 \times 10^{-9}$
	16 × MIC	0.5	$<9.17 \times 10^{-9}$
	64 × MIC	2	$<9.17 \times 10^{-9}$
Vancomycin	4 × MIC	8	$<9.17 \times 10^{-9}$
	16 × MIC	32	$<9.17 \times 10^{-9}$
	64 × MIC	128	$<9.17 \times 10^{-9}$
Fidaxomicin	4 × MIC	0.25	1.47×10^{-7}
	16 × MIC	1	$<9.17 \times 10^{-9}$
	64 × MIC	4	$<9.17 \times 10^{-9}$

Table 7. Post-antibiotic effect of OPS-2071 against *C. difficile* (hours)

	OPS-2071	Vancomycin	Fidaxomicin
4×MIC	3.93	1.03	2.86
8×MIC	4.04	1.25	2.77

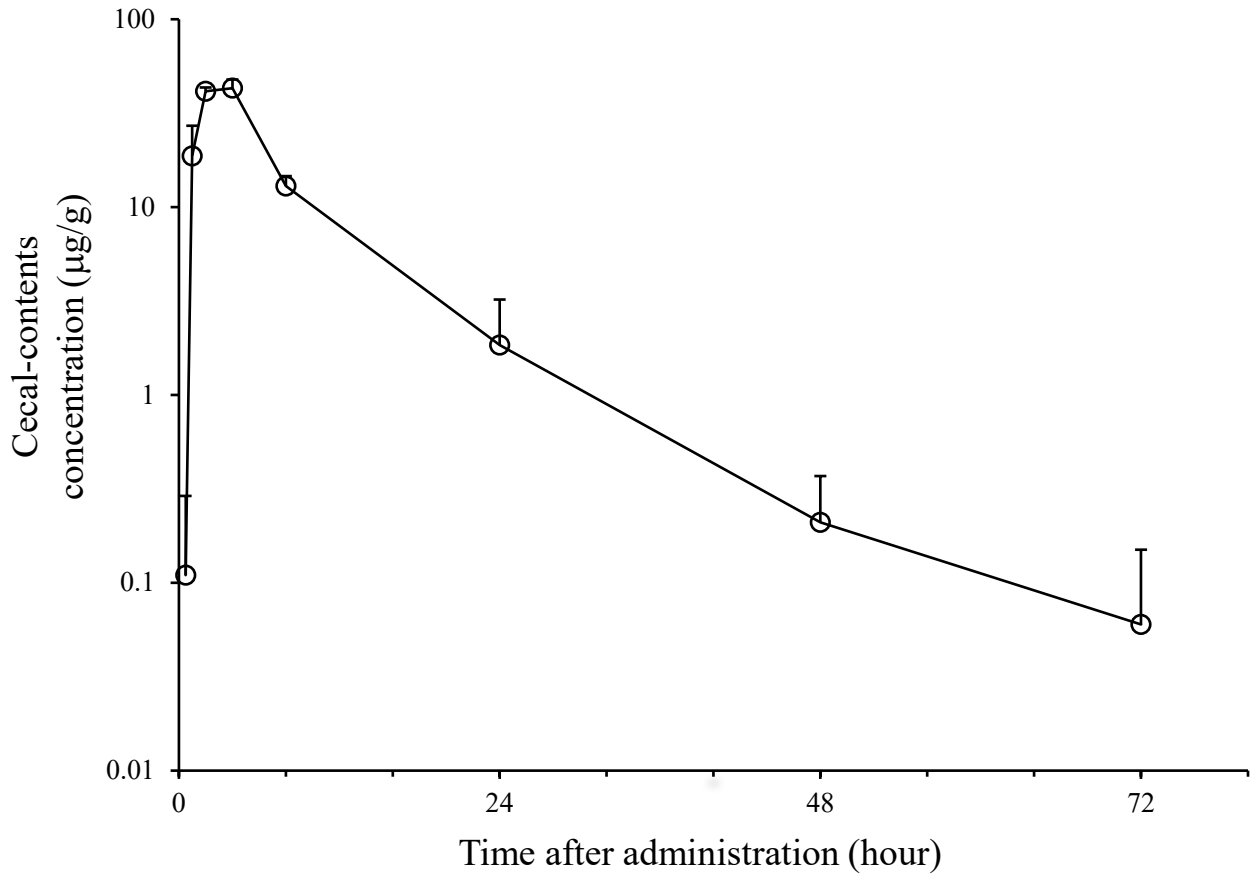

Figure 7. Time-concentration profile of OPS-2071 in cecal contents after oral administration of OPS-2071 to hamsters

OPS-2071 was orally administered at 2 mg/kg to male Syrian hamsters. The concentration in cecal contents was determined after 0.5, 1, 2, 4, 8, 24, 48, and 72 hours postdose (n = 5 for each time point, Mean + SD). C_{max} was 42.95 µg/g at 4 hours post-dose.

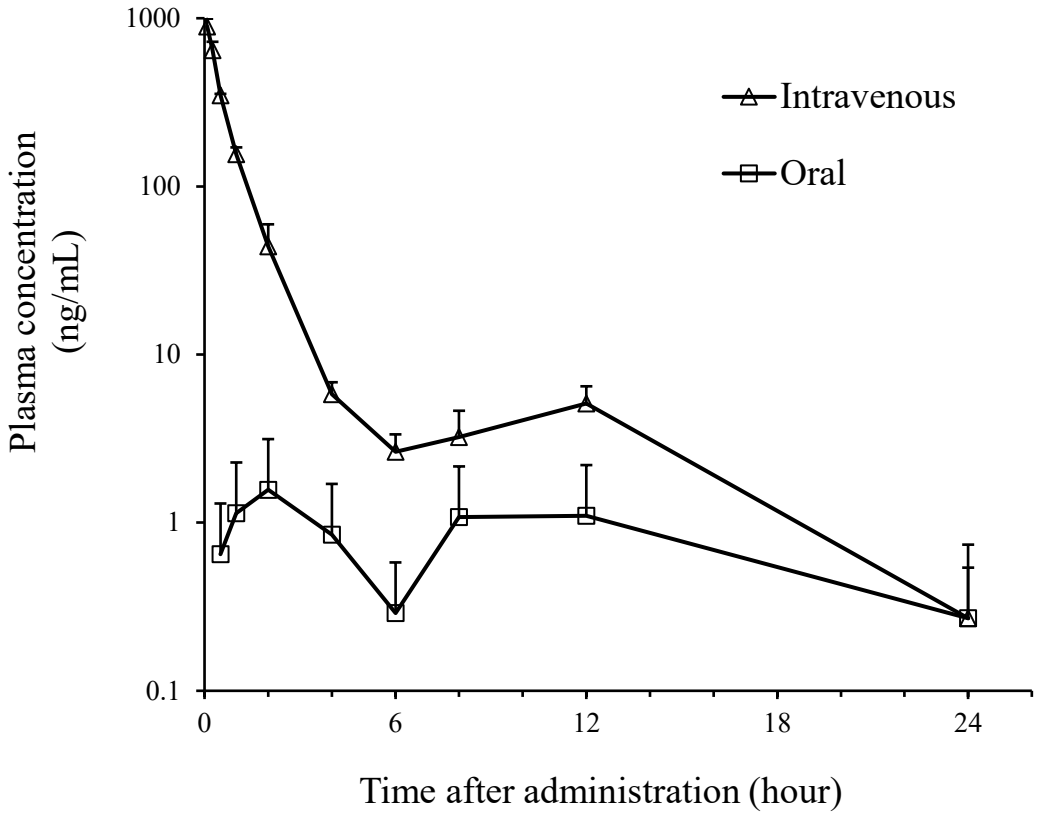

Figure 8. Time-concentration profile of OPS-2071 in plasma after oral and intravenous administration to rats

OPS-2071 was orally or intravenously administered at 1 mg/kg to male SD rats, and then plasma was collected. The concentration of OPS-2071 in plasma was determined by a validated LC-ESI-MS/MS method (Mean + SD, n = 3), and then bioavailability was calculated. The concentrations at 48 and 72 hours after administration were < 0.5 ng/mL. The curve showed a bimodal plasma concentration time profile due to bile excretion.

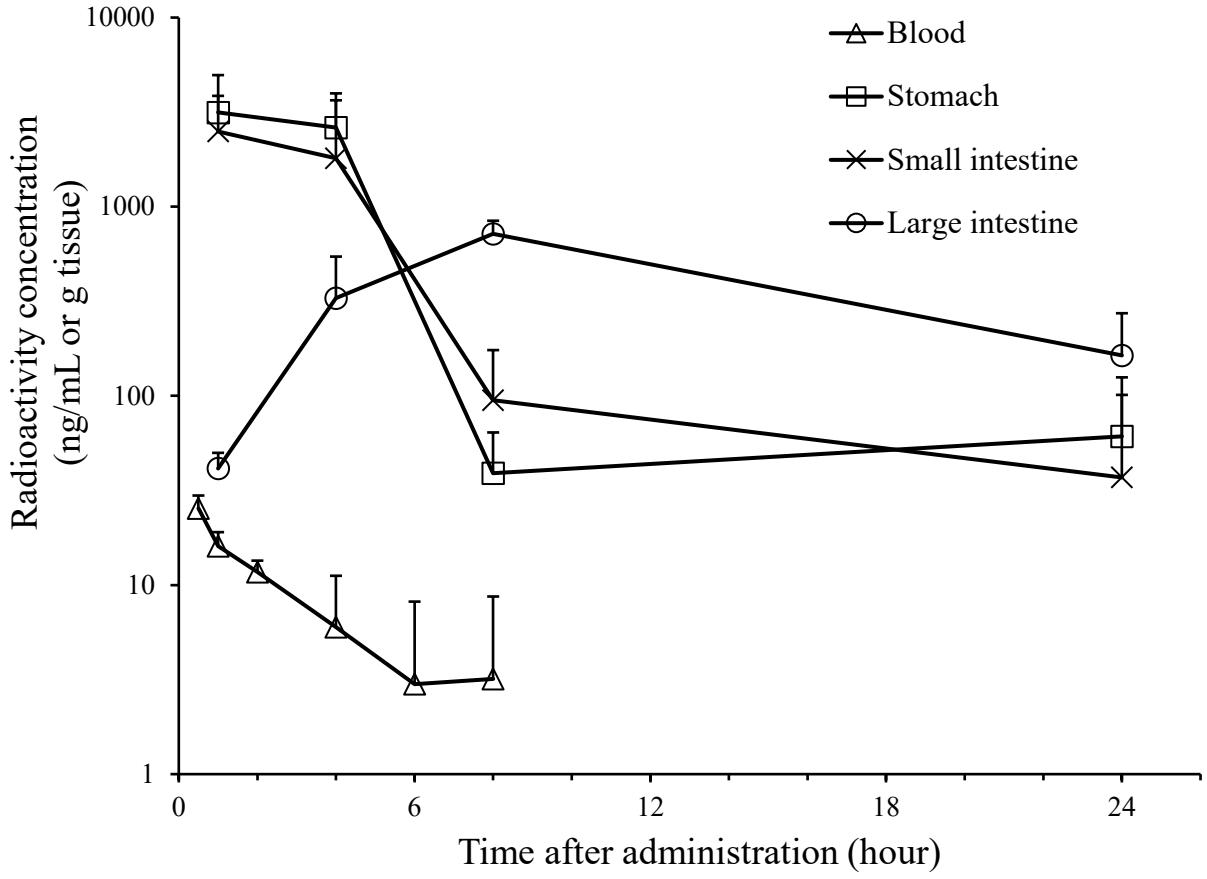

Figure 9. Time-concentration profile of radioactivity in plasma and gastrointestinal tract after oral administration of ^{14}C-OPS-2071 to rats

^{14}C-OPS-2071 was orally administered at 3 mg/kg to male SD rats, and the blood, stomach, small intestine, and large intestine were collected. Radioactivity was measured using a liquid scintillation counter to calculate the concentration (Mean + SD, n = 3). No radioactivity was detected at 24 hours post-dose (< 3 × background radioactivity) for all matrices.

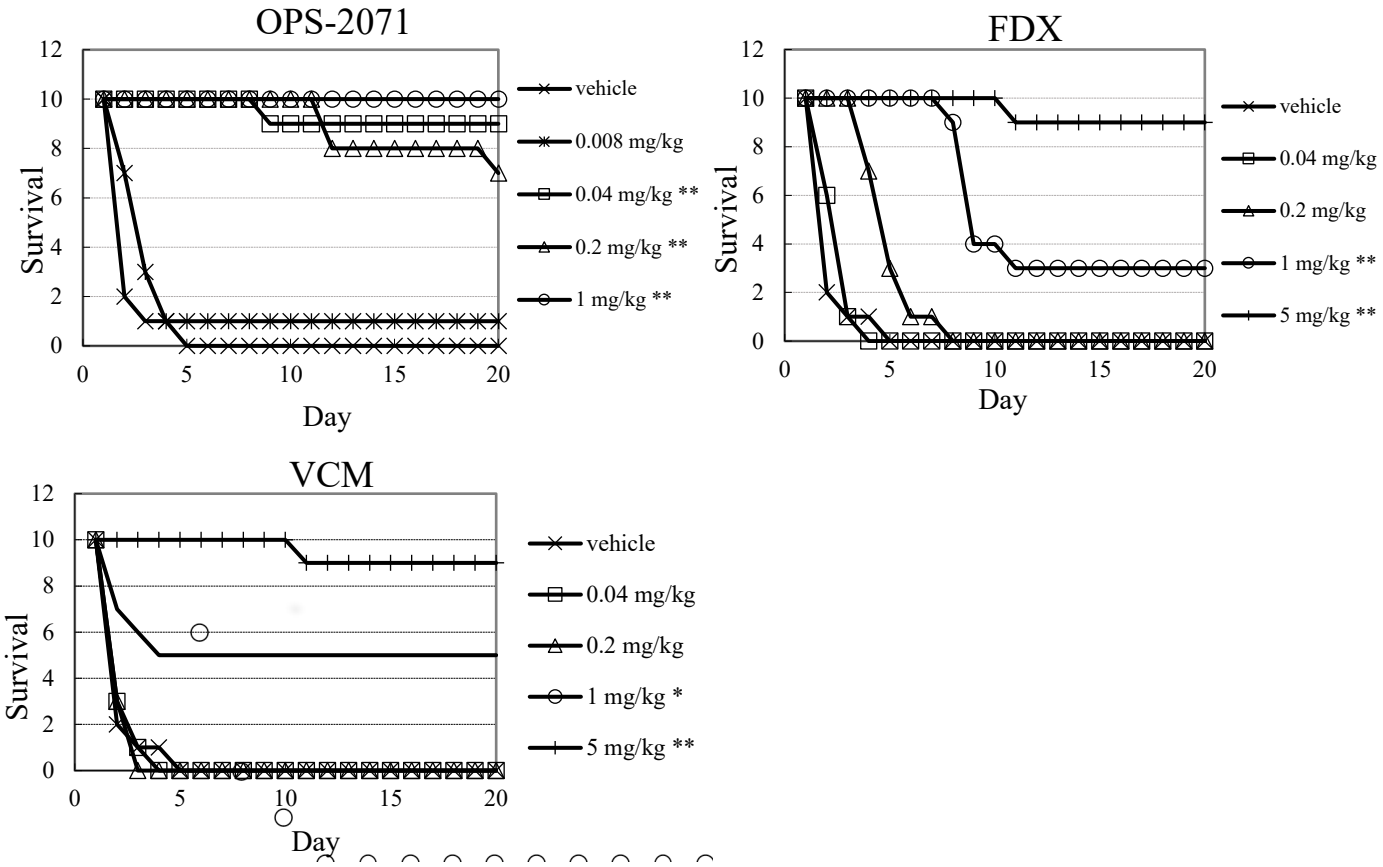

Figure 10. Efficacy of OPS-2071 and other drugs in a hamster model of CDI

Survival curve of hamsters after *C. difficile* ATCC 43255 infection followed by administration of clindamycin and subsequent treatment. Results of a log-rank test with Dunnett-type comparison, *: vs. vehicle, $p<0.05$, **: vs. vehicle, $p<0.01$. ED_{50} on day 20 was calculated by the probit method.

Discussion

C. difficile has been reported as an urgent threat to human health by CDC in the United States in 2019 [9]. To date, new compounds and new therapies have been developed to treat CDI [35, 50, 53]. However, there is no drug presently able to overcome such problems as fulminant life-threatening colitis characterized by high mortality (35 to 50%) and a high rate of recurrence, for which CDI is the cause in 15 to 25% of initial episodes [29]. FMT is a new approach showing high cure rates for recurrent CDI [36]; however, wide clinical use of FMT is still difficult because the preferred method for FMT administration is yet to be defined [36]. Therefore, it is necessary to develop an easy-to-use CDI treatment such as antibiotics used for common infectious diseases.

Fluoroquinolone has been used in clinical practice for over 30 years and is well known to be an antibiotic with high killing efficiency against both Gram-positive and Gram-negative bacteria. Regarding adverse events, it is generally well tolerated, and most adverse effects are mild [1]. Though fluoroquinolone is still considered to be an important antibiotic for patients with serious infections, the FDA issued changes to the safety labeling for systemic fluoroquinolone in 2018. Apart from the existing major well-known central nervous system (CNS)-related adverse effects (phototoxicity, QT interval prolongation, and diarrhea), the FDA required that the risk of serious blood sugar disturbances and psychiatric side effects be added to the labeling [59]. It is important to note that most of these adverse events, including the newly added risks, are caused by the systemic absorption of antibiotics. Fluoroquinolones are considered to be one of the major antibiotic classes that can induce CDI because predominant strains are resistant to common fluoroquinolones such as ciprofloxacin and levofloxacin [43, 47, 52, 57]. We approached the development of a new antibiotic with the aim of increasing antibacterial activity against *C. difficile* by utilizing the quinolone structure and decreasing the oral absorption in order to reduce the risk of adverse events associated with fluoroquinolone class antibiotics. Based on our experience in the area of structure-activity relationships, we succeeded in developing OPS-2071 as a novel fluoroquinolone that demonstrates potent antibacterial activity against *C. difficile*.

The *in vitro* activity against clinical isolates was similar to that of fidaxomicin and more potent than that of vancomycin. Furthermore, while there is no difference in the activity of OPS-2071 between hypervirulent and non-hypervirulent strains, a significant difference in susceptibility to fidaxomicin was observed. Also, MICs of OPS-2071 are not influenced by pH

(Table 3). The intraluminal pH changes rapidly from highly acid in the stomach to about pH 6.0 in the duodenum, and the pH gradually increases in the small intestine from pH 6.0 to about pH 7.4 in the terminal ileum. The activity of OPS-2071 is not affected by differences in pH in the gastrointestinal tract.

In addition, OPS-2071 demonstrated bactericidal activity against *C. difficile* at concentrations close to MIC, rapidly reducing the number of the organisms with no regrowth observed after 48 h (Figure 6). Although each time point represents a single sample, these killing kinetics are reasonable because the trajectories of the kinetics depend on the concentration (MIC values) of these compounds. Since OPS-2071 showed the longest PAE among the tested compounds, this and the other characteristics of OPS-2071 contributed to the ideal bactericidal activity curve portrayed by its inhibition of bacterial regrowth *in vitro*.

With regard to resistance, the frequency of spontaneous resistance was extremely low for OPS-2071, despite inoculation with very high concentrations of *C. difficile*. No spontaneous resistant mutants grew at 4-, 16- or 64-fold the MIC. Furthermore, the MPC value, which has been employed in the evaluation of an antibiotic's ability to minimize or limit the development of resistant organisms, was the lowest among all tested compounds. The data suggest that *C. difficile* may not readily develop resistance to OPS-2071 in the clinical setting and that the drug is suitable as a monotherapy for the treatment of CDI.

Regarding the pharmacokinetics of OPS-2071, the bioavailability at 1 mg/kg in rats was 2.9%, indicating that OPS-2071 has a low absorption profile in rats. We speculated that this profile may also apply to hamsters, because a high concentration of OPS-2071 was observed in the cecal contents of hamsters (Figure 7). This characteristic of low absorption of OPS-2071 is desirable for increasing therapeutic efficacy against the onset of *C. difficile* while at the same time reducing the risk of systemic adverse events, reinforcing the evidence that OPS-2071 should be an effective agent for treating CDI.

With respect to the efficacy in animal models, many therapeutic agents have been tested in the hamster model of clindamycin-induced CDI [50, 60, 66, 74]. Specifically, we organized our animal model with reference to an animal model in which clindamycin is administered 1 day after the *C. difficile* infection [60], because CDI is normally induced by the administration of antibiotics with preexisting *C. difficile* in the intestine. In addition, the MIC of *C. difficile* ATCC

43255 to clindamycin is reported as 8 μg/ml [42]. Since test compounds were administered for 5 days after 1 mg/kg clindamycin administration, the effect of the clindamycin on other test compounds appears to be very low.

OPS-2071 showed significant efficacy in this model with an effective dose, which was 39.0-fold and 52.1-fold lower than those of vancomycin and fidaxomicin, respectively. Although the *in vitro* antibacterial activity of OPS-2071 against *C. difficile* was similar to that of fidaxomicin, *in vivo* efficacy was demonstrated at a lower dose than that of fidaxomicin. Similar to OPS-2071, absorption of vancomycin and fidaxomicin is also low, and both reach the colon efficiently. The oral bioavailability of vancomycin is <10%, and fidaxomicin is minimally absorbed, with plasma concentrations in the low nanogram per milliliter range or lower [45, 69]. The pharmacokinetic profiles of these three drugs are fairly similar. These results suggested the possibility that the cecal contents affect the antibacterial activity among these three drugs in different manners.

For the data lacking biological replicates in this study, the limitation of a single data set must be considered. However, the data are sufficiently reliable considering that they have dose-response, time course-dependent changes, or large data sets.

Summary

OPS-2071 demonstrated potent *in vitro* activity against clinical isolates and hypervirulent strains. *In vivo* efficacy using hamsters was shown at doses 39.0- and 52.1-fold lower than those of vancomycin and fidaxomicin. The data support that OPS-2071 has potential as an agent for treating CDI. The current challenge in treating CDI is the frequency of recurrence [24]. One of the main causes of recurrence is the dormancy of *C. difficile* as spores [32], which can revert back to being an active infection. It is unknown if OPS-2071 eliminates *C. difficile*, including its spores, an important property central to reducing infection recurrence. Follow-up studies are needed to explore this matter in more depth.

CHAPTER II:

In Vitro Antibacterial Activity of OPS-2071 against Gram-positive and Gram-negative Enteropathogenic Bacteria

Introduction

In developing countries in Africa and Asia, enteric infections are still a major cause of death in children aged under five years [2, 41, 58]. Moreover, the Centers for Disease Control and Prevention (CDC) has classified some enteric infections in the US, such as drug-resistant *Campylobacter*, *Salmonella*, and *Shigella*, as serious threats [9] and rising levels of antimicrobial resistance represent a new challenge. Ciprofloxacin is widely used for intestinal infections, however, quinolone-resistant bacteria are reported in *Salmonella*, *Shigella*, enteroaggregative *E. coli* (EAEC)/enterotoxigenic *E. coli* (ETEC), and *Campylobacter* [33, 70, 71, 73, 78]. *Campylobacter* is a leading cause of bacterial gastroenteritis and while azithromycin is currently recommended for *Campylobacter* enteritis, there has also been an increase in macrolide-resistant *Campylobacter* [67, 78].

Quinolone antibiotics are widely used for various infections including enteric infections because of their broad-spectrum, potent antibacterial activity, and favorable bioavailability [59]. However, quinolone antibiotics are also associated with adverse events, such as QTc prolongation, dysglycemia, and adverse central nervous system reactions [25]. Due to a number of life-threatening adverse effects, the FDA notified in 2018 that additional warnings were to be included in the prescribing information, with the consequence that fluoroquinolones are not recommended for patients with aortic aneurysm [72]. Because OPS-2071 is a novel quinolone antibacterial agent with low oral absorption in animals [54], its therapeutic effects are mainly confined to the gastrointestinal tract. For this reason, known quinolone side effects may be minimized. We previously reported its potent *in vitro* and *in vivo* antibacterial activity against *Clostridioides difficile*, a major cause of antibiotic-associated diarrhea [54].

In order to assess the potential utility of OPS-2071 against enteric pathogenic bacteria, the antibacterial activity of OPS-2071 against clinically isolated enteropathogenic bacteria was investigated. In addition, the inhibitory activity of OPS-2071 against DNA gyrase and

topoisomerase IV in *E. coli*, *Staphylococcus aureus*, and *Campylobacter jejuni* was also evaluated. Moreover, the frequency of spontaneous resistance and the mutant prevention concentration against *E. coli*, *S. aureus*, and *C. jejuni* were evaluated to assess the risk of emergence of drug resistance.

Materials and methods

Antibiotics

OPS-2071 was synthesized by Otsuka Pharmaceutical Co., Ltd. (Tokushima, Japan). Ciprofloxacin and levofloxacin were purchased from Sigma-Aldrich (St. Louis, MO). Azithromycin was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Vancomycin was purchased from Fujifilm Wako Pure Chemical Corp. (Osaka, Japan).

Microorganisms

Clinically isolated strains were obtained from Aino Hospital (Osaka, Japan), Osaka Institute of Public Health, and Miroku Medical Laboratory (Nagano, Japan). This study was conducted on bacteria available in Japan and cause intestinal tract infections.
S. aureus (MRSA and MSSA) and *Bacillus cereus* were tested as Gram-positive bacteria, and *C. jejuni*, *E. coli*, *Salmonella enterica*, *Shigella sonnei*, *Shigella flexneri*, *Aeromonas hydrophila*/sp, *Viblio fluvialis*, and *Plesiomonas shigelloides* were tested as Gram-negative bacteria. *S. aureus* ATCC 29231, *E. coli* ATCC 25922, and *C. jejuni* ATCC 33560 were tested as reference strains. They were purchased from the ATCC (Manassas, VA).

Determination of antibacterial activity

MICs obtained using the agar dilution method were determined visually as described for the CLSI method [15, 17-19]. Briefly, bacterial suspensions were inoculated at 10^4 CFU/spot on Mueller Hinton Agar (MHA) plates containing the test agents for all the tested organisms except for *C. jejuni*, which was tested on MHA supplemented with 5% defibrinated sheep blood. The plates were incubated at 35–37°C for 48 hours in microaerobic conditions equivalent to 10% CO_2, 5% O_2, and 85% N_2 using a multi-gas incubator for *C. jejuni* and 35–37°C for 18–24 hours in air for the other test organisms. MIC was defined as the lowest concentration of antimicrobial able to inhibit bacterial growth.

Inhibition of DNA gyrase activity

The DNA gyrase and topoisomerase IV enzymes were purchased from Inspiralis Limited (Norwich, UK) except for *E. coli* topoisomerase IV and *C. jejuni* DNA gyrase. *E. coli* topoisomerase IV and all the gyrase enzymes of *C. jejuni* were cloned and overexpressed in *E. coli* and each subunit was purified [11)]. The supercoiling activity of DNA gyrase and decatenation activity of topoisomerase IV were determined as described previously [30)]. In brief, a substrate DNA was incubated with enzymes and the tested compounds at 37°C. The assay reactions were analyzed by electrophoresis in agarose gel followed by ethidium bromide staining. The inhibitory effect of compounds was assessed by quantifying the DNA products using densitometry and determining the concentration required to inhibit 50% of the enzyme (IC_{50}). The IC_{50} and corresponding 95% confidence intervals were determined by regression analysis using a 4-parameter logistic model using SAS software (Release 9.3, SAS Institute Japan).

Frequency of spontaneous resistance

The frequency of spontaneous resistance in *E. coli*, *S. aureus*, and *C. jejuni* was measured by inoculating an agar plate with a bacterial suspension containing 4×MIC, 16×MIC, and 64×MIC of the test agents. The frequency of spontaneous resistance to each agent was calculated as the number of resistant colonies formed per the inoculated number of bacteria [14)].

Mutant prevention concentration

The mutant prevention concentration (MPC) of OPS-2071 against *E. coli*, *S. aureus*, and *C. jejuni* was measured by inoculating an agar plate with a bacterial suspension containing the test agents. The MPC was defined as the minimal concentration at which there is no emergence of resistant bacteria [48)].

Results

Antibacterial activities of OPS-2071 against clinically isolated enteropathogenic bacteria

Antibacterial activities of OPS-2071 and other relevant compounds against clinically isolated enteropathogenic bacteria are shown in Table 8. OPS-2071 showed potent broad-spectrum antimicrobial activity against these bacteria. The antibacterial activity of OPS-2071 against Gram-positive bacteria, such as MSSA/MRSA, and *B. cereus*, was more potent than existing quinolones. This was especially true for MRSA, against which existing quinolones were less effective. *C. jejuni* is a Gram-negative microaerophilic bacterium, against which OPS-2071 showed superior activity compared to existing quinolones, while its activity against other Gram-negative bacteria was comparable. The activities of OPS-2071 against MRSA and *C. jejuni* were more potent than those of existing quinolones. These bacterial strains included quinolone-resistant strains according to the MIC breakpoints for antimicrobial resistance listed by EUCAST (Table 9) [27].

Inhibition of DNA gyrase and topoisomerase IV

To investigate the mechanism involved, the inhibitory activity of OPS-2071 against DNA gyrase and topoisomerase IV in *E. coli*, Gram-negative bacteria, *S. aureus*, Gram-positive bacteria, and *C. jejuni*, Gram-negative microaerophilic bacteria, was evaluated (Table 10). In *Campylobacter*, due to the absence of topoisomerase IV [55, 56], only DNA gyrase was evaluated. The inhibitory activities against both wild type DNA gyrase and wild type topoisomerase IV were comparable in *E. coli* among all tested compounds (OPS-2071, ciprofloxacin, and levofloxacin). While these compounds showed comparable activity against topoisomerase IV in *S. aureus*, OPS-2071 showed 61- and >1667-fold lower IC_{50} than ciprofloxacin and levofloxacin against wild type DNA gyrase in *S. aureus*, >200-fold lower against quinolone-resistant DNA gyrase in *S. aureus*, 4- and 6-fold lower against wild type DNA gyrase in *C. jejuni*, and 7- to 68-fold and 7- to 21-fold lower against quinolone-resistant DNA gyrase in *C. jejuni*, respectively.

Frequency of spontaneous resistance

Since quinolone-resistant strains have been reported in enteric infections, the frequency of spontaneous resistance to OPS-2071 in *E. coli*, *S. aureus*, and *C. jejuni* was evaluated (Table

11). No spontaneous resistance was observed for OPS-2071 in *S. aureus* and *E. coli* at all tested exposures, in a range from 4×MIC to 64×MIC. In *C. jejuni*, compared with existing quinolones, the frequency of spontaneous resistance to OPS-2071 at 4×MIC was comparable to that of ciprofloxacin and levofloxacin. However, the frequency of spontaneous resistance to OPS-2071 at 16×MIC was 270 and 226 times lower than those of ciprofloxacin and levofloxacin, respectively. In addition, no spontaneous resistance was observed at 64×MIC of OPS-2071, whereas spontaneous resistance was observed at 64×MIC of ciprofloxacin and levofloxacin. Spontaneous resistance to azithromycin did not develop at concentrations exceeding 16×MIC.

Mutant prevention concentration

The MPC is defined as the minimal concentration at which there is no emergence of resistant bacteria [48)]. Resistant mutants are enriched when bacteria are exposed to concentrations that fall within the mutant selection window (MSW), i.e., concentrations between the MIC and MPC [31)]. Treatments under the MPC will lead to the emergence of resistant bacteria. The MPCs of OPS-2071 and other related drugs were evaluated against *S. aureus*, *E. coli*, and *C. jejuni* (Table 12). In *S. aureus*, the MPC of OPS-2071 was the lowest among the tested compounds. In *E. coli*, the MPC of OPS-2071 was slightly higher than those of ciprofloxacin and levofloxacin. In *C. jejuni*, the MPC of OPS-2071 was lower than those of ciprofloxacin and levofloxacin and comparable to that of azithromycin.

Table 8. MICs for clinically isolated strains of enteropathogenic bacteria

Organism (number of isolates)	Drug	MIC (μg/mL)		
		MIC_{50}	MIC_{90}	Range
MSSA (26)	OPS-2071	0.004	0.008	0.002 - 0.063
	ciprofloxacin	0.25	4	0.125 - 64
	levofloxacin	0.25	1	0.25 - 16
	azithromycin	1	>128	0.5 - >128
	vancomycin	1	1	0.5 - 1
MRSA (25)	OPS-2071	0.125	2	0.004 - 2
	ciprofloxacin	64	>128	0.5 - >128
	levofloxacin	16	>128	0.25 - >128
	azithromycin	>128	>128	1 - >128
	vancomycin	1	1	1
Bacillus cereus (6)	OPS-2071	0.004	0.008	0.004 - 0.008
	ciprofloxacin	0.063	0.125	0.063 - 0.125
	levofloxacin	0.125	0.25	0.063 - 0.25
	azithromycin	1	1	0.5 - 1
	vancomycin	0.5	1	0.5 - 1
Campylobacter jejuni (50)	OPS-2071	0.016	0.25	0.008 - 0.25
	ciprofloxacin	0.5	16	0.063 - 16

	levofloxacin	0.25	8	0.063 - 8
	azithromycin	0.125	0.25	0.016 - >128
Escherichia coli (57)[a]	OPS-2071	0.063	0.125	0.031 - 8
	ciprofloxacin	0.031	0.031	0.008 - 2
	levofloxacin	0.063	0.063	0.031 - 4
	azithromycin	8	16	4 - 64
Non-typhoidal *Salmonella enterica* (34)[b]	OPS-2071	0.125	0.5	0.063 - 8
	ciprofloxacin	0.031	0.5	0.008 - 16
	levofloxacin	0.063	0.5	0.031 - 16
	azithromycin	16	16	4 - 16
Salmonella enterica serovar Typhi (11)	OPS-2071	0.031	0.25	0.031 - 0.25
	ciprofloxacin	0.016	0.5	0.016 - 0.5
	levofloxacin	0.016	0.5	0.016 - 0.5
	azithromycin	8	8	4 - 16
Salmonella enterica serovar Paratyphi A (5)	OPS-2071	1	1	0.5 - 1
	ciprofloxacin	1	1	0.5 - 1
	levofloxacin	1	1	1
	azithromycin	8	8	8
Shigella sonnei (27)	OPS-2071	0.063	0.25	0.031 - 0.25

	ciprofloxacin	0.016	0.25	0.008 - 0.25
	levofloxacin	0.031	0.5	0.031 - 0.5
	azithromycin	16	16	4 - 16
Shigella flexneri (13)	OPS-2071	0.031	0.5	0.031 - 4
	ciprofloxacin	0.016	1	0.008 - 16
	levofloxacin	0.031	1	0.031 - 8
	azithromycin	4	8	1 - 8
Yersinia enterocolitica (9)	OPS-2071	0.063	0.5	0.031 - 0.5
	ciprofloxacin	0.016	0.5	0.016 - 0.5
	levofloxacin	0.031	1	0.031 - 1
	azithromycin	4	16	2 - 16
Aeromonas hydrophila/sp. (12)[c]	OPS-2071	0.031	0.031	0.016 - 0.25
	ciprofloxacin	0.002	0.008	<0.001 - 0.5
	levofloxacin	0.008	0.016	0.002 - 0.25
	azithromycin	2	4	1 - 4
Klebsiella oxytoca (50)	OPS-2071	0.125	16	0.125 - 64
	ciprofloxacin	0.016	16	0.008 - 64
	levofloxacin	0.063	16	0.031 - 32
	azithromycin	32	32	8 - >128
Vibrio fluvialis (1)	OPS-2071	0.016	-	-

	ciprofloxacin	0.04	-	-
	levofloxacin	0.016	-	-
	azithromycin	2	-	-
Plesiomonas shigelloides (1)	OPS-2071	0.25	-	-
	ciprofloxacin	0.25	-	-
	levofloxacin	0.25	-	-
	azithromycin	4	-	-

[a]: includes 56 strains of enterohemorrhagic *E. coli*, a strain of enterotoxigenic *E. coli*. [b]: includes 2 strains of *S. enterica* serovar Enteritidis, a strain of *S. enterica* serovar Muenster, a strain of *S. enterica* serovar Schwarzengrund, a strain of *S. enterica* serovar Infantis, a strain of *S. enterica* serovar Thompson, a strain of *S. enterica* serovar Typhimurium, and 27 strains of *S. enterica*, [c]: includes 11 strains of *A. hydrophila* and a strain of *Aeromonas* sp.

Table 9. Breakpoints for tested antibiotics against *S. aureus*, *E. coli,* and *C. jejuni*

	Breakpoints (µg/mL)					
	Ciprofloxacin		Levofloxacin		Azithromycin	
	S ≤	R >	S ≤	R >	S ≤	R >
S. aureus	0.001	1	0.001	1	2	2
E. coli	0.25	0.5	0.5	1	-	-
C. jejuni	0.001	0.5	-	-	0.25 [a]	0.25 [a]

a: EUCAST breakpoints are not established for azithromycin, but CDC and FDA listed breakpoints based on epidemiological cutoff values established by EUCAST.

Table 10. Inhibitory activities of OPS-2071 against DNA gyrase and topoisomerase IV in *E. coli*, *S. aureus,* and *C. jejuni*

	Derived bacteria	Enzymes	IC_{50} and corresponding 95% confidence intervals (µg/ml)		
			OPS-2071	CPFX	LVFX
DNA gyrase	*E. coli*	Wild type	0.01 (0.01 - 0.03)	0.01 (0.01 - 0.02)	0.02 (0.01 - 0.02)
		S83L mutant	0.14 (0.10 - 0.21)	2.68 (1.78 - 4.76)	0.73 (0.52 - 1.15)
		S83W mutant	>100	>100	>100
	S. aureus	Wild type	0.06 (0.04 - 0.11)	3.67 (2.26 - 8.21)	>100
		S84L mutant	0.50 (0.26 - 2.30)	>100	>100
	C. jejuni	Wild type	0.13 (0.11 - 0.15)	0.49 (0.38 - 0.62)	0.75 (0.63 - 0.89)

		T86I	1.47 (1.17 - 1.90)	>100	31.36 (23.30 - 45.30)
		T86K	1.75 (1.34 - 2.29)	19.15 (12.61 - 33.45)	15.20 (11.61 - 20.79)
		T86A	2.68 (2.00 - 3.67)	18.08 (13.71 - 25.49)	27.66 (19.10 - 52.30)
		D90N	2.09 (1.68 - 2.63)	36.80 (27.05 - 55.17)	14.34 (9.74 - 23.06)
		D90Y	1.25 (1.04 - 1.51)	21.75 (15.23 - 33.32)	15.56 (12.32 - 20.08)
Topoisomerase IV	*E. coli*	Wild type	0.15 (0.13 - 0.19)	0.26 (0.22 - 0.31)	0.32 (0.20 - 0.60)
	S. aureus	Wild type	0.12 (0.10 - 0.15)	0.40 (0.36 - 0.43)	0.58 (0.47 - 0.72)

Table 11. Frequency of spontaneous resistance in *S. aureus*, *E. coli*, and *C. jejuni*

		MIC (μg/mL)	Frequency at the multiple of MIC		
			4×MIC	16×MIC	64×MIC
S. aureus ATCC 29231	OPS-2071	0.004	$<8.02 \times 10^{-9}$	$<8.02 \times 10^{-9}$	$<8.02 \times 10^{-9}$
	ciprofloxacin	0.25	2.79×10^{-7}	$<8.02 \times 10^{-9}$	$<8.02 \times 10^{-9}$
	levofloxacin	0.25	$<8.02 \times 10^{-9}$	$<8.02 \times 10^{-9}$	$<8.02 \times 10^{-9}$
E. coli ATCC 25922	OPS-2071	0.03	$<1.05 \times 10^{-8}$	$<1.05 \times 10^{-8}$	$<1.05 \times 10^{-8}$
	ciprofloxacin	0.016	$<1.05 \times 10^{-8}$	$<1.05 \times 10^{-8}$	$<1.05 \times 10^{-8}$
	levofloxacin	0.03	$<1.05 \times 10^{-8}$	$<1.05 \times 10^{-8}$	$<1.05 \times 10^{-8}$
C. jejuni ATCC 33560	OPS-2071	0.03	1.99×10^{-7}	7.25×10^{-10}	$<7.25 \times 10^{-12}$
	ciprofloxacin	0.25	2.14×10^{-7}	1.96×10^{-7}	1.74×10^{-8}
	levofloxacin	0.25	1.99×10^{-7}	1.64×10^{-7}	3.62×10^{-11}
	azithromycin	0.5	1.88×10^{-10}	$<7.25 \times 10^{-12}$	$<7.25 \times 10^{-12}$

Table 12. Mutant prevention concentration against *S. aureus*, *E. coli*, and *C. jejuni*

	Mutant prevention concentration (μg/mL)			
	OPS-2071	ciprofloxacin	levofloxacin	azithromycin
S. aureus ATCC 29231	0.004	2	1	N.T
E. coli ATCC 25922	0.25	0.06	0.125	N.T
C. jejuni ATCC 33560	2	32	16	4

N.T: not tested

Discussion

Correlation between antimicrobial activity and inhibitory activity against DNA gyrase

In this study, the potency of OPS-2071 against both Gram-positive and Gram-negative bacteria that cause enteric infections was investigated. OPS-2071 showed broad and potent antibacterial activity against these bacteria. In particular, the activity of OPS-2071 against MRSA and *C. jejuni* was more potent than that of existing quinolones. These bacterial strains against which OPS-2071 showed potent antibacterial activity included quinolone-resistant strains according to the MIC breakpoints for antimicrobial resistance in the EUCAST guidelines (Table 9) [10, 27]. This result correlated with the inhibitory activity of DNA gyrase and topoisomerase IV. For OPS-2071, a small increase in IC_{50} values against quinolone-resistant DNA gyrase compared with wild type DNA gyrase was observed, while a large increase in the IC_{50} values of ciprofloxacin and levofloxacin were observed. The results of our study in relation to the target molecules of quinolone compounds indicate that OPS-2071 has potent antibacterial activity against quinolone-resistant strains as well as quinolone-susceptible strains.

Double mutations, affecting both DNA gyrase and topoisomerase IV, are common in the clinical setting, especially in highly resistant strains [37, 63]. Although quinolone-resistant mutations in clinical isolates were not assessed in this study, MICs of clinical isolates of MRSA revealed a high degree of resistance to existing quinolone antimicrobials, suggesting double mutations in DNA gyrase and topoisomerase IV. The potency of OPS-2071 against MRSA was substantially lower when compared with the MICs against MSSA, but OPS-2071 retained sufficient potency when compared with existing quinolone antimicrobials. These results suggest that OPS-2071 has potent activity against double mutant strains of *S. aureus*, a Gram-positive bacterium. As for *C. jejuni*, a Gram-negative microaerophilic bacterium, it does not possess topoisomerase IV, so the inhibitory activity of DNA gyrase may contribute to the antimicrobial activity. In contrast, for *E. coli*, a Gram-negative bacterium, both the antimicrobial activity against clinical isolates and the inhibitory activity against DNA gyrase showed a similar trend to that of existing quinolones, although there was only one resistant strain among the clinical isolates evaluated in this study and the number of cases was too small for a comprehensive evaluation of resistant strains. The results of this study showed that OPS-2071 has potent

antibacterial activity against existing quinolone-resistant strains with respect to *S. aureus* and *C. jejuni*, and this can probably be attributed to the potent inhibitory activity against existing quinolone-resistant DNA gyrase.

Evaluation of the risk of the development of drug-resistant bacteria

As antimicrobial resistance increases worldwide, there is an urgent need to limit its further spread. In this study, we investigated the frequency of spontaneous resistance and the MPC in order to evaluate the potential for spontaneous drug resistance to appear. Ciprofloxacin and levofloxacin showed a higher frequency of spontaneous resistance in *C. jejuni* than that of azithromycin. This result correlates with the drug resistance rate of quinolone and azithromycin in *Campylobacter* seen in clinical practice [9)]. Despite the low frequency of spontaneous resistance to existing quinolones in *S. aureus* and *E. coli*, resistant strains have been observed in clinical practice. This can be understood with reference to breakpoints. Clinical breakpoints are the MICs of antibiotics used to define whether infection by a particular bacterial strain/isolate is likely to be treatable [27)]. The breakpoints of ciprofloxacin and levofloxacin for *E. coli* are 0.25 μg/mL and 0.5 μg/mL, respectively, which are higher than the MPCs of quinolone antibiotics obtained in this study. Although MPC studies are widely used, they are subject to the same caveats that similar studies have in that there is variation due to strain response and lack of standardized testing methods; nevertheless, they are useful as a measurable guide. When the drug concentration during treatment falls within the MSW (the difference between the MPC and MIC), the treatment will lead to the emergence of resistant bacteria. However, for ciprofloxacin and levofloxacin, the absence of MSW (due to equivalence of MPC and MIC) suggests that *E. coli* are unlikely to develop resistance to ciprofloxacin or levofloxacin. In our study containing 57 clinical isolates, only one quinolone-resistant strain was found. Conversely, the breakpoint of the quinolone antibiotics for *S. aureus* is 0.001 μg/mL, which is much lower than their MPCs. In fact, many quinolone-resistant *S. aureus* strains have been clinically isolated. Similarly, the MPCs (32 μg/mL and 16 μg/mL, see Table 12) of quinolone antibiotics for *C. jejuni* are much higher than their clinical breakpoints (0.001 μg/mL) for *C. jejuni*, and 28% of clinically isolated *Campylobacter* had reduced susceptibility to ciprofloxacin [9)].

MPCs and pharmacokinetic profile of OPS-2071

OPS-2071 has a lower MPC compared to other major antibiotics. It was developed for intestinal infections, optimized to suppress the rate of oral absorption and increase drug concentration in the intestine to enhance effectiveness against intestinal pathogens. OPS-2071 showed low systemic exposure and high distribution and concentration in the large intestine and cecal contents in an *in vivo* study [54)]. The MPC is sometimes systemically unachievable without undue toxicity due to limited bioavailability. Our MPC data are limited and variability in the data should be considered; however, as the concentration of OPS-2071 in intestinal flora is much higher than the MPC against pathogenic bacteria, it could prevent the development of resistant bacteria. Indeed, the C_{max} in the cecal contents reached 42.95 mg/g at 4 hours postdose and then decreased to 1.84 mg/g at 24 hours postdose in a hamster PK study [54)]. This concentration is much higher than the MIC_{90} and MPC of OPS-2071 and is expected not only to exhibit antimicrobial activity but also to inhibit the emergence of resistant bacteria. In addition to drug concentrations in the intestinal tract, OPS-2071 also exhibits higher concentrations in the intestinal mucosa than its MIC_{90} and MPCs. In a mouse PK study, the C_{max} of OPS-2071 reached 4719 ng/g [65)]. This is important because some enteric pathogens invade the intestinal mucosa and cause infections.

Summary

OPS-2071 showed potent antibacterial activity against clinical isolates of enteric infection-causing bacteria, including *C. jejuni* and MRSA, which are resistant to existing quinolones. The antibacterial activity was strongly correlated with the inhibitory activity of DNA gyrase, a target molecule of quinolones, suggesting that these antibacterial activities are due to DNA gyrase inhibition. The risk of emergence of resistant bacteria was evaluated by assessing the frequency of spontaneous bacterial resistance and MPC, and the results of both studies showed that OPS-2071 was comparable to or lower than existing quinolone drugs. In addition to these results, the low oral absorption and high intestinal drug concentration pharmacokinetics of OPS-2071 suggest that it can be used for treatment at much higher concentrations than MPC.

Given these results, OPS-2071 may be considered a promising therapeutic option for enteric infections including quinolone-resistant *Campylobacter*.

CONCLUSION

In intestinal infections, oral treatment drugs with low absorption can directly distribute them to the intestine and systemic absorption is not necessary for their treatment. Drugs with low absorption PK profile can also achieve a low risk for their systemic side effects and lower risks for the emergence of drug resistance because they are easily able to achieve their higher concentration in the intestine. However, the development of these drugs is rare because most intestinal infections are common in developing countries but in developed countries. Fidaxomicin is a rare example of this because *C. difficile* infection is also common in developed countries. Fidaxomicin and vancomycin, the first-line drug for *C. difficile* infection, have low oral absorption and they are suitable for intestinal infections. However, their antibacterial spectrum is very limited. Therefore, they are available only for *C. difficile* infection.

OPS-2071 is a novel new quinolone with low oral absorption, like fidaxomicin and vancomycin, but the antibiotic spectrum is broad like other existing quinolone drugs as I show in this thesis. In addition to its suitable PK profile for intestinal infections, OPS-2071 showed potent antibacterial activity against existing quinolone-resistant bacteria, such as *C. difficile*, MRSA, and *C. jejuni*. OPS-2071 would be available for intestinal infections including that caused by quinolone-resistant bacteria.

The most important issue for antibiotics is the emergence of antibiotic-resistant strains. Many new drugs have been introduced to the market, but eventually, resistance to them emerged, requiring the development of additional new antimicrobial agents, which is considered a never-ending challenge. One of the reasons to emerge the resistant strains is the inappropriate usage of antibiotics. As I discussed in CHAPTER II, it is important to treat at or above the MPC concentration to inhibit the emergence of resistant bacteria. However, the breakpoint, defined as the MIC at which treatment is possible, is often below the MPC. Thereby, treatment with concentrations that exceed the MIC but are below the MPC, the so-called MSW, results in the emergence of resistant bacteria. Depending on the drugs or bacterial strains, it may be difficult to treat with a concentration exceeding the MPC at the site of infection. In particular, it may be difficult to maintain a high concentration exceeding the MPC at the site of infection for a certain time at a dose that does not cause side effects, as is the case with like respiratory infections and urinary tract infections. In the case of intestinal infections, however, it is possible to distribute

the drug into the intestinal tract at a high concentration exceeding the MPC for a long period of time with a low-absorbable drug. In conclusion, OPS-2071 is a promising therapeutic candidate with high safety and efficacy for the treatment of intestinal infections in general by narrowing its indication to intestinal infections.

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