ANTIBACTERIAL ACTIVITY OF A NEW PARENTERAL CEPHEM, CEFCLIDIN, AGAINST ANAEROBIC BACTERIA AND *Gardnerella vaginalis*

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Abstract

The activity of a new parenteral cephalosporin, cefclidin (CFCL), was compared with those of ceftazidime (CAZ), cefotaxime (CTX), cefpiramide (CPM), and cefotiam (CTM) against anaerobic bacteria and *Gardnerella vaginalis*. In general, CFCL showed a broad spectrum against Gram-positive and -negative reference strains of anaerobic bacteria although weak activity of this compound was seen against *Bacteroides fragilis* group organisms. Against recent clinical isolates, CFCL revealed moderate or weak activity against members of the *B.fragilis* group as did other antimicrobials tested. CFCL showed poor activity against *Prevotella bivia* and *Clostridium difficile* as well. Against most of other members of anaerobic bacteria, CFCL showed good activity, especially against *Mobiluncus* spp. and *G.vaginalis*. Enzymatically, CFCL was more stable than CPM, CTX, and CTM, but less stable than CAZ to hydrolysis by oxyiminocephalosporinases type I derived from *B.fragilis*. Time-kill study demonstrated bactericidal activity without regrowth at concentrations of two times or more the MIC of CFCL for *B.fragilis*. Although CFCL had bactericidal potency in rat pouch infection with *B.fragilis*, regrowth was seen 48h after starting administration of this compound. CFCL seemed to cause little overgrowth of *C.difficile* in a mouse model.

Key words : cefclidin, anaerobic bacteria, β-lactamase, rat pouch, *Bacteroides fragilis*, *Clostridium difficile*

Introduction

Cefclidin (CFCL) is a novel semisynthetic parenteral cephalosporin. The molecular weight of this compound is 550.619.

In the present study, we extensively evaluated the *in vitro* and *in vivo* activity of CFCL in comparison with related agents against anaerobic bacteria and a fastidious facultative anaerobe, *Gardnerella vaginalis*, which is suspected to be one of the causative pathogens of bacterial vaginosis. The stability of CFCL to hydrolysis by β-lactamases produced by *Bacteroides fragilis* strains and the inducibility of overgrowth of *Clostridium difficile* in murine cecum by 7-day administration of this compound were also evaluated.

Materials and Methods

Organisms —The 41 reference strains (12 genera, 37 species) of anaerobic bacteria and fastidious aerobic bacterium, *G.vaginalis*, were obtained from the American Type Culture Collection (ATCC), Rockville, Md., U.S.A., the Gifu Anaerobic Institute (GAI), Gifu, the National Collection of Type Cultures (NCTC), London, the United Kingdom, the Virginia Polytechnic Institute and State University (VPI), Blacksburg, Va, U.S.A., and the Wadsworth Anaerobic Laboratory (WAL), Los Angeles, Calif., U.S.A. A total of 248 recent clinical isolates of anaerobic bacteria and *G. vaginalis* were tested. Isolates were collected from numerous clinical sources in Japan at the Institute of Anaerobic Bacteriology or GAI, Gifu. Organisms were identified by standard methods.

Antimicrobial agents —Laboratory standard

powders for *in vitro* activity study were provided as follows : CFCL from Eisai Co., Ltd., Tokyo ; ceftazidime (CAZ) from Nihon Glaxo Co., LTd., Tokyo ; cefotaxime (CTX) from Hoechst Japan, Tokyo ; cefpiramide (CPM) from Sumitomo Pharmaceuticals, Tokyo ; and cefotiam (CTM) from Takeda Chemical Industries, Osaka. Cefazolin (CEZ) used for β-lactamase assay was supplied by Fujisawa Pharmaceutical Co., Osaka. Cefotetan (CTT) was obtained from Yamanouchi Pharmaceutical Co. for inducibility of overgrowth of *C.difficile* in murine cecum.

Susceptibility testing —The minimal inhibitory concentrations (MICs) were determined by an agar dilution method. An inoculum size of 10^6 colony forming units (CFU) of organisms per ml was applied onto agar plates containing each antimicrobial with a multipoint inoculator (Microplanter Sakuma Seisakusho, Tokyo). Brucella HK agar (Kyokuto Phamaceutical Co., Tokyo) supplemented with 5% laked sheep blood was used for reference strains and modified GAM agar (Nissui Pharmaseutical Co., Tokyo) was for clinical isolates except for *G.vaginalis* and *Mobiluncus* spp.; columbia agar (Oxoid, Basingstoke, the United Kingdom) supplemented with 5% sheep blood and 1% proteose peptone No.3 (Difco Laboratories, Detroit, Mich., USA) was used for *G.vaginalis* and Brucella HK agar supplemented with 5% laked rabbit blood was for *Mobiluncus* spp. *B.fragilis* GAI 5562 was included in all MIC determinations as a control organism. All anaerobic cultures were incubated at 37℃ in an anaerobic chamber (Hirasawa, Tokyo) in an atmosphere composed of 82% N_2, 10% CO_2, and 8% H_2. Plates inoculated organisms were incubated with for 48h except *Porphyromonas gingivalis* and *Mobiluncus* spp. which were incubated for 72h. The MICs were determined as the lowest concentrations of antimicrobial agents that prevented visible growth of organisms.

Time-kill study —A forty eight-h culture of *B. fragilis* GAI 5562 was inoculated into supplemented GAM broth containing CFCL or CAZ at concentrations of 1/2, 1, 2 or 4 MIC. Tubes were subcultured under anaerobic condition for colony counts at 0, 2, 4, 6, and 24h of incubation.

Stability to hydrolysis by β-lactamases from *B. fragilis* —Crude β-lactamase were obtained from three strains of B.fragilis as described previously[1] : Strain GAI 0558 produces oxyiminocephalosporinase type I strain GAI 7955 is highly resistant to cefoxitin (MIC, 100μg/ml) and strain GAI 10150 produces oxyiminocephalosporinase type I and is characterized to be ampicillin-highly-resistant (MIC, 1,600μg/ml). Hydrolysis of the compounds by β-lactmases was assayed by a spectrophotometric technique of the change in absorption at the absorption maximum of each substrate[2]. Relative hydrolysis rate of each compound was expressed as a percentage obtained in comparison with the hydrolysis rate of cephaloridine.

In vivo activity in rat pouch infection —Four-week old male Wistar rats (ca.200g of body weight) were used. Pouch was made on the back of rats by 20 ml of air injection followed by infusing 1% croton oil in olive oil[3]. Intraperitoneal administration of antimicrobials (200mg per kg of body weight, *d.i.d.*) was initiated 24h after inoculation of *B.fragilis* GAI 5562 culture into the pouch. Bacterial counts in the pouch were performed at intervals of 0, 3, 6, 9, 24, 48 and 72h after antimicrobial administration. Concentration of antimicrobials was measured 3,6 and 9h after the first administration of antimicrobials by bioassay method using *Escherichia coli* E01174 strain.

Inducibility of overgrowth of *C.difficile* in murine cecum —Four-week old ICR mice (ca.20g) were used. Antimicrobials were subcutaneously administered twice a day at a dose of 100mg per kg of body weight for 7 days. Cecum contents were cultured quantitatively for *C.difficile* counts on a *C.difficile* selective medium, cefoxitin-cycloserine mannitol agar (CCMA) medium, in an anaerobic chamber.

Results

In vitro activity against reference strains —CFCL had a broad spectrum against anaerobic bacteria while this compound showed weak or little activity against *B.fragilis* group organisms, *Fusobacterium varium*, *Eubacterium lentum*, and *C.difficile*, which were also highly resistant to CAZ (Tables 1 and 2). In general, CFCL was less active than the other 4 antimicrobials tested against these reference

Table 1. Antibacterial spectrum of cefclidin against reference strains of gram-negative anaerobic bacteria

Organism	MIC (μg/ml)				
	Cefclidin	Ceftazidime	Cefotaxime	Cefpiramide	Cefotiam
Bacteroides					
B. fragilis GAI5562	50	12.5	3.13	12.5	25
B. fragilis GAI0558	>200	>200	200	>200	>200
B. distasonis ATCC8503	12.5	6.25	0.05	3.13	6.25
B. ovatus ATCC8483	>200	>200	50	50	100
B. thetaiotaomicron ATCC29741	>200	>200	50	50	100
B. uniformis GAI5466	200	50	1.56	6.25	12.5
B. eggerthii ATCC27754	25	12.5	0.20	6.25	3.13
B. ureolyticus NCTC10941	0.20	0.05	≦0.025	0.39	0.20
Prevotella					
P. oris ATCC33573	12.5	3.13	0.20	3.13	0.78
P. oralis ATCC33269	6.25	3.13	0.10	3.13	1.56
P. melaninogenica ATCC29147	3.13	0.39	0.10	0.05	0.39
P. bivia ATCC29303	12.5	6.25	0.20	0.39	0.39
P. intermedia ATCC25611	3.13	0.39	0.10	0.78	0.20
Porphyromonas					
P. asaccharolytica ATCC25260	3.13	0.10	≦0.025	≦0.025	≦0.025
Fusobacterium					
F. nucleatum ATCC25586	12.5	3.13	0.78	0.05	0.39
F. varium ATCC8501	200	200	50	6.25	6.25
F. mortiferum GAI5576	0.78	1.56	0.20	1.56	1.56
Veillonella					
V. parvula ATCC10790	3.13	3.13	0.20	6.25	0.39

Inoculum size : 10^6 CFU/ml.

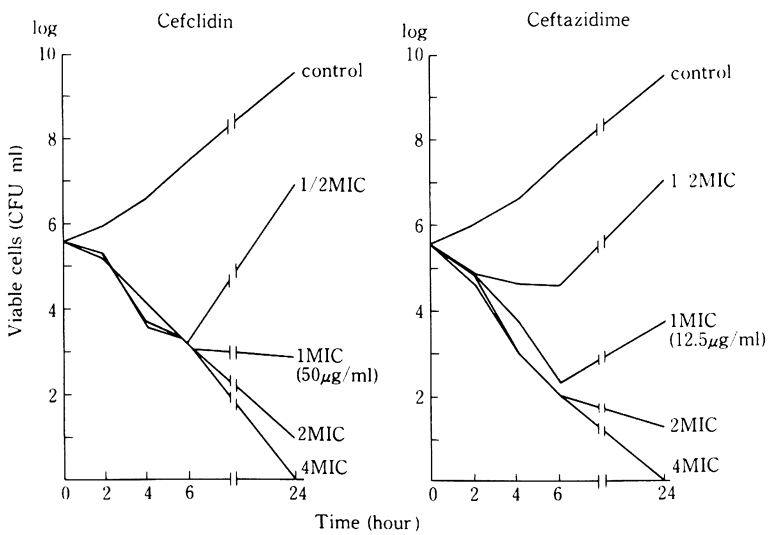


Fig. 1. Killing-kinetics of cefclidin and ceftazidime against *B. fragilis* GAI 5562

Table 2. Antibacterial spectrum of cefclidin against reference strains of gram-positive anaerobic bacteria and *Gardnerella vaginalis*

Organism	MIC (μg/ml)				
	Cefclidin	Ceftazidime	Cefotaxime	Cefpiramide	Cefotiam
Peptostreptococcus					
P. anaerobius ATCC27337	12.5	0.78	0.20	0.20	0.78
P. asaccharolyticus WAL3218	0.78	0.39	0.20	0.39	0.78
P. indolicus GAI0915	0.10	0.10	≦0.025	0.39	0.10
P. magnus ATCC29328	3.13	3.13	1.56	1.56	0.78
P. micros VP15464-1	0.10	0.10	0.10	≦0.025	0.10
P. prevotii ATCC9321	0.39	0.05	0.10	≦0.025	0.20
P. tetradius GAI0608	0.39	3.13	0.20	0.10	1.56
Streptococcus					
S. intermedius ATCC27335	3.13	1.56	0.20	0.78	1.56
S. saccharolyticus ATCC13953	3.13	3.13	0.78	0.39	0.10
S. parvulus VPI0546	25	12.5	1.56	0.78	1.56
Propionibacterium					
P. acnes ATCC11828	12.5	3.13	0.39	0.78	0.39
P. granulosum ATCC25564	12.5	0.20	0.05	0.20	0.78
Bifidobacterium					
B. adolescentis ATCC15703	0.20	1.56	0.20	0.20	3.13
Eubacterium					
E. lentum ATCC25559	>200	>200	200	100	50
Mobiluncus					
M. curtisii subsp. *curtisii* ATCC35241	1.56	3.13	0.20	0.20	0.78
M. curtisii subsp. *holmesii* ATCC35242	3.13	12.5	0.39	0.39	0.78
M. mulieris ATCC35240	0.39	0.78	0.025	0.025	0.10
M. mulieris ATCC35243	0.20	1.56	0.025	0.025	0.20
Clostridium					
C. difficile GAI10029	200	25	100	50	>200
C. difficile GAI10038	>200	50	100	25	>200
C. perfringens ATCC13123	0.78	3.13	0.78	≦0.025	0.20
C. septicum ATCC12464	6.25	100	6.25	1.56	3.13
C. ramosum ATCC25582	1.56	3.13	0.78	0.78	1.56
Gardnerella					
G. vaginalis NCTC10287	0.39	0.78	0.20	0.10	1.56
G. vaginalis NCTC10915	0.39	0.78	0.10	0.10	1.56

inoculum size : 10^6 cfu/ml.

strains.

In vitro activity against clinical isolates —The ranges of MICs and MICs for 50 and 80% of the clinical isolates tested (MIC_{50} and MIC_{80}, respectively) are given in Tables 3 and 4. CFCL showed activity similar to those of CTM against *B.fragilis*, with MIC_{50} and MIC_{80} of 50 and > 200μg/ml, respectively. CFCL was also comparable in activity to CTM against *Bacteroides uniformis*, *Porphyromonas* spp. and *Prevotella bivia*. Although CFCL revealed moderate activity against 3 species of the genus *Peptostreptococcus*, its strong activity was observed against *Mobiluncus* spp. and *G.vaginalis* with MIC_{80} of 3.13 and 1.56μg/ml, respectively, an activity which was higher than those of CAZ. Against *Bacteroides thetaiotaomicron* and *C.difficile*, CFCL showed little activity as did CAZ and CTM.

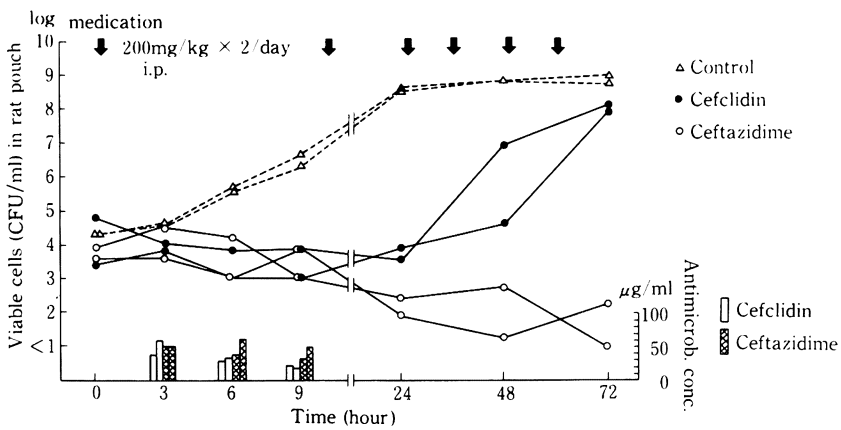
Time-kill study —The bactericidal kinetics of CFCL and CAZ against *B.fragilis* are illustrated in Fig.1. CFCL demonstrated bactericidal activity at concentrations of 1/2 or more of the MIC (50μg/ml) and no regrowth was seen 24h after exposure to concentrations of the MIC or more of CFCL. Comparable kinetics were obtained for CAZ.

Stability to hydrolysis by β-lactamases from *B. fragilis* —CFCL was more stable to hydrolysis by all

Table 3. Comparative activities of cefclidin and other related agents against clinical isolates of gram-negative anaerobic bacteria

Organism (no. of isolates)	Agent	MIC (μg/ml)		
		Range	50%	80%
B. fragilis (38)	Cefclidin	12.5～>200	50	>200
	Ceftazidime	6.25～>200	25	100
	Cefpiramide	3.13～>200	25	100
	Cefotaxime	1.56～>200	6.25	100
	Cefotiam	6.25～>200	100	>200
B. thetaiotaomicron (11)	Cefclidin	>200	>200	>200
	Ceftazidime	100～>200	200	>200
	Cefpiramide	12.5～>200	25	50
	Cefotaxime	12.5～>200	25	>200
	Cefotiam	50～>200	100	100
B. uniformis (13)	Cefclidin	12.5～>200	200	>200
	Ceftazidime	6.25～>200	25	>200
	Cefpiramide	3.13～>200	12.5	50
	Cefotaxime	0.78～>200	6.25	100
	Cefotiam	12.5～>200	25	200
Pigmented gram-negative rods spp.[a] (21)	Cefclidin	0.78～200	6.25	25
	Ceftazidime	0.39～50	0.78	6.25
	Cefpiramide	0.39～6.25	1.56	3.13
	Cefotaxime	0.10～6.25	0.39	1.56
	Cefotiam	0.20～100	6.25	50
P. bivia (28)	Cefclidin	3.13～200	50	100
	Ceftazidime	0.39～100	3.13	12.5
	Cefpiramide	0.39～12.5	0.78	6.25
	Cefotaxime	≦0.025～12.5	0.78	6.25
	Cefotiam	0.39～100	25	100

a) Pigmented gram-negative rods spp. consisted of 12 isolates of *P. intermedia*, 7 isolates of *P. corporis* and 2 isolates of *P. asaccharolytica*.



Bacteria used : *B. fragilis* GAI 5562.
MIC : Cefclidin, 50μg/ml; Ceftazidime, 12.5μg/ml.

Fig. 2. Effect of cefclidin and ceftazidime on the *B. fragilis* rat pouch infection

Table 4. Comparative activities of cefclidin and other related agents against clinical isolates of gram-positive anaerobic bacteria and *Gardnerella vaginaris*

Organism (no. of isolates)	Agent	MIC (μg/ml)		
		Range	50%	80%
P. anaerobius(20)	Cefclidin	0.39～200	12.5	25
	Ceftazidime	0.39～25	0.78	0.78
	Cefpiramide	≦0.025～100	0.78	1.56
	Cefotaxime	0.10～6.25	0.20	0.20
	Cefotiam	0.20～50	0.78	0.78
P. magnus(41)	Cefclidin	0.20～12.5	6.25	6.25
	Ceftazidime	0.78～12.5	3.13	6.25
	Cefpiramide	≦0.025～3.13	1.56	1.56
	Cefotaxime	0.10～3.13	1.56	1.56
	Cefotiam	0.20～1.56	0.78	1.56
P. asaccharolyticus(29)	Cefclidin	0.10～6.25	1.56	3.13
	Ceftazidime	0.20～12.5	0.39	12.5
	Cefpiramide	0.05～3.13	0.20	0.39
	Cefotaxime	0.05～1.56	0.10	0.20
	Cefotiam	0.20～3.13	0.39	1.56
Mobiluncus spp. (9)	Cefclidin	≦0.025～3.13	1.56	3.13
	Ceftazidime	0.05～6.25	3.13	6.25
	Cefpiramide	≦0.025～0.20	0.20	0.20
	Cefotaxime	≦0.025～0.39	0.20	0.20
	Cefotiam	≦0.025～0.78	0.78	0.78
C. difficile(24)	Cefclidin	100～>200	>200	>200
	Ceftazidime	25～>200	50	>200
	Cefpiramide	12.5～50	25	50
	Cefotaxime	50～200	100	200
	Cefotiam	100～>200	>200	>200
G. vaginalis(23)	Cefclidin	0.39～25	0.78	1.56
	Ceftazidime	0.78～50	1.56	3.13
	Cefpiramide	0.10～6.25	0.20	0.39
	Cefotaxime	0.10～6.25	0.20	0.39
	Cefotiam	1.56～25	3.13	6.25

Table 5. Stability of cefclidin, ceftazidime, cefpiramide, cefotaxime and cefotiam to the hydrolysis by β-lactamase dervied from *B. fragilis* as compared with that of cefazolin

Source of β-lactamase	Relative hydrolysis rate[a]				
	Cefclidin	Ceftazidime	Cefpiramide	Cefotaxime	Cefotiam
B. fragilis GAI 0558	7.8	5.1	115.5	47.1	107.5
B. fragilis GAI 7955	6.9	6.6	26.8	32.9	26.8
B. fragilis GAI 10150	1.8	0.7	11.9	23.0	13.9

a) cefazolin = 100.

Table 6. Appearance of *C. difficile* in murine cecum after the 7 days dosing of cefclidin, cefotaxime and cefotetan

Agent	After final dosing													
	1day							5days						
	<10^2	10^2	10^3	10^4	10^5	10^6[a]		<10^2	10^2	10^3	10^4	10^5	10^6	10^7
Cefclidin	4	1						5						
Cefotaxime				2	2	1					NT			
Cefotetan			NT[b]										1	4
Control	5							5						

a) Number (CFU/g) of *C. difficile* detected.

b) Not tested.

β-lactamases tested than CPM, CTX and CEZ but less stable than CAZ (Table 5).

In vivo activity in rat pouch infection —Although CFCL had bactericidal potency *in vivo* also, regrowth was seen 48h after exposure to the compound ; in contrast, CAZ was effective at keeping the organism in low number in the pouch (Fig.2). Drug concentration in the pouch was measured only 3, 6, and 9h after the first administration of antimicrobials. There was a tendency that CAZ persisted in the pouch longer than CFCL ; the concentration of CAZ in the pouch covered the MIC for the organism used by 9h after administration, while that of CFCL lowered below the MIC for the organism by 6h after administration (Fig.3).

Inducibility of overgrowth of *C.difficile* in murine cecum —Appearance of *C.difficile* in murine cecum after 7days dosing of CFCL, CTX, and CTT was examined. Although CTX and CTT caused overgrowth of *C.difficile*, which was detected 1-day and 5days, respectively, after ceasing administration of antimicrobials, a low number of *C.difficile* was detected 1day after ceasing in a mouse of 10 mice which were given CFCL (Table 6).

Discussion

CFCL is expected to have strong activity against Gram-negative aerobic bacteria, particularly against *Pseudomonas aeruginosa*. In recent years, some β-lactam antibiotics, such as CPM, CAZ, cefpirome, and cefepime (CFPM), have been developed ; their properties of which have especially emphasized *in vitro* strong anti-*P.aeruginosa* activity. In contrast, these β-lactams often show moderate or weak activity against members of the *B. fragilis* group[4-6]. In the present study, CFCL revealed weak activity against these anaerobic organisms as did other anti-pseudomonal β-lactams. CFCL showed poor activity against *P.bivia* and *C. difficile* ; such an activity was proved in CFPM as well. Against most of the other members of anaerobic bacteria, CFCL showed good activity, especially against *Mobiluncus* spp. and *G.vaginalis*.

Enzymatically, CFCL was relatively stable to hydrolysis by oxyiminocephalosporinase type I derived from *B.fragilis* in comparing CPM, CTX, and CTM. Oxyiminocephalosporinase type I is known as a common β-lactamase produced by *B. fragilis* strains. The meaning of this difference in the stability for the treatment of infectious diseases is not explained. It may be too little a contrast in the stability to bring dissimilar consequences among these antimicrobials for the treatment of polymicrobial infections including β-lactamase-highly producing *B.fragilis* or we may expect some different outcome among these agents. Treatment of rat pouch infection with CFCL resulted in regrowth of the tested organism 48h after starting the administration of this compound. The antibiotic level in the pouch was below the MIC of CFCL for the organism 6h after the administration. However, good *in vitro* killing kinetics of CFCL against *B.fragilis* demonstrated in this study may suggest that increased dosage of CFCL shows constant depression and eradication of the organism in the pouch.

It is well known that many β-lactams can cause antibiotic-associated colitis and diarrhea. We demonstrated that the administration of CFCL did not generate obvious appearance of *C.difficile* in the mouse model, even when *C.difficile* was examined just after ceasing medication or long after. The results suggest that CFCL would be relatively free from *C.difficile*-associated colitis and diarrhea.

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新注射用セファロスポリンcefclidinの嫌気性菌および*Gardnerella vaginalis*に対する抗菌力

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新注射用セファロスポリンcefclidin(CFCL)の嫌気性菌および*Gardnerella vaginalis*に対する抗菌力をceftazidime(CAZ), cefotaxime(CTX), cefpiramide(CPM), ceftotiam(CTM)と比較して検討した。CFCLは*Bacteroides fragilis* groupの嫌気性菌に対して弱い抗菌力しか示さなかったが, 一般的にはグラム陽性および陰性の嫌気性菌に対して幅広い抗菌力を有していた。新鮮臨床分離株に対しては, 他の比較薬剤と同様に*B.fragilis* groupに対しては中程度か弱い抗菌力しか示さなかった。また, *Prevotella bivia*と*Clostridium difficile*に対しては抗菌力はあまりみられなかった。その他の嫌気性菌に対してはよい抗菌力が認められ, ことに*G.vaginalis*と*Mobiluncus* spp.には強い抗菌力がみられた。CFCLは*B.fragilis*の産生するoxyiminocephalosporinase I型に対してCTX, CPM, CTMよりは安定で, CAZよりはわずかに不安定であった。増殖曲線に対するCFCLの効果の検討では, 2 MICもしくはそれ以上の薬剤濃度で再増殖のみられない殺菌的効果が認められた。CFCLはラット・パウチを用いた感染実験においても*B.fragilis*に対して殺菌的効果が認められたが, 48時間後には菌の再増殖がみられた。CFCLはマウス実験モデルを用いた検討において, *C.difficile*の腸管内異常増殖は引き起こしにくい薬剤であると思われた。

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