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Stability and low induction propensity of cefiderocol against chromosomal AmpC β-lactamases of *Pseudomonas aeruginosa* and *Enterobacter cloacae*

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Objectives: The siderophore cephalosporin cefiderocol possesses *in vitro* activity against MDR Gram-negative bacteria. The stability of cefiderocol against serine- and metallo-type carbapenemases has been reported previously, but little is known about how cefiderocol interacts with chromosomal AmpC β-lactamases. We investigated a number of features of cefiderocol, namely antibacterial activity against AmpC overproducers, stability against AmpC β-lactamases and propensity for AmpC induction using *Pseudomonas aeruginosa* and *Enterobacter cloacae*.

Methods: MICs were determined by broth microdilution according to CLSI guidelines. The MIC of cefiderocol was determined in iron-depleted CAMHB. Hydrolysis of the antibiotics was determined by monitoring the changes in the absorbance in the presence of AmpC β-lactamase, and AmpC induction was evaluated by double disc diffusion and nitrocefin degradation assays.

Results: The MICs of ceftazidime and cefepime for PAO1 increased 4- to 16-fold with inactivation of either *ampD* or *dacB*, whereas cefiderocol MICs were little affected by these inactivations (<2-fold increase). Cefiderocol has 40- and >940-fold lower affinity (higher K_i) to AmpCs of *P. aeruginosa* SR24-12 and *E. cloacae* P99, respectively, compared with ceftazidime. Both disc diffusion and nitrocefin degradation assays indicated that cefiderocol did not induce AmpC β-lactamases of *P. aeruginosa* PAO1 and ATCC 27853 and *E. cloacae* ATCC 13047, whereas imipenem did.

Conclusions: Cefiderocol showed *in vitro* activity against the AmpC-overproducing strains, low affinity for chromosomal AmpC β-lactamases, and a low propensity of temporal induction of AmpC β-lactamases of *P. aeruginosa* and *E. cloacae*. These features relating to chromosomal AmpC could explain the potent antibacterial activity of cefiderocol against drug-resistant strains producing AmpC β-lactamases.

Introduction

Genes for AmpC β-lactamases are commonly found on the chromosomes of several family members of Enterobacteriaceae such as *Enterobacter* spp. and non-fermenting bacteria such as *Pseudomonas aeruginosa*,[1] and AmpC overproduction provides these pathogens with resistance to penicillin, cephalosporins and monobactams as well as carbapenems especially in the presence of porin loss.[2,3] These AmpC-producing isolates are problematic because they are reported to become resistant during antibiotic therapy owing to AmpC overproduction.[4]

Cefiderocol (CFDC, S-649266), a novel catechol-substituted siderophore cephalosporin, possesses *in vitro* activity against a wide variety of Gram-negative bacteria including carbapenem-resistant Enterobacteriaceae and MDR non-fermenting bacteria that produce various β-lactamases such as KPC or NDM carbapenemases, ESBLs and AmpC β-lactamases.[5–8]

Cefiderocol possesses potent activity with MIC_{90}s of ≤1 mg/L for clinical isolates of *P. aeruginosa* and *Enterobacter cloacae* collected in North America and Europe between 2014 and 2015, and also shows a comparable MIC_{90} for meropenem-non-susceptible isolates.[5,6] We have revealed that cefiderocol activity against carbapenemase-resistant bacteria is owing to its relatively high stability against carbapenemases as well as its efficient penetration through the outer membrane via active iron-transport systems, but the degree to which cefiderocol is hydrolysed by AmpC β-lactamases has not been investigated.[7–9] Here, we reveal cefiderocol features relating to antibacterial activity against AmpC overproducers, stability against AmpC β-lactamases, and propensity for AmpC induction for *E. cloacae* and *P. aeruginosa*.

Materials and methods

Bacterial strains and antibiotics

P. aeruginosa SR24883 and SR24 were clinical isolates from Japan; *E. cloacae* 1480700 was kindly provided by GlaxoSmithKline plc (USA). *P. aeruginosa* ATCC 27853 and *E. cloacae* ATCC 13047 were obtained from the ATCC. *P. aeruginosa* SR24-12 was obtained by selecting the cefotaxime-resistant mutant from SR24. *E. cloacae* 1480700-4 was obtained by selecting the ceftazidime-resistant mutant from 1480700. The increases in β-lactamase activity of these mutants were detected by nitrocefin degradation. Transposon-insertion mutant strains of *P. aeruginosa* PAO1 were provided by the University of Washington.[10] The *ampC*-deficient mutant strain AC2064 was constructed from *P. aeruginosa* PAO1 by homologous recombination.[11] Cefiderocol was synthesized at Shionogi & Co., Ltd (Osaka, Japan). Commercial-grade antibiotics aztreonam, cefepime, cefotaxime, ceftazidime, cefalotin, imipenem and meropenem were obtained from commercial sources.

MICs

MICs were determined by broth microdilution according to CLSI guidelines.[12,13] For the determination of cefiderocol MIC, iron-depleted CAMHB (ID-CAMHB) was prepared as previously described and used according to the CLSI's recommendation.[9] The quality-control MIC ranges of cefiderocol using ID-CAMHB approved by the CLSI were 0.06–0.5 mg/L for both *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853.[14]

Kinetic parameters of AmpC β-lactamase

Hydrolysis of β-lactam antibiotics was detected by monitoring the changes in the absorbance of β-lactam solution by spectrophotometry (U-3010, Hitachi, Japan). The wavelengths and molar extinction coefficients ($\Delta\varepsilon$) have been reported previously.[8] The steady-state kinetic parameters (k_{cat} and K_m) were determined using the Hanes–Woolf plot of the Michaelis–Menten equation.[15] For poorly hydrolysing substrates, the competitive inhibition constant (K_i) instead of K_m was determined in the presence of 100 μM cefalotin as a reporter substrate. k_{cat} was determined at >5-fold higher concentration than used for the K_i value. The AmpC β-lactamases purified from *E. cloacae* P99 and *P. aeruginosa* SR24-12 were used.

AmpC induction

The propensity for AmpC induction was evaluated by double disc diffusion assay and nitrocefin degradation assay (for details of the procedure, see the Supplementary data available at *JAC* Online). 3-Aminophenylboronic acid (APB; Tokyo Chemical Industry, Japan) was used for the inhibition of AmpC β-lactamase.[1]

Results and discussion

In *P. aeruginosa*, overproduction of chromosomal AmpC is known to be the cephalosporin resistance mechanism that occurs during exposure to β-lactam antibiotics which is caused by the inactivation of genes that regulate the expression of AmpC, such as *ampD* and *dacB*.[16] To evaluate the effect of overproduction of chromosomal AmpC on the *in vitro* activity of cefiderocol, MICs for isogenic mutant strains of *P. aeruginosa* were determined [Table 1 and Table S1 (available as Supplementary data at *JAC* Online)]. MICs of ceftazidime and cefepime for PAO1 were increased 4- to 16-fold by inactivation of *ampD* (PW8615) and *dacB* (PW6111), whereas cefiderocol MICs were little affected by these inactivations (≤2-fold increase for either *ampD* or *dacB*). The effect of the *ampC* gene inactivation on the MICs of cefiderocol as well as ceftazidime and cefepime was limited (≤2-fold decrease), which was different from imipenem, which showed an 8-fold MIC decrease. These results suggest that the activity of ceftazidime, cefepime and imipenem is reduced by the induced levels of AmpC, whereas the activity of cefiderocol is not. Similar results were demonstrated against the AmpC-overproducing mutants isolated from clinical isolates of *P. aeruginosa* and *E. cloacae* (Table 1). The difference between MICs of cefiderocol for parental strains and those for their derivative isolates were ≤4-fold (*P. aeruginosa* SR24 and *E. cloacae* 1480700). On the other hand, MICs of ceftazidime, cefepime and aztreonam for AmpC-producing isolates were 16-fold higher or more than those for parental strains.

To understand the differences in antibacterial activity against AmpC-overproducing strains between cefiderocol and ceftazidime, the kinetic parameters of AmpC β-lactamases derived from *P. aeruginosa* SR24-12 and *E. cloacae* P99 were examined (Table 2). In the case of *P. aeruginosa* AmpC, although hydrolysis of cefiderocol, ceftazidime and cefepime was not detected, the affinities (K_i) of cefiderocol were 40- and 17-fold lower than those of ceftazidime and cefepime, respectively. In the case of *E. cloacae* AmpC, the affinities of cefiderocol were >940- and >8-fold lower than those of ceftazidime and cefepime, respectively, and the hydrolysis of cefiderocol was not detected whereas that of ceftazidime and cefepime was detected. These results indicate that the relatively lower affinity of cefiderocol for the AmpC β-lactamase of *P. aeruginosa* and *E. cloacae* may contribute to the excellent antibacterial activity of cefiderocol against AmpC-overproducing strains.

The induction of chromosomal AmpC β-lactamase in Gram-negative bacteria is known to be one of the resistance mechanisms to β-lactam antibiotics that are hydrolysed by overproduced AmpC.[2,3] The β-lactam antibiotics differ in their AmpC-inducing abilities, and imipenem and cefoxitin are known to be strong inducers of AmpC.[1,17] The effect of possessing AmpC-inducing ability on clinical outcomes is unclear, but this inducing ability could lead to a limited choice of antibiotics because overproduced AmpC would cause resistance to a wide variety of β-lactam antibiotics, including third/fourth-generation cephalosporins as well as carbapenems. To evaluate the risk of AmpC induction by cefiderocol itself, a double disc diffusion assay was conducted (Table S2 and Figures S1–S4). The assay revealed that cefiderocol did not cause a D-shaped inhibition zone of ceftazidime (indicator disc), though such an inhibition zone was observed with imipenem in *P. aeruginosa* PAO1, *P. aeruginosa* ATCC 27853 and *E. cloacae* ATCC 13047 (Table S2 and Figures S1–S4). This D-shaped inhibition zone was not observed when APB was added in the medium nor in the AmpC deletion mutant strain of *P. aeruginosa* PAO1 (AC2064). These results indicated that the formation of a D-shaped inhibition zone with imipenem was owing to the induction of AmpC by imipenem itself. The nitrocefin degradation activity of cell-free extract after exposure to cefiderocol was also compared with that after exposure to imipenem (Figures S5 and S6). It was confirmed that the level of β-lactamases induced by cefiderocol was quantitatively lower than that induced by imipenem in all the experiments with *P. aeruginosa* PAO1, *P. aeruginosa* ATCC 27853 or *E. cloacae* ATCC 13047, and the induction of β-lactamases in *P. aeruginosa* AC2064 was low even in the presence of cefiderocol and imipenem. These results were consistent with the disc diffusion assay and indicated that cefiderocol has a low propensity to induce the AmpC β-lactamases of *P. aeruginosa* and *E. cloacae*.

Table 1. MICs for clinical strains and isogenic mutant strains

Species	Strain	Characteristic	MIC (mg/L)					
			cefiderocol	ceftazidime	cefepime	imipenem	meropenem	aztreonam
P. aeruginosa	PAO1		0.125	1	1	1	0.5	4
P. aeruginosa	PW8615	Δ*ampD* (Tn)	0.25	16	4	1	1	8
P. aeruginosa	PW6111	Δ*dacB* (Tn)	0.25	16	8	1	0.5	16
P. aeruginosa	PW7953	Δ*ampC* (Tn)	0.063	1	2	0.125	0.5	2
P. aeruginosa	SR24		0.125	1	1	1	0.063	4
P. aeruginosa	SR24-12	AmpC high producer	0.125	>32	16	0.5	0.125	>32
E. cloacae	1480700		0.25	0.5	0.063	0.5	0.063	0.125
E. cloacae	1480700-4	AmpC high producer	1	32	1	0.5	0.063	32

Tn, transposon insertion.
MICs were determined by the broth microdilution method using ID-CAMHB for cefiderocol and CAMHB for the other antibiotics.

Table 2. Kinetic parameters of AmpC β-lactamase

β-Lactamase	Antibacterial agent	k_{cat} (s^{-1})[a]	K_m or K_i (μM)[a]	k_{cat}/K_m or K_i ($\mu M^{-1}\ s^{-1}$)
AmpC (*P. aeruginosa*)	cefiderocol	NH[b]	214±49.6[c]	ND
	ceftazidime	NH[b]	5.3±1.0[c]	ND
	cefepime	NH[b]	12.2±0.1[c]	ND
	cefalotin	95.4±9.3	24.5±3.2	3.89
	meropenem	NH[b]	1.7±0.4[c]	ND
P99 (*E. cloacae*)	cefiderocol	NH[b]	>1700[d]	ND
	ceftazidime	$(2.7 \pm 0.49) \times 10^{-3}$	1.8±0.099[c]	0.0015
	cefepime	0.69±0.028	210±21	0.0033
	cefalotin	86±1.5	5.8±0.44	15
	meropenem	NH[b]	0.26±0.0096[c]	ND

NH, no hydrolysis detected; ND, not determined (because no hydrolysis was detected).
[a]Each k_{cat} and K_m or K_i value is the mean ± SD of three different measurements.
[b]Hydrolysis was too weak to determine the k_{cat} value.
[c]K_i values were obtained using 100 μM cefalotin.
[d]This value was the detection limit of the spectrophotometer and no hydrolysis was observed at this concentration.

In summary, this study showed the stability of cefiderocol against chromosomal AmpC β-lactamases of *P. aeruginosa* and *E. cloacae* and the low propensity for induction of AmpC β-lactamase by cefiderocol. These results suggest that cefiderocol could be an effective treatment option for Gram-negative bacterial infections where resistance is mediated by the induction of AmpC β-lactamase.

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Transparency declarations

All authors are employees of Shionogi & Co., Ltd. A. I., M. O., T. I.-H., N. I., T. S. and Y. Y. own stocks of Shionogi & Co., Ltd.

Supplementary data

Tables S1 and S2 and Figures S1–S6 are available as Supplementary data at *JAC* Online.

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