AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy

In Vitro Antimicrobial Activity of a Siderophore Cephalosporin, S-649266, against *Enterobacteriaceae* Clinical Isolates, Including Carbapenem-Resistant Strains

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S-649266 is a novel siderophore cephalosporin antibiotic with a catechol moiety on the 3-position side chain. Two sets of clinical isolate collections were used to evaluate the antimicrobial activity of S-649266 against *Enterobacteriaceae*. These sets included 617 global isolates collected between 2009 and 2011 and 233 β-lactamase-identified isolates, including 47 KPC-, 49 NDM-, 12 VIM-, and 8 IMP-producers. The MIC_{90} values of S-649266 against the first set of *Escherichia coli*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Citrobacter freundii*, *Enterobacter aerogenes*, and *Enterobacter cloacae* isolates were all ≤1 μg/ml, and there were only 8 isolates (1.3%) among these 617 clinical isolates with MIC values of ≥8 μg/ml. In the second set, the MIC values of S-649266 were ≤4 μg/ml against 109 strains among 116 KPC-producing and class B (metallo) carbapenemase-producing strains. In addition, S-649266 showed MIC values of ≤2 μg/ml against each of the 13 strains that produced other types of carbapenemases such as SME, NMC, and OXA-48. The mechanisms of the decreased susceptibility of 7 class B carbapenemase-producing strains with MIC values of ≥16 μg/ml are uncertain. This is the first report to demonstrate that S-649266, a novel siderophore cephalosporin, has significant antimicrobial activity against *Enterobacteriaceae*, including strains that produce carbapenemases such as KPC and NDM-1.

According to a recent global report of the World Health Organization (WHO), antibiotic resistance is now defined as a major threat to public health (1). Frequent occurrences of resistance to third-generation cephalosporins and carbapenems are reported for *Enterobacteriaceae* especially in *Escherichia coli* and *Klebsiella pneumoniae*, which have various antibiotic-resistant mechanisms, such as β-lactamases, decreased outer membrane permeability, efflux pumps, and target modification (2–7). Of these, distinct β-lactamases such as extended-spectrum β-lactamases (ESBLs) and carbapenemases are the most important resistance mechanisms that cause the greatest impact to public health (8, 9). As ESBLs confer resistance to penicillins, oxyimino-cephalosporins, and monobactams, treatment options for infections by ESBL-producing *Enterobacteriaceae* are limited, even though β-lactam/β-lactamase inhibitor combinations (i.e., piperacillin-tazobactam), carbapenems, tigecycline, and colistin are available treatment options. Carbapenems, which show robust antimicrobial activity against ESBL-producing *Enterobacteriaceae*, are considered a last-resort therapy, and there are concerns that their increased consumption will lead to the spread of carbapenem resistance (10).

Carbapenem-resistant *Enterobacteriaceae* (CRE) have been identified in many countries. According to the 2013 Centers for Disease Control and Prevention (CDC) report on the threat of drug-resistant bacteria and the related statement of the European Centre for Disease Prevention and Control (ECDC), CRE have been recognized as a serious and emergent problem and are defined as an urgent risk (11, 12). The increase of CRE, such as KPC- or NDM-type carbapenemase-producing strains, is of great concern, as there are few antibiotics that are active against CRE. Therefore, there is an urgent need for novel antibiotics that are active against *Enterobacteriaceae*, including carbapenemase-producing strains.

S-649266 (see Fig. 1) is a new siderophore cephalosporin antibiotic with a catechol moiety on the 3-position side chain and binds mainly to PBP 3, which is similar to other cephalosporins (13). This catechol moiety contributes to potent antimicrobial activity against Gram-negative bacteria by functioning as a siderophore to form a chelating complex with ferric iron (14). As reported for other β-lactam antibiotics with iron chelating siderophore moiety, the enhanced antimicrobial activity is related to the active outer membrane transport into the periplasmic space where the β-lactam antibiotic efficiently inhibits cell wall synthesis (15–18). In addition, S-649266 is more stable against various β-lactamases, including KPC-3 and NDM-1 carbapenemases, than β-lactam agents such as cefepime (FEP) and meropenem (MEM) (19). In this study, the antimicrobial activities of S-649266 against a variety of *Enterobacteriaceae* clinical isolates, including carbapenemase-producers, were examined.

MATERIALS AND METHODS

Bacterial strains. Two sets of strain collections, a total of 850 clinical *Enterobacteriaceae* isolates, were used to evaluate the antimicrobial activity of S-649266. The first set included 617 clinical isolates collected by

Received 15 July 2015 **Returned for modification** 11 August 2015
Accepted 6 November 2015
Accepted manuscript posted online 16 November 2015
Citation Kohira N, West J, Ito A, Ito-Horiyama T, Nakamura R, Sato T, Rittenhouse S, Tsuji M, Yamano Y. 2016. *In vitro* antimicrobial activity of a siderophore cephalosporin, S-649266, against *Enterobacteriaceae* clinical isolates, including carbapenem-resistant strains. Antimicrob Agents Chemother 60:729–734. doi:10.1128/AAC.01695-15.
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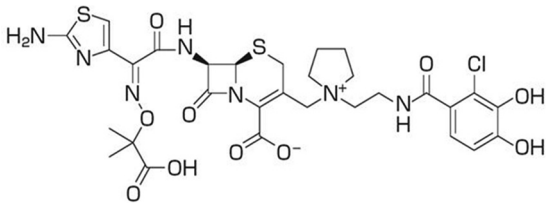

FIG 1 Chemical structure of S-649266.

International Health Management Associates (IMHA) (Schaumburg, IL) between 2009 and 2011. These isolates were obtained from the clinical specimens of hospitalized patients from diverse regions, including North America, Europe, Africa, Asia, Latin America, the South Pacific, and the Middle East. Each isolate was a unique, nonreplicate isolate, with no more than one isolate per patient represented. They consisted of 106 *E. coli* isolates, 105 *K. pneumoniae* isolates, 103 *Serratia marcescens* isolates, 100 *Citrobacter freundii* isolates, 100 *Enterobacter aerogenes* isolates, and 103 *Enterobacter cloacae* isolates. Another set included 233 strains collected by JMI Laboratories (North Liberty, IA) and IHMA, which were obtained from diverse regions, including North America, Europe, Asia-Pacific, and Latin America, between 2000 and 2009. These strains are characterized by their resistance to various β-lactam antibiotics with defined mechanisms related to the production of class A ESBL, noncarbapenemase OXA-type β-lactamases, or various types of carbapenemases. This set consisted of 78 *E. coli* strains, 81 *K. pneumoniae* strains, 20 *S. marcescens* strains, 9 *C. freundii* strains, and 45 *E. cloacae* strains. These strains included 129 carbapenemase-producing strains, consisting of 47 KPC-producing strains, 49 NDM-1-producing strains, 12 VIM-producing strains, 8 IMP-producing strains, 6 OXA-48-producing strains, 6 SME-producing strains, and 1 NMC-A-producing strain. There were 92 strains producing noncarbapenemase class A ESBLs, including 62 CTX-M types, 25 SHV types, 3 TEM types, 1 PER-2, and 1 GES-1. Finally, there were 12 strains that produced noncarbapenemase OXA-type β-lactamases.

MIC determination. MICs were determined by a broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) recommendations; however, to evaluate the antimicrobial activity of S-649266, the cation-adjusted Mueller-Hinton broth (CAMHB) was supplemented with 20 μM human apo-transferrin (Sigma-Aldrich, St. Louis, MO) (20). The apo-transferrin was added to mimic the ferric-iron-limiting condition in human biological fluids in which free iron is tightly bound to proteins such as transferrin (21). Determination of MIC values in iron-deficient conditions has been used to evaluate the activities of other siderophore antibiotics (14, 16, 17, 22). S-649266 was synthesized at Shionogi & Co., Ltd. (Osaka, Japan). Other antimicrobial agents used in this study were obtained from U.S. Pharmacopeia (Rockville, MD) and include FEP, ceftazidime (CAZ), and MEM and from Sigma-Aldrich (St. Louis, MO), including levofloxacin (LVX). FEP, CAZ, MEM, and LVX were used as reference compounds for MIC determinations against the first set of isolates, while FEP and MEM were used for MIC comparison against the second set of strains. All of the MIC values of the compounds except for S-649266 were measured by a broth microdilution method using CAMHB. The resistance breakpoints of these compounds were defined as recommended by CLSI—16 μg/ml for FEP and CAZ, 4 μg/ml for MEM, and 8 μg/ml for LVX (23).

RESULTS

Antimicrobial activity against global clinical isolates collected from 7 regions between 2009 and 2011. The MIC_{50}, MIC_{90}, and MIC ranges of S-649266 and the reference compounds against 617 global clinical isolates of *Enterobacteriaceae* collected from 2009 to 2011 are summarized in Table 1. The overall rates of resistance to FEP, CAZ, MEM, and LVX were 9.2%, 24.5%, 2.6%, and 13.3%, respectively. The MIC_{90} values of S-649266 against these 6 species were ≤1 μg/ml, and there were only 8 isolates (1.3%) among the 617 isolates tested with S-649266 MICs of ≥8 μg/ml. Among the 16 MEM-resistant isolates, 9 isolates (7 *K. pneumoniae* isolates, 1 *E. aerogenes* isolate, and 1 *C. freundii* isolate) were resistant to FEP and CAZ, and 2 isolates (2 *E. aerogenes* isolates) were resistant to only CAZ; all isolates showed MIC values to S-649266 of ≤2 μg/ml. The other 5 MEM-resistant isolates (5 *S. marcescens* isolates) were susceptible to S-649266 as well as to FEP and CAZ with MIC values ranging from ≤0.063 to 1 μg/ml. All of the 5 isolates of *S. marcescens* and the single isolates of *C. freundii*, *E. aerogenes*, and *E. cloacae* that showed S-649266 MICs of ≥8 μg/ml were resistant to FEP and CAZ but were susceptible to MEM, with the exception of 2 *S. marcescens* isolates where the FEP MIC values were 1 and 8 μg/ml. The MICs of LVX against the 8 isolates with S-649266 MICs of ≥8 μg/ml ranged from 0.125 to >8 μg/ml, and 4 of them (2 *S. marcescens* isolates, 1 *C. freundii* isolate, 1 *E. cloacae* isolate) were LVX-resistant isolates.

Antimicrobial activity against β-lactamase-producing strains. Since there were few multidrug-resistant strains in the first set of 617 clinical isolates, the second set was used to investigate the antimicrobial activity of S-649266 against various β-lactamase-producing strains, including carbapenemase-producing strains such as KPC- and NDM producers. The MIC distributions and resistance rates against each β-lactamase type by species of *E. coli*, *K. pneumoniae*, and other *Enterobacteriaceae*, including *S. marcescens*, *C. freundii*, and *E. cloacae*, are shown in Table 2. Most of the strains producing class A carbapenemase KPC and class B carbapenemases, such as NDM-1, VIM, and IMP, were resistant to MEM and FEP. On the other hand, S-649266 showed MIC values ranging from ≤0.125 to 4 μg/ml against all of the KPC-producing strains regardless of species. Also, the MIC values of S-649266 were ≤4 μg/ml against 62 of 69 class B carbapenemase-producing strains, including NDM-1, VIM, and IMP. Among these 233 clinical strains, only 7 strains showed an S-649266 MIC of ≥16 μg/ml; these included 5 NDM-1-producing *E. coli* strains, 1 IMP-1-producing *S. marcescens* strain, and 1 VIM-1-producing *E. cloacae* strain. Against class A carbapenemases-producing strains such as the 6 SME-producing *S. marcescens* strains and 1 NMC-A-producing *E. cloacae* strain, S-649266 and FEP showed MIC values of ≤0.25 μg/ml while the MIC values of MEM were ≥16 μg/ml. S-649266 also showed activity against class D carbapenemase OXA-48-producing strains of *K. pneumoniae* and *E. cloacae* with MIC values of ≤0.125 to 2 μg/ml. The cumulative distributions of the MIC values of S-649266, FEP, and MEM against all of the MEM-resistant carbapenemase-producing strains are summarized in Fig. 2. Against these strains, S-649266 showed antimicrobial activity with MIC_{50} and MIC_{90} values of 0.25 and 2 μg/ml, respectively, which were significantly lower than those of FEP and MEM (MIC_{50} and MIC_{90} values of these two antimicrobial agents were >16 μg/ml). Against the 92 ESBL-producing strains that were mainly CTX-M-type or SHV-type producers, the MIC values of S-649266 were ≤4 μg/ml, including 3 strains with MEM MIC values of ≥16 μg/ml. The MIC values of S-649266 against 12 strains producing OXA-type class D β-lactamases other than OXA-48 ranged from ≤0.125 to 0.5 μg/ml, while the MIC values of FEP ranged from 1 to >16 μg/ml.

DISCUSSION

The breadth of clinically relevant β-lactamase enzymes in a wide range of *Enterobacteriaceae* species continues to contribute to a

TABLE 1 MICs and resistance rates of S-649266 and other antimicrobial agents against 617 clinical isolates of *Enterobacteriaceae*[a]

Species (no. of isolates)	Test compound	MIC (μg/ml)			
		Range	MIC_{50}	MIC_{90}	R%[b]
Total, 6 species (617)	S-649266	≤0.063 to >64	≤0.063	0.5	
	Cefepime	≤0.125 to >32	≤0.125	8	9.2
	Ceftazidime	≤0.125 to >32	0. 25	>32	24.5
	Meropenem	≤0.063 to >16	≤0.063	0.125	2.6
	Levofloxacin	≤0.031 to >8	0.125	>8	13.3
Escherichia coli (106)	S-649266	≤0.063–4	0.125	1	
	Cefepime	≤0.125 to >32	≤0.125	8	8.5
	Ceftazidime	≤0.125 to >32	0.25	2	9.4
	Meropenem	≤0.063–0.125	≤0.063	≤0.063	0
	Levofloxacin	≤0.031 to >8	0.063	>8	26.4
Klebsiella pneumoniae (105)	S-649266	≤0.063–2	≤0.063	0.125	
	Cefepime	≤0.125 to >32	≤0.125	>32	17.1
	Ceftazidime	≤0.125 to >32	0.25	>32	19.0
	Meropenem	≤0.063 to >16	≤0.063	0.125	6.7
	Levofloxacin	0.063 to >8	0.125	>8	21.0
Serratia marcescens (103)	S-649266	≤0.063 to >64	≤0.063	≤0.063	
	Cefepime	≤0.125 to >32	≤0.125	1	5.8
	Ceftazidime	≤0.125 to >32	≤0.125	1	8.7
	Meropenem	≤0.063 to >16	≤0.063	2	4.9
	Levofloxacin	≤0.031 to >8	0.25	2	7.8
Citrobacter freundii (100)	S-649266	≤0.063 to >64	≤0.063	0.125	
	Cefepime	≤0.125 to >32	≤0.125	8	9.0
	Ceftazidime	≤0.125 to >32	1	>32	36.0
	Meropenem	≤0.063 to >16	≤0.063	≤0.063	1.0
	Levofloxacin	≤0.031 to >8	0.125	8	12.0
Enterobacter aerogenes (100)	S-649266	≤0.063–8	≤0.063	0.5	
	Cefepime	≤0.125 to >32	≤0.125	4	5.0
	Ceftazidime	≤0.125 to >32	1	>32	43.0
	Meropenem	≤0.063–8	≤0.063	≤0.063	3.0
	Levofloxacin	≤0.031 to >8	0.063	0.5	6.0
Enterobacter cloacae (103)	S-649266	≤0.063–16	0.125	1	
	Cefepime	≤0.125 to >32	≤0.125	8	9.4
	Ceftazidime	≤0.125 to >32	0.5	>32	32.0
	Meropenem	≤0.063–1	≤0.063	0.125	0
	Levofloxacin	≤0.031 to >8	0.063	4	5.8

[a] Cation-adjusted Mueller-Hinton broth was used as a medium but was supplemented with 20 μM human apo-transferrin for S-649266 to obtain free iron-deficient condition.
[b] Resistance rate (%) based on CLSI resistance breakpoints: 16 μg/ml for cefepime and ceftazidime, 4 μg/ml for meropenem, and 8 μg/ml for levofloxacin (23).

growing global clinical challenge, where even drugs of last resort such as the carbapenems are no longer predictably reliable. S-649266 is a novel siderophore cephalosporin antibiotic with a catechol moiety on the 3-position side chain. The addition of the catechol moiety enables the active transport of S-649266 into bacterial cells via iron transport systems. This molecular technique of harnessing the cell's own essential survival mechanisms to increase the penetration of the antimicrobial agent has been described as a Trojan horse approach (14). Since the iron transporters are strongly regulated by the surrounding iron concentrations (24), the culture medium for the MIC determination of S-649266 was supplemented with an iron chelator (human apo-transferrin) to mimic the limited free iron condition present in human biological fluids (21).

In the first susceptibility study using 617 global clinical isolates, the resistance rates of CAZ, FEP, and MEM against *Enterobacteriaceae* isolates were similar to those recently reported in United States/North American and European surveillance studies where the resistance rates of CAZ, FEP, MEM, and LVX ranged from 9.3% to 21.4%, 6.0% to 14.9%, 1.5% to 2.6%, and 15.8% to 80.6%, respectively (25–27). Although the number of isolates used in our study was less than that of these large surveillance studies, S-649266 showed potent antimicrobial activity with MIC_{90} values of ≤1 μg/ml against clinical isolates of *Enterobacteriaceae* regardless of species.

With regard to the characterized β-lactamase-producing strains, S-649266 has excellent antimicrobial activity against problematic strains that produce carbapenemases such as KPC and class B metallo-β-lactamases, including NDM-1, as well as ESBL-producing strains. In addition to the novel mechanism of

TABLE 2 MIC distributions of S-649266, cefepime, and meropenem against various β-lactamase-producing strains[a]

Species and β-lactamase (no. of isolates)	Test compound	No. of strains inhibited at test compound MIC (μg/ml): ≤0.125	0.25	0.5	1	2	4	8	16	>16	R%[b]
Escherichia coli											
KPC types[c] (7)	S-649266	6					1				
	Cefepime					3		2	1	1	28.6
	Meropenem					3	3	1			57.1
NDM-1 (19)	S-649266		1		6	5	2		1	4	
	Cefepime									19	100
	Meropenem							1	2	16	100
ESBL[d] (49)	S-649266	41	3	4	1						
	Cefepime	1	2		3	5	3	2	4	29	67.3
	Meropenem	48			1						0
Other[e] (3)	S-649266	3									
	Cefepime	1	1			1					0
	Meropenem	1	2								0
Klebsiella pneumoniae											
KPC types[f] (20)	S-649266	15	3	1	1						
	Cefepime				1		1		2	16	90.0
	Meropenem						1	4	4	11	100
NDM-1 (24)	S-649266	2	7	7	7	1					
	Cefepime									24	100
	Meropenem					1		1	5	17	95.8
VIM-1 (4)	S-649266	4									
	Cefepime							1	1	2	75.0
	Meropenem				2					2	50.0
ESBL[g] (24)	S-649266	17	2	4		1					
	Cefepime		1		1	3	3	3	1	12	54.2
	Meropenem	20	1			1			1	1	8.3
Other[h] (9)	S-649266	6	2	1							
	Cefepime		1	1	1	1	1	1		3	33.3
	Meropenem	7		1			1				11.1
Serratia marcescens, *Citrobacter freundii*, and *Enterobacter cloacae*											
KPC types[i](20)	S-649266	15	2	1		1	1				
	Cefepime					1	2	3	4	10	70.0
	Meropenem				2	2	5	6	2	3	80.0
NDM-1 (6)	S-649266	2			2	2					
	Cefepime								1	5	100
	Meropenem					1		2	1	2	83.3
VIM types[j] (8)	S-649266	6	1						1		
	Cefepime					1		1	2	4	75.0
	Meropenem				1	2	3	2			62.5
IMP types[k] (8)	S-649266	3	2	1	1				1		
	Cefepime					1			1	6	87.5
	Meropenem					1	1	2	3	1	87.5
ESBL[l] (19)	S-649266	13	1	1	1		3				
	Cefepime				3	2	4	1	2	7	47.4
	Meropenem	15	2	1					1		5.3
Other[m] (13)	S-649266	11		1		1					
	Cefepime	6	1			2	2			2	15.4
	Meropenem	3	1					1	1	7	69.2

[a] Cation-adjusted Mueller-Hinton broth was used as a medium but was supplemented with 20 μM human apo-transferrin for S-649266 to obtain free iron-deficient condition.
[b] Resistance rate (%) based on CLSI resistance breakpoints: 16 μg/ml for cefepime and 4 μg/ml for meropenem (23).
[c] 4 KPC-2, 2 KPC-3, and 1 KPC-like.
[d] 1 CTX-M-2, 4 CTX-M-3, 14 CTX-M-14, 20 CTX-M-15, 2 CTX-M-24, 1 CTX-M-28, 1 SHV-5, 2 SHV-12, 1 SHV-30, 1 TEM-10, 1 TEM-12, and 1 TEM-26.
[e] 2 OXA-48 and 1 OXA-1.
[f] 13 KPC-2 and 7 KPC-3.
[g] 2 CTX-M-2, 1 CTX-M-3, 3 CTX-M-14, 4 CTX-M-15, 1 CTX-M-24, 3 SHV-5, 2 SHV-7, 3 SHV-12, 1 SHV-27, 2 SHV-30, 1 SHV-31, and 1 SHV-40.
[h] 1 OXA-1, 1 OXA-2, 1 OXA-2-like, 1 OXA-10, 2 OXA-10-like, and 3 OXA-48.
[i] 8 KPC-2, 11 KPC-3, and 1 KPC-4.
[j] 5 VIM-1, 2 VIM-2, and 1 VIM-23.
[k] 7 IMP-1 and 1 IMP-21.
[l] 1 CTX-M-2, 3 CTX-M-3, 2 CTX-M-14, 1 CTX-M-15, 1 CTX-M-24, 1 CTX-M-like, 2 SHV-5, 2 SHV-7, 1 SHV-12, 3 SHV-30, 1 PER-2, and 1 GES-1.
[m] 1 SME-1, 5 SME-2, 1 NMC-A, 4 OXA-2, 1 OXA-10-like, and 1 OXA-48.

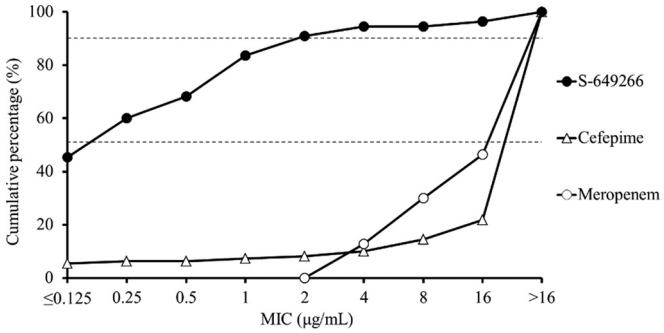

FIG 2 Cumulative percentage of MICs against meropenem-resistant carbapenemase-producing *Enterobacteriaceae*. The test strains were 110 *E. coli*, *K. pneumoniae*, *S. marcescens*, *C. freundii*, and *E. cloacae* isolates that consisted of 40 KPC-type-, 47 NDM-1-, 7 VIM-type-, 7 IMP-type-, 6 SME-type-, 1 NMC-A-, and 2 OXA-48-producing strains. Cation-adjusted Mueller-Hinton broth was used as a medium, but 20 μM human apo-transferrin was supplemented for the MIC determination of S-649266 to obtain a free iron-deficient condition. The broken lines represent 50% and 90% lines.

cell entry provided by the siderophore moiety, S-649266 has been found to be quite stable to various β-lactamases, including ESBLs and class B (NDM-1, VIM, and IMP) and class A (KPC, NMC, and SME) carbapenemases (19). To overcome the threat of KPC-producing CREs, novel β-lactamase inhibitors such as avibactam and RPX7009, broad-spectrum inhibitors for class A, C, and D β-lactamases, have been strategically selected for clinical development as combination therapy with β-lactam agents; however, these β-lactam/β-lactamase inhibitors do not have antimicrobial activity against strains producing class B β-lactamases such as NDM-1 (28). BAL30072, a siderophore-conjugated monosulfactam, displays antimicrobial activity against class B β-lactamase-producing strains, including *P. aeruginosa*, but BAL30072 shows weak antimicrobial activity against KPC-producing *K. pneumoniae* (29). Importantly, S-649266 showed potent antimicrobial activity against various types of carbapenemase-producing strains without the addition of a β-lactamase inhibitor.

Several strains with reduced susceptibility to S-649266 were found among the first set of global clinical isolates and among the second set of β-lactamase-producing strains tested. There were 5 NDM-1-producing *E. coli* strains with reduced susceptibility to S-649266, but most of the NDM-1-producing *Enterobacteriaceae*, including *E. coli* strains were susceptible to S-649266. The reason why some strains of NDM-1-producing *E. coli* strains have reduced susceptibility to S-649266 is unclear, but the deficiency or loss of the influx routes relating to iron transport systems may contribute to the reduced susceptibility to S-649266 as has been reported with other siderophore β-lactams (15, 18). These resistance mechanisms to S-649266 need to be evaluated in the further studies.

This study reveals the potent antimicrobial activity of S-649266 especially against many of the problematic multidrug-resistant *Enterobacteriaceae*. The striking features of S-649266 are the Trojan horse transport and the high stability against hydrolysis by β-lactamases, including ESBLs and carbapenemases (19). To better understand the relationship of MICs and efficacy, S-649266 has shown potent *in vivo* efficacy against ESBL-producing or KPC-producing strains in various animal infection models (30). In conclusion, S-649266 is a promising siderophore cephalosporin to treat bacterial infections caused by *Enterobacteriaceae*, including CRE such as KPC- and NDM-1-producing strains.

ACKNOWLEDGMENT

We declare no conflicts of interest.

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