Antimicrobial Agents and Chemotherapy®

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In Vitro Activity of LYS228, a Novel Monobactam Antibiotic, against Multidrug-Resistant *Enterobacteriaceae*

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ABSTRACT LYS228 is a novel monobactam with potent activity against *Enterobacteriaceae*. LYS228 is stable to metallo-β-lactamases (MBLs) and serine carbapenemases, including *Klebsiella pneumoniae* carbapenemases (KPCs), resulting in potency against the majority of extended-spectrum β-lactamase (ESBL)-producing and carbapenem-resistant *Enterobacteriaceae* strains tested. Overall, LYS228 demonstrated potent activity against 271 *Enterobacteriaceae* strains, including multidrug-resistant isolates. Based on MIC_{90} values, LYS228 (MIC_{90}, 1 μg/ml) was $\geq$32-fold more active against those strains than were aztreonam, ceftazidime, ceftazidime-avibactam, cefepime, and meropenem. The tigecycline MIC_{90} was 4 μg/ml against the strains tested. Against *Enterobacteriaceae* isolates expressing ESBLs ($n = 37$) or displaying carbapenem resistance ($n = 77$), LYS228 had MIC_{90} values of 1 and 4 μg/ml, respectively. LYS228 exhibited potent bactericidal activity, as indicated by low minimal bactericidal concentration (MBC) to MIC ratios (MBC/MIC ratios of $\leq$4) against 97.4% of the *Enterobacteriaceae* strains tested (264/271 strains). In time-kill studies, LYS228 consistently achieved reductions in CFU per milliliter of 3 $\log_{10}$ units ($\geq$99.9% killing) at concentrations $\geq$4$\times$ MIC for *Escherichia coli* and *K. pneumoniae* reference strains, as well as isolates encoding TEM-1, SHV-1, CTX-M-14, CTX-M-15, KPC-2, KPC-3, and NDM-1 β-lactamases.

KEYWORDS *Enterobacteriaceae*, LYS228, monobactams

Over the past 2 decades, the increasing prevalence of Gram-negative bacterial isolates expressing extended-spectrum β-lactamases (ESBLs) has eroded the effectiveness of many β-lactams, the most widely used class of antibiotics (1, 2); this has increased the usage of the carbapenem class of β-lactams as first-line therapy. Consequently, carbapenem-resistant *Enterobacteriaceae* (CRE) now pose yet another significant challenge. In the United States, isolates producing *Klebsiella pneumoniae* carbapenemase (KPC) are now endemic, and numerous large-scale outbreaks have been reported (3–6). The emergence of the New Delhi metallo-β-lactamase (NDM-1) in *K. pneumoniae* (7), and then in *Escherichia coli*, has also contributed to the reduced effectiveness of carbapenems (8–10). Such strains are frequently also resistant to other classes of antibiotics, including aminoglycosides, fluoroquinolones, and tetracyclines, and require treatment with poorly tolerated antibiotics, such as colistin, as a last resort (11, 12). Therefore, the recent identification of CRE strains that are colistin resistant, via transmissible elements such as the mobilized colistin resistance gene (e.g., MCR-1), is alarming (13). The inability to effectively treat infections caused by antibiotic-resistant Gram-negative bacteria has translated into increased morbidity and mortality rates (14–17).

Monocyclic β-lactams, such as monobactams, are a class of β-lactam antibiotics that are intrinsically stable to metallo-β-lactamases (MBLs). Unfortunately, aztreonam, the only monobactam that is clinically available in the United States and Europe, is
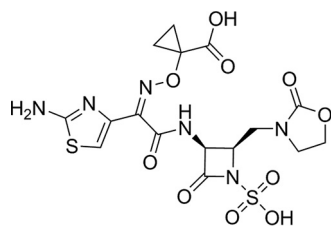
FIG 1 Chemical structure of LYS228.

susceptible to many serine β-lactamases (SBLs) (18); this limits the clinical use of aztreonam, since MBLs and SBLs are frequently found together in the same clinical isolates (2). Given the intrinsic stability of monobactams to MBLs and the reported liability of monobactams to inactivation by SBLs, we initiated a program to enhance the intrinsic stability of monobactams to SBLs through structural modification of the monobactam ring. Our efforts led to the identification of LYS228 (Fig. 1). LYS228 is a single-agent monobactam that is effective against *Enterobacteriaceae* strains, including those expressing ESBLs, SBLs, and MBLs (19, 20). This study describes the *in vitro* antibacterial potency and bactericidal activity of LYS228 against *E. coli*, *K. pneumoniae*, and other members of *Enterobacteriaceae*.

(This work was presented, in part, at the 2017 American Society of Microbiology Microbe Meeting, New Orleans, LA.)

RESULTS AND DISCUSSION

LYS228 is a novel monobactam antibiotic. Here, we tested the *in vitro* antibacterial activity of LYS228 against strains with different resistance genotypes and phenotypes; some isolates produce β-lactamase enzymes with narrow substrate specificity (TEM-1), while others produce broad-spectrum enzymes (including KPCs, CTX-M, and NDM-1). LYS228 was active against a majority of the isolates tested, with MIC_{50} and MIC_{90} values of 0.25 and 1 μg/ml, respectively (Table 1). The MICs determined for LYS228 and comparators against *Enterobacteriaceae* are summarized in Table 2. Based on MIC_{90} comparisons, LYS228 was ≥32-fold more active than aztreonam, ceftazidime, ceftazidime-avibactam, cefepime, and meropenem (Table 2). LYS228 was 4-fold more potent than tigecycline, for which a MIC_{90} value of 4 μg/ml was observed. Overall, 95.9%, 97.9%, and 98.9% of the 271 isolates were inhibited by LYS228 concentrations of ≤2, ≤4, and ≤8 μg/ml, respectively (Table 1).

Among the 271 isolates tested, 37 strains encoded the following ESBLs: TEM-10, TEM-12, TEM-52, SHV-4, SHV-5, SHV-7, SHV-12, CTX-M-2, CTX-M-3, CTX-M-5, CTX-M-14, and CTX-M-15, as well as ESBLs that were not annotated but were identified by phenotypic screening (21). LYS228 MIC values ranged from ≤0.06 to 8 μg/ml, with MIC_{50} and MIC_{90} values of 0.12 and 1 μg/ml, respectively. A group of 46 molecularly characterized KPC-producing isolates, including 1 *Citrobacter freundii* isolate, 4 *Enterobacter cloacae* isolates, 1 *Enterobacter hormaechei* isolate, 4 *E. coli* isolates, and 36 *K. pneumoniae* isolates, were tested. These isolates were inhibited by LYS228 with MICs ranging from ≤0.06 to 4 μg/ml (MIC_{50}, 0.5 μg/ml; MIC_{90}, 2 μg/ml). Strains encoding molecularly characterized enzymes from the IMP, NDM, and VIM classes, as well as strains with uncharacterized genotypes that were positive for carbapenemase activity via both modified carbapenem inactivation method (mCIM) and eCIM carbapenemase identification tests (21), were defined as MBL producers. Against the 33 MBL-producing isolates included in this study, the LYS228 MIC_{50} and MIC_{90} values were 0.5 and 4 μg/ml, respectively.

The activity of LYS228 was evaluated against 77 strains that were defined as CRE and were resistant to meropenem (MICs of ≥4 μg/ml) under the current CLSI resistance breakpoint (21). LYS228 retained activity (MIC_{50}, 0.5 μg/ml; MIC_{90}, 4 μg/ml) against the CRE isolates tested (Table 2). The *in vitro* activity of LYS228 was superior to that of all

TABLE 1 LYS228 MIC distribution among 271 *Enterobacteriaceae* isolates, by resistance genotype and species

Isolate	No. (cumulative %) of isolates inhibited at MIC of:											MIC (μg/ml)	
	≤0.06 μg/ml	0.12 μg/ml	0.25 μg/ml	0.5 μg/ml	1 μg/ml	2 μg/ml	4 μg/ml	8 μg/ml	16 μg/ml	32 μg/ml	64 μg/ml	MIC_{50}	MIC_{90}
All (n = 271)	70 (25.8)	48 (43.5)	49 (61.6)	44 (77.9)	35 (90.8)	14 (95.9)	5 (97.9)	3 (98.9)	2 (99.6)	0 (99.6)	1 (100)	0.25	1
ESBL producers (n = 37)[a]	13 (35.1)	8 (56.8)	8 (78.4)	1 (81.1)	4 (91.9)	1 (94.6)	1 (97.3)	1 (100)				0.12	1
KPC producers (n = 46)[b]	4 (8.7)	3 (15.2)	4 (23.9)	22 (71.7)	8 (89.1)	4 (97.8)	1 (100)					0.5	2
MBL producers (n = 33)[c]	1 (3.0)	4 (15.2)	7 (36.4)	8 (60.1)	6 (78.8)	2 (84.8)	2 (90.9)	1 (93.9)	2 (100)			0.5	4
Citrobacter spp. (n = 22)[d]	2 (9.1)	2 (18.2)	4 (36.4)	3 (50.0)	8 (86.4)	3 (100)						0.5	2
Enterobacter spp. (n = 40)[e]	1 (2.5)	2 (7.5)	4 (17.5)	14 (52.5)	11 (80.0)	6 (95.0)	2 (100)					0.5	2
E. coli (n = 59)	14 (23.7)	17 (52.5)	13 (74.6)	6 (84.7)	4 (91.5)		1 (93.2)	2 (96.6)	2 (100)			0.12	1
K. pneumoniae (n = 81)	13 (16.0)	13 (32.1)	18 (54.3)	20 (79.0)	9 (90.1)	5 (96.3)	1 (97.5)	1 (98.8)			1 (100)	0.25	1
M. morganii (n = 14)	10 (71.4)	1 (78.6)	2 (92.9)	1 (100)								≤0.06	0.25
P. mirabilis (n = 22)	20 (90.9)	2 (100)										≤0.06	≤0.06
S. marcescens (n = 13)	1 (7.7)	6 (53.8)	4 (84.6)		1 (92.3)		1 (100)					0.12	1
Salmonella spp. (n = 16)	7 (43.8)	4 (68.8)	3 (87.5)		2 (100)							0.12	1

[a] Strains tested included C. freundii (n = 2), C. koseri (n = 1), E. aerogenes (n = 1), E. coli (n = 11), K. pneumoniae (n = 8), M. morganii (n = 2), P. mirabilis (n = 6), Salmonella spp. (n = 5), and S. marcescens (n = 1).
[b] Strains tested included C. freundii (n = 1), E. cloacae (n = 4), E. hormaechei (n = 1), E. coli (n = 1), and K. pneumoniae (n = 36).
[c] Strains tested included C. freundii (n = 1), E. aerogenes (n = 1), E. cloacae (n = 10), E. coli (n = 6), K. pneumoniae (n = 13), P. mirabilis (n = 1), and S. marcescens (n = 1).
[d] Strains tested included C. freundii (n = 21) and C. koseri (n = 1).
[e] Strains tested included E. aerogenes (n = 4), E. cloacae (n = 35), and E. hormaechei (n = 1).

TABLE 2 *In vitro* activity of LYS228 against *Enterobacteriaceae* isolates

Isolate type and test agent	MIC (μg/ml)			% susceptible[a]
	Range	MIC$_{50}$	MIC$_{90}$	
Enterobacteriaceae (n = 271)				
LYS228	≤0.06 to 64	0.25	1	NA[b]
Aztreonam	≤0.06 to >64	16	>64	45.0
Ceftazidime	≤0.06 to >64	16	>64	42.8
Ceftazidime-avibactam	≤0.06/4 to >64/4	0.5/4	>64/4	87.1
Cefepime	≤0.06 to >64	2	>64	52.0
Meropenem	≤0.06 to >64	0.12	32	68.6
Tigecycline	0.12 to 8	1	4	86.0
CRE isolates (n = 77)[c]				
LYS228	≤0.06 to 64	0.5	4	NA
Aztreonam	≤0.06 to >64	>64	>64	5.2
Ceftazidime	1 to >64	>64	>64	3.9
Ceftazidime-avibactam	≤0.06/4 to >64/4	2/4	>64/4	66.2
Cefepime	≤0.06 to >64	>64	>64	3.9
Meropenem	4 to >64	32	>64	0.0
Tigecycline	0.125 to 8	1	4	87.0
Citrobacter spp. (n = 22)[d]				
LYS228	≤0.06 to 2	0.5	2	NA
Aztreonam	0.12 to >64	64	>64	27.7
Ceftazidime	0.5 to >64	64	>64	27.3
Ceftazidime-avibactam	≤0.06/4 to >64/4	0.5/4	2/4	95.5
Cefepime	≤0.06 to >64	1	>64	72.7
Meropenem	≤0.06 to >64	≤0.06	4	86.4
Tigecycline	0.25 to 2	0.5	2	100
Enterobacter spp. (n = 40)[e]				
LYS228	≤0.06 to 4	0.5	2	NA
Aztreonam	0.25 to >64	>64	>64	17.5
Ceftazidime	0.25 to >64	>64	>64	15.0
Ceftazidime-avibactam	0.12/4 to >64/4	2/4	>64/4	65.0
Cefepime	≤0.06 to >64	16	>64	35.0
Meropenem	≤0.06 to >64	0.5	64	57.5
Tigecycline	0.25 to 8	1	4	77.5
E. coli (n = 59)				
LYS228	≤0.06 to 16	0.12	1	NA
Aztreonam	≤0.06 to >64	0.25	>64	59.3
Ceftazidime	0.12 to >64	0.5	>64	62.7
Ceftazidime-avibactam	≤0.06/4 to >64/4	0.12/4	>64/4	89.8
Cefepime	≤0.06 to >64	0.25	>64	62.7
Meropenem	≤0.06 to >64	≤0.06	32	83.1
Tigecycline	0.12 to 1	0.25	0.5	100
K. pneumoniae (n = 81)				
LYS228	≤0.06 to 64	0.25	1	NA
Aztreonam	≤0.06 to >64	>64	>64	27.2
Ceftazidime	0.12 to >64	>64	>64	25.9
Ceftazidime-avibactam	≤0.06/4 to >64/4	1/4	>64/4	84.0
Cefepime	≤0.06 to >64	32	>64	32.1
Meropenem	≤0.06 to >64	8	64	38.3
Tigecycline	0.12 to 8	1	4	86.4
M. morganii (n = 14)				
LYS228	≤0.06 to 0.5	≤0.06	0.25	NA
Aztreonam	≤0.06 to 32	0.12	16	78.6
Ceftazidime	0.12 to 32	1	32	57.1
Ceftazidime-avibactam	≤0.06/4 to 1/4	0.12/4	0.5/4	100
Cefepime	≤0.06 to >64	≤0.06	0.5	92.9
Meropenem	≤0.06 to 0.5	0.12	0.25	100
Tigecycline	0.5 to 8	2	8	78.6

(Continued on next page)

TABLE 2 (Continued)

Isolate type and test agent	MIC (μg/ml)			% susceptible[a]
	Range	MIC$_{50}$	MIC$_{90}$	
P. mirabilis (n = 22)				
LYS228	≤0.06 to 0.12	≤0.06	≤0.06	NA
Aztreonam	≤0.06 to >64	≤0.06	2	90.9
Ceftazidime	≤0.06 to 32	0.25	8	81.8
Ceftazidime-avibactam	≤0.06/4 to 4/4	≤0.06/4	0.12/4	100
Cefepime	≤0.06 to >64	0.5	>64	63.6
Meropenem	0.12 to 16	0.12	0.25	95.5
Tigecycline	1 to 8	4	8	36.4
S. marcescens (n = 13)				
LYS228	≤0.06 to 4	0.12	1	NA
Aztreonam	0.12 to >64	2	>64	69.2
Ceftazidime	0.12 to >64	1	32	61.5
Ceftazidime-avibactam	0.12/4 to >64/4	0.25/4	2/4	92.3
Cefepime	≤0.06 to >64	0.25	>64	61.5
Meropenem	≤0.06 to 64	0.25	32	69.2
Tigecycline	0.5 to 8	1	2	92.3
Salmonella spp. (n = 16)				
LYS228	≤0.06 to 1	0.12	1	NA
Aztreonam	≤0.06 to >64	0.25	>64	56.3
Ceftazidime	0.25 to >64	0.5	>64	56.3
Ceftazidime-avibactam	0.12/4 to 2/4	0.25/4	1/4	100
Cefepime	≤0.06 to >64	0.5	>64	62.5
Meropenem	≤0.06 to 0.12	<0.06	0.12	100
Tigecycline	0.25 to 2	0.5	1	100

[a]Susceptibility was defined by CLSI document M100 (21). In the absence of CLSI breakpoints, U.S. FDA breakpoints were applied (30, 31).
[b]NA, not applicable. Susceptibility has not been defined for LYS228.
[c]Strains tested included *C. freundii* (n = 3), *E. aerogenes* (n = 1), *E. cloacae* (n = 11), *E. hormaechei* (n = 1), *E. coli* (n = 10), *K. pneumoniae* (n = 47), *P. mirabilis* (n = 1), and *S. marcescens* (n = 3).
[d]Strains tested included *C. freundii* (n = 21) and *C. koseri* (n = 1).
[e]Strains tested included *E. aerogenes* (n = 4), *E. cloacae* (n = 35), and *E. hormaechei* (n = 1).

β-lactam agents tested, as well as ceftazidime-avibactam (MIC$_{90}$ values of >64 μg/ml). The tigecycline MIC$_{90}$ with these strains was 4 μg/ml.

A very narrow range of LYS228 MIC values were recorded against the *Citrobacter* sp. isolates (n = 22), with the highest recorded value being 2 μg/ml. This group consisted of 2 species, namely, *C. freundii* (n = 21) and *Citrobacter koseri* (n = 1). LYS228 demonstrated potent activity, with MIC$_{50}$ and MIC$_{90}$ values of 0.5 and 2 μg/ml, respectively, against this genus (Table 2). Tested *Enterobacter* sp. isolates (n = 40) consisted of the following 3 species: *Enterobacter aerogenes* (n = 4), *E. cloacae* (n = 35), and *E. hormaechei* (n = 1). The MIC$_{50}$ and MIC$_{90}$ values for LYS228 against this group were 0.5 and 2 μg/ml, respectively; LYS228 MIC values ranged between ≤0.06 and 4 μg/ml (Table 1). LYS228 MIC$_{50}$ and MIC$_{90}$ values against 59 *E. coli* isolates were 0.12 and 1 μg/ml, respectively (Table 1); LYS228 was ≥128-fold more active than ceftazidime, ceftazidime-avibactam, and cefepime (MIC$_{90}$ values of >64 μg/ml) and 32-fold more potent than meropenem (MIC$_{90}$, 32 μg/ml), while the tigecycline MIC$_{90}$ value against the *E. coli* strains tested was 0.5 μg/ml (Table 2). Against 81 *K. pneumoniae* isolates, LYS228 MIC$_{50}$ and MIC$_{90}$ values were 0.25 and 1 μg/ml, respectively (Table 1); LYS228 was 64-fold more potent than meropenem (MIC$_{90}$, 64 μg/ml), and the tigecycline MIC$_{90}$ value was 4 μg/ml (Table 2). LYS228 was very active against the 4 *Klebsiella oxytoca* isolates tested (MIC range, ≤0.06 to 0.12 μg/ml). The susceptibility profile for 1 of the 4 strains showed intermediate resistance to ceftazidime and cefepime and resistance to aztreonam, based on CLSI breakpoints (21). All 4 isolates were susceptible to ceftazidime-avibactam, meropenem, and tigecycline. Fourteen isolates of *Morganella morganii* were tested, and LYS228 was highly potent against those isolates, with MIC values ranging between ≤0.06 and 0.5 μg/ml. Five of those 14 isolates of *M. morganii* were resistant to ceftazidime (MIC$_{90}$, 32 μg/ml) but susceptible to meropenem (MIC$_{90}$, 0.25 μg/ml),

TABLE 3 Bactericidal activity of LYS228 against 271 *Enterobacteriaceae* isolates

Test agent	No. (%) of isolates with MBC/MIC ratio[a] of:				
	1	2	4	8	>8
LYS228	196 (72.3)	52 (19.2)	16 (5.9)	7 (2.6)	
Aztreonam	216 (79.7)	37 (13.7)	10 (3.7)	8 (3.0)	
Ceftazidime	223 (82.3)	29 (10.7)	12 (4.4)	7 (2.6)	
Ceftazidime-avibactam[b]	196 (72.3)	51 (18.8)	16 (5.9)	8 (3.0)	
Cefepime	194 (71.6)	45 (16.6)	16 (5.9)	16 (5.9)	
Meropenem	197 (72.7)	40 (14.7)	21 (7.7)	13 (4.8)	
Tigecycline	46 (17.0)	20 (7.4)	12 (4.4)	14 (5.2)	179 (66.1)

[a]For MBC/MIC ratio calculations, MIC and MBC values of ≤ 0.06 μg/ml were set to 0.06 μg/ml and MIC and MBC values of >64 μg/ml were set to 128 μg/ml.
[b]Avibactam was tested at a fixed concentration of 4 μg/ml.

ceftazidime-avibactam (MIC_{90}, 0.5 μg/ml), and cefepime (MIC_{90}, 0.5 μg/ml). The tige-cycline MIC_{90} against *M. morganii* was 8 μg/ml (Table 2). LYS228 was highly active against the 22 isolates of *Proteus mirabilis* tested (MIC_{90}, ≤ 0.06 μg/ml); the highest MIC value obtained for this group was 0.12 μg/ml (Table 1). Against *Serratia marcescens* ($n = 13$), LYS228 MIC values ranged from ≤ 0.06 to 4 μg/ml (MIC_{90}, 1 μg/ml) (Table 1). While the MIC_{90} values for ceftazidime-avibactam and tigecycline were 2 μg/ml, the MIC_{90} values for the other tested comparators were high (MIC_{90} values of 32 to ≥ 64 μg/ml) (Table 2). LYS228 demonstrated potent activity, with MIC values ranging from ≤ 0.06 to 1 μg/ml and MIC_{50} and MIC_{90} values of 0.12 and 1 μg/ml, respectively, against 16 isolates of *Salmonella* spp.

The preclinical pharmacokinetic (PK) profile of LYS228 predicts a human PK profile similar to that of aztreonam (19). Based on the current CLSI resistance breakpoint for aztreonam (21), LYS228 showed elevated MIC values (MICs ranging from 16 to 64 μg/ml) against 3 of the 271 *Enterobacteriaceae* isolates tested (Table 1), i.e., 2 *E. coli* strains and 1 *K. pneumoniae* strain. The highest LYS228 MIC observed was 64 μg/ml for 1 strain of *K. pneumoniae* producing CTX-M-15, OXA-48, and VEB-1 β-lactamases. This finding is consistent with the instability of LYS228 to VEB-1, which we observed previously when LYS228 was profiled against a wide panel of isogenic strains producing individual β-lactamases (19). LYS228 MIC values of 16 μg/ml were obtained for 2 strains of *E. coli* producing NDM-1, and the MICs did not appear to be associated with the β-lactamases expressed in those isolates. Both isolates harbored a mutation in *ftsI*, encoding the YRIN amino acid insertion in penicillin-binding protein 3 (PBP3), which is known to reduce susceptibility to some monobactams in clinical isolates of *E. coli* expressing NDM-1 (22). This mechanism likely accounts, at least in part, for the elevated LYS228 MIC values observed for those 2 isolates.

The minimal bactericidal concentration (MBC) was defined as the lowest concentration of compound that produced a reduction in the CFU per milliliter of $\geq 99.9\%$ (i.e., ≥ 3 $\log_{10}$ units) in 24 h, compared to the starting inoculum. Bactericidal activity was defined as a MBC/MIC ratio of ≤ 4. MBC values for LYS228 against the 271 isolates tested ranged from ≤ 0.06 to 64 μg/ml, with MBC_{50} and MBC_{90} values of 0.25 and 2 μg/ml, respectively. LYS228 MBC/MIC ratios for all isolates tested were equal to 1 (72.3%), 2 (19.2%), 4 (5.9%), or 8 (2.6%) (Table 3). For the 7 strains with MBC/MIC ratios of 8 (1 *C. freundii*, 1 *E. cloacae*, 1 *E. coli*, 2 *K. pneumoniae*, and 2 *Salmonella* sp. isolates), LYS228 MICs ranged from ≤ 0.06 to 1 μg/ml. These strains produced either a class A or a class C β-lactamase enzyme; none of them was a KPC or MBL producer. Overall, LYS228 was bactericidal against 264 (97.4%) of the 271 strains tested, as indicated by MBC/MIC ratios of ≤ 4 (Table 3). The other β-lactam agents tested had similarly low MBC/MIC ratios. In comparison, tigecycline, a bacteriostatic agent, had MBC/MIC ratios of >4 for 193 (71.2%) of the 271 strains tested. Time-kill studies were performed to characterize further the bactericidal activity of LYS228. Tested isolates included reference strains and clinical isolates encoding the following β-lactamases: TEM-1, SHV-1, CTX-M-14, CTX-M-15, KPC-2, KPC-3, and NDM-1. At concentrations $\geq 4\times$ MIC, LYS228 produced reductions

in CFU per milliliter of ≥ 3 $\log_{10}$ units for all 10 *E. coli* and *K. pneumoniae* strains tested (Table 4). Against an *E. coli* reference strain (ATCC 25922) and *E. coli* NB27236, expressing NDM-1, LYS228 was bactericidal at 2× MIC at 4 and 8 h; however, bacterial regrowth was observed at 24 h (Table 4). Reductions in CFU per milliliter of ≥ 3 $\log_{10}$ units were achieved by LYS228 at all concentrations tested ($\geq 2\times$ MIC) for strains of *E. coli* expressing TEM-1, CTX-M-15, and KPC-3. For the *K. pneumoniae* isolates tested, LYS228 conferred reductions in CFU per milliliter of ≥ 3 $\log_{10}$ units at concentrations of ≥ 0.5 μg/ml ($\geq 4\times$ MIC) for strain ATCC 43816 and strain NB29254, expressing SHV-1 (Table 4). Reductions in CFU per milliliter of ≥ 3 $\log_{10}$ units were achieved by LYS228 against *K. pneumoniae* strains NB29084 (CTX-M-14), NB29082 (KPC-2), and ATCC 1100975 (NDM-1) at all concentrations tested (Table 4).

In conclusion, LYS228 is a novel monobactam with potent *in vitro* activity against a broad panel of clinically relevant *Enterobacteriaceae* strains, including CRE strains. LYS228 is stable to MBLs, such as NDMs, and to most characterized SBLs, such as KPCs. These findings are consistent with data for a panel of isogenic strains expressing various β-lactamases (19). Here, we show that the stability of LYS228 translates to potent activity against a panel of multidrug-resistant *Enterobacteriaceae* clinical isolates, including CREs, which clearly differentiates LYS228 from other reported monobactams (23–25). In recent studies, LYS228 demonstrated efficacy *in vivo*, in a neutropenic murine thigh infection model, against *E. coli* and *K. pneumoniae*, including strains expressing KPC or NDM carbapenemases (26); the PK/pharmacodynamic driver for LYS228 efficacy in this model of infection was the time that the unbound drug concentration exceeded the MIC (27). Together, these *in vitro* and *in vivo* findings support the continued development of this new agent for the treatment of serious infections due to ESBL-producing and carbapenem-resistant *Enterobacteriaceae* strains. LYS228 is currently under evaluation in phase 2 studies among patients with complicated urinary tract infections (ClinicalTrials registration no. NCT03377426) and patients with complicated intra-abdominal infections (ClinicalTrials registration no. NCT03354754).

MATERIALS AND METHODS

Antimicrobial agents. LYS228 and avibactam were synthesized at Novartis. Aztreonam, ceftazidime, cefepime, meropenem, and tigecycline were obtained from commercial sources.

Bacterial strains. A total of 271 *Enterobacteriaceae* clinical isolates from the Novartis collection, which had been obtained from various geographic locations, were used in these studies. The isolates were acquired between 2000 and 2016 and included the following bacterial species: *Citrobacter freundii*, *C. koseri*, *Enterobacter aerogenes*, *E. cloacae*, *E. hormaechei*, *Escherichia coli*, *Klebsiella oxytoca*, *K. pneumoniae*, *Morganella morganii*, *Proteus mirabilis*, *Serratia marcescens*, and *Salmonella* spp. The *E. coli* ATCC 25922, *K. pneumoniae* ATCC 700603, and ATCC 43816 reference strains used in this study were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). *E. coli* BW25113 was obtained from the Keio collection (Institute for Advanced Biosciences, Keio University, Tsuruoka City, Yamagata, Japan).

Antibiotic susceptibility testing. Susceptibility testing was performed using a broth microdilution assay, following the recommended CLSI methodology (28). The MIC_{50} and MIC_{90} were defined as the MIC values at which 50% and 90% of the isolates, respectively, were inhibited.

PCR and sequencing analysis of the *ftsI* gene encoding *E. coli* PBP3. Oligonucleotide primers (Table 5) were synthesized by IDT (Coralville, IA). The *ftsI* gene, which encodes *E. coli* PBP3, was PCR amplified and sequenced from 2 clinical isolates (NB27307 and NB27326; LYS228 MICs, 16 μg/ml), *E. coli* ATCC 25922 (LYS228 MIC, 0.25 μg/ml), and *E. coli* BW25113. PCR amplification was conducted in several sections using Phusion high-fidelity polymerase, according to the supplied protocol. DNA sequencing was performed by ELIM Biopharmaceuticals, Inc. (Hayward, CA). The sequencing results were analyzed for mutations using Vector NTI 11.5.1 (Thermo-Fisher, Waltham, MA) and Sequencher 5.0 (GeneCodes, Ann Arbor, MI), according to the manufacturer's instructions, by comparison to *E. coli* MG1655 *ftsI* reference sequences obtained from the EcoGene database (ecogene.org), as well as gene sequences obtained for *E. coli* ATCC 25922.

Bactericidal activity. For MBC determinations, 2 μl was removed from each well and aseptically plated on a tryptic soy agar plate. The plates were incubated at 35°C in ambient air for 24 h. After incubation, the number of colonies that grew from each well was counted and recorded. The MBC was defined as the lowest concentration of drug that resulted in $\geq 99.9\%$ reductions (≥ 3 $\log_{10}$ units) in titers from the original inoculum. The MBC_{50} and MBC_{90} were defined as the MBCs at which 50% and 90% of the isolates, respectively, were killed.

Time-kill experiments were performed against 10 strains of *E. coli* and *K. pneumoniae* with various β-lactamases in cation-adjusted Mueller-Hinton broth, following CLSI methodology (29). Antibiotics were added to the culture medium at concentrations equivalent to multiples of the MIC for each organism tested. Tubes were inoculated with early log-phase cultures of bacteria, which were diluted to yield a

TABLE 4 Kill kinetics of LYS228 and comparators against *E. coli* and *K. pneumoniae* strains expressing various β-lactamases

Organism (β-lactamase) and test agent	MIC (µg/ml)	Concentration tested (µg/ml)	Δlog₁₀ CFU/ml at:			
			2 h	4 h	8 h	24 h
E. coli ATCC 25922						
LYS228	0.25	0.5	−2.0	−4.6	−4.1	2.0
		1	−2.2	−4.6	−4.6	−4.6
		2	−2.1	−4.6	−4.6	−4.6
Aztreonam	0.12	0.25	−2.1	−3.8	−3.8	−4.4
		1	−1.7	−4.1	−4.4	−4.4
Meropenem	0.03	0.06	−1.6	−3.8	−4.4	−4.4
		0.25	−3.6	−4.1	−4.4	−4.4
E. coli ATCC 35218 (TEM-1)						
LYS228	0.06	0.12	−2.8	−3.8	−4.6	−4.9
		0.25	−2.8	−4.1	−4.9	−4.9
		0.5	−2.9	−3.9	−4.1	−4.9
Meropenem	0.03	0.06	−2.2	−2.9	−4.1	−4.1
		0.25	−2.5	−3.0	−4.1	−4.1
E. coli NB27235 (CTX-M-15)						
LYS228	0.25	0.5	−3.0	−3.1	−4.1	−4.8
		1	−3.1	−3.2	−4.2	−4.5
		2	−3.5	−3.4	−4.2	−4.8
Meropenem	0.03	0.06	−3.0	−3.6	−1.7	3.7
		0.25	−3.3	−4.1	−4.8	−4.8
E. coli NB27169 (KPC-3)						
LYS228	0.5	1	−0.2	−2.1	−2.6	−4.2
		2	−1.0	−2.2	−2.9	−4.9
		4	−1.2	−2.0	−2.9	−4.3
E. coli NB27236 (NDM-1)						
LYS228	4	8	−4.4	−4.4	−4.4	−2.1
		16	−2.9	−4.1	−4.4	−4.4
		32	−4.4	−3.9	−4.4	−4.4
K. pneumoniae ATCC 43816						
LYS228	0.06	0.12	−0.7	−1.9	−0.9	2.7
		0.25	−1.0	−1.7	−3.2	−4.6
		0.5	−1.1	−2.1	−3.3	−4.6
Aztreonam	0.06	0.12	−0.6	−2.0	−0.5	0.7
		0.5	−0.3	−2.1	−3.3	−4.6
Meropenem	0.06	0.12	−2.3	−4.9	−4.9	−4.9
		0.5	−3.0	−4.9	−4.9	−4.9
K. pneumoniae NB29254 (SHV-1)						
LYS228	0.12	0.25	−1.7	−2.6	−4.9	−1.4
		0.5	−1.9	−2.9	−4.9	−4.9
		1	−1.7	−2.7	−4.9	−4.9
Meropenem	0.03	0.06	−2.3	−3.4	−4.9	−1.8
		0.25	−2.8	−3.7	−4.9	−4.9
K. pneumoniae NB29084 (CTX-M-14)						
LYS228	0.5	1	−0.3	−1.5	−2.7	−4.9
		2	−0.9	−2.0	−3.2	−4.9
		4	−0.8	−2.4	−3.7	−4.9
Meropenem	0.03	0.06	−1.4	−2.8	−0.7	4.0
		0.25	−3.2	−3.5	−1.6	3.9
K. pneumoniae NB29082 (KPC-2)						
LYS228	0.5	1	−1.4	−2.4	−4.5	−5.0
		2	−1.6	−2.5	−4.2	−5.0
		4	−1.6	−2.8	−4.1	−5.0
K. pneumoniae ATCC 1100975 (NDM-1)						
LYS228	1	2	−2.0	−1.9	−3.8	−5.0
		4	−1.9	−2.9	−4.1	−5.0
		8	−2.0	−1.6	−4.5	−5.0

TABLE 5 Primers and primer pairs used in this study

Primer or primer pair	Sequence (5′ to 3′)	PCR product size (bp)
ftsI (PBP3) primer		
SR170	GCATGTTGATCCGTCACAAG	
SR171	ATGGCTAACAGCCCGTCATA	
SR172	ACGGCTGTCGAGTGTCATCT	
SR173	TAACGCCAGCTTGGAAACAC	
SR174	CGAGACTCTTCACGCAGATG	
SR175	TTATCGCCCACTGTCGATTAC	
Primer pair		
SR170-SR174		555
SR170-SR173		1,165
SR175-SR172		524
SR171-SR172		1,021
SR170-SR172		1,901

final cell density of 1×10^6 CFU/ml. The samples taken at that time constituted the 0-h time point. Cultures were then incubated at 35°C in ambient air for 24 h, with constant agitation using an orbital shaker (Innova 43; New Brunswick Scientific, Enfield, CT), and were sampled at various times. Prior to each sampling, tubes were mixed carefully. Viable cell counts were determined by performing 10-fold serial dilutions in sterile saline; 0.1 ml of undiluted sample and diluted samples was applied directly to the Mueller-Hinton agar using sterile glass beads. Colonies were counted after 24 h of incubation at 35°C in ambient air.

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