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SUPPLEMENTARY INFORMATION

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A broad-spectrum antibiotic adjuvant reverses multidrug-resistant Gram-negative pathogens

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Tables

Supplementary Table 1 Strains of bacteria used in this study.

Strains	Source/Reference
Gram-positive bacteria	
Bacillus subtilis	ATCC 6051
Enterococcus faecalis VRE A4	1
Staphylococcus aureus	ATCC 29213
S. aureus 215 (LZD^R + *cfr*)	1
MRSA T144	1
Gram-negative bacteria	
Acinetobacter baumannii 2-38 (MCR-1)	In this study
Aeromonas veronii 172 (MCR-3)	2
Citrobacter freumdii 2-4 (MCR-1)	In this study
Escherichia coli	ATCC 25922
E. coli C3 (NDM-1)	In this study
E. coli G6 (NDM-5)	In this study
$^{\#}$*E. coli* B2 (NDM-5 + MCR-1)	In this study
E. coli 3CZ20E (MCR-1 + FLO^R)	In this study
E. coli I1 (NDM-5 + MCR-1)	In this study
E. coli L14 (ESBL + MCR-1)	In this study
E. coli F9 (ESBL + MCR-1)	In this study
E. coli 267 (MCR-1)	In this study
E. coli 1347 (MCR-1)	In this study
E. coli DH5α (pUC19)	3
E. coli DH5α (pUC19-*mcr-1*)	3
*Enterobacter cloacae*16-15 (MCR-1)	In this study
Klebsiella pneumoniae 16-80(MCR-1)	In this study
K. pneumoniae WNX2	In this study
K. pneumoniae 4-20 (MCR-1)	In this study
K. pneumoniae 16-87 (MCR-1)	In this study
K. pneumoniae 16-63 (MCR-1)	In this study
Pseudomonas aeruginosa PAO1	ATCC 47085
Raoultella ornithinolytica 16-68 (MCR-1)	In this study
Raoultella planticola 16-15 (MCR-1)	In this study
Serratia marcescens 16-99 (MCR-1)	In this study
Salmonella enterica	ATCC 13076
S. enteritidis SH30 (MCR-1)	In this study

ATCC, American Type Culture Collection; VRE, vancomycin-resistant *Enterococcus*; *cfr,* florfenicol/chloramphenicol resistance gene; MRSA, methicillin-resistant *Staphylococcus aureus*; LZD^R, linezolid resistance. FLO^R, fluoroquinolone resistance. MCR, mobile colistin resistance.

Supplementary Table 2 All antimicrobial resistance genes in *E. coli* B2.

	Gene	Phenotype
Chromosome (4667 kb)	*mdfA*	Cationic or zwitterionic lipophilic compounds such as ethidium bromide, tetraphenylphosphonium, rhodamine, rifampin, tetracycline and puromycin
	tetA	Tetracyclines
Plasmid 1 (224 kb)	*mcr-1*	Colistin
	sul2	Sulfonamides
	flo^R	Florfenicol
	$bla_{\text{CTX-M-14}}$	β-lactams, especially cefotaxim
	fosA3	Fosfomycin
	ahp(4)-Ia	Hygromycin B
	aac(3)-IV	Aminoglycosides
	aac(6')-Ib3	Aminoglycosides
Plasmid 2 (133 kb)	*dfrA14*	Trimethoprim
	dfrA17	Trimethoprim
	sul2	Sulfonamides
	flo^R	Florfenicol
	oqxA	Quinolones
	oqxB	Quinolones
	$bla_{\text{OXA-10}}$	Carbapenems
	aadA1	Aminoglycosides
	aadA5	Aminoglycosides
	arr-2	Rifampin
	cmlA1	Chloramphenicol
Plasmid 3 (44 kb)	$bla_{\text{NDM-5}}$	Carbapenems
Plasmid 4 (20 kb)	$bla_{\text{TEM-1B}}$	Penicillins
	tetA	Tetracyclines
	*tetA**	Tetracyclines
Plasmid 5 (7 kb)	/	/

* another copy of *tetA* in plasmid 4

Supplementary Table 3 Antibacterial activities of SLAP-S25 against different bacteria.

Strains	SLAP-S25 (μg/mL)
Bacillus subtilis 6051	8
Staphylococcus aureus ATCC 29213	64
S. aureus 215 (LZD^R + *cfr*)	8
MRSA T144	64
Enterococcus faecalis VRE A4	8
Escherichia coli ATCC 25922	12
E. coli C3 (NDM-1)	12
E. coli G6 (NDM-5)	12
E. coli B2 (NDM-5 + MCR-1)	12
E. coli 3CZ20E (MCR-1 + FLO^R)	12
E. coli I1 (NDM-5 + MCR-1)	12
E. coli L14 (ESBL + MCR-1)	12
E. coli F9 (ESBL + MCR-1)	12
E. coli 267 (MCR-1*)*	12
E. coli 1347 (MCR-1*)*	12
Salmonella enterica ATCC 13076	1
Pseudomonas aeruginosa PAO1	4
Klebsiella pneumoniae WNX2	16

Supplementary Table 4 Potency of SLAP-S25 combined with antibiotics against antibiotic-sensitive *E. coli.*

Antibiotics	MIC without S25 (μg/mL)	FIC index	MIC with S25[a] (μg/mL)	Potentiation (fold)[b]
E. coli 267				
Cefepime	0.0625	0.25	0.0313	2
Colistin	4	0.375	0.5	8
Ofloxacin	0.5	0.094	0.0625	8
Rifampicin	8	0.093	≤ 0.0625	≥ 128
Tetracycline	0.5	0.125	0.0625	8
Vancomycin	128	0.375	32	4
E. coli 1347				
Cefepime	0.0625	0.078	0.0156	4
Colistin	4	0.25	0.5	8
Ofloxacin	0.5	0.188	0.0625	8
Rifampicin	8	0.038	≤ 0.0625	≥ 128
Tetracycline	0.5	0.25	0.125	4
Vancomycin	128	0.25	8	16

MIC of different antibiotics ([a]) and degree of antibiotic potentiation ([b]) in the presence of 4 μg/mL SLAP-S25.

Supplementary Table 5 Synergistic activity of pentamidine (PTM) with rifampicin or colistin against *E. coli* B2.

Antibiotics	MIC without PTM (μg/mL)	FIC index	MIC with PTM[a] (μg/mL)	Potentiation (fold)[b]
Rifampicin	128	0.156	4	32
Colistin	8	2	8	None

MIC of different antibiotics ([a]) and degree of antibiotic potentiation ([b]) in the presence of 100 μg/mL pentamidine.

Supplementary Table 6 FIC indices of colistin and SLAP-S25 against different bacterial species (MIC, μg/mL) harboring colistin resistant gene *mcr*.

Strains	Colistin		SLAP-S25		FIC index
	MIC	FICc	MIC	FICs	
Acinetobacter baumannii 2-38	8	0.008	4	0.016	0.024
Aeromonas veronii 172	2	0.125	4	0.250	0.375
Citrobacter freumdii 2-4	8	0.031	2	0.063	0.094
Escherichia coli B2	8	0.031	12	0.016	0.047
*Enterobacter cloacae*16-15	8	0.125	4	0.125	0.250
Klebsiella pneumoniae 16-80	16	1.000	2	0.500	1.500
*Providencia alcalifaciens*16-1	16	0.001	16	0.001	0.002
Raoultella ornithinolytica 16-68	4	0.063	4	0.008	0.071
Raoultella planticola 16-15	16	0.031	8	0.015	0.046
Serratia marcescens 16-99	8	0.008	8	0.063	0.071
Salmonella enteritidis SH30	16	0.016	4	0.063	0.079

Fractional inhibitory concentration (FIC) indices were calculated based on chequerboard broth microdilution assays. FIC index is the sum of FICc and FICs. FICc is the FIC of colistin. FICs is the FIC of SLAP-S25. All strains carry plasmid mediated *mcr-1* genes, except *Aeromonas veronii* 172, which has *mcr-3* in chromosome.

Supplementary Table 7 SLAP-S25 potentiates the antibacterial activity of rifampicin, ofloxacin and tetracycline against *Klebsiella pneumoniae*.

Strains	FIC index		
	Ofloxacin	Rifampicin	Tetracycline
K. pneumoniae WNX2	0.012	0.017	0.034
K. pneumoniae 16-63 (*mcr-1*)	0.0059	0.064	0.129
K. pneumoniae 4-20 (*mcr*)	0.127	0.033	0.065
K. pneumoniae 91 (*mcr*)	0.010	0.006	0.017

Different *K. pneumoniae* strains were treated with SLAP-S25 in the presence of ofloxacin, or rifampicin or tetracycline. *K. pneumoniae* WNX2 is sensitive to colistin (MIC $\leq$ 2 μg/mL), while the other strains are resistant to colistin (MIC $\geq$ 8 μg/mL).

Supplementary Table 8 Effects of additional LPS, Mg^{2+}, PG and NAC on the antibacterial activity of antibiotics and SLAP-S25 against *E. coli*.

Antibiotics	MIC (μg/mL)				
	MHB	+ LPS	+ PG	+ Mg^{2+}	+NAC
SLAP-S25	16	64	32	64	32
Cefepime	0.03	0.03	0.03	0.03	/
Colistin	0.25	2	16	8	/
Ofloxacin	0.03	0.03	0.03	0.5	/
Rifampicin	8	8	8	8	/
Tetracycline	2	2	4	32	/
Vancomycin	2	2	2	2	/

The final concentrations of additional LPS, PG, Mg^{2+} and NAC in MHB broth were 128 μg/mL, 16 μg/mL, 480 μg/mL and 6 mmol/L, respectively. *E. coli* ATCC 25922 was tested for antibacterial activities.

Supplementary Table 9 Exogenous LPS abolished the synergy between SLAP-S25 and non-LPS targeting antibiotics.

Antibiotics	MIC[a] (μg/mL)	FIC index[a]	MIC[b] (μg/mL)	FIC index[b]
Cefepime	1	0.156	32	>0.5
Vancomycin	8	0.094	128	>0.5

MICs of antibiotics or FIC index in the absence ([a]) and presence ([b]) of LPS with 4 μg/mL SLAP-S25. The LPS of *E. coli* O111:B4 was purchased from Sigma, and 128 μg/mL LPS was added.

Supplementary Table 10 Exogenous Mg^{2+} counteracted the synergistic activity between SLAP-25 and non-LPS targeting antibiotics.

Antibiotics	MIC[a] (μg/mL)	FIC index[a]	MIC[b] (μg/mL)	FIC index[b]
Cefepime	1	0.156	32	>0.5
Vancomycin	8	0.094	128	>0.5

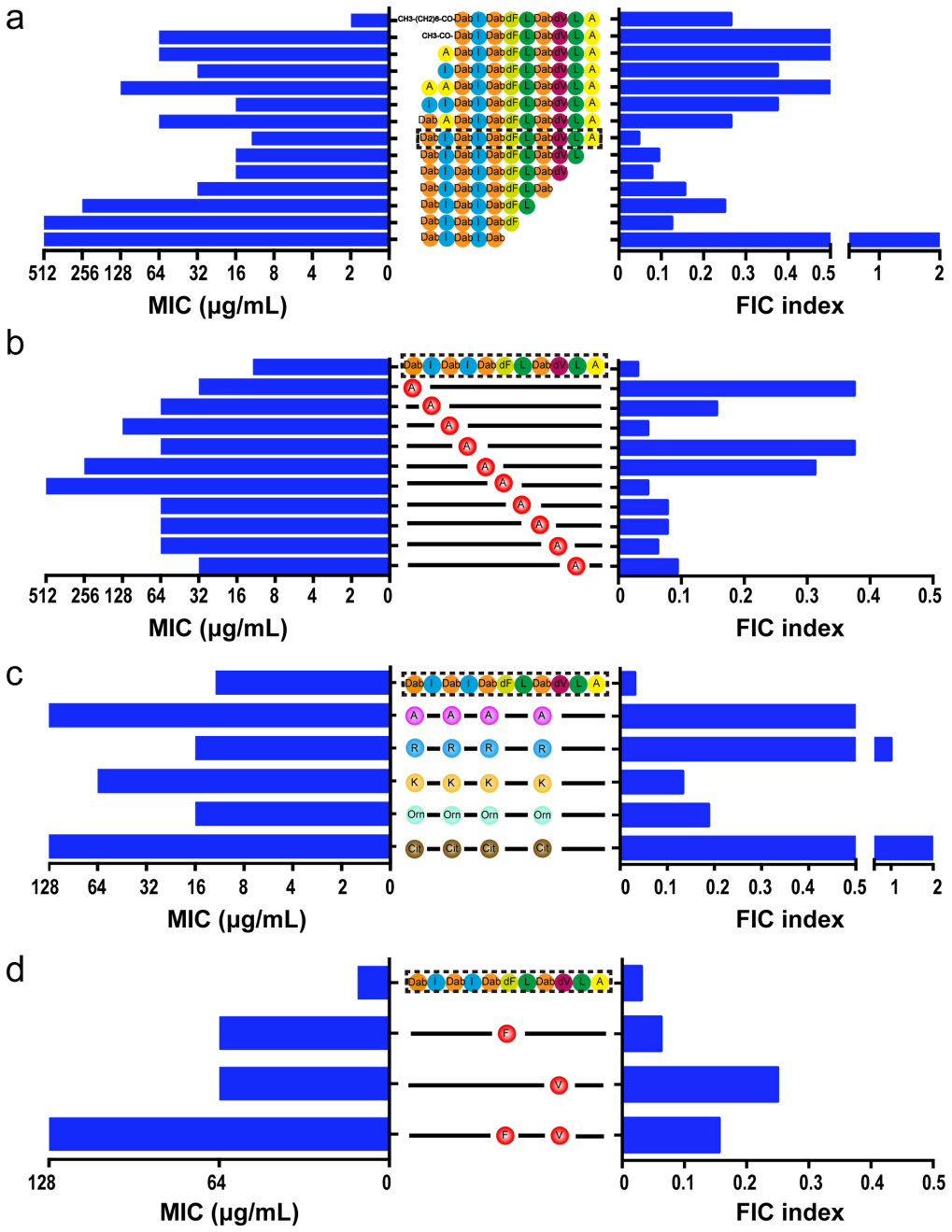
MICs of antibiotics or FIC index in the absence ([a]) and presence ([b]) of Mg^{2+} (480 μg/mL) with 4 μg/mL SLAP-S25.

Supplementary Table 11 MRM parameters for the determination of antibiotics by LC-MS/MS.

Compound	Precursor ion (*m/z*)	Product ions (*m/z*)	Q1 pre bias (V)	Collision energy (eV)	Q3 pre bias (V)
Cefepime	481.05	396.00*	-18	-14	-28
		166.90	-14	-24	-18
Tetracycline	445.25	410.15*	-22	-20	-22
		428.10	-22	-20	-23
Vancomycin	725.40	100.10*	-26	-44	-21
		144.20	-26	-16	-29
Ofloxacin	362.20	318.20*	-25	-18	-24
		261.10	-26	-29	-28
Rifampicin	823.40	151.20*	-24	-19	-30
		791.45	-24	-36	-30
Colistin	585.35	227.20*	-28	-35	-26
		228.25	-28	-36	-28

*, quantitative ion.

Figures



Supplementary Figure 1 Rationally engineered and structure-activity relationship of SLAP-S25.

Antibacterial activity (MIC, μg/mL) and synergistic activity with colistin (FIC index) of SLAP-S25 and its substitutions were determined, including modification or deletion from *N*- or *C*-terminal (**a**), alanine screening library (**b**); Replacement of Dab in SLAP-S25 with Ala (A), Arg (R), Lys (K), Orn and Cit (**c**); Conformation changes of *D*-Phe and/or *D*-Val (**d**); Synergy is defined as an FIC index of ≤ 0.5. Data were representative of two biological replicates (n = 2).

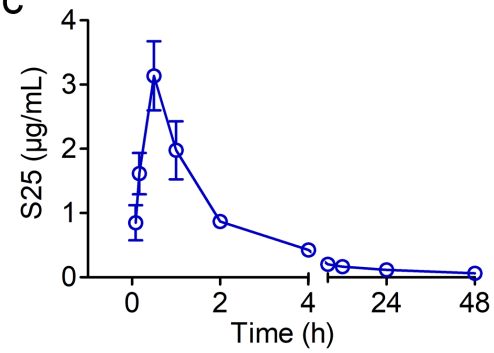
a

Treatments	Colistin		SLAP-S25		FIC index
	MIC	FICc	MIC	FICs	
E. coli B2	8	0.031	12	0.016	0.047
E. coli B2 + 50% fetal bovine serum	16	0.125	16	0.031	0.156
E. coli B2 + 50% rabbit serum	8	0.031	12	0.031	0.062

b

Proteases (20 mg/mL)	Activity sites	SLAP-S25 (μg/mL)
PBS	—	12
Trypsin	Arg, Lys	12
Pepsin	Tyr, Phe, Trp	12
Papain	Arg, Lys, Gly	16

c

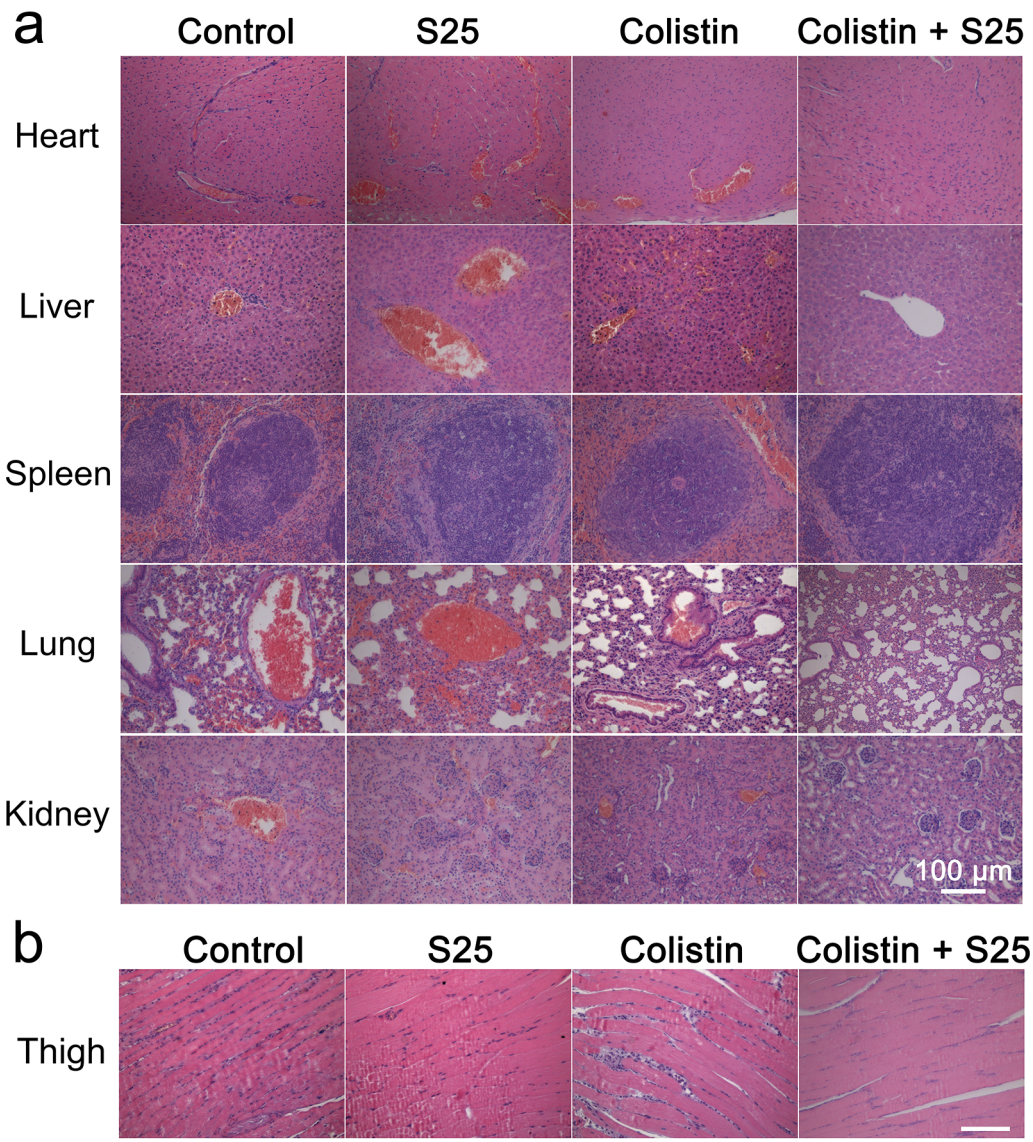


d

Parameters	Mean ± SD
C_{max} (μg/mL)	3.14±1.32
T_{max} (hours)	0.5
$t_{1/2}$ (hours)	1.55±0.26
AUC (μg-hour/mL)	10.71±3.20
CL (L/hour/kg)	1.01±0.29
V_{ss} (L/kg)	2.17±0.40

Supplementary Figure 2 Serum stability, proteolytic stability and pharmacokinetic analysis of SLAP-S25.

(**a**) SLAP-S25 retained its antibacterial activity and synergy effect with colistin against *E. coli* B2 in the presence of either 50% fetal bovine serum or 50% rabbit serum. (**b**) Proteolytic stability of SLAP-S25 on the antibacterial activity of *E. coli* B2 (MIC, μg/mL). (**c**) Mean plasma concentrations of SLAP-S25 after a single intraperitoneal injection of 8 mg/kg SLAP-S25 during 48 h. All data were presented as means ± SD (n = 6). (**d**) Pharmacokinetic parameters of SLAP-S25 calculated with a non-compartmental analysis model based on WinNonlin 6.4 (n = 6). C_{max}, maximal plasma concentration; T_{max}: time to maximal plasma concentration; $t_{1/2}$, half-life; AUC, area under the concentration curve; CL, total plasma clearance; V_{ss}, volume of distribution.



Supplementary Figure 3 Histologic analysis of the tissues in two mice infection models using hematoxylin-eosin (H&E) staining.

Histopathological changes of different organs (**a**) in the mouse peritonitis-sepsis model and of muscles (**b**) in the thigh infection model treated with PBS (control), SLAP-S25 (8 mg/kg), colistin (8 mg/kg) or combination used (8 mg/kg + 8 mg/kg). Magnification, × 400. The results were representative of three biological repeats.

References

1. Liu, Y., Ding, S., Dietrich, R., Märtlbauer, E. & Zhu, K. A biosurfactant-inspired heptapeptide with improved specificity to kill MRSA. *Angew. Chem. Int. Ed.* **56**, 1486-1490 (2017).
2. Ling Z. *et al.* Chromosome-mediated *mcr-3* variants in *Aeromonas veronii* from chicken meat. *Antimicrob. Agents. Chemother.* **61**, e01272-01217 (2017).
3. Li H., et al. Molecular insights into functional differences between *mcr-3*- and *mcr-1*-mediated colistin resistance. *Antimicrob. Agents. Chemother*. **62**, e00366-18 (2018).