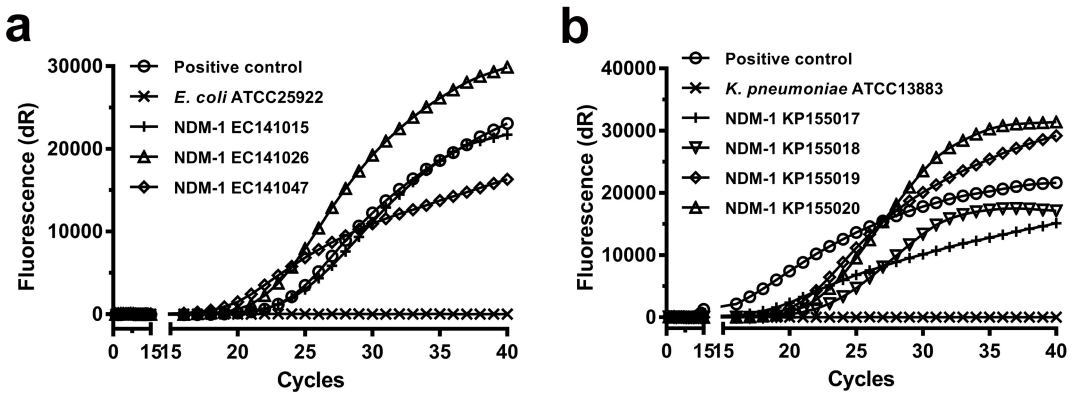
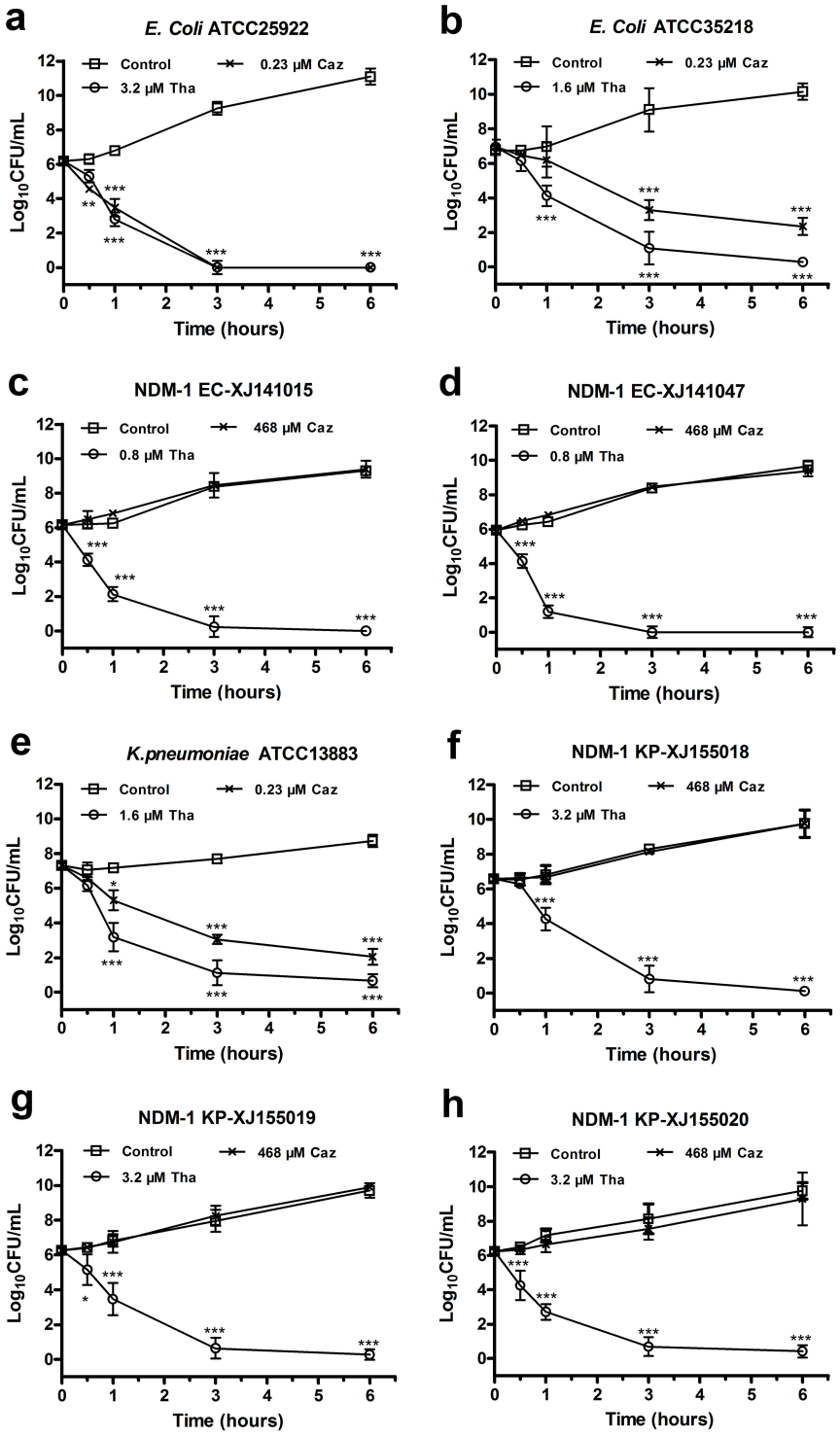
Supplementary information

The antimicrobial peptide thanatin disrupts the bacterial outer membrane and inactivates the NDM-1 metallo-β-lactamase

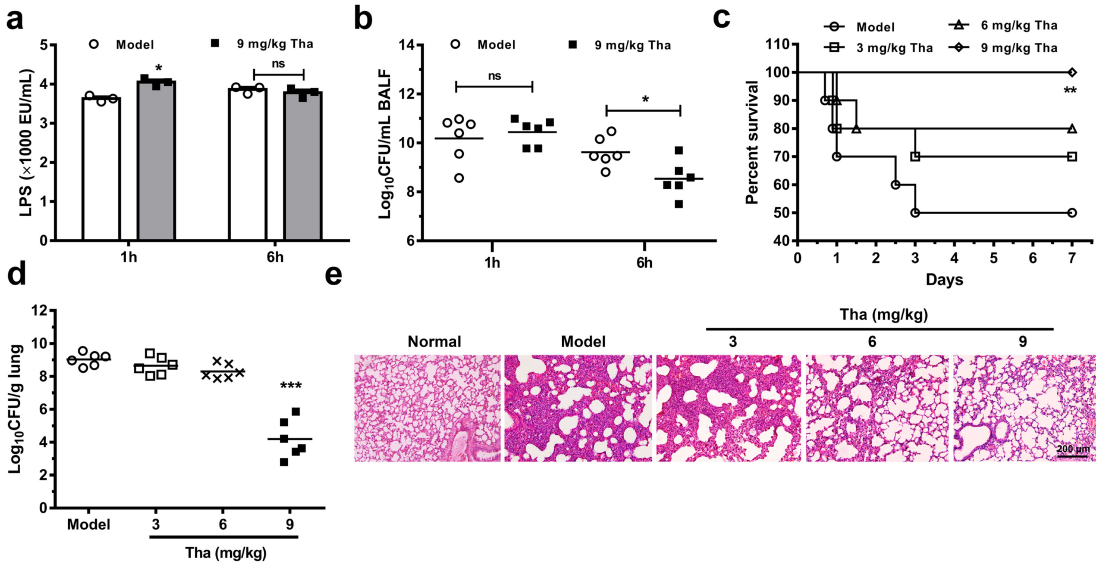
Ma et al.



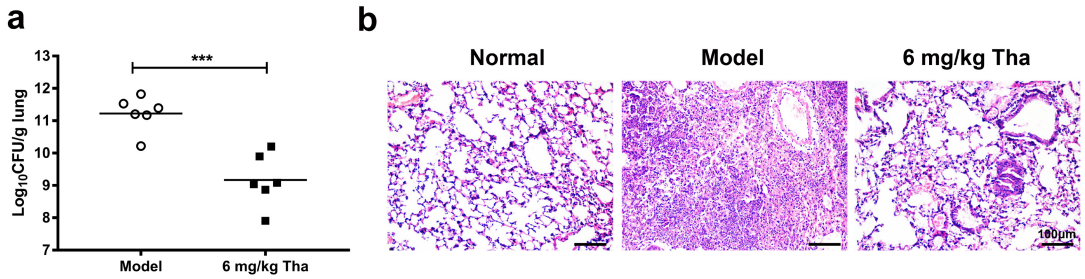
Supplementary Figure 1. Examination of *bla*$_{\text{NDM-1}}$ gene expression. The *bla*$_{\text{NDM-1}}$ gene was detected in clinical isolates of *E. coli* (**a**) and *K. pneumonia* (**b**), with *E. coli* ATCC25922 and *K. pneumonia* ATCC13883 as negative controls. Source data are provided in Source Data file.



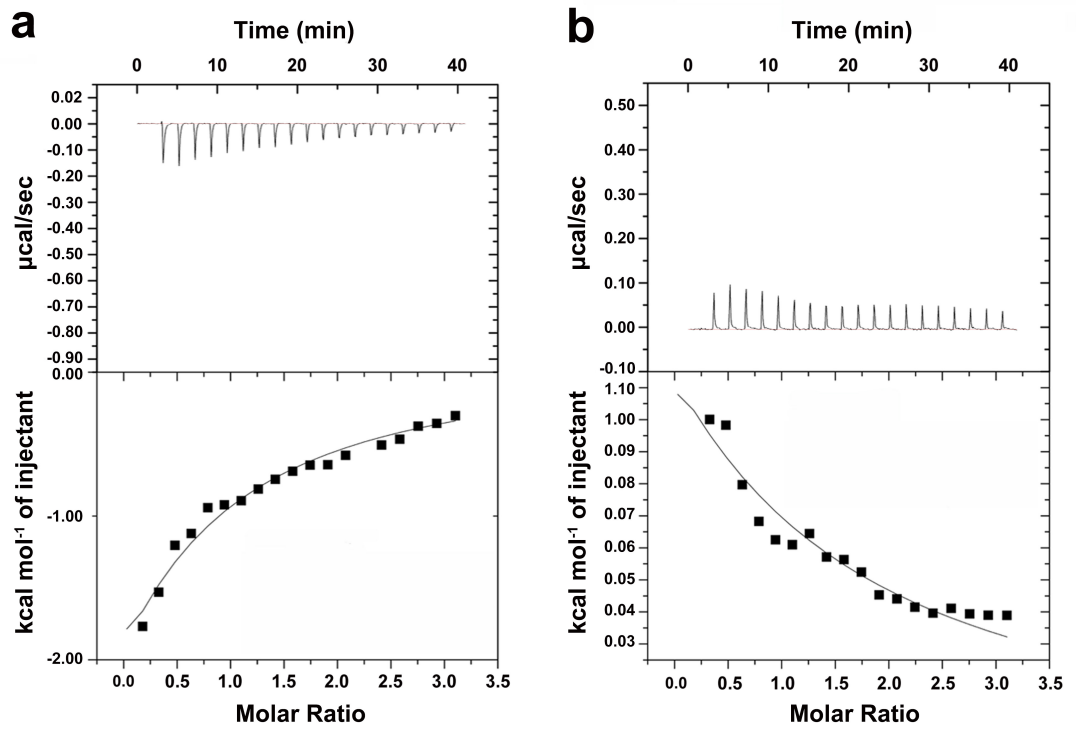
Supplementary Figure 2. Kill curves of thanatin and ceftazidime against clinical NDM-1 strains. NDM-1-producing *E. coli* (**a-d**) and *K. pneumoniae* (**e-h**) were treated with thanatin (Tha) and ceftazidime (Caz). Data are presented as the mean ± s.e.m. from three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus control (determined by two-way ANOVA with Bonferroni's comparison). Source data are provided in Source Data file.



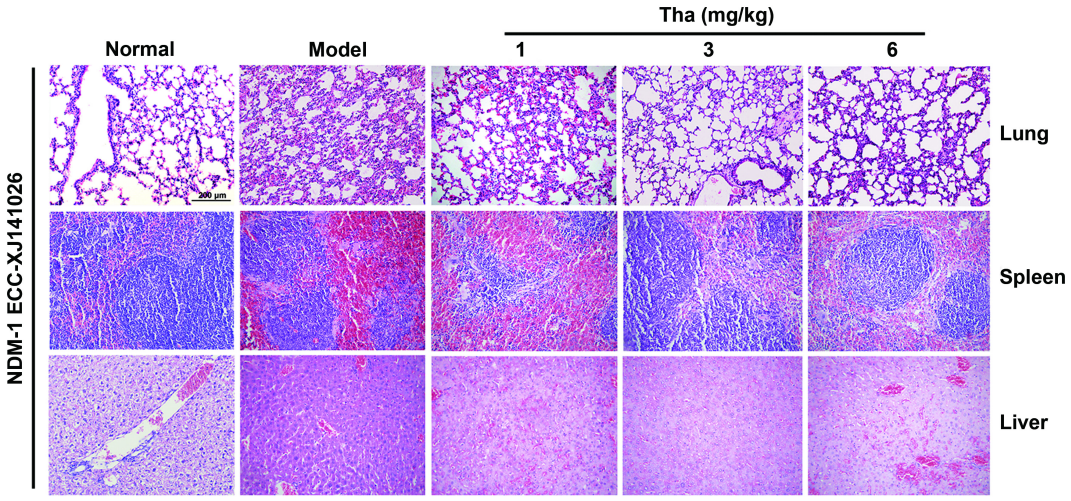
Supplementary Figure 3. Thanatin promotes the release of Ca^{2+} and LPS from the outer membrane of NDM-1-producing *K. pneumoniae* in vivo. a, b LPS levels (**a**) and bacterial loads (**b**) in the BALF of *K. pneumoniae* XJ155017-infected mice were analyzed at 1 and 6 h after 9 mg/kg thanatin treatment (n=6 per group). **c** Survival curves for the *K. pneumoniae* XJ155017 pneumonia model. BALB/c mice were intranasally administered with a sublethal dose of *K. pneumoniae* XJ155017 and treated with three doses of thanatin via intraperitoneal injection (n=10 per group). **d** Bacterial loads in the lungs of thanatin-treated *K. pneumoniae* XJ155017-infected mice were analyzed 24 h after infection (n=6 per group). **e** Lung morphology was examined with H&E staining in the pneumonia model after thanatin treatment. Scale bar = 200 μm. All data are shown as the mean ± s.e.m. *P* values were determined by two-way ANOVA (**a, b**), log-rank test (**c**), or one-way ANOVA with Bonferroni's comparison test (**d**). ns means not significant; **P* < 0.05, ***P* < 0.01, ****P* < 0.001 versus model. Source data are provided in Source Data file.



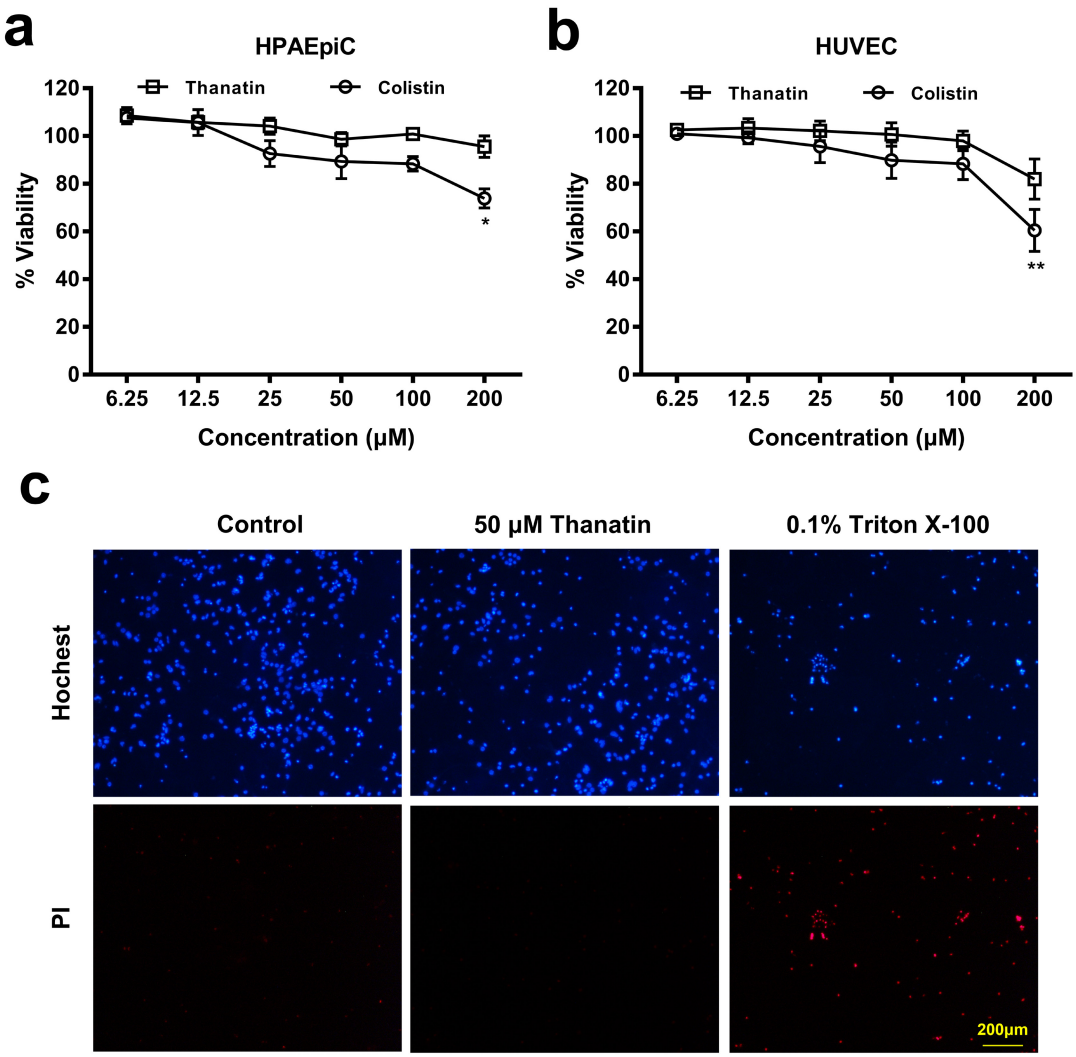
Supplementary Figure 4. Thanatin protects NDM-1-producing *E. coli*-infected pneumonia mice. a Bacterial loads in the lungs of thanatin-treated *E. coli* XJ141026-infected mice were analyzed 24 h after infection (n = 6 per group). Data are shown as the mean ± s.e.m. **b** Lung morphology was examined with H&E staining in the pneumonia model after thanatin treatment. Scale bar = 100 μm. *P* values were determined by two-tailed unpaired t test (**a**). ***$P < 0.001$ versus model. Source data are provided in Source Data file.



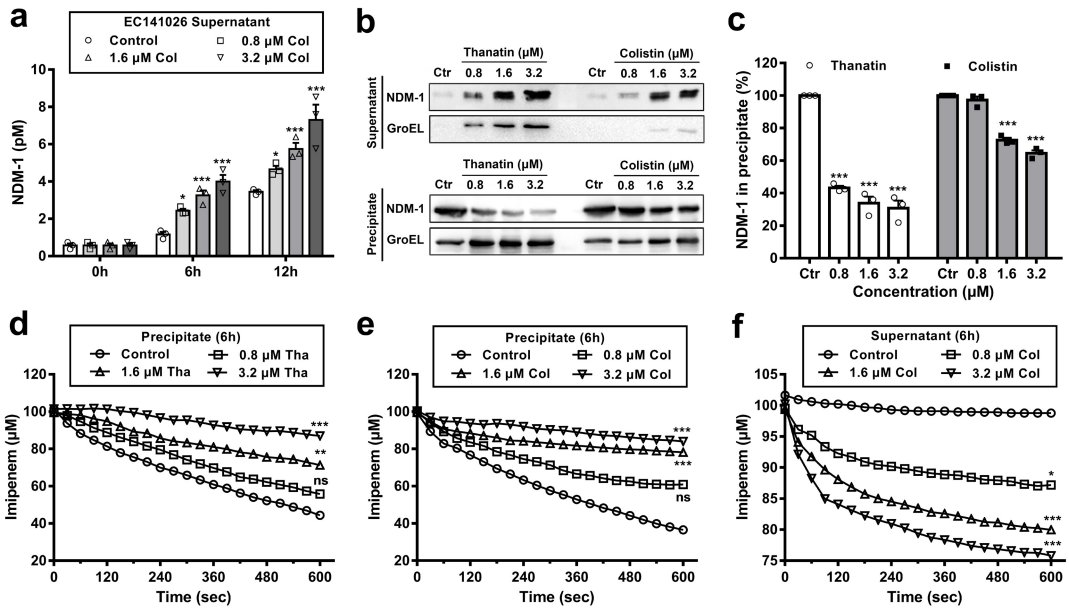
Supplementary Figure 5. ITC thermograms for the binding of LPS with Ca^{2+} (a), Mg^{2+} (b). The downward and upward peaks indicate the exothermic and endothermic process, respectively.



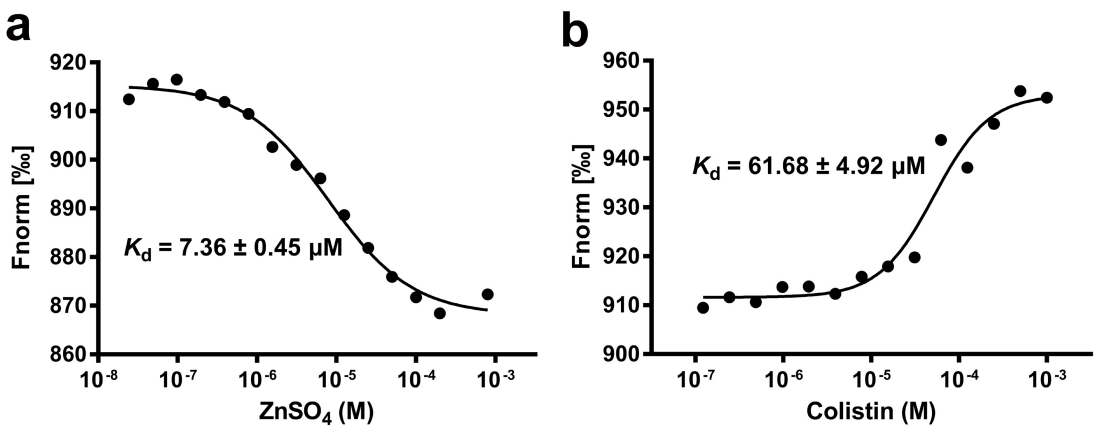
Supplementary Figure 6. H&E staining. Tissue morphology was examined with H&E staining in *E. coli* XJ141026 sepsis models after thanatin treatment. Scale bar = 200 μm.



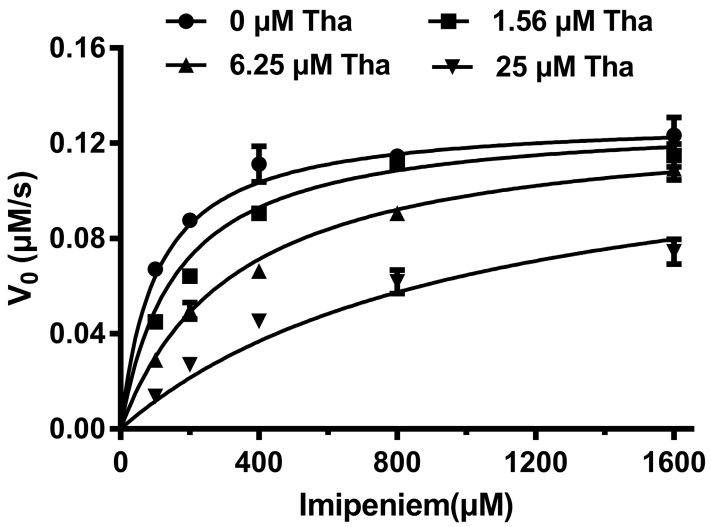
Supplementary Figure 7. Cytotoxicity of thanatin. a, b HPAEpiCs (**a**) and HUVECs (**b**) were treated with thanatin or colistin for 24 h, and cell viability was calculated on the basis of absorbance readings at 450 nm at 4 h after adding WST-8. **c** Representative photomicrographs of mouse neurons stained with Hoechst 33342 and PI fluorescent dye after exposure to 50 μM thanatin or 0.1% Triton X-100 for 24 h. Scale bar = 200 μm. Data are presented as the mean ± s.e.m. from at least three independent experiments. *$P < 0.05$, **$P < 0.01$ (determined by two-way ANOVA). Source data are provided in Source Data file.



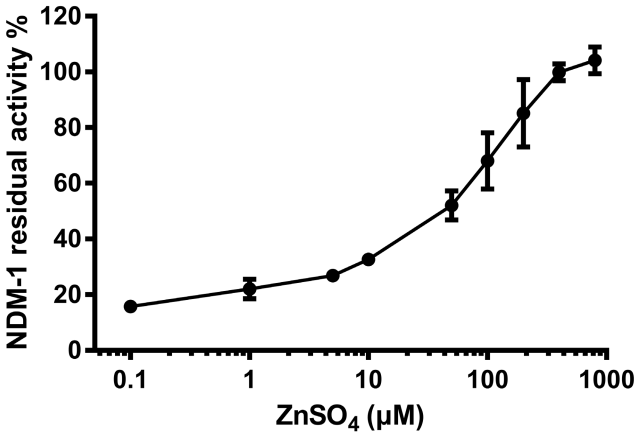
Supplementary Figure 8. NDM-1 protein levels and its hydrolytic activity to imipenem. a Increase in NDM-1 release to supernatants after treatment with colistin (Col). **b** Western blot analysis of NDM-1 in thanatin- and colistin-treated *E. coli* XJ141026 cell supernatants and precipitates for 6 h. GroEL is used as a loading control. **c** Relative expression of NDM-1 in thanatin- and colistin-treated *E. coli* XJ141026 cell precipitates for 6 h. **d** Hydrolytic effect of the cell precipitates obtained from thanatin-treated NDM-1 *E. coli* XJ141026 on imipenem. **e, f** Hydrolytic effect of the cell precipitates (**e**) and supernatants (**f**) obtained from colistin-treated NDM-1 *E. coli* XJ141026 on imipenem. The supernatants and cell precipitates were collected 6 h after incubation with thanatin or colistin. Data are presented as the mean ± s.e.m. from three independent experiments. *P* values were determined by two-way ANOVA (**a**, **d**–**f**), or by one-way ANOVA with Bonferroni's comparison test (**c**). ns means not significant; **P* < 0.05, ***P* < 0.01, ****P* < 0.001. Original western blot images for **b** are shown in **Supplementary Figure 13.** Source data are provided in Source Data file.



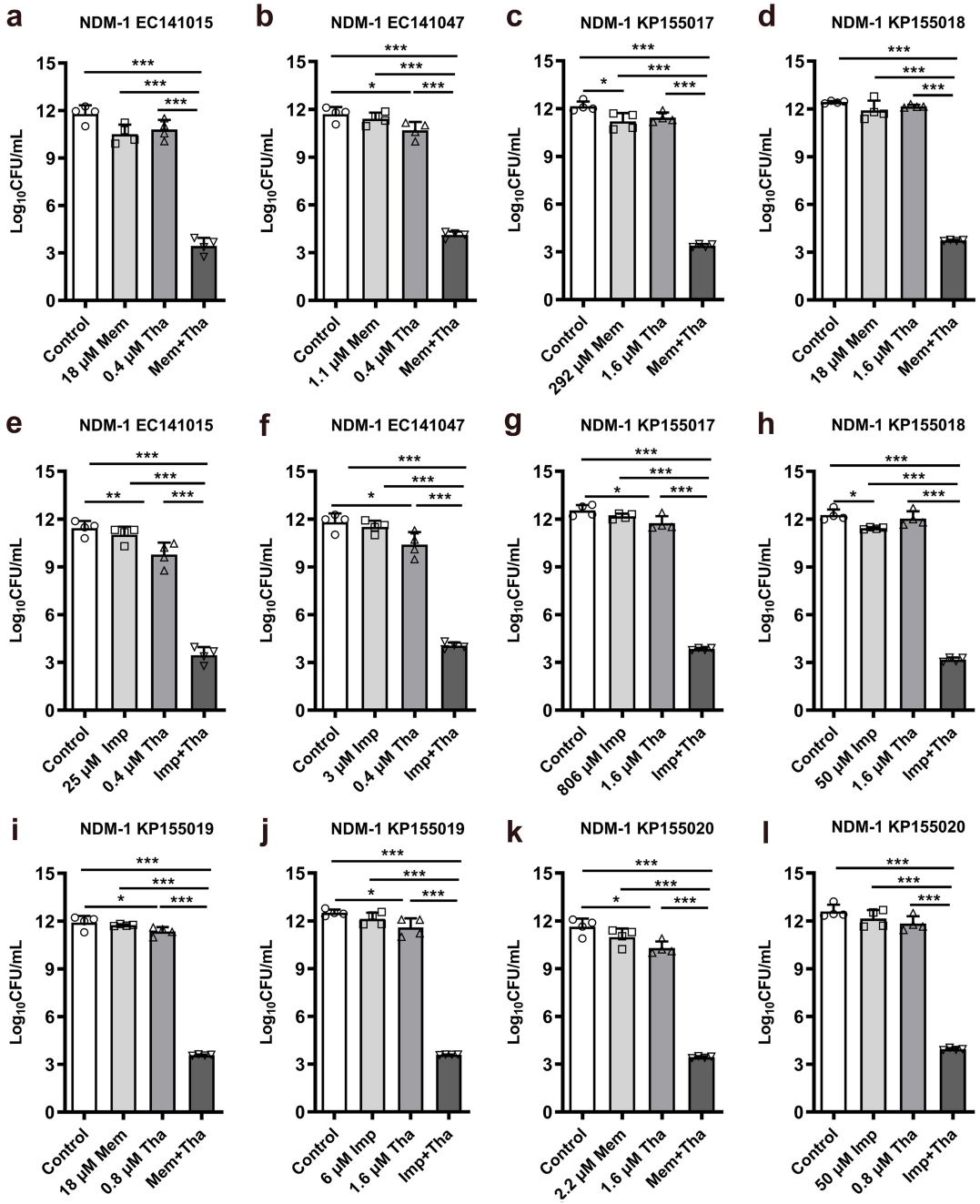
Supplementary Figure 9. MST assay. Interaction between RED-tris-NTA second-generation dye-labeled purified apo-NDM-1with $ZnSO_4$ (**a**) or colistin (**b**). Data are presented as the mean ± s.e.m. from three independent experiments. Source data are provided in Source Data file.



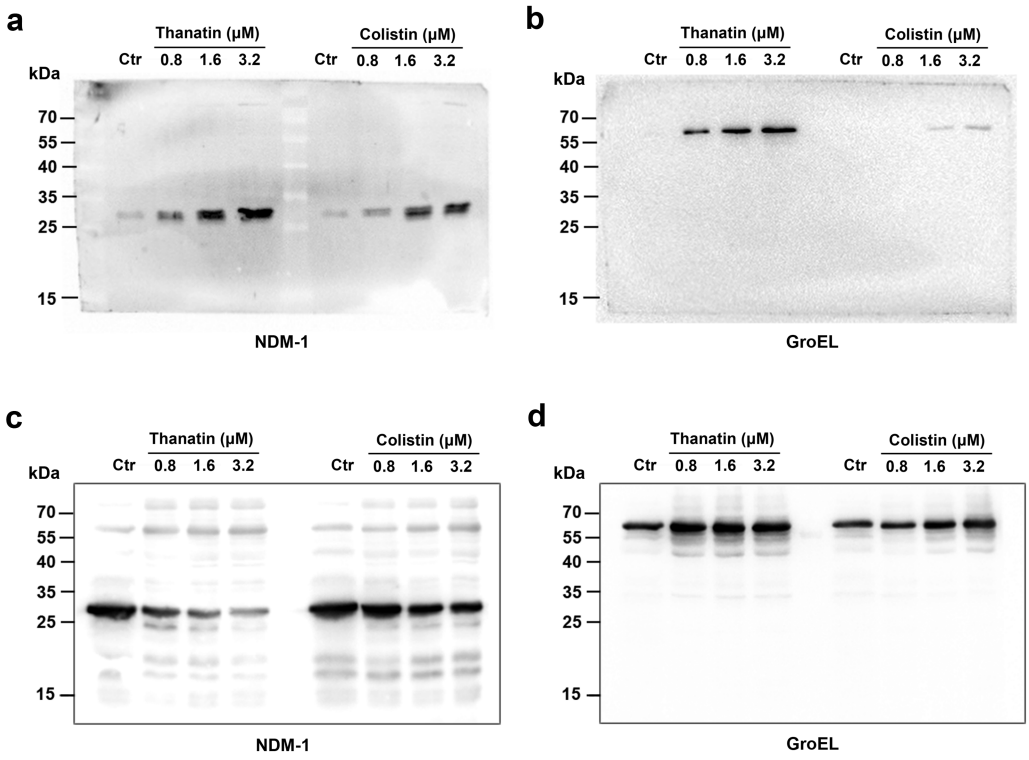
Supplementary Figure 10. In vitro inactivation of NDM-1 by thanatin. The initial rate of hydrolysis of imipenem (100, 200, 400, 800, and 1600 μM) by 1 nM NDM-1 was evaluated in the absence and presence of three concentrations of thanatin. The curves show the fits to the competitive inhibition model. Data are shown as the mean ± s.e.m. from three independent experiments. Source data are provided in Source Data file.



Supplementary Figure 11. Zn^{2+} restoration assays. NDM-1 activity inhibited by thanatin was gradually rescued with the increasing concentrations of Zn^{2+}. Data are shown as the mean ± s.e.m. from three independent experiments. Source data are provided in Source Data file.



Supplementary Figure 12. Sub-MICs of thanatin reverse carbapenem resistance in vitro. Sub-MICs of thanatin restored the activity of meropenem (**a-d, i, k**) and imipenem (**e-h, j, l**) against NDM-1-producing *E. coli* and *K. pneumonia*. All data are shown as the mean ± s.e.m. from at least three independent experiments. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (determined by one-way ANOVA with Bonferroni's comparison). Source data are provided in Source Data file.



Supplementary Figure 13. Original western blot images for Supplementary Figure 8b. a, b Western blot analysis of NDM-1 (**a**) and GroEL (**b**) in cell supernatants of thanatin- and colistin-treated *E. coli* XJ141026. **c, d** Western blot analysis of NDM-1 (**c**) and GroEL (**d**) in cell precipitates of thanatin- and colistin-treated *E. coli* XJ141026.

Supplementary Table 1. Identification and antibiotic susceptibility of NDM-1-producing *E. coli* strains.

Antibiotics		Strains							
		E. coli **ATCC25922**		**NDM-1 *E. coli*** **XJ141015**		**NDM-1 *E. coli*** **XJ141026**		**NDM-1 *E. coli*** **XJ141047**	
Imipenem	**MIC**	<=1	（S）	>8	（R）	>8	（R）	>8	（R）
Aztreonam	**MIC**	<=2	（S）	>16	（R）	>16	（R）	>16	（R）
Cefotaxime	**MIC**	<=1	（S）	>32	（R）	>32	（R）	>32	（R）
Ciprofloxacin	**MIC**	<=0.5	（S）	>2	（R）	>2	（R）	<=0.5	（S）
Ampicillin	**MIC**	<=4	（S）	>16	（R）	>16	（R）	>16	（R）
Meropenem	**MIC**	<=1	（S）	>8	（R）	>8	（R）	>8	（R）
Piperacillin	**MIC**	<=4	（S）	>64	（R）	>64	（R）	>64	（R）
Tetracycline	**MIC**	<=2	（S）	<=2	（S）	>8	（R）	<=2	（S）
Cefepime	**MIC**	<=2	（S）	>16	（R）	>16	（R）	>16	（R）
Moxifloxacin	**MIC**	<=1	（S）	>4	（R）	>4	（R）	<=1	（S）
Cotrimoxazole	**MIC**	<=0.5/9.5	（S）	<=0.5/9.5	（S）	>2/38	（R）	<=0.5/9.5	（S）
Aoxicillin/ clavulanic acid	**MIC**	8/4	（S）	>16/8	（R）	>16/8	（R）	>16/8	（R）
Chloramphenicol	**MIC**	<=4	（S）	>16	（R）	<=4	（S）	<=4	（S）
Gentamicin	**MIC**	<=2	（S）	<=2	（S）	>8	（R）	>8	（R）
Cefazolin	**MIC**	<=4	（S）	>16	（R）	>16	（R）	>16	（R）
Ampicillin/ sulbactam	**MIC**	<=4/2	（S）	>16/8	（R）	>16/8	（R）	>16/8	（R）
Piperacillin/ tazobactam	**MIC**	<=4/2	（S）	>64/4	（R）	>64/4	（R）	>64/4	（R）
Amikacin	**MIC**	<=8	（S）	<=8	（S）	<=8	（S）	<=8	（S）
Ceftazidime	**MIC**	<=1	（S）	>16	（R）	>16	（R）	>16	（R）
Levofloxacin	**MIC**	<=1	（S）	>8	（R）	>8	（R）	<=1	（S）
Cefoperazone/ sulbactam	**KB**	—	—	7	（R）	7	（R）	7	（R）
Nitrofurantoin	**KB**	—	—	14	（R）	17	（R）	19	（S）
Cefuroxime	**KB**	—	—	7	（R）	7	（R）	7	（R）

R, resistant; S, sensitive; MIC, minimum inhibitory concentration; KB, Kirby Bauer disc diffusion method. The unit of MIC is “μg/mL”. The unit of KB is “mm”.

Supplementary Table 2. Identification and antibiotic susceptibility of NDM-1-producing *K. pneumonia* strains.

Antibiotics		Strains									
		K. pneumoniae ATCC13883		NDM-1 *K. pneumoniae* XJ155017		NDM-1 *K. pneumoniae* XJ155018		NDM-1 *K. pneumoniae* XJ155019		NDM-1 *K. pneumoniae* XJ155020	
Imipenem	**MIC**	<=1	(S)	>8	(R)	>8	(R)	>8	(R)	>8	(R)
Aztreonam	**MIC**	<=2	(S)	>16	(R)	>16	(R)	>16	(R)	>16	(R)
Cefotaxime	**MIC**	<=1	(S)	>32	(R)	>32	(R)	>32	(R)	>32	(R)
Ciprofloxacin	**MIC**	<=0.5	(S)	>2	(R)	>2	(R)	>2	(R)	>2	(R)
Ampicillin	**MIC**	16	(R)	>16	(R)	>16	(R)	>16	(R)	>16	(R)
Meropenem	**MIC**	<=1	(S)	>8	(R)	>8	(R)	>8	(R)	>8	(R)
Piperacillin	**MIC**	8	(S)	>64	(R)	>64	(R)	>64	(R)	>64	(R)
Tetracycline	**MIC**	<=2	(S)	4	(S)	>8	(R)	>8	(R)	>8	(R)
Cefepime	**MIC**	<=2	(S)	>16	(R)	>16	(R)	>16	(R)	>16	(R)
Moxifloxacin	**MIC**	<=1	(S)	>4	(R)	>4	(R)	>4	(R)	>4	(R)
Cotrimoxazole	**MIC**	=0.5/9.5	(S)	>1/19	(S)	>2/38	(R)	>2/38	(R)	>2/38	(R)
Aoxicillin/ clavulanic acid	**MIC**	<=4/2	(S)	>16/8	(R)	>16/8	(R)	>16/8	(R)	>16/8	(R)
Chloramphenicol	**MIC**	<=4	(S)	>16	(R)	>16	(R)	>16	(R)	>16	(R)
Gentamicin	**MIC**	<=2	(S)	<=2	(S)	<=2	(S)	<=2	(S)	<=2	(S)
Cefazolin	**MIC**	<=4	(S)	>16	(R)	>16	(R)	>16	(R)	>16	(R)
Ampicillin/ sulbactam	**MIC**	8/4	(S)	>16/8	(R)	>16/8	(R)	>16/8	(R)	>16/8	(R)
Piperacillin/ tazobactam	**MIC**	<=4/4	(S)	>64/4	(R)	>64/4	(R)	>64/4	(R)	>64/4	(R)
Amikacin	**MIC**	<=8	(S)	<=8	(S)	<=8	(S)	<=8	(S)	<=8	(S)
Ceftazidime	**MIC**	<=1	(S)	>16	(R)	>16	(R)	>16	(R)	>16	(R)
Levofloxacin	**MIC**	<=1	(S)	——	-	——	-	>8	(R)	>8	(R)
Cefoperazone/ sulbactam	**KB**	32	(S)	7	(R)	7	(R)	7	(R)	7	(R)
Nitrofurantoin	**KB**	21	(S)	7	(R)	9	(R)	10	(R)	9	(R)
Cefuroxime	**KB**	23	(S)	7	(R)	7	(R)	7	(R)	7	(R)

R, resistant; S, sensitive; MIC, minimum inhibitory concentration; KB, Kirby Bauer disc diffusion method. The unit of MIC is “μg/mL”. The unit of KB is “mm”.

Supplementary Table 3. Thermodynamic parameters for the binding of LPS with thanatin, Ca^{2+}, and Mg^{2+} were estimated by ITC.

	K_d (μM)	△H (Cal/mol)	△S (Cal/mol/deg)
Thanatin + LPS	1.09 ± 0.11	-6.46×10^{3}	5.62
Ca^{2+} + LPS	114.24 ± 26.05	-9.42×10^{4}	-2.98×10^{2}
Mg^{2+} + LPS	208.46 ± 52.28	2.83×10^{6}	9.50×10^{3}

Supplementary Table 4. Kinetic parameters for imipenem hydrolysis catalyzed by NDM-1.

Kinetic parameters	Enzyme/substrate
	NDM-1/Imipenem
K_m (μM)	103.80 ±10.34
V_{max} (μM·s^{-1})	0.13 ± 0.03
K_{cat} (s^{-1})	130.40 ± 2.67