An antimicrobial peptide as a potential therapy for bacterial pneumonia that alleviates antimicrobial resistance

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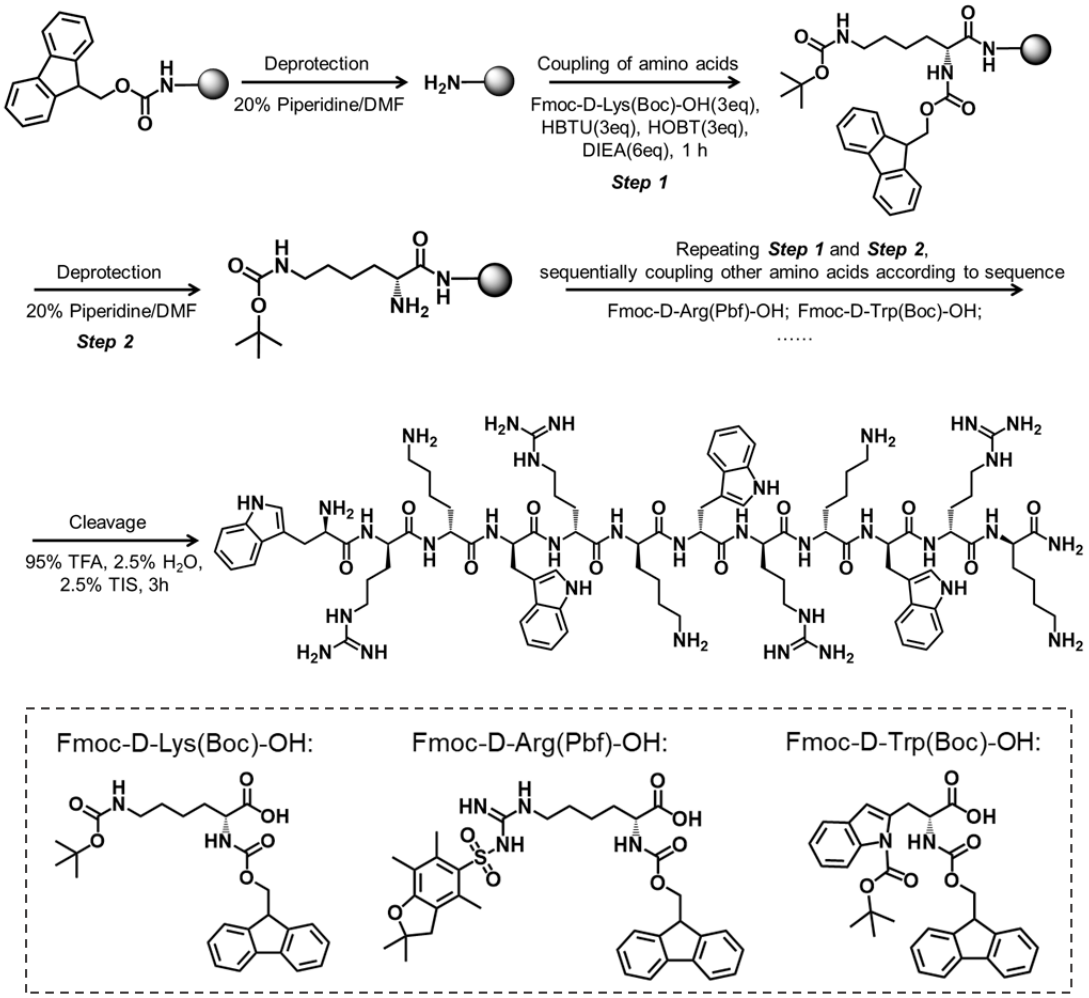
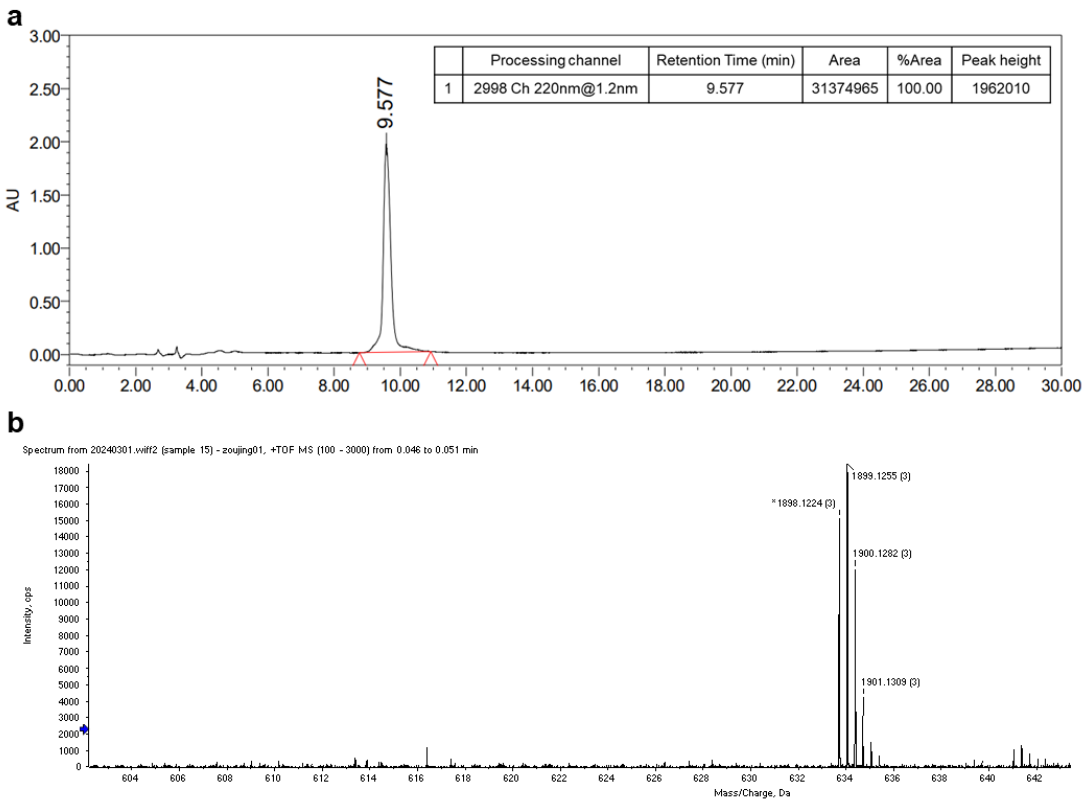
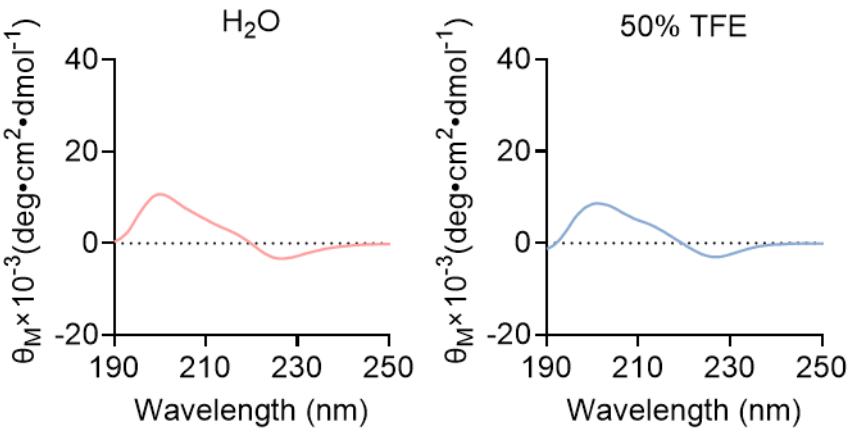
Supplementary Figures



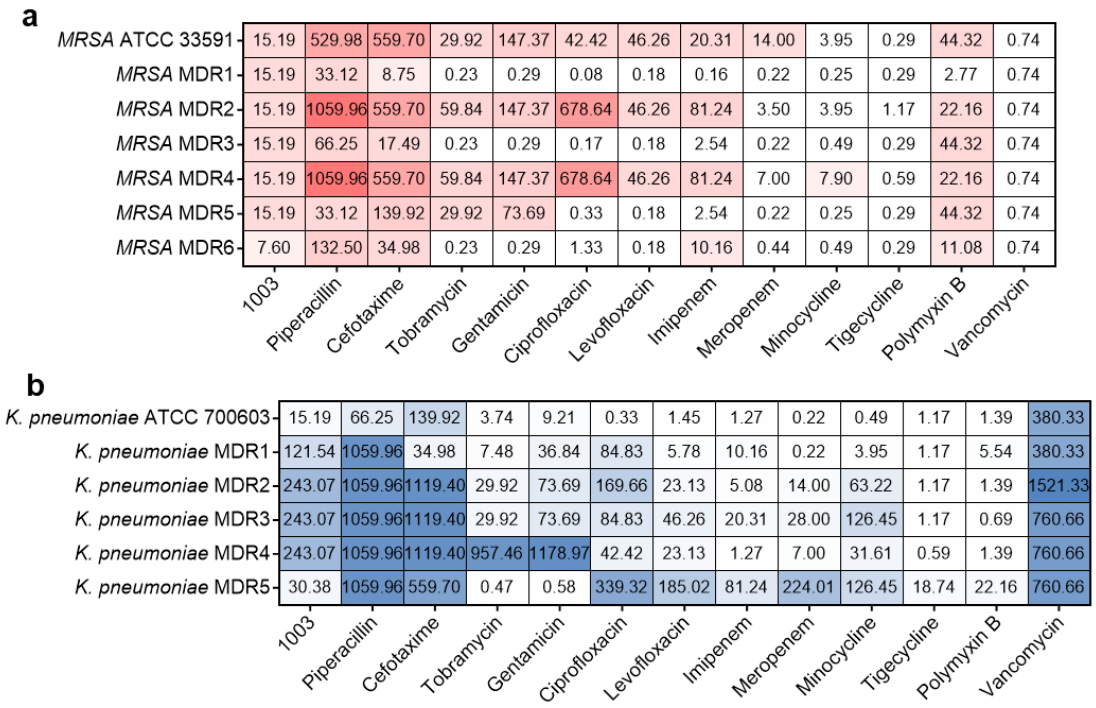
Supplementary Fig. 1 The synthetic scheme of AMP 1003 and the amino acid structure used in the synthesis process. All amino acids are in the D-configuration unless otherwise specified.



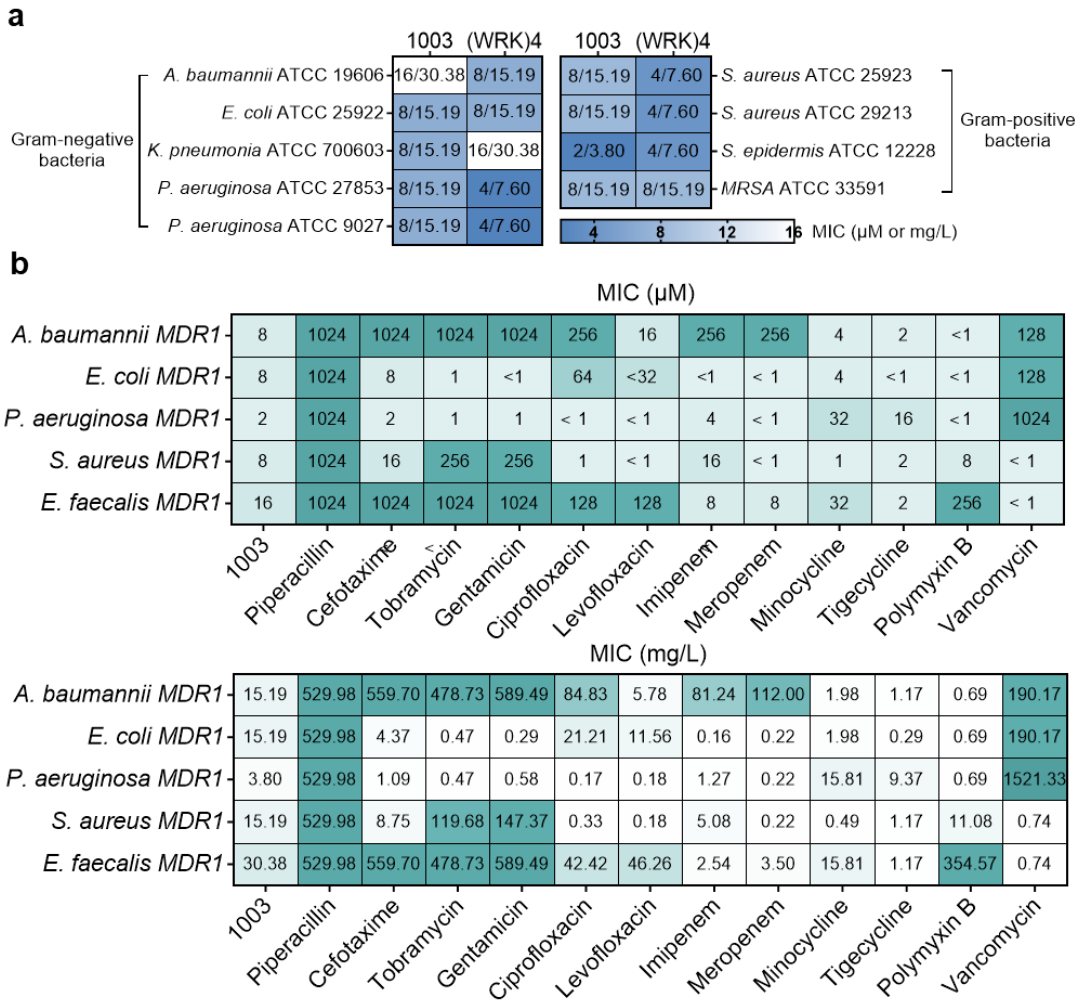
Supplementary Fig. 2 a Purity of AMP 1003 analyzed by RP-HPLC on a C18 column using a gradient of buffer B (0.1% TFA in CH_3CN/H_2O 95:5 v/v) in buffer A (0.1 %TFA in CH_3CN/H_2O 5:95 v/v) for 30 min with a flow rate of 1.0 mL/min. **b** Mass spectrum of AMP 1003 determined by EI-MS.



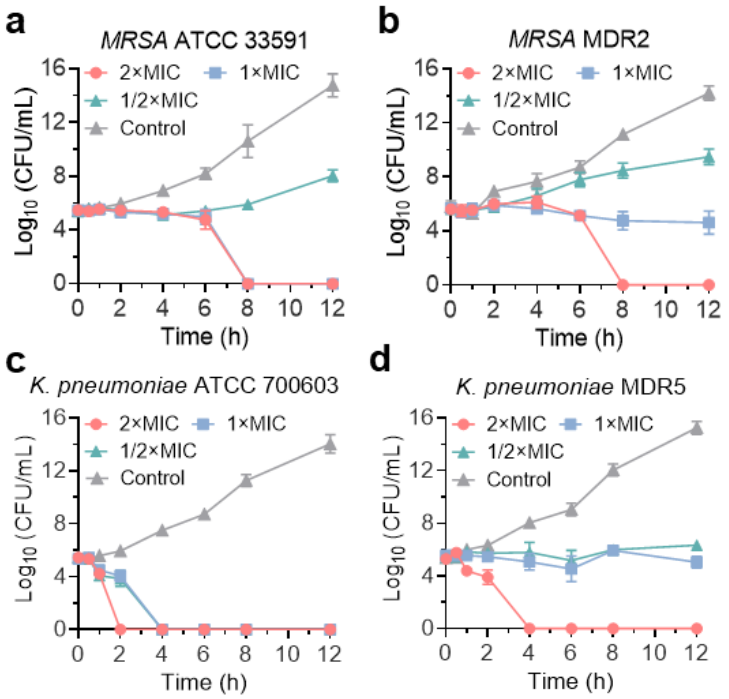
Supplementary Fig. 3 Circular dichroism (CD) spectrum of AMP 1003 in H_2O or 50% TFE. The mean residual ellipticity was employing by the formula: $\theta_M = (\theta_{obs} \times 1000) / (c \times l \times n)$. Where θ_M is the residue ellipticity ($deg \cdot cm^2 \cdot dmol^{-1}$), θ_{obs} is the observed ellipticity corrected for the buffer at a given wavelength (mdeg), c is the peptide concentration (mM), l is the path length (mm), and n is the number of amino acids.



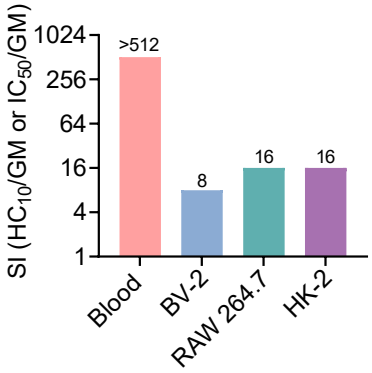
Supplementary Fig. 4 a-b Antimicrobial activity of AMP 1003 against standard and clinical isolates of multidrug-resistant *MRSA* (**a**) or *K. pneumoniae* (**b**), represented by minimum inhibitory concentration (MIC (mg/L)).



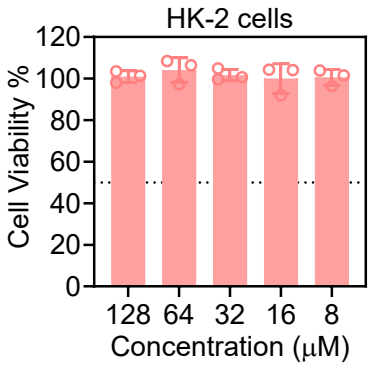
Supplementary Fig. 5 a MICs (μM or mg/L) of AMP 1003 and $(WRK)_4$ against standard bacteria. **b** MICs (μM or mg/L) of AMP 1003 and antibiotics against clinically isolated multidrug-resistant bacteria.



Supplementary Fig. 6 a and **b** Killing kinetics of vancomycin against *MRSA* ATCC 33591 (**a**) and *MRSA* MDR2 (**b**). **c** and **d** Killing kinetics of polymyxin B against *K. pneumoniae* ATCC 700603 (**c**) and *K. pneumoniae* MDR5 (**d**), n = 3 independent replicates. Data are presented as mean values ± SD. Data points show the median, and the error bars indicate the range.

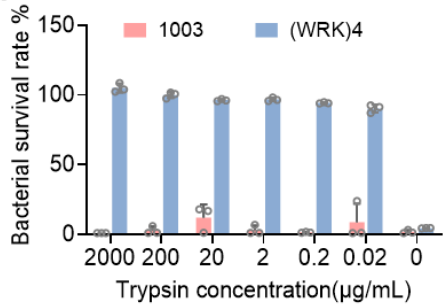


Supplementary Fig. 7 Selectivity index (SI) of AMP 1003 towards blood cells, BV-2 cells, RAW 264.7 cells and HK-2 cells.



Supplementary Fig. 8 Cytotoxicity of polymyxin B for HK-2 cells. n = 3 independent replicates. Data are presented as mean values ± SD. Data points show the individual values, the central line represents the median, and the error bars indicate the range.

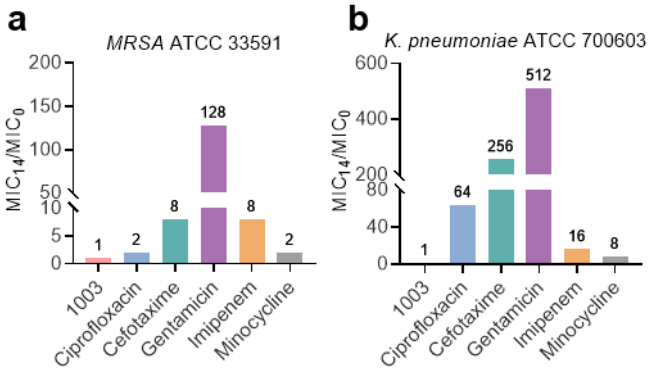
a



b

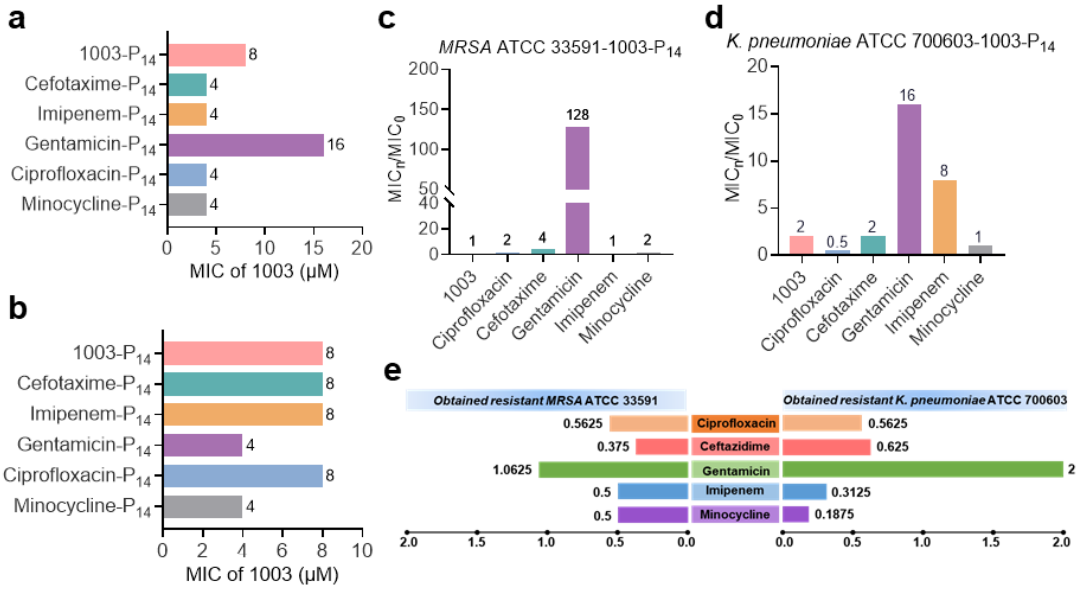
	Protease concentration (mg/mL)						
	8	4	2	1	0.5	0.25	0
	MIC against *K. pneumonia* ATCC 700603 (μM)						
Trypsin	>128	>128	>128	>128	>128	>128	16
Chymotrypsin	>128	>128	>128	>128	>128	>128	16
Pepsin	>128	>128	>128	>128	>128	>128	16
	MIC against *MRSA* ATCC 33591 (μM)						
Trypsin	>128	>128	>128	>128	>128	>128	8
Chymotrypsin	>128	>128	>128	>128	>128	>128	8
Pepsin	>128	>128	>128	>128	128	128	8

Supplementary Fig. 9 a The bacterial survival rate of AMP 1003 and (WRK)4 against *MRSA* ATCC 33591 is detected after incubation with different concentrations of trypsin for 6 h, n = 3 independent replicates. Data are presented as mean values ± SD. Data points show the individual values, the central line represents the median, and the error bars indicate the range. **b** MICs of (WRK)4 against *K. pneumoniae* ATCC 700603 and *MRSA* ATCC 33591 after incubation with different concentrations of trypsin, chymotrypsin or pepsin for 6 h.

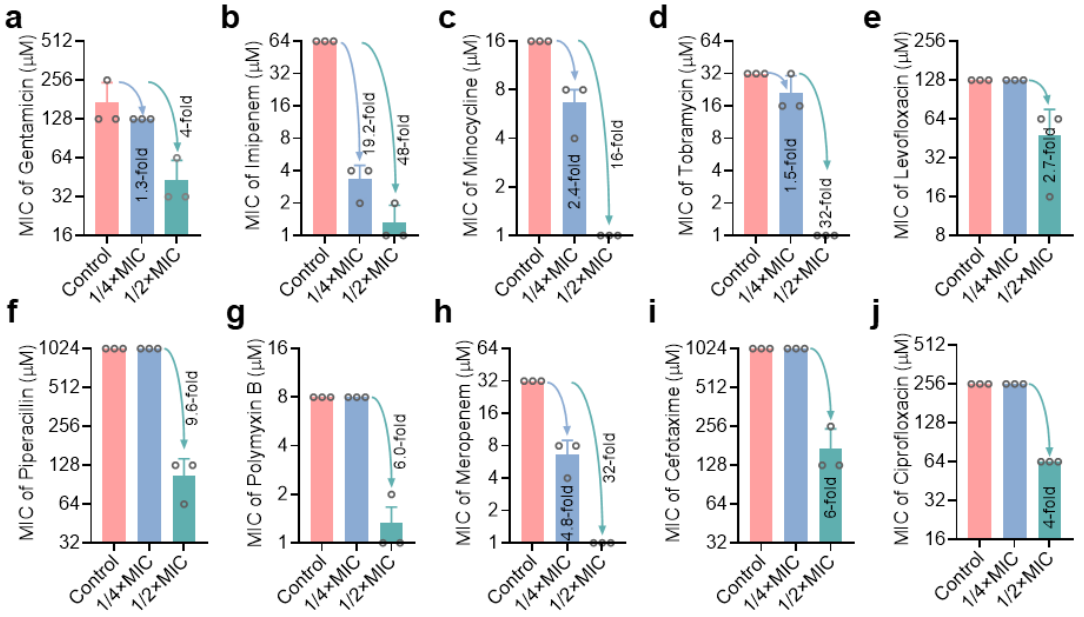


Supplementary Fig. 10 a The changes in MIC values of AMP 1003 and antibiotics against *MRSA* ATCC 33591 after treating with 1003 or antibiotics continuously at 1/2 × MIC for 14 passages compared to that for 0 passage. **b** The changes in MIC values of AMP 1003 and

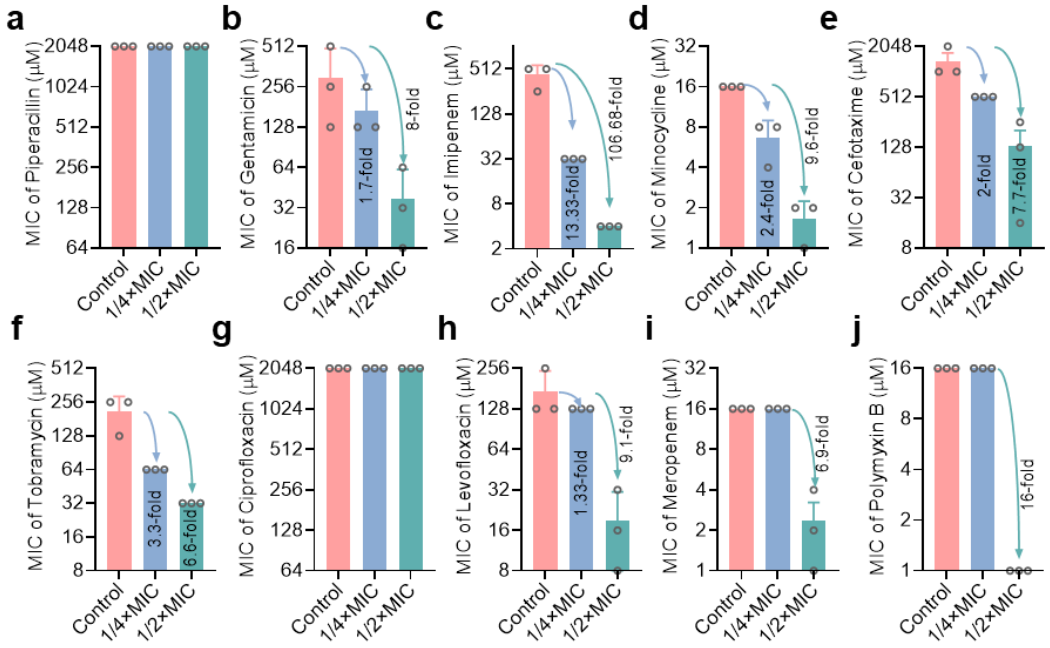
antibiotics against *K. pneumoniae* ATCC 700603 after treating with 1003 or antibiotics continuously at 1/2 × MIC for 14 passages compared to that for 0 passage.



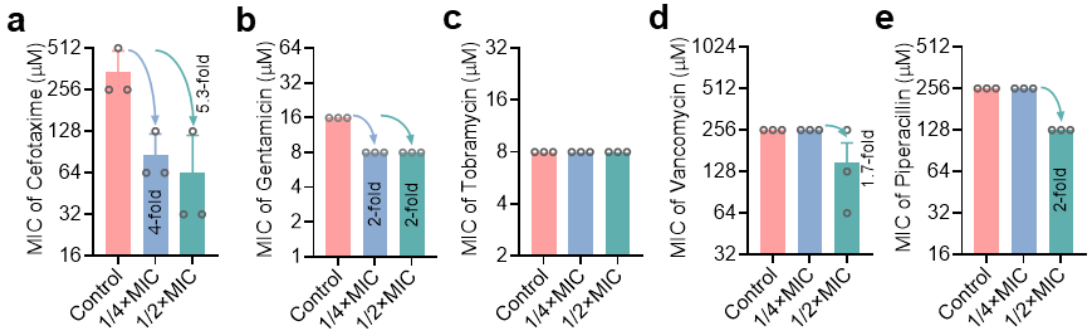
Supplementary Fig. 11 a and **b** The MIC values of AMP 1003 against the final-generation *MRSA* ATCC 33591 (**a**) and *K. pneumoniae* ATCC 700603 (**b**) after treating with 1003 or antibiotics continuously at 1/2 × MIC for 14 passages. **c** and **d** The changes in MIC values of AMP 1003 and antibiotics against the final-generation *MRSA* ATCC 33591 (**c**) and *K. pneumoniae* ATCC 700603 (**d**) after treating with 1003 continuously at 1/2 × MIC for 14 passages compared to that for 0 passage. **e** The FICIs of AMP 1003 in combination with antibiotics against the obtained resistant *MRSA* ATCC 33591 and *K. pneumoniae* ATCC 700603.



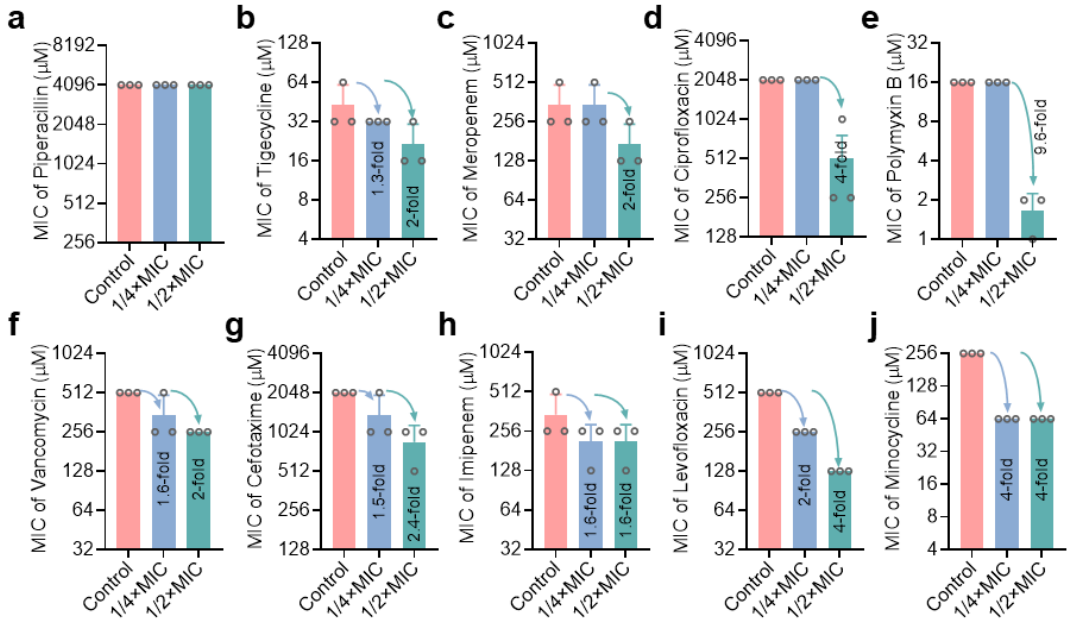
Supplementary Fig. 12 a-j Chemical sensitizations of AMP 1003 to gentamicin(**a**), imipenem (**b**), minocycline (**c**), tobramycin (**d**), levofloxacin (**e**), piperacillin (**f**), polymyxin B (**g**), meropenem (**h**), ceftazidime (**i**), or ciprofloxacin (**j**) against *MRSA* ATCC 33591, n = 3 independent replicates. Data are presented as mean values ± SD. Data points show the individual values, the central line represents the median, and the error bars indicate the range.



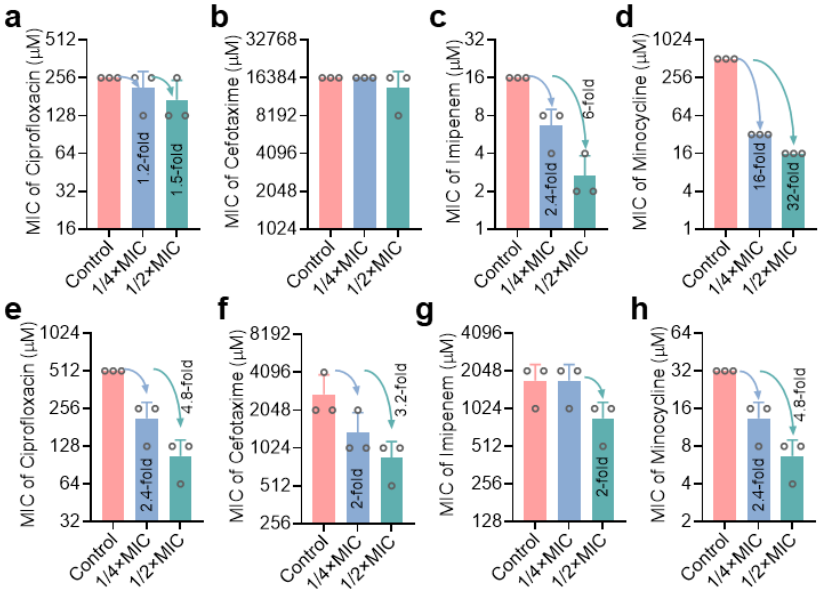
Supplementary Fig. 13 a-j Chemical sensitizations of AMP 1003 to piperacillin (**a**), gentamicin (**b**), imipenem (**c**), minocycline (**d**), ceftazidime (**e**), tobramycin (**f**), ciprofloxacin (**g**), levofloxacin (**h**), meropenem (**i**), or polymyxin B (**j**) against *MRSA* MDR2, n = 3 independent replicates. Data are presented as mean values ± SD. Data points show the individual values, the central line represents the median, and the error bars indicate the range.



Supplementary Fig. 14 a-e Chemical sensitizations of AMP 1003 to ceftazidime (**a**), gentamicin (**b**), tobramycin (**c**), vancomycin (**d**) or piperacillin (**e**) against *K. pneumoniae* ATCC 700603, n = 3 independent replicates. Data are presented as mean values ± SD. Data points show the individual values, the central line represents the median, and the error bars indicate the range.

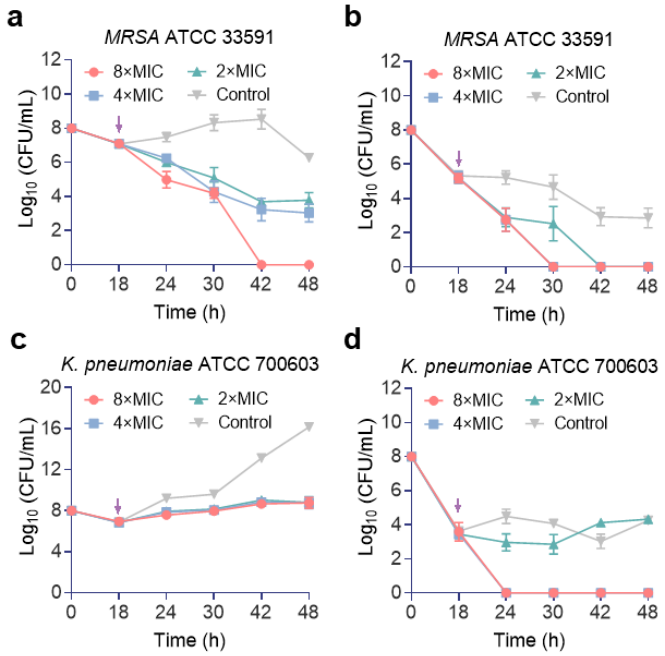


Supplementary Fig. 15 a-j Chemical sensitizations of AMP 1003 to piperacillin (**a**), tigecycline (**b**), meropenem (**c**), ciprofloxacin (**d**), polymyxin B (**e**), vancomycin (**f**), ceftazidime (**g**), imipenem (**h**), levofloxacin (**i**), or minocycline (**j**) against *K. pneumoniae* MDR5, n = 3 independent replicates. Data are presented as mean values ± SD. Data points show the individual values, the central line represents the median, and the error bars indicate the range.

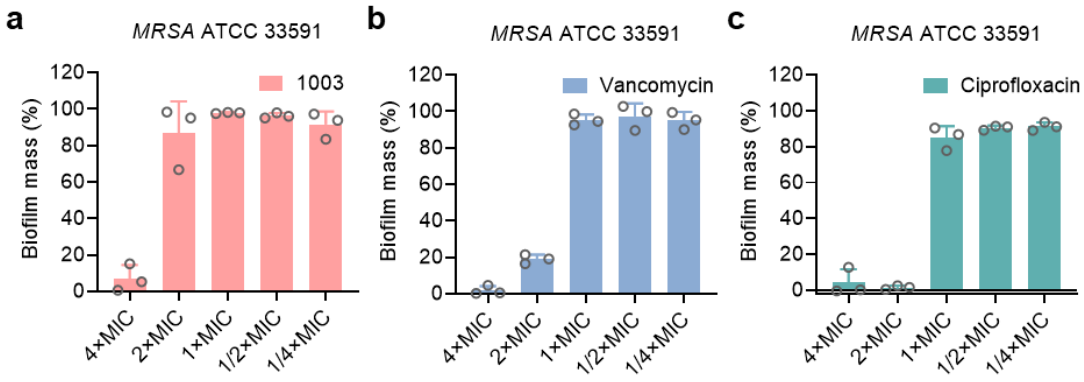


Supplementary Fig. 16 a-h Chemical sensitizations of AMP 1003 to ciprofloxacin (**a**), ceftazidime (**b**), imipenem (**c**), or minocycline (**d**) against the obtained resistant *MRSA* ATCC 33591; Chemical sensitizations of AMP 1003 to ciprofloxacin (**e**), ceftazidime (**f**), imipenem (**g**), or minocycline (**h**) against the obtained resistant *K. pneumoniae* ATCC 700603, n = 3

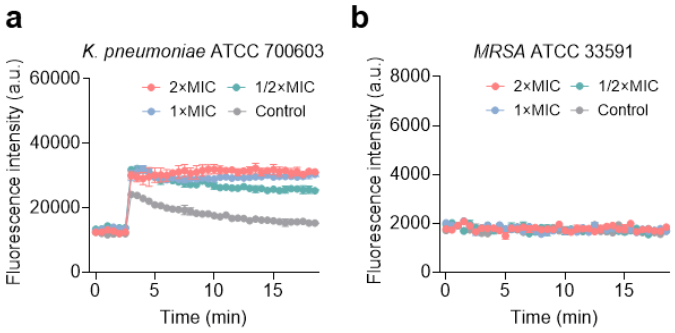
independent replicates. Data are presented as mean values ± SD. Data points show the individual values, the central line represents the median, and the error bars indicate the range.



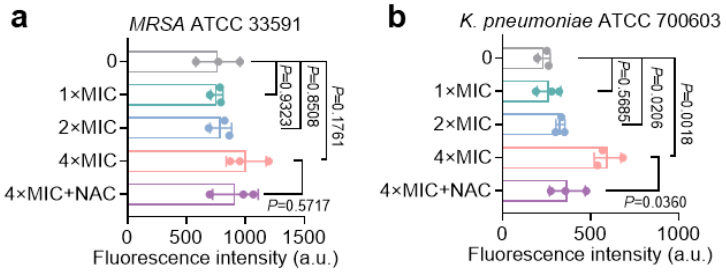
Supplementary Fig. 17 a-d Killing kinetics of AMP 1003 against *MRSA* ATCC 33591 persister cells escaped treatment with 10 × MIC of ceftazidime (**a**) or vancomycin (**b**); Killing kinetics of AMP 1003 against *K. pneumoniae* ATCC 700603 persister cells escaped treatment with 10 × MIC concentrations of ceftazidime (**c**) or polymyxin B (**d**), n = 3 independent replicates. Data are presented as mean values ± SD. Data points show the median, and the error bars indicate the range.



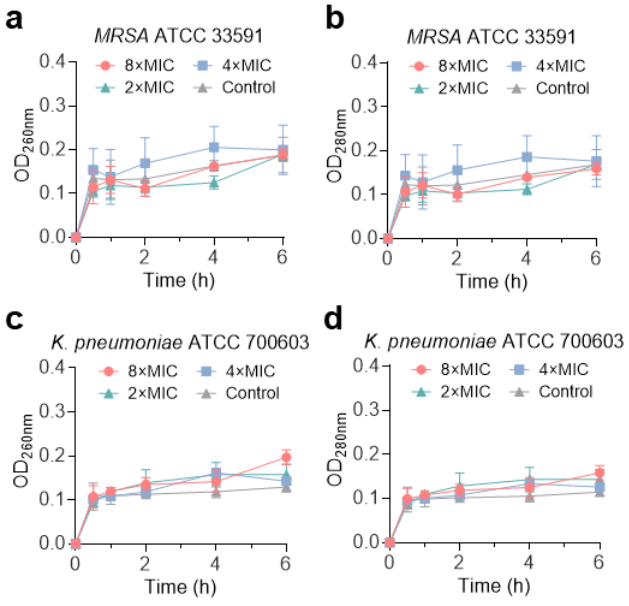
Supplementary Fig. 18 a-b The biofilm inhibition activities of AMP 1003 (**a**), vancomycin (**b**), and ciprofloxacin (**c**) against *MRSA* ATCC 33591, n = 3 independent replicates. Data are presented as mean values ± SD. Data points show the individual values, the central line represents the median, and the error bars indicate the range.



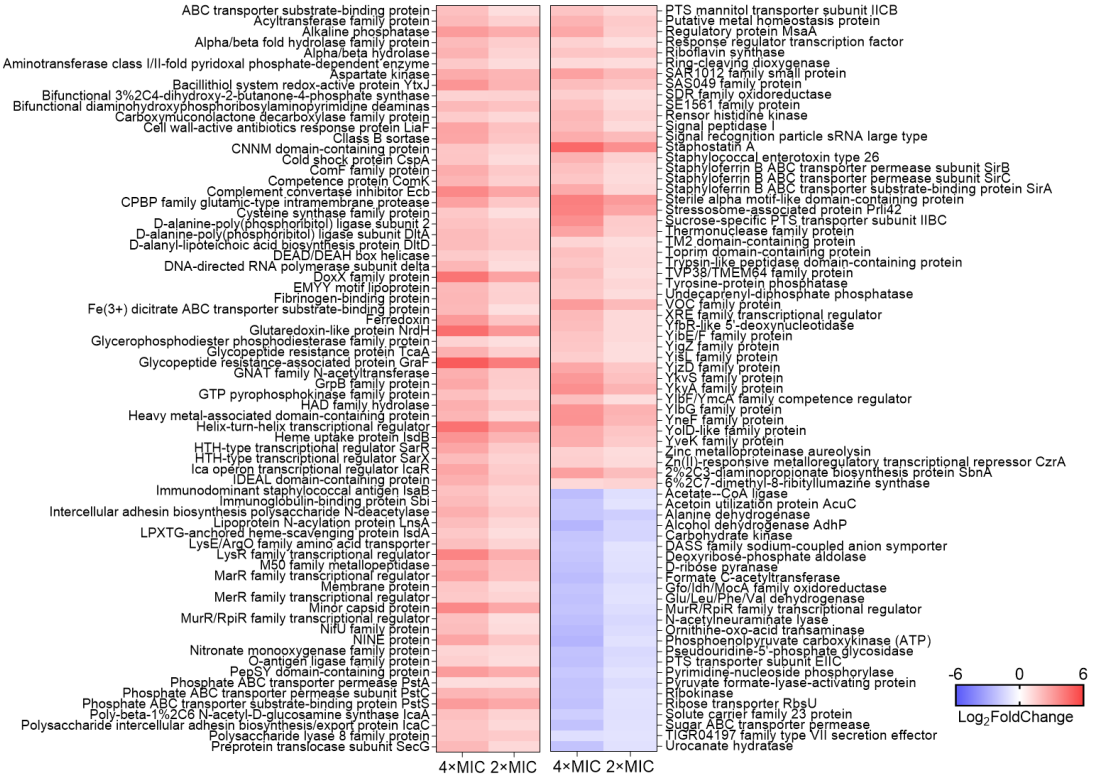
Supplementary Fig. 19 a Cytoplasmic membrane electrical potential of *K. pneumoniae* ATCC 700603 treated with polymyxin B at 2 × MIC, 1 × MIC and 1/2 × MIC concentrations. **b** Cytoplasmic membrane electrical potential of *MRSA* ATCC 33591 treated with vancomycin at 2 × MIC, 1 × MIC and 1/2 × MIC concentrations, n = 3 independent replicates. Data are presented as mean values ± SD. Data points show the median, and the error bars indicate the range.



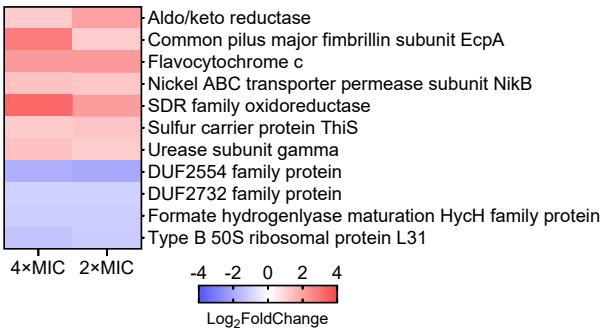
Supplementary Fig. 20 a ROS accumulation of *MRSA* ATCC 33591 treated with vancomycin. **b** ROS accumulation of *K. pneumoniae* ATCC 700603 treated with polymyxin B, n = 3 independent replicates. Data are presented as mean values ± SD. Data points show the individual values, the central line represents the median, and the error bars indicate the range. Statistical analysis: two-tailed *t*-test.



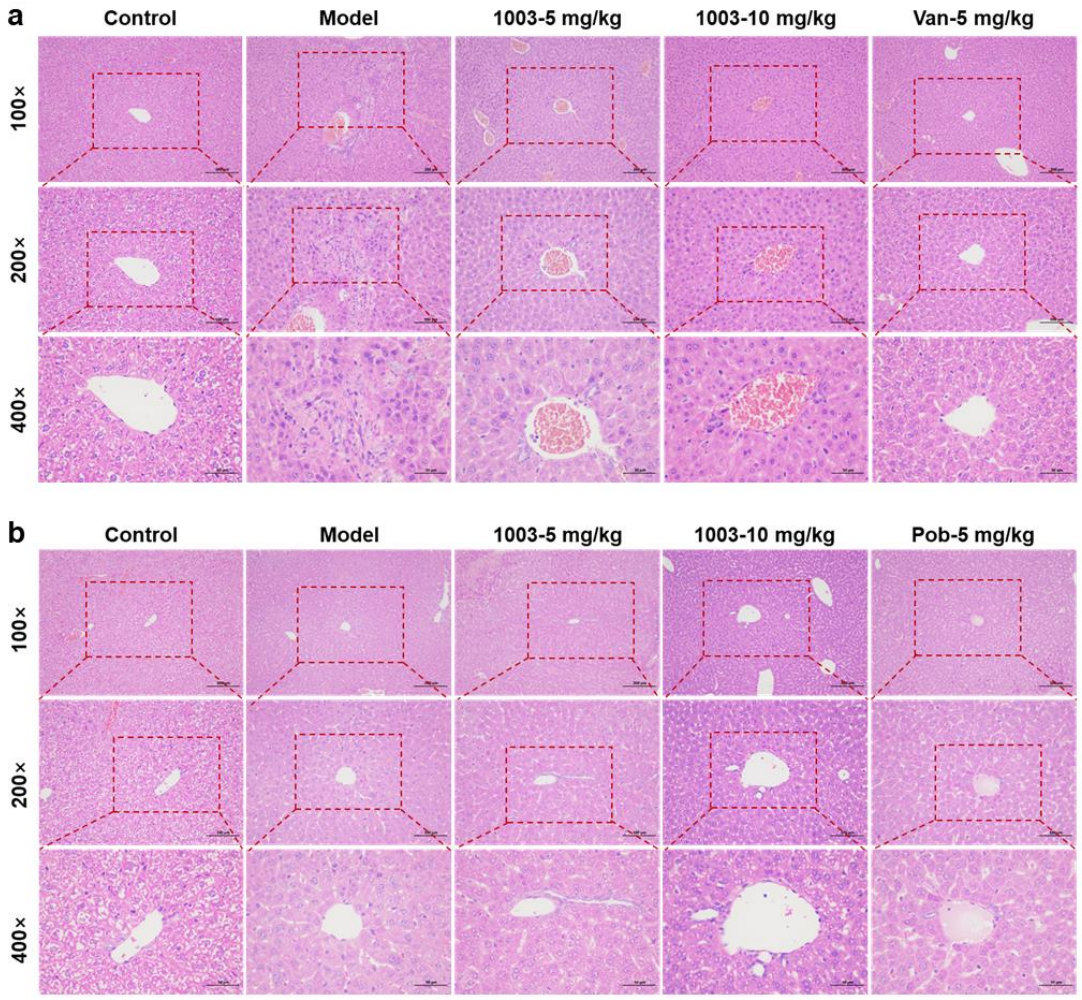
Supplementary Fig. 21 a and **b** Nucleic acid and protein leak of *MRSA* ATCC 33591 treated with vancomycin. **c** and **d** Nucleic acids and proteins leak from *K. pneumoniae* ATCC 700603 treated with polymyxin B, n = 3 independent replicates. Data are presented as mean values ± SD. Data points show the median, and the error bars indicate the range.



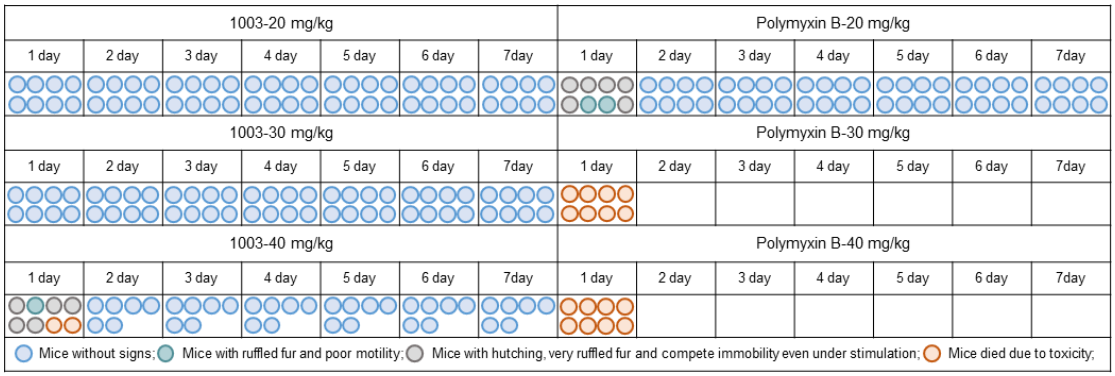
Supplementary Fig. 22 Differently expressed genes of *MRSA* ATCC 33591 under treatment with AMP 1003 at 4 × MIC and 2 × MIC concentrations *vs.* control. n = 3 biological replicates. Data are presented as means.



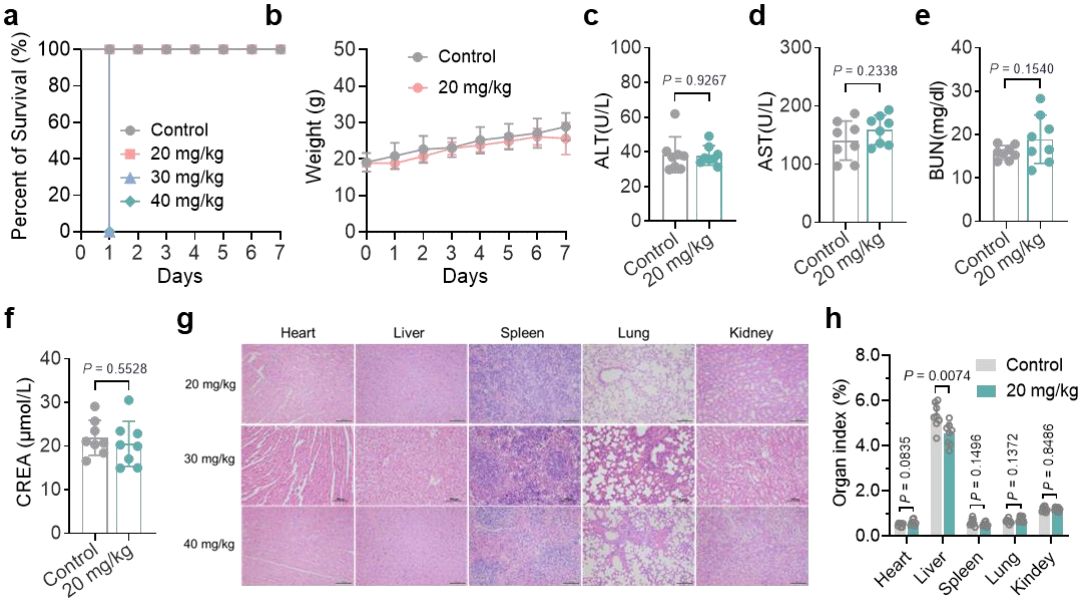
Supplementary Fig. 23 Differently expressed genes of *K. pneumoniae* ATCC 700603 under treatment with AMP 1003 at 4 × MIC and 2 × MIC concentrations *vs.* control. n = 3 biological replicates. Data are presented as means.



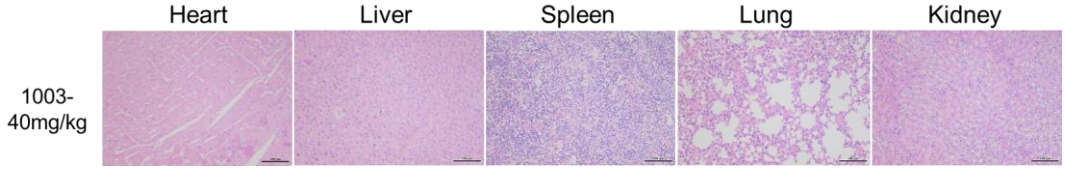
Supplementary Fig. 24 a and **b** Pathological sections stained by H&E of the liver in the *MRSA* ATCC 33591-infected (**a**) or *K. pneumoniae* ATCC 700603-infected (**a**) pneumonia model treated with saline, AMP 1003 (5 mg/kg or 10 mg/kg), Van (5 mg/kg) or Pob (5 mg/kg). Van stands for vancomycin. Pob stands for polymyxin B. n = 3. Scale bar: 100×: 200 μm, 200×: 100 μm, 400×: 50 μm.



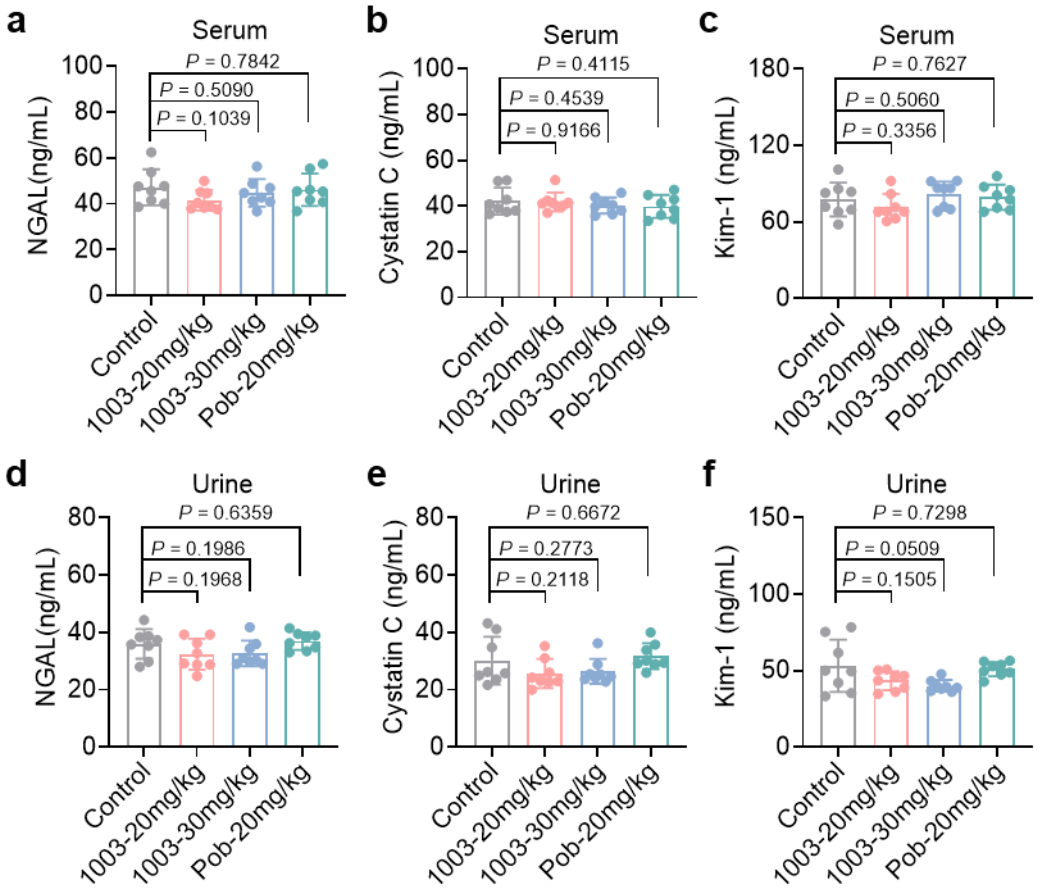
Supplementary Fig. 25 The abnormal behavior and survival of mice administered with AMP 1003 or polymyxin B for 7 days. Each circle represents one mouse, and the toxicity score was indicated by different circle shading as outlined in the legend.



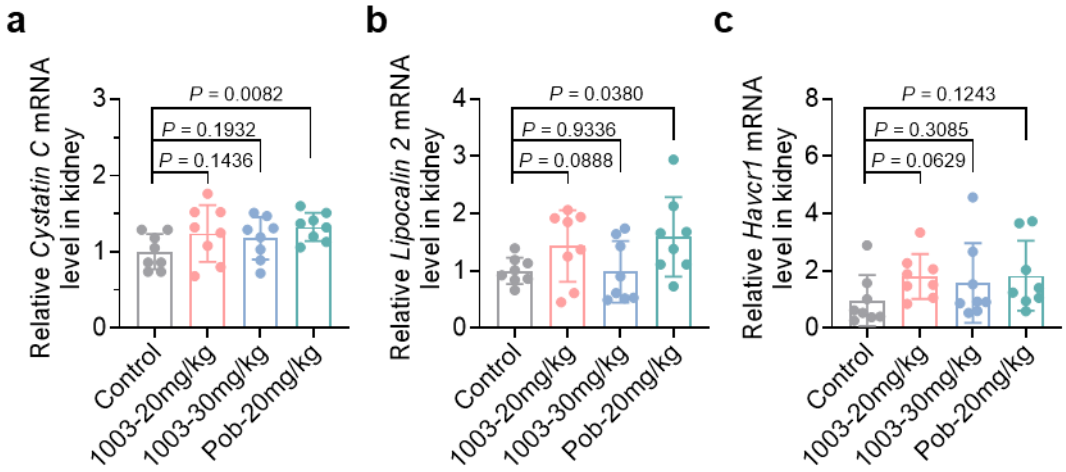
Supplementary Fig. 26 a Survival curves of mice treated with a single intraperitoneal injection of polymyxin B at 20 mg/kg, 30 mg/kg or 40 mg/kg. n = 8 mice per group. **b** The weight changes of mice administered with polymyxin B at a dose of 20 mg/kg for 7 days, n = 8 mice per group. Data are presented as mean values ± SD. Data points show the median, and the error bars indicate the range. **c-f** Blood biochemical analysis (ALT (**c**), AST (**d**), BUN (**e**), CREA (**f**)) of mice at 7 days post-treatment with polymyxin B at a dose of 20 mg/kg, n = 8 mice per group. Data are presented as mean values ± SD. Data points show the individual values, the central line represents the median, and the error bars indicate the range. Statistical analysis: two-tailed *t*-test. **g** Pathological sections stained by H&E of the heart, liver, spleen, lung, and kidney of the mice at 7 days after the single dose of intravenous injection with polymyxin B at a dose of 20 mg/kg, 30 mg/kg or 40 mg/kg. n = 3 mice per group. Scale bar: 100 μm. **h** Organ index of the mice at 7 days after the single intravenous injection dose with polymyxin B at 20 mg/kg, n = 8 mice per group. Data are presented as mean values ± SD. Data points show the individual values, the central line represents the median, and the error bars indicate the range. Statistical analysis: two-tailed *t*-test.



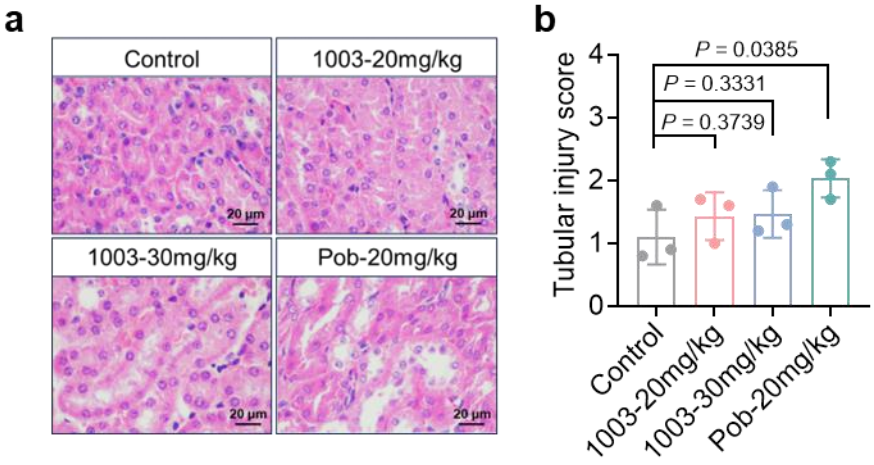
Supplementary Fig. 27 Pathological sections stained by H&E of the heart, liver, spleen, lung, and kidney of the mice died after a single dose of intravenous injection with AMP 1003 at a dose of 40 mg/kg. n = 3 mice per group. Scale bar: 100 μm.



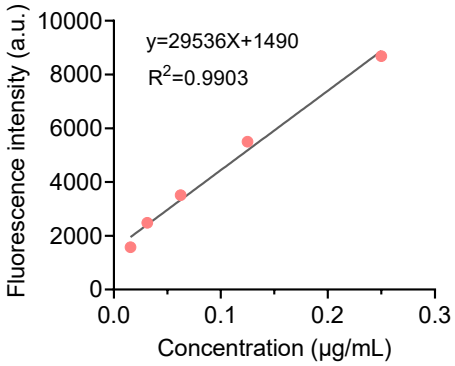
Supplementary Fig. 28 a-f The NGAL (**a** and **d**), Cystatin C (**b** and **e**) or Kim-1 (**c** and **f**) level in the serum or urine of mice at 7 days post-treatment with a single intraperitoneal injection of AMP 1003 at a dose of 20 mg/kg or 30 mg/kg and polymyxin B at 20 mg/kg, n = 8 mice per group. Data are presented as mean values ± SD. Data points show the individual values, the central line represents the median, and the error bars indicate the range. Statistical analysis: two-tailed *t*-test.



Supplementary Fig. 29 a-c The *Cystatin C* (**a**), *Lipocalin 2* (**b**), *Havcr1* (**c**) mRNA level in kidney of mice at 7 days post-treatment with a single intraperitoneal injection of AMP 1003 at a dose of 20 mg/kg or 30 mg/kg and polymyxin B at 20 mg/kg, n = 8 mice per group. Data are presented as mean values ± SD. Data points show the individual values, the central line represents the median, and the error bars indicate the range. Statistical analysis: two-tailed *t*-test.



Supplementary Fig. 30 a Representative (H&E) stained renal sections of mice at 7 days post-treatment with a single intraperitoneal injection of AMP 1003 at a dose of 20 mg/kg or 30 mg/kg and polymyxin B at 20 mg/kg. n = 3 mice per group. Scale bar: 20 μm. **b** Mean tubular injury scores of ten randomly chosen high-power fields in kidney sections of mice at 7 days post-treatment with a single intraperitoneal injection of AMP 1003 at doses of 20 mg/kg or 30 mg/kg and polymyxin B at 20 mg/kg, n = 3 mice per group. Data are presented as mean values ± SD. Data points show the individual values, the central line represents the median, and the error bars indicate the range. Statistical analysis: two-tailed *t*-test.



Supplementary Fig. 31 Standard curve of fluorescamine.

Supplementary Tables

Supplementary Table 1: The primer for the RT-qPCR.

Gene name	Forward oligonucleotide (5'-3')	Reverse oligonucleotide (5'-3')
Tnf-α	CGTCAGCCGATTTGCTATCT	CGGACTCCGCAAAGTCTAAG
iNOS	CCCTTCCGAAGTTTCTGGCAGCAGC	GGCTGTCAGAGCCTCGTGGCTTTGG
Il-1β	GAGAGCCGGGTGACAGTATC	TGACAAACTTCTGCCTGACG
Il-17	CTCCTGCTTCTAGGCTGGTTG	CCACCTGGCACTTCGAGTTAG
Il-12	GTGGACCAAACAATCTGACCTG	AACACGGACTATGAACCTGGA
Il-18	AGACTACTTCCTGAGCACAAGA	TGTCCTTACCAATGGTTCTCACT
Cd86	CTGGACTCTACGACTTCACAATG	AGTTGGCGATCACTGACAGTT
Mcp1	TTAAAAACCTGGATCGGAACCAA	GCATTAGCTTCAGATTTACGGGT
Cystatin C	TGGTGAGAGCTCGTAAGCAG	CATCAGATGGGGCTGGTCAT
Havcr1	GTTAAACCAGAGATTCCCACACG	TCTCATGGGGACAAAATGTAGTG
Lipocalin 2	GACTTCCGGAGCGATCAGTT	CTGATCCAGTAGCGACAGCC
β-actin	CAGGATGCAGAAGGAGATTAC	AACGCAGCTCAGTAACAGTC