Article

Discovery Medicine 2026; 38(212): 2906–2917
https://doi.org/10.24976/Discov.Med.202638212.224

A Macromolecule npEt20 Exerts Broad-Spectrum Synergistic Effects With Meropenem Against Multidrug-Resistant Gram-Negative Pathogens

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Submitted: 10 July 2026 Revised: 1 August 2026 Accepted: 24 August 2026 Published: 23 September 2026

Background: The widespread emergence of multidrug-resistant (MDR) Gram-negative bacteria severely compromises the clinical efficacy of carbapenem antibiotics, leading to refractory nosocomial infections. Novel antibacterial agents that can reverse carbapenem resistance are urgently needed. Herein, this study aimed to evaluate the antibacterial activity of copolymer npEt20 and its synergistic effect with meropenem against clinical carbapenem-resistant isolates, and verify its potential as a meropenem adjuvant.

Methods: The antibacterial activity and underlying mechanism of npEt20 against clinically isolated carbapenem-resistant strains, including *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*, were systematically characterized. Synergistic bactericidal effects of the npEt20-meropenem combination were assessed both *in vitro* and *in vivo*.

Results: The npEt20 copolymer exhibited intrinsic broad-spectrum bactericidal activity against all three tested carbapenem-resistant isolates. Biofilm inhibition assays revealed that npEt20 significantly reduces mature biofilm biomass across all three pathogens in a concentration-dependent manner. Mechanistic studies demonstrated that npEt20 disrupted bacterial outer membrane integrity by increasing membrane permeability and inducing intracellular nucleic acid leakage. Repeated treatment with npEt20 could not induce detectable resistance phenotypes. Moreover, both *in vitro* and *in vivo* experiments demonstrated that npEt20 exhibited broad-spectrum synergistic performance and effectively reversed meropenem resistance in all three tested carbapenem-resistant pathogens.

Conclusion: This work demonstrates that npEt20 acts as a broad-spectrum adjuvant capable of restoring meropenem activity against carbapenem-resistant *A. baumannii*, *K. pneumoniae* and *P. aeruginosa in vitro* and in a mouse infection model, which provides a promising preclinical strategy that requires further clinical validation for treating refractory carbapenem-resistant Gram-negative infections.

Keywords: antimicrobial copolymer; carbapenem-resistant; synergistic antimicrobial activity; bacterial infections

Introduction

Antimicrobial resistance has become one of the most pressing threats to global health, contributing to an estimated 4.95 million deaths in 2019 and projected to increase further over the coming decades [1,2]. The greatest concern is posed by multidrug-resistant (MDR) Gram-negative bacteria. In the 2024 WHO Bacterial Priority Pathogens List, carbapenem-resistant *A. baumannii*, and carbapenem-resistant *Enterobacterales* are classified as critical-priority pathogens, whereas carbapenem-resistant *P. aeruginosa* is re-classified as a high-priority pathogen [3]. Carbapenems such as meropenem have long served as last-line agents for severe Gram-negative infections, yet their efficacy is eroding rapidly [4]. Carbapenem resistance arises through multiple, frequently coexisting mechanisms, including carbapenemase production, loss of outer membrane porins, overexpression of efflux pumps, and alterations in penicillin-binding proteins [5]. Because reduced drug influx can amplify the effects of weakly hydrolytic enzymes, resistance is typically multifactorial rather than attributable to a single determinant [5]. This complexity limits the ef-

fectiveness of inhibitors targeting any one enzyme and renders the discovery of entirely new scaffolds slow and costly. Reversing existing resistance to restore meropenem activity has therefore emerged as a timely and economically attractive alternative [6].

Antibiotic adjuvants—agents with little or no intrinsic activity that potentiate a partner antibiotic or reverse resistance—offer a practical route to prolong the useful life of existing drugs [6]. Synergy is conventionally quantified by the fractional inhibitory concentration index (FICI), with values ≤0.5 defining a synergistic interaction [7]. Several notable candidates have been described. The short linear peptide SLAP-S25 binds lipopolysaccharide and phosphatidylglycerol, inducing membrane damage, broadly potentiating antibiotics and reversing resistance in MDR Escherichia coli *in vivo* [8]. Likewise, the repurposed drug disulfiram and its derivatives restore carbapenem and colistin activity against New Delhi metallo-β-lactamase (NDM)- and mobile colistin resistance (MCR)-producing pathogens by inhibiting the corresponding enzymes or aggravating membrane damage [9]. Despite this progress, the efficacy of most adjuvants remains limited to specific species or resistance mechanisms: enzyme inhibitors are ineffective against non-enzymatic resistance, and many potentiators have been validated in only a single organism or under *in vitro* conditions. A broadly applicable adjuvant that is effective against the major Gram-negative pathogens is still lacking.

A recurring feature of successful potentiators is disruption of the Gram-negative outer membrane. This asymmetric bilayer, whose outer leaflet consists almost entirely of lipopolysaccharide, forms a formidable permeability barrier that excludes many antibiotics and limits β-lactam entry [7]. Permeabilizing the outer membrane can thus markedly increase the intracellular accumulation and potency of a partner drug. Polymyxins exemplify this principle: by binding lipid A and displacing the divalent cations that stabilize lipopolysaccharide, polymyxin B nonapeptide and related molecules permeabilize the outer membrane and resensitize otherwise resistant bacteria to co-administered antibiotics [10]. However, the clinical utility of polymyxins is constrained by dose-limiting nephrotoxicity and neurotoxicity [10]. Even chemically modified derivatives engineered to lower toxicity while preserving permeabilization must trade safety against spectrum [11]. These limitations highlight the need for outer membrane–targeting potentiators that couple broad permeabilization with a substantially improved safety profile.

An ideal broad-spectrum potentiator would combine four attributes: activity against *A. baumannii*, *K. pneumoniae*, and *P. aeruginosa*; low host toxicity; a low propensity to select for resistance; and demonstrable efficacy *in vivo*. Membrane-active cationic macromolecules that mimic host-defense peptides align closely with these criteria. By targeting the universal bacterial membrane rather than a specific protein, such polymers achieve broad-spectrum, rapid killing with a low tendency to induce resistance, while offering greater stability and lower toxicity than small-molecule quaternary ammonium compounds [12]. However, existing candidates have notable limitations: most have been assessed primarily against Gram-positive bacteria or only under *in vitro* conditions, and some lose activity in serum due to nonspecific protein binding [13]. Critically, few membrane-active macromolecules have been positioned explicitly as carbapenem adjuvants and validated for both synergy and safety across multiple Gram-negative pathogens *in vivo*.

To bridge this gap, we developed npEt20, a cationic copolymer conceived as a broad-spectrum macromolecular adjuvant. We show that npEt20 exerts intrinsic, fast-acting bactericidal activity against carbapenem-resistant clinical isolates of *A. baumannii*, *K. pneumoniae*, and *P. aeruginosa*, disrupts the bacterial outer membrane, triggers intracellular content leakage, and does not readily select for resistance during serial passage. Most importantly, npEt20 synergizes with meropenem to reverse carbapenem resistance both *in vitro* and in a murine peritonitis model, without discernible organ toxicity, providing a feasible strategy for treating refractory carbapenem-resistant Gram-negative infections.

Methods

Synthesis of npEt20

npEt20, a macromolecular copolymer, originates from our previously published report, where full monomer composition, molecular weight, dispersity and purity characterization of this copolymer were comprehensively presented [14]. The copolymer was lyophilized from water to obtain a white, transparent solid. A single batch was used for all experiments reported in this study.

Bacterial Strains and Culture Conditions

Clinical multidrug-resistant isolates of *A. baumannii*, *K. pneumoniae* and *P. aeruginosa* (20 isolates of each species) were recovered from clinical specimens obtained from hospitalized patients at the First Affiliated Hospital, Zhejiang University, between January 2010 and December 2013 and identified to the species level using routine microbiological methods. Stock cultures were maintained in glycerol at −80 °C; before each assay, isolates were streaked onto Mueller–Hinton (MH) agar and grown at 37 °C. Three representative carbapenem-resistant isolates—*A. baumannii 9968*, *K. pneumoniae 8649* and *P. aeruginosa 26139*—were selected for the mechanistic, combination and animal studies. All procedures were conducted in accordance with the principles of the Declaration of Helsinki. Because the isolates were obtained from residual, de-identified diagnostic specimens collected during routine clinical care, the Ethics Committee granted a waiver of written informed consent.

Minimum Inhibitory Concentration (MIC) Determination

MIC values were determined by broth microdilution in accordance with the recommendations of the Clinical and Laboratory Standards Institute (CLSI) [15]. Each agent was prepared as a two-fold serial dilution in MHB across the wells of a 96-well microplate. Colonies harvested from overnight MH agar plates were suspended in saline and brought to a turbidity matching a 0.5 McFarland standard (approximately 1×10^8 colony-forming unit [CFU] mL^{-1}), then further diluted in MHB so that the final inoculum in each well was 5×10^5 CFU mL^{-1}. Plates were incubated at 37 °C for 18 h, after which the MIC was determined visually as the lowest concentration yielding no discernible turbidity. Wells containing bacteria without any agent served as growth controls, and all measurements were performed in triplicate.

Time-Kill Kinetics Assay

Exponential-phase cells of each representative isolate were diluted in MHB to roughly 1×10^6 CFU mL^{-1} and challenged with npEt20 or meropenem at 1×, 2× or 4× MIC, with drug-free cultures included in parallel as controls. The suspensions were maintained at 37 °C, and samples were collected at predefined intervals throughout the monitoring window (up to 60 min for *A. baumannii 9968* and up to 180 min for *K. pneumoniae 8649* and *P. aeruginosa 26139*). Each sample was subjected to ten-fold serial dilution, plated on MH agar and incubated at 37 °C for 24 h before viable colonies were counted. All assays were conducted in triplicate, and counts were reported as mean lg (CFU mL^{-1}) ± SD.

Antibiofilm Assay

Antibiofilm assay was performed following a standard microplate crystal violet protocol. Briefly, bacterial suspensions (1×10^7 CFU/mL) were seeded into 96-well plates and incubated statically at 37 °C for 24 h to form mature biofilms. After treatment with gradient concentrations of npEt20 for another 24 h, wells were washed with PBS, air-dried, stained with 0.1% crystal violet, rinsed and dissolved in MBDS solution. The absorbance at 580 nm was measured to quantify relative biofilm biomass, and biofilm cell viability was detected simultaneously. All assays were conducted in triplicate.

Nucleic Acid Leakage Assay

Efflux of intracellular nucleic acids was inferred from the ultraviolet absorbance of cell-free supernatants. Bacterial suspensions (approximately 2×10^9 CFU mL^{-1} in PBS) were treated with npEt20 at 1×, 2×, 4× or 16× MIC, with PBS-treated cells serving as the baseline. After incubation at 37 °C for 2 h, the mixtures were clarified through 0.22-μm filters, and the absorbance of the filtrate at 260 nm was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Values were normalized to those of untreated reference and expressed as A_{260} ratios, with each condition assayed in triplicate.

Outer Membrane Permeabilization (NPN Uptake) Assay

Perturbation of the outer membrane was monitored with the environmentally sensitive fluorophore NPN (MedChemExpress, Monmouth Junction, NJ, USA; Cat. No.HY-W009756). Cells were resuspended in PBS to approximately 2×10^9 CFU mL^{-1} and mixed with NPN (8 μL from a 500 μM stock in acetone) for 30 min. Baseline fluorescence was recorded, after which npEt20 was added to a final concentration of 2× MIC for each isolate. Fluorescence was quantified on SpectraMax® iD3/iD5 multi-mode microplate readers (Molecular Devices, San Jose, CA, USA; Ex = 350 nm, Em = 420 nm) at 0, 30, 60, 120, 180 and 240 min.

In Vitro Resistance Development

The capacity of each isolate to acquire resistance was probed by serial passaging against npEt20, meropenem and ceftazidime. The MIC was measured at each passage as described above; organisms surviving at one-half of the prevailing MIC were recovered, regrown on MH agar and carried forward to the next MIC determination. This procedure was repeated over 10 successive passages, and emergence of resistance was assessed as the ratio of the MIC at passage n to the initial MIC (MIC_n/MIC_0).

Checkerboard Assay and Colony-Count Synergy Assay

Interactions between npEt20 and meropenem were assessed by two-dimensional checkerboard titration, in which orthogonal two-fold dilution gradients of the two agents were arranged in a 96-well format and incubated at 37 °C for 18 h. For each well, the fractional inhibitory concentration index was calculated as FICI = (MIC_combination/MIC_alone) for npEt20 + (MIC_combination/MIC_alone) for meropenem. Combinations were categorized as synergistic (FICI ≤0.5), additive ($0.5 < FICI \leq 1$), indifferent ($1 < FICI \leq 4$) or antagonistic (FICI >4).

To corroborate the combined effect, log-phase cultures of each isolate were either left untreated or treated with npEt20 alone, meropenem alone, or the two agents in combination at the sub-inhibitory concentrations specified in Fig. 4. Following incubation for 18 h at 37 °C, surviving organisms were enumerated by ten-fold serial dilution and MH agar plating, and bacterial loads were expressed as lg (CFU mL^{-1}).

Table 1. Cumulative distribution of MIC values (μg mL^{-1}) of npEt20 against clinically isolated multidrug-resistant *A. baumannii*, *K. pneumoniae*, and *P. aeruginosa* (n = 20).

Antimicrobial agents	Cumulative % of 20 clinically isolated multidrug-resistant strains at indicated MICs							
	4	8	16	32	64	128	256	≥512
A. baumannii								
npEt20			10	60	80	100		
meropenem			5	40	85	100		
K. pneumoniae								
npEt20				5	60	80	90	100
meropenem			5	40	90	90	100	
P. aeruginosa								
npEt20				10	40	80	80	100
meropenem			10	30	50	70	80	100

Note: Resistance breakpoints (μg/mL): meropenem ≥4 for *K. pneumoniae*, ≥8 for *A. baumannii* and *P. aeruginosa* [15]. MIC, minimum inhibitory concentration.

Table 2. MIC values (μg mL^{-1}) of npEt20 and antibiotics against clinically isolated multidrug-resistant bacterial strains.

Isolates	npEt20	Imipenem	Meropenem	Ceftazidime	Levofloxacin
A. baumannii 9968	16	128	128	256	64
K. pneumoniae 8649	32	64	64	>512	64
P. aeruginosa 26139	32	128	128	256	4

Note: Resistance breakpoints (μg/mL): imipenem and meropenem, ≥4 for *K. pneumoniae*, ≥8 for *A. baumannii* and *P. aeruginosa*; ceftazidime ≥16 for *K. pneumoniae*, ≥32 for *A. baumannii* and *P. aeruginosa*; levofloxacin ≥2 for *K. pneumoniae*, ≥4 for *P. aeruginosa*, ≥8 for *A. baumannii* [15].

In Vivo Synergistic Efficacy in a Murine Peritonitis Model

Animal ethics in this study was approved by the independent Ethical Committee of the First Affiliated Hospital, Zhejiang University (approval number: 202024). All mice were housed in SPF facilities at 22 ± 2 °C with a 12 h light-dark cycle, with standard chow and sterile water provided ad libitum. A total of 180 eight-week-old female ICR mice (24–30 g) received intraperitoneal cyclophosphamide (200 mg/kg, Hengrui, Lianyungang, China) 4 days pre-infection to induce neutropenia prior to bacterial challenge. Peritonitis was induced by intraperitoneal inoculation of 0.5 mL of a suspension (approximately 2 × 10^8 CFU mL^{-1}) of each carbapenem-resistant isolate. Infected animals were randomly assigned to four arms—vehicle, meropenem, npEt20, or the npEt20–meropenem combination (15 mice per arm)—and treated intraperitoneally at 1 h and 6 h post-infection with an injection volume of 0.2 mL per dose. The administered doses varied by bacterial species: for A. baumannii, npEt20 at 0.5 mg/kg and meropenem at 3.0 mg/kg; for K. pneumoniae, npEt20 at 2 mg/kg and meropenem at 4.0 mg/kg; for P. aeruginosa, npEt20 at 2 mg/kg and meropenem at 6.0 mg/kg. At 24 h after infection, five mice from each arm were euthanized, and blood, peritoneal lavage fluid, spleen, liver and kidney were collected; the specimens were homogenized as appropriate, serially diluted and plated on MH agar for colony enumeration, with burdens reported as lg (CFU mL^{-1}). The remaining animals were followed for survival over the ensuing 7 days. Mice were humanely euthanized by slow-flow carbon dioxide inhalation in accordance with institutional animal care guidelines.

In Vivo Toxicity Evaluation

Briefly, an independent batch of uninfected mice (5 mice per group) was used. Mice were randomly divided into four groups: PBS control group, npEt20 monotherapy group (4 mg/kg), meropenem monotherapy group (8 mg/kg), and npEt20 combined with meropenem group (4 mg/kg npEt20 + 8 mg/kg meropenem). All treatments were administered via intraperitoneal injection once daily for 3 consecutive days. Then, serum was collected from the periorbital plexus of mice anesthetized with 2% isoflurane delivered in 0.5 L/min O_2 via a nose cone mask using a small animal anesthesia machine (R500, RWD Life Science Co., Ltd., Shenzhen, China). Hepatic and renal function markers (alanine transaminase [ALT], aspartate transaminase [AST], creatinine, urea nitrogen), as well as serum sodium and potassium levels, were quantified using routine biochemical assays for each treatment group.

Statistical Analysis

Data were analyzed using GraphPad Prism 9.5 (GraphPad Software, San Diego, CA, USA). All data were first assessed for normality using the Shapiro–Wilk test. Data are presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used for multi-group comparisons, followed by Tukey's multiple comparisons post-hoc test for pairwise analysis. Comparisons between two groups were performed using an unpaired two-tailed Student's *t*-test. A two-sided *p*-value below 0.05 was considered statistically significant.

Results

npEt20 Exhibits Rapid, Broad-Spectrum Bactericidal Activity Against Carbapenem-Resistant Gram-Negative Isolates

To evaluate the intrinsic antibacterial potential of npEt20, its MICs were determined against a panel of 20 clinically isolated multidrug-resistant strains from each of three Gram-negative species. npEt20 displayed potent and reproducible inhibitory activity across all isolates, with the cumulative MIC distributions comparable to or lower than those of meropenem for *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* (Table 1, Ref. [15]). Three representative isolates—*A. baumannii 9968*, *K. pneumoniae 8649*, and *P. aeruginosa 26139*—were selected for subsequent mechanistic and combinatorial studies. These strains were confirmed to be carbapenem-resistant according to the CLSI guidelines (Table 2, Ref. [15]).

To characterize the killing dynamics of npEt20, time-kill assays were performed against the three carbapenem-resistant isolates at 1×, 2×, and 4× MIC (Fig. 1). npEt20 exerted rapid, concentration-dependent bactericidal activity against all three pathogens. For *A. baumannii 9968*, npEt20 reduced viable counts to undetectable levels within 60 min at 1× MIC and within ~20–30 min at 4× MIC (Fig. 1A–C). Comparable concentration-dependent eradication was observed for *K. pneumoniae 8649* and *P. aeruginosa 26139*, with complete clearance achieved at 4× MIC (Fig. 1D–I). In striking contrast, meropenem produced negligible reductions in viable counts across all three isolates, consistent with their carbapenem-resistant phenotype, while the untreated controls proliferated throughout the assay period. Together, these data demonstrate that npEt20 possesses intrinsic, fast-acting, broad-spectrum bactericidal activity against carbapenem-resistant Gram-negative bacteria that are refractory to meropenem monotherapy.

npEt20 Suppresses Biofilm Viability and Biomass in a Concentration-Dependent Manner

Because biofilm formation is a major driver of persistent and recalcitrant nosocomial infections, we next assessed the activity of npEt20 against pre-established biofilms of the three carbapenem-resistant isolates. Mature biofilms were treated with npEt20 at 1×, 2×, or 4× MIC for 24 h, after which the viability of embedded cells and the total biofilm biomass were quantified. npEt20 reduced the percentage of viable biofilm-embedded cells in a clear concentration-dependent manner across all three species (Fig. 2A). A parallel, concentration-dependent reduction was also observed in total biofilm biomass (Fig. 2B). These reductions were statistically significant at all tested concentrations for each pathogen ($p < 0.05$). These results indicate that npEt20 not only eliminates planktonic bacteria but also effectively disrupts established biofilms, simultaneously reducing the metabolic viability and structural biomass of biofilms formed by carbapenem-resistant Gram-negative pathogens.

npEt20 Eliminates Bacteria by Permeabilizing the Outer Membrane and Inducing Intracellular Content Leakage

To elucidate the bactericidal mechanism of npEt20, we examined its effect on bacterial membrane integrity. Leakage of intracellular nucleic acids—a hallmark of membrane rupture—was quantified by measuring the absorbance of cell-free supernatants at 260 nm. npEt20 induced a concentration-dependent increase in the A_{260} ratio across all three isolates, increasing from baseline under the control condition to approximately 2.8-fold at 16× MIC, indicating substantial release of intracellular contents (Fig. 3A). To directly probe outer membrane permeabilization, we monitored the uptake of the hydrophobic fluorophore 1-N-phenylnaphthylamine (NPN), which fluoresces upon partitioning into a compromised outer membrane. npEt20 elicited a rapid and time-dependent increase in NPN fluorescence in *A. baumannii 9968*, *K. pneumoniae 8649*, and *P. aeruginosa 26139*, with signals significantly exceeding those of untreated controls at every time point from 30 to 240 min ($p < 0.05$) (Fig. 3B–D). Collectively, these findings demonstrate that npEt20 acts through a membranelytic mechanism, permeabilizing the outer membrane, triggering leakage of intracellular macromolecules, and ultimately leading to bacterial death.

npEt20 Does not Readily Select for Resistance Upon Serial Passage

A critical limitation of many antibacterial agents is the rapid emergence of resistance under selective pressure. To evaluate the resistance-inducing potential of npEt20, the three carbapenem-resistant isolates were serially passaged for 10 generations in the presence of sub-inhibitory concentrations of npEt20, meropenem, or ceftazidime, and the fold change in MIC (MIC_n/MIC_0) was monitored at each passage (**Supplementary Fig. 1**). Whereas ceftazidime provoked a steep escalation in MIC, reaching ~128-fold by the end of the passaging series, and meropenem produced a more modest but appreciable increase, npEt20 elicited es-

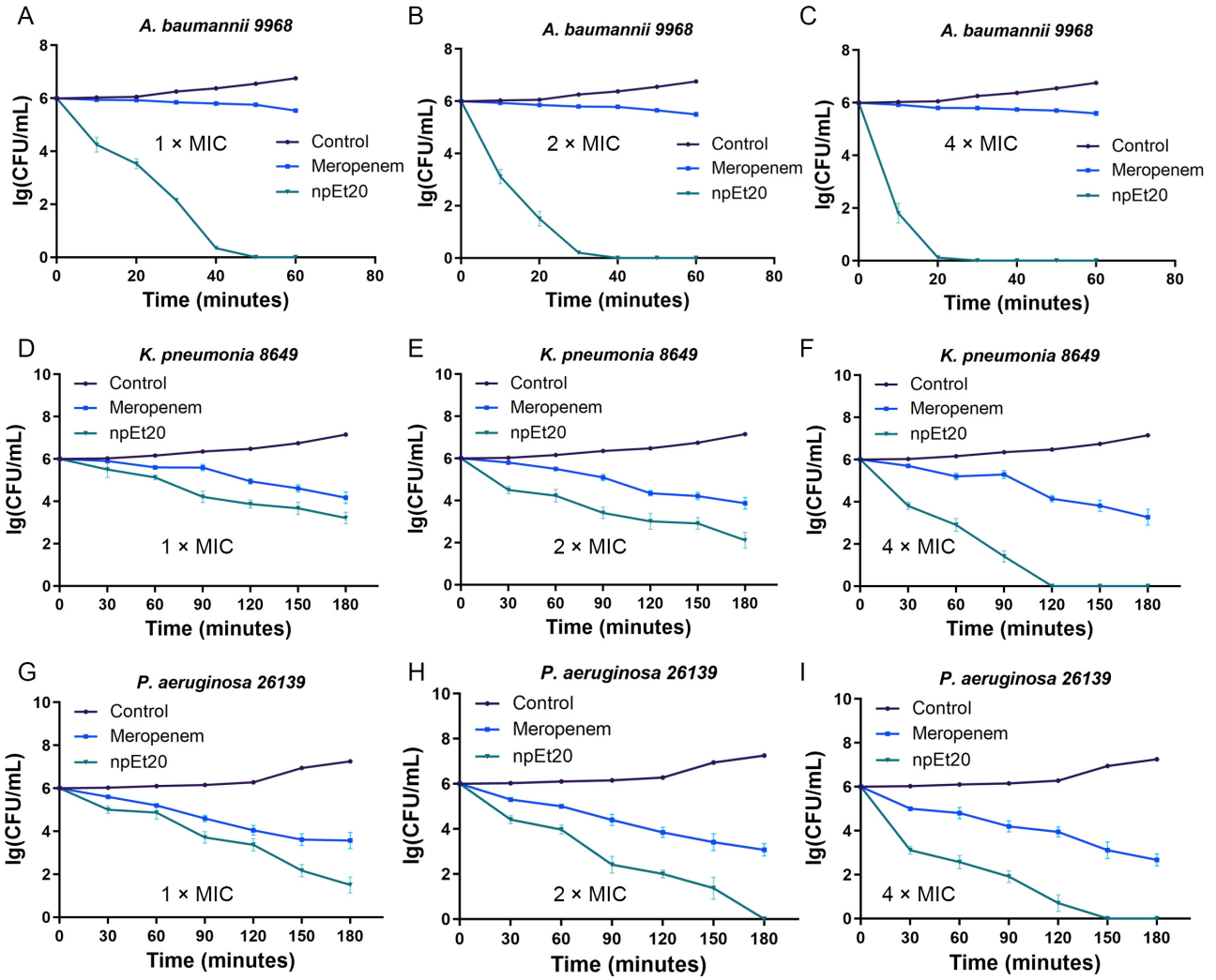

Fig. 1. Killing kinetics of npEt20 against three carbapenem-resistant clinical isolates *in vitro*. Time-kill curves showing CFUs over time for *A. baumannii 9968* (A–C), *K. pneumoniae 8649* (D–F), and *P. aeruginosa 26139* (G–I) following treatment with vehicle control, meropenem, or npEt20 at 1× MIC (A,D,G), 2× MIC (B,E,H), and 4× MIC (C,F,I). Error bars denote standard deviation for n = 3. Note: Some error bars are too small to be visible in the graph.

sentially no detectable change in MIC across all 10 generations in any of the three species (**Supplementary Fig. 1A–C**). The stable susceptibility profile under sustained npEt20 pressure indicates a low propensity for resistance development, a feature consistent with its membrane-targeting mode of action and advantageous for long-term therapeutic utility.

npEt20 Synergizes With Meropenem and Reverses Carbapenem Resistance In Vitro

Given that outer membrane permeabilization can facilitate antibiotic penetration, we hypothesized that npEt20 might potentiate meropenem against otherwise resistant strains. Checkerboard assays revealed clear synergy between npEt20 and meropenem for all three carbapenem-resistant isolates, with fractional inhibitory concentration indices (FICI) of 0.375 for *A. baumannii 9968*, *K. pneumoniae 8649*, and *P. aeruginosa 26139* (FICI ≤0.5 defined as synergy; Fig. 4A–C). Notably, in the presence of npEt20, the effective meropenem concentration required for inhibition was reduced several-fold below its MIC against each resistant strain. This synergistic effect was corroborated by colony-count assays, in which the npEt20–meropenem combination—using sub-inhibitory concentrations of each agent—significantly reduced viable bacterial loads relative to untreated controls ($p < 0.05$), far exceeding the marginal reductions achieved by either agent alone (Fig. 4D–F). These results demonstrate that npEt20 acts as a potent adjuvant that restores the bactericidal activity of meropenem against carbapenem-resistant Gram-negative pathogens *in vitro*.

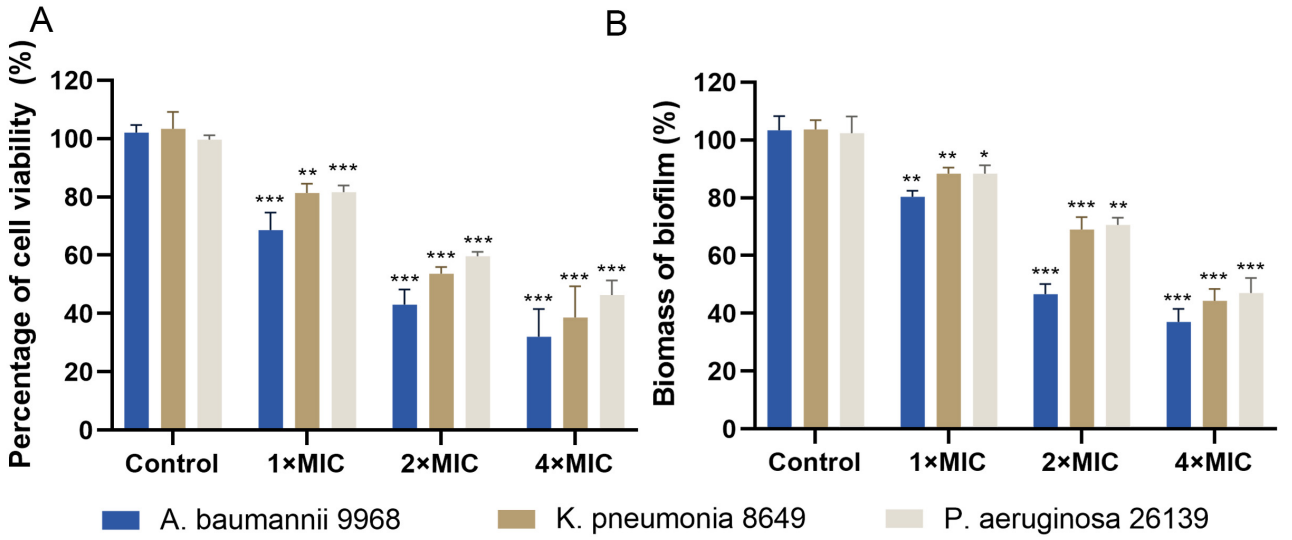

Fig. 2. Antibiofilm effects of npEt20 against three carbapenem-resistant clinical isolates *in vitro*. Quantification of relative viable cell percentage (A) and total biofilm biomass percentage (B) following 24-hour exposure to 1×, 2×, or 4× MIC npEt20. Statistical significance: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ versus the Control group. Error bars denote standard deviation for n = 3.

The npEt20–Meropenem Combination Restores Therapeutic Efficacy Against Systemic Infection In Vivo

To determine whether the *in vitro* synergy translated into therapeutic benefit, we employed a murine peritonitis model in which mice were infected via intraperitoneal injection with each carbapenem-resistant isolate and randomized to receive vehicle, meropenem, npEt20, or the npEt20–meropenem combination. At 24 h post-infection, bacterial burdens in blood, peritoneal fluid, spleen, liver, and kidney were quantified. For all three pathogens, the combination therapy produced a significant reduction in bacterial loads across all organ compartments compared with vehicle and either monotherapy ($p < 0.05$), whereas meropenem or npEt20 alone conferred only limited benefit (Fig. 5A–C). Consistent with the improved bacterial clearance, the npEt20–meropenem combination markedly prolonged survival: combination-treated mice exhibited high survival probabilities throughout the 7-day follow-up, while animals in the control and monotherapy groups succumbed to infection (Fig. 5D–F). Importantly, the combination regimen was well tolerated. Serum biochemical markers reflecting liver and renal function (ALT, AST, creatinine, urea nitrogen), as well as serum sodium and potassium levels, showed no statistically significant differences compared with the vehicle control group (**Supplementary Fig. 2**), indicating no evidence of acute hepatic or renal injury at the doses used in this model. A comprehensive toxicological assessment was beyond the scope of the present study. Taken together, these *in vivo* data confirm that npEt20 serves as a robust, broad-spectrum adjuvant capable of reversing meropenem resistance and protecting mice from lethal carbapenem-resistant Gram-negative infection.

Discussion

This study positions npEt20 as an agent with a dual identity—an intrinsic antibacterial and a broad-spectrum carbapenem adjuvant. The intrinsic bactericidal activity of npEt20 distinguishes it from most reported resistance-reversing agents. Current strategies for restoring carbapenem activity can be broadly divided into two categories. The first comprises adjuvants with little intrinsic antibacterial activity that act on defined enzymatic targets: disulfiram and its derivatives potentiate carbapenems by inhibiting New Delhi metallo-β-lactamase [9]; thiol-based small molecules resensitize metallo-β-lactamase–producing isolates to meropenem [16]; and clinically approved β-lactamase inhibitors, such as vaborbactam and relebactam, restore β-lactam activity against serine-carbapenemase producers [17,18]. Because these agents depend on a specific enzyme, their utility narrows when resistance is enzyme-independent or driven by a β-lactamase that they do not inhibit. The second category comprises membrane-active antibacterial polymers, which target the universal bacterial membrane and typically display broad-spectrum, rapid bactericidal activity with a low propensity to induce resistance [12]. npEt20 combines features of both categories. It killed all three carbapenem-resistant species within minutes and achieved complete clearance at 4× MIC, with MICs of 16–32 μg/mL that were substantially lower than those of meropenem (64–128 μg/mL) against the same isolates. Moreover, npEt20 also functioned as a meropenem adjuvant.

Mechanistically, the synergy between npEt20 and meropenem is consistent with disruption of the Gram-negative outer membrane. This lipopolysaccharide-rich

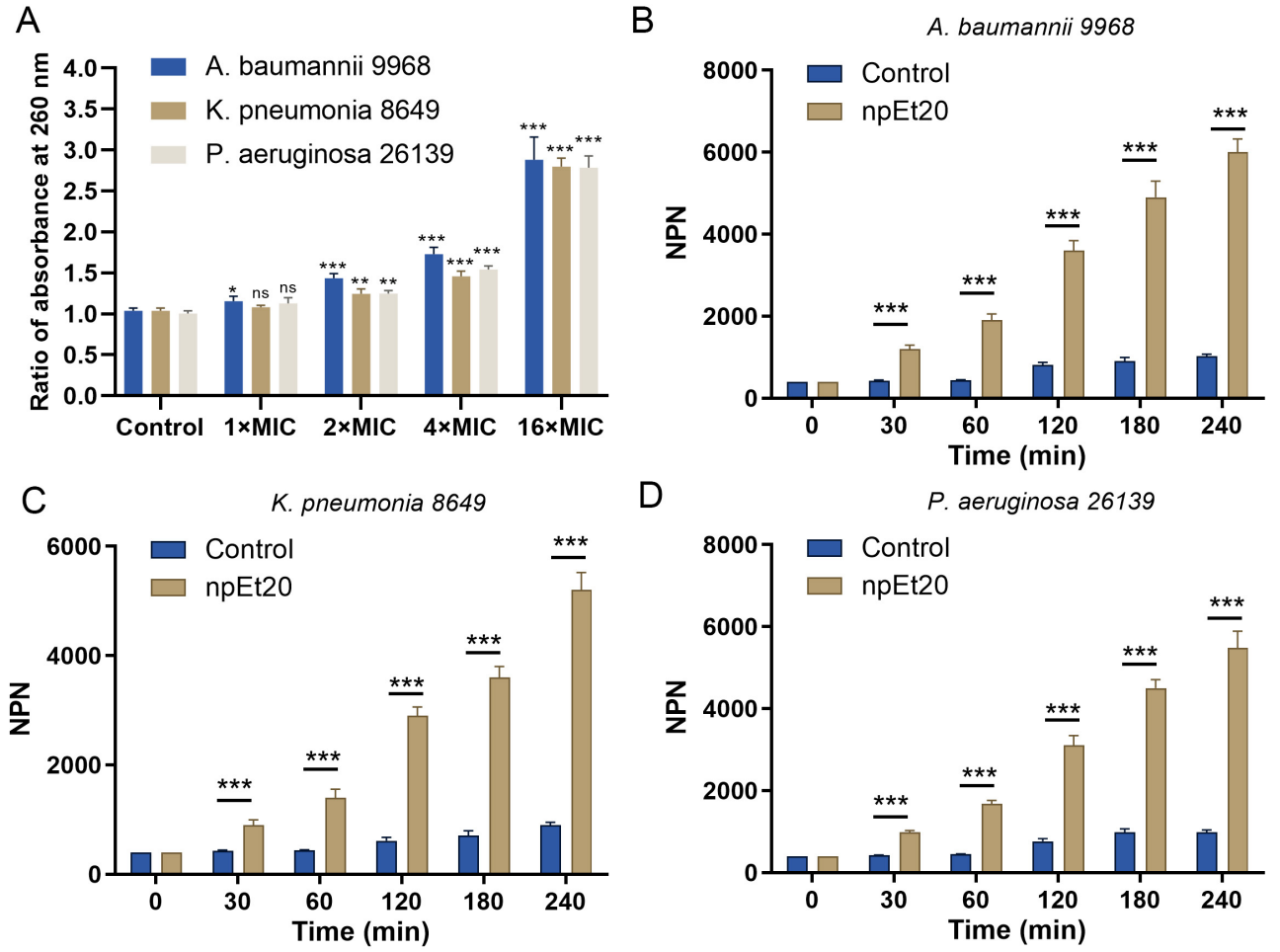

Fig. 3. Membrane-disrupting effects of npEt20 against three carbapenem-resistant clinical isolates. (A) Quantification of extracellular nucleic acid release (A_{260} absorbance ratio) across four npEt20 concentrations. (B–D) Time-dependent outer membrane permeabilization assessed by NPN fluorescence for each strain over 240 min. NS: not significant. Statistical significance: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus the Control group. Error bars denote standard deviation for n = 3.

bilayer limits β-lactam entry and contributes to both intrinsic and acquired resistance [7]. Permeabilizing agents can therefore increase intracellular antibiotic accumulation. Polymyxin B nonapeptide, the prototypical outer membrane permeabilizer, binds lipid A and displaces the divalent cations that stabilize lipopolysaccharide [10]; anionic adjuvants that sequester the divalent cations required for metallo-β-lactamases and lipopolysaccharide restore carbapenem activity across Gram-negative ESKAPE pathogens [19]. Two independent lines of evidence localize the action of npEt20 to the membrane: a concentration-dependent increase in the A_{260} ratio, reaching approximately 2.8-fold at 16× MIC and indicating nucleic acid leakage, and a time-dependent increase in NPN fluorescence, indicating outer membrane permeabilization. This mechanism offers a coherent explanation for the observed synergy, in which permeabilization facilitates meropenem penetration and lowers its effective concentration below the MIC of each resistant strain, yielding a consistent fractional inhibitory concentration index of 0.375 across the three species.

A further consideration concerns the emergence of resistance. Target-specific agents—including carbapenems, which acylate penicillin-binding proteins, and inhibitors directed at a single enzyme—are vulnerable to point mutations, enzyme variation, and efflux upregulation, mechanisms that underlie the rapid spread of carbapenem resistance [18]. Agents that act on the bacterial membrane are less readily circumvented by such adaptations. Consistent with this expectation, serial passage over ten generations resulted in a pronounced increase in the ceftazidime MIC, reaching approximately 128-fold, and an appreciable increase in the meropenem MIC, whereas the MIC of npEt20 remained essentially unchanged in all three species. A low propensity for resistance is not exclusive to membrane-active molecules; adjuvant combinations can also constrain resistance evolution, as reported for disulfiram combined with meropenem or colistin [9]. The contribution of this

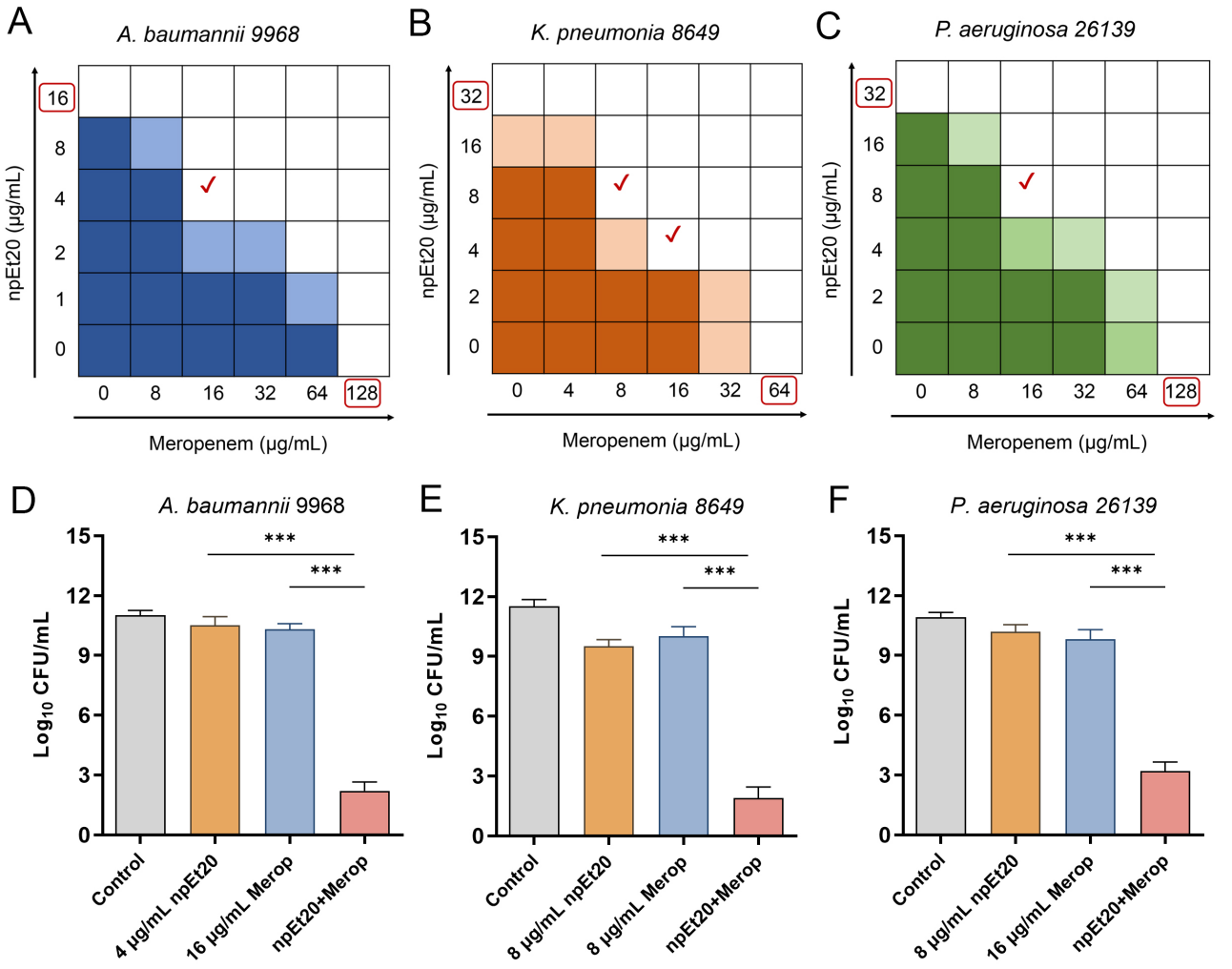

Fig. 4. Synergistic activity of npEt20 plus meropenem against three carbapenem-resistant clinical isolates *in vitro*. (A–C) Checkerboard assay heatmaps illustrating dose-dependent combinatorial inhibition of each strain by npEt20 and meropenem. Gradient color shades indicate the extent of bacterial growth inhibition; darker color corresponds to weaker inhibitory activity. Red-boxed squares denote the MIC values for individual drugs. Check marks indicate drug-combination points showing synergistic effects. (D–F) Colony count assays comparing bacterial loads following single-agent or combination treatment. ***$p < 0.001$. Error bars denote standard deviation for n = 3.

work lies in the consistency of the effect: npEt20 did not select for detectable resistance across three phylogenetically distinct pathogens, an outcome consistent with its membrane-lytic mechanism that supports its utility as a durable therapeutic component. Notably, membrane-targeted macromolecules also exert potent inhibitory effects on bacterial biofilm architecture [20], a critical reservoir of persistent resistant populations where conventional antibiotics often fail to achieve full bactericidal efficacy [21].

The translational value of a resistance-reversing agent ultimately depends on *in vivo* efficacy and safety, criteria that many candidates do not meet. Some have crossed this threshold: a macromolecular adjuvant reversed resistance in combination with rifampicin in a murine bacteremia model [22]; disulfiram combinations were effective in animal models of MDR infection [9]; and a conjugated polymer promoted clearance of carbapenem-resistant *K. pneumoniae* in a murine wound model [23]. The present data extend this evidence in two respects. First, the npEt20–meropenem combination reduced bacterial burden across blood, peritoneal fluid, spleen, liver, and kidney and prolonged survival in three separate carbapenem-resistant infection models, whereas either monotherapy conferred only limited benefit. Second, the combination was well tolerated, with serum ALT, AST, creatinine, and urea nitrogen, together with sodium and potassium, comparable to those of vehicle-treated animals. Given the group size of five, these data indicate the absence of gross acute hepatic or renal injury in this model but are not powered to exclude smaller effects. A formal preclinical safety evaluation will

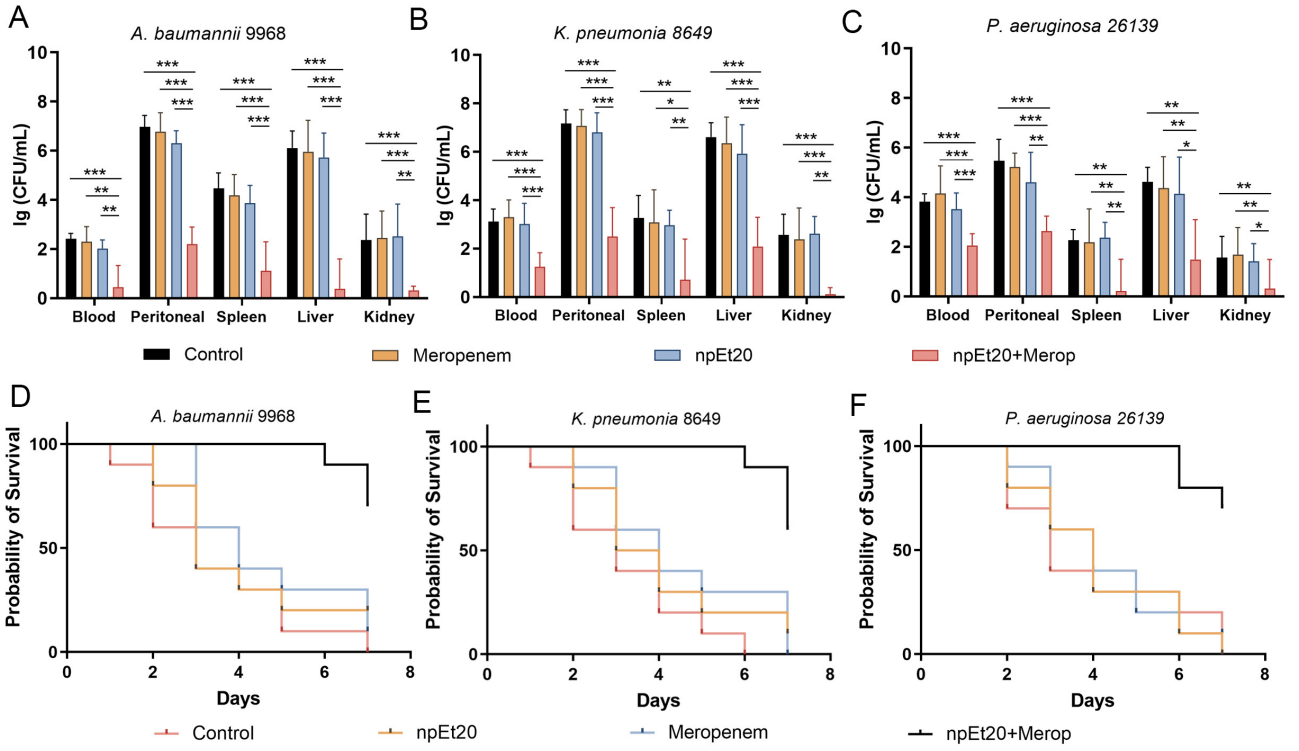

Fig. 5. Synergistic activity of npEt20 plus meropenem against three carbapenem-resistant clinical isolates *in vivo*. (A–C) Quantification of bacterial colony counts in major organs of infected mice at 24 h post-infection for all four treatment groups (n = 5 mice per group). Statistical significance: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus the PBS control group. Error bars denote standard deviation. (D–F) 7-day survival probability curves for infected mice receiving single-agent or combination therapy (n = 10 mice per group). Drugs were administered intraperitoneally at 1 h and 6 h post-infection, and survival was monitored daily for 7 days with no censored cases.

be required in future studies. The activity of npEt20 against biofilms further supports its therapeutic potential. Eradication of mature Gram-negative biofilms by antimicrobial peptides and polymers often requires high concentrations; for example, the minimum biofilm eradication concentration of cecropin Cec4 against carbapenem-resistant *A. baumannii* reaches 256–512 μg/mL [24,25]. By comparison, npEt20 reduced both the viability and the biomass of mature biofilms formed by all three pathogens at concentrations no higher than 4× MIC, highlighting its unique advantages over existing polymeric antibiofilm agents.

Conclusion

npEt20 is a membrane-active cationic copolymer that combines intrinsic broad-spectrum bactericidal activity with a low propensity to select for resistance, and it functions as a broad-spectrum adjuvant that restores meropenem activity against carbapenem-resistant *A. baumannii*, *K. pneumoniae*, *and P. aeruginosa* both *in vitro* and *in vivo*. By coupling effective outer membrane permeabilization with a favorable safety profile, npEt20 represents a promising preclinical candidate for combating refractory carbapenem-resistant Gram-negative infections in mouse models. Further clinical investigation is still required.

Availability of Data and Materials

The data used or analyzed during the current study are available from the first authors upon reasonable request (Yingjiao Zhang: yingying23456789@163.com; Nongyan Wang: 1258561756@qq.com).

Author Contributions

YJZ, NYW, HJL, GSZ contributed to the conception and design. YJZ, NYW, KY and MYL contributed to the data acquisition, data analysis, and manuscript drafting. All authors have been involved in revising it critically for important intellectual content. All authors have read and approved the final version of this manuscript. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

Animal ethics in this study was approved by the independent Ethical Committee of the First Affiliated Hospital, Zhejiang University (approval number: 202024). All

animal procedures were conducted in accordance with the ARRIVE 2.0 guidelines. Human ethics in this study were approved by the independent Ethical Committee of the First Affiliated Hospital, Zhejiang University (approval number: 202044). All procedures were conducted in accordance with the principles of the Declaration of Helsinki. Because the isolates were obtained from residual, de-identified diagnostic specimens collected during routine clinical care, the Ethics Committee granted a waiver of written informed consent.

Acknowledgment

Not applicable.

Funding

This research was supported by the Huadong Medicine Joint Fund of Zhejiang Provincial Natural Science Foundation of China (Grant No. LHDMZ24H300001).

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.24976/Discov.Med.202638212.224.

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