A glycosylated cationic block poly (β -peptide) reverses intrinsic antibiotic resistance in all ESKAPE Gram-negative bacteria

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Title: A glycosylated cationic block poly(beta-peptide) reverses intrinsic antibiotic resistance in Gram-negative bacteria

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Abstract: Carbapenem-resistant Gram-negative bacteria (GNB) are heading the list of pathogens for which antibiotics are the most critically needed. Many antibiotics are either unable to penetrate the outer-membrane or are excluded by efflux mechanisms. Here, we report a cationic block beta-peptide (PAS8-*b*-PDM12) that reverses intrinsic antibiotic resistances in GNB by two distinct mechanisms of action. PAS8-*b*-PDM12 does not only compromise the integrity of the bacterial outer-membrane, it also deactivates efflux pump systems by dissipating the transmembrane electrochemical potential. As a result, PAS8-*b*-PDM12 sensitizes carbapenem- and colistin-resistant GNB to multiple antibiotics *in vitro* and *in vivo*. The beta-peptide allows the perfect alternation of cationic versus hydrophobic side chains, representing a significant improvement over previous antimicrobial alpha-peptides sensitizing agents. Together, our results indicate that it is technically possible for a single adjuvant to reverse innate antibiotic resistance in all pathogenic GNB of the ESKAPE group, including those resistant to last resort antibiotics.

Introduction

The ESKAPE pathogens are commonly associated with antimicrobial resistance ^[1]. Of particular concern are the Gram-negative bacteria (GNB) ESKAPE pathogens *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter spp.* that are often recalcitrant to antibiotic treatment, with an emergence of resistance to carbapenems and colistin ^[2]. The high usage of carbapenems in hospitals has favoured the rapid emergence and spread of carbapenemases such as the New-Delhi metallo-beta-lactamase ^[3].

Transmission by horizontal gene transfer facilitates the dissemination of carbapenem-resistant GNB, which are recalcitrant to most therapeutic options. More recently, dissemination of mobilized colistin resistance (*mcr-1*) gene leading to plasmid-mediated colistin resistance has magnified the threat of carbapenem-resistant GNB infection ^[4]. Despite various efforts to develop new antibiotics ^[5], there has been no new class of antibiotics approved for the treatment of GNB infections in over 50 years ^[6]. Amongst various reasons, the primary challenge lies in the intrinsic antibiotic resistance of GNB mediated by the presence of both outer-membrane impermeability and efficient efflux pump systems, limiting antibiotic accumulation in the bacteria ^[5]. Repurposing Gram-positive agents is an attractive and cost-effective approach to develop new antibiotic treatment options.

Cationic antimicrobial peptides (AMPs) are ideal membrane permeabilization agents and are thought to be the last frontier in antibacterial development ^[7]. More than 3,000 AMPs have been discovered and listed in the Antimicrobial Peptide Database ^[8]. Though AMPs have been widely studied and often tested as direct antibacterial agents, their complex synthetic routes, proteolytic instability and lack of selectivity have hindered their translation to clinical use ^[9]. The GNB outer membrane is a fairly impermeable barrier that typically allows only small hydrophilic molecules to pass through. Due to the presence of the outer membrane, a high concentration of AMPs is required to cause cell death when used as direct antibacterial agent. On the other hand, a well-designed peptide may cause membrane pores even at sub-MIC concentrations ^[10], thereby increasing penetration of classical antibiotics. The use of AMPs as potentiating agents rather than direct killing agents may reduce their dosage and fill the gap as membrane-active agents that not many other small-molecule drugs could fill. Though many synergistic combinations of AMPs with antibiotics for treatment of bacterial infection have been reported ^[11], they act mainly by disrupting the outer-membrane integrity, and typically have a limited efficacy. Very few combinations have been reported to disrupt

efflux pump systems ^[11a, 11d]. The only report on a dual mechanistic peptide is oligo-acyl-lysyl. However the molecule has a limited spectrum of activity and poor *in vivo* potentiation effect ^[11d, 12].

To avoid degradation *in vivo*, beta-peptides seem to be an ideal choice because their biological activity is similar to natural alpha-peptides, but they are more resistant to proteolysis and are commonly non-mutagenic ^[13]. Further, beta-peptides possess both β^3 -position and β^2 -position in their backbone units so that hydrophobicity and cationic residues may be arranged in these respective positions to form a balanced alternating sequence. Thus, the cationic residues would cause the co-peptide electrostatic adsorption and its neighboring hydrophobic side chain would enhance its membrane insertion. Until now, a few beta-peptides have been synthesized based on the general design principles derived from alpha AMPs, but they have been evaluated only as direct bactericidal agents, with proof of concept studies performed only under *in vitro* conditions ^[14]. Grinstaff *et al.* reported a series of poly-amido-saccharide (PAS) amphiphiles that can modulate *P. aeruginosa* biofilm formation ^[15]. A beta-peptide (MM-TM) displays strong synergy against fungi with antifungal drugs ^[16], but there is no report on the use of beta-peptides as membrane-perturbing agents for potentiating antibiotics for effective bacterial infection treatment.

Herein, we report a block co-beta-peptide PAS8-*b*-PDM12, composed of a glycosylated block of PAS and another block of cationic poly-dimethyl beta-lactam (PDM) that effectively sensitize the highest priority ESKAPE GNB bacteria to classical antibiotics with high efficacy in multiple mouse models of bacterial infection.

Results

We employed a facile synthesis strategy - one-shot anionic ring opening block copolymerization - involving simultaneous addition of glucose-derived beta-lactam ^[17] and cationic beta-lactam monomers ^[18] with contrasting reactivities, followed by a one-pot global deprotection strategy, to achieve the glycosylated cationic block poly(beta-peptide) PASm-*block*-PDMn with high efficiency^[19] (Detailed in Supplementary section Part I). Biological profiling of the series revealed weak antibacterial activity, with MICs ranging from 16 to >512 µg/ml against representative GNB and Gram-positive bacteria. (Table S1). We focused our detailed studies on PAS8-*b*-PDM12 (Figure 1a), which displayed the most balanced selectivity, with MICs of 64 µg/ml against both *P. aeruginosa* PAO1 and *S. aureus* 29213, while being non-hemolytic (HC₅₀ >5,000 µg/ml) and relatively non-cytotoxic (IC₅₀ >500 µg/ml) (Table S1). Attempts to select for escape mutants resistant to PAS8-*b*-PDM12 in *P. aeruginosa* PAO1 were unsuccessful, giving an estimated frequency of spontaneous resistance below 1×10⁻⁹, which is similar to colistin and better than imipenem. PAS8-*b*-PDM12 displayed no acute toxicity when administered intravenously in mice at a daily dose of 10 mg/kg/day for 7 days, with no significant change in biomarkers for hepatotoxicity and nephrotoxicity nor blood electrolytes (Table S2). PAS8-*b*-PDM12 was stable in mouse plasma, whereas the antimicrobial peptides LL-37 and melittin lost their activity after 24 h incubation, illustrating a benefit of beta-peptides over natural peptides for therapeutic applications (Table S3).

Given the cationic nature of PAS8-*b*-PDM12, we hypothesized that the compounds interfere with GNB by destabilizing the bacterial cell envelope. 1-N-phenylnaphthylamine (NPN) uptake fluorescence assays were performed to evaluate the effect of PAS8-*b*-PDM12 on outer-membrane permeability. PAS8-*b*-PDM12 induced a dose-proportional increase in fluorescence intensity in all GNB bacteria tested, including *P. aeruginosa* PAO1 (Figure 1b)

and the clinical isolates *A. baumannii* ACBAS, *E. coli* ECOS and *K. pneumoniae* KPNS (Figure S1) indicating an increase permeability of the outer-membrane. The results revealed that PAS8-*b*-PDM12 compromised the impermeability of the outer-membrane at concentrations well below its growth inhibitory concentration. Cryo-Transmission Electron Microscopy (Cryo-TEM) and Stimulated emission depletion microscopy (STED) were also used to visualize the effect of glycosylated cationic block poly(beta-peptide) on the integrity of the cell envelope in *P. aeruginosa* PAO1. The images revealed significant membrane distortion (Figure 1d, 1g and 1h), and at places complete disruption of the samples treated with PAS8-*b*-PDM12 (Figure 1d and 1h). Fluorescence microscopy was used to study the integrity of mammalian cell membrane using ethidium homodimer-1 (EthD-1) as a membrane integrity indicator. The images showed that PAS8-*b*-PDM12 did not affect the integrity of mammalian cell membranes (Figure S2). Conversely, the non-selective AMP gramicidin caused significant mammalian cell membrane destabilization, as reflected by the high fluorescence detected (Figure S2)

Rifampicin is a high-molecular weight hydrophobic antibiotic that is largely inactive against GNB due to its inability to cross the outer-membrane ^[20]. Combination experiments were performed to test whether destabilization of the outer-membrane by PAS8-*b*-PDM12 at sub-inhibitory concentrations would sensitize GNB to killing by rifampicin. Checkerboard experiments revealed a dose-dependent potentiation of rifampicin by PAS8-*b*-PDM12 in *P. aeruginosa* PAO1 (Figure 2a). Moreover, the combination of PAS8-*b*-PDM12 and rifampicin was synergistically bactericidal against *P. aeruginosa* PAO1 (Figure 2b). We next tested if the potentiation of rifampicin by PAS8-*b*-PDM12 also applied to antibiotic-resistant GNB

clinical isolates (Table S4). The results showed that PAS8-*b*-PDM12 potentiated rifampicin by a factor of 4 to 512 in the tested GNB strains, including carbapenem- and colistin-resistant clinical isolates (Figure 2c and Table S5). The lack of synergy between PAS8-*b*-PDM12 and rifampicin in the Gram-positive bacterium *S. aureus* (Figure 2d) supports the hypothesis that the synergy between PAS8-*b*-PDM12 and rifampicin is mediated by destabilization of the outer-membrane, thus favoring rifampicin penetration in GNB. Measurements of drug accumulation inside bacteria confirmed that rifampicin level was significantly higher in PAS8-*b*-PDM12-treated *P. aeruginosa* PAO1 compared to untreated controls (Figure 2e). Synergy between PAS8-*b*-PDM12 and rifampicin was maintained in *P. aeruginosa* PAO750, which is a *P. aeruginosa* mutant strain deficient in the expression of all efflux pump systems (Figure 2f), showing that drug efflux is not involved in the positive interaction between PAS8-*b*-PDM12 and rifampicin.

The rifampicin-PAS8-*b*-PDM12 combination was next tested in murine sepsis models against four antibiotic-resistant GNB strains. PAS8-*b*-PDM12 or rifampicin alone were ineffective when tested alone (Figure 3a-3c and Figure S3). When co-administered, a single dose of drug-peptide combination administered by intraperitoneal injection triggered a reduction of several orders of magnitude in bacterial load in the four bacterial infection models tested (Figure 3a-3c and Figure S3). Bacterial reduction ranged from above 99.9% against *P. aeruginosa* PAER to near eradication in the pathogenic *E. coli* ECOR infection model (Figure 3a-3c and Figure S3). Reduction in bacteria number was observed not only in the peritoneal cavity but also in distal organs such as the liver, spleen and kidneys (Figure S4-S7). We observed damaged bacterial membranes in *P. aeruginosa* PAO1 harvested from PAS8-*b*-

PDM12-treated animals (Figure S8), suggesting that the synergistic interaction between PAS8-*b*-PDM12 and rifampicin *in vivo* involves outer-membrane permeabilization. Additionally, 100% of mice infected with antibiotic-resistant *A. baumannii* MDRAB treated with a single injection of PAS8-*b*-PDM12 + rifampicin survived up to 7 days vs. 24 hours for the untreated control animals (Figure 3d). In this model, the infection had spread in the blood and distal organs before drug treatment (Figure S9), showing the curative potential of the drug combination even for an established infection. Taken together, the studies demonstrate that PAS8-*b*-PDM12 sensitizes GNB to killing by rifampicin by a mechanism involving destabilization of the outer-membrane.

The observation that PAS8-*b*-PDM12 is also a growth inhibitor against *S. aureus* (Table S1) suggested that part of its biological activity is independent of the presence of an outer-membrane. Since PAS8-*b*-PDM12 has structural similarities to alpha-AMPs targeting bacterial cytoplasmic membranes, we tested its effect on the electrochemical potential gradient across the cytoplasmic membrane in *P. aeruginosa* PAO1. We used the DiSC₃(5) dye assay to detect depolarization of the cytoplasmic membrane. The DiSC₃(5) dye accumulates in energized plasma membranes where its fluorescence is quenched [21]. Upon dissipation of the transmembrane electrochemical gradient, fluorescent DiSC₃(5) is released. Fluorescence spectrometry was used to record the fluorescence intensity in a batch bacterial culture. We observed that PAS8-*b*-PDM12 triggered a dose-dependent depolarization of the cytoplasmic membrane in *P. aeruginosa* PAO1 at a dose as low as 4 µg/ml (Figure 4a). PAS8-*b*-PDM12 at 16 µg/ml had a stronger effect on membrane depolarization than the positive control gramicidin S (Figure 4a). Consistent with the requirement of the

transmembrane electrochemical gradient to transport aminoglycosides into bacteria ^[22], PAS8-*b*-PDM12 at sub-inhibitory concentration was slightly antagonistic with gentamycin, streptomycin, and neomycin (Table S6). Furthermore, transmembrane electrochemical potential is the source of energy for multiple biological processes, including motility ^[23], active transport and drug efflux ^[24]. PAS8-*b*-PDM12 at sub-inhibitory concentration drastically inhibited motility in *P. aeruginosa* PAO1 (Figure 4b), a process powered by the proton-motive force ^[23].

Since the electrochemical potential is required for efflux pumps to work, we tested drug efflux in PAS8-*b*-PDM12-treated *P. aeruginosa* PAO1. Using ethidium bromide as a broad efflux pump substrate, we showed by flow cytometry and microscopy that PAS8-*b*-PDM12 inhibited ethidium bromide efflux (Figure 4c-4d and Figure S10), at a fraction of the growth inhibitory concentration of the compound.

To validate the clinical relevance of the effect of PAS8-*b*-PDM12 on efflux, we tested whether the compound sensitized *P. aeruginosa* PAO1 to antibiotics known to be efflux substrates. In combination experiments, PAS8-*b*-PDM12 increased the sensitivity of *P. aeruginosa* PAO1 to several antibiotics which are efflux substrates, including trimethoprim (Figure 4e), moxifloxacin (Figure S11a), tetracycline, sulfamethoxazole, ciprofloxacin and novobiocin (Table S5). The synergy between PAS8-*b*-PDM12 and trimethoprim or moxifloxacin was abrogated in the efflux-pump deficient strain *P. aeruginosa* PAO750, confirming that the synergistic effect is linked to deactivation of the bacterial efflux pumps (Figure 4f and Figure S11b). Novobiocin (a Gram-positive antibiotic) is thought to lack

efficacy against GNB due to both restricted penetration through the outer-membrane^[25] and efflux mechanisms^[26] (Figure S12). Consistent with this, we found that novobiocin was more potent against the efflux pump deficient strain *P. aeruginosa* PAO750 (MIC₅₀= 20.9 µg/ml) compared to the parental *P. aeruginosa* PAO1 strain (MIC₅₀> 200 µg/ml). PAS8-*b*-PDM12 potentiated novobiocin against antibiotic-sensitive as well as antibiotic-resistant GNB strains (Figure 5a-5c and Table S5).

Given the high degree of intrinsic resistance to novobiocin, the drug was selected for further synergistic validation in *in vivo* sepsis models of GNB infections. The combination of PAS8-*b*-PDM12 and novobiocin was highly synergistic against three antibiotic-resistant GNB clinical isolates in murine *in vivo* studies (Figure 5d-5f and Figure S13-S15). A single co-administration of both drugs was highly potent, triggering a reduction in bacterial load ranging from 99.99% to 99.999%, whereas treatment with the individual agent had no significant efficacy. Proof of efficacy was further demonstrated by showing that the combination of PAS8-*b*-PDM12 + novobiocin protected mice from a lethal challenge with clinical antibiotic-resistant *A. baumannii* MDRAB (Figure 5g).

Conclusion

Given the slow pace at which new antibiotic classes are discovered and the high mortality rate associated with carbapenem-resistant GNB infections, strategies to rescue the activity of older classes are promising approaches^[27]. With the ubiquity of genome sequencing, target-based approaches for antibiotic development have been widely explored in the last two decades^[28]. However, many programs for GNB failed due to limited drug penetration or

active efflux from the cytoplasm. Therefore, new strategies for overcoming the two mechanisms of innate resistance to antibiotics have the potential to rescue a plethora of antibiotic drug candidates.

The glycosylated cationic block poly(beta-peptide) PAS8-*b*-PDM12 sensitized all GNB tested to numerous antibiotics, including some considered specific for Gram-positive bacteria. The observed synergistic effect of PAS8-*b*-PDM12 with antibiotics against GNB results from increased intracellular concentration of the drugs that is facilitated by two mechanisms -- the increase in outer-membrane permeability and prevention of antibiotic efflux. This dual mechanism of potentiation may be explained by the formation of hydrogen bonds with lipopolysaccharide of the outer membrane of GNB, and electrostatic/hydrophobic interaction with the cytoplasmic membrane. We hypothesize that the optimum alternation of the cationic ($-\text{CH}_2\text{NH}_2$) and hydrophobic ($(-\text{CH}_3)_2$) moieties: at the β^2 and β^3 positions, together with their almost similar lengths, improve their cytoplasmic membrane disruption. According to this model, the cationic moiety would cause electrostatic adsorption, while its neighboring hydrophobic side chain would enhance membrane insertion. For alpha-peptides which have only one dangling side chain, it is impossible to synthesize a perfect cationic-hydrophobic alternating peptide. Hence our beta-peptide effectively disturbed the outer leaflets of the cytoplasmic membranes of GNB at sub-MIC concentrations.

Unlike the recent report of pentamidine ^[20], PAS8-*b*-PDM12 not only increases the outer-membrane permeability but also depolarizes the cytoplasmic membrane, causing a dissipation of the electrochemical gradient which is the source of energy for drug efflux ^[24]. Loperamide is one of very few compounds that shares a common dual mechanism of action with PAS8-*b*-

PDM12 in GNB since it sensitizes GNB to tetracyclines by compromising the integrity of the outer-membrane and by dissipating the plasma membrane electrochemical gradient ^[21b]. However, the safety and pharmacokinetic properties of loperamide do not seem compatible with clinical use, especially for disseminated infections ^[21b, 29]. Remarkably, unlike pentamidine ^[20], the potency of PAS8-*b*-PDM12 was conserved against *P. aeruginosa*, a species with a particularly impermeable outer-membrane ^[30] and efficient efflux pump systems. The breadth of potentiation *in vivo* is a significant improvement since PAS8-*b*-PDM12 sensitized all antibiotic-resistant ESKAPE GNB to rifampicin or novobiocin. Development of antibodies to the block copolymer was not determined. Albeit development of antibodies against peptide antibiotics such as colistin is not reported, it is still possible that immune response may limit the efficacy of long-term or repeated dosing.

Many AMPs have been reported to synergize with antibiotics *in vitro* but translation to *in vivo* potency has been challenging because typical AMPs suffer from degradation by host proteolytic enzymes ^[31]. The intrinsic resistance to proteolytic degradation of PAS8-*b*-PDM12 facilitates the translation of *in vitro* synergistic potency into *in vivo* potency. Though colistin is a last-line treatment option for carbapenem-resistant GNB, the emergence of *mcr-1* mediated colistin resistant GNB has raised concerns ^[32]. It is therefore important to note that PAS8-*b*-PDM12 retained potency against *mcr-1 E. coli* NMT1833, a clinical isolate with increased resistance to colistin.

Together, our results demonstrate that PAS8-*b*-PDM12 disables the two major mechanisms of intrinsic antibiotic resistance in the highest WHO priority antibiotic-resistant GNB of the ESKAPE group, therefore sensitizing them to numerous antibiotic classes. Considering the

great risk posed by carbapenem and colistin-resistant GNB, the ease of synthesis of the PAS8-*b*-PDM12 coupled with its favorable toxicological profile and the wide range of GNB susceptibility, the glycosylated cationic block poly(beta-peptide) is a promising candidate for further evaluation as adjunct antibiotic therapy for the treatment of difficult to treat GNB infections.

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Figure 1. *In vitro* membrane activity of PAS8-*b*-PDM12 against *P. aeruginosa* PAO1. (a) Chemical structure of the glycosylated cationic block poly(β -peptide) PAS8-*b*-PDM12. (b) PAS8-*b*-PDM12 triggered a dose-dependent increase in outer-membrane permeability of *P. aeruginosa* PAO1 using NPN as an indicator of outer-membrane permeability. Polymyxin B was used as positive control. (c)-(d) Super resolution STED microscopy images of *P. aeruginosa* PAO1 without treatment (c) and treated with 100 μ g/ml of PAS8-*b*-PDM12 (d), scale bar 2 μ m. (e)-(h) Cryo-TEM images of *P. aeruginosa* PAO1 without treatment (e), (f) and in the presence of 100 μ g/ml PAS8-*b*-PDM12 (g), (h), scale bar = 200 nm.

Figure 2. PAS8-*b*-PDM12 potentiates the *in vitro* activity of rifampicin in GNB. (a) Checkerboard broth microdilution assays showing the dose-dependent potentiation of rifampicin by PAS8-*b*-PDM12 against *P. aeruginosa* PAO1. The bacterial density was measured by optical density at 600 nm. Darker regions represent higher bacterial density. (b) *In vitro* kill-kinetics against *P. aeruginosa* PAO1 of rifampicin (4 µg/ml), PAS8-*b*-PDM12 (16 µg/ml) and a combination of PAS8-*b*-PDM12 + rifampicin. Data were expressed as mean $\pm$ standard deviation (n=3). (c) Checkerboard broth microdilution assays showing the dose-dependent potentiation of rifampicin by PAS8-*b*-PDM12 against *mcr-1 E. coli* NMT1833. (d) Checkerboard broth microdilution assays showing absence of potentiation of rifampicin by PAS8-*b*-PDM12 against the Gram-positive bacterium *S. aureus* MRSA-BAA40. (e) Rifampicin accumulation inside *P. aeruginosa* PAO1 treated by rifampicin alone (5 µg/ml) or with a combination of PAS8-*b*-PDM12 (30 µg/ml) + rifampicin (5 µg/ml) quantified by LC-MS. Data were expressed as mean $\pm$ standard deviation (n=4); Student's t-test was used for

statistical analysis, $P=0.0001$. (f) Checkerboard broth microdilution assays showing the dose-dependent rifampicin potentiation by PAS8-*b*-PDM12 in *P. aeruginosa* efflux pumps deficient strain PAO750.

Figure 3. PAS8-*b*-PDM12 potentiates the *in vivo* activity of rifampicin against different antibiotic-resistant GNB. (a) to (c) PAS8-*b*-PDM12 dependent potentiation of rifampicin in a murine systemic infection model of clinical antibiotic-resistant GNB: *P. aeruginosa* PAER (a); *E. coli* ECOR (b) and *A. baumannii* MDRAB (c). Mice received either PBS control (A, blue circles), PAS8-*b*-PDM12 alone (B, green diamonds), rifampicin alone (C, red stars) or a combination of PAS8-*b*-PDM12 + rifampicin (D, purple triangles). PAS8-*b*-PDM12 was used at 25 mg/kg against PAER (a) and ECOR (b) and at 10 mg/kg against MDRAB (c). Rifampicin was used at 10 mg/kg against PAER (a), 5 mg/kg against ECOR (b), and 0.2 mg/kg against MDRAB (c). Five animals per condition were used. Bacterial counts in the intraperitoneal fluid were determined by CFU determination on LB agar plates. Symbols represent the bacterial count in one mouse, and black lines represent the geometric mean of the individual count (n=5). Statistical analysis was done by one-way ANOVA followed by

Dunnett test. (d) Survival of mice in a murine systemic infection model of clinical antibiotic-resistant GNB *A. baumannii* MDRAB. Mice received either PBS control, 10 mg/kg PAS8-*b*-PDM12, 0.2 mg/kg rifampicin or a combination of PAS8-*b*-PDM12 + rifampicin. Mice viability was followed up to 7 days post-infection.

Figure 4. PAS8-*b*-PDM12 dissipates the membrane electrochemical gradient and deactivates efflux mechanism in *P. aeruginosa*. (a) Dose-dependent dissipation of the cytoplasmic membrane potential by PAS8-*b*-PDM12 in *P. aeruginosa* PAO1 using the DiSC₃(5) assay. Bacteria were treated with 1 $\mu\text{g/ml}$ (red line), 4 $\mu\text{g/ml}$ (green line), 16 $\mu\text{g/ml}$ (purple line), 64 $\mu\text{g/ml}$ (orange line), 256 $\mu\text{g/ml}$ (black line) PAS8-*b*-PDM12, Gramicidin (blue line) was used as positive control at 1 mg/ml. Data were expressed as mean $\pm$ standard deviation (n=3). (b) PAS8-*b*-PDM12 decreased the swim motility of *P. aeruginosa* PAO1 at sub-inhibition concentration. Scale bar=7 mm. (c) PAS8-*b*-PDM12 triggered a dose-dependent increase in

ethidium bromide accumulation in *P. aeruginosa* PAO1. The efflux pumps deficient strain *P. aeruginosa* PAO750 was used as control. Fluorescence intensity was measured by flow cytometry. (d) Quantification of ethidium bromide (EtBr) positive bacteria in *P. aeruginosa* PAO1 (Blue bars) and in the efflux pumps deficient strain *P. aeruginosa* PAO750 (red bars). Percentage of ethidium bromide positive bacteria was measured by flow cytometry. Data were expressed as mean $\pm$ standard deviation (n=3). Checkerboard broth microdilution assays showing a dose-dependent potentiation of trimethoprim by PAS8-*b*-PDM12 in *P. aeruginosa* PAO1 (e), and absence of potentiation of trimethoprim by PAS8-*b*-PDM12 in the efflux pumps deficient strain *P. aeruginosa* PAO750 (f). Darker regions represent higher bacterial density.

Figure 5. PAS8-*b*-PDM12 potentiates the *in vitro* and *in vivo* activity of novobiocin against different antibiotic-resistant GNB. Checkerboard broth microdilution assays showing the dose-dependent potentiation of novobiocin by PAS8-*b*-PDM12 against clinical antibiotic-

resistant GNB: *E. coli* ECOR (a); *K. pneumoniae* KPNR (b) and *A. baumannii* MDRAB (c). Darker regions represent higher bacterial density. PAS8-*b*-PDM12 dependent potentiation of novobiocin in a murine systemic infection model of clinical antibiotic-resistant GNB: *E. coli* ECOR (d); *K. pneumoniae* KPNR (e) and *A. baumannii* MDRAB (f). Mice received either PBS control (A, blue circles), PAS8-*b*-PDM12 alone (B, green diamonds), novobiocin alone (C, red stars) or a combination of PAS8-*b*-PDM12 + novobiocin (D, purple triangles). PAS8-*b*-PDM12 was used at 25 mg/kg. Novobiocin was used at 20 mg/kg against ECOR (d), 15 mg/kg against KPNR (e), and 10 mg/kg against MDRAB (f). Five animals per condition were used. Black lines represent the geometric mean of the individual count (n=5). Statistical analysis was done by one-way ANOVA followed by Dunnett test. (g) Survival of mice in a murine systemic infection model of clinical antibiotic-resistant clinical isolates *A. baumannii* MDRAB. Mice received either PBS control, 25 mg/kg PAS8-*b*-PDM12, 10 mg/kg novobiocin, or a combination of both drugs. Mice viability was followed up to 7 days post-infection.

Part I: Chemical Synthesis Section

General methods and instrumentation

All commercially available chemical compounds were purchased from Sigma-Aldrich, Alfa-Aesar, Tokyo Chemical Industry, Cambridge Isotope Laboratories or Aik Moh Paints & Chemicals. Anhydrous toluene was freshly distilled from sodium under argon. Anhydrous tetrahydrofuran (THF) was freshly distilled from sodium and benzophenone was added as indicator and oxygen scavenger under argon. Anhydrous dichloromethane (DCM) was distilled from calcium hydride under argon. Analytical thin-layer chromatography (TLC) was performed on Merck 60 F254 pre-coated silica gel plate. Visualization was performed under UV lamp, potassium permanganate stain or iodine stain. Column chromatography was carried out using Merck silica gel 60. High-purity water with a resistivity of $> 15 \text{ M}\Omega \text{ cm}$ was obtained from a Merck Millipore Integral 3 water purification system. Proton and carbon nuclear magnetic resonance (^1H NMR and ^{13}C NMR) spectroscopy were recorded on a Bruker Avance DPX-300 spectrometer at 300MHz for ^1H and 75 MHz for ^{13}C . ^1H chemical shift was recorded relative to CDCl_3 (7.26 ppm), or HOD (4.79 ppm). ^{13}C chemical shift was recorded relative to CDCl_3 (77.0 ppm). Monomer conversion was calculated by Agilent 6890N gas chromatography (GC) equipped with a HP-5 column using anhydrous dodecane as internal standards. The molecular weight (M_n and M_w) and molecular weight distribution ($\mathcal{D}$) of protected polymers were measured by gel permeation chromatography (GPC) on a Shimadzu liquid chromatography system equipped with a Shimadzu refractive index detector

(RID-10A) and two Polargel columns operating at 40 °C using DMF (with 1 mg/ml LiBr) as the mobile phase at a flowrate of 1 ml/min. Narrow dispersed polystyrene standards was used as reference.

Beta-lactam monomer preparation

Glucose derived beta-lactam was synthesized according to the reported procedure ^[1]. In order to deprotect all the protecting group in one pot, β -lactam DM(Cbz) protected by carboxybenzyl (Cbz) instead of *tert*-butyloxycarbonyl (Boc) group was synthesized by a modification of published method ^[2]. 2.58 g (10 mmol, 1 eqv.) ($\pm$)-trans-3-phthalimidylmethyl- 4,4-dimethyl azetidin-2-one (Phth-DM) and 20 ml methanol were added into a 50 ml round-bottom flask. 1.87 ml (30mmol, 3 eqv.) hydrazine hydrate were injected into the mixture. The resulting solution was stirred at room temperature overnight under argon protection. The slurry precipitate was filtered off through celite and washed with copious methanol. The collected solvent was removed by rotary evaporation. The collected intermediate was dissolved into a 60 ml mixture of 1:1 THF: saturated sodium bicarbonate, to which 2.9 ml (15mmol, 1.5 eqv.) benzyl chloroformate was adding. The reaction mixture was refluxed for 5 h. The concentrated residue was extracted with ethyl acetate and the organic phase was washed with 1M HCl, 1M NaOH and saturated NaCl solution. The combined organic solution was dried by anhydrous MgSO₄ and then purified by chromatography using ethyl acetate and hexane as eluent (1:1). Beta-lactam DM(Cbz) was first obtained as a sticky transparent liquid and was solidified by sticking. The obtained beta-lactam DM(Cbz) was further purified by recrystallization from dichloromethane and hexane. ¹H NMR (300 MHz,

CDCl₃) δ 1.38 (s, 3H), 1.46 (s, 3H), 3.01 (dd, J = 8.8, 6.9 Hz, 1H), 3.39 (t, J = 11.5 Hz, 1H), 3.68 (dt, J = 13.8, 6.9 Hz, 1H), 5.13 - 5.09 (m, 2H), 5.28 (s, 1H), 6.14 (s, 1H), 7.38-7.28 (m, 5H). ¹³C NMR (75 MHz, CDCl₃) δ 22.9, 28.6, 37.6, 54.8, 58.1, 66.9, 128.1, 128.5, 136.4, 156.3, 168.7. HRMS (ESI, m/z) calcd. for C₁₄H₁₈N₂O₃H⁺: 263.1396, found: 263.1393. Melting point 71-73 °C.

General procedure for one-shot polymerization

The polymerization process was carried out in a glovebox. Solutions of monomer (0.4 mmol), 4-*tert*-butylbenzyl chloride co-initiator (0.02 mmol), and bis(trimethylsilyl)amide (LiHMDS) catalyst (0.02 mmol), were prepared separately in anhydrous THF. Taking PAS8-*b*-PDM12 as an example: into an oven-dried 10 ml reaction flask were added 600 μ l of DM(Cbz) monomer, and 400 μ l of AS(Bn) monomer solutions and 1 ml of co-initiator solution. The mixture was stirred for 3 min at room temperature, followed by addition of 2.5 ml of catalyst solution (2.5 equiv relative to the co-initiator) to initiate the reaction. The reaction was stirred at room temperature for 1 hour and then was quenched with a few drops of methanol. The polymer solution, approximately 4.5 ml in volume, was added into a 50 ml centrifuge tube containing 40 ml hexane to precipitate the protected polymer product. The protected polymers were collected by centrifugation and decantation of the supernatant. The collected polymers were re-dissolved in 3 ml dichloromethane, and the precipitation/centrifugation steps were performed two further times. Finally, the polymers were dried under argon and further dried under vacuum provided by an oil pump. The protected polymers were analyzed by NMR and by GPC with DMF containing 1mg/ml LiBr as mobile phase.

General procedure for one-pot global deprotection

140 mg protected polymers and 54 mg potassium tert-butoxide (KOt-Bu) were dissolved in 5.0 ml of anhydrous THF. The polymer solution was added dropwise to a rapidly stirring solution of sodium (120 mg) in liquid ammonia (~15 ml) at -78 °C under nitrogen. The sodium was washed with anhydrous toluene and hexane and cut into small pieces before addition. The reaction was stirred at -78 °C for 1 h, after which saturated ammonium chloride was added drop by drop until the blue color disappeared. The remaining ammonia was evaporated at room temperature. The resulting solution was filtered, washed with DI water and dialyzed with 1000 Da cutoff dialysis bag for 2 days with frequent changes of water. Finally, the white powder product was obtained by dry freezing.

Scheme S1. General chemical scheme of one shot AROP polymerization and one pot of global deprotection strategies for synthesizing glycosylated cationic block beta peptides

^1H NMR of cationic dimethyl-derived β -lactam (DM(Cbz))

^{13}C NMR of cationic dimethyl-derived β -lactam (DM(Cbz))

Gel permeation chromatography (GPC) of PAS m -*b*-PDM n

^1H NMR of PASm-*b*-PDMn (protected) in CDCl_3

^1H NMR of PASm-*b*-PDMn in D_2O

^1H NMR of PDM20 in D_2O

^1H NMR of PAS2-*b*-PDM18 in D_2O

^1H NMR of PAS4-*b*-PDM16 in D_2O

^1H NMR of PAS6-*b*-PDM14 in D_2O

PAS8-*b*-PDM12

¹H NMR of PAS8-*b*-PDM12 in D₂O

PAS10-*b*-PDM10

¹H NMR of PAS10-*b*-PDM10 in D₂O

PAS20

^1H NMR of PAS20 in D_2O

HMQC NMR of PAS8-*b*-PDM12

COSY NMR of PAS8-*b*-PDM12

Part II: Biological Section

Microbial Strains, culture medium and inoculums preparation

The test microorganism strains selected to evaluate the antibacterial activity of glycosylated cationic poly(beta-peptides) and their synergistic effect with antibiotics was *Pseudomonas aeruginosa* PAO1 (gift from Scotts Rice at Nanyang Technology University). The isogenic multiple Mex pump mutant, *P. aeruginosa* PAO750 ($\Delta(\text{mexAB-oprM})$, $\Delta(\text{mexCD-oprJ})$, $\Delta(\text{mexEF-oprN})$, $\Delta(\text{mexJK})$, $\Delta(\text{mexXY})$, ΔopmH , ΔpscC) ^[3] was kindly contributed by Ayush Kumar at University of Manitoba. Clinical isolates, PAER (multi-drug resistant *P. aeruginosa*), ACBAS (PAN-sensitive *Acinetobacter baumannii*), MDRAB (multi-drug resistant *A. baumannii*), KPNS (PAN-sensitive *K. pneumonia*), KPNR (carbapenem resistant *K. pneumoniae*), ECOS (PAN-sensitive *E. coli*), ECOR (multi-drug resistant *E. coli*) and *mcr-1* colistin resistant *E.coli* NMT 1833 were from Tan Tock Seng Hospital. Gram-positive strains *Staphylococcus aureus* (ATCC #29213), and *S. aureus* (ATCC #BAA 40) were purchased from ATCC. All the microbial strains were streaked on a fresh Luria broth (LB) agar plate from the glycerol stock and incubated overnight at 37 °C. Inocula were prepared by transferring single colony from each strain to sterile Muller Hilton Broth (MHB) and incubated at 37°C with vigorous shaking (200 rpm) to obtained culture in the logarithmic growth phase. The culture was then washed and diluted to the desired optical density (OD).

Minimum inhibitory concentration (MIC)

The MIC values of the glycosylated cationic poly(beta-peptides) against the tested microorganisms were determined by broth microdilution method using 96-well microtiter plates as recommended by the CLSI guideline with slight modification. The poly(beta-peptide) stock solutions of 10 mg/ml were prepared in deionized water (DI water). The MHB (50 µl) was added into a 96-well plate and used to dilute the poly(beta-peptides). 50 µl of bacterial suspension was then added into each well and incubated at 37 °C for 18 h. Bacterial growth was determined by measuring the optical density at a wavelength of 600 nm (OD₆₀₀) on a TECAN, infinite F200, or by visual examination of the turbidity. MIC values were defined as the lowest concentration that leads to a reduction in bacterial growth by more than 90%, or by the absence of a defined bacterial pellet.

***In vitro* cytotoxicity measurement**

Mammalian cell biocompatibility test

Cell lines: The fibroblasts 3T3 cell line obtained from ATCC (#CRL-1658™) were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) and antibiotics (glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 µg/ml). The cells were maintained at 37 °C in a humidified incubator with 5% CO₂ until a monolayer with greater than 80% confluence, was obtained.

Cytotoxicity assay: Synthetic glycosylated cationic poly(beta-peptides) were tested for *in vitro* cytotoxicity, using 3T3 fibroblast cell lines by colorimetric assay the 3-(4,5-

dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (4). Briefly, the cultured cell lines were harvested from the flask by trypsinization and pooled into a 50 ml centrifuge tube. Then, the cells were plated at a density of 2×10^4 cells/ml per well (200 μ l) into a 96-well tissue culture plate. The plate was incubated at 37 °C in a humidified with 5% CO₂ incubator for 24 h without agitation. The poly(beta-peptide) stock solution was prepared in sterilized phosphate buffer saline (PBS) at a concentration of 10 mg/ml and diluted to desired concentrations in DMEM complete medium. poly(beta-peptides) in DMEM solution was added into the 96-well plate (cell seeded plate) and control (without compounds) was added as a comparison. The plate was incubated at 37 °C in a humidified with 5% CO₂ incubator for 24 h without agitation. After 24 h, the culture medium containing the compounds was discarded and the cells were rinsed with PBS. MTT (200 μ l of 5 mg/ml) was added into each well and the plate was incubated in a CO₂ incubator for additional 4 h. The MTT was aspirated and dimethyl sulfoxide (DMSO) was added into each well and the plate was kept in the shaker (at 150 rpm) until the intracellular formazan crystals were completely solubilized. The absorbance of each well was measured at 570 nm using microplate reader spectrophotometer (BIO-RAD, Benchmark Plus). The percentage of the cell viability was calculated by the formula:

$$\% \text{ Cell viability} = (\text{Average abs of treated cells}) / (\text{Average abs of controls}) \times 100\%$$

Hemolytic activity test

Fresh human blood (5 ml) was collected from a healthy donor (age 28, male). Erythrocytes were separated by centrifugation at 1,000 g for 10 min, washed three times with Tris buffer

(10 mM Tris, 150 mM NaCl, pH 7.2) and diluted to a final concentration of 5% vol/vol. 50 μ l of poly(beta-peptide) solution at different concentrations were mixed with 50 μ l of erythrocytes suspension, followed by addition into a 96-well microplate and incubated for 1 h at 37 °C with shaking at 150 rpm. The microplate was centrifuged at 1000 g for 10 min. 80 μ l aliquots of the supernatant were then transferred into a new 96-well microplate and diluted with another 80 μ l of Tris buffer. Hemolytic activity was determined at 540 nm with a 96-well plate spectrophotometer (Benchmark Plus, BIO-RAD). Triton X-100 (0.1% in Tris buffer), which is able to lyse RBCs completely, was used as positive control, while Tris buffer was used as negative control. The hemolysis percentage was calculated from the following equation:

$$\text{Hemolysis percentage} = [(O_p - O_b)/(O_t - O_b)] \times 100\%$$

where O_p is the absorbance for the poly(β -peptides), O_b is the absorbance for the negative control (Tris buffer), and O_t is the absorbance for the positive control (Triton X-100).

The study and protocol were approved by the Nanyang Technological University (NTU INSTITUTIONAL REVIEW BOARD APPROVAL), under the approved protocol No. IRB-2015-03-040-02.

***In vivo* systemic toxicity test**

All animal experiments were carried out in accordance with the Code of Practice for the Institutional Care and Use of Animals for Scientific Purposes approved by the Ethics Committee of Ningbo University.

10 female BALB/c mice (6–9 weeks, 16–20 g) were selected randomly and put into two cages with 5 mice per cage. The mice were injected with PAS8-*b*-PDM12 at dosage of 10 mg/kg/day for 7 days. Injection was given intravenously through the tail vein of mice. The liver and kidney function as well as balance of electrolytes were determined before treatment (0 day) and after 7 days' repetitive treatment using celecare M (MNCHIP, Tianjin).

Plasma stability study

10 mg/ml PAS8-*b*-PDM12, colistin, LL-37 and melittin were incubated in fresh mice plasma at 37 °C for 24 hours. The plasma was then diluted and subjected to standard MICs tests.

Resistance study

2.6×10^7 CFU/ml log phase *P. aeruginosa* PAO1 was incubated with 10×MICs PAS8-*b*-PDM12, colistin or imipenem, in 20 ml fresh MHB solution in duplicates (total number of bacteria: 1.04×10^9 CFUs). The tubes were incubated for up to 7 days, or until turbidity was observed. On the fourth day, the tube of bacteria exposed to imipenem became gradually turbid. Conversely, the tubes of bacteria exposed to PAS8-*b*-PDM12 or colistin remained clear for 7 days. Selection of putative spontaneous resistant mutant was further verified by the standard MIC testing after isolation on agar plates. No resistant mutants could be obtained for PAS8-*b*-PDM12 and for colistin.

Checkerboard Assay

The checkerboard method ^[4] was used to assess antibacterial synergism between the activities of antibiotics and glycosylated cationic poly(beta-peptides). Briefly, serial twofold dilutions of each antibiotic and glycosylated cationic poly(beta-peptide) were prepared separately. After dilution, a total of 12.5 µl of antibiotic in Mueller Hinton Broth (MHB) and 12.5 µl of

poly(beta-peptide) in MHB (increasing concentrations along y- and x-axes, respectively) was dispensed into the wells of a 384-well plate, followed by addition of 25 μ l of bacterial inoculum of 1×10^6 CFU/ml in MHB into each well. The plate was incubated at 37 °C with shaking at 200 rpm for 18 h under aerobic conditions. The bacterial growth was assessed by measuring the OD₆₀₀.

Time-kill Assay

Time-kill study was conducted by incubating bacteria with poly(beta-peptide), antibiotic and their combination, and bacteria viability was determined by CFU count on LB agar plates at various time points. Briefly, 100 μ l 2 X target concentration of poly(beta-peptide), antibiotic and their combination were added into a 96-well plate, followed by addition of 100 μ l of *P. aeruginosa* PAO1 at a density of 1×10^6 CFU/ml in MHB medium. Bacteria in MHB suspension without poly(beta-peptide) and antibiotic was used as negative control. The plate was incubated aerobically with shaking at 37 °C. At t = 0 h, 1 h, 3 h, 7 h, 12 h and 18 h; bacteria viability was determined by CFU plating on LB agar plates.

Stimulated emission depletion microscopy (STED)

To prepare samples for super resolution STED confocal microscopy, bacterial cells were pelleted down by centrifugation at 3,000 X g for 10 min, resuspended in culture media at a concentration of 10^8 CFU/ml and incubated for 1 h with PAS8-*b*-PDM12. Membrane stain FM1-43FX (5 μ g/ml; Life Technologies) was added into the samples before washing with sterilized phosphate buffer saline (PBS) and resuspension in a fixing solution of 2% paraformaldehyde in PBS. Bacterial cells were fixed for 2 h at room temperature, washed with PBS and spotted onto a sterile glass bottom collagen coated dish (MatTek Corporation).

STED super resolution microscopy was performed with a Leica TCS SP8 STED-3X (Leica Microsystems, Wetzlar, Germany) at SingHealth Advanced Biomaging Core, Singapore. Further image processing requires deconvolution using Huygens Professional software (Scientific Volume Imaging, Hilversum, Netherlands).

Cryo-TEM microscopy

Mid-log phase bacterial cells were centrifuged at 3,000 X g for 10 min, resuspended in culture medium at a concentration of 10^8 CFU/ml and incubated for 1 h with PAS8-*b*-PDM12. Bacteria suspension was centrifuged and resuspended in PBS to prepare sample for cryo-TEM imaging at IMB-IMCB Joint Electron Microscopy Suite, A*STAR, Singapore.

Outer-membrane permeability assay

1-N-phenylnaphthylamine (NPN) assay was conducted on Gram-negative bacteria to determine the ability of the poly(beta-peptides) to permeabilize outer-membranes [5]. Bacteria overnight cultures were prepared by inoculating a few colonies in MHB. A pre-culture of bacteria was prepared by diluting overnight culture to an OD₆₀₀ of 0.01 in fresh MHB; this was incubated with shaking at 37°C, 200 rpm to mid-log phase (OD₆₀₀= 0.5). Bacterial cells were then collected by centrifugation at 3,000 X g for 10 min, washed twice with 5 mM HEPES buffer and resuspended to a final concentration of 10^7 CFU/ml in 5 mM HEPES buffer. 50 µl of 40 µM NPN solution was added to 100 µl of bacteria suspension in a 96-well black plate (Corning Costar). 50 µl of poly(beta-peptide) in HEPES buffer solution (at desired concentration) was added, and fluorescent signal was immediately measured (TECAN, infinite F200) at an excitation wavelength of 350 nm and an emission wavelength of 420 nm. The following negative controls were also recorded: 100 µl of bacteria + 100 µl of

HEPES buffer; 50 μ l of 40 μ M NPN + 150 μ l of HEPES buffer; 100 μ l of bacteria + 50 μ l of HEPES buffer + 50 μ l of 40 μ M NPN.

Mammalian cell membrane stability assay

3T3 cells were seeded at a density of 2×10^4 cells/well in a 96-well tissue culture plate and incubated at 37°C with 5% CO₂ for 24 h. The cells were treated with 100 μ g/ml PAS8-*b*-PDM12 or gramicidin in DMEM culture medium. DMEM culture medium without compound was added as negative control. After 1h incubation, the medium was discarded and the cells were rinsed with tissue culture-grade Dulbecco's phosphate-buffered saline (D-PBS). 150 μ l of a solution of 4 μ M ethidium homodimer-1 (EthD-1) was added into each well and incubated for 30 min. The prepared cell samples were imaged by fluorescence microscopy.

Rifampicin accumulation in *P. aeruginosa*

Sample preparation for endometabolome extraction: A pre-culture of PAO1 was prepared by diluting overnight culture to an OD₆₀₀ of 0.01 in fresh MHB, then incubating with shaking at 37°C, 200 rpm, to mid-log phase (OD₆₀₀= 0.5). The bacteria culture was harvested by centrifugation at 3,000 X g for 10 min and was washed once with fresh MHB. Rifampicin with or without PAS8-*b*-PDM12 was added to a bacteria culture and incubated with shaking at 37°C, for 30 min at 200 rpm. 1 ml aliquot was quickly spun down at 10,000 rpm for 3 min, supernatant was discarded, and the pellet was washed with same volume of ice-cold sterile PBS. The pellet was collected by centrifugation at 10,000 rpm for 3 min, and immediately snap frozen in liquid nitrogen. Samples were stored at -80°C until extraction of metabolites.

Extraction of bacterial-associated metabolites: The frozen pellets were thawed on ice followed by addition of 1ml of cold 80% (v/v) aqueous methanol, and vortexed for 20 s. Samples were sonicated in ice-water bath for 30 min at 50-60 Hz, 100% power. Samples were centrifuged at 14,000 X g for 10 min at 4°C. 900 µL of the supernatant was collected for each sample, diluted with 3 ml of ultrapure water in a Falcon tube and stored at -80°C before further processing. The frozen samples were lyophilized at -105°C for 24 hours, reconstituted in 200 µl of 50% (v/v) aqueous methanol, and sent for targeted LC-MS studies. DMSO-treated sample was used as a reference control to normalize the accumulation of rifampicin in the bacteria.

Membrane depolarization assay

Bacteria in mid-log phase of growth were centrifuged and washed twice with 5 mM HEPES buffer (pH 7.8) containing 20 mM glucose and 0.1 M KCl and diluted to a final concentration of 10^7 CFU/ml. The bacteria cells were treated with 0.2 mM EDTA (pH 8.0) to permeabilize the outer-membrane. In a 96-well plate, DiSC₃₅ solution was added to 200 µl bacteria suspension to a final concentration of 1 µM. The DiSC₃₅ dye was allowed to gradually quench at room temperature. The poly(beta-peptide) was added into the solution with stirring to a desired concentration. Changes in fluorescence due to the disruption of the membrane potential gradient across the cytoplasmic membrane were recorded using a spectrometer (TECAN fluorescence spectrometer) at an excitation wavelength of 622 nm and an emission wavelength of 670 nm.

***In vivo* bacteria membrane effect**

Nine-week-old female C57B/6 mice (weight ~20 g) (Invivos, Singapore) were used. Inoculation was performed by intraperitoneal injection of 1.5×10^6 CFUs of *P. aeruginosa* PAO1 with 5% mucin. 25 mg/kg PAS8-*b*-PDM12 or 200 μ l PBS control was administered by intraperitoneal injection at 1 h post-infection. Mice were euthanized at 10 min and 60 min post-treatment. Peritoneal washes were performed by injecting 3.0 ml of sterilized PBS in the intraperitoneal cavity followed by abdominal massage. Intraperitoneal fluid was then collected and filtered through 5 μ m membrane to remove eukaryotic cells and debris. The bacteria were washed and resuspended in DI water for cryo-TEM imaging.

Dark-field/fluorescence dual-mode microscope imaging

Bacteria in mid-log phase of growth were adjusted to a cell density of 10^8 CFU in 1.5 ml micro tubes. The bacterial were loaded with 16 μ g/ml ethidium bromide in PBS without glucose to let the ethidium bromide reach maximum accumulation. The compounds in PBS containing glucose were added at the desired concentration and incubated for 5 min. The samples were washed with PBS and imaged with dark-field/fluorescence dual-mode microscope (Olympus IX71).

Efflux pump inhibition assay.

Efflux pump inhibition was assessed using the flow cytometry method following a published procedure ^[6]. Bacteria in mid-log phase of growth were centrifuged and washed twice with sterilized PBS. Bacteria at a density of 10^8 CFU/ml were incubated with 16 μ g/ml ethidium bromide in PBS without glucose to reach maximum ethidium bromide accumulation. Subsequently, bacterial cells were treated with poly(beta-peptide) solution at desired

concentration. Glucose was added to a final concentration of 0.4%. Assay was performed in a 96-well black plate (Corning Costar) with final volume of 200 μ l. Intracellular ethidium bromide accumulation was followed by measuring the fluorescence with a BD Accuri™ C6 Plus flow cytometer. Bacterial cells without ethidium bromide loaded were used as negative control. Ethidium bromide was excited at 488 nm and fluorescence was measured through the FL-2 channel. Data were collected for at least 100,000 events per sample.

Swimming motility assay

The motility plates were prepared as previously described ^[7]. Bacteria in mid-log phase of growth were centrifuged and washed twice with sterilized PBS. 10 μ l of 10^8 CFU/ml bacterial solution was spotted onto the motility plate and incubated for 16 h before imaging.

***In vivo* efficacy study in systemic infection models**

Bacteria count. Eight-week-old female C57B/6 mice (weight ~20 g) (Invivos, Singapore) were used. All mice were housed in a 12-hour light-dark cycle at room temperature for one week prior to the experiment. Before infection, mice were divided from a housing cage to different treated cages with 5 mice per cage. No mouse was excluded from the analyses. Sample dosage was determined based on the results of a preliminary infection trial (n=3). Inoculation was performed by intraperitoneal injection of 1.5×10^6 CFUs of multi-drug resistant *P. aeruginosa* PAER, *K. pneumonia* KPNR, *E. coli* ECOR and *A. baumannii* MDRAB, with 5% mucin (Sigma-Aldrich). Infections were allowed to establish for 1 hour (PAER, KPNR and ECOR) or 2 hours (MDRAB), and treatment was administered by intraperitoneal injection of sterilized PBS (untreated control), PAS8-*b*-PDM12, antibiotics and PAS8-*b*-PDM12/antibiotics combinations. Mice were euthanized by CO₂ asphyxiation

followed by cervical dislocation at 11 hours (PAER, KPNR and ECOR) or 22 hours (MDRAB) post treatment. Peritoneal washes were done by injecting 3.0 ml of sterilized PBS in the intraperitoneal cavity followed by a massage of the abdomen. Subsequently, intraperitoneal fluid was extracted from the abdomen for CFU count determination. Livers, kidneys and spleens of all mice were harvested and stored in 800-900 μ l ice-cold PBS. Organs were homogenized using a high-throughput tissue homogenizer (Omni). The bacteria present in the organs and abdomen were determined by CFU plating of serially diluted samples on LB agar plates.

Survival study. Eight-week-old female C57B/6 mice (weight $\sim$ 20 g) (Invivos, Singapore) were used. All mice were housed in a 12-hour light-dark cycle at room temperature for one week prior to the experiment. Before infection, mice were divided from a housing cage to different treated cages with 5 mice per cage for multi-drug resistant *A. baumannii* MDRAB. No mouse was excluded from the analyses. Inoculation was performed by intraperitoneal injection of 1.5×10^6 CFUs of *A. baumannii* MDRAB, with 5% mucin (Sigma-Aldrich). Infections was allowed to establish for 2 hours, and treatment was administered by intraperitoneal injection of 200 μ l sterilized PBS (untreated control), PAS8-*b*-PDM12, antibiotics and PAS8-*b*-PDM12/antibiotics combinations. Mice were monitored for 7 days. Five-point body condition score according to the body weight, body temperature, respiration, motility and posture was used to determine the clinical endpoint of mice. Mice were euthanized when endpoints were reached according to Nanyang Technological University Institutional Care and Use Committee (IACUC) guidelines.

All studies and protocols were approved by the Nanyang Technological University IACUC Under the approved protocol No. ARF-SBS/NIE-A0363.

Statistical analysis. Statistical analysis used either a two-tailed Student's t-test for two groups' comparison or one-way ANOVA followed by Dunnett test for more than two groups' comparison, where the difference is considered as statistically significant with probability $P < 0.05$.

Figure S1. PAS8-*b*-PDM12 induced a dose-dependent increase in outer membrane permeability in clinically-relevant GNB including *A. baumannii* ACBAS, *E. coli* ECOS and *K. pneumoniae* KPNS using NPN as an indicator of outer membrane permeability.

Figure S2. PAS8-*b*-PDM12 does not destabilize mammalian cell membranes. Fluorescence microscopy images of untreated 3T3 fibroblasts (upper row), treated with 100 $\mu\text{g/ml}$ PAS8-*b*-PDM12 (middle row), or treated with 100 $\mu\text{g/ml}$ gramicidin (bottom row). Ethidium homodimer-1 (EthD-1) was used as a membrane integrity indicator. Scale bar=50 μm .

Figure S3. PAS8-*b*-PDM12 dependent potentiation of rifampicin in a murine systemic infection model of clinical antibiotic-resistant *pneumonia* KPNR . Mice received either PBS control, 25 mg/kg PAS8-*b*-PDM12, 5 mg/kg rifampicin, or a combination of PAS8-*b*-PDM12 + rifampicin. Five animals per condition were used. Bacterial count in the intraperitoneal fluid was determined by plating on LB agar plates. Symbols represent the bacterial count in one mouse, and black lines represent the geometric mean of the individual count (n=5).

Figure S4. *In vivo* synergistic effect of PAS8-*b*-PDM12 with rifampicin in antibiotic-resistant GNB *P. aeruginosa* PAER in distant organs following a systemic infection. Bacterial load was determined in the liver (a); spleen (b) and kidneys (c).

Figure S5. *In vivo* synergistic effect of PAS8-*b*-PDM12 with rifampicin in antibiotic-resistant GNB *E. coli* ECOR in distant organs following a systemic infection. Bacterial load was determined in the liver (a); spleen (b) and kidneys (c).

Figure S6. *In vivo* synergistic effect of PAS8-*b*-PDM12 with rifampicin in antibiotic-resistant GNB *A. baumannii* MDRAB in distant organs following a systemic infection. Bacterial load was determined in the liver (a); spleen (b) and kidneys (c).

Figure S7. *In vivo* synergistic effect of PAS8-b-PDM12 with rifampicin in antibiotic-resistant GNB *K. pneumonia* KPNR in distant organs following a systemic infection. Bacterial load was determined in the liver (a); spleen (b) and kidneys (c).

Figure S8. PAS8-*b*-PDM12 damages bacterial membranes *in vivo* (a) Cryo-TEM images of *P. aeruginosa* PAO1 harvested from the peritoneal cavities of mice untreated (UNT) (a) or treated with 25 mg/kg PAS8-*b*-PDM12 for 10 min (b) and 60 min (c) after infection. The images shown in right panel (ii) were the zoom-in area in the red box in the left panel (i).

Figure S9. Bacteria count in blood, liver, spleen and kidneys of animals after intraperitoneal infection with antibiotic-resistant GNB *P. aeruginosa* PAER (a); *E. coli* ECOR (b); *K. pneumoniae* KPNR (c) and *A. baumannii* MDRAW (d) at 1 hour (a-c) or 2 hours post-infection (d).

Figure S10. Dark-field/fluorescence dual-mode microscopic image of *P. aeruginosa* PAO1 (upper row), PAO1 treated with 16 $\mu\text{g/ml}$ PAS8-*b*-PDM12 (middle row) and efflux pumps deficient strain *P. aeruginosa* PAO750 (below row). Efflux pump substrate ethidium bromide was used to show fluorescence in bacterial cells, scale bar=8 μm .

Figure S11. Checkerboard broth microdilution assays showing a dose-dependent potentiation of moxifloxacin by PAS8-*b*-PDM12 in *P. aeruginosa* PAO1 (a), and absence of potentiation

of moxifloxacin by PAS8-*b*-PDM12 in the efflux pumps deficient strain *P. aeruginosa* PAO750 (b). Darker regions represent higher bacterial density.

Figure S12. Checkerboard broth microdilution assays show dose-dependent novobiocin potentiation by PAS8-*b*-PDM12 in *P. aeruginosa* PAO1 (a) and its pump deficient strains PAO750 (b), MICs of novobiocin is much lower for efflux pumps deficient strain *P. aeruginosa* PAO750 than normal strain *P. aeruginosa* PAO1. Darker regions represent higher bacterial density.

Figure S13. *In vivo* synergistic effect of PAS8-*b*-PDM12 with novobiocin in multi-drug resistant GNB *E. coli* ECOR in distant organs following a systemic infection. Bacterial load was determined in the liver (a); spleen (b) and kidneys (c).

Figure S14. *In vivo* synergistic effect of PAS8-*b*-PDM12 with novobiocin in multi-drug resistant GNB *K. pneumonia* KPNR in distant organs following a systemic infection. Bacterial load was determined in the liver (a); spleen (b) and kidneys (c).

Figure S15. *In vivo* synergistic effect of PAS8-*b*-PDM12 with novobiocin in multi-drug resistant GNB *A. baumannii* MDRA in distant organs following a systemic infection. Bacterial load was determined in the liver (a); spleen (b) and kidneys (c).

Table S1. Antimicrobial activity, hemolysis and mammalian cell biocompatibility of selected glycosylated cationic block poly(β -peptides)

No.	Entry	M_n^a g/mol	$\bar{D}$	MIC ^b (μ g/ml)		HC ₅₀ (μ g/ml)	IC ₅₀ (μ g/ml)
				<i>P. aeruginosa</i>	<i>S. aureus</i>	RBC	3T3
1	20DM	7050	1.32	16	32	312.5	144
2	PAS2- <i>b</i> -PDM18	6675	1.25	32	32	625	323
3	PAS4- <i>b</i> -PDM16	6594	1.26	64	32	625	364
4	PAS6- <i>b</i> -PDM14	7167	1.26	64	64	1250	456
5	PAS8- <i>b</i> -PDM12	7538	1.24	64	64	>5000	>500
6	PAS10- <i>b</i> -PDM10	7850	1.22	128	64	>5000	>500
7	PAS20	8907	1.08	>512	>512	>5000	>500

^aDetermined based on the protected glycosylated cationic block poly(β -peptides) with GPC using DMF (containing 1mg/ml LiBr) as eluent and narrow polystyrene standards used as reference. ^bThe strains used in this study were: *P. aeruginosa* (PAO1) and *S. aureus* (ATCC 29213).

Table S2. *In vivo* safety profile of PAS8-*b*-PDM12 after intravenous injection.

Post Treatment	Hepatotoxicity		Hepatotoxicity	Nephrotoxicity	Kidney function and Blood electrolyte		
	ALT(Ul ⁻¹)	AST (Ul ⁻¹)	TBIL (μmol ⁻¹)	Creatinine (μmol ⁻¹)	Urea nitrogen (mmol ⁻¹)	Potassium ion (mmol ⁻¹)	Sodium ion (mmol ⁻¹)
0 day	44.60±5.103	93.10±18.28	3.650±1.041	59.70±23.29	8.207±1.488	5.154±0.348	147.9±4.012
7 days	42.30±4.218	103.4±18.31	3.583±0.943	58.80±19.16	7.387±1.323	4.923±0.284	147.4±4.993

The PAS8-*b*-PDM12 do not cause significant acute damage to liver and kidney function, nor do they interfere with the electrolyte balance in the blood at 10 mg/kg injection. Data are expressed as mean ± standard deviation (n=10). $P>0.05$ for comparison after 7 day repetitive treatment to 0 day group (before treatment); statistical analysis was performed using Student's t-test; differences are considered statistically significant with probability $P<0.05$. ALT, alanine transaminase; AST, aspartate transaminase; TBIL, total bilirubin; U, international unit.

Table S3. Antimicrobial activity of PAS8-*b*-PDM12, colistin, LL-37 and melittin against *P. aeruginosa* PAO1 after 24-hour incubation with blood plasma.

Drug	MIC (µg/ml)	
	before incubation	after incubation
PAS8- <i>b</i> -PDM12	64	64
Colistin	2	2
LL-37	64	>256
Melittin	64	>256

Table S4. Antimicrobial resistance profile of the bacterial strains used in this study.

Bacteria		MIC (µg/ml)				Source
		CST	MEM	CIP	AMK	
<i>P. aeruginosa</i>	PAO1	2	1	0.125	2	ATCC
	PAER	2	>256	>64	16	NCID
<i>E. coli</i>	ECOS	2	<0.03	<0.03	2	NCID
	ECOR	2	4	2	16	NCID
	NMT1833	8	32	0.25	4	NCID
<i>A. baumannii</i>	ABACS	2	16	0.06	1	NCID
	MDRAB	4	256	64	>64	NCID
<i>K. pneumoniae</i>	KPNS	4	0.06	<0.03	2	NCID
	KPNR	4	32	64	2	NCID

NCID: National Centre for Infectious Diseases, Singapore. CST= Colistin; MEM=Meropenem; CIP= Ciprofloxacin; AMK=Amikacin.

Table S5. Synergy between PAS8-*b*-PDM12 and selected antibiotics against different GNB.

Bacteria	Antibiotics	MIC alone (µg/ml)		Combination with PAS8- <i>b</i> -PDM12			
		PAS8- <i>b</i> -PDM12	Antibiotics	MIC _A (µg/ml)	FIC	Fold decrease	
Antibiotic-sensitive GNB	<i>P. aeruginosa</i> PAO1	64	CHL	128	4	0.28	32
			CAR	32	4	0.38	8
			AMP	2048	64	0.28	32
			CAZ	2	0.12	0.31	16
			TET	25	3.12	0.38	8
			SMZ	128	4	0.28	32
			CIP	0.125	0.016	0.38	8
			NOV	1024	8	0.26	128
	<i>A. baumannii</i> ABACS	512	RIF	3.125	0.012	0.25	256
			NOV	12.5	0.39	0.28	32
	<i>K. pneumoniae</i> KPNS	512	RIF	12.5	0.05	0.25	256
			NOV	25	0.78	0.28	32
	<i>E. coli</i> ECOS	256	RIF	6.25	0.048	0.26	128
			NOV	50	0.195	0.25	256

Antibiotic-Resistant GNB	<i>P. aeruginosa</i> PAER	RIF	32	25	6.25	0.5	4
		NOV		>200	25	<0.38	>8
	<i>A. baumannii</i> MDRAB	RIF	512	3.125	0.012	0.25	256
		NOV		6.25	0.4	0.31	16
	<i>K. pneumoniae</i> KPNR	RIF	512	25	0.05	0.25	512
		NOV		200	0.78	0.25	256
	<i>E. coli</i> ECOR	RIF	256	12.5	0.024	0.25	512
		NOV		100	0.195	0.25	512
	<i>mcr-1</i> GNB <i>E. coli</i> NMT1833	RIF	256	8	0.062	0.26	128
		NOV		32	0.062	0.25	512

MIC_A=MIC of antibiotic in combination. MIC_b=MIC of PAS8-*b*-PDM12 in combination. FIC=MIC_A (in combination)/MIC_A (alone)+ MIC_B (in combination)/MIC_B (alone); Fold decrease=MIC of antibiotic alone/ MIC of antibiotic in combination. The concentration of PAS8-*b*-PDM12 in combination is ¼ MICs. CHL=chloramphenicol; CAR= carbenicillin; AMP=ampicillin; CAZ=ceftazidime; TET= tetracycline; SMZ= Sulfamethoxazole; CIP=ciprofloxacin; NOV=novobiocin. RIF=rifampicin;

Table S6. PAS8-b-PDM12 antagonizes the potency of aminoglycosides in *P. aeruginosa* PAO1

Antibiotics	MIC alone ($\mu\text{g/ml}$)	Combination with PAS8-b-PDM12		
		MIC _A ($\mu\text{g/ml}$)	FIC	Fold Increase
Gentamicin	0.5	2	4.5	4
Streptomycin	16	32	2.5	2
Neomycin	8	16	2.5	2

Fold increase= $\text{MIC}_A / \text{MIC alone}$. MIC alone means MIC of antibiotics, MIC_A=MIC of antibiotics in combination. $\text{FIC} = \text{MIC}_A (\text{in combination}) / \text{MIC}_A (\text{alone}) + \text{MIC}_B (\text{in combination}) / \text{MIC}_B (\text{alone})$; The concentration of PAS8-*b*-PDM12 in combination is $\frac{1}{4}$ MICs.

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