Designer co-beta-peptide selectively targets Gram-negative bacteria

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Introduction

In the last 50 years or so, there has been no new class of antibiotics approved against Gram-negative bacteria. This is a global health concern given the rapid spread of carbapenem-resistant *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacteriaceae*, which have been classified by the World Health Organization (WHO) as critical pathogens and for which new antibiotics are urgently needed[1]. Antimicrobial peptides (AMPs) that target the bacterial membrane are thought to be the last frontier in the development of antibacterial agents as they do not easily evoke resistance in bacteria[2,3]. Natually occurring AMPs are alpha peptides and most research, whether on natural or synthetic AMPs, has focused on alpha peptides. Most AMPs studied to date are unselective, killing both Gram-positive and Gram-negative bacteria and associated with cytotoxicity. Only few AMPs selectively target Gram-negative bacteria, of which the most successful is probably Polymyxine B (PMB), and they are sequence-defined peptides that have amino acids with precise order[4].

AMPs typically contain both cationic and hydrophobic alpha-amino acid residues, which cause them to interact unselectively with both bacterial and mammalian membranes via two driving forces: electrostatic and hydrophobic interactions. The interaction of AMPs with (almost neutral) mammalian cell membranes is largely mediated by hydrophobic interactions. Hence, AMPs with reduced hydrophobicity usually result in less toxicity to mammalian cells at the potential cost of their antibacterial potency. As Gram-positive bacteria have only the cytoplasmic membrane consisting of phospholipids[5,6], many AMPs are highly effective against these Gram-positive bacteria[7,8].

The Gram-negative bacterial envelope is complex, consisting of both an outer membrane and a cytoplasmic membrane[5], and fewer antibiotics and AMPs are effective against these bacteria compared to Gram-postive bacteria[9]. The outer membrane contains lipopolysaccharide (LPS) in the outer leaflet, forming a tight barrier that prevents the translocation of hydrophobic molecules through the impermeable outer membrane[9]. However, since LPS is the outermost layer of the Gram-negative bacterial envelope, it may be possible to achieve selective targeting of Gram-negative bacteria with rationally-designed molecules that specifically target LPS molecules. LPS comprises negatively charged lipids decorated with long chains of polysaccharides with phosphate and hydroxyl groups. This suggests that molecules rich in their ability to engage in electrostatic and hydrogen bonding interactions hold the potential to selectively bind to LPS molecules. However, up to now, this principle of LPS targeting has not been translated. Even PMB's interaction with Gram-negative bacterial envelop is based on the electrostatic and hydrophobic interaction with the LPS[10] and the displacement of divalent ions holding the LPS together.

Beta-peptides are much less explored than natural alpha(α) AMPs though they may be promising alternatives because their biological activity is similar to that of natural α-peptides with the added properties of being more resistant to proteolysis and commonly non-mutagenic[11,12]. Importantly, the existence of an extra carbon in the backbone of β-peptides makes them more flexible than α-peptides, which may potentially facilitate their binding to LPS via both electrostatic interactions and hydrogen bonding. However, Gram-negative-selective beta peptides have not yet been reported. Advances in the synthetic chemistry of β-amino acids and peptides make it possible to design and synthesize various antimicrobial β-peptides[13-19]. The most potent antimicrobial β-peptide yet synthesized is a poly(dimethyl β-lactam)-*co*-poly(cyclohexyl β-lactam) copolymer (P(DM-*co*-CH) which has a tert-butylbenzoyl group at the N-terminus[20] (called "t-BuBenzoyl(N)-

P" hereafter; "P" represents the copolymer backbone and the prefix is the N-terminus group identifier) that contributes to the local hydrophobicity of the N-terminal of the tapered blocky co-beta-peptide (**Scheme 1a**). However, its hydrophobicity entails toxicity to erythrocytes and mammalian cells[21].

Herein, we report the modulation of the N-terminal hydrophobicity of the tapered blocky P(DM-*co*-CH) co-β-peptide through the replacement of the bulky N-terminal hydrophobic tert-butylbenzoyl group with a hydrophilic ammonium group N-terminal through the use of a new perfluorinated aminated co-initiator in the synthesis (**Scheme 1b**). We show that the resulting ammonium-terminated co-β-peptide (called "NH_3(N)-P" hereafter) (**Scheme 1b**) maintains its backbone interaction with the LPS of Gram-negative bacteria via electrostatic and hydrogen bonding while having reduced/no hydrophobicity-driven interaction with Gram-positive bacteria and mammalian cells.

Results

Synthesis and chemical characterization of copolymers. To prepare the control co-beta-peptide polymer (t-BuBenzoyl(N)-P), which possesses the bulky hydrophobic group t-butylbenzoyl at the N-terminus (**Scheme 1a**), 5 mole% t-butylbenzoyl chloride (relative to the total beta-lactam monomers content) was used as the co-initiator. To make the new co-β-peptide NH_3(N)-P, a new protected amine-containing active ester of perfluorophenyl (tert-butoxycarbonyl)-glycinate(Boc-Gly-OPFP) was employed as the co-initiator (**Scheme 1b**). It was synthesized (**Scheme S1 and Figure S1**) by the efficient coupling between (tert-butyloxycarbonyl) glycine (Boc-Gly-OH) and pentafluorophenol via *N, N′*-dicyclohexylcarbodiimide (DCC) activation[22]. Under base-catalyzed conditions (lithium bis(trimethylsilyl)amide (LiHMDS)), the active ester (Boc-Gly-OPFP) reacts

with the β-lactam monomers, generating the corresponding imide initiator *in situ*, to initiate the anionic ring-opening polymerization (AROP) of the cationic (DM) and hydrophobic (CH) beta-lactams fed at the designed ratio of 1:1 (mole: mole), to result in the new co-beta-peptide of P(DM-*co*-CH) with the residual N-terminal amine group from the new co-initiator (**Scheme 1b)**. The obtained co-beta-peptide polymers were treated with trifluoroacetic acid (TFA) to remove the tert-butyloxycarbonyl (Boc) protecting group, producing the water-soluble N-terminal aminated β-peptides (*i.e.* NH_3(N)-P) (**Scheme 1b**). The copolymer length was controlled to be around 20-mer via controlling the co-initiator Boc-Gly-OPFP relative to the total beta-lactam monomers content to be 5 mole%. The molecular structure of the obtained polymers was confirmed by NMR spectroscopy (**Figure S2-S3**). The molecular weight of the polymers measured by using GPC was found to be in good agreement with the stoichiometric values (**Figure S4**). The obtained degree of polymerization (DP) is 22 for both polymers with a monomer/initiator feed ratio of 20:1. Both polymers exhibited relatively narrow molecular weight distributions (*Đ*=1.20-1.29). The GPC results thus confirm that the polymerization process is well-controlled.

Copolymerization Kinetics. The copolymerization using Boc-Gly-OPFP as the new co-initiator was tracked using gas chromatography (GC) following a reported procedure[20]. The hydrophobic cyclohexyl (CH) beta-lactam monomers were found to be consumed faster than the cationic dimethyl (DM) beta-lactam monomers (**Figure S5**), indicating that the N-terminus of the β-peptides was significantly richer in the hydrophobic (CH) residues while the C-terminus was richer in the cationic (DM) residues (**Figure 1**), which is similar to the reported distribution of DM and CH residues in the original polymerization using t-butylbenzoyl chloride as the co-initiator[20]. The faster copolymerization of CH versus DM results in a tapered blocky structure in the resulting co-beta-peptides with a greater accumulation of the hydrophobic CH units at the N-terminals.

Replacement of the bulky hydrophobic residual N-terminal group t-butylbenzoyl (LogP=3.49) in the original co-initiator with a highly hydrophilic ammonium group (LogP=-1.21) from the new co-initiator (Boc-Gly-OPFP) would significantly decrease the local hydrophobicity at the N-terminus of the new co-beta-peptide (**Figure 1b**). The higher hydrophobicity near the N-terminus in t-BuBenzoyl(N)-P compared to NH_3(N)-P was supported by dynamic light scattering (DLS) measurements in phosphate-buffered saline (PBS) solution; the critical aggregation concentration (CAC) of t-BuBenzoyl(N)-P is about 0.5 mg/mL and the hydrodynamic radius of the t-BuBenzoyl(N)-P increased significantly beyond 0.5 mg/mL while NH_3(N)-P remained as solvated single polymer chains up to 16 mg/mL (**Figure S6**).

Antimicrobial potency and biocompatibility. The minimum inhibitory concentrations (MICs) of the synthesized β-peptides against a series of bacteria were evaluated. Consistent with a previous report[20], t-BuBenzoyl(N)-P exhibited broad-spectrum antimicrobial potency (**Table 1**). However, the high toxicity of t-BuBenzoyl(N)-P against both erythrocytes (HC_{50}=312.5μg/ml) and 3T3 mouse fibroblasts (IC_{50}=100 μg/ml) resulted in low selectivity index (HC_{50}/MIC) of 19.5 and (IC_{50}/MIC) of 6.25, respectively when tested against *E coli* 8739 (**Table 1**). Replacement of the N-terminal group with ammonium preserves the efficacy of the NH_3(N)-P β-peptide against Gram-negative bacteria *P. aeruginosa*, *A. baumannii*, and *E. coli*, but reduces efficacy against Gram-positive bacteria (**Table 1**). Excellent antimicrobial activity was retained against various Gram-negative clinical isolates, including carbapenem-resistant strains, with MICs against multi-drug resistant (MDR) *P. aeruginosa*, *A. baumannii* and *E. coli* ranging from 4 to 32 μg/ml. The potency of NH_3(N)-P against Gram-positive bacteria was lower than t-BuBenzoyl(N)-P, which might result from the new polymer's reduced hydrophobicity at the N-terminus. Further, the NH_3(N)-P polymer has significantly reduced toxicity against both erythrocytes (HC_{50}=5000 μg/ml) and fibroblasts

(IC_{50}>500 μg/ml) resulting in improved selectivity index (HC_{50}/MIC =312.5 and IC_{50}/MIC> 31.25).

NH_3(N)-P exhibited rapid killing kinetics against various multi-drug resistant (MDR) Gram-negative strains. At 2×MIC, it completely eradicated MDR *A. baumannii* MDRAB and *P. aeruginosa* PAER within 1 hour and MDR *E. coli* ECOR in 5 hours (**Figure 2**). Because of the unnatural amino acids of the beta-peptide, the copolymer displayed much higher proteolytic resistance than conventional AMPs in physiological conditions. NH_3(N)-P was stable after 24 h incubation with mouse blood plasma (**Table S1**), while the conventional AMP melittin totally lost its potency, representing a significant improvement over classical antimicrobial alpha-peptides as therapeutical agents.

Cryo-TEM shows that, compared with the untreated *E. coli* K12 (**Figure 3a**) and *P. aeruginosa* PAO1 (**Figure 3c**) that have intact outer and cytoplasmic membrane, remarkable wrinkling and lysis occurred in both the outer and cytoplasmic membranes of NH_3(N)-P treated *E. coli* K12 (**Figure 3b**) and *P. aeruginosa* PAO1 (**Figure 3d**). It is known that the outer membrane of Gram-negative bacteria is stabilized by salt-bridges between divalent counter-ions (*e.g.*, calcium ions) and the phosphate groups of the LPS that can compensate for the electrostatic repulsion between the negatively charged LPS molecules[23,24]. Interaction of NH_3(N)-P polymer with LPS would perturb the salt-bridges and destroy the outer membrane integrity, resulting in LPS release and bacterial lysis. Our Cryo-TEM imaging of NH_3(N)-P-treated *E. coli* and *P. aeruginosa* supports the hypothesis of bacterial membrane lysis mechanism due to the polymer (**Figure 3**).

Isothermal titration calorimetry and interaction with model liposomes. To delineate the selectivity mechanism of NH_3(N)-P versus t-BuBenzoyl(N)-P (control) at the molecular level,

isothermal titration calorimetry (ITC) was applied to study the interaction of the β-peptides with liposome models of the mammalian plasma membrane (POPC)[25], Gram-positive cytoplasmic membrane (POPC: POPG=4:1)[25] and Gram-negative bacterial outer membrane (LPS)[26]. The ITC titration thermograms are shown in supplementary **Figure S7** and the derived thermodynamic interaction parameters are shown in **Table 2**. The t-BuBenzoyl(N)-P (control) strongly interacted with all 3 liposomes (mammalian model liposome (ΔG=-25.1 kJ/mol, K_A=16694 M^{-1}), Gram-positive bacteria model liposome (ΔG=-21.2 kJ/mol, K_A=3817 M^{-1}) and Gram-negative LPS liposome (ΔG=-26.3 kJ/mol, K_A=26954 M^{-1}) (**Figure S7a**, **7c and 7e**)), which corroborates its non-selective toxicity to all three types of cells. On the other hand, NH_3(N)-P showed no interaction with the mammalian model liposome and weak interaction with Gram-positive model liposome (ΔG=-14.8 kJ/mol, K_A=311 M^{-1}), but strong interaction with Gram-negative LPS (ΔG=-26.5 kJ/mol, K_a=29325 M^{-1}) (**Figure S7b**, **7d and 7f**), which is consistent with its observed selectivity towards Gram-negative bacteria.

The (control) β-peptide t-BuBenzoyl(N)-P displayed slight unfavourable (positive) enthalpic interactions with both the mammalian model liposome (ΔH=1.04 kJ/mol) and Gram-positive model liposome (ΔH=5.48 kJ/mol). However, the t-BuBenzoyl(N)-P showed a large favorable (positive) entropic gain with the mammalian (ΔS =84.2 J/mol/K) and Gram-positive liposomes (ΔS =86.2 J/mol/K), which may arise from the release of ordered water molecules, as well as the decrease in lipid order, during the penetration of the tert-butylbenzoyl group into the mammalian cell and bacterial cytoplasmic membrane. On the other hand, with the Gram-negative LPS model, the exothermic process associated with t-BuBenzoyl(N)-P binding (ΔH=-50.2 kJ/mol) suggests that the heat release associated with the electrostatic interaction and hydrogen bonds between the cationic groups of the β-peptide and phosphate groups of LPS was larger than the unfavorable

entropic contribution associated with desolvation (excluding the surrounding water molecules around LPS) and lipid perturbation. Hence, the favorable control β-peptide (t-BuBenzoyl(N)-P) interactions with mammalian cell, Gram-positive bacteria and Gram-negative bacteria are governed by entropic, entropic, and enthalpic interactions respectively (**Table 2**).

In the ITC study of NH_3(N)-P with the mammalian model liposome, no detectable heat flow were found (**Figure S7b**), suggesting no interaction of NH_3(N)-P with the mammalian membrane, which is consistent with its low toxicity against mammalian cells. With the Gram-positive model liposome, the titration of NH_3(N)-P results in relatively weak but still favorable affinity (ΔG=-14.8 kJ/mol, K_A=311 M^{-1}), which is consistent with its weak antimicrobial potency against Gram-positive bacteria. As with t-BuBenzoyl(N)-P, the NH_3(N)-P interaction with the Gram-positive bacteria is governed by entropic interaction (ΔS =59.1 J/mol/K), most reasonably due to water displacement. With NH_3(N)-P, the interaction with Gram-negative LPS (ΔG=-26.5 kJ/mol, K_A=29325 M^{-1}) is much more favorable than with mammalian cell and Gram-positive bacteria. When titrating NH_3(N)-P against the anionic LPS, a large enthalpy release (ΔH=-48.6 kJ/mol) was observed, which appears to result from enthalpic interaction, probably from electrostatic interactions and the formation of hydrogen bonds between the peptide amine and LPS phosphate groups.

In summary, for the control peptide interaction with the mammalian and Gram-positive model liposomes, the positive entropy changes (which are favorable) and positive enthalpy changes (which are unfavorable) indicate that the binding of the β-peptide to the two membranes was driven by entropy due to hydrophobic insertion[27]. A change of the bulky hydrophobic tert-butylbenzoyl group at the N-terminus of the control t-BuBenzoyl(N)-P beta-peptide to hydrophilic ammonium

makes NH_3(N)-P insufficiently hydrophobic to interact with mammalian cell membrane and with Gram-positive cytoplasmic membrane. With the Gram-negative LPS and both NH_3(N)-P and control t-BuBenzoyl(N)-P), the negative entropy (unfavorable) and negative enthalpy changes (favourable) indicate that the interactions of both β-peptides with LPS are driven by enthalpic effects resulting from electrostatic and hydrogen bonding interactions[28]. The selectivity of NH_3(N)-P is achieved from the retained enthalpic interactions with the Gram-negative LPS with the removal/reduction of entropic interactions with the mammalian and Gram-positive model liposomes.

Molecular dynamics simulations. To gain further insights into the mechanism of action of the β-peptides with the different membranes at the atomic level, we carried out molecular dynamics (MD) simulations of NH_3(N)-P and t-BuBenzoyl(N)-P with model membranes of mammalian cells, Gram-positive bacteria and.Gram-negative bacteria. We first simulated the mode of interactions of NH_3(N)-P and t-BuBenzoyl(N)-P with a model POPC bilayer mimicking the mammalian membrane. **Figure 4a and 4b** show the snapshots of the two β-peptide interactions with the model mammalian membrane. As the simulation progresses, the distance between the N-terminal residue of t-BuBenzoyl(N)-P (orange peptide terminal, **Figure 4a**) and the lipid bilayer center gradually decreases (**Figure S8a**), suggesting penetration into the bilayer. The final distance is around 0 nm, corresponding to the bilayer center. The C-terminal segment of t-BuBenzoyl(N)-P still locates at the head group region, forming an amphiphilic conformation that enables t-BuBenzoyl(N)-P to perturb both head groups and lipid tails (**Figure 4a**). In contrast, NH_3(N)-P, due to its lack of hydrophobic groups at the N-terminus, primarily locates at the head group region of the membrane without insertion (**Figure 4b**), suggesting low membrane toxicity. We conclude that the toxicity of t-BuBenzoyl(N)-P towards mammalian membrane mainly originates from the hydrophobic

insertion of the tert-butylbenzoyl group into the membrane, which is entropically driven (**Table 2**).

We subsequently examined the modes of interaction of the two β-peptides with a model Gram-positive cytoplasmic membrane consisting of POPC and POPG lipids at a ratio of 4:1. **Figure 4c and 4d** show that the N-terminal segment of t-BuBenzoyl(N)-P (orange) penetrates into the lipid tail region of the bacterial cytoplasmic membrane (**Figure S8b**), while the C-terminal segment locates at the head group region, demonstrating a similar mode of interaction to the case of the mammalian membrane (**Figure 4c**). For NH_3(N)-P, both the N-terminal and C-terminal segments of the β-peptides were found to stay on the membrane surface without penetration during the entire simulation (**Figure 4d**), suggesting low membrane activity against the Gram-positive bacterial cytoplasmic membrane. It is known that the water molecules around a hydrophobic surface have restricted freedom[29]. Upon penetration into the membrane (**Figure 4c**), the dehydration of the hydrophobic groups restores the translational and orientational degrees of freedom of the surface water molecules, which results in an entropy gain. Because of the high hydrophobicity at the N-terminus of t-BuBenzoyl(N)-P, the control peptide experiences a much larger entropy gain than NH_3(N)-P, which is consistent with the ITC results (ΔS=86.2 J/mol/K and 59.1 J/mol/K, respectively).

To further understand the action mechanism of the two β-peptides against Gram-negative bacteria, we carried out MD simulations of the two β-peptides with a model lipid A, an essential compontent of LPS, bilayer mimicking the outer membrane of the Gram-negative bacteria[30]. It has been shown that lipid A membrane is stabilized by divalent cations that are engaged in salt-bridges with the phosphate groups of the membrane[30]. **Figure 4e, 4f** and **Figure S8c-8f** show that both peptides destabilize the lipid A membrane in a similar manner and release the divalent Ca^{2+} (yellow

spheres). As both peptides are cationic and the lipid A membrane is anionic, it is the electrostatic interactions that steer the β-peptides to the membrane. Upon adsorption onto the membrane, the amine groups in the side chains start to engage in hydrogen bonding with the phosphate groups (**Figure 4g, 4h, and Figure S8g, 8h**), resulting in the disruption of the salt-bridges and release of calcium ions from the membrane surface. As both the electrostatic interactions and the formation of amine-phosphate hydrogen bonds are exothermic, the binding of the peptide to LPS results in significant enthalpy release. As the simulation progresses, the membrane is significantly distorted, with some lipid tails becoming exposed to the aqueous phase. The hydration of the exposed lipid tails has to pay an entropic penalty, consistent with the entropy loss (ΔS=-77.4 J/mol/K and ΔS=-70.4 J/mol/K, respectively) observed in the ITC experiments (**Table 2**).

NH_3(N)-P eradicates persisters and biofilm. Bacteria become tolerant of conventional antibiotic treatment by either generating genetic mutants (resistance)[31] or going into a dormant state (persister cells)[32]. Persister cells are a special state of bacteria with inactive metabolism and are mostly un-treatable by classical antibiotics, which usually eradicate only metabolically active cells[33,34]. Since NH_3(N)-P kills bacteria by disrupting their membrane, we hypothesized that it would be able to eradicate persister bacteria. *A. baumannii* ATCC 19606 was taken as a model strain for this study. Persister *A. baumannii* cells were obtained by treatment of stationary-phase bacteria with the antibiotic ofloxacin for 4 hours to kill metabolically active bacteria. Increasing the dosage of ofloxacin did not result in further decrease in bacteria counts (**Figure S9**), suggesting that antibiotic-induced persisters were generated[33]. NH_3(N)-P killed persister *A. baumannii* ATCC 19606 at 1× its MIC (**Figure 5a**), whereas the conventional antibiotic gentamicin was totally ineffective at concentrations as high as 64× its MIC (**Figure 5b**). Persister cells are mainly responsible for the recurrence of bacterial infection and they have been associated with an

increased likelihood of emergence of antibiotic resistance; this ability of NH_3(N)-P seems promising. We further demonstrated that NH_3(N)-P eradicated more than 99% of biofilms associated *A. baumannii* ATCC19606 and MDR clinical isolate MDRAB (**Figure 5c-5d**), indicating that this class of antibacterial may prevent infection relapse by targeting both persisteres and biofilm-associated subpopulations.

Resistance study. To explore the vulnerability of NH_3(N)-P to the rapid emergence of resistance in the target pathogen, we initially tried to generate spontaneous mutants by challenging *E. coli* K12 with a high concentration of NH_3(N)-P. This attempt to generate spontaneous mutants of *E. coli* K12 to NH_3(N)-P was unsuccessful, giving a frequency of resistance of less than 4.4×10^{-9}, which is less than that of the conventional antibiotic ciprofloxacin. The low possibility of evolving resistant bacteria to NH_3(N)-P was further supported by repetitively testing its antimicrobial activity against *E. coli* K12 cultured with a sub-inhibitory concentration of NH_3(N)-P. NH_3(N)-P remained effective after 18 passages, while ciprofloxacin showed a 2048-fold increase in MIC (**Figure 6**). These results indicate that NH_3(N)-P does not rapidly elicit the emergence of resistance.

***In vivo* biocompatibility and efficacy.** The *in vivo* toxicological profile of NH_3(N)-P was measured by intravenous injection in a murine model. NH_3(N)-P was injected via the tail vein at a dosage of 10 mg/kg body weight, and the mouse condition was continuously monitored for 7 days. At 7 days post-injection, all the mice were alive and active. No obvious illness or lassitude was found in visual observation of the mice. The levels of biomarkers related to liver and kidney function and electrolyte balance did not show significant change at 24 h and 7 days after intravenous injection (**Table S2**). The data indicate that NH_3(N)-P did not cause acute toxicity to the liver and kidney or influence the blood chemistry.

Given the good *in vivo* toxicological profile, we tested the *in vivo* antimicrobial performance in murine superficial biofilm wound infection. An excision wound was created and infected with multi-drug resistant clinical isolate *A. baumannii* MDRAB for 24 hours, by which time bacterial biofilm was established in the wound site, resulting in a serious wound infection. The treatment by NH_3(N)-P reduced the bacterial burden by more than 99.2% in the wound areas, which was superior to the last-resort antibiotic meropenem (**Figure 7**).

Discussion

The copolymerization of CH and DM gave rise to a tapered blocky structure of the copolymer beta-peptide (P(DM-*co*-CH)) with more hydrophobic CH residues at the N-terminal. Monitoring of the kinetics of the copolymerization showed that the hydrophobic CH is consumed early. The modest structural change at the N-terminal from the bulky hydrophobic tert-butylbenzoyl group of the highly potent but toxic original β-peptide (t-BuBenzoyl(N)-P) to a hydrophilic ammonium group for the new co-beta-peptide (NH_3(N)-P) significantly improved biocompatibility without sacrificing on potency against multiple Gram-negative bacteria. Changing the co-initiator residue from the tert-butylbenzoyl to the ammonium group significantly reduced the local hydrophobicity at the N-terminus as well as the amphiphilicity of the entire β-peptide. Importantly, the interaction with Gram-negative bacterial LPS is maintained as this interaction is dominated by the electrostatic and hydrogen bonding between the co-beta peptide and the phosphate group of LPS.

The decreased local hydrophobicity at the N-terminus and low amphiphilicity of NH_3(N)-P appears to be responsible for the improvement in its biocompatibility over that of t-BuBenzoyl(N)-P. The ITC results elucidate the basis of the selectivity of NH_3(N)-P: the interaction of the beta-peptides with the mammalian cell membrane and Gram-positive cytoplasmic membranes is

entropy-driven while the interaction with Gram-negative bacteria LPS is enthalpy-driven. The entropy-driven interactions of the control t-BuBenzoyl(N)-P β-peptide measured by ITC with the model mammalian cell membranes and model Gram-positive cytoplasmic membranes corroborates the inference that the lack of local hydrophobicity of the new NH_3(N)-P results in low affinity for mammalian and Gram-positive cytoplasmic membranes. Molecular dynamics simulations further revealed that the tert-butylbenzoyl group of the control t-BuBenzoyl(N)-P could penetrate deeply into the lipid tail region of both the mammalian and Gram-positive cytoplasmic membranes, corroborating the ITC measurements. The lower amphiphilicity of the NH_3(N)-P molecule is associated with weak binding affinity with mammalian and Gram-positive membranes and hence it remains largely at the surface of these model membranes. However, for Gram-negative bacteria LPS, the driving force for the control peptide interaction is enthalpic, which suggests that the driving force is mainly due to electrostatic interaction and hydrogen bonding between the polymer side chains and LPS. In the NH_3(N)-P interaction with the Gram-negative bacterial outer membrane model, ITC measurements and molecular simulation show that the electrostatic and hydrogen bonding interactions between the side-chain amine groups of the β-peptides and the phosphate groups of LPS is the primary driving force. The high flexibility of the beta-peptide would likely aid to promote intimate interactions between the peptide side chain amines and the phosphate head groups of the LPS, allowing strong electrostatic/hydrogen bonding interactions. As both β-peptides share the same side-chain amine groups, NH_3(N)-P, like the control t-BuBenzoyl(N)-P peptide, retains an excellent antimicrobial potency against Gram-negative bacteria.

The few peptides/antibiotics that are selective against Gram-negative bacteria include PMB and darobactin[10,35,36]. PMB selectively kills Gram-negative bacteria by targeting the LPS

molecules via electrostatic and hydrophobic interactions[10] and is also sequence-dependent. However, the hydrophobic lipid tail in PMB also imparts toxicity[37]. Darobactin selectively kills Gram-negative bacteria by targeting the β-barrel assembly machinery (Bam)A, which is an outer membrane protein[35]. However, darobactin-resistant bacteria have emerged after only a few passages, which is possibly due to the fact that it targets a specific protein that is easily mutated. In contrast to PMB, the NH_3(N)-P β-peptide copolymer made by copolymerization is not sequence dependent and can be made in large scale at low cost. Further, our copolymer that interacts with LPS via electrostatic and hydrogen bonding interactions has reduced toxicity associated with the non-selective hydrophobic interaction with mammalian cells. As NH_3(N)-P targets the general structure of the outer membrane of Gram-negative bacteria, the freqeucy of spontaneous resistance is low.

Metabolically dormant persister bacteria are tolerant to antibiotics[32]. However, persisters still need an intact membrane for survival. They are vulnerable to agents that can perturb or disrupt membrane integrity or function, making the membrane an ideal target for the treatment of persisters[38]. The efficacy of NH_3(N)-P against Gram-negative persisters and biofilm is attributable to its ability to interact with the outer membrane constituent LPS. Small cationic polymers/peptides are able to penetrate deep into biofilms[39], thus NH_3(N)-P successfully removes biofilm bacteria by attacking persisters in the deep layers of the biofilm.

Conclusion

We have successfully designed and synthesized a new biocompatible β-peptide copolymer (NH_3(N)-P) that selectively eradicates Gram-negative bacteria without being cytotoxic towards mammalian cells. This is the first report of a copolymer that is not sequence-defined that shows

LPS selectivity. Both the control and new beta peptides have flexible backbones and are able to intimately interact electrostatically and via hydrogen bonding with the phosphate head groups of Gram-negative LPS, without the need for a hydrophobic interaction driving force. To minimize local hydrophobicity that leads to toxicity to mammalian cells, we used a new co-initiator that leaves behind a hydrophilic ammonium group at the hydrophobic segment of the tapered blocky copolymer of DM and CH beta-lactams. The decreased local hydrophobicity at the N-terminus of the new NH_3(N)-P copolymer makes the co-beta-peptide less amphiphilic, resulting in reduced hydrophobic interactions with both mammalian cells and Gram-positive bacteria. The excellent *in vitro* bactericidal activity against carbapenem-resistant *A. baumannii* MDRAB, the most critical pathogen in the WHO list, was demonstrated in an *in vivo* mouse study.

The design of tapered blocky co-beta-peptides without amphilicity (*i.e.* molecules with low local hydrophobicity) and high propensity to engage in electrostatic and hydrogen bonding interactions with LPS provides a new approach for the design of non-toxic Gram-negative selective agents.

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