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Title: Short Synthetic β-Sheet Antimicrobial Peptides for the Treatment of Multidrug-Resistant *Pseudomonas aeruginosa B*urns Wound Infections

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Abstract text: *Pseudomonas aeruginosa* is often implicated in burn wound infections; its inherent drug-resistance often renders these infections extremely challenging to treat. This is further compounded by the problem of emerging drug-resistance and the dearth of novel antimicrobial drug discovery in recent years. In our perennial search for effective antimicrobial compounds, we identified short synthetic β-sheet folding peptides, IRIKIRIK (IK8L), IRIkIrIK (IK8-2D) and irikirik (IK8D) as prime candidates owing to their high potency against Gram-negative bacteria. In this study, the peptides were first assayed against 20 clinically-isolated multidrug-resistant *P. aeruginosa* strains in comparison with the conventional antibiotics imipenem and ceftazidime, and IK8L was demonstrated to be the most effective. IK8L also exhibits superior antibacterial killing kinetics compared to imipenem and ceftazidime. From transmission electron microscopy, confocal microscopy and protein release analyses, IK8L showed membrane-lytic antimicrobial mechanism. Repeated

use of IK8L does not induce drug resistance, while the bacteria develop resistance against the antibiotics after several times of treatment at sublethal doses. Analysis of mouse blood serum chemistry reveals that the peptide does not induce systemic toxicity. The potential utility of IK8L in the *in vivo* treatment of *P. aeruginosa*-infected burn wounds is further demonstrated in a mouse model.

1. Introduction

Pseudomonas aeruginosa is a Gram-negative pathogen that is commonly implicated in infections resulting from burn wounds, particularly amongst critically ill and immunocompromised patients.[1, 2] These infections pose a significant therapeutic dilemma to attending plastic surgeons; this is in large part due to the rising rates of antimicrobial resistance.[3] *P. aeruginosa* is innately-resistant to antibiotics; this is mediated by the low permeability of its outer membrane by efflux pumps and the secretion of alginate during biofilm formation. [4-8] This innate resistance is further compounded by the ease of mutation and accumulation of resistance genes.[9] As a result, multidrug-resistant (MDR) *P. aeruginosa* infections are fast posing a significant healthcare threat, with an ever-increasing incidence of 10-17%. It is associated with a 24% increased mortality risk compared to susceptible strains, with all-cause mortality standing at 34%.[10] In the US alone in 2013, multidrug resistance was found in 13% of all *P. aeruginosa* infections and accounted for close to 440 deaths.[11] The Communicable Disease Centre in 2013 placed the direct economic burden of such multidrug resistance at USD 20 billion, with additional costs relating to loss of productivity amount to an additional staggering USD 35 billion per annum.[11] Without the discovery of newer and more effective classes of antimicrobials, these figures are only set to burgeon.

The lack of effective antimicrobials further limits treatment options, with clinicians increasingly forced to resort to last-line antibiotics with undesirable adverse effects (e.g.

neurotoxicity and nephrotoxicity). The fact that even resistance has been reported for last-line antibiotics such as colistin and polymyxin B is of grave concern.[12] This is compounded by the dearth of antibiotic drug discovery in the past 5 decades.[13, 14] Only 2 novel classes of antibiotics e.g. lipopetides and oxazolidinones have been developed compared to the more than 20 novel classes between 1930 and 1960.[15] Taken together, with increasing antimicrobial resistance and the scarcity in antibiotic drug discovery, the need for a new effective and safe class of antimicrobials has never been more pressing.

To this end, antimicrobial peptides (AMPs) have been developed and are increasingly emerging as a promising class of antimicrobials with good antibacterial efficacy and yet, low propensity for resistance owing to its nonspecific mechanisms of action.[16-20] Cationic AMPs interact with negatively charged bacteria electrostatically, and its hydrophobic regions subsequently integrate within cell membranes, eventually resulting in membrane destabilization, lysis and death. [21, 22] Resistance development is circumvented due to the non-specific electrostatic and hydrophobic interactions that are utilized in membrane disruption and lysis. [23] Despite such promise, the translation of these AMPs into clinical use is hindered by various inherent shortcomings and limitations. Manufacturing costs are high, and AMPs are often associated with high systemic toxicity and low selectivity.[24] These limitations need to be overcome in order for the therapeutic utility of AMPs to be fully realized. Short synthetic AMPs have recently been synthesized to achieve maximum selectivity and efficacy, whilst minimizing systemic toxicity and production costs. [25-30] In particular, we previously reported a series of short synthetic peptide amphiphiles, which demonstrated in vitro efficacy against a broad spectrum of pathogenic microbes including Gram-negative bacteria.[31, 32] These peptides adopted a random coil structure in aqueous solution, while they self-assembled into a β-sheet secondary structure in a microbial membrane-mimicking environment, which is believed to facilitate microbial membrane disruption. They were specially designed to confer

good selectivity, cost-efficiency and ease of scale. In an independent head-to-head comparison of 30 short linear AMP sequences reported from different sources in the literature, it was found that the designed β-sheet forming AMPs were the most potent against *P. aeruginosa* among peptides of 8 amino acids or less.[33] Of these, the candidate peptides IRIKIRIK (IK8L, all L-amino acids), IRIkIrIK (IK8-2D, k, r: D-amino acids), and irikirik (IK8D, all D-amino acids) exhibited the most promising *in vitro* anti-microbial efficacy, and IK8D and IK8-2D were more stable in the presence of enzymes.[32] As such, this study was aimed to validate the *in vitro* and *in vivo* efficacy of these peptides against clinically isolated MDR *P. aeruginosa* in the planktonic and biofilm states. Firstly, the minimum inhibitory concentrations (MICs) of the peptides were determined against 20 clinical isolates of MDR *P. aeruginosa* in comparison with clinically used antibiotics imipenem, ceftazidime, and polymyxin B, as well as an α-helical AMP with similar charge and hydrophobiciy. Bactericidal efficiency was subsequently evaluated *via* time kill kinetics studies. For greater insight into the peptide's bactericidal mechanisms, mechanistic studies were performed *via* transmission electron microscopy (TEM), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and confocal microscopy. The antibacterial and biomass disruption capabilities of the peptide were evaluated using 7-day old biofilms grown from the clinically isolated MDR *P. aeruginosa* strain. Finally, *in vivo* anti-bacterial efficacy was evaluated in a mouse burn wound model. Systemic toxicity was also assessed by means of mouse blood serum chemistry.

2. Results and Discussion

2.1. *In vitro* Antibacterial Efficacy of Peptides Against Clinically Isolated MDR *P. aeruginosa*

We have previously reported a series of short synthetic β-sheet forming peptide amphiphiles and identified IK8L, IK8-2D and IK8D with the best demonstrated *in vitro* efficacy against a

standard strain of *P. aeruginosa* from ATCC (U.S.A.). This work was taken further by assaying the peptides against clinically-isolated MDR *P. aeruginosa* strains. The MICs of IK8L were determined against 20 clinical isolates of MDR *P. aeruginosa* and compared against two conventional first-line antibiotics (ceftazidime, imipenem),[34] one last-line cyclic peptide antibiotic (polymyxin B) used in clinical practice as well as one previously reported α-helical AMP, $(C(LLKK)_2C)$[25] (**Table 1**). Interestingly, IK8L was more potent than both IK8-2D and IK8D at inhibiting clinically isolated MDR *P.* aeruginosa, with inhbition of growth in 100% of the bacterial strains tested at a MIC value of 32 μg/mL compared to inhibition of only 75% of tested strains for the last two AMPs. The β-sheet forming peptides were also more effective than the α-helical peptide against the clinically isolated MDR *P. aeruginosa*. This result was in agreement with the findings reported by Jin *et al.*, which showed that the amphiphatic β-sheet conformation in AMPs of equal charge and hydrophobicity was more effective than the α-helix at mediating antimicrobial activity especially against *P. aeruginosa* and with lower hemolytic potentials.[35] Moreover, IK8L was convincingly superior to ceftazidime, with 100% of strains inhibited at a much lower concentration (32 μg mL^{-1} *vs.* 256 μg mL^{-1}). The MICs of imipenem against 10 strains were determined to be ≥ 8 μg mL^{-1}, which confirmed imipenem resistance.[36] Against a particular MDR strain *P. aeruginosa* 118 (**Table 2**), IK8L demonstrated superior efficacy with lower MIC values (8 μg mL^{-1}) compared to both imipenem (16 μg mL^{-1}) and ceftazidime (64 μg mL^{-1}). At their respective MICs, they eliminated more than 99.9% *P. aeruginosa* 118 (more than 3 lg reuction in the colony forming units (CFU) of the bacteria), hence demonstrating bactericidal activity (Figure S1, Supporting Information). Although polymyxin B had stronger antibacterial activity (Table 1), it showed higher cytotoxicity towards human HaCaT keratinocytes at concentrations of 31.3 μg $mL^{-1}$and above (**Figure 1**). The various β-sheet forming AMPs, imipenem and ceftazidime, on the other hand, did not induce overt cellular toxicity, with more than 90% cell viability up to 500 μg mL^{-1}. These results, taken together,

demonstrate the efficient antibacterial activity and excellent biocompatibility of the designed β-sheet forming AMPs.

Table 1. Cumulative distribution of MIC values (μg mL^{-1}) against clinically isolated MDR *P. aeruginosa* (n=20)

Agents	Cumulative % of 20 *P. aeruginosa* strains at indicated MICs									
	1	2	4	8	16	32	64	128	256	⩾512
IK8L			15	85	90	100				
IK8-2D				40	65	75	80	100		
IK8D					45	75	80	95	100	
$C(LLKK)_2C$						10	25	95	100	
Polymyxin B	55	95	100							
Imipenem		15	50	85	100					
Ceftazidime						20	80	95	100	

Table 2. MIC values of IK8L and antibiotics against *P. aeruginosa* 118.

Strain	Antimicrobial agents	MIC (μg/mL)
P. aeruginosa 118	IK8L	8
	IK8-2D	8
	Ceftazidime	64[a]
	Levofloxacin	8[a]
	Imipenem	16[a]

[a]Defined as resistant when MICs of ceftazidime, levofloxacin and imipenem were ⩾32, 8 and 16, respectively.

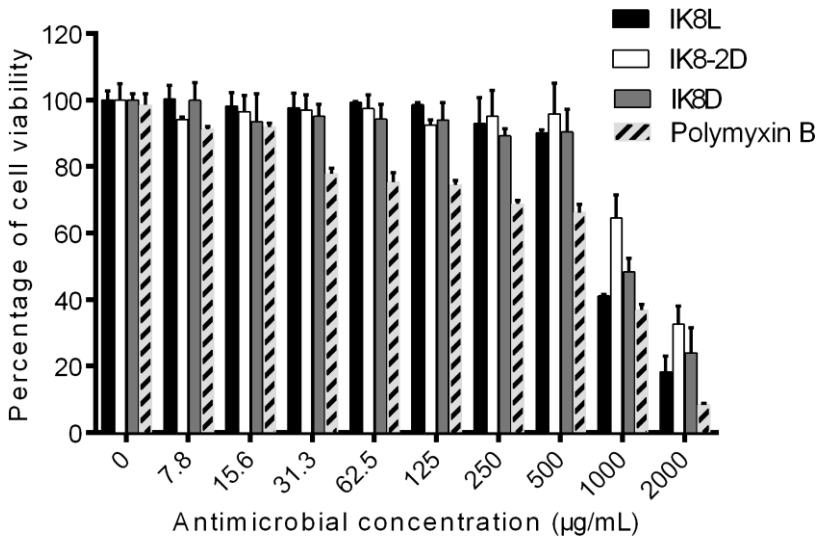

Figure 1. Viability of human HaCaT keratinocytes after incubation with the peptides or polymyxin B for 16 h at 37°C.

In addition to its superior antibacterial efficacy among the various β-sheet forming AMPs, IK8L also demonstrated more rapid bactericidal kinetics against *P. aeruginosa* 118 (**Figure 2**). At 2×MIC concentration, IK8L was able to completely eradicate all bacteria within 2 h, whereas viable bacteria were still observed with ceftazidime and imipenem-treated samples within the same time frame. Its killing kinetics was also demonstrated to be dose-dependent; killing time was halved (2 h to 1 h) when polymer concentration doubled from 2× to 4× MIC (Figure 2B-D). In contrast, even at higher concentrations of up to 4× MIC, imipenem and ceftazidime were unable to completely eradicate *P. aeruginosa* 118. With time often the essence in the successful management of sepsis, IK8L's superior bactericidal kinetics certainly stands it in good stead as a promising modality of treatment for MDR infections. Its rapid elimination of bacteria potentially limits the secretion and circulation of bacterial endo- and exo-toxins, preventing septic shock and other complications. [37, 38]

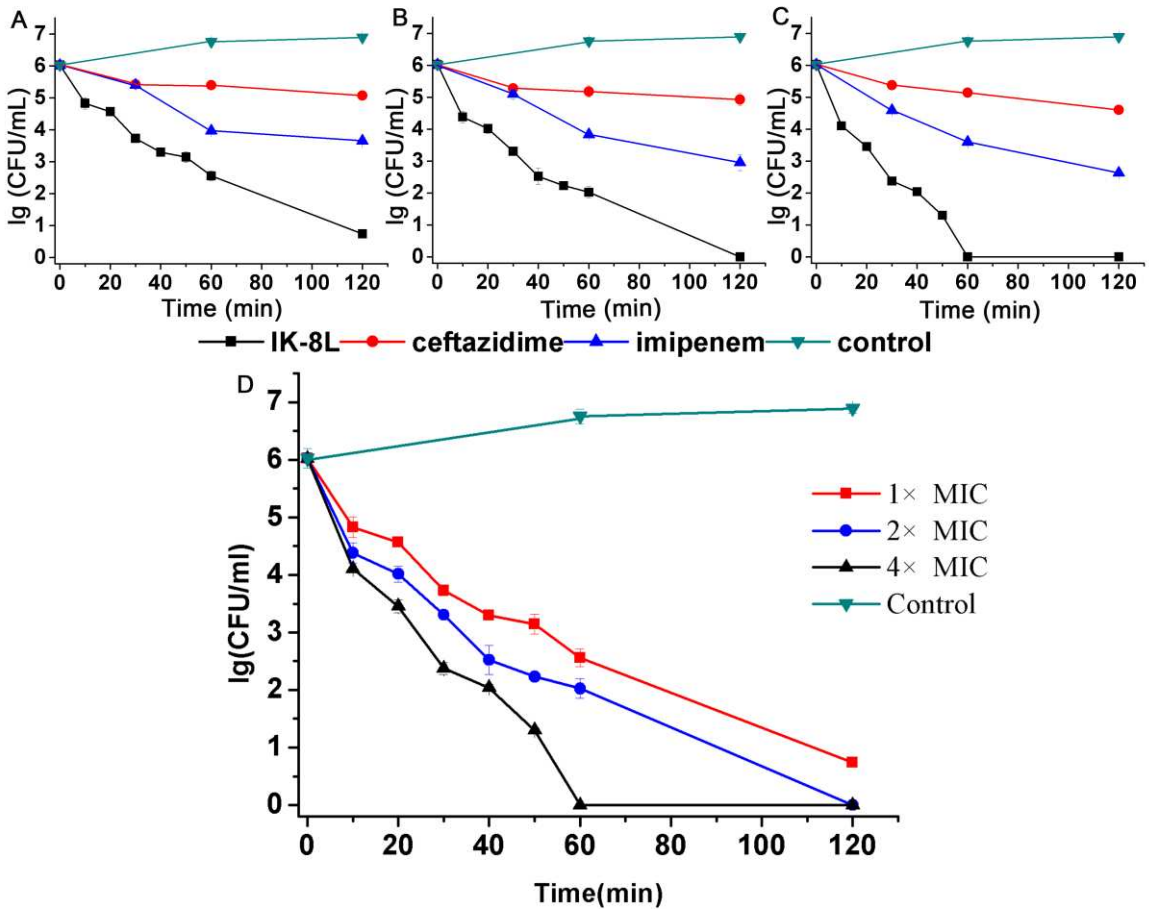

Figure 2. *In vitro* killing kinetics of IK8L, ceftazidime and imipenem. CFUs of *P. aeruginosa 118* from the different treatment arms were compared at varying concentrations A) 1 × MIC, B) 2 × MIC, and C) 4 × MIC. (D) Effect of varying concentrations of IK8L on killing kinetics. Error bars indicate respective standard deviations.

2.2. IK8L eradicated *P. aeruginosa via* membrane-lysis

AMPs have been reported to exert their bactericidal effects *via* the rapid perturbation and disruption of bacteria cellular membranes, leading to the leakage of cytoplasmic contents and eventual cell death. [39] We wanted to ascertain if a similar mechanism of action was operative for IK8L. *P. aeruginosa* 118 was treated with IK8L at 4× MIC concentration for 1 h; phosphate-buffered saline (PBS, pH 7.4) was used as a negative treatment control. Mechanistic evaluation was performed qualitatively *via* confocal and TEM microscopy, and quantitatively *via* SDS-PAGE.

Membrane-lysis was qualitatively demonstrated on confocal microscopy, with IK8L-treated bacteria exhibiting obvious fluorescein isothiocyanate (FITC)-labelled dextran (100 kDa) uptake compared to negative treatment controls (**Figure 3**). This was likely mediated through the loss of membrane integrity induced by peptide treatment, which permitted the entry of the otherwise membrane impermeable FITC-labelled dextran macromolecule into the cell. In concordance, TEM microscopy further revealed the corrugated and disrupted morphology of peptide-treated bacteria. Corresponding decreases in cytoplasmic electron density suggests the loss of cytoplasmic material through disrupted cellular membranes (**Figure 4**A-D). Quantitative evaluation with SDS-PAGE revealed a significant 4.4-fold increase in protein release with IK8L-treated bacteria compared to negative untreated controls (**Figure 4**E). This is likely again, mediated by the loss of membrane integrity, resulting in the leakage and release of cytoplasmic proteins. Taken together, both qualitative and quantitative evaluations convincingly suggest that IK8L exerted its bactericidal effects *via* a membrane-

lytic mechanism.

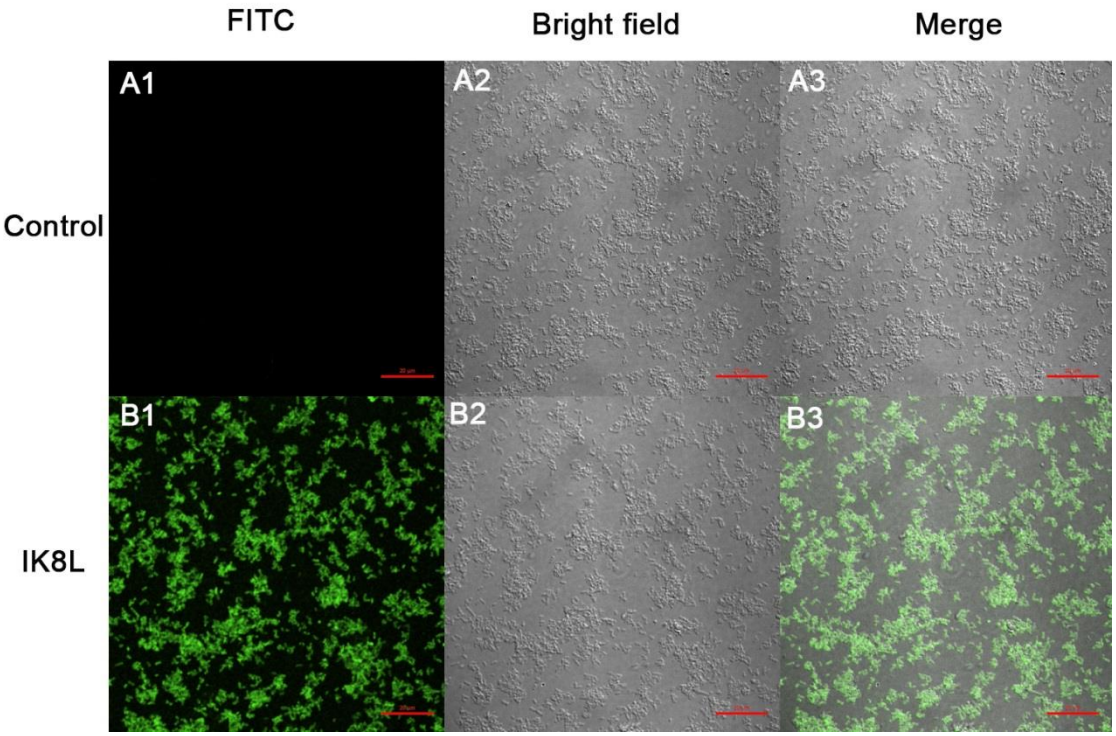

Figure 3. Confocal microscopic images of *P. aeruginosa* 118 incubated with FITC-conjugated dextran (100 kDa, 250 μg mL^{-1}) in the presence of A) PBS or B) IK8L for 1 h. A1, B1) Green represents fluorescence of FITC-conjugated dextran. A2, B2) Bright field. A3, B3: Merged images. Scale bar: 20 μm.

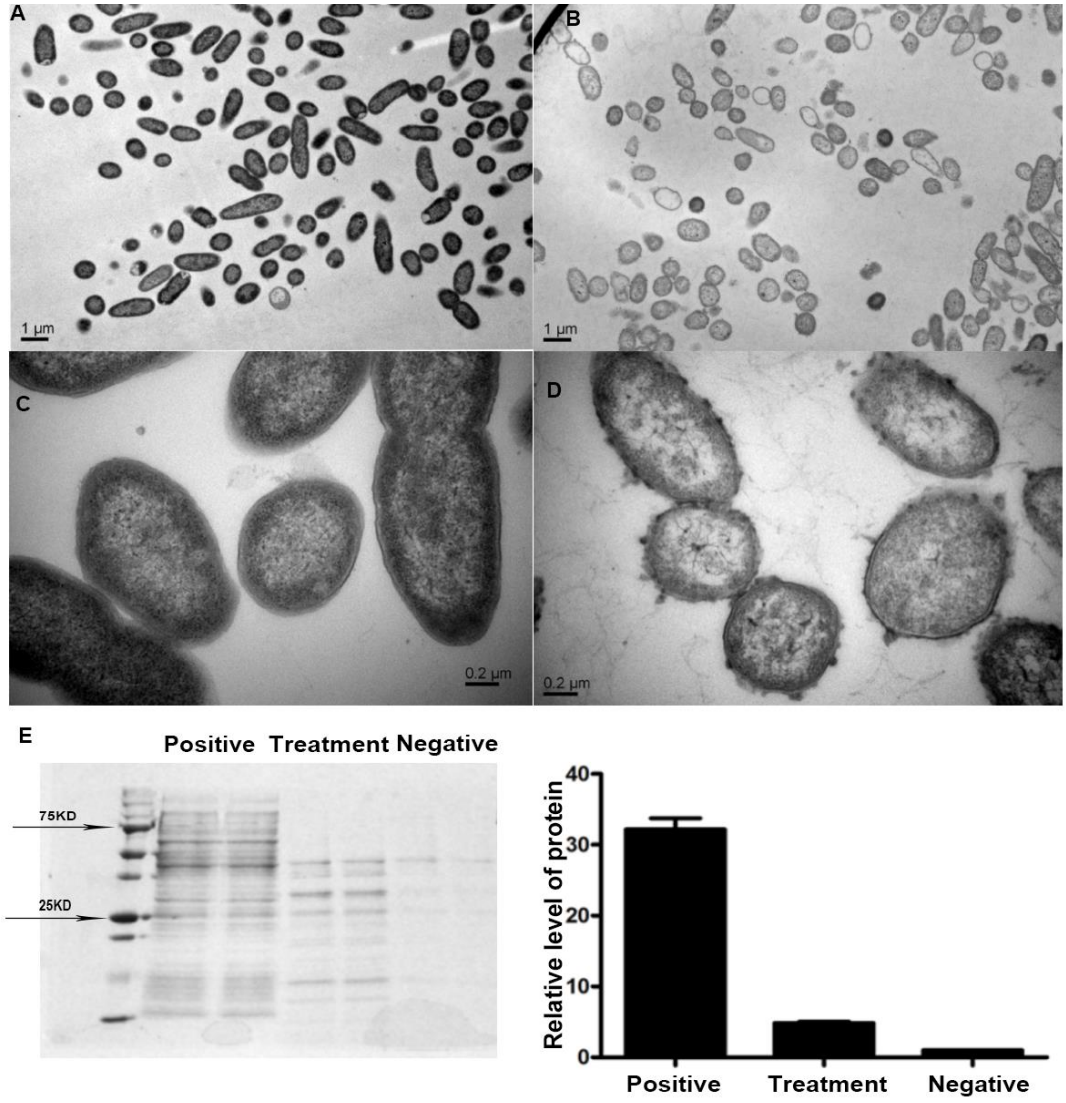

Figure 4. Membrane-lytic mechanism of IK8L. TEM images of *P. aeruginosa* 118 A, C) before and B, D) after treatment with IK8L for 1 h at 37°C. Size of the scale bar: 1 μm for (A) and (B) and 0.2 μm for (C) and (D). SDS-PAGE and semi-quantitative release of proteins by bacteria with and without treatment (E). The respective standard deviations were represented by error bars.

2.3. IK8L Treatment Effectively Prevented the Development of Drug Resistance

Prolonged repeated exposures of bacteria to sublethal doses of antibiotics invariably results in the development of antibiotic resistance. Antibiotic resistance occurs through a variety of means, chiefly through the accumulation of resistance genes within bacteria plasmids.[40] However, owing to its novel physiolytic mechanism of action, β-sheet forming peptides possessed the unique ability to avoid such resistance development against *S. aureus* and *E. coli*. [34] We thus sought to investigate if this unique property extended to the activity of IK8L

against *P. aeruginosa* as well. Resistance studies were conducted to evaluate IK8L's propensity towards resistance development. *P. aeruginosa 118* was used as a representative MDR strain. Antibiotics conventionally used in the treatment of MDR infections i.e. levofloxacin, ceftazidime, and imipenem were used as controls. Briefly, *P. aeruginosa 118* was treated with subtherapeutic doses of IK8L and the various antibiotics and passaged daily. MIC measurements were determined for up to ten passages. Negligible resistance was observed in bacteria treated with IK8L, with MICs remaining unchanged even up till the 10^{th} passage (**Figure 5**). In contrast, exponential increases in resistance were seen in bacteria treated with levofloxacin and ceftazidime, with MICs increasing 128 and 256-fold respectively, by the 10^{th} passage. Resistance development was also rapid, with observable differences in MIC noted as early as after the 2^{nd} passage (ceftazidime) (Figure 5). Whilst imipenem was most resilient against resistance development amongst the antibiotic controls, even it exhibited a 16-fold increase in MIC after 10 serial passages (Figure 5). The bacterial sample taken from imipenem-resistant *P. aeruginosa* 118 at the 10^{th} passage was cultured for 20 passages in the absence of imipenem, and the MIC of imipenem remained similar, indicating that imipenem-resistance of the bacterial cultures was stable. IK8L had the same MIC value against imipenem-resistant *P. aeruginosa* 118 at the 10^{th} passage as compared to *P. aeruginosa* 118 without imipenem treatment. These results convincingly demonstrate that IK8L can effectively inhibit the growth of imipenem-resistant *P. aeruginosa* and prevent resistance development.

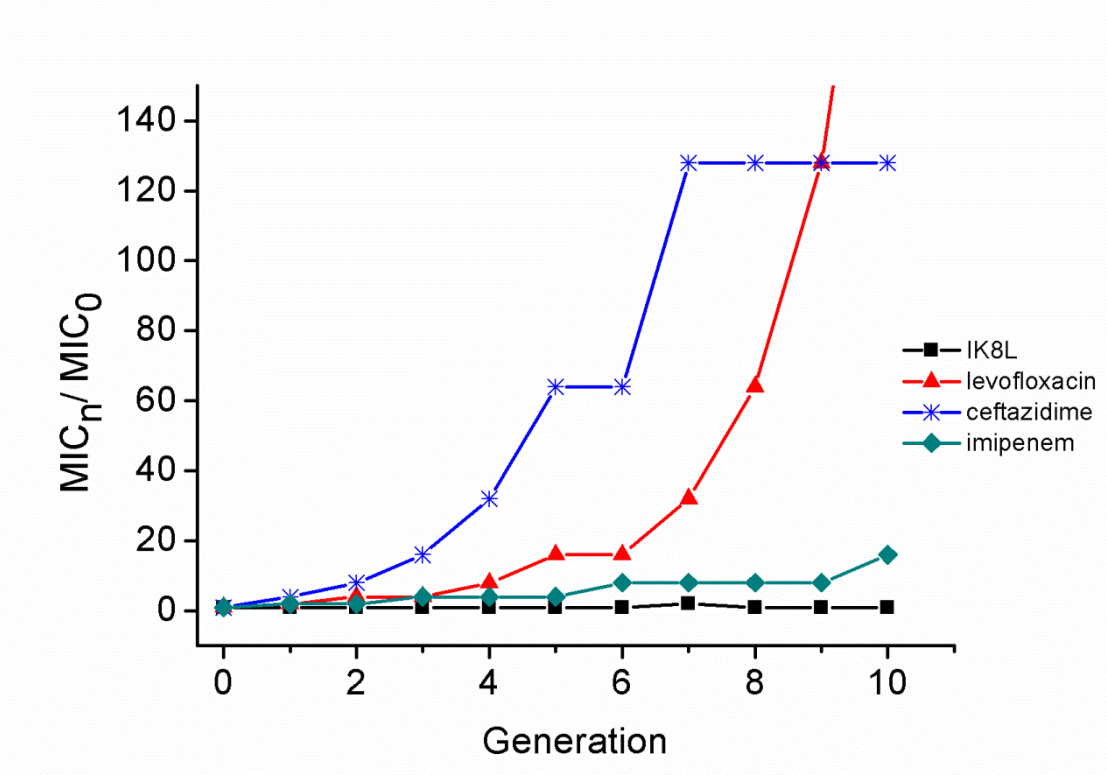

Figure 5. *In vitro* bacteria resistance studies. *P. aeruginosa* 118 were serially passaged together with IK8L, levofloxacin, ceftazidime, and imipenem at subtherapeutic concentrations for up to ten passages. After each passage, MICs were measured.

2.4. Antibiofilm Activity

Biofilms formed from *P. aeruginosa* are notorious for causing persistent and chronic infections clinically due to the presence of a dense matrix formed from extracellular polymeric substances which limit antibiotic penetration and promote higher levels of antibiotic resistance compared to free planktonic cultures.[41] To study the antibiofilm activity of IK8L, *P. aeruginosa* 118 biofilm was formed after 7 days of culture. The biofilm was treated with IK-8L for 24 h at different concentrations. As shown in **Figure 6**, the peptide showed dose-dependent anti-biofilm efficacy. The viability of *P. aeruginosa* 118 in the biofilms decreased to ~10%, and the amount of biomass reduced to ~35% after a single treatment at 8× MIC. These results demonstrated that the peptide effectively eliminated the bacteria in the biofilm and dispersed the biofilm matrix.

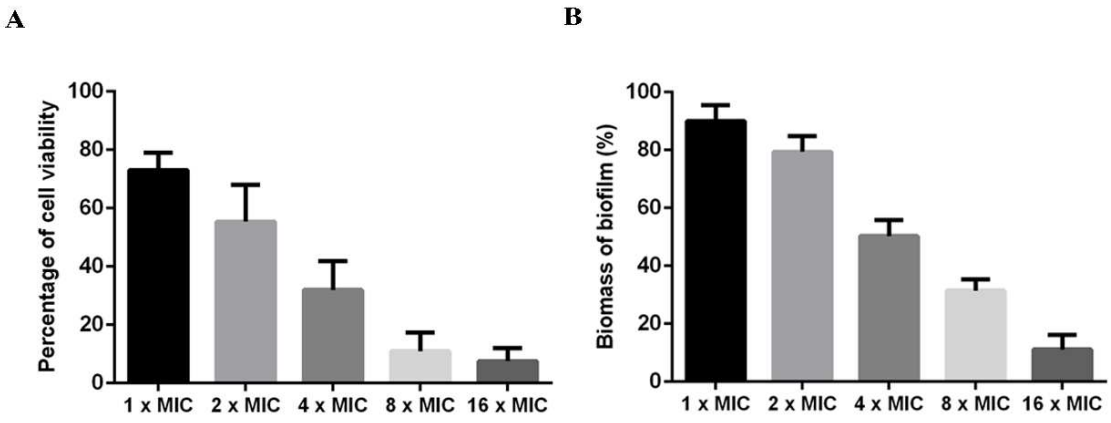

Figure 6. Antibiofilm activity of IK8L. A) Cell viability and B) biomass of *P. aeruginosa* 118 biofilm after peptide treatment for 24 h at various concentrations.

2.5. IK8L Effectively Treated MDR *P. aeruginosa*-Infected Burn Wounds with Negligible Toxicity

Given the prevalence of *P. aeruginosa* in the infection of burn wounds, [42, 43] and against the backdrop of our promising *in vitro* results, the utility of IK8L in the treatment of infected burns wounds was further explored in an *in vivo* mouse burns model. Briefly, dorsal mice skin was thermally injured at 100°C and subsequently infected with MDR *P. aeruginosa* 118 at a concentration of 1×10^8 CFU mL^{-1} (1 mL). Mice were subsequently treated with topical applications of IK8L (2.56 mg kg^{-1}) or IK8-2D (2.56 mg kg^{-1}); with imipenem (5.12 mg kg^{-1}) and PBS used as positive and negative controls, respectively. Polymyxin B was not used as a positive control as it caused cytotoxicity toward human HaCaT keratinocytes even at low concentrations (Figure 1). Treatment efficacy was determined *via* quantitative outcome measures such as (1) reduction in viable bacterial counts (skin and blood samples) and (2) overall mice survival. All mice were tracked at specific time points at 2, 3, and 7 d post-treatment. The results demonstrated superior treatment efficacy with topical IK8L compared to imipenem and PBS controls. Topical applications of IK8L and IK8-2D resulted in a 4.36 log_{10} and 4.0 Log_{10} (CFU g^{-1} of skin) reduction in viable bacterial counts of the wounds,

respectively, compared to imipenem (2.39 $\log_{10}$ reduction) when normalized against negative PBS controls (**Figure 7A** and Figure S2A). This is possibly because the peptide eradicated the bacteria more rapidly than imipenem (Figure 2). Concordance was also seen with the reduction of viable bacterial counts in blood cultures. IK8L treatment resulted in a further 20.6% reduction in bacterial blood counts compared to imipenem controls (**Table 3**). IK8L treatment led to slightly improved survival than IK8-2D treatment (80% *vs.* 70%, respectively). Importantly, mice treated with IK8L demonstrated much higher survival compared to PBS and imipenem controls 7 days post-treatment (80% *vs.* 40% and 60%, respectively) (Figure 7B and Figure S2B, Supporting Information) likely due to the AMP's faster bacteria killing and greater antibacterial potency (Figure 2 and Table 2).

Table 3. Efficacy of IK8L and imipenem against *P. aeruginosa* infection in a mouse burn wound model at 3 days postinfection.

Treatment	Survival (%)	Log_{10}(CFU g^{-1} of skin)[a]	%(CFU ml^{-1} of blood)[b]
Control	40	8.93	100
IK8L	80	4.69	13.6
imipenem	60	6.54 ($p<0.05$)	34.2 ($p<0.05$)

[a] Viable counts of *P. aeruginosa* from skin cultures of the surviving mice in the control and treated groups; [b]% CFU/mL of blood versus the control group.

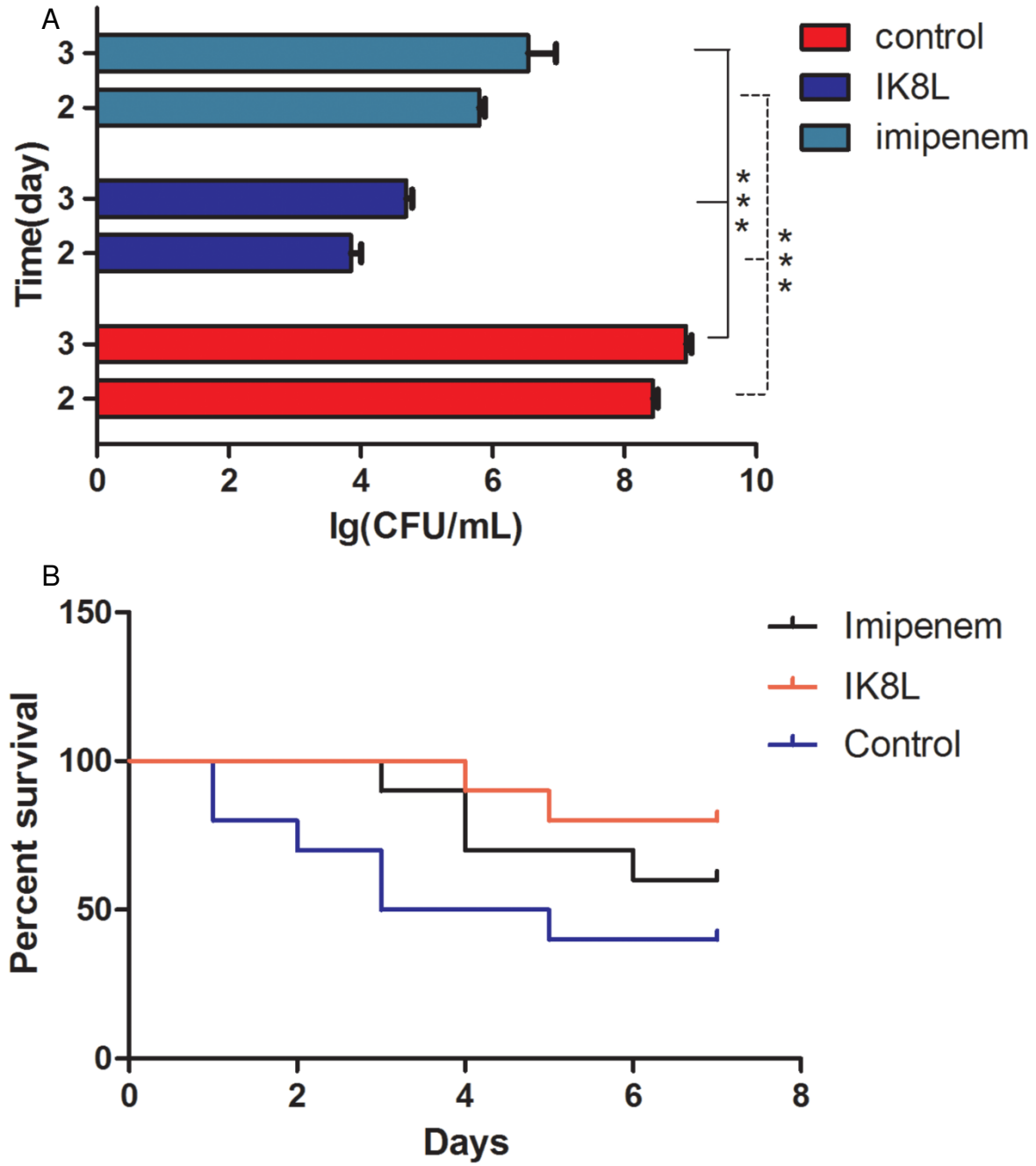

Figure 7. *In vivo* antibacterial efficacy of IK8L in a mouse MDR *P. aeruginosa* 118-induced burn wound infection model. A) Reduction of viable bacterial colonies in mice skin specimens taken on the 2nd and 3rd day post-infection. *** p< 0.001, IK8L versus imipenem, IK8L versus PBS, imipenem versus PBS. B) Mice survival. Tracked for up to 7 days post-infection. Respective standard deviations were represented by error bars.

Such treatment efficacy was achieved remarkably, at negligible mice toxicity (Table S1, Supporting Information). Liver and renal function studies performed on IK8L-treated mice were largely unremarkable compared to the negative PBS control. No major electrolyte

disturbances (potassium/sodium) were detected. This observation is important as systemic toxicity is the major limiting factor in the development of new membrane-active AMPs.[18] In this study, the peptide was applied directly as a solution. The peptide can also be incorporated into hydrogels or creams for easy application as it is highly water soluble. Thus, our results convincingly demonstrate the promising utility of IK8L in the efficacious and yet, safe treatment of MDR *P. aeruginosa* burn wounds.

3. Conclusion

We have demonstrated that the short synthetic β-sheet folding peptide IK8L possesses the advantages of a lower propensity for resistance development with faster killing kinetics against MDR *P. aeruginosa* than conventional antibiotics. The peptide is also capable of inhibiting MDR *P. aeruginosa* growth within biofilms and leads to a significant reduction in biomass after treatment. The antibiofilm activity of the β-sheet forming peptide is clinically relevant as biofilm development is a common and extremely difficult-to-treat complication in *P. aeruginosa* infected wounds. The *in vivo* antibacterial efficacy of the β-sheet forming peptides was demonstrated to be superior to the clinically used antibiotic imipenem in a MDR *P. aeruginosa* infected mouse burn wound model. Importantly, this was achieved with improved mice survival and negligible systemic toxicity. Our mechanistic studies posit a membrane-lytic mode of action to be likely operative. Taken together, our results convincingly show the potential of IK8L in the treatment of MDR *P. aeruginosa* infected burn wounds.

4. Experimental Section

Peptides: The peptides were synthesized in GL Biochem (Shanghai, China) with more than 95% purity as ascertained by reverse phase high performance liquid chromatography.[30] The successful synthesis of the peptides was confirmed by matrix-assisted laser

desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF MS) (Autoflex II, Bruker Daltonics Inc., U.S.A).

Bacterial strains: Twenty clinically-isolated strains of multidrug-resistant *P. aeruginosa* were obtained from body fluid samples (blood/phlegm) of patients warded in The First Affiliated Hospital of Medical College, Zhejiang University (Hangzhou, China). All bacteria isolates were identified by routine laboratory methods and stored in 20% (v v^{-1}) glycerol at -80°C prior to usage.

MIC measurements: *P. aeruginosa* isolates were obtained in mid-exponential growth phase after 24 h incubation in Mueller-Hinton (MH) agar plates at 37°C. The broth microdilution method [30] was employed to determine the respective MICs of the peptides, ceftazidime and imipenem (Meck Sharp & Dohme Corp, American). Briefly, the anti-microbial agents were diluted to varying concentrations with MH broth (MHB). Peptide solution was serially diluted from 256 to 0.5 μg mL^{-1}, imipenem from 512 to 0.5 μg mL^{-1}, ceftazidime from 2048 to 8 μg mL^{-1}. Bacterial suspensions were diluted with 0.45% sodium chloride solution until an optical density reading of 0.07 was achieved, which corresponds approximately to 1×10^8 CFU mL^{-1}. This was further diluted 100-fold in MHB to obtain the final working concentration of 10^6 CFU/mL. Equal volumes (100 μL) of bacterial suspension were added to the agent solutions at varying concentrations in each well (96-well plates), and subsequently incubated for 18 h at 37°C. The concentration at which no bacterial growth was observed (clear solution) was then taken to be the MICs of the respective agents. Broth containing untreated bacteria was used as the negative control. Each experiment was repeated in triplicates.

Cytotoxicity assay: The cytotoxicity of the peptides and polymyxin B against HaCaT keratinocytes was studied by MTT assay according to the protocol reported previously.[28]

The cells were seeded onto a 96-well plate (density: 10000 cells per well), and allowed to attach overnight. The medium was then replaced with fresh medium containing the peptides or polymyxin B at different concentrations, and incubated for 16 h at 37°C. Cell viability was measured with and without the antimicrobial treatment.

Killing efficiency: After 24 h of incubation, *P. aeruginosa* 118 suspension treated with IK8L and antibiotics (50 μL) was plated on MH agar plates with or without dilution using the growth medium. The agar plates were incubated at 37°C for 24 h. The number of colony forming units was counted.

Time-kill kinetics assay: *P. aeruginosa* 118 isolate was used to determine the killing kinetics of the various agents according to a protocol reported previously.[31] Briefly, the bacteria were grown in MH agar plate at 37°C. After 24 h, the bacteria were suspended in 0.45% NaCl solution to achieve a turbidity corresponding to McFarland 0.5 standard (1 x 10^8 CFU ml^{-1}). This suspension was further diluted 100 times to obtain a working starting concentration of 10^6 CFU ml^{-1}. The various agents (IK8L, ceftazidime, imipenem, levofloxacin) were then added to this working bacterial suspension at varying concentrations to obtain final concentrations of 1×, 2× and 4× MIC. Untreated bacteria were used as the negative control. The samples were then incubated at 37°C for 2 h, during which samples were pipetted out at 10-minute intervals, and serially diluted in 10-fold. Each diluted bacteria suspension (50 μL) was then plated on MH agar plate. After overnight incubation at 37°C, the number of viable colonies was counted and compared across different time intervals to determine the killing kinetics of each agent. All experiments were performed in triplicates and results expressed as mean lg (CFU ml^{-1}) ±SD.

TEM analysis: The morphology of *P. aeruginosa* 118 pre- and post-treatment with IK8L was observed under a JEM-1230 TEM (JEOL, Japan) at an acceleration voltage of 80 kV based on a method described previously.[31] Bacterial suspension of *P. aeruginosa 118* was prepared and diluted as previously described for MIC measurement. IK8L at 4×MIC concentration was subsequently added to the final working bacterial suspension and incubated for 1 h. Untreated bacterial suspensions were employed as negative controls. After incubation, the bacterial suspension was then centrifuged (5000 rpm, 10 min), and bacteria precipitates were then fixed overnight in 2.5% glutaraldehyde (in PBS) at 4°C. The fixed bacteria were then washed thrice with PBS, and further incubated for 1 h in 1% OsO4 in PBS. After again washing thrice with PBS, dehydration was performed using a graded ethanol series. The samples were then placed in a 1:1 acetone/Spurr resin mixture for 1 h at r.t.p, subsequently transferred to 1:3 acetone/resin mixture for 3 h, and finally in complete Spurr resin overnight. These fixed samples were then processed with a Reichert-Jung Ultracut E Ultra microtome to achieve 70-90 nm thickness, and stained with uranyl acetate and lead citrate for 15 min prior to TEM observations.

Protein release analysis by SDS-PAGE: *P. aeruginosa 118* isolate was cultured overnight in MH agar plate at 37°C, and subsequently suspended in 0.45% NaCl solution to achieve a turbidity corresponding to McFarland 0.5 standard (1 x 10^8 CFU ml^{-1}). This suspension was then treated with IK8L at 4× MIC concentration and incubated at 37 °C for 1 h. Post-incubation, the bacteria samples were then centrifuged (1000g for 5 min, 4°C), supernatants removed and replaced with 4 µL of 5× SDS loading buffer. Untreated bacteria suspensions were used as negative controls; bacteria suspensions were also boiled to obtain positive controls. Samples were then subjected to SDS-PAGE followed by coomassie brilliant blue staining. Images were quantified using a Bio-Rad Gel Doc XR and Image Lab software (Bio-rad).

Confocal microscopy: *P. aeruginosa 118* was inoculated and prepared according to the protocols as afore-stated for MIC measurement. Bacteria suspension (400 µL) was seeded onto a 8-well cover slip chamber and incubated overnight at 37 °C under shaking (100 rpm). Samples were then removed and replaced with an equal volume of MHB media containing IK8L (4× MIC concentration) and FITC-dextran (100 kD, 250 µg mL^{-1}). After further incubation for 1 h, samples were then washed thrice with PBS to remove any free dye. Samples were then fixed and morphology of bacteria was observed under confocal microscopy (Nikon AIR confocal microscope, 1000× (oil) Plan Apochromate Lens).

In vitro resistance development studies: The propensity for *P. aeruginosa 118* to develop resistance against the various therapeutic agents used in this study was evaluated *in vitro*. Briefly, the MICs of the various agents (IK8L, levofloxacin, ceftazidime, imipenem) were determined using the protocols as afore-described. Partially treated bacteria (0.5× MIC) was harvested, washed and cultured overnight in MH agar. MICs were then determined for these bacteria samples for up to 10 similar serial passages. Development of resistance was then monitored by evaluating for any changes in the MICs of the various agents.

Biofilm assays: Anti-biofilm activity of the peptide IK8L was evaluated according the protocols reported previously.[29] *P. aeruginosa* 118 biolfim was formed after 7 days of culture, and treated with the peptide at at MIC, 2 × MIC, 4 × MIC, 8 × MIC and 16 × MIC. Viability of the cells in the biofilm and biomass were measured with or without the peptide treatment.

Preparation of animals: Female ICR mice (8 weeks old, 26-28mg) were used for the *in vivo* studies later described. Immunosuppression was induced by intra-peritoneal injection of 200

mg/kg cyclophosphamide (Hengrui Corp, Jiangsu Province, P. R. China) 4 days prior to bacteria inoculation. Anesthesia was introduced using an intraperitoneal (*i.p.*) injection with 1% pentobarbital (40mg kg^{-1}, sigma). All animal studies were performed in accordance with protocols approved by the Animal Studies Committee, P. R. China.

Mouse burn wound infection model: Mice used were immunosuppressed as described above. First, mice were anesthetized using an *i.p.* injection with 1% pentobarbital (40mg kg^{-1}). Once the mice were fully anesthetized, the dorsa hair was clipped and then the depilatory cream was introduced later to totally shave off the dorsa. Thermal injury was then inflicted unto the mice dorsal skin using 8 layer of gauze (2 cm × 3 cm) at 100°C for 30s. Immediately after thermal injury, PBS was administered *i.p.* for fluid resuscitation.

Evaluation of in vivo anti-bacterial efficacy: The *in vivo* anti-bacterial efficacy of IK8L was determined using the mouse burn wound infection model. Briefly, overnight cultures of *P. aeruginosa 118* were harvested and prepared according to the protocols as afore-described. All mice were scalded as described above. The *P. aeruginosa 118* was then distributed over thermal injury surface at a designated dose of 1×10^8 CFU ml^{-1} (1 mL). A total of 30 mice were used, equally distributed amongst the control, IK8L and imipenem treatment groups. The mouse burn wounds were treated topically with PBS, IK8L, IK8-2D (both at 2.56 mg kg^{-1}) or imipenem (5.12 mg kg^{-1}) an hour after bacterial challenge. In 2 or 3 days later, blood and skin samples were subsequently analysed to determine treatment efficacy. Briefly, blood was taken from the peri-orbital plexus and 0.05 mL dilutions were plated on MH agar plates. Mouse skin was removed and homogenized in 2 mL of sterile PBS solution under aseptic conditions. The homogenates were then serially diluted, and diluents (0.05 mL) were plated on MH agar plates. After overnight incubation, colony forming units (CFU) were subsequently counted after 24 h incubation. Data was expressed as mean lg (CFU g^{-1} of skin)

± SD and % (CFU ml^{-1} of blood). All mice were followed up for 7 days and survival tracked accordingly for each treatment group.

In vivo toxicity studies: Blood serum chemistry was performed to investigate the potential *in vivo* systemic toxicity introduced by IK8L. Briefly, blood samples were obtained from the peri-orbital plexus of anaesthetized mice at 72 h after topical treatment with PBS and IK8L (2.56 mg kg^{-1}) in mice burn wound infection model described above. Liver and renal function tests on mouse blood samples were performed to assess the potential hepato- and nephrotoxicity of IK8L.

Statistical analysis: Statistical analyses were performed using either two-way ANOVA or two-tailed Fisher's exact T-test wherever appropriate. P values < 0.05 were taken to be statistically significant.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Short synthetic β-sheet forming peptides demonstrate potent efficacy against 20 strains of difficult-to-treat clinically-isolated multidrug-resistant *P. aeruginosa*. Repeated use of the peptide does not induce drug resistance. The peptide IRIKIRIK mediates rapid bactericidal activities and efficiently eradicates bacteria in an *in vivo P. aeruginosa* infected mouse burn wound model, leading to enhanced survival rate with no systemic toxicity.

Keyword: antimicrobial, peptides, burns wounds, infections, multidrug resistance

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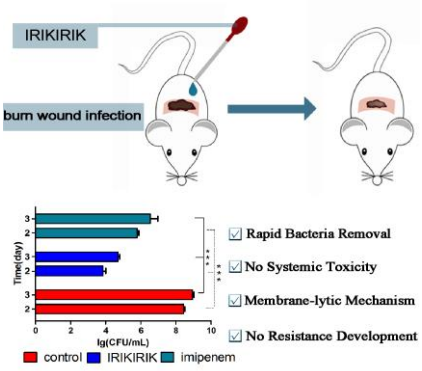
Title: Short synthetic β-sheet anti-microbial peptides for the treatment of multidrug-resistant *Pseudomonas aeruginosa* burns wound infections



Supporting Information

Title: Short synthetic β-sheet anti-microbial peptides for the treatment of multidrug-resistant *Pseudomonas aeruginosa* burns wound infections

Guansheng Zhong, Junchi Cheng, Zhen Chang Liang, Liang Xu, Weiyang Lou, Chang Bao, Zhan Yuin Ong, Huihui Dong, Yi Yan Yang, Weimin Fan**

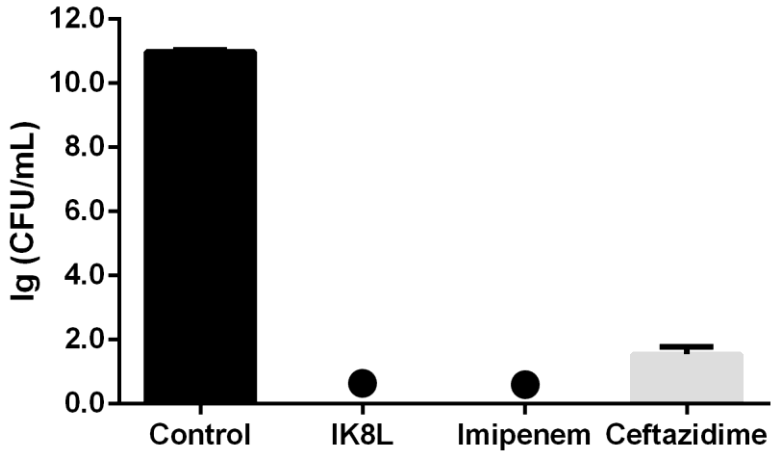


Figure S1. Killing efficiency of IK8L, imipenem and ceftazidime against clinically isolated MDR *P. aeruginosa* 118 at their respective MICs (• denotes no colonies observed).

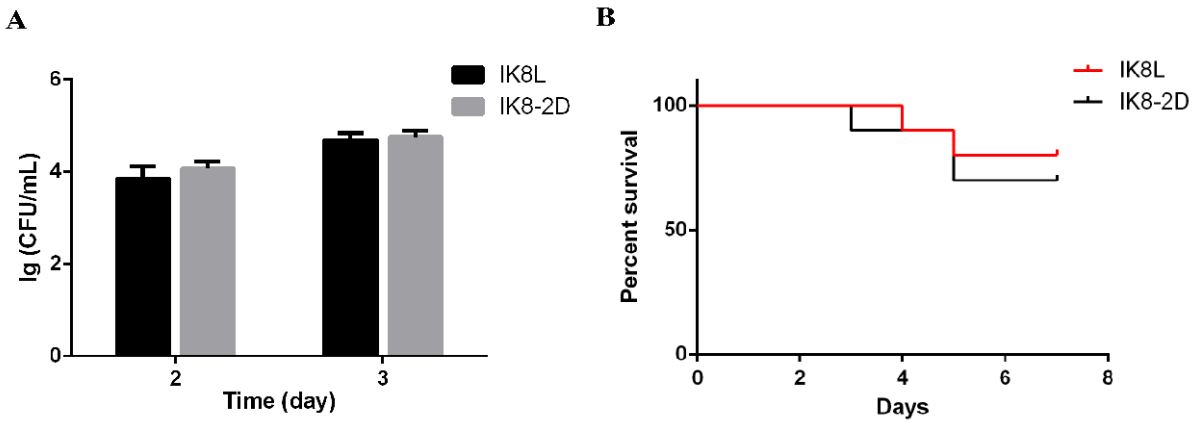


Figure S2. *In vivo* antibacterial efficacy of IK8-2D in a mouse MDR *P. aeruginosa* 118-induced burn wound infection model. (A) Reduction of viable bacterial colonies in mice skin specimens taken on the 2nd and 3rd day post-infection. *** p=0.414 > 0.05, IK8-2D *vs.* IK8L. (B) Mice survival. Tracked for up to 7 days post-infection.

Table S1. *In vivo* toxicity studies (serum chemistry).

Treatment[a]	ALT (U/L)[b]	AST (U/L)	Creatinine (μmol/L)	Urea nitrogen (mmol/L)	Sodium ion (mmol/L)	Potassium ion (mmol/L)

PBS	26.2±5.3	85.5±8.6	16.8±4.8	7.9±1.1	145.5±2.8	4.1±0.6
IK8L	29.4±4.8	76.6±4.6	17.6±5.5	8.4±0.9	148.5±2.9	4.7±0.4

[a]Mice (n=8 in each group) treated with PBS or IK8L
[b]U/L, international units per litre
IK8L *vs.* PBS: $p>0.05$