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ARTICLE

Targeting Intracellular Bacteria with an Extended Cationic Amphiphilic Polyproline Helix

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An extended cationic and amphiphilic polyproline helix (CAPH) is described with a dual mode of action: effective cell penetration of human macrophages, and potent antimicrobial activity *in vitro* against both Gram-positive and negative pathogens, including *Acinetobacter baumanii*, *Escherichia coli* O157 and *Bacillus anthracis.* This dual action was successfully combined to clear pathogenic bacteria (*Brucella* and *Salmonella*) residing within macrophages.

Introduction

The emergence of antimicrobial resistance in recent decades has created a major global health crisis.[1-3] The continuous rise in pathogenic antibiotic resistant strains, such as carbapenem-resistant *Enterobacteriaceae* (CRE) and methicillin- and vancomycin resistant *Staphylococcus aureus* (MRSA and VRSA) has undermined almost all clinically approved antimicrobial agents.[4-7] Even worse, outbreaks of pan-drug resistant (PDR) strains, such as *A. baumannii*, that are resistant to all FDA approved antibiotics have been reported in recent years.[8-10] This problem is further compounded by bacterial strains that thrive or transiently reside inside mammalian cells, such as phagocytic macrophages.[11] A range of pathogens, including *Mycobacterium tuberculosis*, *Salmonella*, *Listeria*, *Legionella* and *Brucella*, seek protection inside these intracellular havens.[12] A significant subset of antibiotics, such as β-lactams and aminoglycosides, fail to rescue cells infected with these pathogens due to their poor cell permeability and/or their susceptibility to drug efflux transporters.

With the goal of eradicating intracellular bacteria, we sought to combine the properties of cell penetrating peptides (CPPs) and antimicrobial peptides (AMPs) within a single designed agent. A few elegant studies have used CPPs to deliver antibacterial agents to cells.[13, 14] However, our efforts have focused on the development of CPPs with intrinsic antibacterial activity. A wide array of cationic and amphiphilic AMPs display antibacterial activity through a membrane-lytic mechanism of action, and in some cases show good selectivity for bacterial versus mammalian cell membranes.[15-18] A subset of cationic, proline-rich AMPs (P-AMPs) exert their antimicrobial action through a non-membrane active pathway.[19-21] These P-AMPs, such as bactenectin, PR-39 and drosocin, are less toxic to mammalian cells and have reduced hemolytic activity when compared to AMPs in general.[19-21]

Our designed dual-action agents, therefore, harnessed a proline-rich polyproline type II (PPII) helical scaffold, but with chemical modifications to introduce amphiphilicity and a net positive charge – termed cationic amphiphilic polyproline helices (CAPHs) (Fig. 1a). One particular compound**, Fl-$P_RP_RP_L$-4** (Fig. 1b), displayed efficient cell penetration and potent anti-lytic antibacterial activity *in vitro* and *in cyto*.[22] A shorter homolog, **Fl-$P_RP_RP_L$-3**, however, provided limited activity against Gram-negative and positive bacteria *in vitro*. With this in mind, we wished to explore an extended PPII helix (**Fl-$P_RP_RP_L$-5**) containing additional cationic and hydrophobic groups within the helix. We postulated that these additional moieties would lead to an increase in the initial interactions between the helix and cell membranes that are crucial for cell penetration. [22, 23] Herein we describe efforts to ultimately target intracellular bacteria with extended CAPHS.

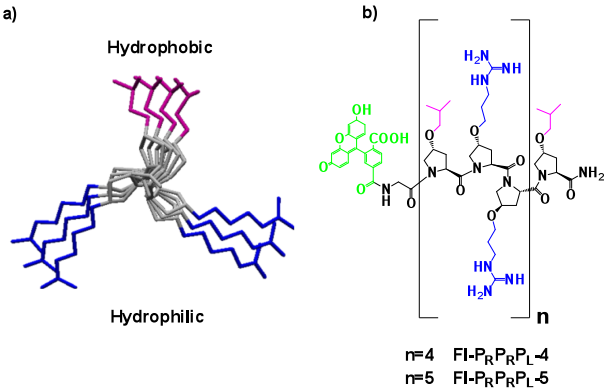

Fig. 1 Cationic amphiphilic polyproline helices (CAPHs). a) Top view of the PPII helix model with hydrophobic face (pink) and hydrophilic face (blue), and b) structures of CAPHs **Fl-$P_RP_RP_L$-4** and **Fl-$P_RP_RP_L$-5** with the fluorescein fluorophore in green.

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Result and Discussion

The CAPH peptides were synthesized on the Rink amide resin using standard Fmoc-based solid phase synthesis methods. The proline-modified unnatural amino acids bearing hydrophobic (P_L) and cationic (P_R) moieties were synthesized as previously described.[24] The peptides were purified to homogeneity using reversed phase HPLC, and characterized using MALDI spectrometry (see Supplementary Information Fig. S1).

Since many pathogenic bacteria reside within human macrophages, effective cell penetration of antibacterial agents is a prerequisite to rescue infected mammalian cells. The ability of **Fl-$P_RP_RP_L$-5** to enter J774A.1 macrophage cells was determined using flow cytometry, and was compared to **Fl-$P_RP_RP_L$-4**. Both CAPHs demonstrated a concentration-dependent entry into cells (Fig. 2). For instance, **Fl-$P_RP_RP_L$-5** displayed a 7-fold increase in cellular accumulation upon increasing the concentration from 5 to 25 μM. At each concentration tested, however, **Fl-$P_RP_RP_L$-5** demonstrated approximately a 2.5- to 3.5-fold increase in cell penetration as compared to its shorter counterpart, demonstrating the significance of increasing the cationic charge and number of hydrophobic groups within CAPHs on cell uptake.[18,22,23] In addition, we evaluated the integrity of the cell membranes using Sytox Red, and found no change in the permeability of the cell membranes with CAPH-treated and untreated cells (see Supplemental Information Fig. S2).

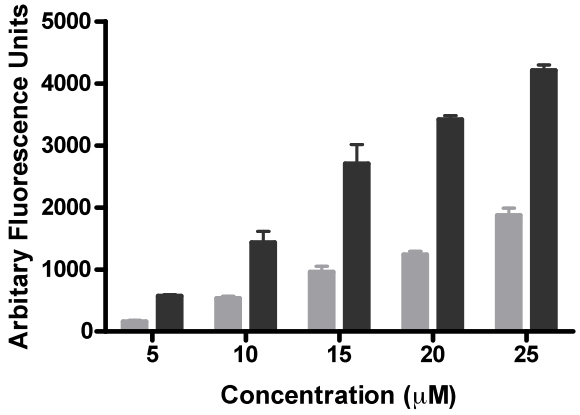

Fig. 2 Cellular fluorescence due to internalization of Fl-$P_RP_RP_L$-4 (gray) and Fl-$P_RP_RP_L$-5 (black) within J774A.1 cells after 1 h incubation as analyzed by flow cytometry.

Intracellular bacteria reside in a number of sub-cellular niches.[11, 12] It is of interest, therefore, to determine the sub-cellular localization of the designed CAPHs. **Fl-$P_RP_RP_L$-4**, for instance, has been reported to associate with both endosomes and mitochondria inside macrophages.[22] The localization of **Fl-$P_RP_RP_L$-5** within live J774A.1 cells was investigated using confocal microscopy. **Fl-$P_RP_RP_L$-5** was found to reside within macrophages and localize to endosomes and mitochondria as demonstrated by co-localization with Lysotracker and Mitotracker dyes, respectively (Fig. 3). However, **Fl-$P_RP_RP_L$-5** localization is more pronounced in mitochondria, as compared to **Fl-$P_RP_RP_L$-4.** Under these conditions both **Fl-$P_RP_RP_L$-4** and **Fl-$P_RP_RP_L$-5** demonstrated no increase in reactive oxygen species within the cells, and both were found to have a limited influence on mitochondrial membrane depolarization (~10 and 20%, respectively), whereas the known the mitochondrial depolarizer FCCP showed strong depolarization (~40%) (see Supplemental Information Fig. S3). This localization inside mitochondria and, to a lesser extent, endosomes suggests that **Fl-$P_RP_RP_L$-5** has potential to access bacteria that reside within cells.

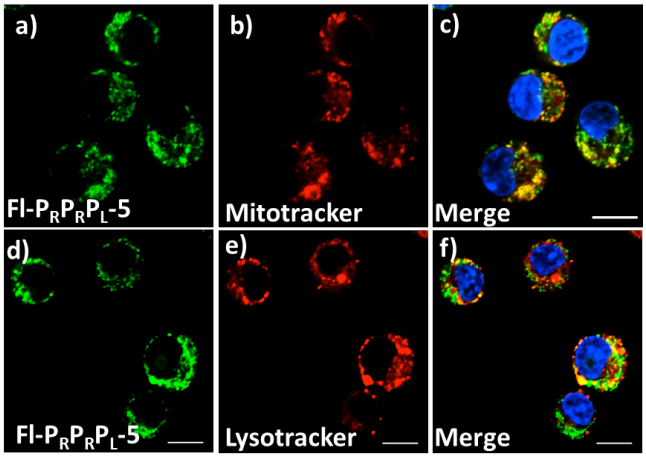

Fig. 3 Confocal images of J774A.1 cells incubated with Fl-PRPRPL-5 (15 μM) for 1 h. Co-localization was studied with a) the mitochondria (a-c) tracking dye, Mitotracker and b) endosome tracking dye, Lysotracker (d-f), the merged images also have cells treated with Hoechst 33342 to highlight the nuclei.

The antibacterial activity of **Fl-$P_RP_RP_L$-5** was next explored with both Gram-positive and negative bacteria using a broth dilution assay. **Fl-$P_RP_RP_L$-5** displayed a minimum inhibitory concentration (MIC) of 2 μM against *E. coli* (ATCC 25922) and 4 μM against *S. aureus*, values that are 2- and 3-fold more potent than those observed with **Fl-$P_RP_RP_L$-4**. Higher electronic interactions between the more positively charged **Fl-$P_RP_RP_L$-5** and negatively charged bacterial membrane may have resulted in the improved antibacterial action. The MIC value of 2 μM for **Fl-$P_RP_RP_L$-5** with *E. coli* is comparable to the activity of other AMPs such as melittin (4 μM).[25]

Many AMPs exert their antimicrobial action via lysis of bacterial membranes. This membrane lytic activity can also be a significant problem with the use of some AMPs, causing human cell toxicity and hemolysis. In *E. coli*, membrane lytic AMPs, such as melittin, disrupt the membrane causing the release of the cytosolic enzyme β-galactosidase.[26,27] To gain insight into the antimicrobial mechanism of action for **Fl-$P_RP_RP_L$-5**, *E. coli* (ATCC 25922) were treated with the CAPH and the release of β-galactosidase was measured (Fig. 4). Melittin was used as a positive control, and, as reported previously, showed significant discharge of β-galactosidase.[26,27] On the other hand, **Fl-$P_RP_RP_L$-5** showed no release of β-galactosidase at its MIC for *E. coli* and up to 10X its MIC value. These data support a non-membrane lytic mode of action for this agent.

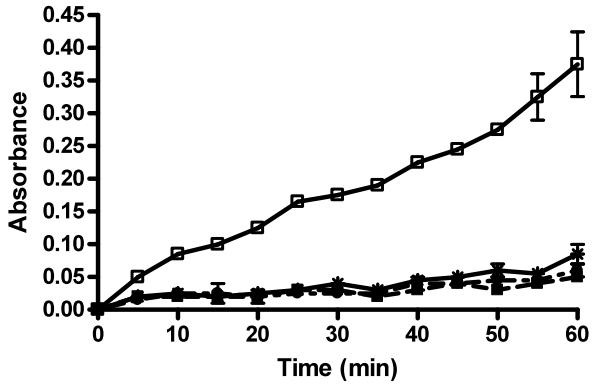

Fig. 4 Leakage of β-galactosidase when *E. coli* (ATCC 25922) were treated for 1 h with **Fl-$P_RP_RP_L$-5**, 2 μM (1X MIC, solid square), 10 μM (5X MIC, solid circle) and 20 μM (10X MIC, asterisk). Melittin, 20 μM (5X MIC, open square), was used as a control. The release was monitored by the cleavage of ortho-nitrophenyl-β-galactosidase (ONPG), a substrate of β-galactosidase

An assay was also performed with **Fl-$P_RP_RP_L$-5** to evaluate cell lysis with human red blood cells (hRBCs) (Fig. 5). hRBCs were treated with various concentration of **Fl-$P_RP_RP_L$-5** for 1 hour and the release of hemoglobin was measured; 1% triton X-100 and melittin were used as controls.[27] Triton X-100 and melittin, as expected, lysed the hRBCs and showed release of hemoglobin. Melittin exhibited concentration dependent hRBC lysis starting from the lowest tested concentration (2 μM), whereas **Fl-$P_RP_RP_L$-5** showed minimal hemoglobin release at all concentrations up to 64 μM. We also monitored the viability of J774A.1 macrophage cells in the presence of **Fl-$P_RP_RP_L$-4** and **Fl-$P_RP_RP_L$-5**. The cells were incubated with the CAPH for 9 hours at a range of concentrations (5-25 μM). **Fl-$P_RP_RP_L$-5** demonstrated only minimal toxicity (<5%) at 15 μM (see Supplemental Information Fig. S4). **Fl-$P_RP_RP_L$-5** presents itself, therefore, as a potent non-hemolytic AMP with minimal toxicity to mammalian cells.

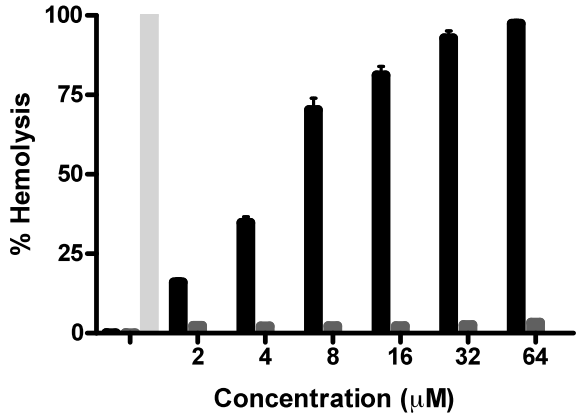

Fig. 5 Hemolytic activity of **Fl-$P_RP_RP_L$-5** (dark gray) and controls (melittin – black, 1% triton X – light gray). hRBC were incubated with varied concentrations for 1 h and the release of hemoglobin was monitored at OD_{405}.

In addition to *E. coli* and *S. aureus*, we investigated the antimicrobial action of **Fl-$P_RP_RP_L$-5** against multidrug resistant pathogens and clinical isolates. This agent displayed exceptional antimicrobial activity against a broad spectrum of pathogenic bacteria, including clinical isolates of *A. baumannii*, an extensively drug-resistant organism, and clinical isolates of *E. coli* O157 that are responsible for haemorrhagic colitis (Table 1). Furthermore, the CAPH was active against MRSA and VRSA, and limited the growth of the biological warfare agents *B. anthracis* and *Brucella*. In addition, **Fl-$P_RP_RP_L$-5** showed activity against intracellularly residing bacterial pathogens, such as *Listeria*, *Salmonella* and *Brucella*. In almost all cases **Fl-$P_RP_RP_L$-5** was 2- to 8-fold more potent against pathogenic bacteria than the shorter **Fl-$P_RP_RP_L$-4**, with the exception of *Brucella* where the MIC values were identical.

Table 1 Antibacterial activity of **Fl-$P_RP_RP_L$-4** and **Fl-$P_RP_RP_L$-5**

Pathogens	Fl-$P_RP_RP_L$-4 (MIC/MBC) μM	Fl-$P_RP_RP_L$-5 (MIC/MBC) μM
Gram negative		
Salmonella enteritidis	16/16	4/4
Acinetobacter baumanii	8/16	4/8
Brucella abortus	8/16	8/8
Escherichia Coli 0157 ATCC 35150	16/>32	8/>32
Escherichia Coli 0157 ATCC 700728	16/>32	8/>32
Gram positive		
Bacillus anthracis	8/16	4/8
Listeria monocytogenes	64/128	8/16
methicillin sensitive Staphylococcus aureus	32/64	16/16
vancomycin resistant Staphylococcus aureus	32/64	16/16

The establishment of **Fl-$P_RP_RP_L$-5** as a dual action antibacterial and cell penetrating agent spurred us to evaluate if these properties could be combined to target pathogenic bacteria located within human macrophages. Clearance of intracellular *Brucella abortus* and *Salmonella enteritidis* in J774A.1 cells by **Fl-$P_RP_RP_L$-5** was studied using an *in cyto* bacterial assay (Fig. 6).[28] **Fl-$P_RP_RP_L$-5** cleared bacterial pathogens within J774A.1 cells by 80% for both *Salmonella* and *Brucella*, a significant increase over the activity of **Fl-$P_RP_RP_L$-4** (Fig. 6). There are a number of interesting trends in these *in cyto* data as compared to the *in vitro* antibacterial activity. For instance, **Fl-$P_RP_RP_L$-5** is 4-times more potent against *Salmonella in vitro* than **Fl-$P_RP_RP_L$-4**, and this longer CAPH is also more potent against this pathogen *in cyto*. With *Brucella*, however, the two CAPHs are equipotent *in vitro*, whereas **Fl-$P_RP_RP_L$-5** is significantly more potent than **Fl-$P_RP_RP_L$-4** *in cyto*. This is likely due to the increased cellular accumulation of the longer CAPH within macrophages. Additionally, when comparing the antimicrobial activity of **Fl-$P_RP_RP_L$-5** against *Salmonella* and *Brucella*, this CAPH is 2-fold more potent against *Salmonella in vitro*, and the activity trends switches *in cyto* to about 2-times more potent against *Brucella*. Both of these pathogens enter macrophages via vacuoles, but ultimately reside in sub-cellular locations such as endosomes and the endoplasmic reticulum, respectively. The ability of CAPHS to co-localize with bacteria within cells will play a significant role in intracellular antimicrobial activity.

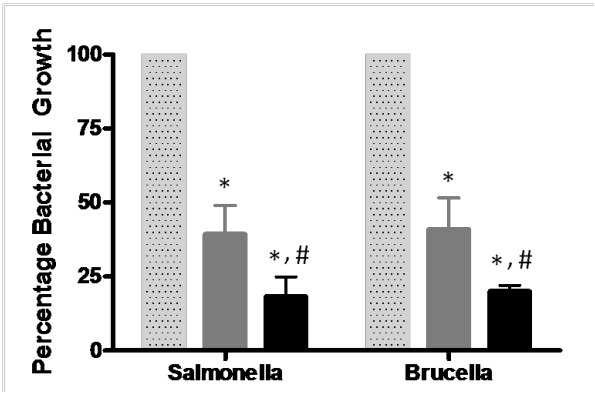

Fig. 6 Intracellular clearance of *Salmonella* and *Brucella* from J774A.1 cells after the infected cells were treated with 15 μM **Fl-$P_RP_RP_L$-4** (gray) and **Fl-$P_RP_RP_L$-5** (black) for 9 h. Buffer (dotted gray) was used as the control. Statistical significance was calculated by unpaired student *t* test. **Fl-$P_RP_RP_L$-5** was compared to controls **(*)** and to **Fl-$P_RP_RP_L$-4 (#).**

Conclusions

Pathogenic bacteria that reside within human cells are notoriously difficult to treat due to the poor accumulation of a number of antibiotic classes within cells. Herein, we have demonstrated that increasing the length of CAPH peptides increases both the ability to enter cells and the antimicrobial activity as compared to a shorter CAPH counterpart. A number of pathogenic bacteria were effectively killed with **Fl-$P_RP_LP_L$-5**, including *A. baumannii*, *E. coli* O157, MRSA and VRSA. Significantly, this agent was able to tackle the problem of intracellular bacteria, and cleared pathogens from within mammalian macrophages. Future studies will seek to track both the CAPH and the bacteria within macrophages to ultimately enable more effective targeting. The non-membrane lytic mechanism of action, minimal hRBC lysis and antibacterial potency make **Fl-$P_RP_LP_L$-5** a very attractive tool to deal with intracellular pathogenic bacteria.

Experimental section

Materials

All chemicals and reagents were purchased commercially and were used without further purification unless mentioned. Sterile media (DMEM, L-glutamine, Penicillin-Streptomycin) and buffers (PBS) used in cell culture were purchased from VWR (VWR Direct, IL).

Synthesis

The proline monomers with either an isobutyl (hydrophobic) or guanidinium (hydrophilic) moiety were synthesized via O-alkylation of hydroxyproline as described previously.[23] **Fl-$P_RP_RP_L$-4** and **Fl-$P_RP_RP_L$-5** were synthesized using standard Fmoc-based solid phase peptide synthesis. Briefly, a 10 mL synthesis flask was loaded with 100 mg (0.052 mmol) of Rink Amide ChemMatrix resin and washed with CH_2Cl_2 (3 X 5 mL) and DMF (3 X 5 mL). Fmoc-protected unnatural proline monomers (2.5 equiv, 0.13 mmol) were added to the reaction flask with O-(7-azabenzotriazole-1-y)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HATU) (2.5 equiv, 0.13 mmol) and diisopropyl amine (DIEA) (5 equiv, 0.26 mmol), and the flask was agitated for 3 h. At the end of coupling, the reaction mixture was drained and the resin was washed with DMF, CH_2Cl_2, MeOH, CH_2Cl_2, and DMF (3 X 5 mL each). The Fmoc-group was removed by agitating the resin with 20 % piperidine in DMF for 20 min. The piperidine solution was drained and the resin was washed with DMF, CH_2Cl_2, MeOH, CH_2Cl_2, and DMF (3 X 5 mL each). These steps were repeated until all proline-based amino acids were coupled to the resin, followed by a glycine residue (5 equiv, 0.26 mmol) to allow the attachment of N-hydroxy-succinimide fluorescein (NHS-Fluorescein). NHS-Fluorescein (1.1 equiv, 0.0572 mmol) was allowed to react with resin for 24 h in the dark. The peptide was cleaved from resin using a trifluoroacetic acid (TFA) cocktail solution (95% TFA, 2.5 % triisopropylsilane, 2.5% H_2O). The resulting TFA solution was concentrated *in vacuo* to remove TFA, triturated with cold anhydrous diethyl ether and the precipitate was collected by centrifugation. The precipitate was dissolved in H_2O and purified to homogeneity using reversed phase HPLC (RP-HPLC). A C18 PrepLC column (Phenomenex) was used for purification of both peptides. The elution times for **Fl-$P_RP_RP_L$-4** and **Fl-$P_RP_RP_L$-5** using a 25-65% gradient of acetonitrile in H_2O (with 0.1% TFA) were 24.0 and 23.2 min, respectively. Each peptide was characterized by matrix-assisted laser desorption ionization mass spectrometry: **Fl-$P_RP_RP_L$-4** 2978.6 (calculated), 2976.4 (observed); **Fl-$P_RP_RP_L$-5** 3569.5 (calculated), 3572.3 (observed).

Cell Culture

Murine macrophage J774A.1 cells were grown under standard conditions. Briefly, J774A.1 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine and 1% penicillin-streptomycin and maintained at 37 °C under a controlled humidified atmosphere containing 5% CO_2. DMEM supplemented with FBS, 1% L-glutamine, and 1 % penicillin-streptomycin was used for all cell assays.

Cell Uptake

J774A.1 cells as cultured above were harvested and 125,000 cells were collected in round bottom tubes (BD Falcon). Next 400 μL of FBS supplemented DMEM containing the CAPHs at the desired concentrations were added to the tubes and incubated for 1 h at 37 °C. Cells treated with media only served as control for the experiment. After incubation, the cells were centrifuged at 1500 rpm, the media was aspirated and the cells were re-suspended in 500 μL PBS. Samples were analyzed using a FACS Calibur Flow cytometer (BD Biosciences). Data obtained from flow cytometry experiment was processed using the FlowJo software. Data was obtained in duplicates from at least two independent experiments.

Sub-cellular localization

J774A.1 cells were seeded at a density of 200,000 cells/well in a 4-well LabtTek chambered slides (Thermo Fisher Scientific 155383) and allowed to grow for 18 h at 37 °C under 5% CO_2. The media was aspirated and the cells were washed 1X with 400 µL PBS. Next 10 µM of the CAPHs was added to each well in 400 µL of DMEM media supplemented with FBS. The cells were allowed to incubate with peptide for 1 h at 37 °C under 5% CO_2. Excess media was aspirated and the cells were washed 1X with 400 µL PBS. The cells were further treated with either 100 nM Mitotracker (Invitrogen 7512) or 300 nM Lysotracker (Invitrogen L7528) for 30 min at 37 °C. The excess dye was aspirated, the cells were washed with PBS and fresh DMEM media was added to each well. Imaging was performed using a Nikon A1R multiphoton inverted confocal microscope under 60X oil objective. Fluorescein and Mitotracker/Lysotracker were excited using 488 nm and 561 nm lasers respectively.

ROS Production Assay

In a 96-well plate, 25,000 J774A.1 cells were cultured for 24 h at 37 °C under 5% CO_2. Spent media was aspirated and the cells were washed with 100 µL PBS. The cells were then incubated with 50 µM 2',7'-dicholrofluorescien diacetate (DCFDA) (Sigma D6883) in PBS for 45 min at 37 °C supplied with 5% CO_2. The DCFDA solution was aspirated and the cells were treated with either media only, antimycin A (Sigma A8674), tert-butyl hydroperoxide (Sigma B2633) or peptides. The cells were allowed to incubate for 4 h at 37 °C. The fluorescence of each well was measured using fluorimeter (TECAN SpectraFluor Plus) using excitation/emission wavelength of 488/525 nm, respectively. Note: CAPHs labelled with a rhodamine dye were used to perform this assay. Data was obtained in duplicates from at least two independent experiments.

Mitochondrial depolarization assay

J774A.1 cells were transferred to BD flow cytometry tubes at a density of 125,000 cells/tube and treated with either **Fl-$P_RP_RP_L$-4** (15 µM), **Fl-$P_RP_RP_L$-5** (15 µM), carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) (50 µM) (Sigma C2920) or media only (control). The cells were incubated at 37 °C under 5% CO_2 for 1 h. Following the incubation, the cells were centrifuged at 15000 rpm for 5 min at 4 ^{0}C. The cells were then treated with tetramethylrhodamine ethyl ester (TMRE) (Life technologies T-699) (25 nM) for 20 min at 37 ^{0}C for 1 h. The cells were centrifuged at 1500 rpm for 5 min at 4 ^{0}C and re-suspended in fresh ice cold PBS. The fluorescence was measured using a BD ARIA SORP flow cytometer using the 561 yellow-green laser with a 585/15 filter to detect the TMRM.

Antibacterial Activity against *E. coli* and *S. aureus*

Escherichia coli (ATCC 25922) or *Staphylococcus aureus* (ATCC 25923) was grown to the mid-exponential phase in Tryptic Soy Broth (TSB) at 37 °C with shaking. An aliquot of the bacterial suspension was centrifuged for 5 min at 3000 rpm, the supernatant aspirated and the pellet re-suspended in Muller Hinton Broth (MHB) to a final optical density of 0.001 as measured by absorbance at 600nm (OD_{600}). Next 90 µL of this culture was added in a sterile 96-well plate (Cellstar 655180) and then supplemented with 10 µL of sterile water or two-fold serial dilutions of the designed peptides in water**.** Amoxicillin (Sigma A8523) and Ampicillin (USB 11259) were used as positive controls for *E. coli* and *S. aureus,* respectively. The plate was then incubated for 6 h at 37 °C. The OD_{590} was determined using a microplate reader (TECAN SpectraFluor Plus). The minimum inhibitory concentration (MIC) was defined as the lowest concentration of peptide at which no growth was observed. Data was obtained in duplicates from at least two independent experiments.

β-galactosidase Release Assay

E. coli (ATCC 25922) was grown to mid-exponential phase (OD_{590}≈0.6) in TSB at 37 °C with shaking. β-Galactosidase expression was induced for 1 h using freshly prepared isopropyl-β-D-thiogalactopyranoside (IPTG) (Gold Biotechnology I2481C5) in PBS (1 mM final concentration). At the end of the induction, an aliquot of the bacterial suspension was centrifuged, washed twice with fresh TSB and plated into a sterile 96-well plate (90 µL). Next 10 µL aliquots of **Fl-$P_LP_RP_R$-5** were added to give final concentrations of 4 µM, 10 µM and 20 µM. Bacteria treated with sterile water and melittin (20 µM final concentration) served as controls. The plate was then incubated for 1 h at 37 °C. At the end of the incubation, the plate was centrifuged at 3000 rpm/4 °C for 10 min. 80 µL of the supernatant from each well was transferred to a new sterile 96-well plate. Next 20 µL of freshly prepared 2-Nitrophenyl-β-D-galactopyranoside (ONPG) (Sigma 73660) in PBS was added to each well (0.8 mg/ml final concentration). The β-galactosidase activity was monitored at OD_{405} every five minutes for a period of 1 h using a micro-plate reader. Data was obtained in duplicates from at least two independent experiments.

Hemolysis assay

Fresh human red blood cells (hRBCs) were obtained from healthy volunteers. hRBCs were collected by centrifugation at 2000 rpm for 5 min followed by washing three times with phosphate buffered saline (PBS, pH 7.4). An 8% suspension (v/v) of hRBCs was prepared in PBS and 50 µL of the solution was added into a 96-well plate. Then 50 µL of a two-fold serial dilution of the designed peptides and controls in PBS were added to give a final suspension of 4% (v/v) of hRBCs. The plate was incubated at 37 °C for 1 h to allow for hemolysis. The plate was subsequently centrifuged at 1000 rpm for 5 min at 4 °C. Next 75 µL aliquots of the supernatants in each well were carefully transferred to a new sterile 96-well plate. Finally the release of hemoglobin was monitored by measuring the absorbance at OD_{405} with a micro-plate reader. As controls, wells treated with PBS and the known hemolysing agents 0.1 % Triton X-100 as well as 5 µM melittin from honey bee venom (Sigma M2272) were used. The hemolysis percentage was calculated based on the 100%

release with 0.1 % Triton X-100. Data was obtained in duplicates from two independent experiments.

Activity of Peptides against Pathogenic Bacteria

The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of **Fl-$P_LP_RP_R$-5** were tested in triplicates against wide spectrum of Gram- positive and negative bacterial pathogens in micro dilution broth method in 96 well-plate and according to Clinical and Laboratory Standards Institute (CLSI).[22] The bacterial cultures were incubated at 37 °C for 20 h. The MIC was defined as the lowest concentration of peptide at which no growth was observed. The MBC of the peptide against each species was defined as the lowest concentration producing more than 99.9% reduction of the initial inoculum.

Cell Viability

Toxicity of **Fl-$P_LP_RP_R$-4 and Fl-$P_LP_RP_R$-5** against J774.A1 macrophage-like cells was carried out using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma M2128). Briefly, 20,000 J774A.1 cells suspended in 200 µL of DMEM supplemented with 10% fetal bovine serum (FBS) were seeded in 96-well plates and incubated at 37 °C in a 5% CO_2 atmosphere for 24 h (60% confluency). The J774A.1 cells were further incubated with a range of concentrations of **Fl-$P_LP_RP_R$-4** and **Fl-$P_LP_RP_R$-5** for 4h and 9h. After incubation, the media was aspirated and 100 µL of fresh media was added to each well. Next 10 µL of 12 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well and allowed to incubate for 4 h at 37 ^{o}C under 5% CO_2. The MTT solution was aspirated and 100 µL of dimethyl sulfoxide (DMSO) was added to each well. The plate was placed on a shaker for 5 min and the absorbance of each well was measured at 490 nm using a microplate reader (TECAN SpectraFluor Plus). Results were expresses as the percentage of viable cells as compared to control with no peptide. Data was obtained in triplicates from at least two independent experiments.

Activity of Peptides against Intracellular Bacteria

To determine the activity of **Fl-$P_LP_RP_R$-4** and **Fl-$P_LP_RP_R$-5** against intracellular bacterial pathogens, J774A.1 murine macrophage-like cells were seeded at a density of 1 x10^5 cells per well in 96-well plates (Corning Incorporated) 22 h and 26 h before infecting with *Salmonella* and *Brucella* respectively. The cells were routinely grown in DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS, Sigma Chemical Co.) in a humidified 5 % CO_2 atmosphere at 37 °C. The J774A.1 cells were infected with *Salmonella* for 30 min at a 1:10 multiplicity of infection (MOI) or *Brucella* for 1 h at a 1:100 MOI. At the end of the infection, the cells were washed three times with DMEM medium containing 50 µg mL gentamicin (Sigma) to kill and wash off non-phagocytized bacteria. Then DMEM medium supplemented with 10% Fetal Bovine Serum with 15 µM **Fl-$P_LP_RP_R$-4** and **Fl-$P_LP_RP_R$-5** was added. The plate was returned to the incubator for 9 more hours. Finally the infected cells were washed three times and lysed to collect the intracellular bacteria. The colony forming units (CFUs) of *Salmonella* and *Brucella* in the lysates were determined by plating a series of 10-fold serial dilutions onto TSA and incubating the plates at 37 °C for 20 h (*Salmonella)* or 72 h (*Brucella*). Experiments were performed in triplicate in two independent experiments. Statistical significance was assessed with Graph Pad Prism 6.0 (Graph Pad Software, La Jolla, CA). *P* values were calculated by the two-tailed unpaired Student *t* test. *P* values of 0.05 were considered as significant.

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