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A Library Approach to Cationic Amphiphilic Polyproline Helices that Target Intracellular Pathogenic Bacteria

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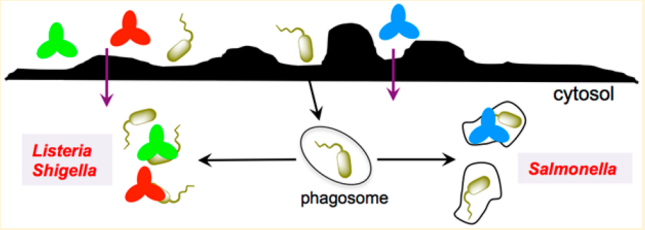
Supporting Information

ABSTRACT: A number of pathogenic bacteria reproduce inside mammalian cells and are thus inaccessible to many antimicrobial drugs. Herein, we present a facile method to a focused library of antibacterial agents known as cationic amphiphilic polyproline helices (CAPHs). We identified three CAPHs from the library with superior cell penetration within macrophages and excellent antibacterial action against both Gram-positive and Gram-negative bacteria. These cell-penetrating antibacterial CAPHs have specific subcellular localizations that allow for targeting of pathogenic bacteria at their intracellular niches, a unique feature that promotes the successful clearance of intracellular pathogens (*Salmonella*, *Shigella*, and *Listeria*) residing within macrophages. Furthermore, the selected CAPHs also significantly reduced bacterial infections in an *in vivo* model of *Caenorhabditis elegans*, with minimal *in vivo* toxicity.



KEYWORDS: *intracellular pathogenic bacteria, antibiotic, cell penetration*

A significant challenge for effective treatment of infectious diseases arises from intracellular pathogens that have evolved to reside inside mammalian cells, including phagocytic macrophages, and establish replication niches. Bacterial pathogens such as *Mycobacterium*, *Salmonella*, *Brucella*, *Shigella*, and *Listeria* thrive within mammalian cells and hence evade the humoral defense system.[1−4] Once sheltered inside host cells, many potent antibiotics fail to effectively clear these pathogens because of either reduced cell penetration or their susceptibility to drug efflux transporters.[5−8] As a result, some intracellular pathogens cause persistent and chronic infections that lead to significant human mortality and morbidity.[9−11]

The difficulties in targeting intracellular pathogens have spurred development of nanomedicine strategies.[12−15] More recently, elegant studies have conjugated antibiotics with peptides or proteins to target intracellular pathogens,[16,17] including an antibiotic and anti-*Staphylococcus aureus* (*S. aureus*) monoclonal antibody conjugate,[18] a methotrexate and cell penetrating peptide construct,[16,19] and peptide nucleic acids conjugated to cell penetrating peptides.[17] Our efforts to target intracellular bacteria led to the development of a class of cell-penetrating, antibacterial peptides based on a cationic amphiphilic polyproline helix (CAPH) scaffold.[20,21] CAPHs such as **P14LRR** (Figure 1) exhibited broad spectrum antibacterial activity and demonstrated modest reduction in *Brucella* and *Salmonella* levels within macrophages.[20] In an effort to improve the intracellular potency of CAPHs, we prepared a cleavable conjugate of the amino glycoside antibiotic kanamycin and CAPHs, resulting in significantly improved performance against intracellular pathogenic bacteria.[22] We wished to investigate the critical question, however, of whether the structural features of CAPH peptides could be more effectively engineered for enhanced clearance of pathogens from mammalian cells.

Once intracellular pathogenic bacteria undergo phagocytosis, they reside in different subcellular locations, such as vacuoles (*Salmonella* and *Mycobacterium*) and the cytosol (*Listeria*, *Shigella*, *Rickettsia*).[3] Peptide sequences have been reported with a range of subcellular localizations, but these peptides do not possess intrinsic antimicrobial activity.[23−25] A novel antibacterial agent targeting intracellular pathogens should, therefore, penetrate mammalian cells and localize to a desired subcellular site with bacteria. Herein, we present our efforts to engineer the hydrophobic face of CAPHs through a focused library approach to develop antibacterial agents that exhibit superior cell penetration with specific subcellular localization, excellent antibacterial activities, and targeting of intracellular pathogens, both *in cyto* and *in vivo*.

In an effort to prepare highly potent CAPHs against intracellular bacteria, we developed a library approach to more easily access new agents (Scheme 1). CAPHs contain a polyproline scaffold that forms a type II polyproline helix with hydrophobic groups along one face of the helical structure. The guanidinium groups of the cationic face are an essential feature of CAPHs, but the nature of the hydrophobic group

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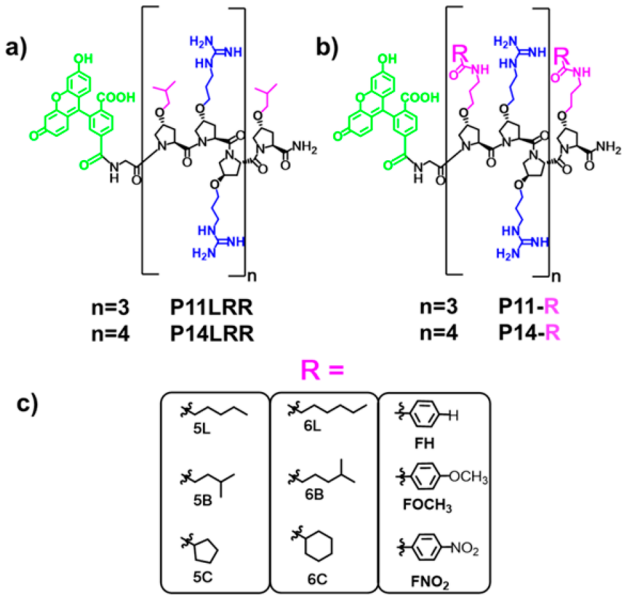
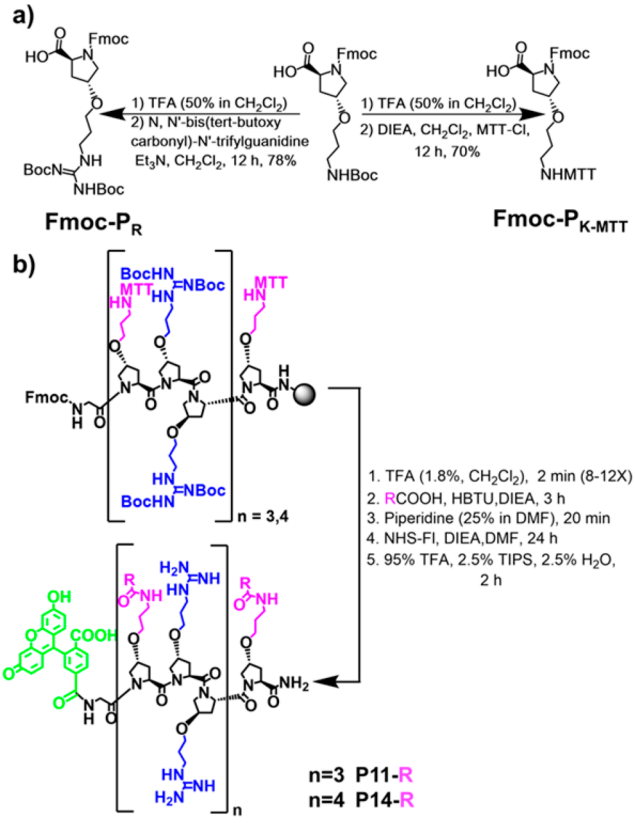


Figure 1. Cationic amphiphilic polyproline helices (CAPHs). (a) Structure of the CAPHs **P11LRR** and **P14LRR** with the hydrophobic groups in pink, the cationic moiety in blue, and fluorescein in green, (b) structure of the modified proline residues within CAPHs for the designed library with the proline modification in pink, and (c) hydrophobic moieties (R) used to create the library.

Scheme 1. (a) Synthesis of Unnatural Amino Acids for the Construction of CAPH Peptides; (b) On-Resin Installation of Hydrophobic Groups onto the CAPH Scaffold



has not been explored. In preparing CAPHs such as **P14LRR**, the hydrophobic groups were attached to the proline residues through a direct ether linkage (Figure 1a). However, the ether-based approach limited the ability to prepare a diverse library of CAPHs quickly, as a new amino acid was needed for the synthesis of each peptide. Therefore, to allow for the facile preparation of focused libraries of CAPHs, we developed the use of an amide linkage between the hydrophobic group and an amino-modified proline residue, **Fmoc-P$_{K\text{-}MTT}$**, that was derived from a common intermediate, **Fmoc-P$_{K\text{-}Boc}$**, used to construct **Fmoc-P$_R$** (Scheme 1a, Figures S8 and S9). Two general classes of hydrophobic modifications were prepared: (1) aliphatic groups with 5- to 6-carbons and (2) phenyl moieties (Figure 1c). Straight, branched, and cyclic aliphatic groups were designed, whereas electron rich and poor aromatic groups were investigated. Further, the effect of different lengths of the CAPHs was probed with **P11-R** and **P14-R** (Figure 1b, Scheme 1b). Overall, a library of CAPHs with two chain lengths and a range of hydrophobic modifications was prepared on resin. The peptides were cleaved from the resin with a trifluoroacetic acid (TFA) cocktail, purified to homogeneity by HPLC, and characterized by matrix-assisted laser desorption ionization (MALDI) mass spectrometry (Figure S7 and Tables S1−S3).

One of the major obstacles in treating intracellular pathogens is the inability of therapeutics to accumulate in sufficient concentrations inside of cells. Therefore, we assessed the cell accumulation of the CAPH library in J774A.1 macrophage cells using flow cytometry. Cells were treated with the individual library components, and the cellular fluorescence was measured (Figures 2a and S1). Ideally, we were interested in CAPHs that were about equipotent with **P14LRR** (Figure 2a blue bar) or better for cell uptake. We identified about ten compounds that fell into this category, most of which were based on the P14 scaffold with hydrophobic alkyl groups, with **P14-6C** and **P14-5L** demonstrating about 3-fold higher cellular accumulation as compared to **P14LRR**.

In order to develop effective therapies targeting intracellular pathogens, acceptable mammalian cell viability is desired. Hence, we screened the CAPHs library for cell viability against J774A.1 cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay under conditions that would be used for pathogen-infected cell experiments (Figures 2b and S2). The **P11** and **P14** series of CAPHs modified with 5-carbon alkyl chains exhibited minimal or no toxicity to the macrophages. However, lengthening the hydrophobic groups attached to the CAPHS to 6-carbons significantly decreased the cell viability except for **P11-6C**, but this compound had poor cell accumulation. Similarly, when the CAPHs were modified with aryl groups, only modest cell viability was observed (30−64%). The substantial drop in cell viability observed with these subtle 5- to 6-carbon side chain modifications reveals the significance of hydrophobic chain length on the biocompatibility of CAPHs. On the basis of our screening of the CAPHs library, we concluded that the peptides modified with 5-carbon alkyl chains possessed the most favorable characteristics overall to move forward, with acceptable cell viability and cellular uptake that was comparable or better than **P14LRR**, specifically for the longer P14 variants. Therefore, we selected three CAPHs, **P14-5L**, **P14-5B**, and **P14-5C** (Figure 2 red bars), and the 15 μM concentration to limit cytotoxicity for our continuing studies.

As discussed above, intracellular pathogens reside at a number of subcellular locations. Therefore, to understand which intracellular pathogens would make suitable targets for these peptides, the subcellular localization of **P14-5L**, **P14-5B**, and **P14-5C** was determined via confocal microscopy. J774A.1 cells were

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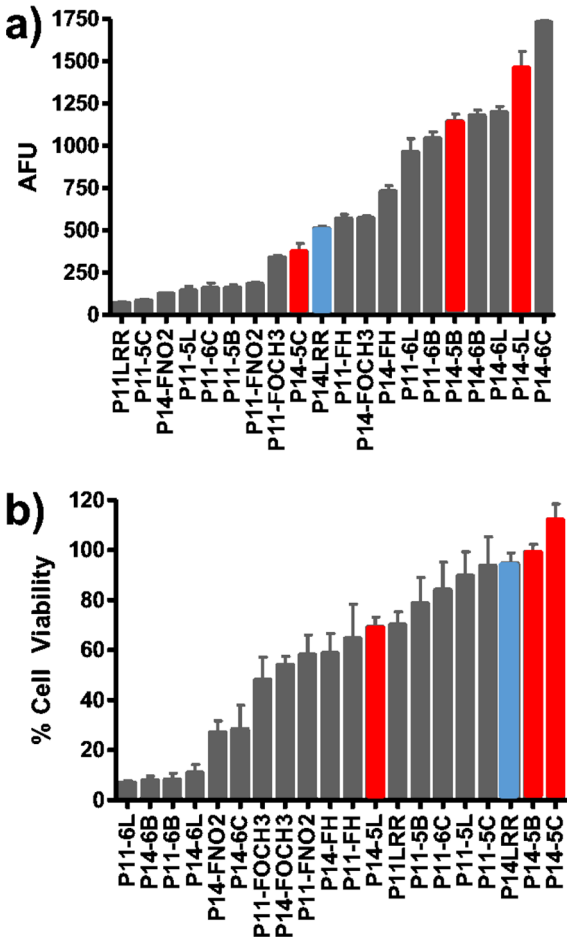

Figure 2. Screening of the CAPHs library for cell accumulation and cytotoxicity in J774A.1 cells. (a) Cellular fluorescence after addition of peptides (15 μM) for 1 h was measured with flow cytometry. (b) Cell viability was monitored by treating J774A.1 cells with the CAPHs library (15 μM) for 9 h using the MTT assay. **P14LRR** data are shown in blue, and data for library members chosen for further studies are shown in red.

treated with the CAPHs (15 μM), and the cells were further treated with Lysotracker or Mitotracker to label endosomes or mitochondria, respectively. As early as 15 min, **P14-5L** and **P14-5B** were found localized in the cytosol of the macrophages, with some endosomal, mitochondrial, and nuclear localization also observed, and this was maintained through 3 to 9 h (Figure S3). At 15 min and 1 h time points, **P14-5C**, however, mostly showed endosomal localization. Some release into the cytosol was only observed after 3 and 9 h. These data demonstrate that varied subcellular localization of CAPHs can be achieved through modification of the hydrophobic groups. This feature may provide a means to target specific intracellular pathogens at a subcellular level. For instance, **P14-5L** and **P14-5B** may be useful in clearing cytosolic pathogens, such as *Listeria* and *Shigella*, whereas **P14-5C** may be used for bacteria that reside in phagosomes, such as *Salmonella*.

With these data in hand, we first tested the *in vitro* antibacterial activity of the three CAPHs against *Salmonella enteritidis*, *Listeria monocytogens*, and *Shigella flexneri* using an *in vitro* broth dilution assay as compared to **P14LRR** (Table 1). The modified CAPHs displayed antibacterial activity that was 2-fold more potent than **P14LRR** against *Salmonella*, equipotent against *Shigella*, and 2- to 4-fold less active than **P14LRR** against

Table 1. *In Vitro* Antibacterial Activity of CAPHs against Pathogenic Intracellular Bacteria

	minimum inhibitory concentration (MIC), μM		
compounds	*Salmonella*	*Shigella*	*Listeria*
P14LRR	32	8	8
P14-5L	16	8	16
P14-5B	16	16	32
P14-5C	16	8	32

Listeria in vitro. We also evaluated the activity of the peptides lacking the fluorophore and found that these peptides were either equipotent or somewhat more potent (2- to 4-fold) depending on the bacteria and the compound (Table S4). Investigation of the mode of action of these peptides against bacteria using a hemolysis assay and a β-galactosidase release assay, used commonly to monitor membrane integrity in bacteria,[26] demonstrated that the antibacterial activity was not due to membrane lysis (Figures S4 and S5) as has been shown for **P14LRR**.[20]

The establishment of **P14-5L**, **P14-5B**, and **P14-5C** as dual cell penetrating and antibiotic peptides inspired us to combine these properties to target intracellular pathogens hiding within mammalian macrophages. Cellular clearance of *Shigella*, *Listeria*, and *Salmonella* was studied *in cyto* with J774A.1 macrophages (Figure 3). All three of the CAPHs derived from the

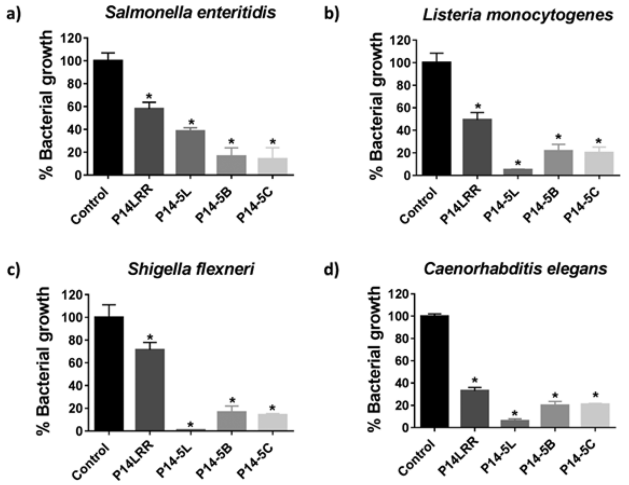

Figure 3. Reduction of pathogenic bacteria from within J774A.1 macrophages with CAPHs (15 μM) after 9 h. Cells were infected with (a) *Salmonella*, (b) *Listeria*, and (c) *Shigella* and washed with gentamycin to remove extracellular bacteria, and the levels of bacteria remaining were quantified. (d) Bacteria levels within *Listeria*-infected *C. elegans* treated with CAPHs (64 μM) for 12 h. *P values of $\leq$0.05 are considered significant.

library outperformed the starting peptide, **P14LRR**, in reducing levels of the three intracellular bacteria. **P14-5B** and **P14-5C** both demonstrated fairly similar clearance across the three intracellular pathogens, with about 80−85% reductions observed. After 9 h, the subcellular localization of **P14-5B** and **P14-5C** is fairly similar (Figure S3), so it is reasonable that the intracellular clearance would be complementary. **P14-5L** demonstrated the most striking results with 95% and 99% clearance of *Listeria* and *Shigella*, respectively, from the macrophages but only about 60% reduction of *Salmonella*. These intracellular results are due to a combination of variables, including CAPH mammalian cell penetration, antibacterial activity, and subcellular

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localization within the macrophages. For instance, the CAPHs **P14-5L** and **P14-5C** are equipotent *in vitro* against *Shigella* and *Salmonella* (Table 1), but **P14-5L** enters macrophages more effectively than **P14-5C**. **P14-5C** mostly localizes within endosomes, with endosomal escape observed after 9 h, whereas **P14-5L** is found mostly within the cytosol and the nucleus even after 9 h (Figure S3). Taking these data into account, if subcellular localization was not an issue, **P14-5L** should have been more potent than **P14-5C** against intracellular *Salmonella*, but the reverse is observed. The more effective subcellular localization of **P14-5C** within endosomes may allow this CAPH to colocalize with endosome-residing *Salmonella* (*vida infra*), thus resulting in an improved intracellular clearance of the pathogen. **P14-5L** accumulates in the cytosol of macrophages, and this CAPH was able to significantly lower the population of the cytosol-dwelling *Listeria* and *Shigella* from J774A.1 cells. These findings provide support for the importance of delivering therapeutics at the subcellular level for enhanced clearance of intracellular pathogens.

The above data suggests that colocalization of bacteria and CAPHs translates to more effective pathogenic bacteria clearance from macrophages. To more specifically address this issue, we used confocal microscopy to monitor the intracellular location of green fluorescent protein (GFP)-labeled *Shigella flexneri* within J774A.1 macrophages with the addition of rhodamine-labeled **P14-5L** and GFP-labeled *Salmonella* within J774A.1 macrophages with the addition of rhodamine-labeled **P14-5C**. After 1 h, minimal interaction was observed between the **P14-5L** (red) and *Shigella* (green) (Figure 4a), but after 3 h, definite

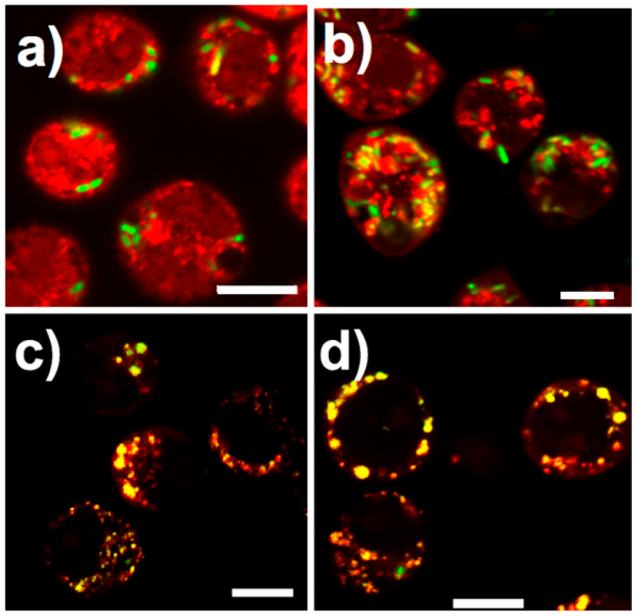

Figure 4. Cellular colocalization of rhodamine labeled CAPHs with bacteria: (a and b) *Shigella* (green) infected J774A.1 cells were treated with **P14-5L** (15 μM, red) for either 1 h (a) or 3 h (b) and (c and d) *Salmonella* (green) infected J774A.1 cells were treated with **P14-5C** (15 μM, red) for either 1 h (c) or 3 h (d). Samples were visualized with confocal microscopy, and yellow regions in the merged panels represent colocalization (scale bar 10 μm).

interactions between the peptide and *Shigella* were seen, with colocalization observed in the merged image (Figure 4b). With **P14-5C** and *Salmonella*, colocalization was observed at both 1 and 3 h time points (Figure 4c,d). This study clearly demonstrates that CAPHs possess the potential to interact with these bacteria within cells and may have the potential for greater potency when colocalized with bacteria within cells.

After the encouraging cell-based results with **P14-5L**, **P14-5B**, and **P14-5C**, we evaluated these agents for *in vivo* antibacterial activity within *Caenorhabditis elegans* (*C. elegans*). First, the viability of *C. elegans* in the presence of the CAPHs was evaluated. Worms were treated with **P14-5L**, **P14-5B**, and **P14-5C** (64 μM), and their survival rate was monitored over 48 h (Figure S6). After 24 h, very limited toxicity was observed with the three peptides; after 48 h of treatment, very close to 100% *C. elegans* survival was found with **P14-5B** and **P14-5C**, and 90% survival was observed with **P14-5L**. Since the peptides showed minimal toxicity to *C. elegans*, we investigated bacterial clearance from infected worms. *C. elegans* were infected with *Listeria* and then treated with **P14-5L**, **P14-5B**, and **P14-5C** for 12 h at 64 μM. All three CAPHs demonstrated reductions in bacteria levels within *C. elegans* and were more effective than **P14LRR** (Figure 3d). As observed with the *in cyto* studies, **P14-5L** was the most effective peptide in reducing the population of *Listeria* within *C. elegans* with a 95% reduction in bacteria levels (Figure 3d). Overall, the selected CAPHs were able to substantially reduce the bacterial infection in an *in vivo C. elegans* model with minimal toxicity to the worms.

In conclusion, intracellular pathogens represent a particularly difficult challenge in the development of anti-infective therapies. In our studies, we designed a focused library of CAPHs and identified three promising agents that exhibited potent cell penetrating and antibacterial activities that were harnessed to reduce levels of *Salmonella, Listeria,* and *Shigella* within macrophages. Interestingly, the CAPH **P14-5L** that localized in the cytoplasm was found to clear cytosolic *Listeria* and *Shigella* more effectively but was much less effective in reducing phagosome-residing *Salmonella* than the endosome-localizing **P14-5C**. Issues of cell penetration, antibacterial potency, and subcellular localization all play a role in the overall activity of CAPHs against intracellular pathogenic bacteria. CAPHs also demonstrated a notable reduction in *Listeria* levels in an *in vivo* model with infected *C. elegans* with minimal toxicity. On the basis of their potent activity against intracellular bacteria, the CAPHs described herein provide an excellent platform to develop therapies to treat intracellular pathogenic bacterial infection.

METHODS

Cell Uptake. J774A.1 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and at 37 °C under 5% CO_2. Briefly, J774A.1 cells (125 000) were harvested and transferred to round-bottom tubes (BD Biosciences). These cells were treated with CAPHs (15 μM) in 10% FBS supplemented DMEM (300 μL) and were allowed to incubate for 1 h at 37 °C. Cells with no treatment (DMEM only) served as the control for the experiment. Upon completion of the incubation period, the cells were centrifuged and the spent media was aspirated. The cells were resuspended in phosphate buffered saline (PBS; 400 μL), and the fluorescence of the cells was measured using a FACS Calibur Flow Cytometer (BD Biosciences). Data were obtained in duplicates from two independent experiments and were processed using the BD software.

Subcellular Localization. J774A.1 cells were seeded at a density of 200 000 cells per well in 4-well Lab-Tek 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

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the cells were washed with 400 μL of PBS (1×). Next, CAPHs (15 μM, **P14-5L**, **P14-5B**, and **P14-5C**) were added to each well in 400 μL of DMEM supplemented with 10% FBS. The cells were allowed to incubate with the peptides for desired time (15 min, 1 h, 3 h, 9 h) at 37 °C under 5% CO_2. Excess media was aspirated, and the cells were washed with 400 μL of PBS (1×). The cells were further treated with Hoechst 33342 (1000 nM) and either Mitotracker (100 nM) (Invitrogen 7512) or Lysotracker (300 nM) (Invitrogen L7528) for 30 min at 37 °C. The excess dye was aspirated; the cells were washed with PBS, and fresh DMEM was added to each well. Imaging was performed using a Nikon A1R multiphoton inverted confocal microscope under 60× oil objective. Fluorescein, Hoechst 33342, and Mitotracker/Lysotracker were excited using 488, 350, and 561 nm lasers, respectively.

Intracellular Clearance. J774A.1 cells were seeded at a density of 1×10^5 cells per well in 96-well plates (Corning Incorporated) for 22 h before being infected with the bacteria. Following incubation, the cells were washed once with DMEM. Then, the cells were infected with *S. enteritidis*, *S. flexneri*, or *L. monocytogenes* (at a multiplicity of infection of 1:10 for *S. enteritidis* and 1:100 for *S. flexneri* or *L. monocytogenes*) in DMEM supplemented with 10% FBS for 45 min. At the end of the infection, the cells were washed three times with DMEM containing 50 μg/mL gentamicin (Sigma) and were further incubated for 30 min to kill and wash off nonphagocytized bacteria. Then, DMEM supplemented with 10% fetal bovine serum with 15 μM CAPHs was added. The plates were returned to the incubator for 9 h. Finally, the infected cells were washed three times with DMEM and lysed with 100 μL of 0.01% triton X in PBS to collect the intracellular bacteria. The colony forming units (CFUs) of the bacteria in the lysates were determined by plating a series of 10-fold serial dilutions onto tryptic soy agar (TSA) and incubating the plates at 37 °C for 20 h. 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 ≤0.05 were considered as significant.

Bacteria-CAPHs Colocalization Study. Rhodamine-functionalized **P14-5L** and **P14-5C** peptides were prepared for this experiment. J774A.1 cells were seeded at a density of 1.5×10^5 cells/well in 4-well Lab-Tek chambered slides in DMEM supplemented with 10% fetal bovine serum (FBS) and incubated at 37 °C in a 5% CO_2 atmosphere for 20 h. The media was aspirated, and the cells were washed 1× with 400 μL of PBS. The cells were infected with GFP-*Shigella* ATCC 12022GFP or GFP-*Salmonella* ATCC 14028GFP (at multiplicity of infection, 100 bacteria: 1 macrophage cell) in DMEM with 10% FBS for 1 h. After infection, the wells were washed three times with 200 μL of DMEM with gentamicin (final concentration of 50 μg/mL) to kill extracellular bacteria. Next, 15 μM rhodamine-labeled **P14-5L** or **P14-5C** was added to the cells and allowed to incubate for 1 and 3 h at 37 °C under 5% CO_2. The cells were washed 3× with PBS and visualized under a 60× oil objective of a Nikon A1R multiphoton inverted confocal microscope. GFP-*Shigella*/GFP-*Salmonella* and rhodamine-labeled peptides were excited using 488 and 561 nm, respectively.

***In Vivo* Efficacy in a Worm Infection Model.** The infection and treatment of *Caenorhabditis elegans* were performed as reported previously.[17,27] A pathogen-sensitive strain of *C. elegans* {glp-4(bn2) I; sek-1(km4)} was used in this study. Approximately 40 worms infected with *Listeria monocytogenes* (100 μL of PBS) were transferred to 1.5 mL microcentrifuge tubes. The CAPHs (64 μM) were added to tubes in triplicate, with negative control tubes containing only PBS. After 12 h, the tubes were centrifuged, and the supernatant was removed. The worms were washed twice with 1 mL of PBS, and 200 μg of autoclaved silica carbide was added to each tube. The worms were vortexed for 1 min, and an aliquot (100 μM) from each tube was diluted 10-fold serially in PBS. The aliquots were plated and incubated for 16 h at 37 °C, and *Listeria* colonies were counted. Statistical analysis was performed using the two-tailed student *t* test ($P \leq 0.05$ was considered significant).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.8b00124.

Synthetic procedures and compound characterization; cell localization, hemolysis, and β-galactosidase data (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

C. elegans, *Caenorhabditis elegans*; CAPHs, cationic amphiphilic polyproline helices; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMEM, Dulbecco's Modified Eagle Medium; GFP, green fluorescent protein; h, hour; MALDI, matrix-assisted laser desorption ionization; PBS, phosphate buffered saline; TFA, trifluoroacetic acid

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