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Dual Targeting of Intracellular Pathogenic Bacteria with a Cleavable Conjugate of Kanamycin and an Antibacterial Cell-Penetrating Peptide

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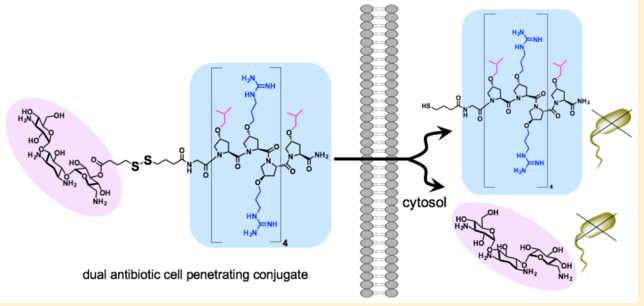
Supporting Information

ABSTRACT: Bacterial infection caused by intracellular pathogens, such as *Mycobacterium*, *Salmonella*, and *Brucella*, is a burgeoning global health epidemic that necessitates urgent action. However, the therapeutic value of a number of antibiotics, including aminoglycosides, against intracellular pathogenic bacteria is compromised due to their inability to traverse eukaryotic membranes. For this significant problem to be addressed, a cleavable conjugate of the antibiotic kanamycin and a nonmembrane lytic, broad-spectrum antimicrobial peptide with efficient mammalian cell penetration, **P14LRR**, was prepared. This approach allows kanamycin to enter mammalian cells as a conjugate linked via a tether that breaks down in the reducing environment within cells. Potent antimicrobial activity of the **P14KanS** conjugate was demonstrated in vitro, and this reducible conjugate effectively cleared intracellular pathogenic bacteria within macrophages more potently than that of a conjugate lacking the disulfide moiety. Notably, successful clearance of *Mycobacterium tuberculosis* within macrophages was observed with the dual antibiotic conjugate, and *Salmonella* levels were significantly reduced in an in vivo *Caenorhabditis elegans* model.



INTRODUCTION

A significant challenge in the development of effective antibacterial agents arises from bacterial pathogens, including *Mycobacterium tuberculosis*, *Salmonella*, *Brucella*, *Listeria*, *Shigella*, and methicillin-resistant *Staphylococcus aureus* (MRSA), which have evolved to inhabit mammalian cells, such as phagocytic macrophages.[1−4] Moreover, most of the bacterial bioterrorism agents monitored by the U.S. Centers for Disease Control and Prevention are intracellular pathogens, such as multidrug-resistant *Mycobacterium* (tuberculosis, TB), *Yersinia pestis* (plague), *Salmonella* species (salmonellosis), *Fancisella tularensis* (tularemia), *Brucella* species (brucellosis), *Chlamydia psittaci* (psittacosis), *Coxiella burnetii* (Q fever), *Rickettsia prowazekii* (typhus fever), *Burkholderia mallei* (glanders), and *Burkholderia pseudomallei* (melioidosis). Within these intracellular safe havens, the bacteria reproduce and form a repository, often causing chronic infections. Infected patients become life-long carriers of the pathogens[5] and chronically suffer from the infection or die from invasive forms of the pathogen. For example, in 2013, 9 million people around the world became sick with TB disease, and there are an estimated 1.5 million TB-related deaths annually. Also, *Salmonella* infection affects >100 million people worldwide, accounting for 370,000 deaths annually.[6,7] In addition, some intracellular pathogens are capable of switching to a dormant stage of growth, thus enabling long-term colonization of the host and relapses even after prolonged antibiotic therapy.[8,9]

Whereas these pathogenic bacteria are internalized within macrophages, many of the commonly used classes of antibiotics, such as aminoglycosides, glycopeptides, and macrolides, do not effectively accumulate within these cells. This is due, in part, to inefficient membrane penetration and susceptibility to drug efflux transporters.[8] As such, the therapeutic value of many antibiotics against intracellular bacteria is severely limited. For instance, it is generally accepted that aminoglycosides have poor internalization within macrophages with activity developing slowly because of the low rate of uptake. For instance, kanamycin has demonstrated only approximately 50% reduction in *M. tuberculosis* levels within THP-1 macrophages after 10 days.[10]

One approach to target intracellular pathogenic bacteria uses delivery vehicles, such as liposomes and micro/nanoparticles,

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that are loaded with antibiotics.[11] Although these vehicles have demonstrated cellular delivery of antibiotics and reduction in intracellular bacteria levels, they suffer from instability to biological fluids and difficulties in drug loading.[12,13] Recently, a noncleavable conjugate of methotrexate and a short cell-penetrating peptide was demonstrated to target intracellular *Listeria*,[14] whereas a cell-penetrating peptide with intrinsic antimicrobial activity, **Fl-P$_R$P$_R$P$_L$-4** (or **P14LRR**), was shown to target intracellular *Salmonella* and *Brucella*.[15] This latter peptide forms a cationic amphiphilic polyproline helix and is a nonmembrane lytic, broad spectrum antibiotic. Although both of these peptide-based agents display a good reduction in intracellular bacteria, much more potent agents are needed to effectively eradicate pathogenic bacteria from mammalian cells. Herein, we describe a cell-permeable, dual antimicrobial agent that reversibly links the antibacterial **P14LRR** with the aminoglycoside antibiotic kanamycin (Figure 1) to achieve an agent with synergistic activity against intracellular pathogenic bacteria, including *M. tuberculosis*.

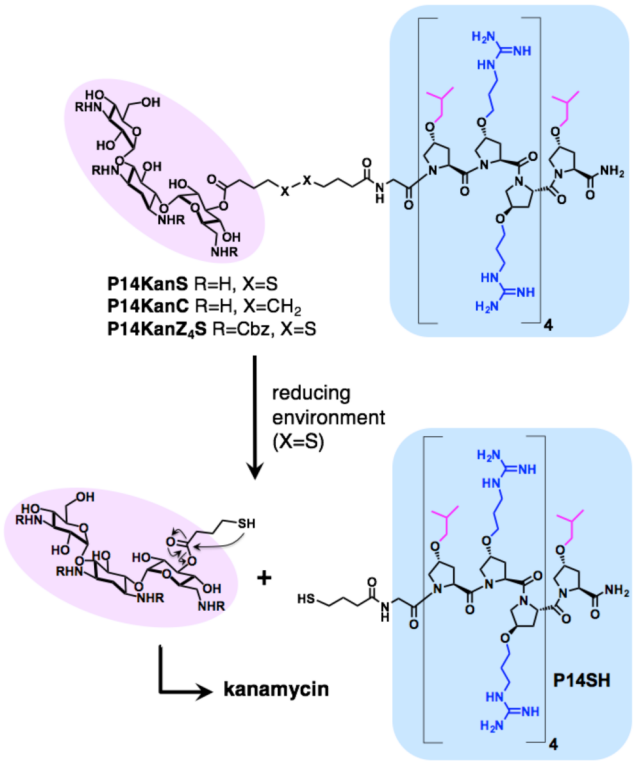

Figure 1. Designed dual agents composed of the antibacterial and cell-penetrating peptide **P14LRR** (blue) and the antibiotic kanamycin (pink). In the reducing environment of mammalian cells, the conjugate containing the disulfide linkage (**P14KanS**) will breakdown to release kanamycin and **P14SH**, whereas the conjugate lacking the disulfide (**P14KanC**) should remain intact. Protected **P14KanZ$_4$S** was designed to monitor the reductive release of kanamycin by HPLC.

In the design of the dual antibiotic agent, we wished to investigate the importance of reversibility of the conjugation chemistry between **P14LRR** and kanamycin. Therefore, we chose to link the peptide and kanamycin using a tether either with or without a disulfide moiety to generate **P14KanS** and **P14KanC**, respectively (Figure 1). We hypothesized that the disulfide linker within **P14KanS** would cleave in the reducing environment within the cell to release free kanamycin and the thiol-modified **P14SH** (Figure 1), whereas **P14KanC** would remain intact.[16−18] The reductive approach with **P14KanS** would allow dual delivery of both of the individual antibiotics to mammalian cells, thereby increasing the therapeutic value of kanamycin and potentially more effectively clearing intracellular pathogens than either agent alone.

RESULTS AND DISCUSSION

For the synthesis of the dual agents, the amino groups of kanamycin were first protected using the acid labile *tert*-butyloxycarbonyl group (Boc) or the carboxybenzyl group (Cbz) to afford Boc$_4$-kanamycin (**1a**) or Cbz$_4$-kanamycin (**1b**), respectively, according to literature procedures.[19,20] Treatment of compounds **1a** and **1b** with either 4,4′-dithiodibutyric acid or sebacic acid and *O*-(7-azabenzotriazole-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) afforded compounds **2a**−**2c** either with or without the disulfide in the tethering group (Scheme 1). A mixture of isomers was obtained, and one isomer was easily separated from the others by HPLC. Detailed NMR analysis of this isomer, including COSY, TOCSY, and HMBC, was used to confirm the site of tether functionalization at the 3′ hydroxyl group of kanamycin (see Supporting Information). For instance, the 3′ H of compound **2a** was found to shift by 1.6 ppm as compared to this proton in **1a** (Figures S2 and S3), and a correlation was observed between the 3′ H and the carbonyl carbon of the tether in the HMBC experiment (Scheme 1 and Figures S5 and S23). Resin-bound and side chain-protected **P14LRR** on the ChemMatrix Rink amide resin was treated with the modified kanamycins **2a**−**2c** and HATU to prepare the resin-bound **P14LRR**-kanamycin conjugates. Treatment of the resins with a trifluoroacetic acid (TFA) cocktail liberated the peptide conjugates from the resin, as well as deprotecting the Boc groups. The dual agents **P14KanS**, **P14KanC**, and **P14KanZ$_4$S** (Figure 1) were purified to homogeneity using reverse-phase HPLC, and their final structures were confirmed using MALDI mass spectrometry (see Supporting Information).

The dual antibiotic conjugate **P14KanS** was designed to enter mammalian cells and release kanamycin and the **P14SH** peptide within an intracellular reducing environment. To evaluate the half-life of release of these agents under reducing conditions, we employed the **P14KanZ$_4$S** construct as it will release the UV-active **KanZ4** (Figure 1). The conjugate **P14KanZ$_4$S** (100 μM in a 1:1 mixture of PBS buffer and DMF) was treated with 1 mM dithiothreitol (DTT), and the accumulation of **KanZ$_4$** was monitored by HPLC and LC−MS. We observed efficient release from the conjugate with a half-life of 1.5 ± 0.2 h and full release after 4 h (Figure S8). Ideally, the ester bond of **P14KanS** linking kanamycin to the **P14LRR** peptide would have sufficient stability to withstand plasma esterases. To investigate whether the ester bond is susceptible to esterase cleavage, we incubated **P14KanS** in the presence of 100 U of porcine liver esterase. Accumulation of the released peptide was monitored by HPLC for up to 120 h; however, only ∼15% of the cleavage product was observed. We conclude, therefore, that **P14KanS** is not susceptible to esterase cleavage, presumably due to steric crowding on kanamycin around the ester bond. These data demonstrate that the dual antibiotic conjugate **P14KanS** is responsive to a reducing environment and produces **KanZ$_4$** and **P14SH** as designed but should be stable against plasma esterases. The conjugate lacking the disulfide linkage, **P14KanC**, is not affected by a reducing environment and should remain intact within mammalian cells. This will allow us to evaluate the two different conjugation strategies for antibacterial potency against intracellular patho-

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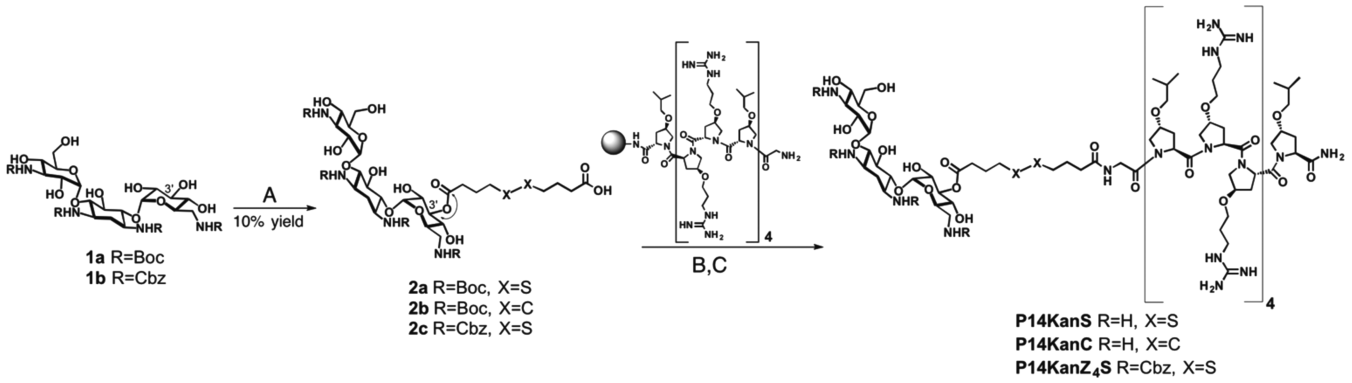
Scheme 1. Synthesis of P14KanS, P14KanC, and P14KanZ$_4$[a]



[a](A) (**2a** and **2c**) 4,4′-Dithiobutyric acid, DIEA, HATU, DMF, 24 h; (**2b**) Sebacic acid, DIEA, HATU, DMF, 24 h. (B) DIEA, HATU, DMF, 24 h. (C) TFA:H_2O:triisoprolylsilane (95:2.5:2.5), 1 h, RT.

gens, as kanamycin analogues lacking the 3′ hydroxyl group have been shown to maintain activity against bacteria.[21]

The addition of kanamycin to **P14LRR** increases the overall net positive charge of the conjugate from +8 to +12, a change that may modulate mammalian cell accumulation efficiency. Therefore, we prepared a fluorescent conjugate, **P14KanC-Fl** (see Supporting Information), to monitor cell uptake within J774A.1 cells as compared to that in **P14LRR**. This fluorescent analogue displayed a potency that was within 2-fold of the activity of **P14KanC** against a selection of bacteria (Table S1). Both peptides displayed a concentration-dependent cell penetration (Figure 2a), and the kanamycin conjugate demonstrated a small increase (~20%) in cell uptake as compared to that of **P14LRR** at 10 μM. Although there is a substantial increase in positive charge in the conjugate, the increase in cell accumulation was not large, as has been shown with Arg-based cell-penetrating peptides.[22]

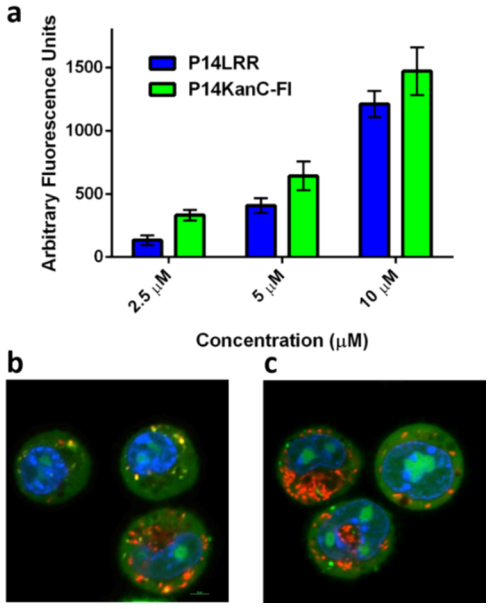


Figure 2. (a) Cellular internalization of **P14KanC-Fl** and **P14LRR**. J774A.1 cells were incubated with the peptides for 1 h and analyzed by flow cytometry. Confocal microscopy images of colocalization studies using **P14KanC-Fl** (10 μM) and (b) LysoTracker or (c) MitoTracker stains within J774A.1 cells after 1 h.

We also investigated the subcellular localization of **P14KanC-Fl** within J774A.1 cells. Whereas **P14LRR** has been shown to localize to endosomes and mitochondria,[15] **P14KanC-Fl** was found throughout the cell, including the cytosol, nucleus, and endosomes (Figure 2b with Lysotracker stain), but did not localize with the mitochondria (Figure 2c with Mitotracker stain).

With the knowledge of mammalian cell penetration, we evaluated the antimicrobial activity of the **P14KanS** and **P14KanC** conjugates with a series of Gram positive and negative bacteria, including a range of intracellular pathogens (Table 1). This activity was compared to that obtained for **P14LRR**, **P14SH**, kanamycin, and a 1:1 mixture of kanamycin and **P14LRR**. Across the series of bacteria, **P14KanS** is highly active with MIC values ranging from 0.12 to 2 μM, including *M. tuberculosis*. With four strains of bacteria, **P14KanS** is 2- to 32-times more active than the noncovalent mixture of **P14LRR** and kanamycin, respectively. With the exception of *E. coli* and *M. tuberculosis*, this conjugate is also 2- to 16-fold more potent than the nonreducible **P14KanC** conjugate. We also investigated the activity of **P14KanS** on clinical isolates of *S. aureus* and *S. epidermis* that form biofilms, and the conjugate was 8- to 16-fold more potent than the **P14LRR** peptide, respectively. The antibacterial activity of **P14LRR** has previously been shown to occur in a nonmembrane lytic fashion.[15] We also investigated if the **P14KanS** and **P14KanC** conjugates disrupted bacteria membranes by monitoring β-galactosidase release from *E. coli* upon addition of the conjugates. At five times their MIC values, no significant release of β-galactosidase was observed (Figure S9), whereas melittin at 5 times its MIC displayed a substantial level of release as has previously been shown.[15] These data demonstrate that the kanamycin-peptide conjugates do not lyse membranes as the mechanism for their antibacterial activity, as was observed for **P14LRR**.

Because the conjugates effectively enter mammalian cells and display antibacterial activity, we investigated the ability of **P14KanS** and **P14KanC** and their components to clear intracellular pathogenic bacteria from J774A.1 macrophage cells, including *S. enteritidis*, *B. abortus*, *M. smegmatis*, *M. tuberculosis*, and *S. flexneri* with an in cyto bacterial assay (Figure 3).[23] We have shown that the conjugates and the components display limited cytotoxicity to J774A.1 cells across a range of concentrations, including the conditions of these assays (Figure S10). This selective toxicity toward bacteria but not mammalian cells is a crucial feature for application of the conjugate against

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Table 1. Minimum Inhibitory Concentration Values [μM] for the Antibacterial Activity of the Dual Conjugates Compared to Those of Individual Components

	E. coli	*S. aureus*	*S. enteritidis*	*B. abortus*	*S. flexneri*	*M. smegmatis*	*M. tuberculosis*	*S. aureus*[b]	*S. epidermis*[b]
P14LRR	4	16	32	16	8	8	16	16	8
P14SH	4	16	4	2	4	4	8		
kanamycin	2	2	16	4	2	8	2	4	>128
P14LRR:kanamycin[a]	2	2	8	4	2	4	1		
P14KanS	2	2	2	0.12	1	1	1	1	1
P14KanC	2	16	8	2	2	2	1		

[a]A 1:1 mixture of **P14LRR** and kanamycin. [b]Clinical isolate that forms biofilms.

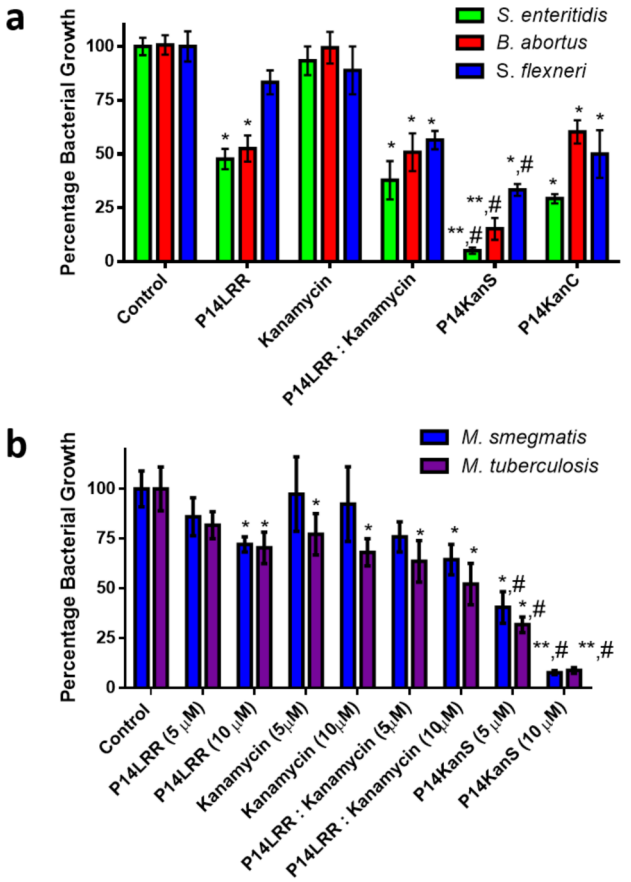

Figure 3. Intracellular antibacterial activity of (a) **P14LRR** (10 μM), kanamycin (10 μM), a 1:1 mix of **P14LRR** and kanamycin (10 μM), **P14KanS** (10 μM), and **P14KanC** (10 μM) in J774A.1 cells infected with *Salmonella enteritidis*, *Brucella abortus*, and *Shigella flexneri* and (b) **P14LRR**, kanamycin, a 1:1 mix of **P14LRR** and kanamycin, and **P14KanS** (each at 5 and 10 μM) in J774A.1 cells infected with *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* after 9 h of treatment. Statistical analysis was calculated using one-way ANOVA with post hoc Tukey's multiple comparisons test. *P* values of <0.05 were considered significant. One asterisk (*) and two asterisks (**) indicate significance from the control of $P < 0.05$ and $P < 0.01$, respectively. Symbol (#) indicates significance from kanamycin, **P14LRR**, and **P14LRR**:kanamycin ($P < 0.05$). Results are expressed as means from three biological replicates ± standard deviation.

intracellular bacteria. Whereas the 1:1 mixture of **P14LRR** and kanamycin demonstrated modest reductions in *Salmonella*, *Brucella*, and *Shigella* levels in the infected cells over those of the components alone, the **P14KanS** conjugate displayed a marked increase in efficacy with significant reduction in these intracellular bacteria (Figure 3a; 95, 85, and 70%, respectively). Interestingly, the nonreducible conjugate **P14KanC** was closer in activity to the 1:1 mixture of components with *Salmonella*, *Brucella*, and *Shigella* than to **P14KanS**, indicating the importance of the reducible linker on intracellular potency. With *Mycobacteria*, the 1:1 mixture of **P14LRR** and kanamycin demonstrated very modest reductions of bacteria levels as compared to those of the individual components. **P14KanS**, however, very effectively lowered levels of these bacteria within macrophages by 95 and 93% for *M. smegmatis* and *M. tuberculosis*, respectively, a significant increase in efficacy over the individual antibiotics or their mixture at two concentrations (Figure 3b). These data illustrate that neither kanamycin nor **P14LRR** is capable of effectively clearing intracellular bacteria on their own, even when coadministered. Only the **P14KanS** conjugate potently clears intracellular pathogens by taking advantage of the nonmembrane lytic, mammalian cell-penetrating activity of **P14LRR** to codeliver kanamycin. When comparing the antimicrobial activity of **P14KanS** against *Salmonella* and *Brucella*, this agent is 16-fold more potent against *Brucella* in vitro (Table 1), but the activity trend switches in cyto with a more effective clearance of *Salmonella* intracellularly (Figure 3a). Both of these pathogens enter macrophages via vacuoles, but ultimately reside in subcellular locations such as endosomes and the endoplasmic reticulum, respectively.[24,25] Because **P14KanS** localizes to endosomes, it is reasonable that this agent is more effective against *Salmonella* within mammalian cells. The notable difference in potency between **P14KanS** and**P14KanC** demonstrates that it is crucial for the two antibiotics to separate and go their own way within macrophages for optimal performance.

Encouraged by the potent antibacterial activity of the peptide conjugate **P14KanS** in vitro and in the in cyto bacterial clearing assay, we investigated the ability of the conjugate to clear *S. enteritidis* using an in vivo *C. elegans* model (Figure 4).[26] First, we evaluated the toxicity of **P14KanS**, **P14LRR**, kanamycin, and melittin at 50 μM against *C. elegans*. After 3 days of treatment with **P14KanS**, **P14LRR**, or kanamycin, the *C. elegans* were highly viable (>90%), whereas no live worms were observed with melittin after this time (Figure S11). These data serve to highlight the nontoxic nature of the kanamycin-peptide conjugate. For antibacterial activity to be monitored, *S. enteritidis*-infected *C. elegans* were treated with a range of concentrations of **P14KanS**, **P14LRR**, kanamycin, and a 1:1 mixture of **P14LRR** and kanamycin for 24 h, and the bacteria levels were monitored. **P14KanS** demonstrated significantly reduced levels of *Salmonella* in the *C. elegans* host as compared to the 1:1 mixture of antibiotics across all concentrations, and at the highest dose, approximately 90% of the bacteria had been cleared in vivo. The *C. elegans* infection model confirms that **P14KanS** does exhibit potent in vivo antimicrobial activity and has significant promise to be used as a novel treatment of intracellular pathogens.

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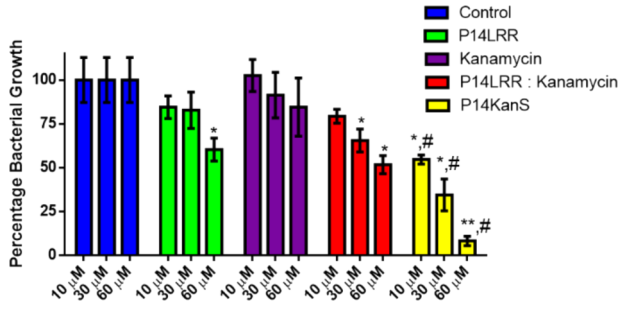

Figure 4. Treatment of *Caenorhabditis elegans* infected with *Salmonella enteritidis* with **P14LRR**, kanamycin, a 1:1 mix of **P14LRR** and kanamycin, and **P14KanS** for 24 h. Statistical analysis was calculated using one-way ANOVA with post hoc Tukey's multiple comparisons test. *P* values of <0.05 were considered significant. One asterisk (*) and two asterisks (**) indicate significance from the control of $P < 0.05$ and $P < 0.01$, respectively. Symbol (#) indicates significance from kanamycin, **P14LRR**, and **P14LRR**:kanamycin ($P < 0.05$). Results are expressed as means from three biological replicates ± standard deviation.

CONCLUSIONS

A number of pathogenic bacteria invade and then reside within mammalian host cells. At the same time, many of the most commonly used antibiotics, such as β-lactams and aminoglycosides, are unable to achieve therapeutic concentrations within these same cells. Therefore, there is a significant need to develop antibiotics with the ability to enter mammalian cells and target intracellular pathogens.[27] In this work, we have found that very effective targeting of intracellular bacteria, including *Salmonella*, *Brucella*, *Shigella*, and *Mycobacterium*, is possible using a dual antibiotic approach, where one of the antibiotics also has mammalian cell-penetrating properties. The reducible nature of the linker within these dual antibiotic conjugates was found to play a significant role in the intracellular activity. **P14KanC** lacks the reducible disulfide moiety, and as such, the kanamycin travels within macrophages to the preferred intracellular sites of the antibiotic peptide. Synergistic antibacterial activity is realized when the dual components of the conjugate are released within the reducing environment of the cell. Herein, we have demonstrated that the **P14KanS** conjugate not only clears notoriously difficult to treat intracellular pathogens such as *S. enteritidis*, *B. abortus*, and *M. tuberculosis* within J774A.1 cells, but also displays a significant reduction of *S. enteritidis* in an in vivo *C. elegans* model. Combining the cell penetrating ability and nonmembrane lytic mechanism of antimicrobial action of **P14LRR** with the kanamycin antibiotic has proven to be a very effective tool in combating intracellular pathogenic bacteria.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b04831.

Experimental procedures, spectra, figures, and supplementary references (PDF)

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Notes

The authors declare no competing financial interest.

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