Intracellular Targeting

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Tuning the Intracellular Bacterial Targeting of Peptidic Vectors**

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Many deadly pathogens live and divide within human cells, an approach that provides protection against the host immune response and antimicrobial treatment. When applied against intracellular pathogens, antibiotics show variable efficacy owing to factors such as low cellular accumulation and deactivation within the cell, making the development of treatments difficult and further narrowing the pipeline of effective antibiotics.[1–3] *Listeria monocytogenes* is an example of an intracellular pathogen that is very dangerous and often deadly.[4]

The search for drugs that can specifically target intracellular pathogens is particularly complex, and not amenable to the in vitro methods that produce leads for extracellular pathogens.[5–7] Efforts in targeting intracellular pathogens have previously focused on the use of nanoparticle delivery vectors such as liposomes and polymer-based nanoparticles to enhance therapeutic potential and overcome cellular barriers.[8–13] The use of liposomes as a method of antimicrobial delivery has been studied extensively[8] and has been shown to enhance uptake of membrane impermeable antibiotics and increase intracellular bacterial clearance.[9] However, instability in biological fluids, low entrapment efficiencies, and difficulties in ensuring consistent formulation highlights the need for additional work in this area.[10] The use of polymer-based nanoparticles as a delivery vector has also been studied as a successful method of targeting intracellular pathogens. These methods provide prolonged, steady release times and increased therapeutic efficacy.[11,12] However, similar to their liposomal counterparts, polymeric nanoparticles display poor entrapment of water soluble cargos, requiring considerable effort being put towards entrapment optimization for each new cargo.[13] Receptor-based methods have also been explored to deliver therapeutics to intracellular bacteria.[14]

Recent work in our laboratory has focused on the use of peptide-based vectors for intracellular targeting of small molecules, particularly to the mitochondria of mammalian cells.[15,16] Given the evolutionary link between bacteria and mitochondria, we hypothesized that similar peptides could be used to target intracellular bacteria. Moreover, previous studies indicated that conjugation of a methotrexate (Mtx) to a peptide specific for the mitochondria of human cells can be used to increase the therapeutic window of this molecule when used as an antimicrobial agent. Mtx, a bacteriostatic dihydrofolate reductase (DHFR) inhibitor, exhibits appreciable toxicity towards human cells, but this activity can be suppressed if the drug is kept away from cytosolic DHFR and directed to mitochondria.[17] Here, we explore whether an Mtx–peptide conjugate engineered to specifically target intracellular bacteria might be active against *Listeria monocytogenes*. We present engineered Mtx–peptide conjugates as antimicrobial delivery vectors for the intracellular clearance of *L. monocytogenes* from mammalian cells (Figure 1 A). This is the first report of peptidic delivery vectors that can target

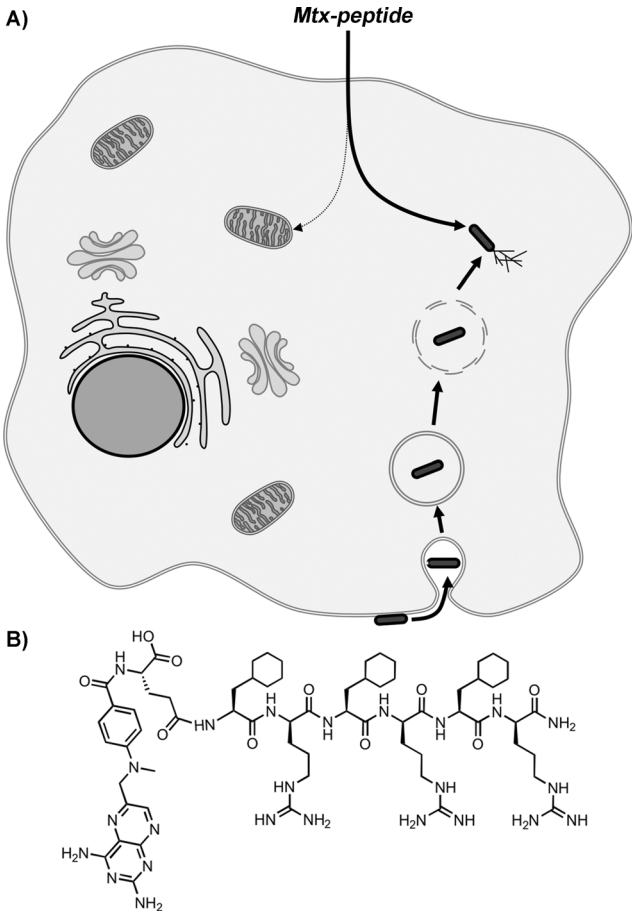

Figure 1. A) Representation of peptide-based Mtx targeting of intracellular *L. monocytogenes*. The bacterium induces formation of a vacuole in non-phagocytic cells.[4] Lysis of the vacuoles release of *L. monocytogenes* into the cytoplasm. Conjugation of Mtx to a peptide allows for specific delivery into *L. monocytogenes* while sequestering excess Mtx into mitochondria at higher concentrations. B) Structure of initial Mtx-peptide conjugate tested against *L. monocytogenes*.

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intracellular pathogens and clear them from mammalian cells through the action of an antibacterial drug.

To evaluate whether a Mtx–peptide conjugate would localize to *L. monocytogenes* within human cells, a sequence containing six amino acids and featuring alternating hydrophobic (cyclohexylalanine) and cationic residues (D-arginine) was tested (Figure 1B). The hydrophobic character of the peptide allows for efficient penetration of lipophilic biological membranes, while the cationic residues promote penetration of energized membranes and localization within the mitochondrial matrix or bacterial cytosol. To test the specific cellular and bacterial localization of the peptide delivery vector, a dye-labeled peptide was developed by N-terminal conjugation of the base peptide with the fluorescent compound tetramethylrhodamine (TAMRA). Fluorescently labeled peptides show specific localization to the mitochondria and, in infected cells, to intracellular *L. monocytogenes* (Figure 2A). *L. monocytogenes* in infected HeLa cells show characteristic actin polymerization activity,[18] indicating bacterial viability (Supporting Information, Figure S1).

The Mtx–peptide conjugate was tested for toxicity against cultured *L. monocytogenes* and exhibited potent growth inhibition with an MIC_{50} of 3.7 ± 0.2 μM. This corresponds to a slight increase in inhibition when compared to unconjugated Mtx, which exhibited an MIC_{50} of 8.8 ± 0.9 μM (Figure 2C). When tested against cells infected with *L. monocytogenes*, the Mtx–peptide maintained growth inhibitory activity against intracellular *L. monocytogenes* with a MIC_{50} of 13.1 ± 1.4 μM (Figure 2D). Unconjugated Mtx also caused growth inhibition of intracellular *L. monocytogenes*, with a MIC_{50} at 19.1 ± 4.2 μM. However, the drug also exhibited considerable toxicity against HeLa cells with an LD_{50} of 14.8 ± 0.1 nM (Figure 2E), indicating that most of the host cells were dead before the effects of the antimicrobial drug could be realized against the pathogen (Figure 2F). Overall, these results indicate that the peptide conjugate is effective against an intracellular pathogen, and unlike the parent drug, has little toxicity towards human cells at concentrations required for clearance of *L. monocytogenes*.

Having successfully applied an Mtx–peptide conjugate to target intracellular *L. monocytogenes*, we conducted a study designed to elucidate the optimal properties of an intracellular antimicrobial delivery vector. Previous studies indicated that charge and hydrophobicity are the main determinants of peptide localization into mitochondria.[15] Therefore, a panel of peptides were designed to determine the effect of charge and hydrophobicity on peptide partitioning between bacteria and mitochondria (Figure 3A). These two parameters have also been suggested to promote entry of peptides into bacteria.[19,20] Using the original peptide tested (**1**) as a starting point, a series of peptides with increased positive charge (and similar hydrophobicity; **5**–**7**) or decreasing hydrophobicity (and consistent positive charge; **2**–**4**) were synthesized. Peptide hydrophobicity was modified through substitution of cyclohexylalanine with less hydrophobic residues, and was characterized by measuring retention time on a C18 reverse-phase column (Supporting Information, Table S1). To study the subcellular localization of the peptides, fluorescently labeled derivatives were generated

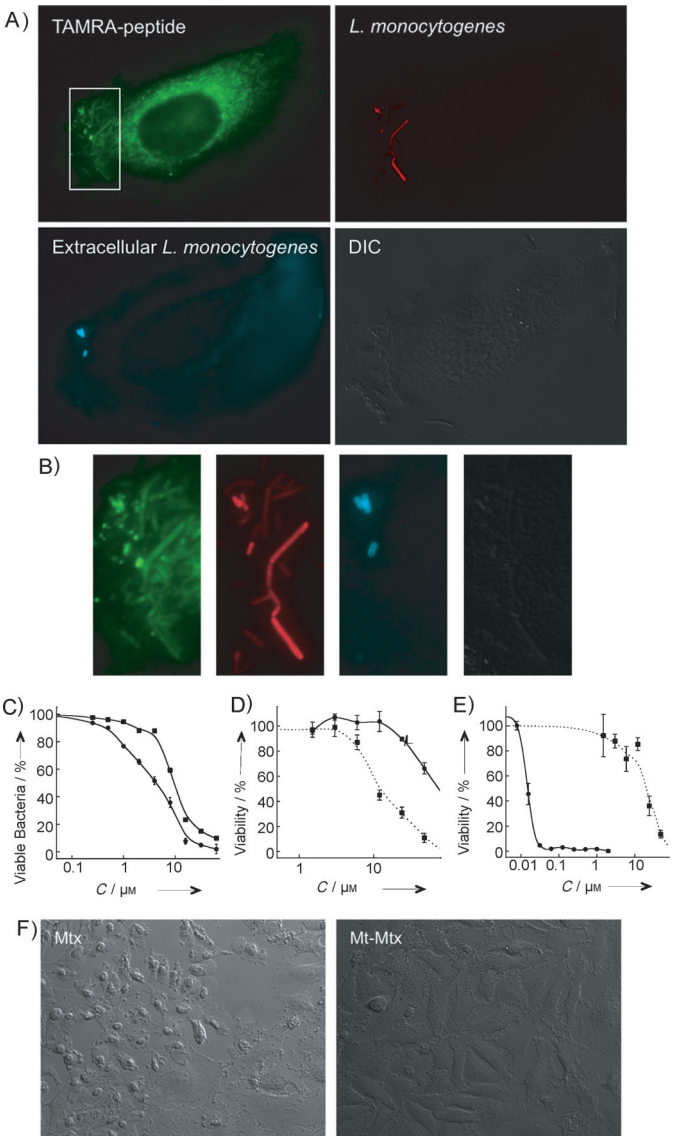

Figure 2. A) Fluorescently labeled peptides localize specifically to mitochondria and *L. monocytogenes* in infected HeLa cells (shown in green). *L. monocytogenes* labeled with commercially available CellTrace Far Red show strong colocalization with peptide fluorescence (shown in red). Addition of a *L. monocytogenes* specific antibody indicates the position of extracellular bacteria (shown in blue). The lack of staining indicates an intracellular bacterium. The boxed region has been expanded in (B) to clearly demonstrate the colocalization between the peptide staining and the *L. monocytogenes* bacterium. C) In vitro antimicrobial activity of Mtx-peptide conjugate **1** (●) and Mtx alone (■) against *L. monocytogenes*. *C* = concentration. D) Antimicrobial activity of **1** against *L. monocytogenes* in HeLa cells (■,•••••). Effect of **1** on HeLa cell viability (●,——). E) Antimicrobial activity of Mtx alone against *L. monocytogenes* in HeLa cells (■,•••••). Effect of Mtx on HeLa cell viability (●,——). F) Differential interference contrast microscopy of HeLa cells infected with *L. monocytogenes* and then treated with 20 μM Mtx and **1**.

by N-terminal conjugation to TAMRA. Fluorescence microscopy of cells treated with TAMRA-conjugated peptides revealed that all of the peptides localized specifically to mitochondria in the absence of internalized bacteria (Supporting Information, Figure S4). Treatment of cells with

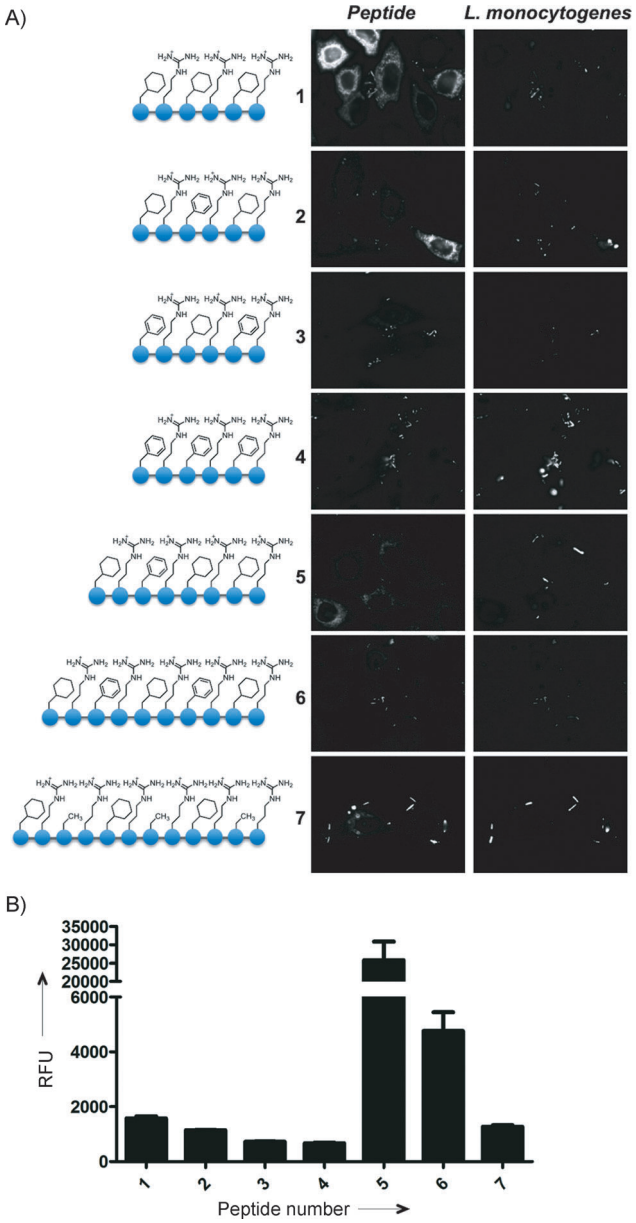

Figure 3. A) Partitioning of fluorescently labeled peptides is affected by modification of peptide chemical properties. HeLa cells were treated with 5 μM of each labeled peptide, with the exception of **3** and **4** which were treated at 20 μM. *L. monocytogenes* labeled with commercially available CellTrace Far Red show similar position to peptide fluorescence (for DIC, see the Supporting Information, Figure S2). B) Summary of uptake measurements made with flow cytometry for HeLa cells treated with 5 μM of each labeled peptide. For representative fluorescence histograms, see the Supporting Information, Figure S3.

TAMRA alone or non-MPP peptide conjugates show no mitochondrial staining at relevant concentrations (Supporting Information, Figure S5). Interestingly, the localization patterns changed in the presence of intracellular bacteria, with increased cationic character resulting in increased partitioning in bacteria (Figure 3A; **1**, **5**–**7**). Similarly to peptide **1**, peptide **5** showed little difference in partitioning between mitochondria and *L. monocytogenes*; however, peptide **7** showed a clear increase in bacterial staining as compared to mitochondrial staining. Furthermore, decreasing hydrophobicity was also found to increase bacterial partitioning, but higher concentrations were necessary to observe fluorescence for the low hydrophobicity peptides (Figure 3A, **2**–**4**). While increasing bacterial partitioning was observed with each peptide series, peptides **4** and **7** showed the most significant effect.

To determine the effect of the peptide sequence changes on intracellular concentrations of peptide, flow cytometry was used to evaluate uptake. Uptake was observed to decrease upon reduction in hydrophobicity with the least hydrophobic peptide **4** having considerably less uptake than **1**. Conversely, increase in charge resulted in an initial increase in uptake between **1** and **5**, followed by a reduction in uptake as charge was increased as shown by **6**–**7**.

Interestingly, peptide **7** was found to have a similar level of overall uptake to **1** (Supporting Information, Table S1), indicating that the increase in bacterial partitioning was due to an increase in the relative uptake of **7** into the bacterium, rather than an increase in overall uptake (Figure 3B). Therefore, peptide **7** was chosen as the ideal candidate to assess whether improvements in the delivery of an antibacterial were realized. An Mtx–peptide conjugate was generated with peptide **7**, and compared to the original compound made with **1**. This compound was found to potently inhibit the growth of cultured *L. monocytogenes*, exhibiting an MIC_{50} of 0.68 ± 0.06 μM, corresponding to a greater than 5-fold increase in antimicrobial activity as compared to **1** (Figure 4A). When tested against intracellular *L. monocytogenes*, the **7** Mtx-peptide conjugate was found to be more potent relative to **1**, with an MIC_{50} of 4.8 ± 0.2 μM (Figure 4B). Conjugate **7** also showed little toxicity towards human cells at concentrations required for clearance (Supporting Information, Figure S7). The observed increase in the efficacy of the **7** conjugate both in mammalian cells and in culture indicates that molecular charge is an important determinant of the activity of a peptide vector targeting an intracellular pathogen. Increased partitioning of the potentiometric dye JC-1 towards *L. monocytogenes* suggests a stronger membrane potential compared to mitochondria, possibly accounting for increased uptake of peptide **7** (Figure 5).

The activity of the Mtx–peptide conjugates was linked to the activity of the appended drug by testing the effects of media containing high levels of nucleotides (Supporting Information, Figure S8). Under these conditions, the pathway methotrexate inhibits is not essential. Indeed, significant decreases in toxicity for both **1** and **7** are observed under these conditions, indicating that it is the effects of the appended drug that underlie the activity. This observation differentiates this approach from work leveraging the effects of antimicrobial peptides that disrupt bacterial membranes.

In summary, we have demonstrated that engineered peptides can provide vectors for efficient delivery of antimicrobial compounds against intracellular *Listeria monocytogenes*. We have also shown that peptides can be chemically modified for greater bacterial specificity while maintaining the mitochondria as a drug reservoir, a feature critical for reduction of cargo toxicity. This work also highlights the importance of strong cationic character in intracellular

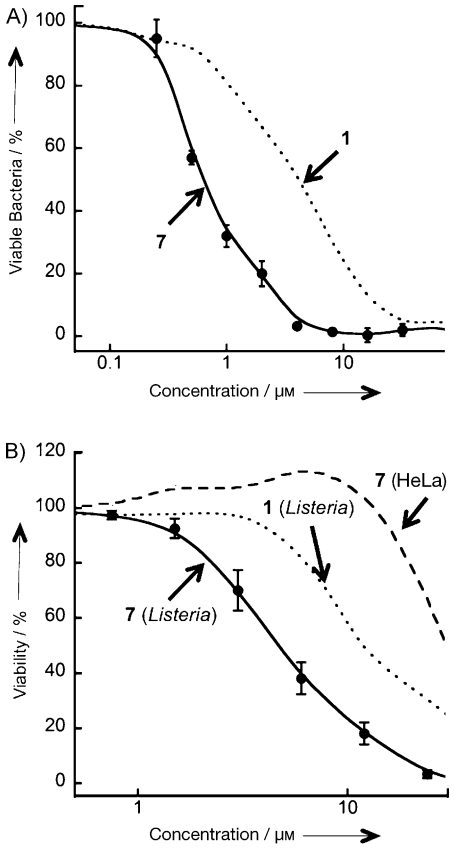

Figure 4. A) In vitro antimicrobial activity of Mtx–peptide **1** and Mtx–peptide **7** against *L. monocytogenes*. Mtx–peptide **7** shows potent inhibition of *L. monocytogenes* growth B) Mtx–peptide inhibition of *L. monocytogenes* growth is maintained in cells. Similarly to in vitro *L. monocytogenes* treatment, Mtx–peptide **7** shows increased inhibition of *L. monocytogenes* growth compared Mtx–peptide **1.** Toxicity curve for Mtx–peptide **7** against HeLa cells is also shown (for raw data, see the Supporting Information, Figure S7).

bacterial targeting with peptide based vectors. The results are the first showing that peptides can facilitate targeting intracellular bacteria and provide evidence that this method can be used to increase antimicrobial efficacy against intracellular pathogens.

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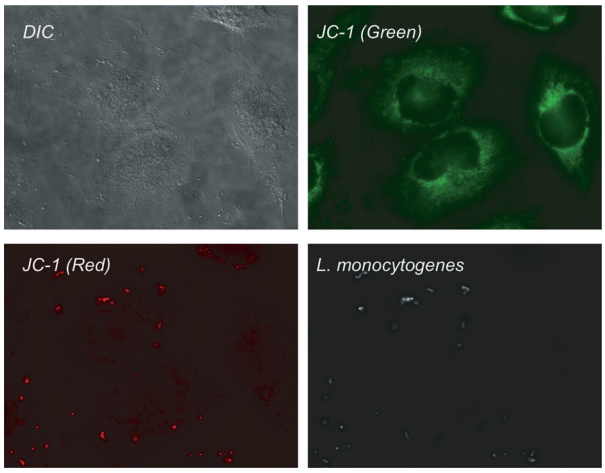

Figure 5. Partitioning of the potentiometric dye JC-1 in HeLa cells infected with *L. monocytogenes* shows preference for bacteria. JC-1 accumulates within areas of high potential and forms J-aggregates upon reaching a critical concentration, resulting in a shift in emission wavelength. Monomeric JC-1 fluorescence is shown in green. Fluorescence from J-aggregates, indicative of membrane potential, is shown in red. *L. monocytogenes* labeled with commercially available CellTrace Far Red show similar position to J-aggregate fluorescence (shown in gray). J-aggregate formation is inhibited by the addition of uncoupling agents such as FCCP (Supporting Information, Figure S6).

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