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Probing length effects and mechanism of cell penetrating agents mounted on a polyproline helix scaffold

Iris Geisler and Jean Chmielewski*

Department of Chemistry, Purdue University, 560 Oval Drive, West Lafayette, Indiana 47907, USA

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Abstract—Cell penetrating peptides (CPP) displaying a type II polyproline helix backbone of different length and amphiphilic character were synthesized and their cellular uptake was compared. The longer CPP sequence, P14LRR, displayed a 7- to 12-fold higher uptake in MCF-7 cells as compared to its shorter counterpart, P11LRR, and a 35-fold higher uptake as compared to Tatp. These results demonstrate that an increased number of cationic and hydrophobic residues can strongly influence the extent of cellular internalization. Mechanistic investigations suggest internalization via a receptor independent endocytotic pathway with these agents.

A number of different approaches have been taken to accomplish the delivery of therapeutic agents into cells. One recent approach is the use of cell penetrating peptides (CPP) that are rich in basic amino acids.[1] CPPs have many advantageous features: generally low toxicity, high efficiency toward a variety of different cell lines, as well as the delivery of diverse cargo to intracellular targets ranging from proteins and oligonucleotides to magnetic nanoparticles.[2] Two of the most well-studied CPPs, derived from transcription factors, include the cationic domain of HIV-Tat (49–57)[3] and the penetratin peptide from the Antennapedia homodomain.[4] Other short peptides previously used for membrane translocation include: cationic nuclear localization signal sequences[5], polylysine or polyarginine peptides,[6] cationic moieties linked to scaffolds, such as peptoids,[7] β-amino acid peptides,[8] oligocarbamates,[9] and loligomers.[10]

To date the mechanism of uptake of CPPs remains debatable and a common consensus has not yet been reached. However, for the Tat peptide (Tatp) early studies using fixed cells showed that the peptide could directly cross the plasma membrane in an energy-independent manner.[11] These initial results have been challenged; live cell imaging studies have shown that internalization of TAT could be inhibited at low temperature or after depletion of ATP using sodium azide and deoxyglucose, indicating an energy-dependent endocytic pathway for TAT uptake.[12] Currently it is generally accepted that endocytosis is the major uptake route for TAT or TAT conjugates.[2a,13] Different endocytotic mechanisms for TAT cellular uptake have been reported, including clathrin-mediated endocytosis,[14] caveolae-mediated endocytosis,[15] and macropinocytosis.[16] Several studies have also attributed TAT-mediated cellular uptake as a consequence of the negatively charged heparan sulfate proteoglycans on the cell surface.[17] However, enzymatic removal of heparan sulfate only partially inhibited the internalization of TAT,[14b] implying the existence of a heparan sulfate independent mechanism for TAT uptake.[18] It has also been proposed that the attached cargo may play a significant role on the actual mechanism for CPP cellular uptake.[19]

In an effort to probe the role of hydrophobic residues and amphiphilicity in cell penetration of CPPs, research has focused on the use of scaffolds with a preorganized structure to position cationic and hydrophobic moieties.[20] One such agent, P11LRR (Fig. 1), used a rigid backbone containing polyproline residues that allow for the formation of a polyproline type II helix (PPII).[20c] This type of helix contains three residues per turn with a pitch of 10 Å, thereby aligning every third ring on the same face of the helix. This scaffold was chosen to allow for the controlled orientation of cationic (blue) and hydrophobic (purple) moieties (Fig. 1).

Previous investigations undertaken in our laboratory with these types of CPPs found significant cellular internalization with MCF-7 breast cancer cells when six

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* Corresponding author. E-mail: chml@purdue.edu

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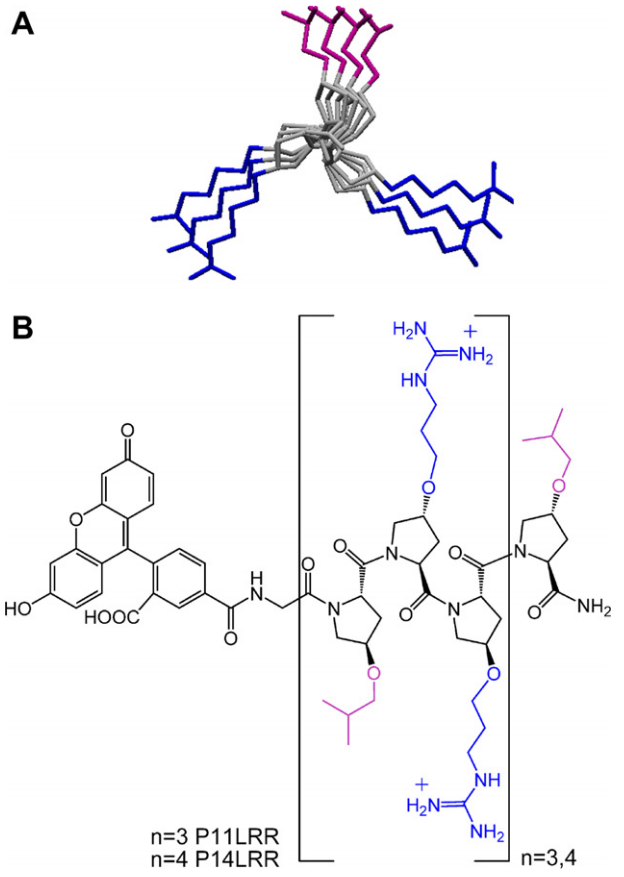

Figure 1. (A) Model of a polyproline helix containing cationic (blue) and hydrophobic residues (purple), fluorescein moiety omitted for clarity. (B) General structure of the modified polyproline oligomers used in this study.

guanidinium groups were positioned along the polyproline backbone as in P11LRR (Fig. 1)[20c]. In comparison, only mediocre cellular internalization was observed with the amine-containing polyproline compounds. Also, scrambled versions of these CPPs, featuring hydrophobic and cationic groups on all faces of the helix, were only as potent as their flexible Arg_n counterparts.[20c] These findings clearly indicate that the ordered phasing of hydrophilic and hydrophobic units, or amphiphilicity, plays a crucial role in the efficiency of cellular internalization.

Herein, we further investigate the effect of increased helical length and cationic charge on cellular internalization. The type of mechanism of cellular uptake was also probed to gain a better understanding of the features influencing the extent of internalization. The representative peptides utilized in this investigation are P11LRR and P14LRR (Fig. 1).

Specific functionalization of the PPII helix was accomplished by O-alkylation of the hydroxyproline monomers, as previously described, with either an isobutyl group or a guanidinium moiety.[20c] After successful synthesis of the desired monomers, P11LRR and P14LRR were synthesized on the Rink solid support using an Fmoc-based strategy with HATU as the coupling reagent. Successful coupling was monitored using the chloranil test.[21] The synthesis of the helical domain was followed by addition of a glycine spacer, followed by fluorescein labeling in order to track cellular uptake by flow cytometry and confocal microscopy. The peptides were deprotected and cleaved from the resin simultaneously. The compounds were purified to homogeneity by reverse phase HPLC and characterized by matrix assisted laser desorption ionization mass spectrometry (MALDI). Each peptide was found to adopt a PPII helical conformation as confirmed by circular dichroism at pH 7.4, and analytical ultracentrifugation confirmed the monomeric nature of each agent (100 μM) at neutral pH (data not shown).

Flow cytometry was used to elucidate the efficiency of P11LRR and P14LRR for cellular internalization in adherent MCF-7 breast cancer cells. Cellular uptake of both peptides was investigated using a range of concentrations (Fig. 2). Cellular fluorescence was found to increase with increasing concentration of the agents. Interestingly, the cell uptake data were found to be non-linear with increasing CPP concentration, indicating that a certain concentration threshold was needed for activity, possibly due to cell membrane binding. The optimum concentration of P14LRR was determined to be 15 μM, as higher concentrations led to precipitation of the peptide. P14LRR was found to be 12- and 7-fold more efficient than P11LRR at concentrations of 10 and 15 μM, respectively. Notably, at a concentration of 15 μM, P14LRR was 35-fold more efficient as compared to Tatp.

Following flow cytometry, confocal microscopy experiments were performed to determine the exact cellular localization of P11LRR and P14LRR within live MCF-7 cells. Confocal microscopy indicated that each compound was internalized within the cells with no evidence of membrane binding (Figs. 3A and D). Confocal microscopy images obtained at identical settings

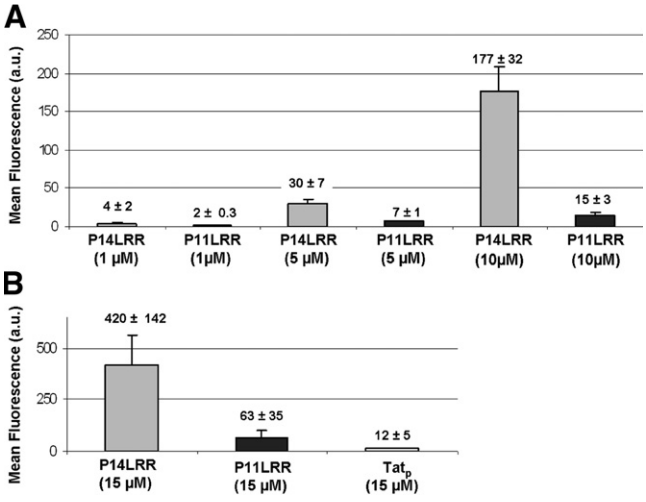

Figure 2. (A) Flow cytometry data for the cellular internalization of P11LRR and P14LRR at 1, 5, and 10 μM. (B) Cellular internalization at 15 μM with comparison to Tat_p. MCF-7 cells were incubated with the compounds for 6 h at 37 °C.

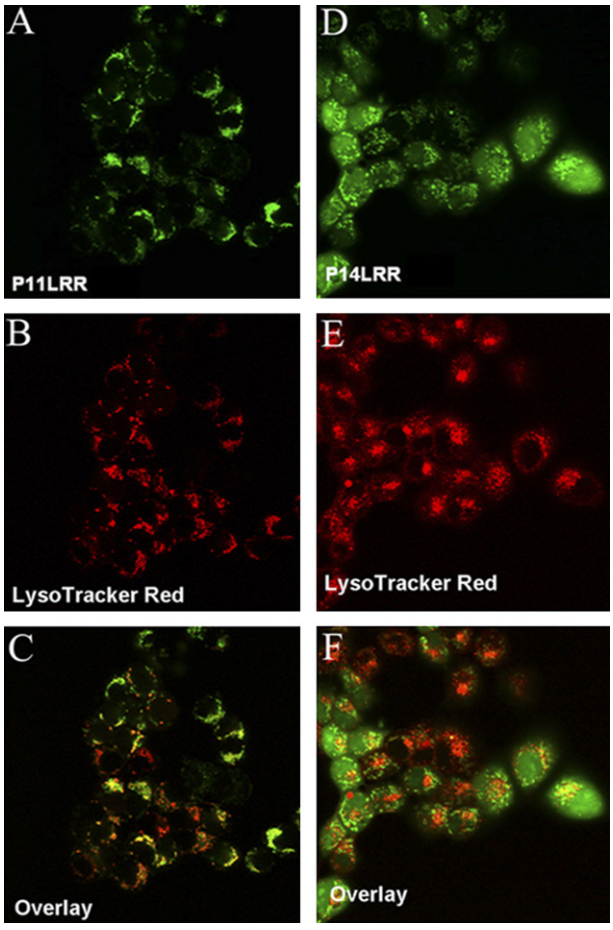

Figure 3. Confocal images of live MCF-7 cells incubated with P11LRR (A) and P14LRR (D) (15 μM) for 6 h. LysoTracker Red images of P11LRR (B) and P14LRR (E). Overlay of images of P11LRR A and B (C) and P14LRR D and E (F).

displayed a higher degree of fluorescence for P14LRR as compared to P11LRR (Figs. 3A and D). This is in compliance with results obtained by flow cytometry. Punctate cytoplasmic staining was observed for each of the compounds. Notably, P14LRR also displayed diffuse cytoplasmic and nuclear localization (Figs. 3A and D).

Punctate fluorescence can be an indication of endosomal localization. In order to determine if the CPPs were located within endosomes, an endosomal co-staining experiment was preformed with LysoTracker Red and the CPPs. Figures 3A and D represent the images of MCF-7 cells incubated with the fluorescein labeled CPPs. Figures 3B and E are images of the same cells treated with LysoTracker Red to visualize endosomes found within the cells. The two images were digitally overlaid in Figures 3C and F. The overlaid image for P11LRR indicates a good correlation between the location of fluorescence associated with the CPP and with the LysoTracker dye, indicating endosomal localization of the CPP. The punctate cellular fluorescence of P14LRR was found to also colocalize with the endosomal dye, however, there was extensive nuclear staining and cytosolic fluorescence that was not endosomal, indicating either release from endosomes or an alternative non-endosomal pathway for cell entry.

To further investigate the possibility of an endocytosis-mediated pathway of entry, temperature-dependent, as well as energy-dependent, studies were performed. The effects of temperature were studied by incubating MCF-7 cells with P11LRR and P14LRR (15 μM) for 6 h at 4 and 37 °C. The data obtained clearly indicated that cellular uptake was temperature-dependent and decreased with lower temperature by 82% and 89%, respectively. A decrease in cell uptake at low temperature has also been observed with Tat[22] and is indicative of a possible endocytotic entry mechanism.

Next, the effect on uptake due to depletion of ATP in MCF-7 cells was examined by incubating cells with P11LRR and P14LRR (15 μM) for 6 h at 37 °C in the presence of 2 mM sodium azide (NaN_3) with analysis by flow cytometry. NaN_3 is an oxidative phosphorylation inhibitor commonly used to abolish ATP production within the cell membrane. In this study, a small decrease in uptake (19%) was evident for P14LRR, whereas for P11LRR essentially no change was observed. A decrease in cellular uptake in these types of studies suggests a receptor-mediated pathway of internalization.[23] In the case of P14LRR the observed small decrease in uptake implies that uptake occurs, in part, by an energy-dependent mechanism. However, the data primarily indicates that uptake occurs via an energy-independent process, since internalization is only diminished but not abolished.

In a final experiment, the potential cytotoxicity of both peptides was determined by flow cytometry using propidium iodide.[24] The data indicated approximately 80% cell viability for both CPPs (15 μM) and approximately 95% for Tatp (15 μM) after a 24 h incubation period.

In conclusion, these data demonstrate that increased CPP length and cationic/hydrophobic character serve to promote cellular penetration. The longer CPP, P14LRR (approximately 45 Å in length), demonstrated a significantly higher internalization efficiency as compared to P11LRR and Tatp. Further studies probing the importance of the hydrophobic moiety within the peptide for optimum internalization are currently underway. Overall, these data show promise toward the employment of a polyproline scaffold in the delivery of therapeutic agents into cells, an endeavor under current examination.

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