Drug Delivery

Potential Peptide Carriers: Amphipathic Proline-Rich Peptides Derived from the N-Terminal Domain of γ-Zein**

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The ability of certain peptides to cross eukaryotic cell membranes is clearly of interest in the drug delivery field. In recent years, this interest has led to the rapid development of peptide carriers for the delivery of antitumoral, antiviral, or antibiotic drugs, which otherwise would be unable to cross the cell membrane and reach their therapeutic target. Additional advantages of the use of peptide carriers include their low toxicity, accessible synthesis, and high flexibility for modification when attaching peptides or small-molecule drugs as cargoes.[1] The ability of a wide variety of short peptides to act as carriers[2] for the delivery of peptides,[3] proteins,[4] or oligonucleotides[5] inside the cell has been demonstrated, while recent studies have identified peptide vectors including: human calcitonin (hCT),[6] fragments of protein-transduction domains (VP22,[7] Tat,[8] or Antp[9]), arginine-rich peptides,[10] β-peptides,[11] peptoids,[12] and loligomers.[13] Although little is known about the mechanism that operates in a translocation process of this nature, the amphipathicity of the carriers, which determines self-assembly, appears to be crucial for the interaction of the molecules with receptors (molecular recognition) or with highly amphipathic environments.[14]

The ability of proline-rich antibiotics to cross the cell membrane has also been demonstrated[15] and recently we reported the surprising result that a peptide containing only proline residues (P_{14}) crossed the cell membrane, albeit with low efficiency.[16] Polyprolines adopt a well defined secondary structure, polyproline II (PPII), in pure water but unlike the α-helix, PPII is left-handed with 3.0 residues per turn. The rules for transforming a PPII helix into an amphipathic helix

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are easily predicted. As we have demonstrated elsewhere, 50% of the proline content in the sequence is sufficient to maintain the PPII structure.[17] Thus, it is possible to alternate helix turns formed by three P residues with amphipathic helix turns formed by polar residues X in positions $i+1$, $i+7$, $i+13\ldots$ and hydrophobic residues Z in positions $i/i+2$, $i+6/i+8$, $i+12/i+14\ldots$ in sequences $(ZXZPPP)_n$, (P = proline residue; Figure 1a, b). Such structures appear in natural protein domains as the N-terminal repetitive domain $(VHLPPP)_n$ (where $n=8$) of maize γ-zein. This and related peptides interact with membranes and do not present antibiotic effects.[17,18]

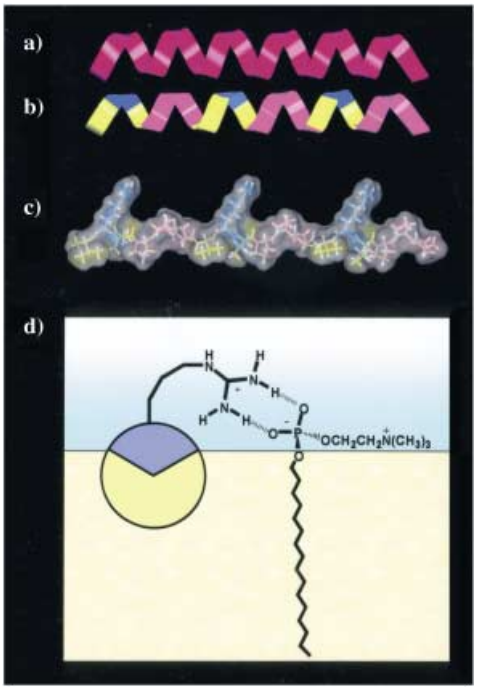

Figure 1. a) Schematic representation of a polyproline helix (PPII), b) the amphipathic Pro-rich helix generated after exchange of Pro residues at the $i+1$, $i+7$, $i+13$ positions by polar residues (blue) and the $i/i+2$, $i+6/i+8$, $i+12/i+14$ positions by hydrophobic residues (yellow), c) water accessible surface of $(VRLPPP)_3$ modeled adopting a PPII conformation using the Connolly algorithm implemented in Insight II v.98. The hydrophobic residues (Val and Leu) are yellow, polar protonated Arg are blue, and neutral Pro residues pink, d) model proposed for the interaction between the hydrophilic Arg residues (represented as the blue region in the circular head, the yellow region represents the hydrophobic residues of the peptide) and the polar heads of the phospholipids located in outer part of the cell membrane (the fatty diacylglycerol chain is schematically represented as a long alkyl chain).

Herein, we present the synthesis of amphipathic peptides $(VXLPPP)_n$ (with $n=1$–3 and X = His (H), Arg (R), Lys (K); Figure 1c) and examine their ability to cross cell membranes. Val (V) and Leu (L) were chosen by analogy with the aforementioned γ-zein domain. To favor the interaction with phosphate diester anionic polar heads, the X residues have lateral cationic chains (or partially cationic as His; Figure 1d). The interface between a cell membrane and its aqueous extracellular environment is an amphipathic ambient. We aimed to determine whether the transformation of a polyproline helix into an amphipathic Pro-rich sequence would generate a new family of peptide shuttles.

The synthesis of $(VXLPPP)_n$ and P_m, where $n=1$, 2, and 3 and $m=6$, 12, and 18, was performed by solid-phase peptide synthesis on a 2-chlorotrityl resin.[19] The choice of this support was particularly appropriate as it completely avoided the PP diketopiperazine side reaction. 5(6)-carboxyfluorescein (CF) was used for the synthesis of the fluorescent labeled peptides required in the cell uptake studies. A new optimized method for the incorporation of the fluorescent label has been achieved with the combined used of PyAOP and HOAt (see Figure 2).[20]

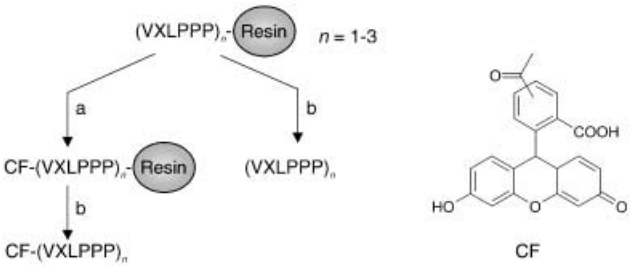

Figure 2. Scheme of synthesis and carboxyfluoresceination reaction on solid support (Resin = 2-chlorotrityl resin); a) 5(6)-carboxyfluorescein (5 equiv), PyAOP (5 equiv), HOAt (5 equiv), DIEA (10 equiv), DMF, 90 min, RT, b) 95% TFA, 2.5% TIS, 2.5% water (15–90 min). PyAOP = 7-azabenzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate, HOAt = 1-hydroxy-7-azabenzotriazole, DIEA = *N*,*N*-diisopropylethylamine, TFA = trifluoroacetic acid, TIS = triisopropylsilane.

Once these series of labeled peptides had been synthesized, their comparative properties for translocation through the cell membrane were checked with HeLa human cell line. First, the extent of the uptake of the monomers, dimers, and trimers at 50-μM concentration in HeLa cells was quantified using a fluorescence microplate reader assay. After incubating the peptides with the cells for 1–3 h, washing in phosphate buffer saline (PBS) and the addition of a lysis buffer containing 0.1% Triton, the fluorescence emitted was measured in the microplate reader (see Supporting Information). As shown in Figure 3a, cells incubated with $CF\text{-}(VRLPPP)_3$ presented the highest fluorescence intensity. This result could be attributed to the ability of guanidinium groups to interact strongly with bidentate anions,[21] present here in the form of phosphate diester in the outer part of the cell membranes (Figure 1d) and, it is in agreement with several reported examples.[22] A dose–response analysis was then undertaken, by incubating HeLa cells with the peptides at different concentrations. Again, the nature of the hydrophilic residues and, specifically, the length of the peptide were shown to have a pronounced effect on uptake (Figure 3b).

The cellular uptake of the new amphipathic Pro-rich peptides was also studied by confocal laser scanning microscopy (CLSM). HeLa cells were incubated for 3 h with each carboxyfluoresceinated peptide (50 μM). After washing with PBS, the cells were fixed with paraformaldehyde, and analyzed by confocal microscopy. As revealed by confocal microscopy images, an intracellular vesicular distribution of

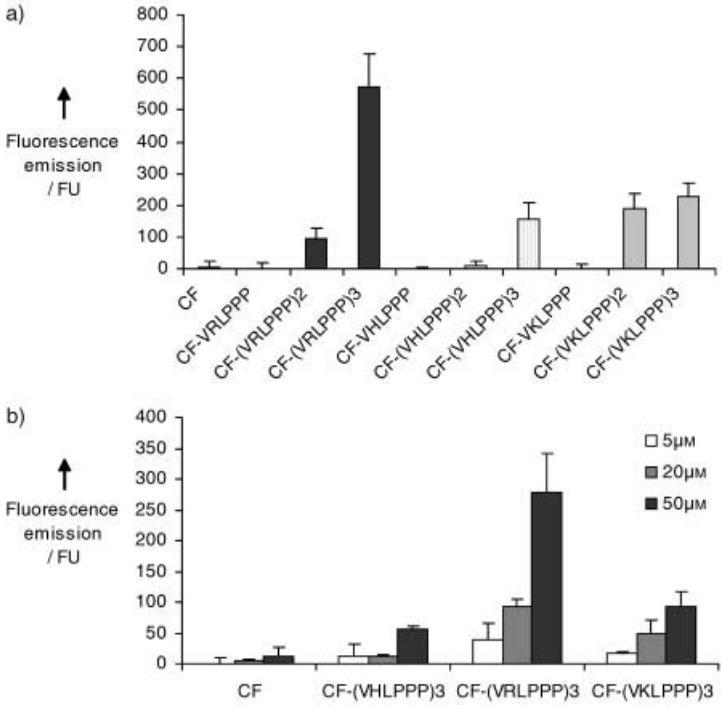

Figure 3. a) Fluorescence emitted after incubating HeLa cells for 3 h with CF-$(VXLPPP)_n$ with X = Arg, Lys, His and $n = 1$–3 at 50 μM concentration, b) comparative representation of fluorescent emission obtained after incubation of HeLa cell line for 1 h at 37 °C with several carboxyfluoresceinated peptides at different concentrations ranging from 5 μM to 50 μM.

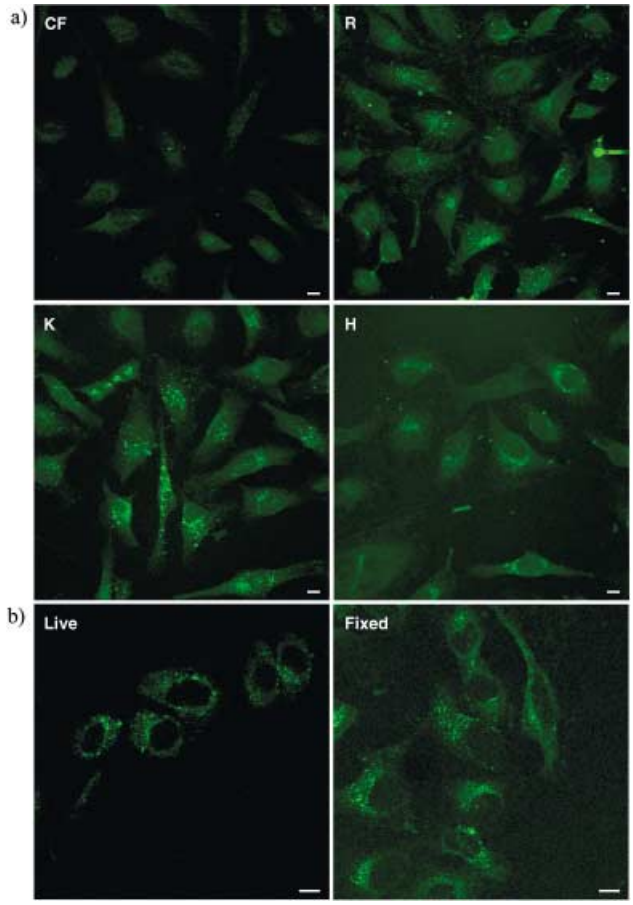

Figure 4. a) CLSM images of HeLa cells incubated for 3 h at 37 °C with 50 μM CF as negative control (CF) and CF-$(VXLPPP)_3$ where X = Arg, Lys, His; Image R = CF-$(VRLPPP)_3$-OH, H = CF-$(VHLPPP)_3$-OH, K = CF-$(VKLPPP)_3$-OH; b) HeLa cells were incubated with CF-$(VRLPPP)_3$ at 20 μM concentration for 3 h at 37 °C in 5% CO_2 atmosphere. Cells were washed with PBS and directly visualized (left) or fixed with paraformaldehyde (right) before observation by fluorescence microscopy. Scale bar = 10 μm.

the fluorescent peptides was found after recording optical sections that allowed the 3D cell reconstruction (Figure 4a). The carboxyfluoresceinated peptides were located inside the cells and were not attached to the cell membrane. In addition, we examined the influence of the fixation step with a 3% paraformaldehyde solution, since recent papers have shown that the fixation step prior to observation by microscopy leads to the presence of artifacts on entry[23] or, otherwise changes the localization of the carrier molecule.[24] As shown in Figure 4b, a punctate cytoplasmic distribution outside the nucleus was observed in vivo and in fixed cells. We conclude that fixation with paraformaldehyde does not influence entry in HeLa cells and nor does it modify the localization of these carrier peptides.

Finally, cell viability assays with (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) were performed to check the toxicity of the amphiphilic Pro-rich peptides. The most promising peptide CF-$(VRLPPP)_3$ was noncytotoxic after incubation for 24 h with HeLa cells in concentrations up to 1000 μM, which highlights its potential as a carrier (see Supporting Information). Comparative cytotoxic studies of other well established cell-penetrating peptides have been carried out. Thus, as already discussed in the literature CF-Tat or CF-Antp were cytotoxic at relatively low concentrations.[25] In our hands, at the concentration used for the internalization studies, that is, 50 μM, CF-Tat reduced the cell viability to 64% and CF-Antp to 75% (see Supporting Information). The viability of HeLa cells was dramatically reduced in the presence of CF-Tat or CF-Antp at higher concentrations (e.g. to 40 and 11%, respectively, at 500 μM concentration). Measured against CF-Tat or CF-Antp, the degree of internalization of CF-$(VRLPPP)_3$ was found to be, respectively, 15- or 20-times lower, however, CF-$(VRLPPP)_3$ showed the absence of cytotoxicity.

In conclusion, $(VXLPPP)_n$ is a new family of peptides that can translocate human cell membranes. Compared with other carrier peptides, this new family presents several advantages including its nonviral origin, amphipathic character, solubility in water, and the absence of a cytotoxic effect at high concentrations. In terms of their synthesis, we report a new and efficient method for labeling resin-bound peptides with 5(6)-carboxyfluorescein marker. We are currently undertaking studies to gain further insight into the translocation process of Pro-rich amphipathic peptides as well as their use in the delivery of plasmid DNA.

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[19] The peptides were synthesized by solid-phase peptide synthesis following the 9-fluorenyl methoxy carbonyl/*tert*-butyl (Fmoc/*t*Bu) strategy.

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