**all-δ proline-rich cell-penetrating peptides: a preliminary in vivo internalization study**

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**Abstract**

Proline-rich cell-penetrating peptides, particularly the SAP (sweet arrow peptide), \((\text{VRLPPP})_3\), have been proposed to be useful intracellular delivery vectors, as a result of their lack of cytotoxicity combined with their capacity to be internalized by cells. A common limitation of the therapeutic use of peptides is metabolic instability. In general, peptides are quickly degraded by proteases upon entry into the bloodstream. The use of all-δ-peptide derivatives is emerging as a fruitful strategy to circumvent this degradation problem. In this context, we report on the internalization behaviour, protease-resistance enhancement and self-assembly properties of an all-δ version of SAP \([(\text{vrlppp})_3]\). The cellular uptake of \((\text{vrlppp})_3\) was evaluated in an in vivo assay in mice. Both flow cytometry and confocal laser-scanning microscopy experiments showed that a carboxyfluoresceinated version of the molecule, carboxyfluorescein–(vrlppp)_3, is internalized rapidly in white blood cells and kidney cells. Significant fluorescence was also detected in other organs such as the spleen and the liver. Finally, the toxicity of \((\text{vrlppp})_3\) was examined, and no significant differences in the main biochemical parameters nor in weight were detected compared with controls.

**Proline-rich peptides in drug delivery: the SAP [sweet arrow peptide, \((\text{VRLPPP})_3]\]**

High-proline content has been identified as a key signature of several families of CPPs (cell-penetrating peptides) which are structurally diverse: linear peptides [1–5], dendrimers [6] and γ-peptides [7,8]. Among proline-rich linear peptides, the SAP family combines a moderate cellular uptake capacity with a remarkable lack of cytotoxicity [3,9–11]. SAP is composed of 50% proline together with three positively charged arginine residues. These residues contain the guanidinium group, which is crucial for cellular uptake in other systems [12–16]. In solution, SAP adopts an amphipathic PPII (polyproline II) helical structure (Figure 1) and has a strong tendency to self-assemble, forming tubular structures.

**Internalization studies of all-δ SAP [(vrlppp)_3]**

Proteolytic stability is a critical requirement for the therapeutic application of CPPs. For this purpose, an enantiomeric version of carboxyfluoresceinated SAP, CF–(vrlppp)_3, was prepared and evaluated (Figure 1). Uptake experiments in HeLa cells using 50 μM peptide solutions in PBS indicated that the all-δ CF–SAP was internalized by cells to the same extent as the protease-susceptible parent peptide.

**Figure 1** | Structural model of (VRLPPP)_3 and (vrlppp)_3

Structures of both peptides adopting a left-handed [(VRLPPP)_3; top] or right-handed [(vrlppp)_3; bottom] PPII structure in aqueous solution. Colour code: valine and leucine, yellow; arginine, blue; proline, pink.

**Protease-resistance and self-assembly behaviour**

The proteolytic stability of all-δ SAP was assessed in response to both a highly concentrated trypsin solution and to human serum. Under both conditions, the (vrlppp)_3 peptide was completely stable after more than 48 h of treatment. This behaviour clearly contrasts with that of SAP, which showed rapid degradation when exposed to the same conditions. Both CD and transmission electron microscopy indicated that (vrlppp)_3 forms stable fibrillar structures that are very similar to those reported previously for SAP [17–19].

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Figure 2 | Uptake of CF–(vrlppp)₃ in mouse leucocytes analysed by flow cytometry

Frequency distributions of the fluorescence intensity in leucocytes after i.p. injection of 400 nmol of CF–(vrlppp)₃ in 100 µl of PBS (red) or the same amount of free CF (blue). Experiments were performed in duplicate. A representative sample is shown in each case. All animal procedures were performed in accordance with institutional guidelines.

Figure 3 | CLSM of mouse organ sections

CF–(vrlppp)₃ was administered as described previously (Figure 2). Tissues were harvested and cryoprotected with a sucrose gradient. After tissues were fixed with paraformaldehyde and embedded in Tissue-Tek® OCT resin, 8-µm-thick sections were cut on a cryostat and analysed by CLSM. (A) Time course of kidney CF–(vrlppp)₃ internalization. (B) Detail of the fluorescence pattern observed in spleen (i) and liver (ii) sections isolated 24 h after i.p. injection. Scale bars, 50 µm.
A preliminary in vivo internalization study

The efficiency observed for in vitro internalization of all-\(D\) SAP prompted us to perform an in vivo internalization study in mice [20]. The toxicity of (vrlppp)\(_3\) was evaluated by administration of the peptide (400 nmol in 100 \(\mu\)l of PBS) to Balb/c mice for one week on alternate days by i.p. (intraperitoneal) injection (\(n = 5\), with PBS injected as a control (\(n = 4\)). No significant differences in the main blood biochemical parameters nor in weight were detected.

For the in vivo internalization studies, a carboxyfluoresceinated version of all-\(D\) SAP was used. The uptake of CF–(vrlppp)\(_3\) was monitored in white blood cells and in major organs in mice. The peptide was efficiently taken up in leucocytes, as demonstrated by flow cytometry 1 h after administration. A significant amount of uptake was still detected 5 h after injection, whereas no differences with controls were observed at 24 h (Figure 2).

CLSM (confocal laser-scanning microscopy) was used to study the distribution of CF–(vrlppp)\(_3\), in mouse organs. Confocal images showed a rapid and intense enhancement of fluorescence in kidney sections (Figure 3A). Moreover, the fluorescence signal in the kidney sections was still detectable 24 h after i.p. injection. A weaker, but significant, uptake was also observed in spleen and liver sections (Figure 3B).

On the basis of these results, we propose that CF–(vrlppp)\(_3\), is a useful tool for intracellular drug delivery.

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