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A New *Antennapedia*-Derived Vector for Intracellular Delivery of Exogenous Compounds

C. García-Echeverría,[a,*] L. Jiang,[b] T. M. Ramsey,[b] S. K. Sharma[b] and Y.-N. P. Chen[b,*]

[a]*Oncology Research, Novartis Pharma Inc., CH-4002 Basel, Switzerland*
[b]*Oncology Research, Novartis Pharmaceuticals Corporation, 556 Morris Avenue, Summit, NJ 07901, USA*

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Abstract—We describe the design, synthesis and cell translocation capacity of a peptide derived from the third α-helix of the homeodomain of *Antennapedia*. The new sequence appears to be an efficient and nontoxic means to deliver a covalently linked peptide cargo into cells. © 2001 Elsevier Science Ltd. All rights reserved.

Introduction

The *Antennapedia* third α-helix peptide encompassing residues ^{43}Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys^{58} (peptide **1**, Table 1)[1] is a cell-membrane translocating vector able to deliver a covalently linked 'molecular cargo' (e.g., peptides or oligonucleotides) to either cytoplasmic or nuclear targets.[2] It has been speculated that the mechanism of internalization of this peptide probably involves the formation of inverted micelles that can be formed due to the intrinsic conformational flexibility of the peptide backbone.[2a,3] An understanding of the basis for this exceptional translocation behavior would be of considerable interest in the identification of alternative intracellular delivery systems.[4] As part of our own studies in this area of research, we have designed an *Antennapedia*-derived peptide and examined its cellular translocating properties by both fluorescence microscopy and flow cytometry. The new vector (peptide **2**, Table 1), which contains some of the structural traits present in the wild-type *Antennapedia* third α-helix sequence, is capable of carrying a covalently linked peptide cargo across cell membranes.

Design, Synthesis and Biological Assays

Peptide design

It has been shown that truncated or mutated versions of the wild-type *Antennapedia* third α-helix are unable to translocate across cell membranes or have a markedly decreased cell internalization capacity.[2a,4] These studies have highlighted the important role of certain residues for internalization. With this information in hand, we decided to keep the motifs Trp^{48}-Phe^{49}, Arg^{52}-Arg^{53} and Trp^{56}-Lys-Lys^{58} of the parent *Antennapedia* third α-helix and link them with 8-amino-3,6-dioxaoctanoic acid (Adoa), which acts as a flexible spacer.[5] The new peptide (peptide **2**, Table 1) was fluorescein-labeled on the N-terminal residue to examine its cell membrane translocation properties by fluorescence microscopy. The utility of peptide **2** as cell internalization vector of exogenous compounds was assessed using Pro-Ala-Lys-Arg-Lys-Leu-Phe-Gly as a molecular cargo in peptide **3** (Table 1). The sequence of this octapeptide is based on the cyclin A/cdk2 binding motif in p21-like cdk inhibitors.[6] Recently, we have shown that a cell membrane-permeable form of such peptide induced transformed cells to undergo apoptosis.[7]

Peptide synthesis

Peptide **2** (Table 1) was synthesized manually starting with a 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin for establishing the required C-terminal

Table 1. Peptide sequences

Entry	Peptide sequence
1	FITC-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys-OH
2	FITC-Adoa-Ser-Gly-Trp-Phe-Adoa-Arg-Arg-Adoa-Trp-Lys-Lys-NH_2
3	FITC-Adoa-Ser-Gly-Trp-Phe-Adoa-Arg-Arg-Adoa-Trp-Lys-Lys-Pro-Ala-Lys-Arg-Lys-Leu-Phe-Gly-NH_2

*Corresponding authors. Tel.: +41-61-696-1094; fax: +41-61-696-6246; e-mail: carlos.garcia-echeverria@pharma.novartis.com (C. García-Echeverría); tel.: +1-908-277-5752; fax: +1-908-277-5006; e-mail: yingnan.chen@pharma.novartis.com (Y.-N. P. Chen)

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carboxamide[8] and using standard solid-phase protocols based on fluorenylmethoxycarbonyl (Fmoc) chemistry.[9] Peptide **3** (Table 1) was synthesized on a Milligen 9050 peptide synthesizer (continuous flow), starting with an Fmoc-PAL-PEG-MBHA resin[10] and employing protocols previously reported from our laboratory.[11] The incorporation of fluorescein to the N-terminus of the resin-bound peptide **2** and **3** was performed using fluorescein isothiocyanate isomer 1 (3.0 equiv) in the presence of diisopropylethylamine (6.0 equiv).[12] The assembled peptide resins were simultaneously deprotected and cleaved by treatment with trifluoroacetic acid–water–1,2-ethanedithiol (76:4:20, v/v/v) for 2 h at room temperature. The filtrate from each cleavage reaction was added to diisopropyl ether–petroleum ether (1:1, v/v) at 0 °C, and the resulting precipitate was collected by filtration. The crude peptides were purified by medium-pressure liquid chromatography (MPLC) on a C_{18}-column using an acetonitrile–water gradient. The purity of the final compounds was verified by reversed-phase analytical HPLC and the identity was assessed by correct mass spectral and amino acid analyses.[13] Peptide **1** was purchased from Research Genetics Inc., Huntsville, AL, USA.

Cell culture

U2OS osteosarcoma cells, which were acquired from American Type Culture Collection, Rockville, MD, USA, were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

Fluorescence microscopy

Cells (3×10^4/well) were seeded in a 24-well plate and allowed to adhere overnight in the presence of medium containing 10% FBS. The cells were treated with the fluorescein (FITC)-labeled peptides for 10 or 24 h in Opti-MEM, rinsed with phosphate-buffered saline (PBS) (10 mM phosphate, 150 mM NaCl, pH 7.4) and fixed with 10% formaldehyde for 5 min, followed by three PBS washes. The cells were visualized under a fluorescent microscope (Axiovert 135, Zeiss) using appropriate filters. All experiments were carried out at room temperature.

Flow cytometry

Cells (0.4×10^6) were cultured in 60 mm dishes overnight and treated with the FITC-labeled peptides for 10 or 24 h in Opti-MEM at room temperature. After treatment, the cells were trypsinized and harvested by centrifugation (1000 rpm for 5 min at 4 °C). The cell pellets were washed once with PBS and fixed with 100 μL of 10% formaldehyde for 5 min, followed by three washings with PBS containing 10% FBS. The cells were then analyzed by a Becton Dickinson FACSort.

Results and Discussion

The *Antennapedia* third α-helix peptide (residues 43–58) is able to deliver functionally active molecules across cell membranes. In spite of the broad use of this approach and vector in cell-based studies, the mechanism by which this peptide translocates across the lipid bilayer of biological membranes remains largely unknown. As part of our own studies in this field, we have designed a peptide (peptide **2**, Table 1) that keeps the basic and aromatic motifs of the wild-type *Antennapedia* sequence and uses 8-amino-3,6-dioxaoctanoic acid as a flexible hydrophilic spacer. Fluorescence microscopy was used to examine if the new *Antennapedia*-derived peptide was internalized by cells in culture. Stock solutions of peptides **1** and **2** (Table 1) in Opti-MEM were freshly prepared and added to human U2OS osteosarcoma cells to achieve a final concentration of 50 μM. Figure 1 shows the phase contrast and fluorescent microscopic views of cells treated with the preceding peptides. Both peptides translocate well as shown by the fluorescence signal (right panels of Fig. 1), though the intensity for peptide **2** is slightly weaker than that for peptide **1**. Under identical experimental conditions, cells incubated with fluorescein alone did not provide any fluorescence signal (data not shown). It was also noted during this experiment that cells treated with peptide **1** started rounding up, suggesting a potential cytotoxic effect of peptide **1** at 50 μM. This effect was not observed for peptide **2** at the same concentration and it disappeared for peptide **1** at 10 μM (data not shown). Flow cytometry analysis was used to confirm and measure peptide uptake. As shown in Table 2, peptides **1** and **2** were equally internalized at 10 and 50 μM, but peptide **2** was less efficiently internalized as compared to peptide **1** at 1 μM. The fluorescence signal remained constant for up to 24 h post incubation.

After confirming that the new *Antennapedia*-derived peptide was internalized by cells, we evaluated if peptide

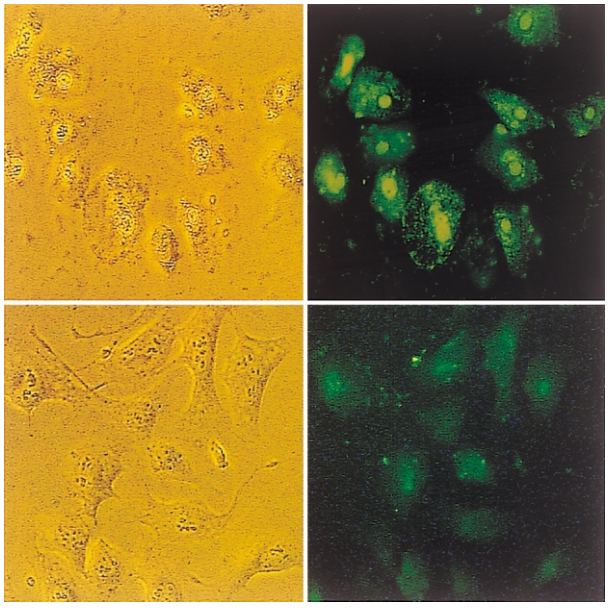

Figure 1. Visualization of the cellular uptake of peptides **1** and **2** in U2OS cells. U2OS cells were treated with 50 μM of peptides **1** and **2** for 10 h and visualized under a microscope. Left top: phase contrast, peptide **1**; right top: fluorescein detection, peptide **1**; left bottom: phase contrast, peptide **2**; and right bottom: fluorescein detection, peptide **2**.

2 was capable of carrying an exogenous compound across the cell membrane. In this study, the octapeptide Pro-Ala-Lys-Arg-Lys-Leu-Phe-Gly, which is derived from the cyclin A/cdk2 binding motif in p21-like cdk inhibitors,[6] was used as molecular cargo and synthesized in tandem with the *Antennapedia*-derived peptide (peptide **3**, Table 1). A strong fluorescence signal was observed in U2OS cells treated with peptide **3** (Fig. 2). Interestingly, the phase contrast figures indicated a change in cell morphology in the presence of peptide **3** (left panel of Fig. 2).[14] The reduced cell number and rounding up cell morphology suggested that the treated cells may undergo apoptosis. In order to confirm this observation, U2OS cells treated with 60 μM of peptides **2** and **3** were subjected to flow cytometric analysis for DNA content. Peptide **3** induced subdiploid DNA contents (26% at $c = 60\,\mu M$) in U2OS cells (Fig. 3B). In contrast, peptide **2**, which does not carry a cargo, caused ca. 3% of sub G1 fraction (Fig. 3A). This result correlates well with the cell lethal activity observed for HIV Tat and *Antennapedia* third α-helix cell membrane-permeable forms of similar peptides.[7]

Table 2. Uptake of peptides **1** and **2** in U2OS cells[a]

c (μM)	Peptide **1** (%)	Peptide **2** (%)
50	>99	>99
10	>99	>99
1	>99	80

[a]U2OS cells were treated with peptides **1** and **2** ($c = 50$, 10 and 1 μM) and analyzed by flow cytometry. The numbers indicate the percentage of FITC positive cells.

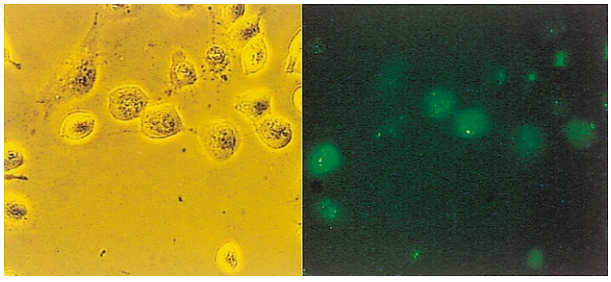

Figure 2. Visualization of the cellular uptake of peptide **3** in U2OS cells. U2OS cells were treated with 30 μM of peptide **3** for 24 h and visualized under a microscope. Left picture: phase contrast, peptide **3**; and right picture: fluorescein detection, peptide **3**.

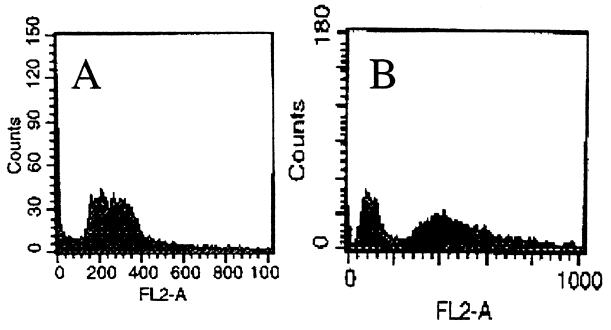

Figure 3. Flow cytometric analysis of U2OS cells treated with peptide (A) **2** and (B) **3**. The subdiploid DNA content observed in (B) confirms that cells undergo apoptosis in the presence of peptide **3**.

In summary, we have identified a new cell-membrane permeable sequence. Based on our data, the new *Antennapedia*-derived vector appears to be an efficient and nontoxic means to translocate peptides into cells. In agreement with previous studies,[4] this work also reveals that only a few of the structural traits present in the parent *Antennapedia* peptide are necessary and sufficient for efficient cell membrane translocation. This finding opens the possibility to identify alternative carrier moieties having an advantageous toxicity, immunogenicity, solubility or clearance profile.

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13. The purity of the peptides was verified by reversed-phase analytical HPLC on a Nucleosil C_{18} column (250×4.0 mm; 5 μM, 100 Å): linear gradient over 10 min of MeCN/0.09% TFA and H_2O/0.1% TFA from 1:49 to 1:0, flow rate 2.0 mL/min, detection at 215 nm; single peak at $t_R = 6.88$ min (peptide **2**); and $t_R = 6.73$ min (peptide **3**). Mass spectral analyses (matrix-assisted laser-desorption ionization time-of-flight mass spectrometry, MALDI-TOF) revealed molecular masses within 0.1% of the expected values (negative-ion mode): 2073.0 (calcd 2073.5; peptide **2**); and 2969.4 (calcd 2969.5; peptide **3**). Quantitative amino acid analyses of the purified peptides revealed the expected amino acid compositions.

14. Peptide **3** also induced cell morphology change in human colon adenoma cell line HCT-116 at 50 μM (data not shown).