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Kinetic uptake profiles of cell penetrating peptides in lymphocytes and monocytes

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ABSTRACT

Background: Nucleolar targeting peptides (NrTPs), resulting from structural minimization of the rattlesnake toxin crotamine, are a novel family of cell-penetrating peptides (CPPs) shown to internalize and deliver cargos into different cell types.
Methods: In this study, we address NrTP kinetics of translocation into primary cells. We used flow cytometry to measure the intracellular uptake of rhodamine B-labeled NrTPs in peripheral blood mononucleated cells (PBMCs).
Results: The kinetic profiles for each peptide are concentration-independent but significantly different among NrTPs, pointing out for the amino acid sequence importance. Arginine-containing peptides (NrTP7 and $Tat_{48–60}$, used for comparison) were found to be more toxic than lysine-containing ones, as expected. On the other hand, one same peptide behaves differently in each of the lymphocyte and monocyte cell populations, suggesting differences in entry mechanism that in turn reflect diversity in cell functionality. Uptake results obtained at 4 °C or using chemical endocytosis inhibitors support the importance of non-endocytic mechanisms in the cellular internalization of NrTP1 and NrTP5, while confirming endocytosis as the main mechanism of NrTPs entry.
Conclusion: Overall, both direct translocation and endocytosis mechanisms play a role in NrTP entry. Yet, there is predominance of endocytosis-mediated mechanisms. NrTPs (especially NrTP6) are an excellent intracellular delivery tool, with efficient internalization and no toxicity.
General significance: This work validates NrTPs as potential therapeutic tools for, e.g., cancer or inhibition of viral replication and establishes a new comparative and quantitative method to test CPP efficiency.

1. Introduction

Cell-penetrating peptides (CPPs) are receiving increasing attention as an innovative concept in therapeutics, for their role as delivery shuttles. They are able to deliver different molecular cargos (e.g., imaging agents, drugs, proteins, plasmid DNA, siRNA, nanoparticles or bacteriophages) both in vitro and in vivo [1,2]. The use of CPPs as drug carriers may overcome limitations such as poor drug bioavailability, low clinical efficacy [3], or undesirable side-effects. There is significant interest on these peptides in areas of application such as skin diseases (CPPs for topical application), myocardial infarction, ischemia, pain, splicing correction, HIV vaccines and several different cancers [4–6]. The first CPP-based drug reached phase II clinical trials in 2003 and, from then on, the number of peptides under trial has not stopped increasing [7,8].

Despite the widespread interest in CPPs, the mechanisms underlying their cellular translocation are poorly understood and subject to discussion. It is generally believed that there is not a universal translocation mechanism for all CPPs; instead, some use endocytosis-mediated mechanisms, others direct translocation by energy-independent mechanisms, while others may use both [9]. HIV-derived CPP $Tat_{48–60}$ is proposed to enter via direct translocation [10], while penetratin ($pAntp_{43–58}$), another well-studied CPP, was shown to use both endocytosis and direct translocation [9]. Other factors influencing CPP entry mechanisms are the nature and size of the cargo, as well as the type of target cell or tissue [11]. For example, when conjugated to large molecules Tat internalizes by endocytosis-mediated mechanisms [12].

The CPPs in this work belong to the family of nucleolar targeting peptides (NrTPs), designed by structural minimization of crotamine, a main component of the venom from the South-American rattlesnake *Crotalus durissus terrificus*. NrTPs were first reported in 2008 as capable of entering HeLa cells and localizing in their nucleolus [13]. The potential of NrTPs to interact with membranes was assessed in studies with lipid model systems [14], and their ability to deliver large cargos in native

Abbreviations: Ahx, 6-aminohexanoic acid; AMP, antimicrobial peptide; CPPs, cell penetrating peptides; HPLC, high-performance liquid chromatography; NrTPs, nucleolar-targeting peptides; PBMCs, peripheral blood mononucleated cells; RhB, rhodamine B; CPZ, chlorpromazine; FITC, fluorescein isothiocyanate; FSC, forward scatter; SSC, side scatter

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form was demonstrated with β-galactosidase, a 465 kDa tetrameric protein that retained enzymatic activity after both NrTP conjugation and cell internalization [15]. More recently, further insights into the uptake mechanism have been obtained from both confocal microscopy and flow cytometry studies [16].

In the present work, in order to further explore the potential of NrTPs as in vivo delivery agents, we have used human primary immune cells, specifically fresh peripheral blood mononuclear cells (PBMCs), as a model. PBMCs can be divided into two main populations: lymphocytes and monocytes, both involved in fighting infection and adapting to intruders, yet with completely different functions in the organism. Different subsets of lymphocytes perform a variety of functions within the immune system: helper T cells regulate other immune cells; cytotoxic T and NK cells lyse virus-infected cells, tumor cells and allografts; B cells secrete antibodies. Monocytes, for their part, are involved in phagocytosis, antigen presentation and cytokine production, and are the precursors of macrophages and dendritic cells [17]. PBMCs can either be directly involved in pathologies such as leukemia and HIV-1 infection, or play an important role in virtually any pathology, as part of the immune system. Studying PBMCs in terms of CPP uptake efficiency may help find applications for CPPs in immunology. To this end, we have developed an efficient method to detect and quantitatively compare differences in PBMC uptake among NrTPs that may reveal details of their translocation mechanism. Furthermore, we have studied the effect of amino acid residue substitutions in NrTP uptake efficiency and toxicity.

2. Material and methods

2.1. Materials

Ficoll-Paque Plus was from GE Healthcare. TO-PRO3, Hoechst 33342 and trypan blue were from Invitrogen. Annexin V-FITC conjugate was from BD Pharmingen, while dynasore and chlorpromazine were from Sigma.

2.2. Peptides

The solid phase synthesis of N-terminal rhodamine B (RhB)-labeled peptides NrTP1 (YKQCHKKGGKKGSG), NrTP2 (with a 6-aminohexanoic acid spacer between GG and KK), NrTP5 (enantiomer of NrTP1), NrTP6 (Cys residue replaced by Ser), NrTP7 (all 5 Lys residues changed to Arg) and NrTP8 (Tyr replaced by Trp) have been described earlier [13,18]. $Tat_{48–60}$ (GRKKRRQRRRPPQ-amide) was similarly synthesized by solid phase methods. All peptides were purified to >95% homogeneity by analytical HPLC and had the expected molecular masses. Sequences are shown in Table 1.

2.3. Isolation of PBMCs

Human blood samples were collected from healthy blood donors, with their previous written informed consent, following a protocol established with the Portuguese Blood Institute (Lisbon), approved by the Ethics Committee of the Faculty of Medicine of the University of Lisbon. Human PBMCs were isolated by density gradient using Ficoll-Paque Plus (GE Healthcare, Little Chalfont, UK). Briefly, the collected blood sample is diluted 1:1 (v/v) with PBS, pH 7.4, and carefully added to Ficoll-Paque, 1:0.6 (v/v), in order to avoid mixing. After centrifugation at 400 *g* for 40 min at 18–20 °C, the cloudy interphase between plasma and Ficoll-erythrocytes corresponds to the PBMCs layer. These cells are gently aspirated, washed with PBS and centrifuged at 100 *g* for 10 min. This step is repeated three times. In the last repeat, cells are resuspended in PBS supplemented with 2% fetal bovine serum (FBS). All reagents were used at room temperature. Experiments started immediately after PBMC isolation.

Table 1
NrTPs and reference CPP used in this study.

Peptide	Sequence[a]	Comments
NrTP1	YKQCHKKGGKKGSG	Derived from crotamine by N- to C-terminal
NrTP2	YKQCHKKGG-Ahx-KKGSG	endpiece splice, with or without Ahx spacer [13]
NrTP5	ykqchkkGGkkGsG	Enantiomer of NrTP1
NrTP6	YKQSHKKGGKKGSG	Cys in NrTP1 replaced by Ser
NrTP7	YRQSHRRGGRRGSG	All Lys in NrTP6 replaced by Arg
NrTP8	WKQSHKKGGKKGSG	Tyr in NrTP6 replaced by Trp
$Tat_{48–60}$	GRKKRRQRRRPPQ	broadly used CPP [10]

[a] Lower case letters denote D-amino acid residues. All sequences were labeled with rhodamine B (RhB) at the N-terminus.

2.4. Flow cytometry

Experiments were done in a BD FACSAria III, using a yellow-green (561 nm) laser to excite rhodamine B, which was detected using a 582/15 filter. 10,000 events were recorded on each measurement. Briefly, PBMCs suspensions were prepared at 2×10^6 cells/mL. Each peptide was added to the corresponding tube immediately after the $t = 0$ min reading. Peptide concentrations used in the kinetic assays were 2, 5 and 15 μM and measurements were made every 10 min. The cells were kept at 37 °C in a thermostatized bath during the entire experiment (2 h) as well as on the detection chamber of the cytometer. Tubes were gently flickered before and after the reading to ensure proper cell homogeneity. At least one control tube of unstained cells was tested per day of experiment. Each concentration of each peptide was tested at least three times. Peptide uptake at 4 °C was also followed for 2 h, with readings every 10 min, as described above.

Dose–response experiments were performed as end point measurements, with readings after 2 h incubation with peptide concentrations ranging from 1 to 20 μM at 37 °C. Measurements with endocytosis inhibitors were also done after 2 h incubation with 5 μM peptide. Chlorpromazine (CPZ) (5 or 50 μM) or dynasore (80 μM) were added to cells 30 min prior to peptide [16,19,20]. Dynasore assays were carried out in the absence of serum [19]. Specific controls were carried out for each independent experiment: cells only, and cells with CPZ (5 or 50 μM), dynasore (80 μM) or DMSO 2% (v/v; dynasore vehicle).

Following 2 h incubation with NrTPs and the corresponding reading, TO-PRO3 (viability dye) was added to the cell suspension to a final concentration of 0.25 μM [18]. After 10 min incubation with the probe, a new measurement was taken using the red (640 nm) laser to excite and the emission filter 670/14 to detect TO-PRO3 fluorescence. The influence of NrTPs on the viability of PBMCs was further studied using annexin V. Samples resulting from the 2 h incubation of 15 μM NrTPs with PBMCs were centrifuged at 150 *g* for 5 min in an Eppendorf miniSpin centrifuge. Supernatant was discarded and cells were resuspended in 100 μL of buffer with 0.1% annexin V and left to stain in the dark, for 15 min, at room temperature. Prior to analysis, samples were further diluted with 100 μL of annexin V buffer. Samples were measured in the same flow cytometer, using a blue (488 nm) laser to excite and a 530/30 filter to detect FITC fluorescence. All flow cytometer results were analyzed using FlowJo Software version 9. Events were gated to the population of lymphocytes and monocytes according to their forward scatter (FSC) and side scatter (SSC). Curve fitting and statistical analysis were done using GraphPad Prism.

2.5. Live cell microscopy

Microscopy experiments were carried out in a Zeiss LSM 510 META confocal point-scanning microscope (Jena, Germany). Diode 405–30 (405 nm; 50 mW), diode-pumped solid-state (DPSS; 561 nm; 15 mW) and HeNe633 (633 nm; 5 mW) lasers were used with a 63× oil-objective of 1.4 numerical aperture. Cells were incubated with NrTPs for 2 h prior to imaging. They were labeled with Hoechst (nuclear