Chem Biol Drug Des 2012; **79:** 907–915

Research Article

doi: 10.1111/j.1747-0285.2012.01377.x

Insights into the Uptake Mechanism of NrTP, A Cell-Penetrating Peptide Preferentially Targeting the Nucleolus of Tumour Cells

Gandhi Rádis-Baptista[1,2,*], Beatriz G. de la Torre[1] and David Andreu[1,*]

[1]*Department of Experimental and Health Sciences, Pompeu Fabra University, Barcelona Biomedical Research Park, Dr. Aiguader, 80, E-08003 Barcelona, Spain*

[2]*Institute for Marine Sciences, Federal University of Ceará, Av. da Abolição, 3207, 60165-081 Fortaleza-CE, Brazil*

**Corresponding authors: Gandhi Rádis-Baptista, gandhi.radis@ufc.br and David Andreu, david.andreu@upf.edu*

Nucleolar targeting peptides are 14–15 residue-long sequences designed by structural minimization of a snake toxin (J Med Chem 2008;50:7041). Peptides such as NrTP1 (YKQCHKKGGKK GSG) and analogues are capable of penetrating human cervix epithelial carcinoma cells and homing into their nucleoli. We now show that NrTP1 similarly penetrates and localizes in the nucleolus of tumour cells derived from human pancreatic (BxPC-3) and human ductal mammary gland (BT-474) carcinomas. Live cell confocal microscopy imaging, combined with flow cytometry analysis of cells arrested to defined phases of their cycle, confirms that NrTP1 uptake and nucleolar homing are independent of cell cycle phase. Peptide uptake is significantly reduced at low temperature. Also, drugs inhibiting chlatrin-mediated endocytosis severely decrease uptake, pointing to a clathrin-dependent route as the primary NrTP1 internalization mechanism. These results highlight nucleolar targeting peptides not only as a novel and efficient class of cell-penetrating peptides but also for their exceptional ability to target preferentially an essential and dynamic subnuclear structure such as the nucleolus.

Key words: cell-penetrating peptide, clathrin-mediated endocytosis, nucleolar localization sequence, theranostic peptide, tumour cell target

Abbreviations: CLSM, confocal laser scanning microscopy; CPPs, cell-penetrating peptides; DIPCDI, *N,N'*-diisopropylcarbodiimide; DMEM, Dulbecco's modified Eagle medium; EDT, ethanedithiol; FBS, foetal bovine serum; FI, fluorescein; MALDI-TOF MS, matrix-assisted laser desorption ionization, time-of-flight mass spectrometry; NrTP, nucleolar targeting peptide; PBS, phosphate-buffered saline; RhB, rhodamine B; TFA, trifluoroacetic acid; TIS, triisopropylsilane.

Received 9 September 2011, revised 1 December 2011 and accepted 25 February 2012

Since the unveiling of the mechanisms of protein trafficking, based on 'zip codes' or 'homing domains' (1), a substantial number of signal sequences capable of driving and delivering biomolecules, drugs and other cargos to specific subcellular compartments have been discovered, opening the way for significant diagnostic or therapeutic opportunities (2). The nucleus, in particular its amenability to genetic disorder correction through non-invasive and non-viral gene therapy, is regarded as an exceptionally desirable target for intracellular delivery systems (3). Several nuclear localization sequences (NLSs) able to transport cargos across the cell membrane and route them to the nucleus have been described and successfully exploited. Such NLSs are found not only in cellular proteins, usually translocated to the nucleus upon metabolic signalling (4), but also in viral proteins, where NLSs assist in the delivery of virus particles or genomes to the nucleus for viral replication (5). In all these situations, NLSs may be present as discrete monopartite or bipartite stretches of amino acid residues or domains along the sequence, as a patch in the protein three-dimensional structure, or even encrypted in the protein chain. In addition to drive a given protein to the nucleus, several NLSs have the intrinsic ability to penetrate into distinct cell types by transposing their cytoplasmic membranes. Well-known examples are residues 43–58 of the *Drosophila* antennapedia (Antp) homeodomain [Antp(43–58), also named penetratin] (6), or residues 47–57 (or 48–60) of the HIV-1 trans-acting transcriptional activator protein [Tat(47–57) or Tat(48–60)] (7), or residues 267–301 of structural protein VP22 of herpes simplex virus type I (8). In the last two decades, these protein-derived NLSs and other natural or synthetic sequences like designed model peptides (9) or oligomers (10) have been added to the fast-growing family of the so called cell-penetrating peptides (CPPs) (11). From a structural viewpoint, CPPs are broadly defined as short (<30 residues), cationic sequences (often though not necessarily amphipathic) that can be bound, covalently or not, to a cargo molecule (e.g. a drug) and achieve its fast trans-membrane internalization (11,12). Numerous reports have demonstrated, both *in vivo* and *in vitro*, the ability of CPPs to deliver assorted cargos such as drugs with poor bioavailability (13), imaging agents (14), biopolymers (nucleic acids, peptides/proteins, peptide nucleic acids) (15–17), nanoparticles or quantum dots (18) at specific cell types, organelles or subcellular structures, all of which has raised considerable medical and pharmaceutical interest (19,20). The mechanisms by which CPPs translocate the lipid bilayers and access the cell interior are diverse but essentially based on fluid phase endocytosis (21), as shown for Tat (22) and other CPPs (12). In the best-documented cases, the endocytic pathways involve clathrin- and caveolin-mediated coated pit and

vesicle endocytosis (23), receptor-independent/actin-dependent macropinocytosis (24), as well as other clathrin-and caveolin-independent mechanisms (25). On the other hand, the retrograde trans-Golgi network, that is, the pathway that allows some internalized molecules to reach the trans-Golgi network from early/recycling endosomes, is regarded as the main route of CPP intracellular traffic (26). Animal venoms are a rich source of bioactive compounds, including a handful of polypeptide families whose members are amenable to turn into drug leads (27). Although many structural templates have been described for peptide venoms, and an increasing number of sequences hitherto have been added to the CPP inventory, the number of animal toxins with membrane translocation and organelle-targeting abilities is rather limited. One of the first described is crotamine (28), a rattlesnake venom peptide of 42 amino acids, rich in Lys residues and folded by three disulphide bonds. Crotamine selectively translocates into cells at the G1/S phase of cell proliferation and localizes to the nucleus both *in vivo* and *in vitro*, after initial binding to heparan sulphate proteoglycans and internalization by endocytosis (29). In NMR solution studies, crotamine has been shown to adopt an $\alpha\beta\beta\beta$-fold, similar to that of the human antimicrobial peptide β-defensin 2, or to scorpion toxins targeting sodium channels (30). Our group has devoted some effort to structure-guided, minimalist downsizing of bioactive peptides (31), and the outcome of such expertise in the case of crotamine is a new group of CPPs characterized by the ability to target the nucleolus (32), hence their acronym nucleolar targeting peptides (NrTPs). Nucleolar targeting peptides result from covalently splicing the spatially close N-(residues 1–9) and C-terminal (residues 38–42) sections of crotamine (5.09 Å between the C^{α}s of Gly-9 and Lys-38). The resulting sequences, such as NrTP1 (YKQCHKKGGKKGSG) and analogues, contain a Lys-rich palindromic hexad (KKGGKK) and exhibit strong membrane-translocating properties, plus a particular ability to localize in the nucleolus (32). The nucleolus is known to be the most prominent functional structure in the eukaryotic nucleus. In addition to its conventional role in ribosomal RNA transcription and ribosome biogenesis, other nucleolar functions potentially amenable to therapeutic development include viral infection, regulation of oncogenesis and tumour suppression, stress sensing, control of ageing or modulation of telomerase activity (33), all of them related to the movement of nucleolar components, the sequestration of regulatory cell cycle-dependent proteins, and the transit of proteins that leave the nucleolus after their respective transactions (34). In this work, using NrTP1 as prototype, we address several important issues regarding the biological action of NrTPs, namely (i) their relative homing preference for several tumoral cell lines, (ii) their non-dependence on the cell cycle for efficient penetration and (iii) their mechanism of internalization, which appears to rely mainly on clathrin-mediated endocytosis.

Materials and Methods

Chemicals and peptides

Reagents used in the cell synchronization and uptake assays [amiloride, bafilomycin A1, chlorpromazine, heparin, Hoechst 33342, methyl-β-cyclodextrin, nocodazole, phenylarsine oxide, propidium iodide (PI) and thymidine] were purchased from Sigma-Aldrich (Madrid, Spain). Fmoc-protected amino acids were from Senn Chemicals (Dielsdorf, Switzerland). 5(6)-carboxyfluorescein and rhodamine B were from Fluka (Madrid, Spain). Solvents for peptide synthesis and HPLC were from SDS (Peypin, France). Peptides [NrTP1, its scrambled version NrTP1scr, and Tat(48–60), sequences in Table 1] were prepared by solid phase peptide synthesis methods (32) in a 433A synthesizer (Applied Biosystems, Foster City, CA, USA) running 0.1-mmol FastMoc protocols. The protected peptide resins were *N*-deblocked and labelled with 5(6)-carboxyfluorescein or rhodamine B, in the presence of DIPCI (10× molar excess of all reagents) in dichloromethane to produce fluorescent derivatives. Final deprotection and cleavage were performed with TFA/H_2O/EDT/TIS (94:2.5:2.5:1 v/v, 90 min, rt) for Cys-containing NrTP1 and NrTP1scr, and with TFA/H_2O/TIS (95:2.5:2.5 v/v, 90 min, rt) for Tat (48–60). Peptides were isolated from the cleavage mixture by precipitation with cold ether and centrifugation, redissolved in water and lyophilized and then purified by RP-HPLC to at least 95% purity. Their identity was confirmed by MALDI-TOF MS. Further details are given in Table 1.

Cell lineages and culture

Human cervix epithelial carcinoma (HeLa), pancreatic adenocarcinoma (BxPC-3), ductal mammary gland carcinoma (BT-474) and murine neuroblastome (N2A) cells were from the Cancer Cell Line Repository of the Institute Municipal d'Investigació Mèdica (Barcelona Biomedical Research Park), Barcelona, Spain. Cells were cultured in flasks (75 cm^2) or poly-L-lysine-coated circular glass slides (2.5 mm diameter) at 37 °C and 5% CO_2 in DMEM/F-12, a maintenance medium consisting of a 1:1 mixture of Dulbecco's modified Eagle medium and F-12 supplemented with 10% (v/v) of heat-inactivated foetal bovine serum (FBS), 2 mM L-glutamine, 10 U/mL penicillin and 100 μg/mL streptomycin. Cells were kept at 60–70% confluence to avoid quiescence by contact inhibition, particularly before manipulations for cell cycle synchronization and cell uptake experiments. Enrichment of HeLa, BT-474 and BxPC-3 cells in their G0 phase populations was achieved by serum deprivation and maintenance in DMEM/F-12 without FBS for 72 h. Synchronization at the G1/S phase was achieved by a double-thymidine treatment (35), namely incubation for 12 h in DMEM/F-12 containing 2 mM thymidine, followed by three PBS washes, incubation for additional 12 h in DMEM/F-12, and by another 12 h in DMEM/F-12 again supplemented with 2 mM thymidine. Synchronization at the G2/M phase was done by supplementing DMEM/F-12 with 60 ng/mL nocodazole and a 16-h incubation period. Prior to the cell uptake experiments, cells (asynchronously replicating or G0, G1/S and G2/M-synchronized) were washed with PBS and incubated for 1 h in DMEM/F-12 (without phenol red) containing 50 μM fluorescently labelled NrTP1.

Table 1: NrTP analogues and control peptides used in this work

Peptide[a]	Amino acid sequence	[M+H+](expected/found)	Purity %[b]
Fl-NrTP1	YKQCHKKGGKKGSG	1863.11/1863.62	98
RhB-NrTP1		1930.02/1930.34	96
Fl-NrTPscr	QKYKSHGKGKSKGG	1848.14/1848.06	98
Fl-Tat	GRKKRRQRRRPPQ	2076.39/2076.27	97

[a]All peptides N-terminally labeled with either fluorescein (Fl) or rhodamine B (RhB).
[b]As determined by RP-HPLC.

Laser confocal scanning microscopy

Laser confocal scanning microscopy (LCSM) imaging was performed with a Leica (Leica Microsystems, Wetzlar, Germany) TCS SP2 adapted to an inverted Leica DM IRBE microscope with a PeCon incubator system for live cell imaging. 25-mm coverslips were mounted in an Attofluor cell chamber (Life Technologies SA, Alcobendas, Spain) and placed into the microscope incubator at 37 °C and 5% CO_2. Images were taken with a 40× (NA 1.25, oil) Leica Plan Apochromatic objective. Fluorescein (Fl) and rhodamine B were excited with the 488-nm line of an Ar laser and a 543-nm He–Ne laser, with emission recorded between 495 and 540 and between 555 and 610 nm, respectively. Phase contrast images were acquired along with fluorescence using the same laser excitation and a photomultiplier for transmitted light. Time-lapse experiments were performed with a maximum length of 1 h and a time interval of 30 seconds. Also, selected cells in the same coverslip were zoomed in to get a Z-stack of the cellular volume (z-step between 1 and 3 μm). Images were 512×512 pixels and were acquired at 400 Hz.

Multi-parametric flow cytometry

For multi-parametric flow cytometry (FC) analysis, NrTP1-incubated HeLa cells were washed with PBS and treated with 500 μL of 0.5% trypsin-EDTA solution for 10 min at 25 °C to release adherent cells and to remove cell surface-bound peptide. Trypsin treatment was halted by addition of 5 mL of maintenance media supplemented with 10% FBS, and cells were collected by centrifugation ($1200 \times g$, 5 min, 4 °C), washed and re-suspended in PBS to a final concentration of 10^6 cells/mL. Then, each cell suspension was treated with Hoechst 33342 (1 μg/mL) or PI (1 μg/mL) to stain either the nucleus or dead cells, respectively, and kept on ice until analysis (within 1 h). A total of 10 000 events were recorded on a LSR FACS instrument (BD Biosciences, Franklin Lakes, NJ, USA). Percentages of Hoechst 33342-and NrTP-labelled cells in the G0, G1/S and G2/M, as well the change in the median fluorescence of samples (asynchronous or enriched in one cell cycle phase) relative to their untreated controls, were determined by means of the CellQuest software (BD Biosciences). All measurements were conducted in triplicate and results expressed as the change of median fluorescence or, alternatively, as the number of cells in each cell cycle phase.

Cell uptake inhibition studies

Exponentially growing subconfluent HeLa cells were cultured in DMEM/F-12 in 75-cm^2 flasks. The medium was replaced with serum-free medium, and the cells were separately treated with 2.5 mM amiloride for 10 min, 2.5 mM methyl-β-cyclodextrin for 10 min, 50 nM bafilomycin A1 for 30 min, 50 μM chlorpromazine for 10 min, 250 μg/mL heparin (avg MW 3000) for 45 min, 3 μM phenylarsine oxide for 10 min. With the exception of heparin, all inhibitors used in the pretreatment step were removed by washing the cells twice with PBS. The pretreated cells were then incubated for 1 h with 50 μM of Fl-labelled NrTP1 in serum-free medium. Next, the peptide-containing medium was aspirated and the cells washed again twice with PBS and then treated with 500 μL of 0.25% trypsin-EDTA solution for 10 min at RT to remove membrane-bound peptides and to detach cells from the flasks. Trypsin was inactivated by addition of maintenance medium. Finally, cells ($\sim 10^6$) were collected by centrifugation ($300 \times g$, 4 °C, for 5 min), resuspended in 1 mL of cold PBS and kept at 4 °C for the duration of FC measurements (about 1 h). For uptake experiments at low temperature, subconfluent HeLa cells cultures were treated with 15 and 50 μM of Fl-labelled NrTP1 in serum-free medium at 4 °C for 60 min. Thereafter, cells were washed, treated with trypsin at RT for 10 min and handled as above.

Results and Discussion

NrTP1 compartmentalizes in the nucleolus of tumour cell lines other than HeLa cells

Our previous work (32), based exclusively on HeLa cells, established the efficient uptake of NrTP1 and its specific localization at the nucleolus. Tumour cells, however, are well known for presenting a variety of phenotypes resulting from differential expression of several classes of molecules including, for example, heparan sulphate proteoglycans that in turn may affect intracellular signalling/response (36), they may also experience altered nuclear traffic of transcription factors as well as other changes in nuclear architecture (37). Thus, it seemed important to confirm that cell type did not bias the intrinsic ability of NrTP1 for membrane translocation and nucleolar compartmentalization. To this end, two additional human tumour cell lines derived from aggressive types of cancer were tested, namely human pancreatic adenocarcinoma (BxPC-3) and ductal mammary gland carcinoma (BT-474). Asynchronously replicating cells from these tumour lines were treated with 50 μM NrTP1 and examined by LCSM, which for BxPC-3 (Figure 1A–C) and BT—474 (Figure 1D,E) showed membrane translocation and nucleolar homing abilities very similar to those found earlier for HeLa. In contrast, murine neuroblastoma (N2A) cells, though partially penetrated by NrTP1, showed a much less intense buildup of peptide at the nucleus (practically negligible), with the peptide accumulated in the membrane and the uptake confined to endocytic structures (Figure 1F–I). To account for the preferential uptake observed for HeLa, BxPC-3 and BT-474 cells, one may assume that certain cell membrane and/or nucleolar components acting as NrTP1 targets are overexpressed in such tumour cells. We are examining such hypothesis by means of affinity capture proteomics experiments currently under way in our laboratory.

NrTP1 uptake and compartmentalization proceed independently of cell cycle

As mentioned, several cellular properties might influence the uptake efficiency and intracellular traffic of CPPs. For instance, structural and compositional changes of membrane glycosaminoglycans (GAGs) and proteoglycans associate with the cell cycle phase (38) or tissue type (39) have been reported. In fact, GAGs were demonstrated to play a role in the internalization of oligoarginine peptides (40), being the uptake efficiency and intracellular traffic of other CPPs, with or without associated cargos, influenced by the cell type (41). Crotamine, the rattlesnake venom peptide from which NrTPs are derived, binds to GAGs on cell membrane, penetrates proliferating active cells and compartmentalizes into their nucleus during the G1/S phase of cell cycle, by interacting with metaphase chromo-

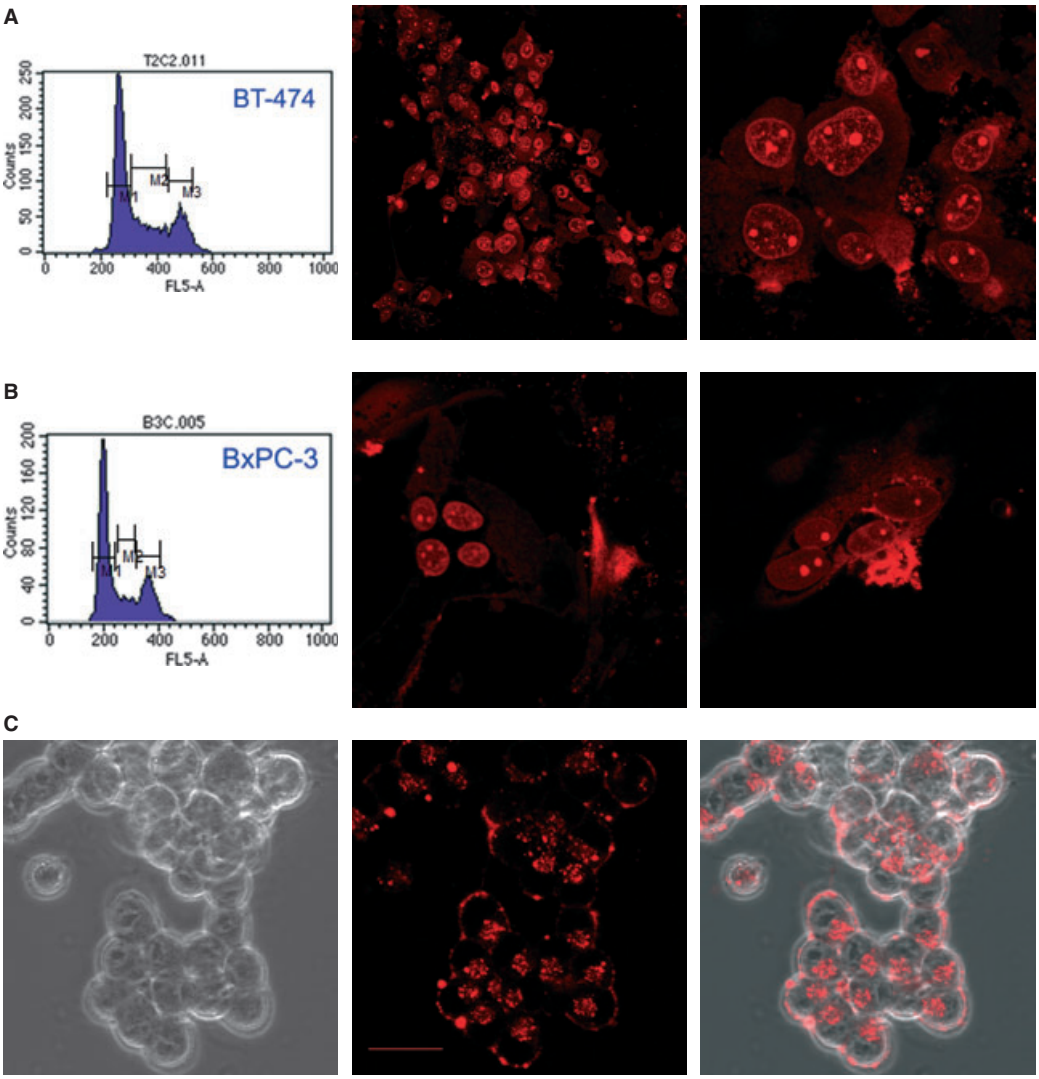

Figure 1: NrTP1 penetrates and targets the nucleolus of asynchronously replicating tumour cell lines. (A) human ductal mammary gland carcinoma (BT-474) cells, left: FC of asynchronously growing cells after incubation with 50 μM RhB-NrTP1 for 45 min, right: live cell confocal laser scanning microscopy (CLSM) showing NrTP1 penetration and accumulation in the nucleolus, bar = 20 μm. (B) as above for human pancreatic adenocarcinoma (Bx-PC3) cells. (C) murine neuroblastome (N2A) cells showing membrane-bind peptide and a punctuate pattern of endocytic accumulation of RhB-NrTP, which is distinct of the human tumour-derived cell lines (c_1, phase contrast; c_2, confocal fluorescence; c_3, merge).

somes in G2/M (28,29). Moreover, the accumulation of certain viral proteins in the nucleolus is also related to the dependence of cell cycle (42). This background prompted us to investigate whether the uptake and nucleolar localization of NrTP1 might be related to cell cycle phase in any similar fashion. To this end, exponentially growing HeLa and BT-474 cell lines were cultured either under conventional asynchronous replicating conditions or arrested at specific stages of the cell cycle. For asynchronous HeLa cultures (Figure 2A–C), FC analysis showed that NrTP1-internalizing cells were distributed among the G0 (*c.* 61% of total population), the S (21%) and the G2/M (18%) phases. Similar trends were observed for asynchronous BT-474 (58%, 23% and 18% for G0, S and G2/M phases, respectively) (Figure 2D–F) and BxPC-3 (75%, 16% and 9%, respectively) cultures. Peptide uptake was next evaluated for HeLa cultures enriched at different cell cycle phases. When replication was arrested at G0 by serum deprivation, NrTP1 uptake was largely concentrated (93%) on the cell population at this G0 phase, with only 4% of peptide-internalizing cells in the S, and 3% in the G2/M phase. For cells under double-thymidine treatment, arrested at the S phase, uptake grew again appreciably (25%) for populations at this stage. Finally, cultures arrested at the G2/M phase by nocodazole treatment showed again increased (48%) uptake for cells at that phase. By analysing these results, one can observe that NrTP1 uptake is roughly proportional to the size of cell population at one given phase of the cell cycle. Indeed, HeLa and BT-474 (Figure 2), as well as for BxPC-3 cells (not shown), the proportion of fluorescently labelled cells because of NrTP1 internalization is the same as the number of cells synchronized at determined cell cycle phase, as quantitatively measured by FC. Moreover, confocal laser scanning microscopy (CLSM) complemented FC data by showing

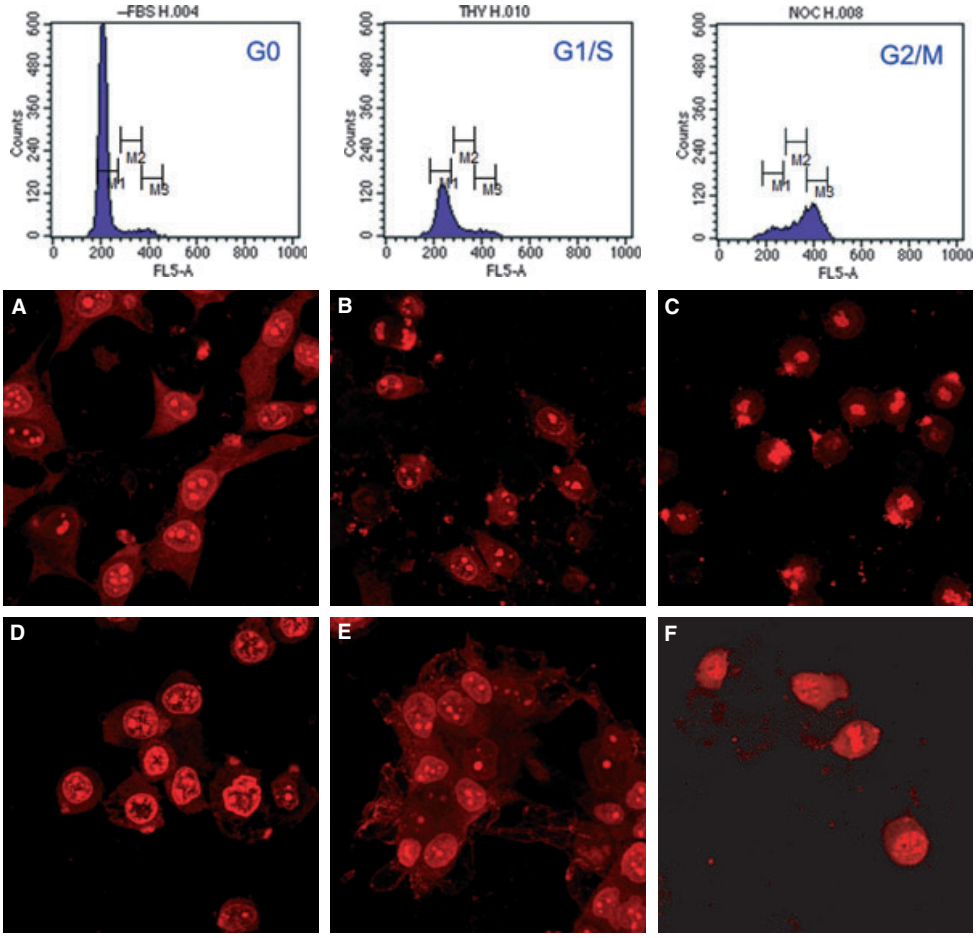

Figure 2: NrTP1 targets the nucleolus of tumour cells independently of cell cycle phase. Top: Sequential FC plots monitoring cell cycle phase enrichment. Bottom, (A–C) confocal laser scanning microscopy (CLSM) of live HeLa cells enriched in the G0, G1/S and G2/M phases of cell cycle, respectively, and incubated with 50 μM RhB-NrTP1 for 45 min. (D–F) CLSM of human ductal mammary gland carcinoma cells (BT-474) treated as above. In image D (cells undergoing mitosis), the nucleoli are disassembled and NrTP1 interacts with metaphasic chromosomes and/or a chromatin component that allows labelling by the peptide.

NrTP1 as having crossed the membrane and localized to the nucleolus of cells arrested at G0 and G1/S phases (Figure 2A,B,D,E). In cells at the G2/M phase, where the nuclear membrane is disrupted and the nucleolus is disassembled, NrTP1 seems to bind somehow to the mitotic chromosome or to other unidentified chromosomal component (Figure 2C,F). These results reveal a mimetic behaviour of NrTP1 in relation to the parental native crotamine, except by the fact that upon internalization NrTP1 localizes preferentially to the nucleoli of tumour cells. Interestingly, some viral proteins containing nucleolar localization sequences are known to gain access to the nucleolus with the property of subvert the cell cycle for the benefit of viral replication (42,43). For NrTP1, however, cell entry and nucleolar targeting show to proceed independent of the cell cycle phase, in at least three cell lines examined.

NrTP1 uptake is concentration-dependent and mediated mainly by endocytosis

To investigate the concentration- and sequence-dependence, as well as the mechanism of NrTP1 uptake, we again resorted to FC analysis. NrTP1 uptake by HeLa cells was shown to be concentration-dependent in the 5–50 μM range (Figure 3A). Although NrTP1 penetrates efficiently into cultured mammalian cells, it was not cytotoxic, as shown by the negligible increase, if any, in the fraction of dead (PI-labelled) cells upon exposure to NrTP1 (Figure 3B,C), in agreement with our earlier MTT assay (32) data. To explore the primary structure requirements for NrTP1 uptake, a sequence-scrambled version, NrTPscr (Table 1), was made and shown to internalize *c.* 80% less efficiently than the parent peptide (Figure 4), pointing to a considerable degree of sequence specificity in the uptake events. The two basic triads (KKG, GKK) at residues 6–11 bear resemblance to some described NLSs (44) and confer NrTP1 a partially palindromic nature, lost in NrTP1scr but preserved in the retro version, earlier shown to internalize like NrTP1 though at higher concentrations (32). Substantial evidence points to fluid phase endocytosis as the main pathway for CPP uptake. Several endocytic mechanisms have been described, including macropinocytosis, caveolin- and clathrin-mediated uptake, as well as other clathrin- and caveolin-independent mechanisms (21,45). In virtually all these pathways membrane fluidity plays a key role, which becomes

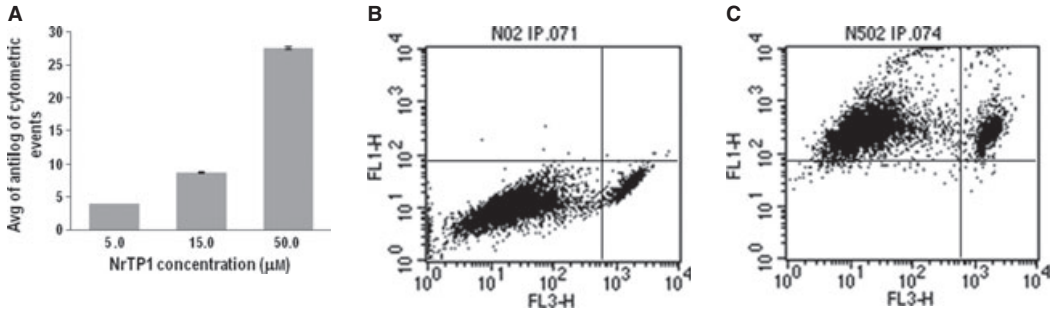

Figure 3: Flow cytometry analysis of Fl-NrTP1 uptake by HeLa cells. (A) Concentration dependence of cell uptake. In panels B and C, channels FL1-H and FL3-H detect green (Fl) and red (propidium iodide, PI) fluorescence, respectively. (B) Control HeLa cells incubated in uptake solution without NrTP1: lower left and lower right quadrants correspond to live and dead (PI-uptaking) cells, respectively. (C) Cells uptaking Fl-NrTP1 (50 μM, plus 1 μg/mL PI) with green (upper left quadrant) and red (upper right quadrant) detection.

significantly hampered at low temperature (46), even if endocytic vesicle formation is not fully impaired (47). In our case, for live Hela cells at 4 °C, NrTP1 uptake was likewise reduced by three- and fivefold, respectively, at 15 and 50 μM (Figure 4). Tat(48–60), used at 15 μM as control, underwent a similar fourfold decrease at 4 °C, whereas for NrTP1scr, low temperature caused a twofold reduction in uptake at 50 μM, but not at 15 μM. Because we noticed from previous works that CPP-to-cell ratio, rather absolute CPP concentration, influences the efficiency on peptide cell uptake (48,49), we used a non-toxic NrTP1 concentration of 50 μM for consistent interpolation of all cell uptake inhibition experiments.

An early step in the internalization mechanism of several CPPs is strong electrostatic binding to anionic glycosaminoglycans/proteoglycans on the cell surface, followed by endocytosis (50). To ascertain whether such model applied to the present case, soluble low molecular weight heparin (LMWH) was added to the uptake solution as a competitor of NrTP1 for GAG binding. At concentrations as high as 250 μg/mL, LMWH inhibited NrTP1 internalization by only *c.* 20% (Figure 5), in contrast to native crotamine (29), Tat protein (51) or Arg-rich peptides (16,40), which are highly dependent of GAGs for internalization. GAG-independent uptake routes have nonetheless been described for Tat(48–60) (24,52), as well as for other CPPs. Having discarded the importance of GAG interaction as a key step for NrTP1 internalization, we explored other plausible entry mechanisms using HeLa cells and specific pharmacological inhibitors of five well-established internalization routes. Amiloride specifically blocks the Na^+/H^+ exchange required for macropinocytosis, a receptor-independent, lipid raft-dependent endocytotic event (53). In our system, at 2.5 mM amiloride did not interfere signifi-

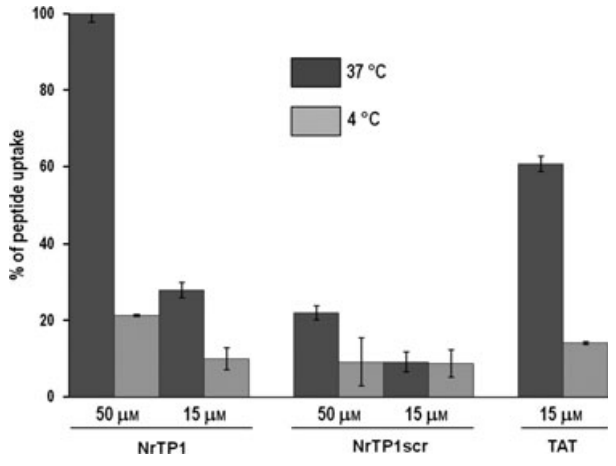

Figure 4: Effect of low temperature on NrTP1 uptake. HeLa cells incubated for 45 min with 50 or 15 μM Fl-NrTP1, Fl-NrtP1scr and Fl-Tat(48–60), at either 37 or 4 °C, were trypsinized and analysed by FC. For clarity, the average antilog of cytometric events was converted to peptide uptake, with 50 μM Fl-NrTP1 uptake taken as 100%.

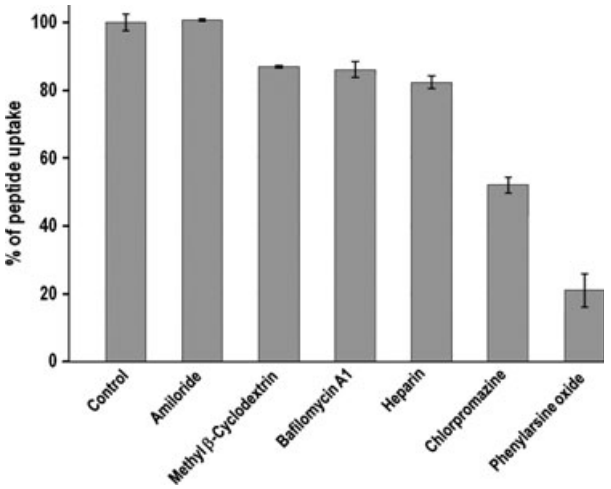

Figure 5: Effect of endocytosis inhibitors on NrTP1 uptake. HeLa cells were pretreated for 10 min with 2.5 mM amiloride, 2.5 mM methyl β-cyclodextrin, 3 μM phenylarsine and 50 μM chlorpromazine, or for 30 min with 50 nM bafilomycin and, after inhibitor removal, incubated for 45 min with 50 μM Fl-NrTP1. Heparin (250 μg/mL) was coincubated with the peptide. Cells were harvested after trypsinization and analysed by FC. Decrease in peptide uptake corresponds to reduction in fluorescence relative to HeLa cells incubated with 50 μM Fl-NrTP1 alone.

cantly with NrTP1 uptake (Figure 5). Methyl β-cyclodextrin, known to deplete cholesterol from raft microdomains and thus disrupt macropinocytosis and caveolae-based pathways of Tat penetration (24), caused only a 13% decrease on NrTP1 uptake at a non-cytotoxic concentration of 2.5 mM. Indeed, these two results contrast with those found for Tat, which enters T cells by macropinocytosis and whose uptake is therefore drastically inhibited by amiloride and β-cyclodextrin (24). Bafilomycin A1 is a specific inhibitor of vacuolar H^+-ATPase, an enzyme responsible for an endosome internal pH decrease that triggers receptor–ligand dissociation after receptor-mediated endocytosis (54). Pretreatment of HeLa cells with this inhibitor at 50 nM had again little effect (only 14% decrease) on the internalization rate of NrTP1 (Figure 5). We finally tested two compounds that interfere with the function of clathrin, the protein that forms coated vesicles in one of the receptor-mediated endocytic pathways (45). Chlorpromazine inhibits this process by causing loss of clathrin-coated pits from the cell surface (55), while phenylarsine oxide prevents clathrin-mediated endocytosis by cross-linking vicinal thiols (54). Treatment of HeLa cells with these inhibitors at 50 and 3 μM, respectively, had a drastic inhibitory effect, with a decrease in uptake of 48% and 79%, respectively (Figure 5). Taken together, the above results strongly suggest that NrTP1 is primarily internalized via a putative receptor or adaptor molecule, through an endocytic pathway dependent on formation of clathrin-coated vesicles, possibly with no need for direct interaction with plasma membrane GAGs prior to endocytosis.

Conclusions

On the face of the above results, NrTP1 appears as a valuable addition to transduction technology, particularly for its remarkable nucleolus-homing ability, which may be harnessed for the specific and precise delivery of diverse cargos, from imaging agents to functional molecules. For instance, NrTP1 or its analogues might find an application as markers of subnuclear organelles in cell biology, already partially addressed by other CPPs (15), or for tailored targeting of gene activators or down-regulators to the nucleolus (56), or for controlled protein compartmentalization as a tool to overcome diseases caused by mislocalized proteins (2). Although a significant number of protein homing domains or 'zip codes', driving proteins to particular subcellular compartments, are already known (2), identification of nucleolus-homing motifs is less obvious, as they are often encrypted into NLSs. One may easily envisage a window of theranostic opportunity for sequences such as NrTP and other congeners (2), engineered to fulfil Paul Ehrlich's goal of 'drugs that go straight to their intended cell-structural targets' (57).

Acknowledgments

Research supported by the Spanish Ministry of Science and Innovation (BIO2008-04487-CO3-02 to D.A.), by the European Union (HEALTH-F3-2008-223414) and by Generalitat de Catalunya (SGR2009-00492). We thank Drs. Xavier Sanjuan and Oscar Fornas (Scientific-Technical Services, Pompeu Fabra University) for expert assistance in confocal microscopy and flow cytometry experiments, respectively, Dr. Xavier Mayol, manager of the Cancer Cell Line Repository of the Institute Municipal d'Investigació Mèdica for kindly supplying cell lines, and Yolanda Tor for help in cell culture. G.R.-B. thanks to The National Council for the Scientific and Technological Development (CNPq), Ministry of Science and Technology, Federal Government of Brazil and Universitat Pompeu Fabra for financial support.

References

1. Blobel G. (2000) Protein targeting (Nobel lecture). ChemBioChem;1:86–102.
2. Davis J.R., Kakar M., Lim C.S. (2007) Controlling protein compartmentalization to overcome disease. Pharm Res;24:17–27.
3. Pouton C.W., Wagstaff K.M., Roth D.M., Moseley G.W., Jans D.A. (2007) Targeted delivery to the nucleus. Adv Drug Deliv Rev;59:698–717.
4. Tkach J.M., Glover J.R. (2008) Nucleocytoplasmic trafficking of the molecular chaperone Hsp104 in unstressed and heat-shocked cells. Traffic;9:39–56.
5. Wu W.W., Pante N. (2009) The directionality of the nuclear transport of the influenza A genome is driven by selective exposure of nuclear localization sequences on nucleoprotein. Virol J;6:68.
6. Derossi D., Joliot A.H., Chassaing G., Prochiantz A. (1994) The third helix of the Antennapedia homeodomain translocates through biological membranes. J Biol Chem;269:10444–10450.
7. Vives E., Brodin P., Lebleu B. (1997) A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. J Biol Chem;272:16010–16017.
8. Elliott G., O'Hare P. (1997) Intercellular trafficking and protein delivery by a herpesvirus structural protein. Cell;88:223–233.
9. Pooga M., Hallbrink M., Zorko M., Langel U. (1998) Cell penetration by transportan. FASEB J;12:67–77.
10. Goun E.A., Pillow T.H., Jones L.R., Rothbard J.B., Wender P.A. (2006) Molecular transporters: synthesis of oligoguanidinium transporters and their application to drug delivery and real-time imaging. ChemBioChem;7:1497–1515.
11. Zorko M., Langel U. (2005) Cell-penetrating peptides: mechanism and kinetics of cargo delivery. Adv Drug Deliv Rev;57:529–545.
12. Heitz F., Morris M.C., Divita G. (2009) Twenty years of cell-penetrating peptides: from molecular mechanisms to therapeutics. Br J Pharmacol;157:195–206.
13. Morris M.C., Deshayes S., Heitz F., Divita G. (2008) Cell-penetrating peptides: from molecular mechanisms to therapeutics. Biol Cell;100:201–217.
14. Kersemans V., Kersemans K., Cornelissen B. (2008) Cell penetrating peptides for *in vivo* molecular imaging applications. Curr Pharm Des;14:2415–2447.
15. Martin M., Rice K. (2007) Peptide-guided gene delivery. AAPS J;9:E18–E29.
16. El-Andaloussi S., Johansson H.J., Holm T., Langel U. (2007) A novel cell-penetrating peptide, M918, for efficient delivery of proteins and peptide nucleic acids. Mol Ther;15:1820–1826.
17. Segura J., Fillat C., Andreu D., Llop J., Millan O., de la Torre B.G., Nikolovski Z., Gomez V., Andreu N., Pinyot A., Castelo R.,

Gispert J.D., Pascual J.A. (2007) Monitoring gene therapy by external imaging of mRNA: pilot study on murine erythropoietin. Ther Drug Monit;29:612–618.
18. Zhou M., Ghosh I. (2007) Quantum dots and peptides: a bright future together. Biopolymers;88:325–339.
19. Stewart K.M., Horton K.L., Kelley S.O. (2008) Cell-penetrating peptides as delivery vehicles for biology and medicine. Org Biomol Chem;6:2242–2255.
20. Deshayes S., Morris M., Heitz F., Divita G. (2008) Delivery of proteins and nucleic acids using a non-covalent peptide-based strategy. Adv Drug Deliv Rev;60:537–547.
21. Doherty G.J., McMahon H.T. (2009) Mechanisms of endocytosis. Annu Rev Biochem;78:857–902.
22. Gump J.M., Dowdy S.F. (2007) TAT transduction: the molecular mechanism and therapeutic prospects. Trends Mol Med;13:443–448.
23. Vendeville A., Rayne F., Bonhoure A., Bettache N., Montcourrier P., Beaumelle B. (2004) HIV-1 Tat enters T cells using coated pits before translocating from acidified endosomes and eliciting biological responses. Mol Biol Cell;15:2347–2360.
24. Wadia J.S., Stan R.V., Dowdy S.F. (2004) Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. Nat Med;10:310–315.
25. Jones S.W., Christison R., Bundell K., Voyce C.J., Brockbank S.M., Newham P., Lindsay M.A. (2005) Characterisation of cell-penetrating peptide-mediated peptide delivery. Br J Pharmacol;145:1093–1102.
26. Fischer R., Kohler K., Fotin-Mleczek M., Brock R. (2004) A stepwise dissection of the intracellular fate of cationic cell-penetrating peptides. J Biol Chem;279:12625–12635.
27. Hodgson W.C., Isbister G.K. (2009) The application of toxins and venoms to cardiovascular drug discovery. Curr Opin Pharmacol;9:173–176.
28. Kerkis A., Kerkis I., Radis-Baptista G., Oliveira E.B., Vianna-Morgante A.M., Pereira L.V., Yamane T. (2004) Crotamine is a novel cell-penetrating protein from the venom of rattlesnake Crotalus durissus terrificus. FASEB J;18:1407–1409.
29. Nascimento F.D., Hayashi M.A., Kerkis A., Oliveira V., Oliveira E.B., Radis-Baptista G., Nader H.B., Yamane T., Tersariol I.L., Kerkis I. (2007) Crotamine mediates gene delivery into cells through the binding to heparan sulfate proteoglycans. J Biol Chem;282:21349–21360.
30. Fadel V., Bettendorff P., Herrmann T., de Azevedo W.F. Jr, Oliveira E.B., Yamane T., Wuthrich K. (2005) Automated NMR structure determination and disulfide bond identification of the myotoxin crotamine from Crotalus durissus terrificus. Toxicon;46:759–767.
31. Vila-Perelló M., Tognon S., Sánchez-Vallet A., García-Olmedo F., Molina A., Andreu D. (2006) A minimalist design approach to antimicrobial agents based on a thionin template. J Med Chem;49:448–451.
32. Radis-Baptista G., de la Torre B.G., Andreu D. (2008) A novel cell-penetrating peptide sequence derived by structural minimization of a snake toxin exhibits preferential nucleolar localization. J Med Chem;51:7041–7044.
33. Shaw P., Doonan J. (2005) The nucleolus. Playing by different rules? Cell Cycle;4:102–105.
34. Emmott E., Hiscox J.A. (2009) Nucleolar targeting: the hub of the matter. EMBO Rep;10:231–238.
35. Harper J.V. (2005) Synchronization of cell populations in G1/S and G2/M phases of the cell cycle. Methods Mol Biol;296:157–166.
36. Su G., Meyer K., Nandini C.D., Qiao D., Salamat S., Friedl A. (2006) Glypican-1 Is frequently overexpressed in human gliomas and enhances FGF-2 signaling in glioma cells. Am J Pathol;168:2014–2026.
37. Meyers S., Hiebert S.W. (2000) Alterations in subnuclear trafficking of nuclear regulatory factors in acute leukemia. J Cell Biochem Suppl;35:93–98.
38. Breton M., Berrou E., Brahimi-Horn M.C., Deudon E., Picard J. (1986) Synthesis of sulfated proteoglycans throughout the cell cycle in smooth muscle cells from pig aorta. Exp Cell Res;166:416–426.
39. Skandalis S.S., Stylianou M., Vynios D.H., Papageorgakopoulou N., Theocharis D.A. (2007) The structural and compositional changes of glycosaminoglycans are closely associated with tissue type in human laryngeal cancer. Biochimie;89:1573–1580.
40. Kosuge M., Takeuchi T., Nakase I., Jones A.T., Futaki S. (2008) Cellular internalization and distribution of arginine-rich peptides as a function of extracellular peptide concentration, serum, and plasma membrane associated proteoglycans. Bioconjug Chem;19:656–664.
41. Mueller J., Kretzschmar I., Volkmer R., Boisguerin P. (2008) Comparison of cellular uptake using 22 CPPs in 4 different cell lines. Bioconjug Chem;19:2363–2374.
42. Cawood R., Harrison S.M., Dove B.K., Reed M.L., Hiscox J.A. (2007) Cell cycle dependent nucleolar localization of the coronavirus nucleocapsid protein. Cell Cycle;6:863–867.
43. Reed M.L., Dove B.K., Jackson R.M., Collins R., Brooks G., Hiscox J.A. (2006) Delineation and modelling of a nucleolar retention signal in the coronavirus nucleocapsid protein. Traffic;7:833–848.
44. Lusk C.P., Blobel G., King M.C. (2007) Highway to the inner nuclear membrane: rules for the road. Nat Rev Mol Cell Biol;8:414–420.
45. Conner S.D., Schmid S.L. (2003) Regulated portals of entry into the cell. Nature;422:37–44.
46. Wadia J.S., Dowdy S.F. (2002) Protein transduction technology. Curr Opin Biotechnol;13:52–56.
47. Illinger D., Poindron P., Kuhry J.G. (1991) Fluid phase endocytosis investigated by fluorescence with trimethylamino-diphenylhexatriene in L929 cells, the influence of temperature and of cytoskeleton depolymerizing drugs. Biol Cell;73:131–138.
48. Hällbrink M., Oehlke J., Papsdorf G., Bienert M. (2004) Uptake of cell-penetrating peptides is dependent on peptide-to-cell ratio rather than on peptide concentration. Biochim Biophys Acta;1667:222–228.
49. Rodrigues M., de la Torre B.G., Rádis-Baptista G., Santos N.C., Andreu D. (2011) Efficient cellular delivery of β-galactosidase mediated by NrTPs, a new family of cell-penetrating peptides. Bioconjug Chem;22:2339–2344.
50. Poon G.M., Gariépy J. (2007) Cell-surface proteoglycans as molecular portals for cationic peptide and polymer entry into cells. Biochem Soc Trans;35(Pt 4):788–793.

51. Tyagi M., Rusnati M., Presta M., Giacca M. (2001) Internalization of HIV-1 Tat requires cell surface heparan sulfate proteoglycans. J Biol Chem;276:3254–3261.
52. Silhol M., Tyagi M., Giacca M., Lebleu B., Vives E. (2002) Different mechanisms for cellular internalization of the HIV-1 Tat-derived cell penetrating peptide and recombinant proteins fused to Tat. Eur J Biochem;269:494–501.
53. West M.A., Bretscher M.S., Watts C. (1989) Distinct endocytotic pathways in epidermal growth factor-stimulated human carcinoma A431 cells. J Cell Biol;109:2731–2739.
54. Yumoto R., Nishikawa H., Okamoto M., Katayama H., Nagai J., Takano M. (2006) Clathrin-mediated endocytosis of FITC-albumin in alveolar type II epithelial cell line RLE-6TN. Am J Physiol;290:L946–L955.
55. Wang L.H., Rothberg K.G., Anderson R.G. (1993) Misassembly of clathrin lattices on endosomes reveals a regulatory switch for coated pit formation. J Cell Biol;123:1107–1117.
56. Zullo A.J., Michaud M., Zhang W., Grusby M.J. (2009) Identification of the small protein rich in arginine and glycine (SRAG). J Biol Chem;284:12504–12511.
57. Strebhardt K., Ullrich A. (2008) Paul Ehrlich's magic bullet concept: 100 years of progress. Nat Rev Cancer;8:473–480.