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Unlocking endosomal entrapment with supercharged arginine-rich peptides

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Abstract

Endosomal entrapment is a common bottleneck in various macromolecular delivery approaches. Recently, the polycationic peptide dfTAT was identified as a reagent that induces the efficient leakage of late endosomes and, thereby, enhances the penetration of macromolecules into the cytosol of live human cells. To gain further insights into the features that lead to this activity, the role of peptide sequence was investigated. We establish that the leakage activity of dfTAT can be recapitulated by polyarginine analogs but not by polylysine counterparts. Efficiencies of peptide endocytic uptake increase linearly with the number of arginine residues present. In contrast, peptide cytosolic penetration displays a threshold behavior, indicating that a minimum number of arginines is required to induce endosomal escape. Increasing arginine-content above this threshold further augments delivery efficiencies. Yet, it also leads to increasing the toxicity of the delivery agents. Together, these data reveal a relatively narrow arginine-content window for the design of optimally active endosomolytic agents.

Keywords

Cell-penetrating peptides; endosomal escape; endocytosis; delivery

Introduction

Delivering macromolecules into human cells is potentially useful in applications ranging from basic cell biology to therapeutic intervention^{1–3}. The plasma membrane of cells is however a barrier to most macromolecules and crossing this barrier efficiently without

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Author contributions

K.N. and J.-P.P. designed experiments. K.N. generated data and processed data. K.N., A.E.O., J.M., M.W., I.R., J.C. contributed reagents. K.N. and J.-P.P. wrote the manuscript. K.N., A.E.O. and J.-P.P. edited and approved the final manuscript.

Supporting Information

The supporting information is available for this manuscript. This includes HPLC and mass spectrometry data, cellular uptake quantification and normalization, monomeric peptide delivery experiments, Bradford assay and western blot analysis for cell lysates and liposome binding assays.

disrupting cell physiology remains a challenging task. A possible solution to this problem consists of using cell-penetrating peptides (CPPs) as delivery agents. CPPs are typically short peptides that have the ability to translocate across biological membranes⁴. CPPs have been used to successfully deliver various cargos into the cytosol of cells, including proteins, nucleic acids, or nanoparticles^{5–9}. However, the cytosolic delivery efficiency associated with CPPs is often low^{10, 11}. For instance, it is well appreciated that TAT, a prototypical CPP from the HIV trans-activator of transcription protein, promotes endocytic uptake^{12, 13}. Yet, TAT-cargo conjugates remain mostly trapped inside endosomes and fail to reach the cytosolic space at levels sufficient to exert a biological effect^{10, 14, 15}.

Unlike TAT, we have recently established that dfTAT, a dimeric fluorescent analog of this CPP, is capable of escaping from endosomes efficiently¹⁶. In particular, dfTAT enters the cytosol of cells by causing the leakage of late endosomes. Membrane leakage appears to require bis(monacylglycerol)phosphate (BMP), an anionic lipid found in these organelles^{17, 18}. As a result of this enhanced endosomolytic activity, dfTAT can deliver a variety of molecules into the cytosol of cells by using a simple co-incubation protocol: dfTAT and a macromolecule of interest (MOI) are taken up by cells simultaneously, dfTAT and MOI follow a similar route in the endocytic pathway, dfTAT and MOI reach the late endosomes, dfTAT promotes leakage and MOI is released into the cytosol of live cells.

The chemical properties that confer dfTAT its robust endosomolytic activity and that distinguish it from its far less active monomeric counterpart are currently not understood. Answering this question is however important as it may provide a foundation for the rational design of next-generation delivery agents. In particular, while dfTAT is effective in tissue cultures, the peptidic nature of this reagent makes it presumably not ideal for *in vivo* use^{19, 20}. For example, CPPs typically display poor pharmacokinetics and are rapidly cleared from circulation^{21–25}. Understanding the features that provide high cell-penetration activity to dfTAT may therefore guide the development of analogs with more therapeutic relevance. Moreover, dfTAT also shares some similarities with other molecules that display enhanced endosomal escape activities. For instance, recent reports have suggested that proteins with a very high density of positive charges can escape from endosomes. This is, for instance, the case for supercharged GFP (scGFP), a GFP variant engineered with 36 surface exposed cationic residues (+1.3/kDa)²⁶. It is also the case for viral capsid proteins that naturally display charge density as high as +2/kDa and that can either directly participate in the viral entry into host cells or that can be used to deliver exogenous cargos²⁷. Notably, dfTAT, with a total of 18 positive charges and a charge density of +4.4/kDa, may also be considered a “supercharged” species.

Structure-activity relationship studies have previously been conducted on the monomeric TAT peptide. However, due to the TAT peptide’s extremely poor activity at escaping endosomes, these results have only allowed for an understanding of the parameters necessary for its endocytic uptake activity^{28–30}. In this report, we aimed to establish the importance of sequence and charge density of the endosomal escape property of dfTAT. The structure of dfTAT is readily amenable to modifications and the endosomal escape activity of the peptide is high enough to be measured accurately. We therefore envisioned that the combination of these properties would facilitate the establishment of new structure-activity

relationships that may shed light on the poorly characterized process of endosomal leakage. In turn, the cell-penetration rules learnt with this study may be applicable to other delivery systems that utilize the endocytic pathway as a route of cellular entry, including other CPPs, supercharged proteins, cationic liposomes and nanoparticles.

Results

A polyarginine construct recapitulates dfTAT's cytosolic penetration but a polylysine analog does not

dfTAT induces leakage from late endosomes by interacting with the lipid BMP¹⁷. dfTAT is cationic while BMP is anionic, suggesting that electrostatic interactions may be important for peptide penetration activity. To establish whether charge alone is a determining factor for endosomal escape, polyarginine or polylysine analogs of dfTAT were synthesized. In particular, given that dfTAT contains 16 basic residues, dfR8 (16 arginine residues) and dfK8 (16 lysine residues) were first produced (Fig. 1A and Fig. S1–3). Live cells were incubated with each peptide for 1 h and the cellular penetration of each compound was assessed by live cell fluorescence microscopy. Our measurements are based on the fact that, when endosomal escape fails, a distinct punctate fluorescence can be observed^{31–33}. This is a result of endosomal entrapment of the fluorescent peptides. In contrast, upon endosomal escape, a diffuse cytosolic fluorescence signal can be detected^{16, 34}. Moreover, because polycationic peptides tend to accumulate at nucleoli upon cytosolic entry, nucleolar staining can also be observed^{35, 36}. While the cytosolic signal is admittedly unreliable because of possible out-of-focus contributions, the nucleolar signal is useful because it decisively validates that the compounds have indeed penetrated cells. Overall, we therefore use a binary assay in which cells are counted as either positive or negative for nucleolar staining. When compared to dfTAT, dfK8 showed a very weak cytosolic penetration activity (Fig. 1B). More precisely, while dfTAT stains the nuclei of a majority of cells in a culture, dfK8 appears trapped in endosomes in 95% or more of the cells, at all concentrations tested (Fig. 1C). In contrast, the percentage of cells displaying nucleolar staining after dfR8 incubation was high (>80% at 5 μ M peptide) and comparable to that of cells incubated with dfTAT (Fig. 1C). Furthermore, the overall level of cellular uptake of dfK8 was approximately half of that of dfTAT (Fig. S4). Together, these data therefore suggest that the endocytic uptake and endosomal escape activities of dfK8 are poor in comparison to those of dfTAT. In contrast, dfR8 enters the cytosol of cells like dfTAT does.

In the previous assays, the nuclear stain Sytox Blue was used to identify dead cells that may be erroneously stained by TMR-labeled peptides (such cells are not included in our cytosolic penetration analyses, as Sytox Blue staining indicates that cells have a disrupted plasma membrane). As shown in Fig. 1D, more than 94% of cells in a culture exclude Sytox Blue after dfTAT treatment, indicating that the peptide is relatively non-toxic under the conditions tested (5 and 10 μ M, 1 h incubation). In contrast, the number of cells positive for SYTOX Blue staining was significantly higher for dfR8; 13% and 47% at 5 and 10 μ M, respectively (Fig. 1D). Notably, while 13% toxicity was sufficiently low to permit the monitoring of the cytosolic penetration of the peptide in the remaining 87% of live cells, the toxicity of the peptide at 10 μ M made this analysis difficult and rather pointless (therefore it is not shown

in Fig. 1C). More importantly, the significant difference in toxicity response observed between dfTAT and dfR8 prompted us to investigate other polyarginine analogs. In particular, we chose to test whether reducing the number of arginine residues may yield reasonable endosomal escape activity while reducing toxicity.

Cytosolic penetration is dependent on the number of arginine residues present

A series of dfRn peptides, where n= 4,5,6 or 7, was synthesized (Fig. S5). The peptide dfR4, the smallest compounds of the series, displays no detectable cell penetration, regardless of the concentration tested (from 1 to 5 μ M). Instead, the peptide appears to exclusively accumulate inside endosomes, as indicated by co-localization with lysotracker Green, a fluorophore that stains acidified endocytic organelles (Fig. 2A, Fig. S6)³⁷. The activity of dfR5 was similarly poor. In contrast, both dfR6 and dfR7, similar to dfTAT and dfR8 displayed robust cytosolic penetration and nucleolar staining (Fig. 2A and 2B). However, the monomeric versions of these peptides showed no detectable cell penetration at 5 μ M concentration (Fig. S7). The toxicity of the dfRn peptides, estimated by the Sytox Blue exclusion assay, was low for dfR4, dfR5, dfR6, and comparable to that of dfTAT (Fig. 2D). In contrast, as previously observed for dfR8, dfR7 showed a sudden increase in toxicity at 10 μ M.

While our cytosolic penetration assay establishes whether a peptide can reach the cytosolic and nucleolar space (above the detection threshold of our imaging set-up), it does not address how much peptide enters cells. To test how the dfRn peptides compare to dfTAT in that regard, the total fluorescence of cells incubated with 5 μ M of each reagent was measured (a condition at which the

peptides show low toxicities, with the exception of dfR8). In particular, to avoid artifacts that could arise from the peptides having different fluorescence intensities depending on whether they localize in endosomes or in the cytoplasm, cell lysates were analyzed. First, the total number of cells present in our samples, which was similar between treated and untreated samples, was determined using flow cytometry (Fig. S8)¹⁶. Cells were subsequently lysed and the fluorescence intensities of lysates were measured by fluorescence spectrometry. Fluorescence intensities were then normalized to the number of cells present in each sample. Western blot analysis and a Bradford assay confirm that beta-actin and total protein content, respectively, were similar between treated and untreated samples (Fig. S8). As shown in Figure 2E, the overall cellular uptake increases with the number of arginine residues, with dfR7 showing 2 fold more peptide inside cells than dfR4. dfR8 showed the highest level of uptake. Yet, in this particular case, peptide sticking to dead cells may contribute significantly to the signal detected (dead cells are typically as bright as live cells (Fig. S9)).

dfRn peptides are endosomolytic

The structural and chemical similarities between dfTAT and the penetration-active dfRn peptide do not guarantee that the compounds follow the same mechanisms for cytosolic access. To test whether, like dfTAT, dfR6/7/8 enter cells by escaping from endosomes, several assays were performed. Cells were incubated with the peptides for 5 min, so as to permit brief endocytic uptake. Cells were then washed to remove excess peptide and imaged

at various time points. At time 0 (immediately after cell washing), more than 95% of cells displayed the distinct punctate distribution indicative of endosomal entrapment (Fig. 3A). However, as time gradually increased in this pulse-chase experiment, the number of cells positive for penetration (i.e. displaying nucleolar staining) also increased. These data therefore indicate that the peptides can redistribute from the lumen of endosomes to the cytosolic and nuclear space. In complementary experiments, the endosomes of cells were preloaded with the fluorescent cargo DEAC-k5. DEAC-k5 is a proteolytically stable poly-D-lysine peptide labeled with a blue fluorescent coumarin derivative (Fig. S10)^{17, 19}. As shown in Figure 3B, pre-incubation of cells with DEAC-k5 leads to accumulation of the peptide within endosomes, where it remains entrapped. However, addition of dfR6 or dfR8 after DEAC-k5 loading causes a redistribution of this peptide into the cytosol of cells (DEAC-k5 also accumulates at nucleoli and displays a lower contrast in other areas of the nucleus). Overall, these data therefore support the notion that, like dfTAT, the dfR6/7/8 peptides can escape from endosomes and, in doing so, also mediate the leakage of other entrapped cargos into the cytosol of cells.

To further evaluate the membrane-disrupting activities of the dfRn compounds, liposomes with a lipid composition mimicking the lipid bilayers of the plasma membrane and late endosomes were prepared. The liposomes were prepared with Phosphatidylcholine (PC), Phosphatidylethanolamine and Cholesterol (chol) (65 PC: 15 PE: 20 chol) to model the plasma membrane and early endosomes (PM/E.E. LUVs) and with BMP:PC:PE (77:19:4) to model late endosomes (L.E. LUVs)^{18, 38, 39}. Calcein was encapsulated inside the liposomes and peptide-induced liposome disruption was quantified by measuring calcein leakage. When incubated with PM/E.E. LUVs, the peptides dfTAT and dfRn did not cause significant leakage. However, when incubated with L.E. LUVs, dfRn peptides induced leakage in a manner linearly proportional to arginine content ($R^2=0.99$) (Fig. 3C). Furthermore, dfTAT and dfR6, the two peptides that showed the most similarities in our *in cellulo* assays, were also comparable in their leakage activity *in vitro*. Notably, these assays were performed at a peptide to lipid ratio of 1:50. Under this condition, all peptides are fully bound to L.E. LUVs (Fig. S11), suggesting that variations in peptide/lipid binding affinities do not account for the differences in leakage activity observed.

Differences in endocytic uptake do not solely account for differences in cytosolic penetration

Conceptually, the peptides dfR4 and dfR5, which stay entrapped inside endosomes, could fail to reach the cytosolic space because their uptake is poor and does not permit sufficient accumulation of peptide inside endosomes for escape. To test whether this is the case, we established peptide incubation concentrations that lead to similar uptake for dfR4, dfR5, and dfR6. We found that cell incubation with 20 μ M dfR4 and 10 μ M dfR5 yielded a peptide uptake comparable to that of 5 μ M dfR6, as measured by the total fluorescence of cell lysates (Fig. 4A). Yet, under these conditions, dfR4 remained unable to reach the cytosol and deliver cargo into cells (Fig. 4B and 4C). dfR5, although slightly more active, was also unable to reproduce the activity of dfR6. Together, these results suggest that differences in how much peptide is endocytosed by cells cannot account for the differences in cytosolic penetration observed between dfR4/5 and dfR6/7/8. Instead, these results suggest that dfR4

and dfR5 have an endosomolytic activity significantly weaker than that of dfR6. This in turn indicates that a simple difference of 2 arginine residues is enough to transform a low-activity peptide to a high efficiency CPP.

Discussion

The polycationic dfK8 performed poorly in our assays while dfR8, a peptide sharing a similar charge, was highly effective. Inducing endosomal escape and cytosolic release may therefore not simply be a charge density effect. It appears to instead be dependent on the presence of arginine residues. Moreover, the activities of dfTAT and dfR6 are very comparable. This suggests that the residues other than arginine, namely 4 lysine residues and 2 glutamine residues, are not contributing significantly to the cytosolic penetration of dfTAT. Our data also indicate that a content of 12 arginine residues is an approximate threshold necessary for high endosomal escape efficiency. Below this threshold, the peptides tested are mostly inactive and trapped inside endosomes. In contrast, above this threshold, a clear increase in cytosolic access is achieved. Interestingly, both the endocytic uptake and the leakage activity of the dfRn peptides are proportional to the number of arginine residues present). It is therefore likely that, in the case of shorter peptide such as dfR4 and dfR5, a relatively lower endocytic uptake and an intrinsically weaker membrane disrupting activity lead to conditions under which there is simply not enough peptide inside endosomes to cause any detectable endosomal leakage. It should be noted however that the peptides tested can be partially degraded during their transit in endocytic organelles¹⁹. In principle, differences in their propensity for proteolysis may also contribute to the non-linear cytosolic release response observed.

Our data suggest that, as far as endosomal escape is concerned, the more arginine residues present in the dfRn series, the more efficient the peptide is. However, as seen with dfR7 and dfR8, cell viability decreases quite dramatically with arginine content. It is currently unclear why dfR7 and dfR8 are more toxic than dfR6 or dfTAT and whether the peptides express their deleterious effects from outside or inside the cell, or both. However, given that the endosomolytic activities of all four compounds are similar, the leakage of endosomes itself is presumably not responsible for the loss of viability observed (the endosomolytic activity of dfTAT does not lead to a reduction in proliferation rates and does not induce a noticeable transcriptional response)¹⁶. Overall, when combining the cytosolic penetration and toxicity data, dfR6, which contains 12 arginine residues, appears optimal.

If dfTAT can be simplified to dfR6, why not simply use R12, a peptide with similar arginine content but with a linear structure? Interestingly, fluorescently labeled R12 CPPs have already been reported to penetrate cells^{40–42}. However, these peptides seem to enter cells by a mechanism that involves direct translocation at the plasma membrane (please note that the importance of arginine residues have also been well appreciated for this activity)^{41–43}. This is a process that we have also detected in our laboratory and that we have attributed in part to the membrane oxidation of cells grown under 20% oxygen (e.g. ambient air)⁴⁰. Overall, it would therefore appear that CPPs with different structures but with similar arginine content take different routes into the cells. Investigating how this is possible may in turn reveal how to favor specific delivery pathways. Notably, it is worth noticing that dfR6 differs from a

linear R12 peptide in several ways. First, a disulfide bond separates two R6 peptides. In the context of dfTAT, we have already established that a non-reducible linker could replace this disulfide bond without causing a loss of activity, indicating that the presence of a disulfide group is not necessary per se¹⁶. However, it is possible that the presence of a spacer between two R6 sequences is making the peptide behave differently than a peptide with 12 contiguous arginine residues. Consistent with this idea, Schepartz and co-workers have reported that arginine topology played a determining role in the intracellular trafficking of minimally cationic proteins, including their endosomal escape⁴⁴. Alternatively, it is important to note that dfR6 contains two tetramethylrhodamine moieties while the R12 peptides previously studied were labeled with only a single copy of this fluorophore. It is therefore possible that the addition of an extra fluorophore contributes to changing how the peptides interact with cells. Notably, spectroscopic analyses of a TAT peptide labeled with TMR suggest that the peptide has a propensity to dimerize in lipid bilayers and that the fluorophores enhance this aggregation process⁴⁵. Moreover, molecular dynamics simulations suggest that the dimeric self-aggregate form of the peptide has an enhanced propensity to destabilize membranes⁴⁶. These results are in agreement with our experiments as dfTAT and, by extension, the dfRn series are preformed versions of the dimeric structures that arise from self-aggregation. In addition, these *in silico* studies suggest that the fluorophores may contribute to slowing down TAT peptides during membrane translocation, thereby extending the lifetime of possible membrane defects. Whether these fluorophore-induced effects are pertinent to the peptides presented herein and to their cell-penetration remains to be tested experimentally, something we aim to pursue in future studies. Overall, it is evident that relatively subtle differences in CPP structures impact where they go, how well they get into cells, and how much damage they produce.

Material and Methods

Peptide design, synthesis and purification

All peptides were synthesized on the rink amide MBHA resin (Novabiochem, San Diego, CA) by solid phase peptide synthesis (SPPS). Fmoc-Lys(Mtt)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Gly-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Cys(tbutio)-OH (Novabiochem) were used to assemble the peptides using standard Fmoc protocols. For each amino acid coupling, a mixture of Fmoc-amino acid (1.6 mmol), HBTU (Novabiochem) (1.5 mmol) and di-isopropylethylamine (DIEA) (sigma) (4.0 mmol) in dimethylformamide (DMF) (Fisher) was added. All reactions were agitated using a stream of dry N₂ in a standard SPPS vessel at room temperature. Fmoc deprotection was performed by addition of 20% piperidine in DMF. The reaction was carried out twice: 1x 5 min followed by 1x 15 min with a DMF washing step between reactions. DEAC-k5 (D-amino acid) was synthesized by coupling five Fmoc-D-Lys(Boc)-OH. The DEAC (Anaspec) fluorophore was conjugated to the N-terminus of the peptide by reaction of DEAC, HBTU and DIEA (4, 3.9 and 10 eq.) in DMF overnight. CK(ϵ -NH-TMR)RnG peptides (n=4, 5, 6, 7 or 8) were generated similar to work described earlier for CK(ϵ -NH-TMR)TATG (L-ftAT)¹⁶. Briefly, the MTT group found on C(tbutio)K(ϵ -NH-MTT)RnG was removed using 1% trifluoroacetic acid (TFA) (Fisher) and 2% triisopropylsilane (TIS) (Sigma) in DCM. The peptidyl-resin was washed with both DMF and DCM between each reaction. The peptidyl-resin was then left to react

with a mixture of TMR, HBTU and DIEA (4, 3.9 and 10 eq.) overnight. The peptidyl-resin was washed and Fmoc deprotection was performed to remove the N-terminal Fmoc moiety. To remove the tbutyl protecting group on the side chain of cysteine, the peptidyl-resin was then incubated in presence of tributylphosphine (PBU₃) (a solution containing: 180 μ L PBU₃, 500 μ L DMF and DCM and 50 μ L H₂O; 3 times the volume is used for a full cleavage resin>100 mg). The peptides and the remaining protecting groups were cleaved from the resin by treatment with 92.5% TFA, 2.5% H₂O, 2.5% TIS and 2.5% ethanedithiol (EDT) (Sigma) for 3h at room temperature. The crude peptides were precipitated in cold anhydrous diethyl ether (Fisher). The precipitates were resuspended in 0.1 %TFA/water and lyophilized. The lyophilized product was dissolved in 0.1% TFA/water. The peptides were analyzed and purified by reverse-phase HPLC. HPLC analysis was performed on a Hewlett-Packard 1200 series instrument and an analytical Vydac C18 column (5 μ m, 4 x 150 mm). The flow rate was 2 mL/min, and detection was at 214 nm and 550 nm. Semi-preparative HPLC was performed on a Vydac C18 10 x 250 mm column. The flow rate was 4 mL/min, and detection was at 214 nm and 550 nm. All runs used linear gradients of 0.1% aqueous TFA (solvent A) and 90% acetonitrile, 9.9% water, and 0.1% TFA (solvent B). The correct identity of the dfRn series was confirmed by electrospray ionization mass spectrometry (ESI), performed with an exactive plus orbitrap mass spectrometer instrument (Thermo Fisher Scientific). The correct identity of the dfTAT and DEAC-k5 was confirmed by MALDI-TOF performed with a Shimadzu/Kratos instrument (AXIMA- CFR, Shimadzu, Kyoto). fTAT, expected mass: 2041.17, observed mass: 2040.66, DEAC-k5, expected mass: 900.59, observed mass: 900.84

Generation of dfRn, dfK8 and dfTAT by dimerization reactions

fRn (where n=4, 5, 6,7 or 8), fK8 and fTAT (0.3 mg, 1.5×10^{-4} mmol) were each dissolved in 5 mL of aerated phosphate buffer saline (PBS) at pH 7.4. The reaction was left to incubate overnight (100% yield based on HPLC analysis). dfRn (where n=4, 5, 6,7 or 8), dfK8 and dfTAT were purified using reverse-phase HPLC. Expected masses were: dfTAT: 4078.27, dfR4: 2683.48, dfR5: 2995.7, dfR6: 3307.92, dfR7: 3620.14, dfR8: 3932.36, dfK8: 3484.25. The observed masses using ESI were dfTAT: 4076.24, dfR4: 2682.36, dfR5: 2994.6, dfR6: 3306.8, dfR7: 3619.28, dfR8: 3932.16, dfK8: 3484.08.

Cell culture

HeLa cells (ATCC CCL-2) were grown in Dulbecco's Minimum Essential Media (DMEM, Fisher) supplemented with 10% fetal bovine serum (FBS) (Fisher) and 1X penicillin/streptomycin (P/S) (Fisher) and incubated in a humidified atmosphere containing 5% CO₂ at 37 °C.

Delivery of peptides into live cells

HeLa cells were seeded in either an 8-well dish or a 48-well dish and were grown to 80–90% confluency in a 37°C humidified atmosphere containing 5% CO₂. The cells were washed three times (3X) with PBS and once with nRL-15. Cells were incubated with each peptide (dfRn, dfKn or dfTAT) in nRL-15 (serum free media) at 37°C for 1 h at concentrations specified in the text. Incubation were performed in the absence of FBS as albumin interacts with polycationic peptides and decreases the activity of CPPs, as

previously reported¹⁶. A time period of 1 h was also chosen because it is the incubation time required to achieve cytosolic delivery of dfTAT (5 μ M) in more than 90% of cells in a culture¹⁶. Since this incubation time is sufficient to achieve efficient cytosolic delivery of macromolecules with dfTAT, longer incubation times were not tested. Following incubation, cells were washed 3X with L-15 supplemented with heparin (1 mg/mL) and an extra nrL-15 wash. Cells were treated with the cell impermeable nuclear stain SYTOX® BLUE or SYTOX® GREEN to take into account cells that had a compromised plasma membrane (i.e. dead cells). Alternatively, the cell permeable dye Hoechst was used for nuclear staining and LysoTracker green (500 nM) was used to stain acidified endocytic organelles. Cells were imaged using an inverted epifluorescence microscope (Model IX81, Olympus, Center Valley, PA) equipped with a heating stage maintained at 37 °C. Images were collected using a Rolera-MGI Plus back-illuminated EMCCD camera (Qimaging, Surrey, BC, Canada). Images were acquired using bright field imaging and three standard fluorescence filter sets: CFP (Ex = 436 $\pm$ 10 nm / Em= 480 $\pm$ 20 nm), RFP (Ex = 560 $\pm$ 20 nm / Em= 630 $\pm$ 35 nm), FITC(Ex = 488 $\pm$ 10 nm / Em= 520 $\pm$ 20 nm). For viewing and processing individual fluorescent images, the SlideBook 4.2 software (Olympus, Center Valley, PA) was used. Similar to previous reports, cells that displayed a cytosolic and nucleolar peptide staining were considered positive for cytosolic penetration (penetration (+)). The percentage of penetration (+) cells was determined by dividing the number of cells that displayed a fluorescent nuclear staining (20X images) by the total number of cells present (acquired using bright field imaging). Cells that displayed a punctate peptide distribution consistent with endosomal entrapment were considered negative for penetration and were not counted. Dead cells (i.e. cells stained with SYTOX dyes) were excluded from the analysis of penetration assays. To determine the percentage dead cells, the total number of cells stained with Sytox blue was divided by the total number of cells per image. For all quantitative experiments performed, an average of at least three 20X pictures were taken, representing 300–400 cells. The reproducibility of all the experiments was assessed by performing experiments with independent batches of cell cultures on three different days (i.e. biological triplicates).

Determination of mechanism of cellular entry

To investigate the process of cellular entry, HeLa cells were incubated in L-15 with DEAC-k5 (20 μ M) for 1 h. Cells were then washed three times with PBS and the incubated with the dfRn peptide (5 μ M) or without peptide (control cells) for 1h. Cells were washed afterwards and then imaged as described earlier.

For the pulse-chase experiments, our goal was to load endosomes with peptide, wash the peptide outside the cell so as to remove extracellular excess fluorescence signal, and monitor whether the release of peptide from endosomes could be observed as time progresses. Consequently, HeLa cells were incubated with the dfRn peptides for only 5 min (dfR6: 5 μ M, dfR7/8: 3 μ M). The cells were washed as previously described and imaged immediately (time point= 0 min). The cells were then imaged at different time points as indicated in the text. Please note that the goal of these experiments is not to achieve maximal delivery efficiencies. As a matter of fact, the short incubation times used here lead to an overall lower

number of cells positive for penetration when compared to the 60 min incubation used in other experiments.

Quantitative determination of peptide uptake

HeLa cells were seeded in a 48-well dish and grown to 80% confluency. Cells were treated with peptides for 1h at various concentrations (range: 1–10 μ M). The total amount of peptide internalized was measured by a whole cell lysate analysis. Cells were washed with heparin (1mg/mL) and then trypsinized (50 μ L) for 3 min. Cells were resuspended in nrL-15 (350 μ L) and centrifuged at 4°C, 2,500 rpm for 10 min. The supernatant was discarded and the cell pellet was resuspended in nrL-15 (50 μ L). An aliquot from the resuspended cells (3 μ L) was removed to determine the total number of cells per sample by analysis using flow cytometry. The remainder of resuspended cells were lysed by addition of 50 μ L of a lysis buffer (components: 50mM Tris, pH7.5, 2 mM EDTA, 4mM DTT, 20% Triton X-100 and protein inhibitor cocktail) and 3 min of vortexing. A volume of 70 μ L of the lysed cells for each condition was placed in a 96 well plate. The fluorescence emission intensity was measured using a plate reader equipped with a fluorescence module (Ex=525, Em=580–640 nm) (GloMax®-Multi+ Detection System, Promega, Fitchburg, WI). To normalize the data, an aliquot of the cells (3 μ L) was resuspended in nrL-15 (197 μ L) and the total amount of cells per samples was determined using flow cytometry.

For fluorescence measurements using the flow cytometer, cells were trypsinized and resuspended in nrL-15 medium. Cells were analyzed using a BD Accuri C6 flow cytometer equipped with the FL2 filter (Ex = 488 nm/Em = 533 $\pm$ 30 nm). All data was acquired at flow rate of 66 μ L/min with detection of a minimum of 40,000 events. The geometric mean of the FL2 signal for each experiment was determined using the Flowjo software. The data reported represent the average and corresponding standard deviations of three independent experiments for each peptide concentration.

For western blot analysis, the cell lysates were spun down at 4°C, 13,000 RPM for 10 min. The supernatant was collected and run on SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane using a transfer apparatus according to the manufacturer's protocols (Bio-Rad). The PVDF membrane was then blocked with 5% nonfat milk in TBST (10 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween 20) for 1 h. The membrane was then incubated with the primary antibody against β -actin (1: 2000, abcam ab8227) at room temperature for 2 h. The membrane was washed three times for 5 min with TBST and incubated with a 1:3000 dilution of horseradish peroxidase-conjugated anti-rabbit antibodies for 1 h. Blots were washed with TBST and then water (three times, 5 min each) and developed with the ECL system (PerkinElmer) according to the manufacturer's protocols. Alternatively, the total protein concentration of the supernatant of the cell lysates was determined using the Bradford protein assay (Bio-Rad).

Liposome Preparation

The lipids used in the experiments consisted of: 1,2-dioleoyl-s-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), sn-(3-oleoyl-2-hydroxy)-glycerol-1-phospho-sn-1'-(3'-oleoyl-2'-hydroxy-glycerol) (BMP), cholesterol

(chol) (Avanti Polar Lipids). Liposomes were prepared by first prepared by transferring the required volumes of lipids dissolved in chloroform (stock solutions of known concentrations) into scintillation vials. For liposomes mimicking the intraluminal vesicles of late endosomes (L.E.) the molar ratios of lipids consisted of 77:19:4 BMP:PC:PE. For liposomes mimicking the plasma membrane, the lipid mixture was 65:15:20 PC:PE:Chol. The lipid film was prepared by evaporating the chloroform from the lipid mixture using a stream of N₂ and placing the vial in a desiccator overnight. The lipid films were hydrated by addition of a buffer composed of 100 mM NaCl, 10 mM NaH₂PO₄ pH7.4, with or without 60 mM calcein. The lipids were mixed vigorously and swelled for 1 h at 42°C under N₂ to obtain multilamellar vesicles (MLVs). For production of unilamellar vesicles (LUVs), the MLVs were extruded (20 passes) through a 100 nm pore size polycarbonate membrane (Whatman) using a Mini-Extruder (Avanti Polar Lipids). Dynamic light scattering was used to determine the average diameter size distribution of the liposomes using a Zeta Sizer (Malvern instrument). The liposomes were purified by gel filtration using a Sephadex G-50 (GE Healthcare) column (2.5 x 17.5 cm) to separate the liposomes from free calcein. The eluate was collected in a 96 well plate and the plate was read using a Promega GloMax-Multi plate reader (Promega) at 450 nm and 750 nm corresponding to the wavelength of detection for calcein and liposome respectively.

Liposome leakage assays

Purified calcein-loaded LUVs were mixed with different peptides at a 1:50 peptide: lipid ratio for 1 h at room temperature in 100 mM NaCl, 10 mM NaH₂PO₄ pH5.5. Samples were centrifuged for 1 min at 4,000 rpm. To measure the amount of leaked calcein and to separate soluble liposomes from released calcein, the supernatants were purified using an illustra NAP-10 Sephadex G-25 column (GE Healthcare) (the elution volumes of liposomes and free calcein were determined independently with pure samples). Fractions were collected in a 96-well plate and the fluorescence of calcein was measured using a Promega GloMax-Multi plate reader (Ex 490nm, Em 520–560nm). The percent leakage was calculated using the following equation:

$$\% \text{ Leakage} = 100 \times \frac{Fl_t - Fl_0}{Fl_{\max} - Fl_0}$$

where Fl_t is the free calcein fluorescence intensity of a sample at a specific peptide:lipid ratio measured after 1 h, Fl_0 is the free calcein fluorescence intensity of an untreated sample and $Fl_{\max}$ is the free calcein fluorescence intensity of a sample after treatment with 0.2 % Triton X-100.

Liposome Binding Assays

To determine the difference in the binding affinity of the peptides to LUVs of different lipid compositions, liposome binding assays were conducted. The LUVs were incubated for 1 h with either peptide at the same peptide to lipid ratio as the leakage assay (1:50) using the buffer composition of 100 mM NaCl, 10 mM NaH₂PO₄ pH 5.5. Samples were centrifuged at 13,000 rpm for 3 min. The supernatant was removed to measure the amount of unbound

peptide (using the fluorescence emission of TMR). To insure that quenching due to the proximity of the TMR fluorophore in the dFTAT constructs would not affect our results, the supernatant was reduced with TCEP (50 mM). The TMR fluorescence was measured using the red channel (Ex= 525nm, Em=580–640 nm) of a Promega GloMax-Multi plate reader (Promega). The amount of peptide bound to the MLVs was determined according to the following equation:

$$P_b = P_{tot} - P$$

Where P_{total} is the amount of peptide from the supernatant in the absence of MLVs, P is the fraction of unbound peptide at a particular lipid concentration and P_b is the fraction of bound peptide at a particular lipid concentration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Deshayes S, Morris MC, Divita G, Heitz F. Cell-penetrating peptides: tools for intracellular delivery of therapeutics. *Cellular and Molecular Life Sciences CMLS*. 2005; 62:1839–1849. [PubMed: 15968462]
2. Torchilin VP. Recent approaches to intracellular delivery of drugs and DNA and organelle targeting. *Annu Rev Biomed Eng*. 2006; 8:343–375. [PubMed: 16834560]
3. Schwarze SR, Dowdy SF. In vivo protein transduction: intracellular delivery of biologically active proteins, compounds and DNA. *Trends Pharmacol Sci*. 2000; 21:45–48. [PubMed: 10664605]
4. Bechara C, Sagan S. Cell-penetrating peptides: 20 years later, where do we stand? *FEBS Letters*. 2013; 587:1693–1702. [PubMed: 23669356]
5. Gupta B, Levchenko TS, Torchilin VP. Intracellular delivery of large molecules and small particles by cell-penetrating proteins and peptides. *Advanced Drug Delivery Reviews*. 2005; 57:637–651. [PubMed: 15722168]
6. Zorko M, Langel Ü. Cell-penetrating peptides: mechanism and kinetics of cargo delivery. *Advanced Drug Delivery Reviews*. 2005; 57:529–545. [PubMed: 15722162]
7. Eguchi A, et al. Efficient siRNA delivery into primary cells by a peptide transduction domain-dsRNA binding domain fusion protein. *Nat Biotechnol*. 2009; 27:567–571. [PubMed: 19448630]
8. Farkhani SM, et al. Cell penetrating peptides: efficient vectors for delivery of nanoparticles, nanocarriers, therapeutic and diagnostic molecules. *Peptides*. 2014; 57:78–94. [PubMed: 24795041]
9. Liu BR, et al. Endocytic trafficking of nanoparticles delivered by cell-penetrating peptides comprised of nona-arginine and a penetration accelerating sequence. *PloS one*. 2013; 8:e67100. [PubMed: 23840594]
10. Lee YJ, Datta S, Pellois JP. Real-time fluorescence detection of protein transduction into live cells. *J Am Chem Soc*. 2008; 130:2398–2399. [PubMed: 18251482]

11. Schwarze SR, Hruska KA, Dowdy SF. Protein transduction: unrestricted delivery into all cells? *Trends in Cell Biology*. 2000; 10:290–295. [PubMed: 10856932]
12. Kaplan IM, Wadia JS, Dowdy SF. Cationic TAT peptide transduction domain enters cells by macropinocytosis. *J Control Release*. 2005; 102:247–253. [PubMed: 15653149]
13. Nakase I, et al. Cellular uptake of arginine-rich peptides: roles for macropinocytosis and actin rearrangement. *Mol Ther*. 2004; 10:1011–1022. [PubMed: 15564133]
14. Wadia JS, Stan RV, Dowdy SF. Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. *Nat Med*. 2004; 10:310–315. [PubMed: 14770178]
15. Trehin R, Krauss U, Beck-Sickinger AG, Merkle HP, Nielsen HM. Cellular uptake but low permeation of human calcitonin-derived cell penetrating peptides and Tat(47–57) through well-differentiated epithelial models. *Pharm Res*. 2004; 21:1248–1256. [PubMed: 15290867]
16. Erazo-Oliveras A, et al. Protein delivery into live cells by incubation with an endosomolytic agent. *Nat Meth*. 2014; 11:861–867.
17. Erazo-Oliveras A, et al. The Late Endosome and Its Lipid BMP Act as Gateways for Efficient Cytosolic Access of the Delivery Agent dfTAT and Its Macromolecular Cargos. *Cell Chem Biol*. 2016; 23:598–607. [PubMed: 27161484]
18. Kobayashi T, et al. Late endosomal membranes rich in lysobisphosphatidic acid regulate cholesterol transport. *Nat Cell Biol*. 1999; 1:113–118. [PubMed: 10559883]
19. Najjar K, Erazo-Oliveras A, Brock DJ, Wang TY, Pellois JP. An l- to d-Amino Acid Conversion in an Endosomolytic Analog of the Cell-penetrating Peptide TAT Influences Proteolytic Stability, Endocytic Uptake, and Endosomal Escape. *Journal of Biological Chemistry*. 2017; 292:847–861. [PubMed: 27923812]
20. Stewart MP, et al. In vitro and ex vivo strategies for intracellular delivery. *Nature*. 2016; 538:183–192. [PubMed: 27734871]
21. Palm C, Jayamanne M, Kjellander M, Hällbrink M. Peptide degradation is a critical determinant for cell-penetrating peptide uptake. *Biochimica et Biophysica Acta (BBA) - Biomembranes*. 2007; 1768:1769–1776. [PubMed: 17499577]
22. Elmquist A, Langel Ü. *Biological Chemistry*. 2003; 384–387
23. Lindgren ME, HÄLLbrink MM, Elmquist AM, Langel Ü. Passage of cell-penetrating peptides across a human epithelial cell layer in vitro. *Biochemical Journal*. 2004; 377:69–76. [PubMed: 12968950]
24. Pujals S, Sabidó E, Tarragó T, Giralt E. all-D proline-rich cell-penetrating peptides: a preliminary in vivo internalization study. *Biochemical Society Transactions*. 2007; 35:794–796. [PubMed: 17635150]
25. Youngblood DS, Hatlevig SA, Hassinger JN, Iversen PL, Moulton HM. Stability of Cell-Penetrating Peptide–Morpholino Oligomer Conjugates in Human Serum and in Cells. *Bioconjug Chem*. 2007; 18:50–60. [PubMed: 17226957]
26. Thompson DB, Cronican JJ, Liu DR. Engineering and identifying supercharged proteins for macromolecule delivery into mammalian cells. *Methods Enzymol*. 2012; 503:293–319. [PubMed: 22230574]
27. Freire JM, Santos NC, Veiga AS, Da Poian AT, Castanho MARB. Rethinking the capsid proteins of enveloped viruses: multifunctionality from genome packaging to genome transfection. *FEBS Journal*. 2015; 282:2267–2278. [PubMed: 25808179]
28. Futaki S, et al. Arginine-rich peptides. An abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery. *J Biol Chem*. 2001; 276:5836–5840. [PubMed: 11084031]
29. Mitchell DJ, Kim DT, Steinman L, Fathman CG, Rothbard JB. Polyarginine enters cells more efficiently than other polycationic homopolymers. *J Pept Res*. 2000; 56:318–325. [PubMed: 11095185]
30. Wender PA, et al. The design, synthesis, and evaluation of molecules that enable or enhance cellular uptake: peptoid molecular transporters. *Proc Natl Acad Sci U S A*. 2000; 97:13003–13008. [PubMed: 11087855]

31. Fuchs SM, Raines RT. Pathway for polyarginine entry into mammalian cells. *Biochemistry*. 2004; 43:2438–2444. [PubMed: 14992581]
32. Al-Taei S, et al. Intracellular traffic and fate of protein transduction domains HIV-1 TAT peptide and octaarginine. Implications for their utilization as drug delivery vectors. *Bioconjug Chem*. 2006; 17:90–100. [PubMed: 16417256]
33. Nishi K, Saigo K. Cellular Internalization of Green Fluorescent Protein Fused with Herpes Simplex Virus Protein VP22 via a Lipid Raft-mediated Endocytic Pathway Independent of Caveolae and Rho Family GTPases but Dependent on Dynamin and Arf6. *Journal of Biological Chemistry*. 2007; 282:27503–27517. [PubMed: 17644515]
34. Angeles-Boza AM, Erazo-Oliveras A, Lee YJ, Pellois JP. Generation of endosomolytic reagents by branching of cell-penetrating peptides: tools for the delivery of bioactive compounds to live cells in cis or trans. *Bioconjug Chem*. 2010; 21:2164–2167. [PubMed: 21043514]
35. Fretz, Marjan M., et al. Temperature-, concentration- and cholesterol-dependent translocation of L- and D-octa-arginine across the plasma and nuclear membrane of CD34+ leukaemia cells. *Biochemical Journal*. 2007; 403:335–342. [PubMed: 17217340]
36. Vivès E, Brodin P, Lebleu B. A Truncated HIV-1 Tat Protein Basic Domain Rapidly Translocates through the Plasma Membrane and Accumulates in the Cell Nucleus. *Journal of Biological Chemistry*. 1997; 272:16010–16017. [PubMed: 9188504]
37. Chazotte B. Labeling lysosomes in live cells with LysoTracker. *Cold Spring Harbor protocols*. 2011 pdb prot5571.
38. Kobayashi T, et al. A lipid associated with the antiphospholipid syndrome regulates endosome structure and function. *Nature*. 1998; 392:193–197. [PubMed: 9515966]
39. Kobayashi T, Startchev K, Whitney Andrew J, Gruenberg J. Localization of Lysobisphosphatidic Acid-Rich Membrane Domains in Late Endosomes. *Biological Chemistry*. 2001; 382:483–485. [PubMed: 11347897]
40. Wang T-Y, et al. Membrane oxidation enables the cytosolic entry of polyarginine cell-penetrating peptides. *Journal of Biological Chemistry*. 2016
41. Kosuge M, Takeuchi T, Nakase I, Jones AT, Futaki S. Cellular internalization and distribution of arginine-rich peptides as a function of extracellular peptide concentration, serum, and plasma membrane associated proteoglycans. *Bioconjug Chem*. 2008; 19:656–664. [PubMed: 18269225]
42. Hirose H, et al. Transient Focal Membrane Deformation Induced by Arginine-rich Peptides Leads to Their Direct Penetration into Cells. *Molecular Therapy*. 2012; 20:984–993. [PubMed: 22334015]
43. Duchardt F, Fotin-Mleczek M, Schwarz H, Fischer R, Brock R. A comprehensive model for the cellular uptake of cationic cell-penetrating peptides. *Traffic*. 2007; 8:848–866. [PubMed: 17587406]
44. Appelbaum, Jacob S., et al. Arginine Topology Controls Escape of Minimally Cationic Proteins from Early Endosomes to the Cytoplasm. *Chemistry & Biology*. 2012; 19:819–830. [PubMed: 22840770]
45. Macchi S, et al. Self-aggregation propensity of the Tat peptide revealed by UV-Vis, NMR and MD analyses. *Physical chemistry chemical physics : PCCP*. 2017; 19:23910–23914. [PubMed: 28836633]
46. Jan, Akhunzada M., et al. The role of Tat peptide self-aggregation in membrane pore stabilization: insights from a computational study. *Physical chemistry chemical physics : PCCP*. 2017

Figure 1.

dfR8 recapitulates the cytosolic penetration activity of dfTAT, while dfK8 does not. A) Schematic representation of the structures of dfTAT (top), dfR8 (middle) and dfK8 (bottom). The link between the cysteine residues represents a disulfide bond. The C-termini of the peptides are amidated. The N-termini are left uncapped and are therefore expected to be protonated. B) Fluorescence microscopy analysis of the cellular distribution of the peptides after incubation. HeLa cells were incubated with dfTAT, dfR8 and dfK8 at 5 μM for 1 h, washed and then stained with the cell permeable Hoechst nuclear stain (1 $\mu\text{g}/\text{mL}$). Live cells were imaged with a 20X and 100X objective. Fluorescence images are overlays of the TMR emission at 560 nm (pseudocolored red) and Hoechst emission at 460 nm (pseudocolored cyan). Co-localization between dfK8 (pseudocolored red) and Lysotracker green (pseudocolored green) is shown in a zoomed-in overlay image. Nucleolar staining by dfR8 and dfTAT is highlighted with white arrows in the insert images. Scale bars, 10 and 100 μm for 100X and 20X respectively. C) Evaluation of the cytosolic delivery efficiency of dfTAT, dfR8 and dfK8 as a function of peptide concentration present in the incubation media. HeLa cells were incubated with peptides (1– 5 μM) for 1 h. The percentage of cells detected as positive for penetration was established by counting the number of cells displaying nucleolar staining by the peptides while excluding the dead/live cell stain SYTOX Blue. ns represents $P > 0.05$ and ****= $P = 0.0001$ compared to dfTAT treatment at the same concentration. D)

Fluorescence microscopy analysis of the cytotoxicity of treatment of cells with dfR8 and dfTAT. HeLa cells were treated with dfR8 or dfTAT (5 and 10 μ M) for 1 h, washed and stained with Sytox Green and Hoechst. Fluorescence images are overlay of Hoechst emission at 460 nm (pseudocolored purple) and Sytox Green at 488 nm (pseudocolored green). The data reported in this figure represent the average and corresponding standard deviations of biological triplicates.

Figure 2.

A threshold number of arginine residues is required for cytosolic penetration. A) Schematic representation of the structures of dfRn (where n=4,5,6 or 7) along with a table of expected charge density of each peptide. B) Comparison of cellular distribution of the polyarginine peptides after 1 h incubation. HeLa cells were incubated with dfTAT or dfRn constructs (n= 4, 5, 6, or 7) at 5 μM for 1 h, washed and then stained with the cell permeable Hoechst nuclear stain (1μg/mL). Live cells were imaged with a 100X objective. Fluorescence images are overlays of the TMR emission at 560 nm (pseudocolored red), Hoechst emission at 460 nm (pseudocolored cyan). Scale bars, 10 μm. C) Quantitative determination of the cytosolic penetration efficiency of dfRn constructs in comparison to dfTAT as a function of peptide concentration present in the incubation media. HeLa cells were incubated with peptides (1, 3 and 5 μM) for 1 h. The percentage of cells detected as positive for penetration was established by microscopy. ns represents P >0.05, *=P 0.05 and ****=P 0.0001 compared to dfTAT treatment at the same concentration. (D) Toxicity of dfRn peptides as determined by a SYTOX Blue exclusion assay. HeLa cells were incubated with 5 or 10 μM peptides for 1h, washed, and treated with SYTOX Blue. The number of cells positive for SYTOX BLUE nuclear staining was established by fluorescence microscopy. ns represents P >0.05, *=P 0.05, **= P 0.01 and ****= P 0.0001. The p-values were determined by comparing each condition to dfTAT treatment at the equivalent concentration. E) Comparison of the total cellular uptake of dfRn peptides as a function of the number of arginine residues. HeLa cells

were incubated with peptides at 5 μ M. The overall amount of peptide internalized by cells (endosomal + cytosolic) was assessed by measuring the bulk fluorescence of cell lysates. All fluorescence measurements were normalized to the lowest averaged fluorescence measurements obtained (ie. dfRn where n=4). ns represents $P > 0.05$, * = $P \leq 0.05$ and ** = $P \leq 0.01$. This data in represent the average and corresponding standard deviations of biological triplicates.

Figure 3.

dfR6, dfR7 and dfR8 enter cells by causing endosomal leakage. A) Pulse-chase experiment monitoring the cellular distribution of dfR6 (5 μ M), dfR7 (3 μ M) and dfR8 (3 μ M) as a function of time. Cells were incubated with the peptides for 5 min, washed, and imaged at different time points. The number of cells positive for penetration, i.e. displaying nucleolar staining, was established by fluorescence microscopy. Representative fluorescence images for the first and last time point are pseudocolored red for TMR emission at 560 nm. ns represents $P > 0.05$, * = $P < 0.05$, ** = $P < 0.01$ and *** = $P < 0.001$. The p-values are determined by comparing each time point to the final time point ($t = 40$ min) for each peptide respectively. B) dfR6/7/8 release into the cytosol a cargo preloaded into endosomes. Cells were pre-incubated with the blue fluorescent peptide DEAC-k5 for 1 h, washed and subsequently treated with the dfRn peptides. Fluorescence imaging was performed to establish the cellular distribution of DEAC-k5 (pseudocolored cyan) before and after peptide treatment. C) Lipid bilayer leakage activity of dfRn peptides. The green fluorophore calcein was encapsulated into the lumen of large unilamellar vesicles (LUV) mimicking the lipid bilayers of the plasma membrane or early endosomes (PM/E.E.) or that of late endosomes (L.E.). Peptides (10 μ M) and lipids (500 μ M) were mixed at a 1:50 ratio and the subsequent leakage of calcein from treated LUV was monitored (100% leakage was established by using the detergent Triton X as a positive control). CK(TMR) (20 μ M) was used as a control and was similarly incubated with calcein loaded L.E. LUVs (500 μ M). ns represents $P > 0.05$, * = $P < 0.05$, ** = $P < 0.01$ and **** = $P < 0.0001$. The p-values were determined by comparing each treatment to that of dfTAT. The data reported represent the average and corresponding standard deviations of biological triplicates.

Figure 4.

Increasing endocytic uptake of dfR4 and dfR5 does not augment endosomal escape. A) Evaluation of the total cellular uptake of dfR4, dfR5 and dfR6. HeLa cells were incubated with either 20 μ M dfR4, 10 μ M dfR5 or 5 μ M dfR6. The overall amount of peptide internalized by cells was assessed by measuring the bulk fluorescence of cell lysates (normalized to cell count and to dfR6 treatment). ns represents $P > 0.05$. B) Determination of the cytosolic penetration efficiency of dfR4, dfR5 and dfR6. HeLa cells were incubated with peptides dfR4, dfR5 and dfR6 as described in part B. The percentage of cells detected as positive for penetration was established by microscopy. **** represents $P < 0.0001$. P-value was determined by comparing the values for the treatments with dfR4/5 to that of dfR6. C) Cellular distribution of dfR4, dfR5 and dfR6 after incubation at 20 μ M, 10 μ M, and 5 μ M, respectively. HeLa cells were incubated with the peptides for 1h in the presence of DEAC-k5 (10 μ M). Cells were washed and imaged with a 20X objective. Fluorescence images of the TMR emission at 560 nm (pseudocolored red) and CFP emission at 436 nm (pseudocolored blue). Scale bars, 100 μ m. The data reported represent the average and corresponding standard deviations of biological triplicates.