Cargo-dependent cytotoxicity and delivery efficacy of cell-penetrating peptides: a comparative study

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The use of CPPs (cell-penetrating peptides) as delivery vectors for bioactive molecules has been an emerging field since 1994 when the first CPP, penetratin, was discovered. Since then, several CPPs, including the widely used Tat (transactivator of transcription) peptide, have been developed and utilized to translocate a wide range of compounds across the plasma membrane of cells both *in vivo* and *in vitro*. Although the field has emerged as a possible future candidate for drug delivery, little attention has been given to the potential toxic side effects that these peptides might exhibit in cargo delivery. Also, no comprehensive study has been performed to evaluate the relative efficacy of single CPPs to convey different cargos. Therefore we selected three of the major CPPs, penetratin, Tat and transportan 10, and evaluated their ability to deliver commonly used cargos, including fluoresceinyl moiety, double-stranded DNA and proteins (i.e. avidin and streptavidin), and studied their effect on membrane integrity and cell viability. Our results demonstrate the unfeasibility to use the translocation efficacy of fluorescein moiety as a gauge for CPP efficiency, since the delivery properties are dependent on the cargo used. Furthermore, and no less importantly, the toxicity of CPPs depends heavily on peptide concentration, cargo molecule and coupling strategy.

Key words: cell-penetrating peptide, cytotoxicity, delivery vector, penetratin, transactivator of transcription (Tat), transport.

INTRODUCTION

The ability to cross the lipid bilayer of cells and access the cell interior is still one of the major obstacles to overcome in order to progress current drug development. Various techniques have therefore been developed in order to improve cellular uptake of bioactive agents [1]. CPPs (cell-penetrating peptides) have, since their discovery in 1994 [2], been widely used to deliver a wide range of bioactive compounds across cellular membranes of several cell types both *in vivo* and *in vitro* (reviewed in [3,4]). Delivery seems to be independent of cell type and can be directed to diverse compartments inside the cell [5]. No proper definition of CPPs has been formulated, but most, if not all, carry a net positive charge, are less than 30 amino acids long and have the ability to rapidly translocate large molecules into cells. Still it is unclear what mechanism is responsible for uptake of CPPs and their cargos. Originally it was believed that CPPs translocated across cell membranes in a receptor- and energy-independent manner. However, more recent studies suggest that the uptake for most CPPs is an energy-dependent process with initial binding of peptides to proteoglycans on the cell surface and concomitant endocytosis of peptides [6–8]. However, there are still reports claiming that membrane translocation is independent of endosome formation and that uptake, in line with early reports, occurs directly through the outer cellular membrane [9,10].

There is a constantly growing number of CPPs introduced in the literature with different chemical properties and abilities to ferry various cargos across cellular membranes. However, studies on CPP-mediated cargo delivery performed so far have focused on achieving high delivery yields, and few studies have focused on the toxicity that these peptides might exhibit. Lindsay and co-workers analysed the toxicity of CPP-mediated peptide delivery in 2005 [11], and Saar et al. [12] studied the toxicity of free CPP uptake. Yet, the results are divergent regarding uptake efficacy and toxicity of single CPPs and different cargo molecules, making comparisons between one study and another very difficult. Furthermore, different cells, cell passages, incubation times, concentrations etc. have been used, making comparisons between studies all the more complicated. Since no comprehensive study has been performed to evaluate the relative efficacy of single CPPs to deliver different cargos, and whether the cargo type might influence the toxicity of peptides, a study using the same conditions would offer a more accurate comparison concerning these aspects.

In the present study, we aimed to evaluate the delivery efficiency and cytotoxicity of three well characterized CPPs, Tat (transactivator of transcription), TP10 (transportan 10) and penetratin [2,13,14] (Table 1), using different cargos. These cargo molecules include carboxyfluorescein that is used on a routine basis to assess the cellular uptake of peptides, dsDNA (double-stranded DNA) that can serve as model for decoy oligonucleotides or possibly siRNAs (small interfering RNAs) and two model proteins: streptavidin and avidin. Proteins have been transported into cells by CPPs and have been shown to be biologically active [3,4], and, in the present study, streptavidin and avidin were utilized as model proteins, as they have been widely studied and have very high affinity for biotinylated peptides, making conjugation less cumbersome. In addition, since different proteins have different pI values, choosing these two proteins as models is appropriate, as their pI values are significantly different

Abbreviations used: CHO, Chinese-hamster ovary; CPP, cell-penetrating peptide; DMF, dimethylformamide; dsDNA, double-stranded DNA; FBS, fetal bovine serum; HKR, Hepes–Krebs–Ringer; HOBt, 1-hydroxybenzotriazole; LDH, lactate dehydrogenase; siRNA, small interfering RNA; Tat, transactivator of transcription; t-Boc, t-butoxycarbonyl; TP10, transportan 10; WST-1, water-soluble tetrazolium salt 1.

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Table 1 Sequences of cell-penetrating peptides and DNA

Bold letters represent nucleobases in DNA. DNA was labelled at the 3′ end with carboxyfluorescein. Fl, fluoresceinyl. Fl[1]-, fluoresceinyl modification at αN^1; Fl[7]-, fluoresceinyl modification at εN^7.

Peptide	Sequence	Abbreviation
Penetratin	RQIKIWFQNRRMKWKK-NH_2	Pen
Fl[1]-Penetratin	Fl-RQIKIWFQNRRMKWKK-NH_2	Fl[1]-Pen
Tat-(47–57)	YGRKKRRQRRR-NH_2	Tat
Fl[1]-Tat-(47–57)	Fl-YGRKKRRQRRR-NH_2	Fl[1]-Tat
Transportan 10	AGYLLGKINLKALAALAKKIL-NH_2	TP10
Fl[7]-transportan 10	AGYLLGK(εNH-Fl)INLKALAALAKKIL-NH_2	$Fl^{\varepsilon N7}$-TP10
Fl[1]-transportan 10	Fl-AGYLLGKINLKALAALAKKIL-NH_2	$Fl^{\alpha N1}$-TP10
Sense DNA	**ACGCCAAAAACATAAAGAAAG**	sDNA
Antisense DNA	**TTCTTTATGTTTTTGGCGTCT**	asDNA

(streptavidin, pI 5.5, and avidin, pI 10.5). The present study does not consider the biological activity of the delivered cargos (see [15] for a thorough review), but rather the cargo-dependent toxicity that the CPP translocation exhibits.

We show that the cellular uptake of CPPs is cargo-dependent, and our results also imply that the cytotoxicity of these peptides is cargo-dependent. Furthermore, we show that the cytotoxicity and internalization level of TP10 vary significantly depending on the cargo-coupling position within the peptide. Taken together, we believe that these results could be useful in future experiments utilizing CPPs to facilitate the choice of peptide depending on cargo and choice of conjugation strategy.

EXPERIMENTAL

Peptide synthesis and purification

The peptides (Table 1) were synthesized in a stepwise manner in a 0.1 mmol scale on an automated peptide synthesizer (Applied Biosystems, Model 433A) using a t-Boc (t-butoxycarbonyl) solid-phase peptide synthesis strategy. t-Boc amino acids were coupled as HOBt (1-hydroxybenzotriazole) esters to a *p*-methylbenzylhydrylamine resin (amino acids and resin purchased from Neosystem) to obtain C-terminally amidated peptides. Deprotection of the formyl protecting group on tryptophan was carried out in 20 % piperidine in DMF (dimethylformamide) for 1 h. Peptides used for uptake studies were N-terminally labelled and TP10 was also labelled orthogonally on Lys[7] with carboxyfluorescein using 5 mol of 5,6-carboxyfluorescein, 5 mol of 1,3-di-isopropylcarbodi-imide, 5 mol of HOBt and 20 mol of *N*,*N*-di-isopropylethylamine dissolved in 1:1 (v/v) DMSO/DMF overnight [16]. Biotin (Sigma) was coupled as a HOBt ester to the N-terminus of Tat and penetratin or to the ε-amino group of Lys[7] of TP10.

The peptide was finally cleaved from the resin using liquid HF at 0 °C for 1 h in the presence of *p*-cresol (1:1). Peptides were purified using reverse-phase HPLC on a C_{18} column, 20–100 % acetonitrile [0.1 % TFA (trifluoroacetic acid)] gradient, and the molecular mass was determined by MALDI–TOF (matrix-assisted laser-desorption ionization–time-of-flight) MS using a PerkinElmer prOTOF™ 2000 MALDI O-TOF mass spectrometer. The peptide purity was > 90 % as determined by analytical HPLC.

Cell culture

HeLa cells were grown in DMEM (Dulbecco's modified Eagle's medium) with GlutaMAX™ supplemented with 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 10 % FBS (fetal bovine serum), 100 units/ml penicillin and 100 mg/ml streptomycin. CHO (Chinese-hamster ovary) cells were grown in MEM (minimal essential medium) with GlutaMAX™ supplemented with 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 10 % FBS, 100 units/ml penicillin and 100 mg/ml streptomycin. Cells were grown at 37 °C in a 5 % CO_2 atmosphere. All media and chemicals were purchased from Invitrogen.

Quantitative cellular uptake

A total of 2×10^5 HeLa or CHO cells were seeded 24 h before experiments in 12-well plates to reach 70 % confluence. In the uptake studies on free peptide, cells were treated with 1 or 5 μM fluorescein-labelled peptides in 500 μl of serum-free medium. In transfection studies of dsDNA, peptides in various concentrations were co-incubated for 30 min in 50 μl of 0.9 % NaCl solution. Cells were then treated with 0.2 μM fluorescein-labelled dsDNA and CPPs in 500 μl of serum-free medium. In the protein transduction experiments, streptavidin or avidin was either complexed with 1 μM biotinylated peptides in serum-free medium or co-incubated with free peptides at various molar ratios in 0.9 % NaCl solution to a final volume of 50 μl. Cells were then treated with 0.2 μM protein and CPPs in 500 μl of serum-free medium. At 1 h after treatment or 90 min after treatment with proteins, cells were washed twice in HKR (Hepes–Krebs–Ringer) buffer before trypsinization. The trypsinization step is crucial in order to remove membrane-associated peptides and/or cargo [17]. Cells were centrifuged at 1000 ***g*** for 5 min at 4 °C, and cell pellets were lysed with 300 μl of 0.1 M NaOH for 60 min, after which 250 μl of lysate was transferred to a black 96-well plate. Fluorescence was measured at 494/518 nm on a Spectra Max Gemeni XS fluorimeter (Molecular Devices) and recalculated to the amount of internalized compound and normalized to the amount of total protein (Lowry assay; Bio-Rad). Fluorescein-labelled peptides, dsDNA and proteins displayed a linear correlation between concentration and fluorescence. DNA was purchased from CyberGene AB, and proteins (streptavidin and avidin) were from Invitrogen. Single-stranded DNA, fluorescein-labelled at the 3′ end of the antisense strand, were hybridized to the sense strand in Milli-Q water containing 0.9 % NaCl for 1 h at 37 °C to create dsDNA with two nucleotides overhanging at the 3′ end. The DNA sequences are presented in Table 1.

LDH (lactate dehydrogenase) leakage assay

Membrane integrity was measured using the Promega CytoTox-ONE™ assay (Promega). In brief, 10^4 HeLa or CHO cells were seeded in 96-well plates 2 days before treatment with 100 μl of the above-mentioned compounds at different concentrations in serum-free medium. After 30 min, 80 μl of medium was transferred to a black fluorescence plate and incubated for 10 min with 80 μl of CytoTox-ONE™ reagent, followed by 40 μl of stop solution. Fluorescence was measured at 560/590 nm. Untreated cells were defined as no leakage and 100 % leakage was defined as total LDH release by lysing cells in 0.18 % Triton X-100 in HKR buffer.

WST-1 (water-soluble tetrazolium salt 1) assay

HeLa or CHO cells were seeded on to 96-well plates, 10^4 cells/well, 2 days before treatment. Cells were treated according to the same procedure as in the LDH leakage assay, but for 24 h. Cells were then exposed to WST-1, according to the manufacturer's protocol (Sigma). Absorbance (450–690 nm) was measured on a Digiscan absorbance reader (Labvision). Untreated cells were defined as 100 % viable. Although the WST-1 assay measures

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Table 2 Long-term toxic effects in HeLa and CHO cells after treatment with CPPs

TP10 reduces proliferation (i.e. mitochondrial activity) significantly at higher concentrations, whereas Tat and penetratin have negligible effects on proliferation up to 50 μM. Overall, CHO cells are less sensitive to CPP treatment. A total of 10^4 cells seeded in 96-well plates 48 h before experiment were treated with the indicated peptide concentrations in 100 μl of serum-free medium for 24 h and analysed using the WST-1 assay. Results are means ± S.E.M.

	Cell viability (%)					
	Tat		Penetratin		TP10	
CPP concentration (μM)	HeLa	CHO	HeLa	CHO	HeLa	CHO
1	96.5 ± 4.5	97.0 ± 1.5	91.3 ± 6.8	112.2 ± 3.8	94.3 ± 7.6	90.4 ± 3.1
2	103.5 ± 6.9	92.7 ± 2.6	86.3 ± 1.1	92.2 ± 4.3	92.9 ± 4.9	85.3 ± 2.4
5	94.8 ± 8.1	87.5 ± 3.6	89.7 ± 6.8	92.7 ± 2.0	85.1 ± 5.9	87.1 ± 3.1
10	92.1 ± 6.6	102.0 ± 3.4	91.1 ± 6.1	112.9 ± 4.1	88.9 ± 5.0	100.0 ± 3.0
20	100.2 ± 6.2	93.5 ± 4.8	86.2 ± 4.5	127.7 ± 5.6	49.7 ± 1.4	76.2 ± 2.3
50	86.6 ± 3.2	95.3 ± 3.4	88.2 ± 7.2	107.7 ± 6.6	16.1 ± 2.0	24.9 ± 1.0

dehydrogenase activity in mitochondria, it is generally distributed and used as a measure of proliferation [18].

Microscopy

A total of 2×10^5 HeLa cells/well were seeded 1 day before exposure with the above-mentioned compounds in 12-well plates. Cells were treated for 1 h and analysed with an Olympus 1×70 microscope (with a DP50 camera) using the Viewfinder Lite V1.0 software.

Statistical analyses

All results are means ± S.E.M. for at least three independent experiments performed in quadruplicate, and statistics were calculated using ANOVA, with Dunnett's post-hoc test. $^{**}P < 0.01$; $^{*}P < 0.05$.

RESULTS

TP10 induces membrane leakage and reduces proliferation dose-dependently

To get a general view of the toxic profile displayed by the selected CPPs, the cytotoxic properties of free (unlabelled) peptides were assessed in HeLa and CHO cells. In order to get a comprehensive analysis of the cytotoxicity, both membrane disturbance and mitochondrial activity were studied. An LDH leakage assay was used to measure the acute membrane disturbance caused by the peptides, and a WST-1 assay was employed to study the more long-term change in proliferation (i.e. mitochondrial dehydrogenase activity). As seen in Table 2, neither penetratin nor Tat affected proliferation at concentrations up to 50 μM. Conversely, TP10 displayed long-term toxic side effects in HeLa and CHO cells at 20 μM. Overall, CHO cells appear to be more resistant to peptide treatment than HeLa cells. Penetratin and Tat have no effect on membrane integrity (Figure 1c, and results not

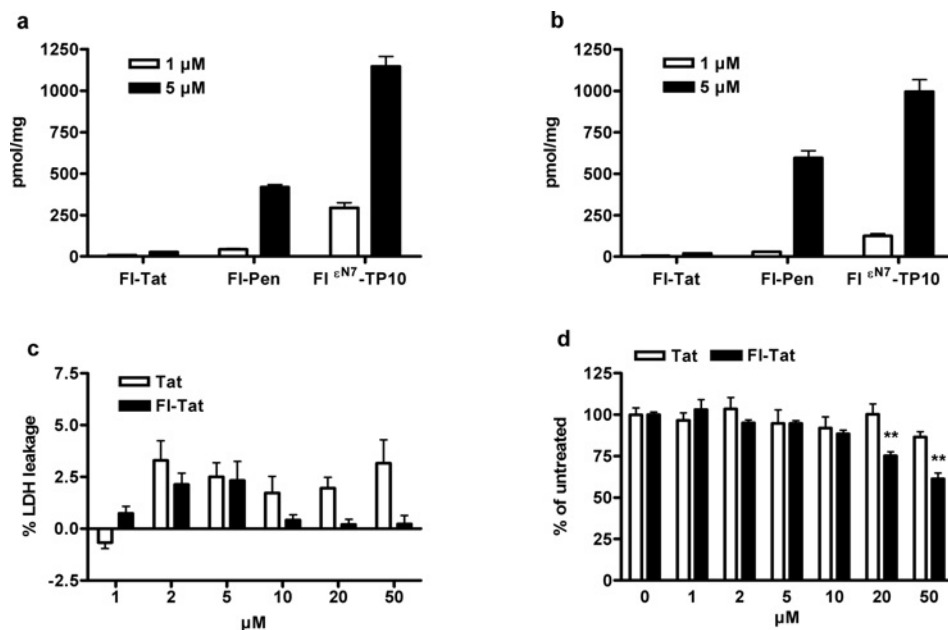

Figure 1 Cellular uptake and toxicity of fluorescein-labelled CPPs

Both TP10 and penetratin are dose-dependently internalized into CHO (**a**) and HeLa (**b**) cells, whereas the uptake of Tat is negligible. Introducing a fluoresceinyl moiety N-terminally on Tat decreases the membrane disturbance (**c**), but increases the long-term toxicity (**d**). In (**a**) and (**b**), 2×10^5 cells/well were seeded 1 day before the experiment in 12-well plates, treated for 1 h with 1 or 5 μM peptide and analysed after washing, trypsinization and centrifugation. Uptake is presented as the amount of internalized peptide (pmol/mg of protein). In (**c**) and (**d**), cells were treated with the same concentrations as in (**a**) and (**b**), but were analysed after different time points. LDH leakage was measured 30 min after treatment (**c**) and WST-1 activity was assayed 24 h after treatment (**d**).

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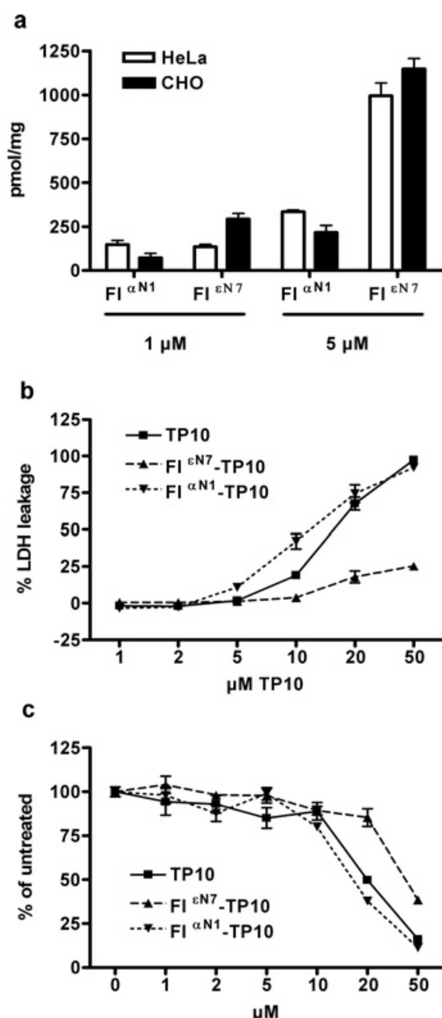

Figure 2 Coupling position within TP10 alters CPP properties

TP10 labelled orthogonally with carboxyfluorescein displays severalfold higher uptake and significantly lower toxicity than N-terminally labelled peptide. (**a**) An uptake study of TP10, labelled at two different positions, in CHO and HeLa cells was conducted as described in Figure 1. (**b**) LDH leakage in HeLa cells after 30 min of treatment with TP10, $Fl^{\varepsilon N7}$-TP10 or $Fl^{\alpha N1}$-TP10 at different concentrations in serum-free medium. (**c**) WST-1 activity in HeLa cells 24 after treatment with TP10, $Fl^{\varepsilon N7}$-TP10 or $Fl^{\alpha N1}$-TP10 at different concentrations in serum-free medium.

shown), whereas TP10 induces LDH leakage (approx. 20 %) at 10 μM in HeLa cells (Figure 2b). The same was observed in CHO cells (results not shown).

Uptake of fluorescein-labelled CPPs: cargo attachment changes the cytotoxic properties of CPPs

When fluorescein-labelled, the selected peptides have vastly different cell-penetrating properties. From Figure 1, the following order of uptake yields was observed: TP10 > penetratin ≫ Tat, where the uptake of Tat is nearly undetectable (Figures 1a and 1b). The yield of internalized peptide is overall slightly higher in CHO cells (Figures 1a and 1b). To see whether the fluorescein moiety alters the toxic properties of the peptides, the membrane integrity and proliferation were compared between free or fluorescein-labelled peptides. Penetratin demonstrated no signs of toxicity, either as a free peptide or when flouresceinylated in any of the two assays (results not shown). Tat, in contrast, displayed slightly higher membrane leakage as a free peptide (Figure 1c), but, interestingly, proliferation was reduced significantly at 20 μM with the labelled peptide (Figure 1d), but not as a free peptide. Since the fluoresceinyl moiety apparently influences the cell viability, it is highly possible that it also alters the uptake levels of some peptides. If this is true, many of the uptake studies performed on CPPs might only show the uptake of the fluorescein-labelled peptide, which do not inevitably correlate with the uptake of the free CPP.

Internalization and cytotoxicity of TP10 are dependent on the cargo-coupling position within the peptide

On a routine basis, TP10 has been orthogonally conjugated with cargo molecules. Although orthogonally coupled TP10 has been shown to deliver various cargos efficiently [19], we wanted to elucidate whether coupling carboxyfluorescein N-terminally to TP10 would change the uptake pattern and cytotoxic profile of the peptide. Surprisingly, internalization of N-terminally labelled TP10 is decreased 4-fold compared with the orthogonally labelled peptide (Figure 2a), suggesting that the cargo-coupling position is an important aspect to consider when designing conjugates, at least in case of TP10. This observation also supports further the hypothesis that uptake of free peptides might deviate significantly from that of fluorescein-labelled ones. Furthermore, orthogonally labelled TP10 displays significantly lower membrane perturbation and long-term toxicity compared with free TP10 or TP10 labelled in the N-terminus (Figures 2b and 2c). Additionally, N-terminally labelled peptide exhibited both higher membrane leakage and long-term toxicity compared with free peptide (Figures 2b and 2c). As seen in Figure 3, both free and N-terminally labelled TP10 slightly altered the morphology and proliferation of HeLa cells treated with 10 μM peptide, while the orthogonally labelled peptide had a negligible effect on cell morphology.

CPPs promote internalization of dsDNA in a relatively non-toxic fashion

As seen in Figure 4(a), all three peptides dose-dependently promoted cellular internalization of dsDNA with the following efficiency order: TP10 > penetratin ⩾ Tat. This pattern is in agreement with the one observed for fluorescein-labelled peptides (Figures 1a and 1b), with the exception of Tat, which seems to be more efficient in translocating dsDNA than when used as a free peptide (Figures 1a, 1b and 4a).

The same fluorescein-labelled dsDNA was used further in complex with increasing amounts of CPPs to examine the cytotoxicity of peptides in non-covalent complexes with dsDNA. Both penetratin and Tat are, together with dsDNA, non-toxic at concentrations of up to 50 μM (Figures 4b and 4c). There is a tendency that Tat in complex with dsDNA decreases the WST-1 activity at concentrations above 20 μM, although this decrease is not significant (Figure 4c). Intriguingly, it appears that the cargo decreases the toxic side effects of TP10, as no sign of toxicity was observed at 10 μM TP10 together with dsDNA (Figures 4b and 4c). This is compared with free TP10, which displays approx. 20 % LDH leakage (Figure 2b) and approx. 11 % decrease in WST-1 activity at 10 μM (Table 2).

Protein uptake varies depending on the CPP and the delivery strategy used

The selected CPPs were studied in two different protein delivery strategies with two dissimilar proteins (streptavidin and avidin).

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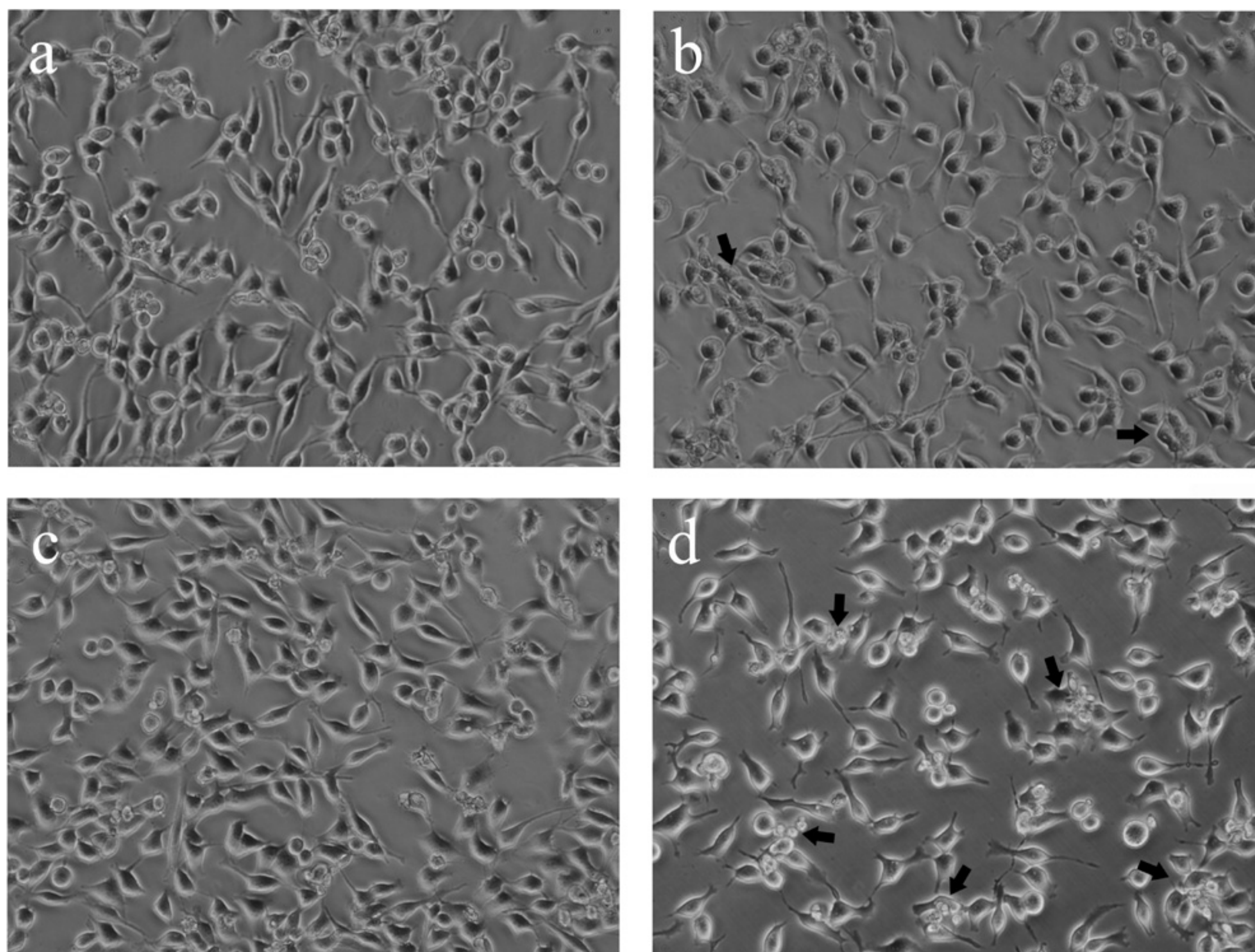

Figure 3 Treatment with TP10 alters cell morphology

Apart from causing membrane perturbation and affecting mitochondrial activity, conjugation of a fluoresceinyl moiety to TP10 also affects cellular morphology. Cells were treated for 1 h with 10 μM peptide. (**a**) Untreated HeLa cells, (**b**) HeLa cells with TP10, (**c**) HeLa cells with $Fl^{\varepsilon N7}$-TP10, and (**d**) HeLa cells with $Fl^{\alpha N1}$-TP10. The total cell count was decreased when treated with TP10 or $Fl^{\alpha N1}$-TP10 compared with untreated or $Fl^{\varepsilon N7}$-TP10 (results not shown). Furthermore, the position of the fluoresceinyl moiety in TP10 affects the morphology of cells after treatment shown by an increased number of cells displaying altered morphology (shown by black arrows). These pictures are representative of at least three independent experiments.

First, the peptides were co-incubated with the proteins and, secondly, the peptides were conjugated to the proteins through a biotin linker.

When comparing data from co-incubation studies on streptavidin, the translocation efficacy is penetratin $\geqslant$ TP10 > Tat (Figure 5a). No significant difference in uptake was observed with avidin with or without peptide co-incubation, most probably since avidin has a cationic nature that prohibits electrostatic interactions with positively charged CPPs (Figure 5b). Again, penetratin and Tat showed no toxicity as determined by LDH leakage (Figure 5d). Also the toxic side effects of TP10 seemed to decrease together with protein compared with free peptide, suggesting that peptide–protein interactions somewhat shield the cellular membrane from perturbation (Figure 5d). However, when measuring the mitochondrial activity of cells 24 h after streptavidin treatment, we observed a strong reduction of approx. 75 % in cell viability, concluding that streptavidin is toxic if applied for longer periods of time (Figure 5e). Interestingly, when co-incubating streptavidin with increasing concentrations of TP10, the long-term toxicity clearly decreases, suggesting that the peptide might form stable complexes with the protein that remain unaltered inside cells and thereby shield the cytotoxic epitopes of the protein (Figure 5e). This pattern was not observed for Tat or penetratin, indicating that these peptides form less stable complexes with streptavidin (results not shown).

The CPP efficacy pattern changes completely when using biotinylated CPPs that form nearly irreversible interactions with streptavidin. Then the observed uptake is as follows: TP10 $\geqslant$ Tat > penetratin (Figure 5c). The same pattern was observed with avidin, but, overall, the level of uptake was higher, most probably since avidin is more cationic, allowing interactions with negatively charged cell surfaces (Figure 5c). Strikingly, Tat efficiently internalizes to cells when conjugated via biotin to proteins, but is poorly internalized as a fluorescein-coupled peptide compared with TP10 and penetratin (Figures 1a, 1b and 5c).

DISCUSSION

In the present study, we compared three of the major CPPs by means of their delivery yield and toxic side effects. We selected the two first discovered CPPs, penetratin and Tat, where the latter has been most extensively used (reviewed in [20]). Both peptides are highly cationic and they have a low amphipathic moment. The

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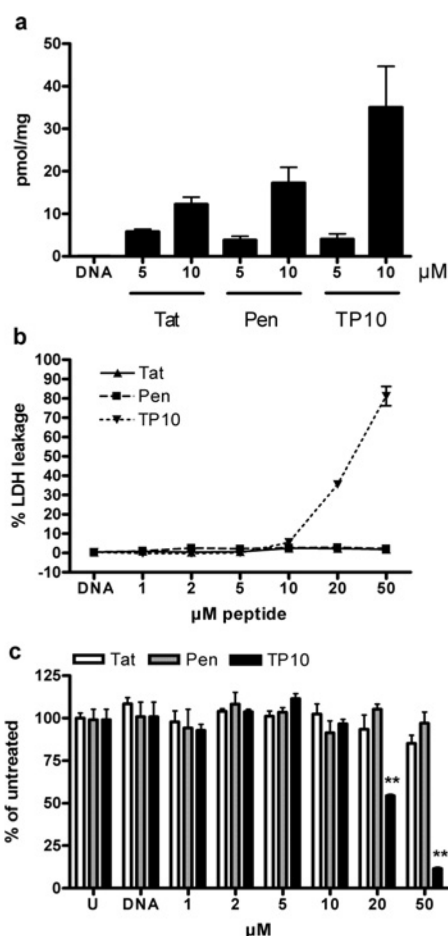

Figure 4 CPPs promote dsDNA internalization

CPPs promote a dose-dependent internalization of fluorescein-labelled dsDNA. (**a**) Fluorescein-labelled dsDNA (0.2 μM) was co-incubated with 5 or 10 μM CPP for 30 min in 0.9 % NaCl solution before addition to cells. Uptake in HeLa cells was measured 1 h after treatment in serum-free medium and displayed as pmol of dsDNA/mg of total protein. (**b**) LDH leakage in HeLa cells after 30 min of treatment with CPP–dsDNA complexes prepared as described previously, but at various peptide concentrations. LDH leakage is presented as percentage of total LDH release. (**c**) WST-1 activity in HeLa cells 24 h post-treatment. Cells were treated as in the LDH leakage assay. Activity is displayed as the percentage of untreated cells. U, untreated control.

third peptide in the present study, TP10, is longer, less cationic and more amphipathic than the other two. Common to all the peptides (Table 1) is the ability to promote cellular uptake of various cargos.

The most common way to determine the cell-penetrating efficiency of different CPPs has so far been through labelling with fluorophores, such as carboxyfluorescein. On a routine basis, peptides have been labelled N-terminally, with the exception of transportan and its analogue TP10, which have been labelled orthogonally on Lys^{13} or Lys^{7} respectively [21]. We wanted to see whether this labelling strategy could influence the cytotoxic properties of different CPPs which thereby also might influence the detected cellular uptake. Free penetratin showed no different in toxicity compared with fluorescein-labelled penetratin. Tat, on the other hand, displays slightly higher membrane perturbation and significantly reduced proliferation as a labelled peptide already at 20 μM (Figure 2b). This difference shows the importance of measuring both acute membrane toxicity, as well as more long-term toxic effects when studying CPPs, since the two do not necessarily correlate.

The striking differences in uptake and toxicity observed between N-terminally and orthogonally labelled TP10 (Figure 1) might be assigned to the hydrophobic property of fluorescein. When coupled N-terminally, it increases the already hydrophobic nature of the peptide tail, resulting in increased membrane destabilization and elevated cytotoxicity. When coupled orthogonally, fluorescein is attached to the cationic part of the peptide, possibly reducing the amphipathicity of the peptide and hence the cytotoxicity. This is just speculation, but it has been shown previously that there might be a correlation between amphipathicity and toxicity of CPPs [12]. This observation also supports further the hypothesis that uptake of free peptides might deviate significantly from that of fluorescein-labelled ones.

The routine use of carboxyfluorescein as a CPP cargo molecule provides a valuable tool for measurements of cellular uptake; however, it is not a biologically relevant cargo. Therefore, to investigate further the potential of using CPPs to facilitate uptake of biologically relevant molecules, dsDNA was utilized as a model cargo for decoy-DNA or siRNA. All CPPs in the present study can promote dsDNA uptake in a dose-dependent manner. Both penetratin and Tat are virtually non-toxic together with dsDNA at concentrations up to 50 μM. The presence of dsDNA seems to decrease the cytotoxic side effects caused by TP10, most probably due to electrostatic and hydrophobic interactions between the peptide and the DNA, which makes cellular membranes less exposed to the peptide.

Introducing proteins to cells offer a great therapeutic potential, as many diseases are caused by deficient protein expression. The major obstacle in protein delivery to date is the poor bioavailability of these molecules. Unlike oligonucleotides, where several transfection reagents have been developed to facilitate the cellular uptake, few delivery vectors exist for proteins. CPPs have been utilized in numerous studies to convey bioactive proteins inside cells [3]. In most cases, peptides have been recombinantly expressed from plasmids as a fusion with the protein, resulting in a conjugate of one peptide per protein [22,23]. Some protein transduction experiments have been conducted with CPPs using the same co-incubation strategy as for oligonucleotides, resulting in non-covalent complexes of several peptides per protein [24,25]. Both of these strategies have been successfully used to transport various proteins inside cells both *in vitro* and *in vivo*.

To get a comprehensive study of protein translocation by CPPs, two diverse methods to promote protein uptake were applied together with two proteins (streptavidin and avidin). Streptavidin and avidin display, as mentioned in the introduction, similar and dissimilar properties, making them suitable as models for whichever protein is desired. Either the CPPs were co-incubated with proteins or biotinylated CPPs were pre-incubated with proteins to form stable CPP–protein complexes.

TP10 and penetratin was found to be the most potent vectors for protein delivery when co-incubated with the proteins (Figure 5a). This is not unexpected, since they both have several hydrophobic residues that can contribute to protein complex formation. Tat, on the other hand, does not promote any protein uptake when utilizing this co-incubation strategy. Since the Tat peptide is a highly cationic with few hydrophobic residues, it might be less prone to forming CPP complexes. Tat is, on the other hand, a potent vector for protein uptake when conjugated to the protein through a stable linker. Then Tat is as efficient as TP10 to promote uptake of both avidin and streptavidin (Figure 5c). These results are in line with several other studies implying that uptake of fluoresceinylated Tat is nearly negligible [26], whereas it is readily internalized when conjugated to a protein [23]. One plausible explanation for this behaviour could be that Tat utilizes different internalization routes depending on coupled cargo. Fluoresceinylated Tat might

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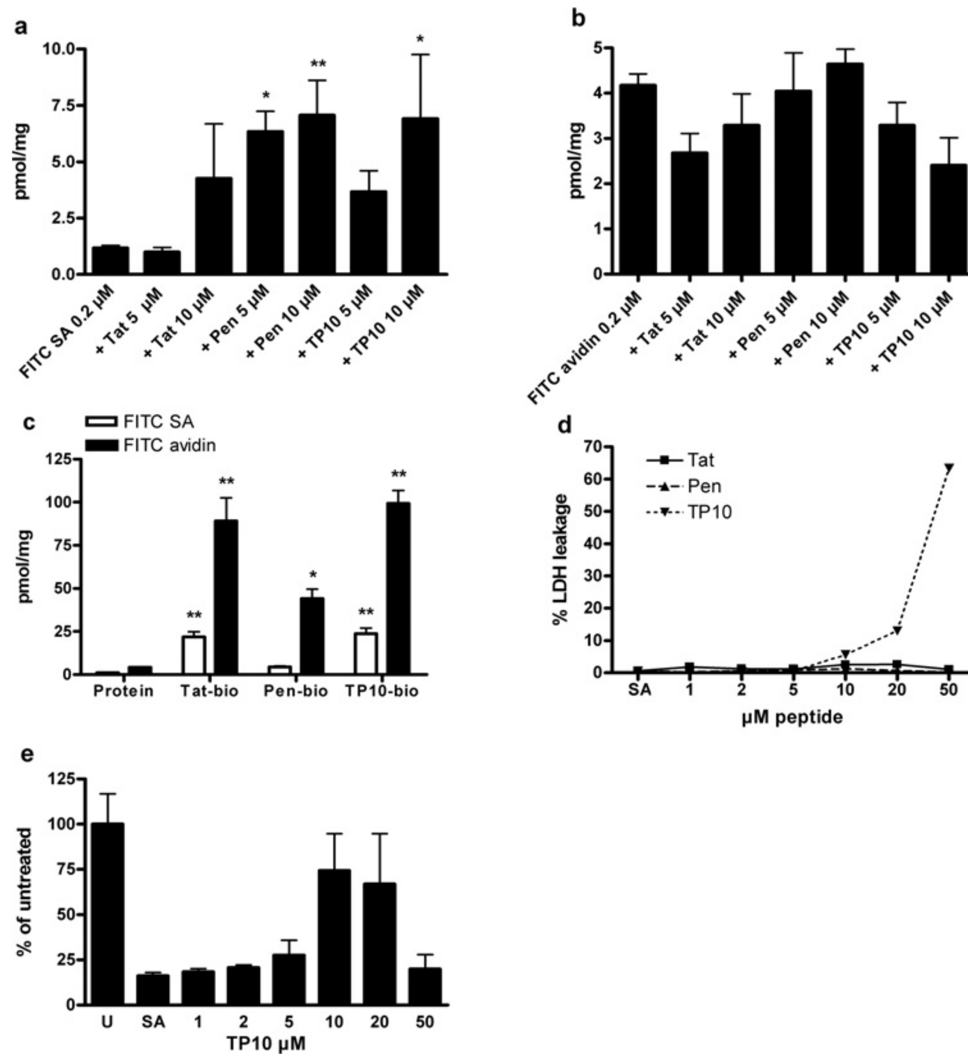

Figure 5 CPPs promote protein internalization

(**a**) CPPs have the ability to deliver 60 kDa proteins into HeLa cells either as non-covalent complexes or conjugated through a stable linker. Tat presents itself as an effective protein transduction vector when coupled to proteins, as compared with fluorescein-labelled peptide. (**a**) Uptake of 0.2 μM FITC–streptavidin (SA) pre-incubated with CPPs (5 or 10 μM) in 50 μl of 0.9 % NaCl and treated for 90 min in serum-free medium. Uptake is displayed as pmol of SA or avidin/mg of total protein. (**b**) Uptake of 0.2 μM FITC–avidin pre-incubated with CPPs (5 or 10 μM) in 50 μl of 0.9 % NaCl. Uptake is displayed as pmol of avidin/mg of total protein. None of the peptides promoted cellular uptake of avidin when co-incubated with the protein. (**c**) Uptake of 0.2 μM FITC–SA and FITC–avidin, pre-incubated with 1 μM biotinylated (bio) CPPs in 50 μl of serum-free medium and treated for 90 min. Uptake results are expressed as pmol of SA or avidin/mg of protein. (**d**) LDH leakage after 30 min of treatment with 0.2 μM FITC–SA complexed with 1–50 μM CPPs, displayed as the percentage of total LDH release. (**e**) WST-1 activity after 24 h of TP10 treatment together with SA. TP10 seemed to shield cells from SA toxicity at 10 and 20 μM concentrations. U, untreated control.

have the ability to pass directly through the plasma membrane, as suggested recently [27], but the uptake is not high enough to be visualized. In contrast, when a large cargo has been attached to the peptide, the uptake mechanism is changed to endocytosis, as suggested previously [8,23,28]. Furthermore, it seems that conjugating peptides to proteins is overall more effective than co-incubating peptides with proteins, at least when using the above-mentioned proteins.

On the whole, TP10 presents itself as the most efficient CPP of the tested peptides to deliver different cargos. As with many other delivery vectors, it seems to be a correlation between efficacy and toxicity at higher concentrations. Therefore, although TP10 is an effective transporter, it cannot be administered to cells at as high concentrations as Tat or penetratin.

Penetratin displays virtually no membrane perturbation or long-term toxicity up to 50 μM concentration, and the uptake yield is average compared with Tat and TP10 for all tested compounds. Tat coupled to fluorescein is, as reported previously [29], poorly taken up by the cell lines tested compared with penetratin and TP10. However, when conjugated to protein, Tat translocation is increased dramatically. Consequently, our results emphasize that it is unfeasible to compare the uptake of fluorescein-labelled CPPs, and we can use these results as a gauge for translocation efficacy for various cargos. Furthermore, certain CPPs may be practical for use with one cargo, but be insufficient to use as a vector for another. Cargos, such as dsDNA and proteins, seem to decrease both the acute and long-term toxicity of CPPs. However, this is not true for all cargo molecules, since the fluorescein moiety increases the toxicity of Tat (Figure 1d), and also TP10 displays completely different cytotoxic properties when conjugated to fluorescein (Figures 2b and 2c).

There are many impediments to overcome before CPPs can be used as efficient and safe pharmaceutical vectors. Obviously, the mechanism of entry must be resolved, but, additionally, it is

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important to use appropriate peptide vectors for desired cargos. We have shown that different peptides are suitable for different cargos and that the uptake yield of one cargo does not assure that the same CPP is effective in delivery for all bioactive molecules. In addition, we have also shown that the cytotoxicity of peptides is highly dependent on the cargo used and the cargo-coupling position within the peptide. In conclusion, we believe that the results presented in this paper can serve as guidelines to select appropriate CPPs for specific cargos.

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