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### ► To cite this version:

Samir El-Andaloussi, Peter Järver, Henrik J Johansson, Ülo Langel. Cargo-dependent cytotoxicity and delivery efficacy of cell-penetrating peptides: a comparative study. *Biochemical Journal*, 2007, 407 (2), pp.285-292. 10.1042/BJ20070507. hal-00478786

**HAL Id: hal-00478786**

**<https://hal.science/hal-00478786>**

Submitted on 30 Apr 2010

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

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Running Title: Cargo dependent toxicity of cell-penetrating peptides

## **Abstract**

The use of cell-penetrating peptides (CPPs) 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 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 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 fluorescein moiety, double stranded DNA, and proteins (ie. avidin and streptavidin), and studied their effect on membrane integrity and cell viability. Our results demonstrate the unfeasibility to use translocation efficacy of fluorescein moiety as a gauge for CPP efficiency since the delivery properties are depending on cargo used. Further, and not less important, toxicity caused by CPPs depend heavily on peptide concentration, cargo molecule, and coupling strategy.

## **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. Therefore, various techniques have been developed in order to improve cellular uptake of bioactive agents [1]. Cell-penetrating peptides (CPPs) 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 cell membranes in a receptor- and energy independent manner. However, 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 is, in line with early reports, occurring 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 et al analyzed the toxicity of CPP mediated peptide delivery in 2005 [11], and Saar et al studied the toxicity of free CPP uptake 2005 [12]. 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. Further, different cells, cell passages, incubation times, concentrations et cetera, 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.

Here we aim to evaluate the delivery efficiency and cytotoxicity of three well characterized CPPs, Tat, transportan 10 (TP10), 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, double stranded DNA (dsDNA) that can serve as model for decoy oligonucleotides or possibly short interfering RNAs (siRNAs), and two model proteins streptavidin and avidin. Proteins have been transported into cells by CPPs and shown to be biologically active [3, 4], and in this 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 isoelectric points (pI), choosing these two proteins as models is appropriate as they have significantly different pI (streptavidin 5.5 and avidin 10.5). The 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 exhibit.

We show here 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 varies significantly depending on 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 Procedures

### 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 *t*-Boc solid-phase peptide synthesis strategy. *tert*-Butyloxycarbonyl amino acids were coupled as hydroxybenzotriazole (HOBT) esters to a *p*-methylbenzylhydramine resin (amino acids and resin purchased from Neosystem, Strasbourg, France) to obtain C-terminally amidated peptides. Deprotection of the formyl protecting group on tryptophan was carried out in 20% piperidine in DMF for 1 h. Peptides used for uptake studies were labeled N-terminally and TP10 was also labeled orthogonally on Lys<sup>7</sup> with carboxyfluorescein using 5 eq. 5,6-carboxyfluorescein, 5 eq. 1,3-diisopropylcarbodiimide, 5 eq. HOBT and 20 eq. N,N-diisopropylethylamine dissolved in dimethyl sulfoxide/dimethylformamide 1:1 over night [16]. Biotin (Sigma, Sweden) was coupled as hydroxybenzotriazole ester to the N-terminus of tat and penetratin or to the  $\epsilon$ -amino group of lys<sup>7</sup> 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 reversed phase HPLC on a C18 column, 20-100% Acetonitrile (0.1% TFA) gradient, and the molecular weight was determined by MALDI-TOF mass spectrometry using Perkin Elmer prOTOF™ 2000 MALDI O-TOF Mass Spectrometer (Perkin Elmer, Sweden). The peptide purity was >90% as determined by analytical HPLC.

### Cell culture

HeLa cells were grown in Dulbecco's Modified Eagle's Media (DMEM) with glutamax supplemented with 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 10

% FBS, 100 U/ml penicillin, and, 100 mg/ml streptomycin. CHO cells were grown in minimal essential media (MEM) with glutamax supplemented with 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 10 % FBS, 100 U/ml penicillin, and, 100 mg/ml streptomycin. Cells were grown at 37 °C in 5 % CO<sub>2</sub> atmosphere. All media and chemicals were purchased from Invitrogen (Sweden).

### **Quantitative cellular uptake**

200.000 HeLa or CHO cells were seeded 24 h prior to experiments in 12-well plates to reach 70 % confluency. In the uptake studies on free peptide, cells were treated with 1 or 5 µM fluoresceinyl labeled peptides in 500 µl serum free media. In transfection studies of dsDNA, peptides in various concentrations were co-incubated for 30 min in 50 µl 0.9 % NaCl solution. Cells were then treated with 0.2 µM fluoresceinyl labeled dsDNA and CPPs in 500 µl serum free media. In the protein transduction experiments, streptavidin or avidin were either complexed with 1 µM biotinylated peptides in serum free media media 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 serum free media. One hour after treatment or 90 min after treatment with proteins, cells were washed twice in Hepses Krebb Ringer (HKR) buffer before trypsination. The trypsination step is crucial in order to remove membrane associated peptides and/or cargo [17]. Cells were centrifuged at 1000g for 5 min at 4 °C and cell pellets were lysed with 300 µl 0.1 M NaOH for 60 min after which 250 µl lysate was transferred to a black 96-well plate. Fluorescence was measured at 494/518 nm on a Spectra Max Gemini XS fluorometer (Molecular devices, USA) and recalculated to the amount of internalized compound and normalized to the amount of total protein (Lowry BioRad, USA). Fluoresceinyl labeled peptides, dsDNA, and proteins displayed linear correlation between concentration and fluorescence. DNA was purchased from CyberGene

AB (Sweden) and proteins (streptavidin and avidin) from Invitrogen (Sweden). Single stranded DNA, fluoresceinyl labeled in the 3' end of the antisense strand, were hybridized to the sense strand in MQ water containing 0.9 % NaCl for 1h at 37 °C to achieve dsDNA with two nucleotides overhang at the 3' end. The DNA sequences are presented in Table 1.

### **LDH leakage assay**

Membrane integrity was measured using the Promega CytoTox-ONE™ assay (Promega, Sweden). In brief, 10<sup>4</sup> HeLa or CHO were seeded in 96-well plates two days before treatment with 100 µl of the above mentioned compounds at different concentrations in serum free media. After 30 min, 80 µl media was transferred to a black fluorescence plate and incubated for 10 min with 80 µl CytoTox-ONE™ reagent followed by 40 µl stop solution. Fluorescence was measured at 560/590 nm. Untreated cells were defined as no leakage and 100% leakage was defined as total lactate dehydrogenase (LDH) release by lysating cells in 0.18% triton X100 in HKR buffer.

### **Wst-1 assay**

HeLa or CHO cells were seeded onto 96-well plates, 10.000 cells/well, two days prior 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 manufacturers protocol (Sigma, Sweden). Absorbance (450-690nm) was measured on absorbance reader Digiscan (Labvision, Sweden). Untreated cells were defined as 100% viable. Although the wst-1 assay measures dehydrogenase activity in mitochondria, it is generally distributed and used as a measure of proliferation [18].

## **Microscopy**

200.000 HeLa cells/well were seeded one day prior to exposure with the above mentioned compounds in 12-well plates. Cells were treated for one hour and analyzed in an Olympus 1X70 microscope (with a DP50 camera) using the Wiewfinder Lite V1.0 software.

## **Statistic analyses**

All results represent the mean of at least three independent experiments done in quadruplicate (mean  $\pm$ SEM,  $n=3$ ) and statistics were calculated using Anova, Dunnett's.

\*\*: $p<0,01$  \*: $p<0,05$ .

## 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 unlabeled peptides was assessed in HeLa and CHO cells. In order to get a comprehensive analysis of the cytotoxicity, both membrane disturbance and mitochondrial activity was studied. A 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 affect proliferation at concentrations up to 50  $\mu$ M. TP10 conversely displays long-term toxic side effects in HeLa and CHO cells at 20  $\mu$ M concentration. Overall, CHO cells appear more resistant to peptide treatment than HeLa cells. Penetratin and Tat have no effect on membrane integrity (Fig. 1c, and data not shown) while TP10 induces LDH leakage (approximately 20 %) at 10  $\mu$ M concentration in HeLa cells (Fig. 2b). The same was observed in CHO cells (data not shown).

### **Uptake of fluoresceinyl labeled CPPs: cargo attachment changes the cytotoxic properties of CPPs**

As fluorescein labeled, the selected peptides have vastly different cell-penetrating properties. From Fig. 1, the following order of uptake yields was observed; TP10 > Penetratin >> Tat, where the uptake of Tat is nearly undetectable (Fig. 1a, b). The yield of internalized peptide is overall slightly higher in CHO cells (Fig. 1a, b). To see whether the fluorescein moiety alters the toxic properties of the peptides, the membrane integrity and proliferation was compared between free or fluoresceinyl labeled peptides. Penetratin shows no signs of toxicity, neither as a free peptide or when fluoresceinylated in any of the two assays (data not shown). Tat, in contrast, displays slightly higher membrane leakage as free peptide (Fig. 1c)

but, interestingly, proliferation is reduced significantly already at 20  $\mu\text{M}$  concentration with the labeled peptide (Fig. 1d) but not as a free peptide. Since the fluoresceinyl moiety apparently influence 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 labeled peptide, which do not inevitably correlate with the uptake of the unlabeled CPP.

### **Internalization and cytotoxicity of TP10 is dependent on 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 efficiently deliver various cargos [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 labeled TP10 is decreased four-fold compared to the orthogonally labeled peptide (Fig. 2a), suggesting that cargo coupling position is an important aspect to consider when designing conjugates, at least in case of TP10. This observation also further supports the hypothesis that uptake of free peptides might deviate significantly from that of fluoresceinyl labeled ones. Furthermore, orthogonally labeled TP10 displays significantly lower membrane perturbation and long term toxicity compared to free TP10 or TP10 labeled in the N-terminus (Fig. 2b, c). Additionally, N-terminally labeled peptide exhibit both higher membrane leakage and long term toxicity compared to unlabeled peptide (Fig. 2b, c). As seen in Fig. 3, both unlabeled and N-terminally labeled TP10 slightly alter the morphology and proliferation of HeLa cells treated with 10  $\mu\text{M}$  peptide, while the orthogonally labeled peptide has negligible effect on cell morphology.

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

As seen in Fig. 4a, all three peptides dose-dependently promote cellular internalization of dsDNA with the following efficiency order: TP10 > Penetratin  $\geq$  Tat. This pattern is in agreement with the one observed for fluoresceinyl labeled peptides (Fig. 1a, b) with the exception for Tat, that seems more efficient in translocating dsDNA than when used as a free peptide (Fig 1a, b, Fig. 4a).

The same fluoresceinyl labeled dsDNA was further used 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 nontoxic at concentrations up to 50  $\mu$ M (Fig. 4b, c). There is a tendency that Tat in complex with dsDNA decreases the wst-1 activity at concentrations above 20  $\mu$ M, although this decrease is not significant (Fig. 4c). Intriguingly, it appears that the cargo decreases the toxic side effects of TP10, as no sign of toxicity was observed at 10  $\mu$ M TP10 together with dsDNA (Fig. 4b, c). This is compared to free TP10, which displays approximately 20 % LDH leakage (Fig. 2b) and about 11 % decrease in wst-1 activity, at 10 $\mu$ M (Table 2).

### **Protein uptake varies depending on CPP and delivery strategy used**

The selected CPPs were studied in two different protein delivery strategies with two dissimilar proteins (streptavidin and avidin). First, the peptides were co-incubated with the proteins and second, 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  $\geq$  TP10 > Tat (Fig. 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 prohibit electrostatic interactions with positively charged CPPs (Fig. 5b).

Again, penetratin and Tat show no toxicity displayed by LDH leakage (Fig. 5d). Also the toxic side effects of TP10 seem to decrease together with protein compared to free peptide, suggesting that peptide-protein interactions somewhat shield the cellular membrane from perturbation (Fig. 5d). However, when measuring the mitochondrial activity of cells 24 h after streptavidin treatment, we observed a strong reduction of approximately 75 % in cell viability, concluding that streptavidin is toxic if applied for longer periods of time (Fig. 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 (Fig. 5e). This pattern was not observed for Tat or penetratin, indicating that these peptides form less stable complexes with streptavidin (data 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  $\geq$  Tat > Penetratin (Fig. 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 (Fig. 5c). Strikingly, Tat efficiently internalizes to cells when conjugated via biotin to proteins but is poorly internalized as fluorescein coupled peptide compared to TP10 and penetratin (Fig. 5c and Fig. 1 a, b).

## Discussion

In this 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 third peptide in this study, TP10, is longer, less cationic and more amphipathic than the other two. Common for all the peptides (Table 1) is their 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 labeling with fluorophores, such as carboxyfluorescein. On a routine basis, peptides have been labeled N-terminally, with the exception of transportan and its analog TP10, that have been labeled orthogonally on Lys<sup>13</sup> or Lys<sup>7</sup>, respectively [21]. We wanted to see whether this labeling strategy could influence the cytotoxic properties of different CPPs which thereby also might influence the detected cellular uptake. Non-labeled penetratin showed no different in toxicity compared to fluorescein labeled. Tat on the other hand displays slightly higher membrane perturbation and significantly reduced proliferation

as a labeled peptide already at 20  $\mu\text{M}$  concentrations (Fig 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 does not necessarily correlate.

The striking differences in uptake and toxicity observed between N-terminally and orthogonally labeled TP10 (Fig. 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 previously been shown that there might be a correlation between amphipathicity and toxicity of CPPs [12]. This observation also further supports the hypothesis that uptake of free peptides might deviate significantly from that of fluoresceinyl labeled 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 further investigate 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 this study can promote dsDNA uptake in a dose-dependent manner. Both penetratin and Tat are virtually nontoxic together with dsDNA at concentrations up to 50  $\mu\text{M}$ . 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 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 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 (Fig 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 co-incubation strategy. Since the Tat peptide is a highly cationic with few hydrophobic residues it might be less prone to form cell penetrating protein 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 (Fig 5c). These results are in line with several other studies implying that uptake of fluoresceinylated Tat is nearly negligible [26] while it is readily internalized when conjugated to a protein [23]. One plausible explanation for this behavior could be that Tat utilizes different internalization routes depending on coupled

cargo. Fluoresceinylated Tat might have the ability to pass directly through the plasma membrane as suggested recently in [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 in [8, 23, 28]. Further, it seems that conjugating peptides to proteins is overall more effective than co-incubating peptides with proteins, at least when using 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 can not 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  $\mu$ M concentration, and the uptake yield is average compared to Tat and TP10 for all tested compounds. Tat coupled to fluorescein is as previously reported poorly taken up by tested cell lines compared to penetratin and TP10 [29]. However, when conjugated to protein, Tat translocation is increased dramatically. Consequently, our results emphasize that it is unfeasible to compare uptake of fluoresceinyl labeled CPPs and later use these results as a gauge for translocation efficacy for various cargos. Further, 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 fluorescein moiety increases the toxicity of Tat (Fig 1d), and also TP10 displays completely different cytotoxic properties when conjugated to fluorescein (Fig 2b, c).

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 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 bio-active molecules. In addition we have also shown that the cytotoxicity of peptides is highly dependent on cargo used and 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.

### **Acknowledgements**

The authors would like to thank the Swedish Research Council (VR-NT, VR-Med) for supporting this work.

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### Figure legends

**Figure 1.** Both TP10 and penetratin are dose-dependently internalized to CHO (a) and HeLa (b) cells, while 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), 200 000 cells/well seeded one day prior experiment in 12-well plates, treated for 1 h with 1 or 5  $\mu$ M peptide and analyzed after washing, trypsination, and centrifugation. Uptakes are presented as amount of internalized peptide (pmol/ mg protein). In c) and d) cells were treated with the same concentrations as in a and b, but analyzed after

different time points. LDH leakage was measured 30 min post treatment (c) and wst-1 activity was assayed 24 h post treatment (d).

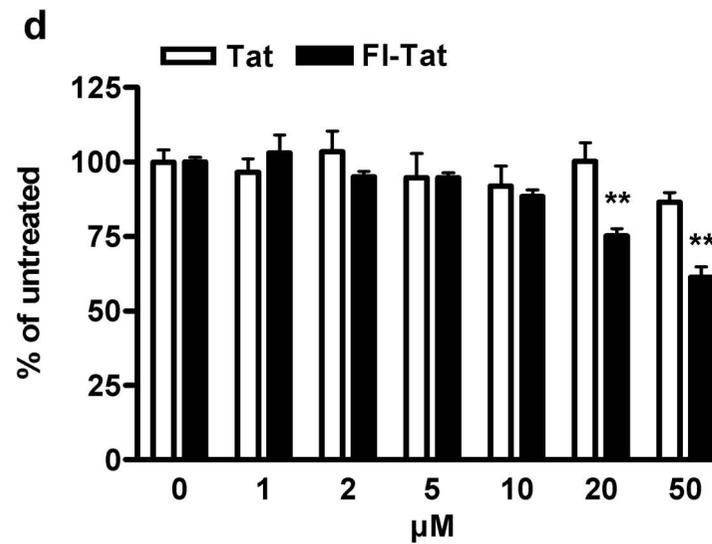
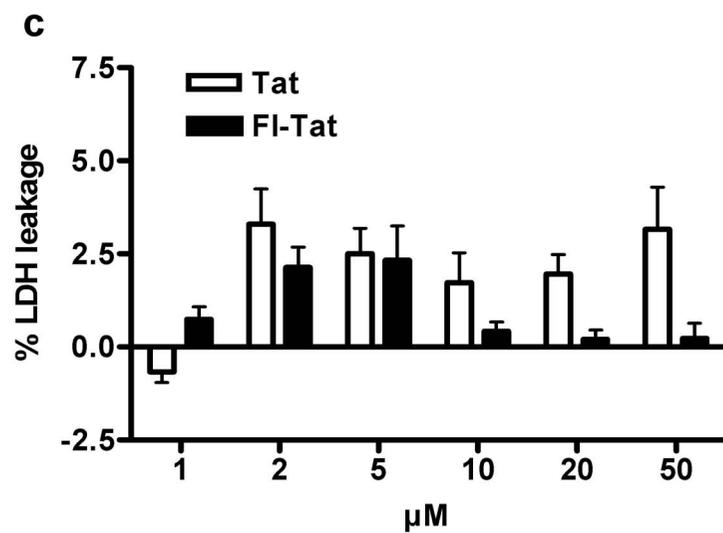
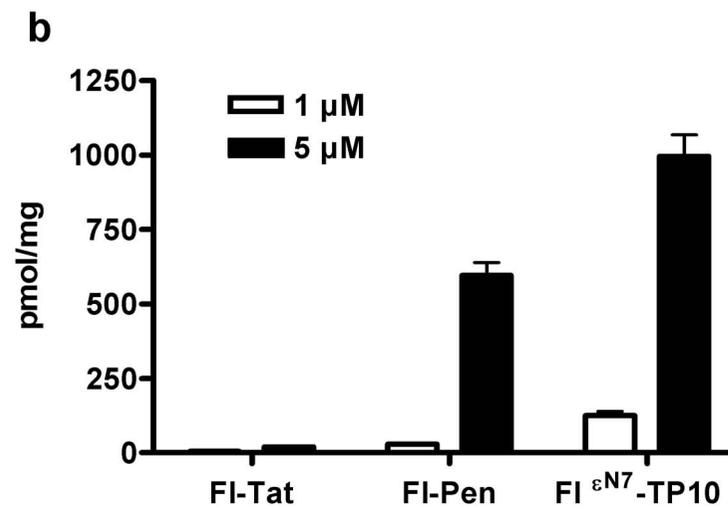
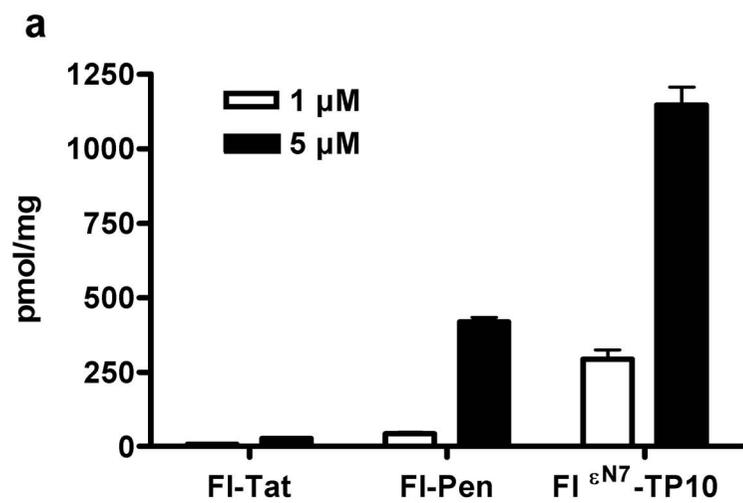
**Figure 2.** TP10 labeled orthogonally with carboxyfluorescein displays several fold higher uptake and significantly lower toxicity than N-terminally labeled peptide. a) Uptake study on TP10, labeled at two different positions, in CHO and HeLa cells was conducted as described in Fig. 1. b) LDH leakage in HeLa cells after 30 min treatment with TP10, Fl<sup>εN7</sup>-TP10, or Fl<sup>αN1</sup>-TP10 at different concentrations in serum free media. c) Wst-1 activity in HeLa cells 24 post treatment with TP10, Fl<sup>εN7</sup>-TP10, or Fl<sup>αN1</sup>-TP10 at different concentrations in serum free media.

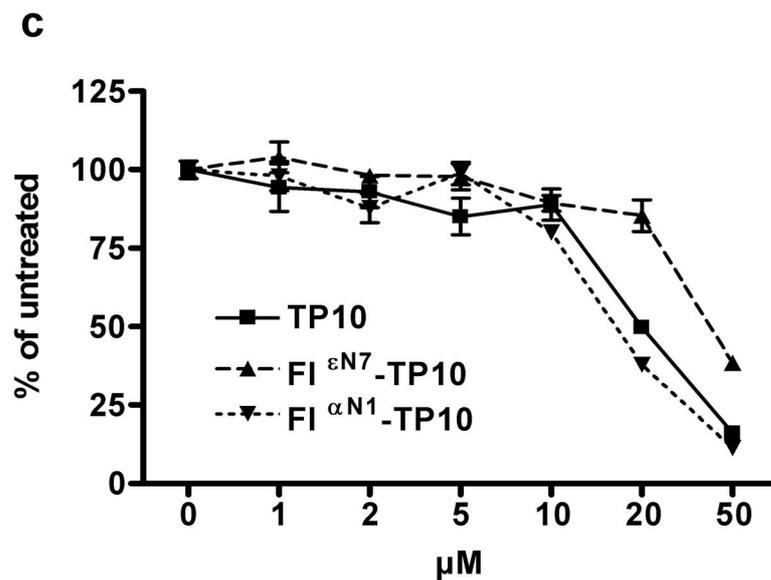
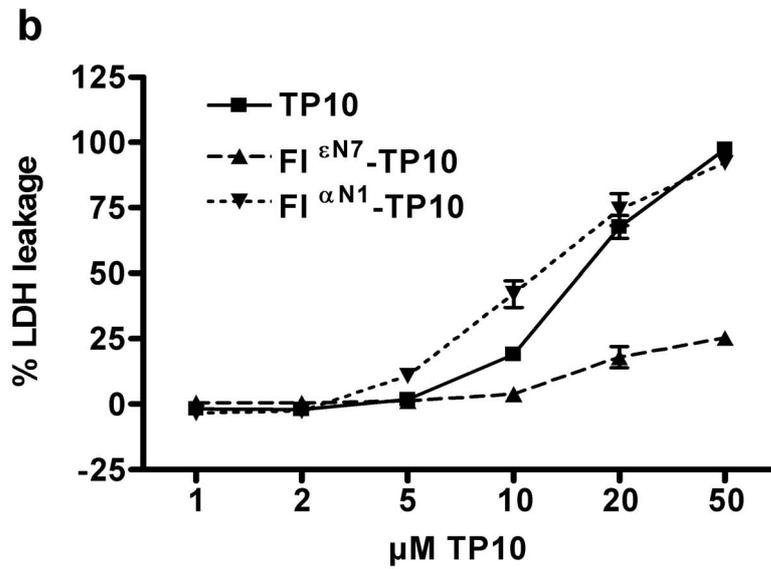
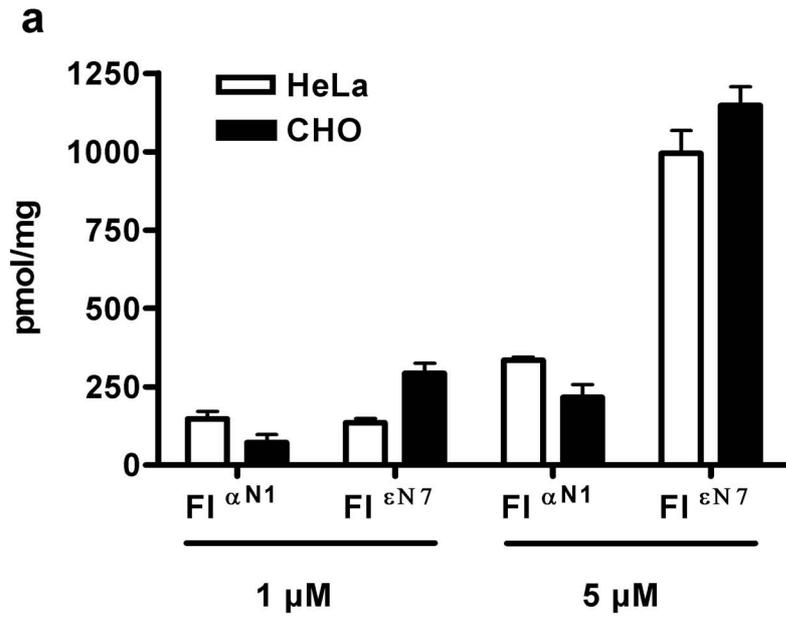
**Figure 3.** Apart from causing membrane perturbation and affecting mitochondrial activity, conjugation of fluoresceinyl moiety to TP10 also affects cellular morphology. Cells were treated for 1 h with 10 μM peptide. a) Untreated HeLa cells b) TP10 , c) Fl<sup>εN7</sup>-TP10 and d) Fl<sup>αN1</sup>-TP10. The total cell count was decreased when treated with TP10 or Fl<sup>αN1</sup>-TP10 compared to untreated or Fl<sup>εN7</sup>-TP10 (data not shown). Further, position of the fluoresceinyl moiety in TP10 affects the morphology of cells after treatment shown by an increased number of cells displaying altered morphology (pointed out by black arrow heads) These pictures are representative from at least three independent experiments.

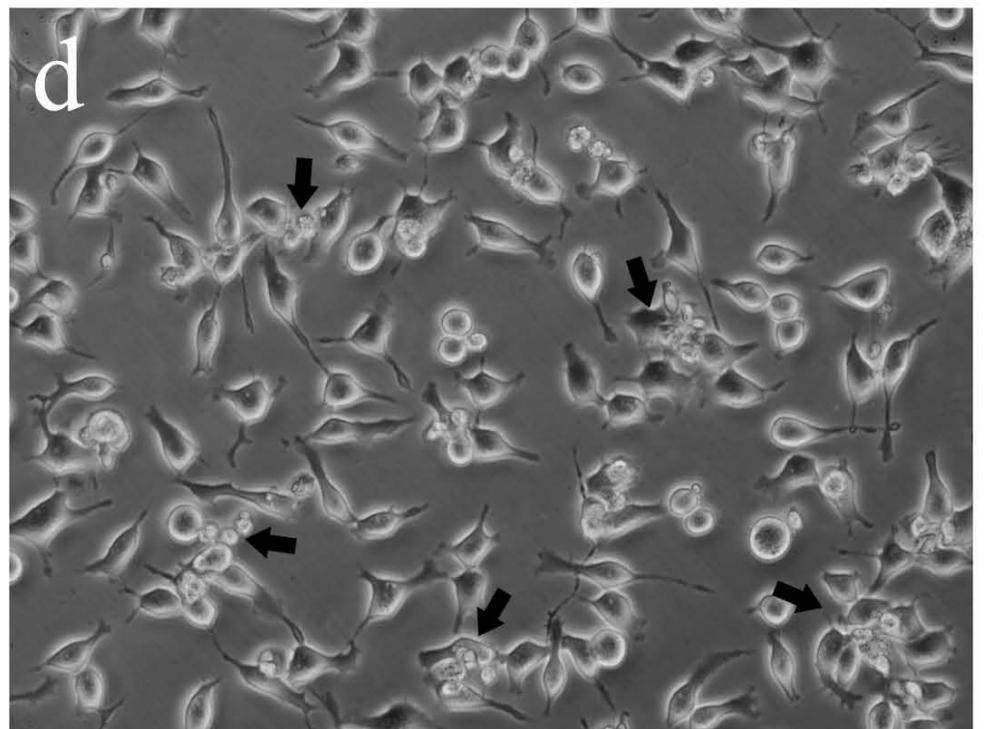
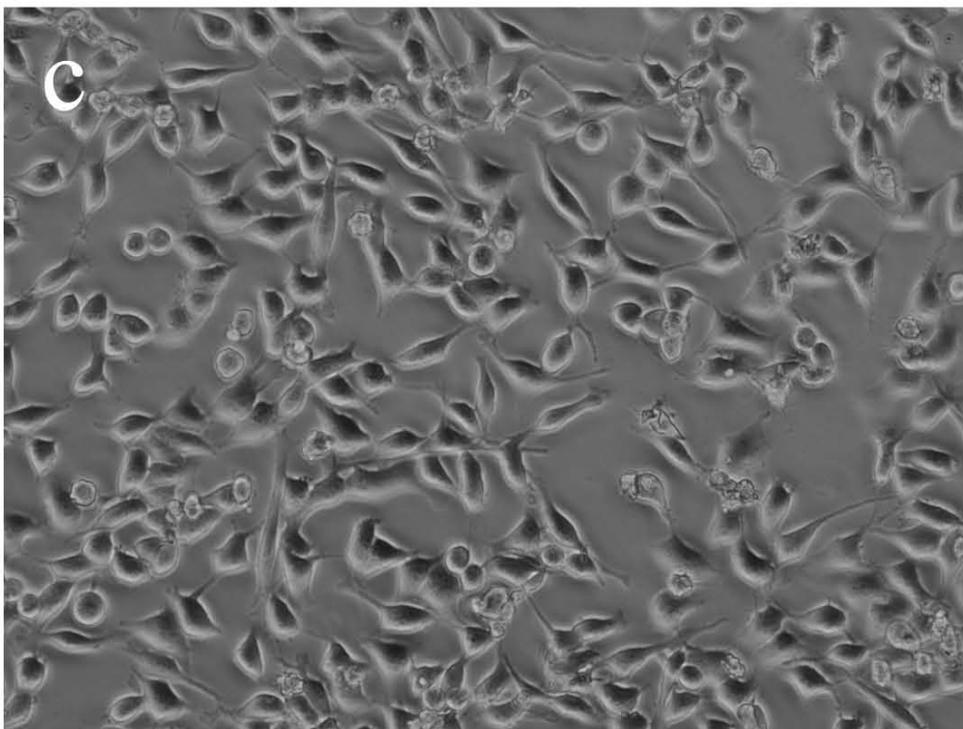
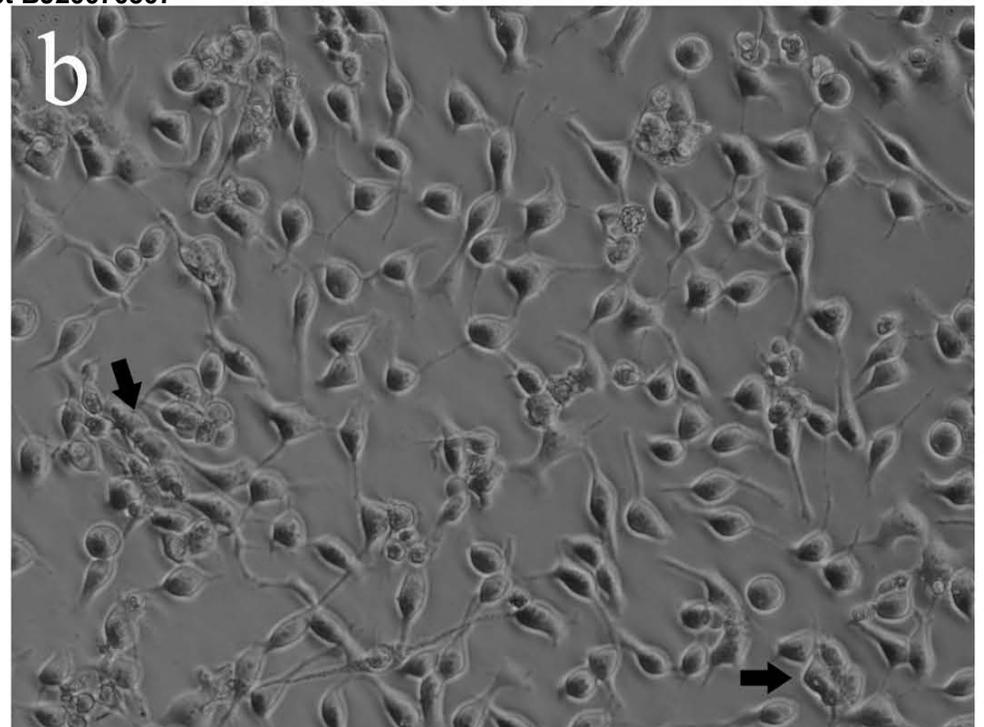
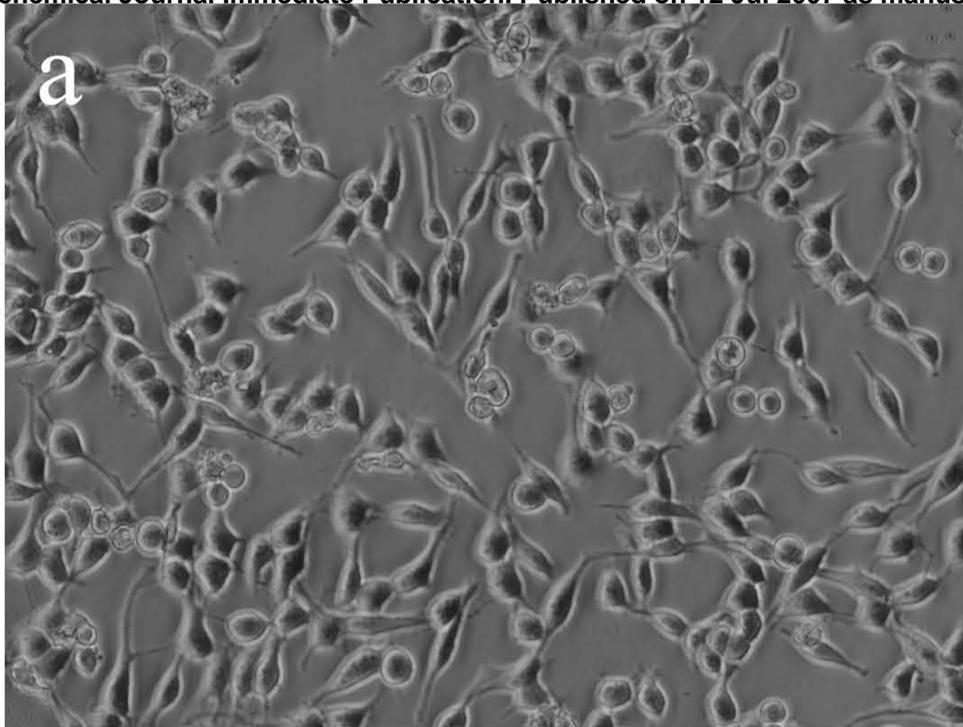
**Figure 4.** CPPs promote a dose-dependent internalization of fluoresceinyl labeled dsDNA. a) 0.2 μM fluoresceinyl labeled dsDNA was co-incubated with 5 or 10 μM CPP for 30 min in 0.9 % NaCl solution prior addition to cells. Uptake in HeLa cells was measured 1 h after treatment in serum free media and displayed as pmol dsDNA/mg total protein. b) LDH leakage in HeLa cells after 30 min treatments with CPP-dsDNA complexes prepared as

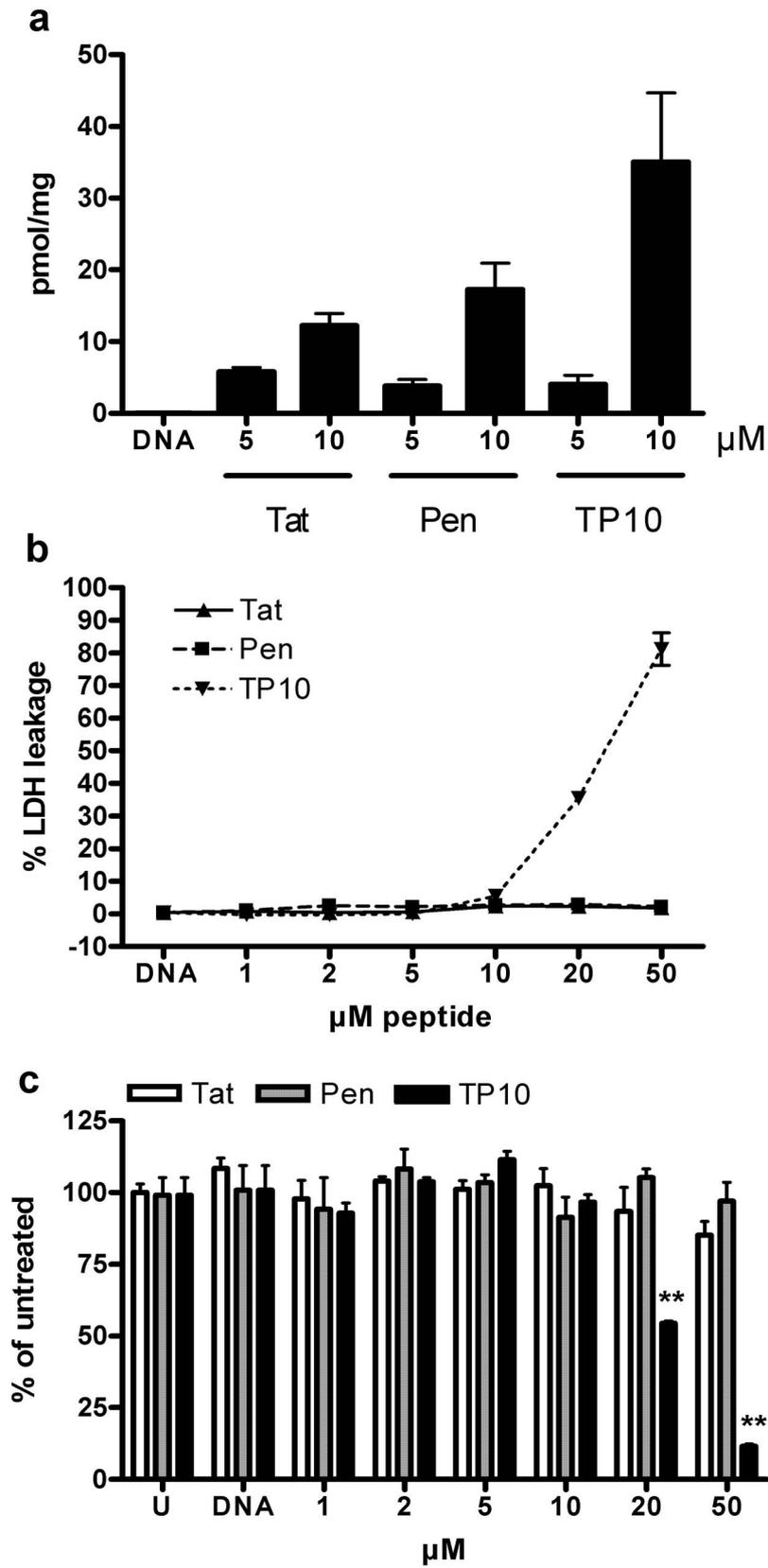
previously described, but at various peptide concentrations. LDH leakage is presented as percentage of total LDH release. c) Wst-1 activity in HeLa cells 24 h post treatment. Cells were treated as in the LDH leakage assay. Activity displayed as percentage of untreated cells.

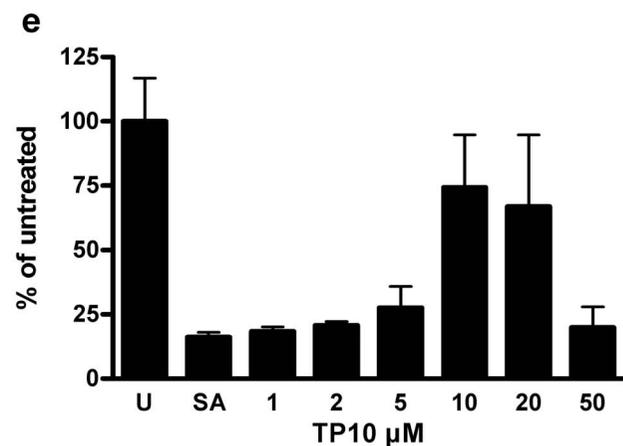
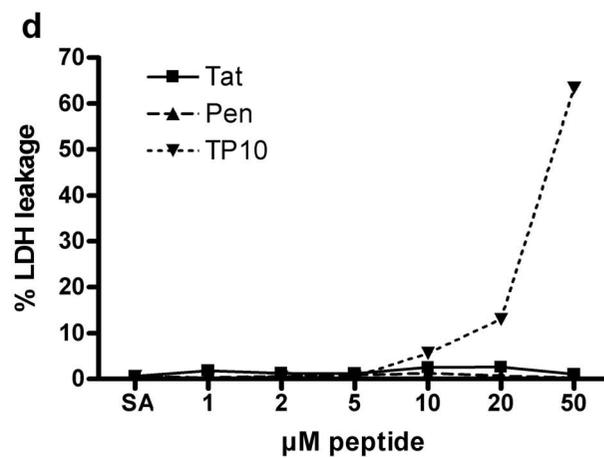
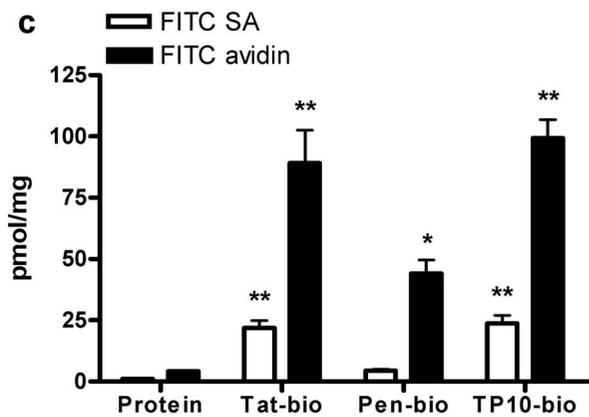
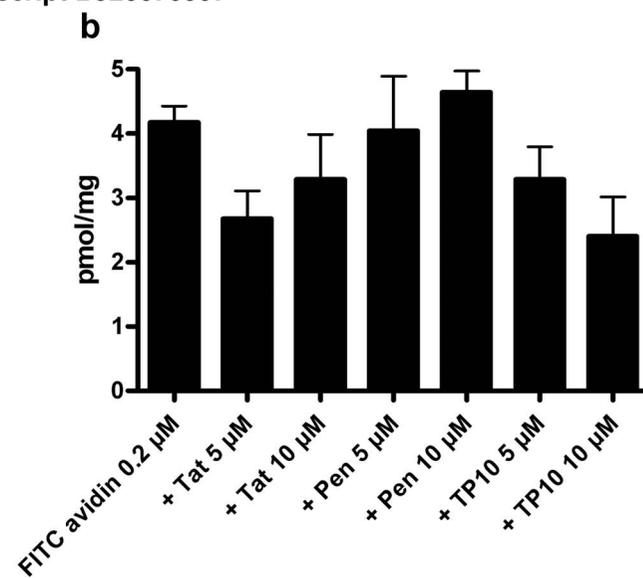
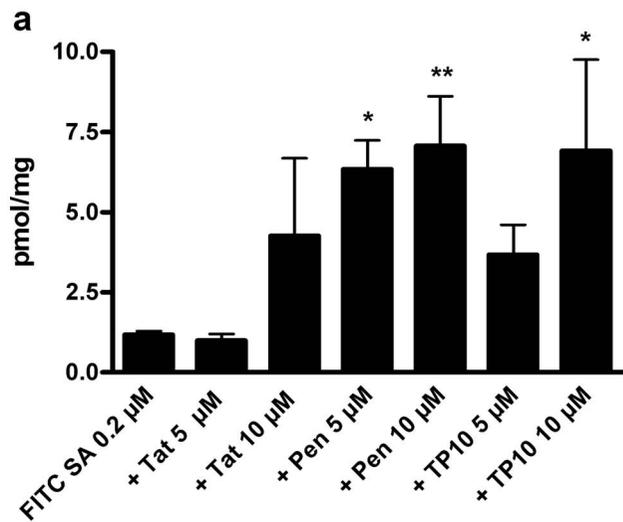
**Figure 5.** a) CPPs have the ability to deliver 60 kDa proteins in to HeLa cells either as non-covalent complexes or conjugated through a stable linker. Tat presents itself as an effective protein transduction vector when coupled to proteins, as compared to fluorescein labeled peptide. a) Uptake of 0.2  $\mu$ M FITC- Streptavidin (SA) pre-incubated with CPPs, (5 or 10  $\mu$ M) in 50  $\mu$ l 0.9% NaCl and treated for 90 min in serum free media. Uptake is displayed as pmol SA, avidin/mg total protein. b) Uptake of 0.2  $\mu$ M FITC- Avidin pre-incubated with CPPs, (5 or 10  $\mu$ M) in 50  $\mu$ l 0.9% NaCl. Uptake is displayed as pmol avidin/mg total protein. Neither of the peptides promote cellular uptake of avidin when co-incubated together with the protein. c) Uptake of 0.2  $\mu$ M FITC-SA and FITC-avidin, pre-incubated with 1  $\mu$ M biotinylated CPPs in 50  $\mu$ l serum free media and treated for 90 min. Displayed as pmol SA, avidin/mg protein d) LDH leakage after 30 min treated with 0.2  $\mu$ M FITC-SA complexed with 1-50 $\mu$ M CPPs, displayed as percentage of total LDH release. e) Wst-1 activity after 24 h TP10 treatment together with SA. TP10 seem to shield cells from SA toxicity at 10 and 20  $\mu$ M concentrations.











**Table 1. Sequences of cell-penetrating peptides and DNA.**

Peptide	Sequence	Abbreviation
Penetratin	RQIKIWFQNRRMKWKK-NH <sub>2</sub>	Pen
Fl <sup>1</sup> -Penetratin	Fl-RQIKIWFQNRRMKWKK-NH <sub>2</sub>	Fl <sup>1</sup> -Pen
Tat (47-57)	YGRKKRRQRRR-NH <sub>2</sub>	Tat
Fl <sup>1</sup> -Tat (47-57)	Fl-YGRKKRRQRRR-NH <sub>2</sub>	Fl <sup>1</sup> -Tat
Transportan 10	AGYLLGKINLKALAALAKKIL-NH <sub>2</sub>	TP10
Fl <sup>7</sup> -Transportan 10	AGYLLGK(εNH-Fl)INLKALAALAKKIL-NH <sub>2</sub>	Fl <sup>εN7</sup> -TP10
Fl <sup>1</sup> -Transportan 10	Fl-AGYLLGKINLKALAALAKKIL-NH <sub>2</sub>	Fl <sup>αN1</sup> -TP10
sense DNA	<b>ACGCCAAAAACATAAAGAAAG</b>	sDNA
antisense DNA	<b>TTCTTTTATGTTTTTGGCGTCT</b> α	asDNA

Bold letters represent nucleobases in DNA. α DNA was labelled at the 3' end with carboxyfluorescein. Fl is the abbreviation for fluoresceinyl.

Fl<sup>1</sup>- : fluoresceinyl modification at αN<sup>1</sup>

Fl<sup>7</sup>- : fluoresceinyl modification at εN<sup>7</sup>

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

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

TP10 reduces proliferation (i.e. mitochondrial activity) significantly at higher concentrations while Tat and penetratin have negligible effects on proliferation up to 50 μM concentrations. Overall, CHO cells are less sensitive to CPP treatment. 10 000 cells seeded in 96-well plates 48 h prior experiment were treated with the indicated peptide concentrations in 100 μl serum free media for 24 h and analyzed using the wst-1 assay.