

Characterisation of cell-penetrating peptide-mediated peptide delivery

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1 Cell-penetrating peptides such as antennapedia, TAT, transportan and polyarginine have been extensively employed for *in vitro* and *in vivo* delivery of biologically active peptides. However, little is known of the relative efficacy, toxicity and uptake mechanism of individual protein transduction domain–peptide conjugates, factors that will be critical in determining the most effective sequence.

2 In the present study, we show by FACS analysis that unconjugated antennapedia, TAT, transportan and polyarginine demonstrate similar kinetic uptake profiles, being maximal at 1–3 h and independent of cell type (HeLa, A549 and CHO cell lines). A comparison of the magnitude of uptake of cell-penetrating peptide conjugates demonstrated that polyarginine = transportan > antennapedia > TAT.

3 However, examination of cellular toxicity showed that antennapedia < TAT < transportan < polyarginine, with antennapedia–peptide conjugates having no significant toxicity even at 100 μ M.

4 Confocal studies of the mechanism of antennapedia- and TAT-peptide uptake showed that the time course of uptake and their cellular distribution did not correlate with transferrin, a marker of clathrin-mediated endocytosis. In contrast, the peptides co-localised with a marker of lipid rafts domains, cholera toxin, which was attenuated following the disruption of these domains using methyl- β -cyclodextrin.

5 Overall, comparison of the uptake and toxicity suggests that antennapedia provides the optimal cell-penetrating peptide for peptide delivery *in vitro* and that both antennapedia- and TAT-mediated peptide delivery occurs predominantly *via* lipid raft-dependent but clathrin-independent endocytosis. *British Journal of Pharmacology* (2005) **145**, 1093–1102. doi:10.1038/sj.bjp.0706279; published online 6 June 2005

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Abbreviations: CPP, cell-penetrating peptide; PKI, protein kinase inhibitor peptide

Introduction

Cell-penetrating peptides (CPPs) are short cationic peptide sequences that have been demonstrated to mediate the intracellular delivery of a range of biological cargos. They were first identified while investigating the ability of the HIV TAT transactivation protein to penetrate cells and activate HIV-1-specific genes (Frankel & Pabo, 1988; Green & Loewenstein, 1988). Subsequent studies revealed that the minimum region required for translocation was a positively charged section between amino acids 47–57, which was associated with DNA binding (Vives *et al.*, 1997). Since these initial observations, a range of additional CPPs have been identified (Lindgren *et al.*, 2000; Wadia & Dowdy, 2002) including antennapedia (Derossi *et al.*, 1996), transportan (Pooga *et al.*, 2001) and polyarginine (Matsui *et al.*, 2003).

Antennapedia and TAT conjugation has been extensively used for the *in vitro* and *in vivo* delivery of biological active peptides and proteins (Lindsay, 2002). Examination of these publications suggests that these antennapedia and TAT conjugates have a number of significant advantages over the alternative viral-based delivery systems including the ability to

transfect primary non-dividing cells, rapid cellular uptake and low toxicity. Furthermore, studies have demonstrated that these conjugates have biological activity in a range of disease models *in vitro* and *in vivo* including asthma (Myou *et al.*, 2003a, b), cardiovascular disease (Rey *et al.*, 2001; Jacobson *et al.*, 2003), inflammation (Bucci *et al.*, 2000; May *et al.*, 2000; Gratton *et al.*, 2003; Jimi *et al.*, 2004), cancer (Fujimoto *et al.*, 2000; Hosotani *et al.*, 2002; Snyder *et al.*, 2004) and stroke (Aarts *et al.*, 2002; Asoh *et al.*, 2002; Cao *et al.*, 2002; Dietz *et al.*, 2002; Kilic *et al.*, 2002; Borsello *et al.*, 2003; Kilic *et al.*, 2003).

Early studies of CPP-mediated delivery showed rapid, receptor- and endosomal-independent uptake of conjugated biological tools into all cell types. However, recent mechanistic studies suggest that these observations were an artefact of their positively charged nature (Green *et al.*, 2003; Richard *et al.*, 2003). Instead, it appears that there is an initial rapid interaction between the positively charged CPPs and the negatively plasma membrane (Rusnati *et al.*, 1998; Rusnati *et al.*, 1999; Rusnati *et al.*, 2001; Tyagi *et al.*, 2001; Silhol *et al.*, 2002; Suzuki *et al.*, 2002; Wadia *et al.*, 2004) followed by endocytosis and subsequent cytoplasmic release (Ferrari *et al.*, 2003; Fittipaldi *et al.*, 2003; Richard *et al.*, 2003; Wadia *et al.*,

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2004). Recent attempts to characterise the intracellular mechanism have focused upon the delivery of protein conjugates by the TAT sequence and have produced contradictory conclusions. There are reports that uptake occurs through lipid raft-dependent endocytosis involving either macropinocytosis (Wadia *et al.*, 2004) or a caveolae (Ferrari *et al.*, 2003; Fittipaldi *et al.*, 2003) pathway. However, a subsequent investigation showed that TAT uptake occurred *via* clathrin-mediated endocytosis (Vendeville *et al.*, 2004). The reasons for these differences are unknown, but may relate to differences in cell type or cargo. In addition, uptake may be influenced by the individual CPPs, since comparative studies have shown that the uptake of TAT but not antennapedia complexes are inhibited by dextran sulphate and that they localise to different intracellular compartments (Console *et al.*, 2003).

Although antennapedia is commonly employed for peptide delivery *in vitro* and *in vivo*, there has been no attempt to determine whether this is the optimal delivery sequence. To this end, we have compared the kinetics and magnitude of uptake of antennapedia-, TAT-, transportan- and polyarginine-conjugated peptides into a range of cell lines (A549, HeLa and CHO). We have also examined their cellular toxicity since this is crucial to their utility as effective delivery vehicles. In addition, we have investigated the effect of CPP sequence upon uptake mechanism by comparing antennapedia- and TAT-mediated peptide delivery.

Methods

Materials

Rhodamine-labelled CPP-PKI peptides (Table 1) were purchased from GeneMed (San Francisco, CA, U.S.A.). Unconjugated CPPs and CPP-NBD conjugates (Table 1) were purchased from Biosynthesis (Lewisville, TX, U.S.A.). HeLa, A549 and CHO K1 cell lines were obtained from the AstraZeneca mammalian cell bank. HeLa and A549 cells were grown in Dulbecco's modified Eagles medium (DMEM) (Sigma, Poole, Dorset, U.K.) supplemented with 10% foetal calf serum (FCS) and containing 1% penicillin, 1% strepto-

mycin and 1% glutamine (PSG). CHO K1 cells were grown in Ham-12 Nutrient Mix supplemented with 10% FCS and containing 1% PSG.

Flow cytometry

CHO, A548 and HeLa cells were grown to approximately 60% confluency in 24-well plates and then incubated with CPP peptides (5 μ M) for the prescribed time periods (Figure 1). Cells were then washed twice in PBS, trypsinised for 5 min and then washed again with PBS before being resuspended in 500 μ l PBS. Cell suspensions were then analysed using a FACScalibur cytometer (Becton Dickinson, Franklin Lakes, NJ, U.S.A.). A total of 10,000 events per sample were counted. The GeoMean fluorescence was recorded and the fold change from a Rho/Rho-PKI control used for standardisation.

Confocal microscopy of live HeLa cells

HeLa cells were seeded at 5×10^4 cells ml⁻¹ in Lab-Tek chamber slides (Nunc, Rochester, NY, U.S.A.) and grown overnight. Cells were then prestained with Hoechst (1 μ g ml⁻¹) for 30 min and then incubated with 5 μ M of either rhodamine labelled antennapedia-PKI or TAT-PKI for 15, 30 min, 1, 2 and 4 h. For co-localisation studies, Rho-CPP peptides were co-incubated with either 10 μ g ml⁻¹ Alexa488 Transferrin (Molecular Probes, 2333 AA Leiden, Netherlands) or 10 μ g ml⁻¹ Alexa488 Cholera Toxin (Molecular Probes) for 2 h. At the prescribed time points the media was aspirated, cells were washed twice with PBS and live cells were viewed using a BioRad confocal microscope following excitation with a green He-Ne (543 nm) and argon ion (488 nm) laser. To examine the effect of disrupting lipid raft endocytosis, cells were pretreated with methyl- β -cyclodextrin (5 mM) for 30 min to strip the membranes of cholesterol.

Cytotoxic *Wst-1* assay

Following standard curve determination of cell density and absorbance, cells were seeded at 10,000 cells well⁻¹ in a 96-well plate in a volume of 100 μ l DMEM containing 10% FCS, 1% PSG. After overnight incubation at 37°C, cells were incubated

Table 1 Amino-acid sequences of protein transduction domains and peptide conjugates

Peptide	Sequence
Antennapedia	RQIKIWFQNRRMKWKK
Antp-PKI	RQIKIWFQNRRMKWKK TYADFIASGRTGRRNAI
Antennapedia-NBD	RQIKIWFQNRRMKWKK TALDWSWLQTE
Antennapedia-Control	RQIKIWFQNRRMKWKK STLWDTAELWQ
TAT	GRKKRRQRRRPPQ
TAT-PKI	GRKKRRQRRRPPQ TYADFIASGRTGRRNAI
TAT-NBD	GRKKRRQRRRPPQ TALDWSWLQTE
TAT-Control	GRKKRRQRRRPPQ STLWDTAELWQ
Transportan	AGYLLGKINLKALAALAKKIL
Transportan-PKI	AGYLLGKINLKALAALAKKIL TYADFIASGRTGRRNAI
Transportan-NBD	AGYLLGKINLKALAALAKKIL TALDWSWLQTE
Transportan-Control	AGYLLGKINLKALAALAKKIL STLWDTAELWQ
Polyarginine	RRRRRRRRRR
Polyarginine-PKI	RRRRRRRRRR TYADFIASGRTGRRNAI
Polyarginine-NBD	RRRRRRRRRR TALDWSWLQTE
Polyarginine-Control	RRRRRRRRRR STLWDTAELWQ

Bold denotes peptide cargo.

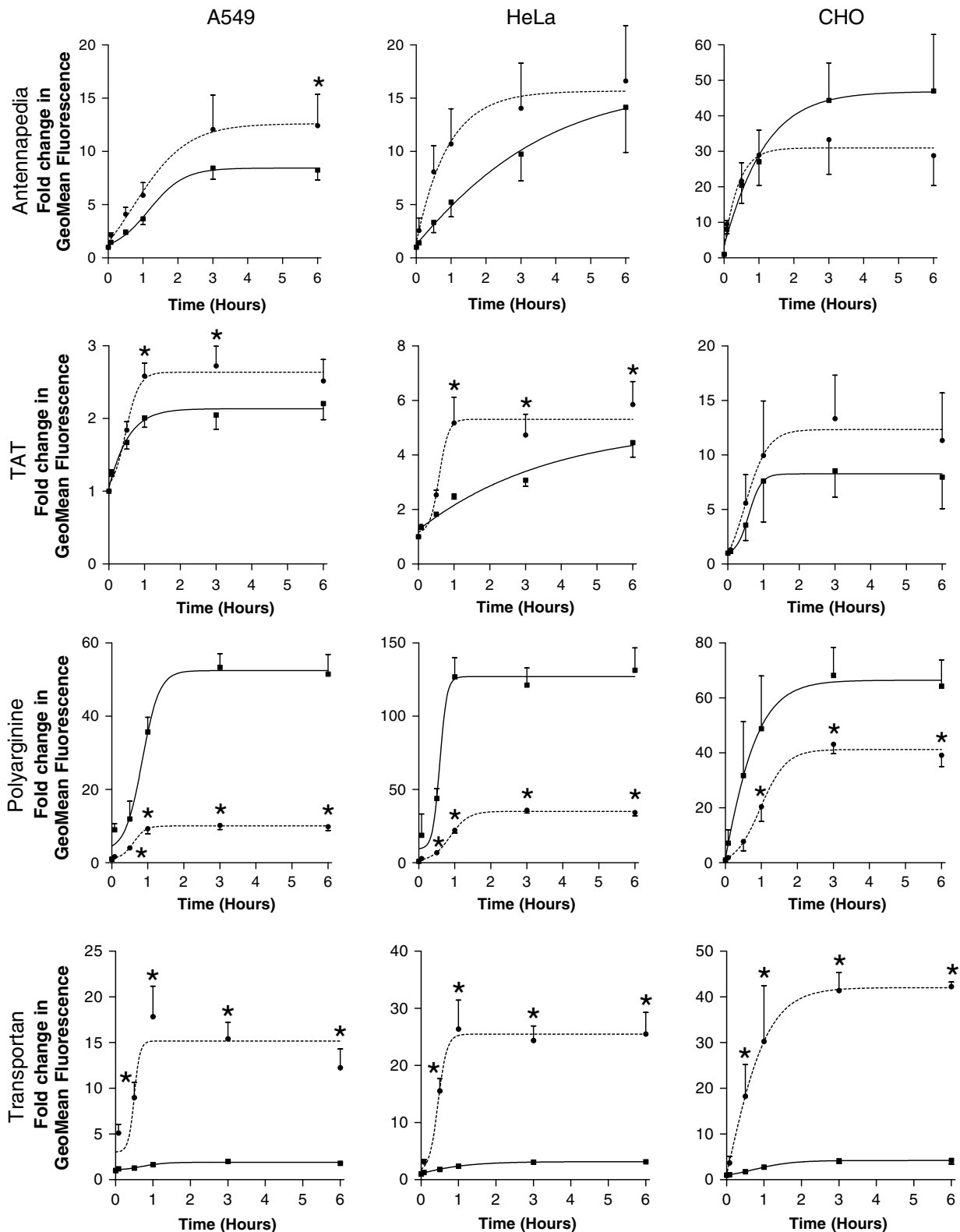


Figure 1 Uptake kinetics of **CPPs** and **CPP-PKI** peptide conjugates in different cell lines. A549, HeLa and CHO cells were incubated with $5\ \mu\text{M}$ rhodamine-labelled antennapedia, TAT, transportan or polyarginine either alone (solid line) or conjugated to a PKI peptide (hatched line) for up to 6 h. At the prescribed time points, cells were washed twice in PBS, trypsinised and washed twice again in PBS. Values represent the fold change in GeoMean Fluorescence from a media control sample and are expressed as mean \pm s.e.m. ($n=3$). * $P<0.05$. Significantly different from the corresponding unconjugated **CPP**.

with the relevant peptides for 7 h before 10 μ M WST-1 reagent (Roche) was added to each well and incubated for 30 min. Measurement at 7- and 24-h peptide incubation under identical experimental conditions showed comparable cellular toxicity. The plate was then shaken thoroughly for 1 min and the absorbance read at 450 and 650 nm using a microplate reader.

Statistical analysis

ANOVA (two-way) was used to examine the uptake of CPPs with and without PKI peptide cargo over time. Where time or cargo effects were observed, *post hoc* tests were performed to examine specific differences. Statistical significance was accepted at the $P < 0.05$ level.

Results

Kinetics and magnitude of CPP-mediated peptide delivery

Initial studies compared the kinetics and magnitude of cellular delivery by rhodamine-labelled antennapedia, TAT, transportan and polyarginine into A549, HeLa and CHO cells by FACS analysis (Figure 1 – solid lines). To eliminate non-specific plasma membrane binding by these CPP conjugates, cells were treated with trypsin for 5 min prior to measurement. In general, maximal uptake with all the unconjugated CPPs occurred between 1 and 3 h and did not appear to be affected by either CPP or cell type. Comparison of maximal uptake by individual unconjugated CPPs showed that polyarginine > antennapedia > TAT = transportan, such that polyarginine delivery was 10–30 times greater than TAT (Figure 1). With the exception of increased uptake by polyarginine into HeLa cells and antennapedia into CHO cells, uptake by individual CPPs into the various cell types was generally equivalent. Conjugation of a short 17 amino-acid sequence derived from the protein kinase A inhibitor 6–22 amide (Glass *et al.*, 1989) peptide (PKI) had no effect upon the kinetics profile and had little effect upon the magnitude of antennapedia- and TAT-mediated delivery. In contrast, the peptide cargo caused a significant increase in the level of transportan uptake and, paradoxically, attenuated the polyarginine response (Figure 1 – hatched lines) such that polyarginine = transportan > antennapedia > TAT.

Cellular toxicity of CPP-mediated peptide delivery

Across all cell types, comparison of the toxicity of the rhodamine-labelled CPPs showed that transportan > polyarginine > antennapedia > TAT, giving mean values for EC_{50} of 6, 10, 17 and > 100 μ M, respectively (Figure 2 – solid line). Peptide conjugation had no significant effect upon the response to transportan ($EC_{50} = 5 \mu$ M), polyarginine ($EC_{50} = 12 \mu$ M) and antennapedia ($EC_{50} = 21 \mu$ M), but increased the toxicity of TAT at 100 μ M, reducing the EC_{50} to 67 μ M (Figure 2 – hatched lines). To examine the effect of rhodamine labelling and the biological action of PKI-derived peptide, we also undertook further studies in A549 cells using unlabelled CPPs (Figure 3 – solid lines) and those conjugated to a peptide derived from the NEMO-binding domain (NBD) (short hatched lines) and its scrambled control (Table 1) (Figure 3 – long hatched lines) (May *et al.*, 2000). Interestingly, these

studies suggested that rhodamine increased toxicity, since in the absence of the label, antennapedia, TAT and transportan alone gave no significant toxicity at concentrations up to 100 μ M, while polyarginine gave 20 and 68% reductions in cell number at 30 and 100 μ M, respectively. The conjugation of either the NBD and scrambled peptide had no effect upon the antennapedia response, but increased the toxicity of TAT at the highest concentration (100 μ M) and reduced the EC_{50} with polyarginine from approximately 80 to 15 μ M. The addition of both these peptide cargos profoundly increased transportan toxicity with a significant 20–30% reduction in cell viability at even at the lowest concentration (10 μ M) employed in this study.

Mechanism of antennapedia- and TAT-mediated peptide delivery

The investigation of the mechanism of CPP-mediated peptide delivery was focused upon the least toxic and most commonly employed sequences, antennapedia and TAT (Lindsay, 2002). These confocal microscopy studies were performed in live HeLa cells, to avoid fixation artefacts (Green *et al.*, 2003), and used 5 μ M rhodamine-labelled CPP-PKI conjugates. Examination of the time course of antennapedia-PKI (Figure 4a) and TAT-PKI (Figure 4b) uptake showed localisation into 'endosomal-like' vesicles within 30 min, which peaked at approximately 2 h, an observation that is in agreement with the FACS measurements (Figure 1). Subsequent studies into the identity of these vesicular structures examined the involvement of clathrin- and lipid raft-mediated endocytosis using the Alexa488-labelled markers transferrin (Schmid, 1997) and cholera toxin (Nichols *et al.*, 2001), respectively. Measurement of transferrin delivery showed that this was more rapid than that of the antennapedia and TAT conjugates, with maximal uptake achieved within 30 min (data not shown). Furthermore, co-incubation of transferrin and CPP peptides resulted in the majority of vesicles containing either red (rhodamine) or green (Alexa488) fluorescence, with only a few vesicles containing yellow fluorescence, indicating poor co-localisation of CPP peptides with transferrin (Figure 5a). In contrast, both antennapedia and TAT peptide conjugates were found to co-localise with Alexa488-labelled cholera toxin, a marker for cellular membrane regions rich in cholesterol (lipid rafts) (Figure 5b). This hypothesis was supported by studies in HeLa cells that had been pre-incubated for 30 min with methyl- β -cyclodextrin (5 μ M) to remove cholesterol from the cell membranes (Figure 6). As would be expected, labelled CPP conjugate was not observed by confocal microscopy, although Alexa488-labelled transferrin vesicles were still visible, demonstrating that the disruption of lipid rafts had specifically blocked CPP peptide uptake but not affected clathrin-dependent endocytosis (Figure 6). Overall, this suggested that both antennapedia and TAT peptide conjugates were being taken into cells *via* lipid raft, but not clathrin-mediated endocytosis.

Discussion

Antennapedia has been widely used for the delivery of short peptides that have been utilised to inhibit protein–protein and protein–substrate interactions in a variety of intracellular

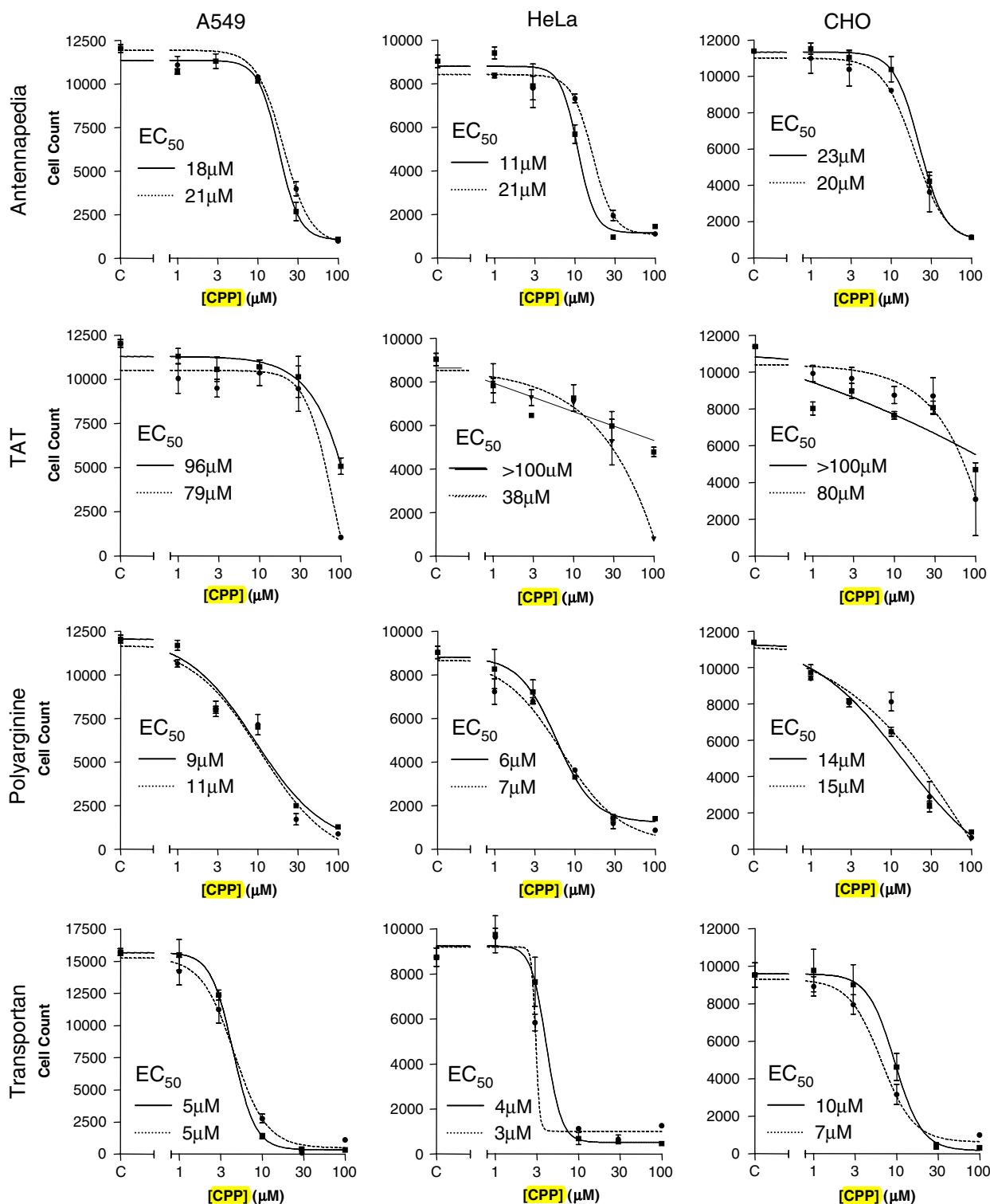


Figure 2 Cytotoxicity of rhodamine-labelled CPPs and CPP-PKI peptide conjugates in different cell lines. A549, HeLa and CHO cells were incubated with the indicated concentration of rhodamine-labelled antennapedia, TAT, transportan or polyarginine either alone (solid line) or conjugation to a PKI peptide (hatched line) for 7 h. Wst-1 assay reagent (10 μl) was then added to each well and incubated for 30 min before the absorbance at 450 and 650 nm was read. Bars represent mean \pm s.e.m. ($n = 3$).

signalling pathways (Lindsay, 2002). However, since there has been no systematic attempt to determine whether antennapedia is the optimal CPP sequence, we undertook studies to compare peptide delivery mediated by antennapedia plus three

commonly utilised CPPs, namely TAT, transportan and polyarginine. These studies were also important since the results of many earlier studies of CPP-mediated peptide delivery failed to take into account their highly positive

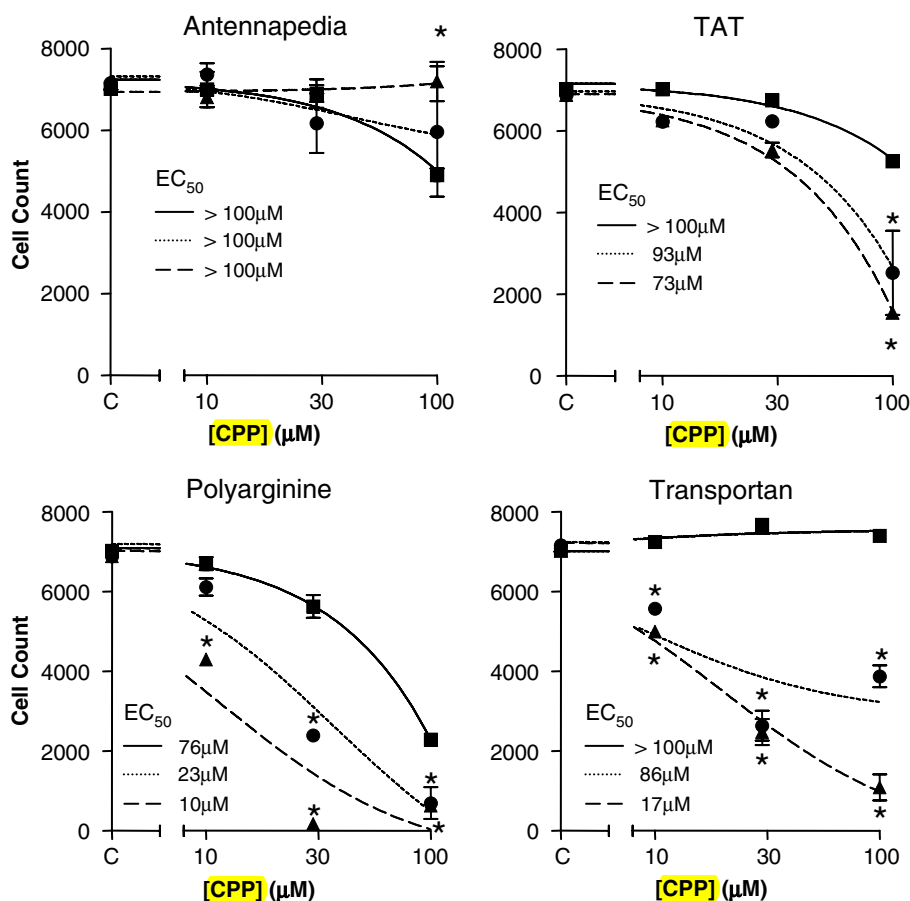


Figure 3 Cytotoxicity of unlabelled CPPs and CPP-NBD peptide conjugates in A549 cells. A549 cells were incubated with the indicated concentration of antennapedia, TAT, transportan or polyarginine either alone (solid line) or conjugated to either a scrambled peptide (long hatched line) or the NBD peptide (short hatched line) for 7 h. Wst-1 assay reagent (10 μl) was then added to each well and incubated for 30 min before the absorbance at 450 and 650 nm was read. Bars represent mean ± s.e.m. ($n = 3$). * $P < 0.05$. Significantly different from the corresponding unconjugated CPP.

(cationic) nature, which can produce artefacts. Thus, the rapid (<5 min) uptake initially reported in previous studies by FACS analysis, is now thought to be due to the nonspecific and electrostatic interaction of the positively charged CPPs and the negatively charged plasma membrane and can be removed by trypsin treatment (Green *et al.*, 2003; Richard *et al.*, 2003). Furthermore, previous confocal studies appeared to show extensive cytoplasmic and nuclear localisation, which it now appears is an artefact of cellular fixation (Green *et al.*, 2003; Richard *et al.*, 2003) since investigation using live cells have shown that CPP conjugates are predominantly seen in vesicular structures.

Following trypsin treatment, examination of the kinetics showed a slow and sustained cellular uptake that peaked at approximately 1–3 h and was unaffected by either CPP or cell type. Practically, this suggests that cells should be incubated with CPP peptide conjugates for a minimum of 1 h before commencing relevant studies, although additional time may be required to permit release from the endosomes. Interestingly, similar uptake kinetics have been reported for *in vitro* and *in vivo* delivery of TAT proteins using Western blotting. For example, the uptake of TAT-catalase/superoxide dismutase (Kwon *et al.*, 2000; Jin *et al.*, 2001) *in vitro* and TAT-Bcl-xL *in vivo* (Cao *et al.*, 2002) was first observed at 1 h before peaking at 4 h.

In the present study, the magnitude of uptake by CPPs in the absence of peptide cargo was polyarginine > antennapedia > TAT = transportan. These initial observations would suggest that polyarginine is the optimum delivery sequence. However, although the addition of the peptide cargo to TAT and antennapedia causes a small increase in uptake, this had profound effects upon delivery by the polyarginine and transportan. In the case of polyarginine, peptide conjugation caused a large reduction, while uptake of transportan conjugates was increased such that the uptake magnitude was in the order polyarginine = transportan > antennapedia > TAT. Whether these changes result from alterations in the physical properties of the conjugates or from the biological action of the PKI peptide is unknown. However, since the effects upon uptake are seen at the earliest time point (30 min) and are not observed with the TAT-PKI/antennapedia-PKI conjugates, this would imply that these effects are due to changes in the physical properties of the conjugate. If this is indeed the case, then considerable work would be required to determine the influence of peptide cargo upon polyarginine- and transportan-mediated cellular uptake, a factor which may ultimately limit their utility as a universal peptide delivery vehicle. Interestingly, there may have been a change in the delivery mechanism since an investigation in

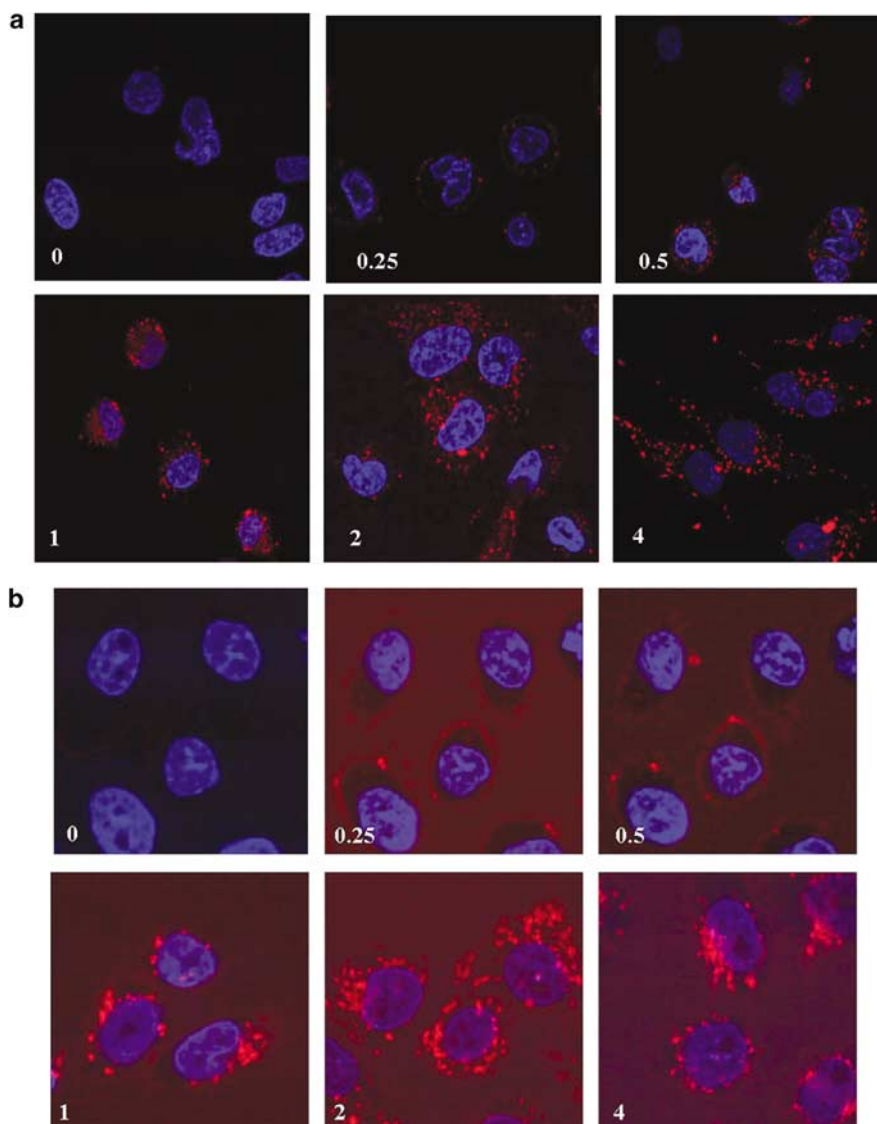


Figure 4 Time course of antennapedia-PKI and TAT-PKI uptake into HeLa cells. HeLa cells pre-incubated for 1 h with Hoescht nuclei stain ($1 \mu\text{g ml}^{-1}$), followed by incubation with either rhodamine-labelled (a) antennapedia-PKI or (b) TAT-PKI peptides ($5 \mu\text{M}$) for 15, 30 min, 1, 2 and 4 h. At the prescribed time points, the cells were washed in PBS and analysed by confocal microscopy ($n = 3$).

PC-12 cells showed that uptake of a hepta-arginine peptide alone was *via* a nonendocytotic route (Thoren *et al.*, 2003). In the case of transportin, it could also be speculated that the increase is secondary to cell death/necrosis, although this is difficult to explain given that the cytotoxicity of the rhodamine-labelled peptides is almost identical either in the absence ($\text{EC}_{50} = 6 \mu\text{M}$) or presence ($\text{EC}_{50} = 5 \mu\text{M}$) of peptide cargo.

The uptake by individual CPPs appears to be similar across all three cell types although, in general, CHO and HeLa cells showed better uptake than A549 cells. These small differences could be due to differential expression of cell surface-expressed glycosaminoglycans, particularly heparin sulphate glycosaminoglycans (HPSGs), which have been reported to act as receptors for both antennapedia and TAT (Tyagi *et al.*, 2001; Console *et al.*, 2003). Indeed, although all tissues express proteoglycans, the level of expression is determined by the state

of differentiation and growth of the cell (Grassel *et al.*, 1995) and specific HPSG isoforms are known to be differentially expressed in different cell types (David, 1993).

A survey of the literature shows that, compared to CPP-protein conjugates (which are biologically active in the submicromolar range), concentrations of $> 10 \mu\text{M}$ are required for CPP-peptide conjugate cellular activity (Lindsay, 2002). Indeed, studies using the antennapedia-NBD peptide were undertaken at $200 \mu\text{M}$ (May *et al.*, 2000). The requirement for such high concentrations means that it is important to examine the overall cytotoxicity of the CPP-peptide conjugates. These studies were undertaken at 7 h since maximal uptake was observed at 1–3 h (Figure 1) and the majority of biological studies using CPP-mediated delivery of peptides are undertaken within this time frame. Thus, inhibition of the NF- κ B pathway was achieved following incubation of human islets for 2 h with a CPP-conjugated NEMO-binding domain peptide

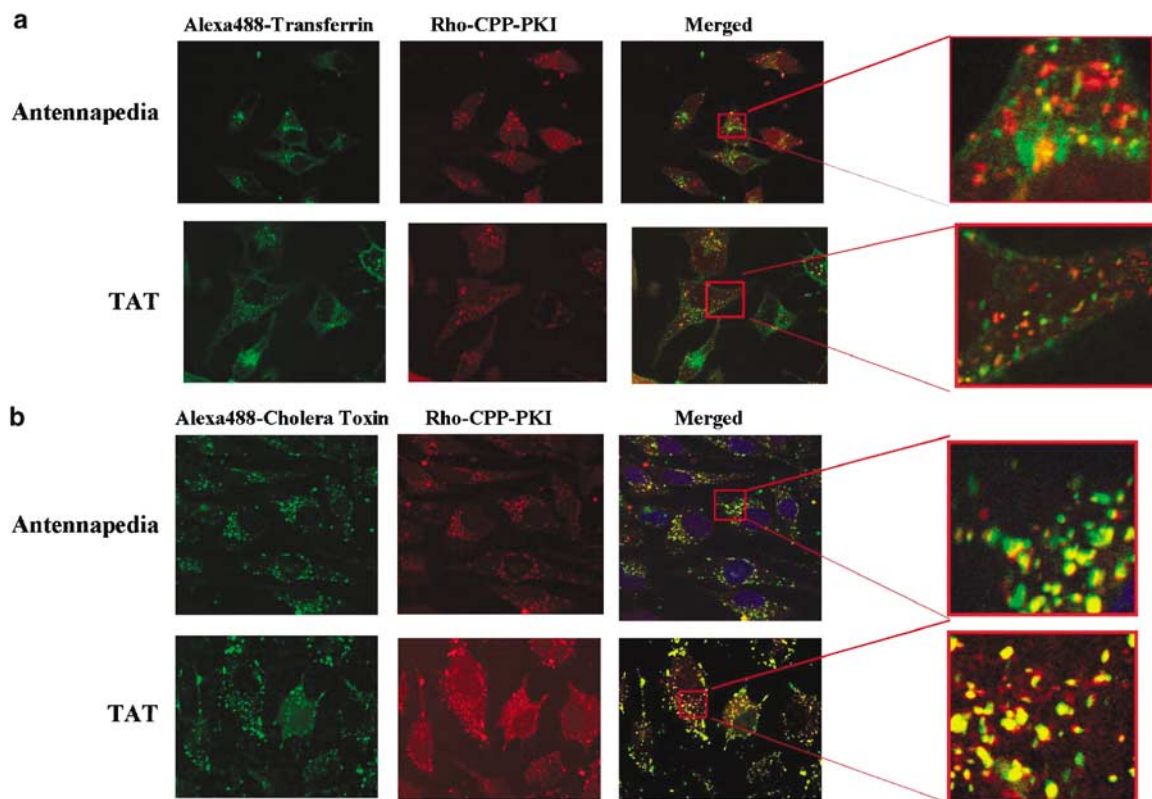


Figure 5 Examination of the co-localisation of antennapedia-PKI and TAT-PKI with markers of clathrin- and lipid raft-dependent endocytosis. HeLa cells were co-incubated for 2 h with either rhodamine-labelled (red stain) antennapedia-PKI or TAT-PKI, and either Alexa488 (green stain) transferrin (a) or cholera toxin (b). Co-localisation (yellow stain) was visualised through overlay of rhodamine and Alexa488 stains ($n = 3$).

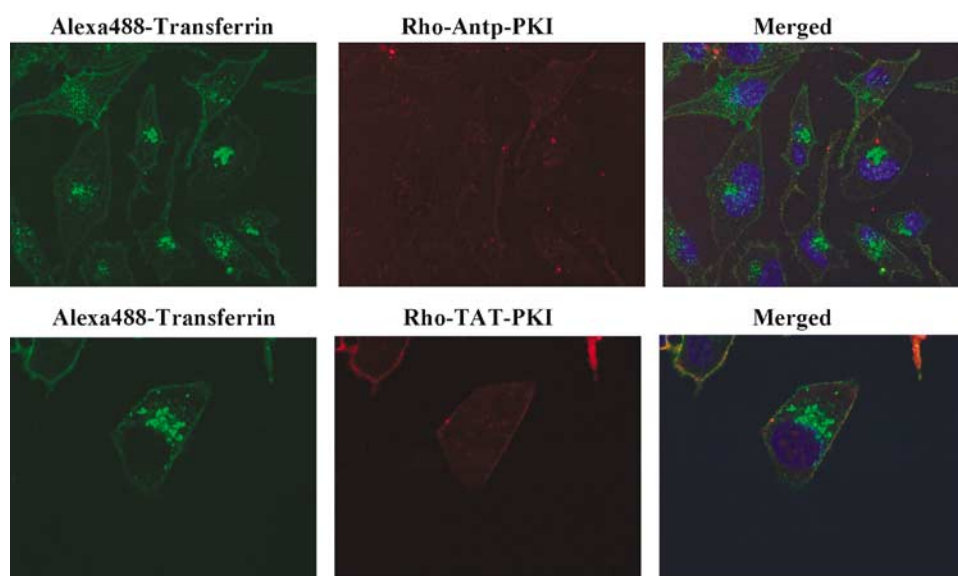


Figure 6 Effect of disruption of the lipid raft domains. HeLa cells pre-incubated for 30 min with methyl- β -cyclodextrin ($5 \mu\text{M}$), followed by co-incubation with either rhodamine-labelled antennapedia-PKI or TAT-PKI ($5 \mu\text{M}$) together with Alexa488-labelled transferrin ($10 \mu\text{g ml}^{-1}$) for 2 h ($n = 3$).

(Rehman *et al.*, 2003), and also following a 3-h incubation in HeLa cells (May *et al.*, 2000).

Initial studies comparing rhodamine-labelled and unlabelled CPPs showed that rhodamine significantly increased cyto-

toxicity (comparison of Figures 2 and 3). For this reason, subsequent investigations on the cytotoxicity of CPP peptide conjugates were undertaken with CPP alone and following the conjugation of NBD and scrambled control (Figure 3). The

optimum CPP was antennapedia, which showed no toxicity in the absence or presence of peptide, even at 100 μM . Tat and transportan alone were relatively nontoxic at concentrations up to 100 μM and polyarginine up to 30 μM . However, the addition of NBD peptide cargo increased toxicity, particularly with transportan. The reason for this effect is unknown. However, it is unlikely to be related to the biological action of NBD peptide since the effect was also observed with the scrambled control.

Finally, studies were undertaken to examine and compare the mechanism of peptide uptake by antennapedia and TAT peptide conjugates. These studies were performed with live HeLa cells to eliminate artefacts caused by cell fixation. Examination of the time course showed a similar profile for both antennapedia and TAT peptide conjugates, which involved rapid initial binding around the plasma membrane, followed by uptake into endosomal like structures. Our finding that the uptake of both CPP constructs is slower than transferrin and poorly co-localised with transferrin suggests that the majority of CPP uptake is not *via* clathrin-dependent endocytosis. Instead, the strong co-localisation of antennape-

dia and TAT peptides with cholera toxin and the inhibition of uptake following the removal of cholesterol indicates that delivery is predominately *via* lipid-raft-dependent endocytosis. Interestingly, this is in agreement with investigations of the mechanism of TAT-protein uptake (Ferrari *et al.*, 2003; Fittipaldi *et al.*, 2003; Wadia *et al.*, 2004) and suggests, at least with TAT, that there is a similar pathway for both protein and peptide uptake.

In summary, the data show that, for *in vitro* peptide delivery, taking into account both the magnitude of uptake and cytotoxicity, antennapedia is the CPP of choice. TAT also appears to be a useful alternative to antennapedia, but concentrations above 100 μM should be avoided where TAT peptide conjugates exhibit some cytotoxicity. Furthermore, we show for the first time that the route of uptake of both TAT- and antennapedia-mediated peptide delivery is similar and predominantly *via* lipid-raft dependent endocytosis.

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