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The role of endocytosis on the uptake kinetics of luciferin-conjugated cell-penetrating peptides

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ABSTRACT

Cell-penetrating peptides (CPPs) are short cationic/amphipathic peptides that can be used to deliver a variety of cargos into cells. However, it is still debated which routes CPPs employ to gain access to intracellular compartments. To assess this, most previously conducted studies have relied on information which is gained by using fluorescently labeled CPPs. More relevant information whether the internalized conjugates are biologically available has been gathered using end-point assays with biological readouts. Uptake kinetic studies have shed even more light on the matter because the arbitrary choice of end-point might have profound effect how the results could be interpreted. To elucidate uptake mechanisms of CPPs, here we have used a bioluminescence based assay to measure cytosolic delivery kinetics of luciferin–CPP conjugates in the presence of endocytosis inhibitors. The results suggest that these conjugates are delivered into cytosol mainly via macropinocytosis; clathrin-mediated endocytosis and caveolae/lipid raft dependent endocytosis are involved in a smaller extent. Furthermore, we demonstrate how the involved endocytic routes and internalization kinetic profiles can depend on conjugate concentration in case of certain peptides, but not in case of others. The employed internalization route, however, likely dictates the intracellular fate and subsequent trafficking of internalized ligands, therefore emphasizing the importance of our novel findings for delivery vector development.

1. Introduction

Cell-penetrating peptides (CPPs) are relatively short cationic and/or amphipathic peptides capable of delivering many types of cargos into mammalian cells. The first CPPs, penetratin [1] and Tat [2], were discovered in 1994 and 1997, respectively, and numerous other CPPs have been reported ever since. The range of CPP-compatible cargo molecules is wide and it includes many types of therapeutic proteins and peptides, nucleic acids, cytotoxic agents and imaging contrast agents [3]. In order to mediate its corresponding biological effect, each cargo type needs to reach the specific intracellular compartment, such as the cytoplasm or the nucleus [4–6]. However, it is still actively debated which mechanisms are involved in CPP-mediated cargo delivery and whether the mechanisms might differ for reaching separate intracellular targets.

While early reports suggested the involvement of direct (energy independent) pathways in the internalization process, the conclusion was based largely on cell fixation artifacts [3]. Thereafter endocytosis has been considered as the predominant uptake route, especially when CPPs are carrying cargo molecules. However, some more carefully controlled recent studies still suggest that direct translocation mechanism cannot be overruled in case of some naked or fluorescently labeled CPPs at certain conditions [7–9].

For both the endocytosis and direct translocation the CPPs interact with the cell membrane prior to being taken up by cells [8]. The cell membrane, however, contains many components that are required for different types of endocytosis [10–14] and different cell lines might express these constituents in various levels. Composite CPP properties could therefore lead to multifaceted membrane interactions, and it is thus not surprising why the results of CPP uptake mechanism studies are not always converging.

It seems that, similarly to uptake of many ligands and receptors [13], different endocytosis sub-types can be involved simultaneously in the CPP uptake process [3,15]. Their relative importance and the subsequent intracellular fate of the internalized material may depend on numerous factors. For example, at low concentration epidermal growth factor (EGF) triggers its receptor (EGFR) internalization via

Abbreviations: CPP, Cell-penetrating peptide; siRNA, Short interfering ribonucleic acid; EGF, Epidermal growth factor; EGFR, Epidermal growth factor receptor; CME, Clathrin-mediated endocytosis; SPPS, Solid phase peptide synthesis; *t*-Boc, *tert*-Butyloxycarbonyl; HOBt, Hydroxyl-benzotriazole; DCC, *N,N′*-Dicyclohexylcarbodiimide; DIEA, *N,N′*-Diisopropylethylamine; DMF, *N,N′*-Dimethylformamide; Cpz, Chlorpromazine; CyD, Cytochalasin D; MP, Macropinocytosis; Nys, Nystatin; C/LR, Caveolae/lipid raft dependent endocytosis; CQ, Chloroquine; TBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate; LDH, Lactate dehydrogenase; AUC, Area-under-curve; RLU, Relative luminescence unit

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clathrin-mediated endocytosis (CME) after which the receptor is recycled back to the membrane. However when the EGF concentration is increased the uptake of EGFR is routed to a non-CME pathway during which the receptor will be degraded [13]. Since many CPPs are derived from proteins it could be hypothesized that similar processes to the previously described example could also occur when delivering cargos with CPPs, thus making it important to study their internalization pathways [13,16].

Most studies in the field regarding CPP uptake mechanisms are end-point studies. While biological readout systems are exploited in some investigations (e.g. the delivery of enzymes or oligonucleotides), mostly fluorophore-labeled peptides have been used. Furthermore, CPP uptake kinetic measurements should be preferred to end-point studies because in an arbitrarily chosen end-point certain effects of endocytosis inhibitors and thus the involvement of certain pathways could be left unregistered. Further, differences between peptides that might display similar overall internalization degree may have completely different kinetic profiles which could not be noticed using end-point assays [17].

In this paper we have used luciferin-conjugated CPPs (luciferin–CPPs) to evaluate CPP uptake mechanisms. Previously we have used a semi-biological real-time uptake kinetics assay [18,19] to characterize CPPs based on their uptake kinetics profiles [17]. In the latter study we showed that CPPs can be divided into two distinct groups —the fast internalization group (TP10, MAP and Tat) whose uptake kinetics profile resembled the behavior of membrane permeable free luciferin, and the slow uptake group (TP10(Cys), *p*Vec, M918, penetratin and EB1) whose uptake profile was more consistent with the uptake rates conventionally observed in case of endocytosis.

Here we used the same assay to assess the effects of selected endocytosis inhibitors on the CPP cytosolic uptake kinetics profiles of the both CPP groups. We will show that endocytosis is extensively involved in the cytosolic delivery of all the tested luciferin–CPP conjugates, even in case of the fast uptake group peptides despite their behavior resembles the membrane permeable free luciferin. We will also discuss how different luciferin–CPP conjugates can utilize preferred endocytosis sub-type depending on the conjugate concentration.

2. Materials and methods

2.1. Peptide synthesis

The peptides used in this study (Table 1) were synthesized using a solid phase peptide synthesis (SPPS) method on an automated peptide synthesizer (ABI 433A, Applied Biosystems, USA). In the synthesis *tert*-butyloxycarbonyl (*t*-Boc) chemistry was used. Shortly, *t*-Boc amino acids (Neosystem, France; Iris Biotech, Germany; Bachem AG, Switzerland) were coupled as hydroxyl-benzotriazole (HOBt) esters (Iris Biotech, Germany) in the presence of *N*,*N*′-dicyclohexylcarbodiimide (DCC; Iris Biotech, Germany) and *N*,*N*′-diisopropylethylamine (DIEA; Iris Biotech, Germany) to a 4-methylbenzhydryl-amine resin (Iris Biotech, Germany). This yielded in C-terminally amidated peptides.

TP10 peptide was modified by manual coupling of the Cys residue to the ε-amino group on its Lys^7 to obtain TP10(Cys) peptide. The Cys residue was activated by HOBt, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyl-aminium tetrafluoroborate (TBTU) and DIEA.

Before being cleaved from the resin, peptides containing His(Dnp) or Trp(For) were deprotected with 20% thiophenol in *N*,*N*′-dimethylformamide (DMF) for 1 h or 20% piperidine in DMF for 1 h, respectively. All peptides were cleaved from the resin using anhydrous hydrogen fluoride/*p*-cresol/*p*-thiocresol (90/5/5) solution for 1 h at 4 °C. After cleavage the peptides were ether-precipitated and finally purified by semi-preparative reverse-phase HPLC column (Discovery®BIO Wide Pore C-18, Supelco®, Sigma-Aldrich, Sweden).

Table 1
Cell-penetrating peptides used in this paper.

CPP	Cys-CPP sequence	Origin	Type	Ref.
MAP	C-KLALKLALKALKAALKLA-amide	Synthetic	Secondary amphipathic	[52]
TP10	C-AGYLLGKINLKALAALAKKIL-amide	Chimeric	Primary amphipathic	[53]
TP10(Cys)	AGYLLGK(C)[a]INLKALAALAKKIL-amide	Chimeric	Primary amphipathic	[53]
EB1	C-LIRLWSHLIHIWFQNRRLKWKKK-amide	Chimeric	Secondary amphipathic	[54]
Penetratin	C-RQIKIWFQNRRMKWKK-amide	Protein derived	Secondary amphipathic	[1]
*p*Vec	C-LLIILRRRIRKQAHAHSK-amide	Protein derived	Secondary amphipathic	[55]
Tat	C-GRKKRRQRRRPPQ-amide	Protein derived	Non-amphipathic/cationic	[2]
M918	C-MVTVLFRRLRIRRASGPPRVRV-amide	Protein derived	Non-amphipathic/cationic	[56]

[a] Luciferin was coupled to the cysteine at the ε-amino group on Lys^7.

Their purity was determined by RP-HPLC analytical column (Discovery® C-18, Supelco®) and correct molecular weight was verified by MALDI-TOF (Perkin Elmer prOTOF™ 2000, Perkin Elmer, Sweden) mass spectrometry.

2.2. Synthesis of luciferin–CPP conjugates

Luciferin-linker was synthesized as previously reported (see Scheme 1 in [20]). Luciferin-linker and Cys-CPPs were mixed in 1:1 molar ratio at 0.88 mM concentration in DMF/acetic acid buffer (pH 5, 50 mM) for 1 h under nitrogen. Luciferin–CPP conjugates were purified by semi-preparative RP-HPLC column, and their purity (>99%) was analyzed by RP-HPLC analytical column. The correct molecular weight was verified by MALDI-TOF mass spectrometry.

2.3. Cell culture

HeLa pLuc 705 cells, kindly provided by Ryszard Kole [21], were grown in DMEM (Dulbecco's modified Eagle's medium) with glutamax and supplemented with 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin (hereafter referred to as complete medium). For uptake kinetics experiments, complete medium without phenol red was used. Cells were grown in a humidified 5% CO_2 environment at 37 °C. Cell culture media and supplements were purchased from Invitrogen, Sweden.

2.4. Uptake kinetics

2×10^5 cells were seeded onto a 6-well cell culture plate (day 1). 24 h later (on day 2) 4 μg luciferase encoding pGL3 plasmid (Promega, Sweden) was complexed with 10 μl Lipofectamine™ 2000 reagent (Invitrogen, Sweden) and the cells were transfected with the plasmid according to the manufacturer's instructions in 2.5 ml complete medium for 4 h. After that the transfection medium was replaced with fresh complete medium and the cells grown for further 20 h. After that, on day 3, 9×10^3 cells were seeded in a white 96-well clear-bottom plate (Greiner Bio-One, Germany) and 24 h after seeding, on day 4, the cells were treated with luciferin–CPP conjugates.

On the day of the experiment the cells were washed once with 100 μl complete cell culture media, after which 30 min pre-incubation with endocytosis inhibitors was conducted. The inhibitors

were dissolved in complete media without phenol red; the pre-treatment was carried out in a humidified 5% CO_2 environment at 37 °C.

For endocytosis inhibitors we used 10 μM chlorpromazine (Cpz) to inhibit clathrin mediated endocytosis (CME), 4 μM cytochalasin D (CyD) to inhibit macropinoctytosis (MP), and 50 μM nystatin (Nys) to inhibit caveolae/lipid raft dependent endocytosis (C/LR) (Table 2).

These inhibitors were chosen to have minimized cross-inhibition effects [22]. In addition 100 μM chloroquine (CQ) was used. CQ is conventionally used in biological assays to inhibit acidification rate of early endosomes and to promote release of the endosomally entrapped material in long term; however as a side effect of decelerated acidification, vesicle recycling is slowed too which could slow down overall endocytosis rate [23,24]. By using CQ we aimed to test which of these mechanisms prevail in early uptake kinetics.

After pre-treating the cells with endocytosis inhibitors, the incubation media was replaced with various concentrations of 120 μl luciferin–CPP solution in complete medium without phenol red. The endocytosis inhibitors were present during luciferin–CPP treatment. All experiments were carried out at RT (25 °C) for 2 h (in complete medium) in atmospheric CO_2. Luminescence was measured by GLOMAX™ 96 microplate luminometer (Promega, Sweden) where data points were recorded in 1.5 min time interval.

To analyze how the used endocytosis inhibitors Nys, Cpz, CyD and CQ affect the overall luciferin–CPP uptake at different incubation time points, we calculated the area-under-curve (AUC, in RLU units) of the registered kinetics curves (measured in units RLU/s) after 15, 30, 60 and 120 min incubation with luciferin–CPP conjugates in the presence of these endocytosis inhibitors. We normalized the calculated AUC values to AUC of the control (no inhibitor treated) sample at the same time points. For example, the value 0.80 marks that the inhibitor decreases the overall uptake by 20% compared to the control, whereas 1.20 states that under current conditions the overall uptake was increased by 20% compared to the control.

2.5. Lactate dehydrogenase (LDH) leakage

To assess possible membranolytic effects and short term toxicity of luciferin–CPP conjugates, LDH leakage from cells was measured using the Promega CytoTox-ONE™ assay (Promega, USA). Cells were seeded and treated similarly to the uptake kinetics experiments and the CytoTox-ONE™ assay was carried out according to the manufacturer's protocol. LDH release from cells lysed with 0.2% Triton X-100 in HKRg was defined as 100% leakage, and LDH release from untreated cells as 0% leakage.

Table 2
Endocytosis inhibitors used in this paper.

Inhibitor	Mechanism	Affected pathway		
		CME*	MP*	C/LR*
Chlorpromazine	Clathrin/AP2 depletion from plasma membrane to endosomal membranes	++ +	+	-
Cytochalasin D	Blocking of actin polymerization, disassembly of actin cytoskeleton	+	++ +	+
Nystatin	Binding to plasma membrane cholesterol	−	−	++ +
Chloroquine	Slows down acidification of endosomal vesicles, promotes endosomal escape	+	+	+

*CME—clathrin mediated endocytosis; MP—macropinocytosis; C/LR—caveolae/lipid raft dependent endocytosis.

2.6. Statistical analysis

All data were processed in GraphPad Prism version 5 for Windows (GraphPad Software Inc., USA) with results displayed as mean ± SEM. The uptake kinetics measurements consist of three independent experiments performed in triplicate. The cytotoxicity measurements include two independent experiments performed in triplicate.

3. Results

In this paper we measured the cytosolic cargo delivery kinetics of eight CPPs Tat, MAP, TP10, TP10(Cys), *p*Vec, M918, penetratin and EB1 (Table 1) using a bioluminescence assay [18]. Shortly, in this assay luciferin is conjugated to a specific thiolated linker [20] that in turn is attached to a cysteine-containing CPP via a disulfide bond. When these conjugates reach cytoplasm the disulfide bridge is reduced by glutathione, the linker self-cyclicizes and is thus removed from the luciferin molecule. During this process free luciferin is released which is the substrate for luciferase enzyme. The enzyme converts luciferin into oxyluciferin and a photon of light is produced (Fig. 1). The rate of this reaction reflects the uptake rate of the luciferin–CPP conjugates at a given time point. By registering it over time, real time uptake kinetics curve can be obtained (Fig. 2).

3.1. Effects of endocytosis inhibitors on the total luciferin–CPP uptake at various incubation times

First we analyzed how the used endocytosis inhibitors nystatin (Nys), chlorpromazine (Cpz), cytochalasin D (CyD) or chloroquine (CQ) (Table 2) affect the overall luciferin–CPP uptake at different incubation time points—15, 30, 60 and 120 min (Table 3, Supplementary Fig. 1).

It seems that the overall uptake of all the peptide conjugates, except luciferin-Tat, is affected most by CyD treatment. Generally this inhibitor has stronger effects at longer incubation times (Table 3, Supplementary Fig. 1). Nys tends to inhibit the overall internalization of the conjugates when their concentration is high, except for Tat, M918 and penetratin in case of which almost no statistically significant inhibition is observed. Rather interestingly, Nys seems to increase the internalization of lower concentrations of Tat, MAP, *p*Vec and EB1 conjugates (Table 3, Supplementary Fig. 1). In some cases also Cpz co-incubation leads to increased uptake, e.g. in case of Tat and MAP conjugates. In case of other peptides Cpz treatment tends to inhibit the overall uptake (Table 3, Supplementary Fig. 1).

CQ, a generally used endosomolytic agent, is able to increase the overall uptake of only Tat, MAP and TP10 conjugates, and not at every concentration (Table 3, Supplementary Fig. 1). However, in case of TP10(Cys), CQ treatment leads to an interesting change of its uptake kinetics profile. In the presence of CQ the uptake rate kinetics curve seems to be bi-phasic as the kinetics profile displays an additional linear increase after having previously reached a temporary plateau value (Fig. 2). This can be observed also in case of lower concentrations of MAP, TP10 and M918 conjugates and at higher concentrations of pVec and penetratin (Fig. 2).

The endocytosis inhibitors do not inhibit the cytosolic delivery of the positive control free luciferin (Fig. 3).

Endocytosis inhibitors can have profound effects on CPP uptake kinetic profiles, as mentioned above. The inhibitor treatment changes the time at which the maximal uptake rate is observed, but clear correlations among peptides, even within the fast and slow uptake group peptides, are difficult to define (Supplementary Table 1). Similarly, the relative maximal uptake rate of luciferin–CPP conjucates depends on the inhibitor treatment as well. While in case of the fast uptake group CPPs the inhibitors tend to affect this parameter less than in

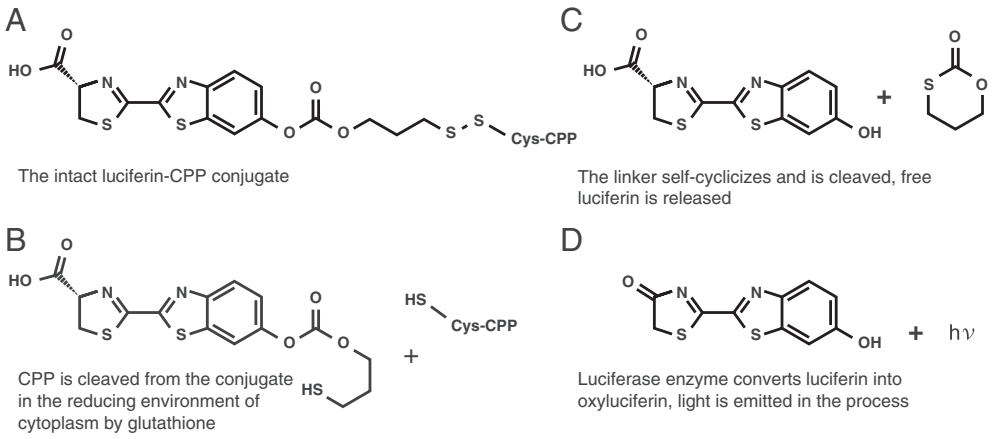

Fig. 1. Intracellular fate of the luciferin–CPP conjugates. The luciferin–CPP conjugates (A) enter cells. In the reducing environment of cytoplasm, the CPP is cleaved from the conjugate by glutathione (B) and the linker goes through spontaneous cyclization and is cleaved from the luciferin molecule and free luciferin is released (C). Free luciferin is a substrate for luciferase enzyme which converts it to oxyluciferin, releasing a photon of light in the process (D).

case of the slow uptake group peptides, however, clear patterns do not emerge (Supplementary Table 2).

3.2. Peptide toxicity by lactate dehydrogenase (LDH) leakage assay

Next we estimated whether the peptides together with the used endocytosis inhibitors affect cell membrane integrity because the luciferin–CPP conjugates might leak into the cells through damaged membranes. At the same time the otherwise cytoplasm-restricted glutathione might potentially leak out of the cells and cleave the CPP from luciferin molecule prematurely. Both of the processes could result in the overestimation of cytosolic delivery capacity of toxic CPPs. We assessed the possible toxic effects by measuring the leakage of intracellular LDH to the extracellular environment in two representative time points, at 30 and 120 min (Fig. 4).

The slow uptake group peptides (TP10(Cys), *p*Vec, M918, penetratin and EB1) can generally be considered non-toxic throughout the experiment even if co-incubated with the used endocytosis inhibitors; miniscule toxicity is observed only in case of TP10(Cys). In case of the latter CPP conjugate the relative LDH leakage remained well below 5% in most cases, however CQ, CyD and Cpz co-treatment leads to approximately 10% LDH leakage at 10 μM conjugate concentration. The toxicity was in the similar level also when high concentration of the EB1 conjugate was incubated together with Nys or CQ. For details, see Fig. 4.

Toxicity profile of the fast internalizing group conjugates (Tat, MAP and TP10) is divergent. 10 μM MAP and TP10 display considerably high LDH leakage at 120 min incubation while it is much less pronounced in 30 min time point and insignificant at lower conjugate concentrations (Fig. 4). This suggests that the toxic effects in the early incubation times are not the cause of the observed uptake. The toxicity of these two latter peptides is increased even further by certain endocytosis inhibitors. When co-incubated with CQ, the LDH leakage is increased at 10 μM conjugate concentration during 30 min incubation; and it becomes significant in case of 5 μM concentration at the 120 min incubation time point. Cpz, and to some extent also Nys, possesses similar effects on the TP10 conjugate but interestingly not on MAP. As opposed to the other fast group CPPs Tat does not mediate any cellular toxicity at any tested concentration according to the LDH leakage assay. Co-incubation of the Tat conjugate with endocytosis inhibitors does not affect its toxicity either.

4. Discussion

There are still controversies regarding which pathways exactly are involved in CPP uptake. It seems that the consensus view is that endocytic routes and vesicular uptake are almost exclusively used at least when CPPs are attached to larger cargos. However, direct cell membrane translocation/non-vesicular transport cannot be ruled out either because CPPs are shown to interact strongly with biological membranes. Some groups have postulated that the latter is activated above a certain CPP concentration threshold [9] whereas according to other models direct penetration is active rather at low concentrations (below 2 μM) [25–27], which adds further controversies.

The extent in which different active transport/endocytosis related internalization routes are involved in the uptake process is actively debated to address the existing controversies. The employed endocytosis route might depend on several factors, including which membrane-bound molecule a certain CPP interacts with. Different CPP types may have distinct cell membrane interaction partners, such as charged/uncharged membrane phospholipids, negatively charged GAGs, syndecans and glypicans, that might take part in various endocytic processes [8,10,11]. Therefore, the comparison of CPP uptake mechanisms studies is often complicated because the membrane composition can differ largely among cell types. This all emphasizes the need for more systematic studies on the latter topic.

It has been hypothesized that different endocytosis routes are simultaneously involved in CPP uptake and that these parallel mechanisms can compensate each other [3,15]. This may lead to data misinterpretation when the overall uptake only in a single time point is measured. We have previously shown that, in certain cases, the inhibitors tend to affect only the uptake rate while the overall total uptake remains unchanged when incubation is carried out long enough, while in other cases the trend is reversed [28]. Further, there can be profound discrepancies among CPPs regarding their uptake kinetics profiles [17] and the effects of endocytosis inhibitors on these profiles are left unregistered in single time point experiments.

Thus kinetic studies could be more informative when studying CPP uptake mechanisms but nevertheless generally the CPP kinetics has not been quantified. Few attempts, however, have been made to address the issue. For example measurement of ^{125}I-biotinyl-transportan kinetics in Bowes' melanoma cells [29], NBD penetratin kinetics in K562 cells [30,31], [^{99m}Tc]Tat [32] and fluoresceinyl-Tat kinetics in Jurkat cells [33] have been reported. Internalization kinetics of rhodamine-labeled Tat, polyarginine [34,35], transportan and penetratin [35] have been estimated as well. More recently, the uptake kinetics of modified fluorescein-labeled polyarginine [36], fluorescein-labeled programmed cell death inducing cyclic hexapeptide conjugated to an arginine rich CPP [37], and fluorescein or TAMRA labeled L- or D-isomer of polyarginine [38] has been reported and we have measured cytosolic delivery kinetics of CPPs using a quenched fluorescence assay [28] and a bioluminescence based assay [17]. These kinetic studies have demonstrated

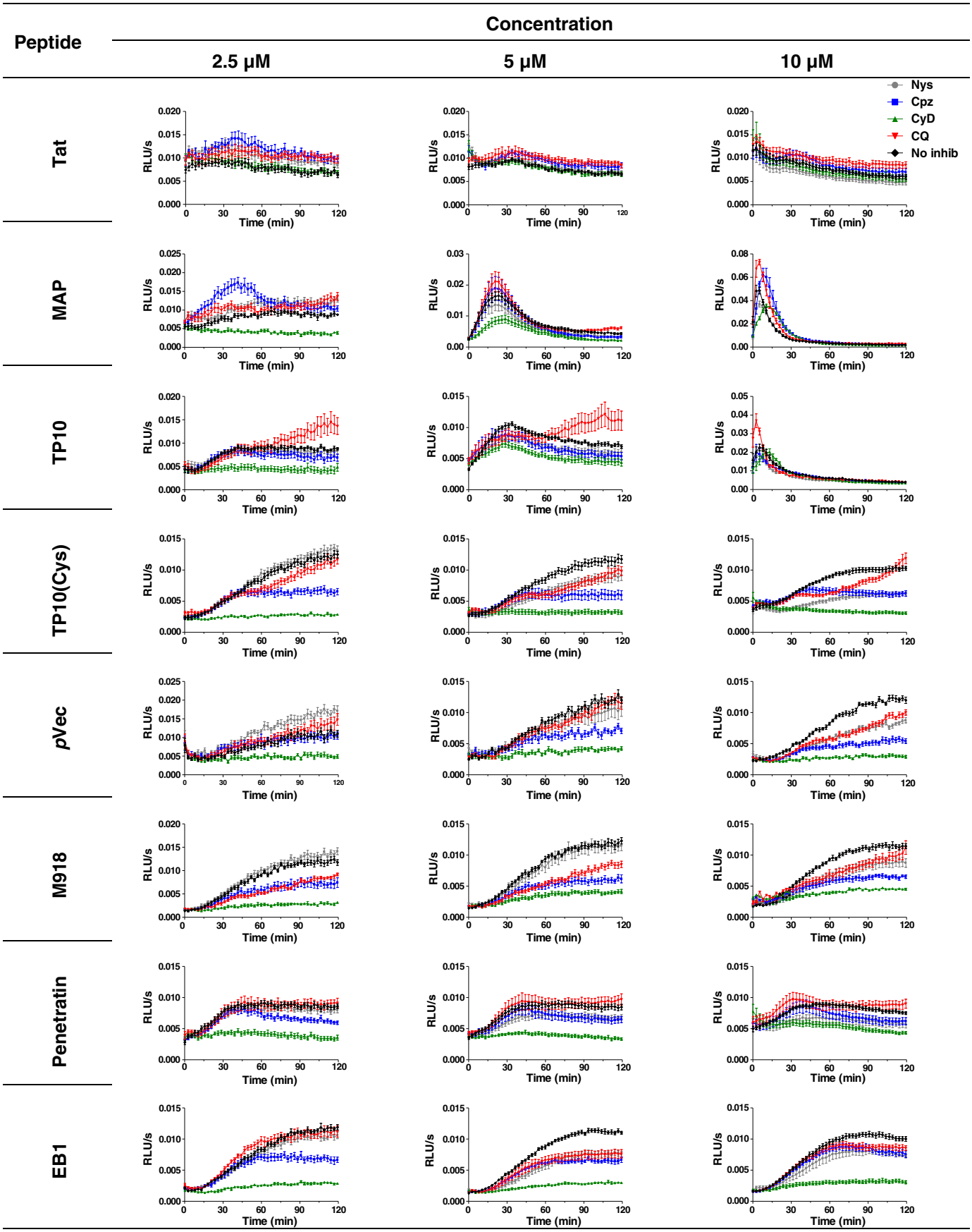

Fig. 2. CPP cytosolic cargo delivery rate kinetic profiles in the presence of endocytosis inhibitors. HeLa pLuc 705 cells were incubated with various concentrations of Luc–CPP conjugates in the presence of endocytosis inhibitors nystatin (Nys), chlorpromazine (Cpz), cytochalasin D (CyD), or chlorquine (CQ). In the control sample no inhibitors were used (No inhib). The figures represent average of three independent experiments, each performed in triplicate, the error bars represent standard error of mean (SEM).

Table 3
Relative inhibition of the overall uptake of Luc-CPP conjugates. The figures represent relative overall uptake compared to the control (no inhibitor) sample ± SEM. Values > 1 indicate that the overall luciferin–CPP uptake in the presence of inhibitors increased. The conditions where the inhibition was statistically not significant, according to the two-way ANOVA using Bonferroni's post-hoc test, are denoted with "–."

CPP	Inc. Time	Nystatin			Chlorpromazine			Cytochalasin D			Chloroquine		
		2.5 μM	5 μM	10 μM	2.5 μM	5 μM	10 μM	2.5 μM	5 μM	10 μM	2.5 μM	5 μM	10 μM
Tat	15 min	1.34 ± 0.05	1.20 ± 0.09	–	–	–	–	–	–	–	–	–	–
	30 min	1.34 ± 0.06	–	–	–	–	–	–	–	–	–	–	–
	60 min	1.34 ± 0.08	–	–	1.43 ± 0.13	–	–	–	–	–	–	–	–
	120 min	1.33 ± 0.08	–	–	1.46 ± 0.12	1.17 ± 0.05	–	–	–	–	1.33 ± 0.09	1.24 ± 0.07	–
MAP	15 min	.46 ± 0.13	–	0.79 ± 0.05	1.47 ± 0.12	–	–	–	0.51 ± 0.07	0.71 ± 0.03	1.42 ± 0.10	–	1.48 ± 0.10
	30 min	–	–	0.86 ± 0.04	1.71 ± 0.16	–	1.52 ± 0.16	–	0.52 ± 0.07	–	1.44 ± 0.11	–	1.48 ± 0.09
	60 min	–	–	–	1.83 ± 0.17	–	1.51 ± 0.13	0.62 ± 0.03	0.58 ± 0.07	–	1.42 ± 0.12	–	1.41 ± 0.08
	120 min	–	–	–	–	–	1.46 ± 0.10	0.49 ± 0.03	0.57 ± 0.07	–	–	–	1.39 ± 0.08
TP10	15 min	–	–	0.76 ± 0.07	–	–	0.79 ± 0.11	–	–	0.78 ± 0.09	–	1.39 ± 0.02	1.22 ± 0.08
	30 min	–	–	0.75 ± 0.04	–	–	–	0.73 ± 0.08	0.80 ± 0.05	–	–	–	–
	60 min	–	–	0.75 ± 0.03	–	–	–	0.61 ± 0.07	0.71 ± 0.04	–	–	–	–
	120 min	–	0.16 ± 0.04	0.77 ± 0.02	–	–	–	0.54 ± 0.07	0.67 ± 0.04	–	1.34 ± 0.08	–	–
TP10(Cys)	15 min	–	–	0.68 ± 0.06	–	–	–	–	–	0.65 ± 0.08	–	–	–
	30 min	–	–	0.62 ± 0.04	–	–	–	0.62 ± 0.02	–	0.44 ± 0.06	–	–	–
	60 min	–	–	–	–	–	–	0.45 ± 0.01	0.60 ± 0.07	–	–	–	–
	120 min	–	–	–	0.69 ± 0.05	0.67 ± 0.08	0.75 ± 0.04	0.32 ± 0.01	0.41 ± 0.04	–	–	–	–
*p*Vec	15 min	–	1.29 ± 0.12	–	–	–	–	–	–	–	–	–	–
	30 min	–	–	0.78 ± 0.06	–	–	–	–	–	0.65 ± 0.02	–	–	0.75 ± 0.02
	60 min	1.35 ± 0.08	–	0.72 ± 0.03	–	–	0.66 ± 0.03	–	0.63 ± 0.04	0.48 ± 0.02	–	–	0.74 ± 0.02
	120 min	1.43 ± 0.08	–	0.70 ± 0.01	–	0.71 ± 0.06	0.53 ± 0.02	0.59 ± 0.03	0.45 ± 0.02	0.34 ± 0.01	–	–	0.73 ± 0.02
M918	15 min	–	–	–	–	–	–	–	–	–	–	–	–
	30 min	–	–	–	–	–	–	0.66 ± 0.06	–	–	–	–	–
	60 min	–	–	–	–	0.71 ± 0.07	–	0.43 ± 0.04	0.53 ± 0.04	0.61 ± 0.03	0.68 ± 0.03	0.67 ± 0.04	–
	120 min	–	–	–	0.66 ± 0.07	0.58 ± 0.05	0.64 ± 0.04	0.30 ± 0.02	0.41 ± 0.03	0.47 ± 0.02	0.66 ± 0.02	0.65 ± 0.03	–
Penetratin	15 min	–	–	–	–	–	–	–	–	–	–	–	–
	30 min	–	–	–	–	–	–	0.76 ± 0.05	0.71 ± 0.03	–	–	–	–
	60 min	–	–	–	–	–	–	0.60 ± 0.04	0.58 ± 0.02	–	–	–	–
	120 min	–	–	–	0.82 ± 0.03	–	–	0.51 ± 0.04	0.50 ± 0.02	0.69 ± 0.05	–	–	–
EB1	15 min	1.19 ± 0.06	–	–	–	–	–	–	–	–	–	–	–
	30 min	–	0.22 ± 0.07	–	–	0.86 ± 0.03	–	0.63 ± 0.02	0.66 ± 0.03	0.73 ± 0.06	–	–	–
	60 min	–	0.33 ± 0.06	–	–	0.78 ± 0.02	–	0.43 ± 0.02	0.42 ± 0.02	0.48 ± 0.04	–	0.80 ± 0.09	–
	120 min	–	0.33 ± 0.05	–	0.74 ± 0.05	0.66 ± 0.02	–	0.31 ± 0.01	0.31 ± 0.01	0.36 ± 0.03	–	0.72 ± 0.07	–

that CPP uptake via endocytic routes can be very fast in certain cases [17], with uptake half-times as low as 2–12 min [32,34,39]. In most cases, however, the uptake is slower, with internalization half-life reaching 60 min [17,28,31,39]. The mentioned studies are informative but they generally tend to have one important drawback. Namely the registered signal often arises from the material still entrapped in endosomes. When CPP mechanisms are studied in these settings then the biologically available material cannot be correctly assessed.

There have been attempts to overcome this problem by designing quenched fluorescence based probes, i.e. by attaching a fluorophore to a CPP and a corresponding quencher to its cargo, or vice versa, and conjugating the two molecules to each other over a disulfide

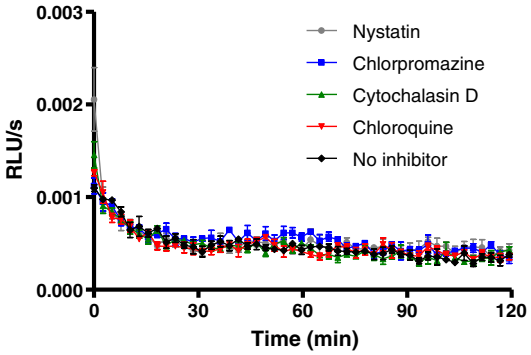

Fig. 3. Example of the uptake kinetics curve of the positive control (membrane permeable free luciferin). The uptake is fast and the used endocytosis inhibitors do not lower the uptake rate. HeLa pLuc 705 cells were incubated with 10 μM free luciferin and the luminescence was measured over time.

bridge [28,39,40]. In the reducing environment of cytoplasm the disulfide bridge is cleaved and quenching is lost, after which the conjugates start to fluoresce.

Disulfide bonds that are used in the mentioned methods are conventionally believed to be cleaved by intracellular concentration of glutathione which represents the major component of cellular redox system [41]. Nevertheless, there are enzymes responsible for controlled disulfide cleavage reactions as well, such as protein disulfide isomerase (PDI) and other Trx family enzymes. This might raise a question whether these proteins could facilitate disulfide bridge cleavage on a cell membrane. However this does not seem to be a case as we have not observed any increase of extracellular free thiols when incubating luciferin–CPPs with cells [17], similarly to the original works conducted with this assay [18]. Indeed, PDI system requires an efficient thiol regenerating system, such as glutathione, but this is not found on the outer surface of cell membranes [41]; additionally PDI seems to be excluded from the membranes of macropinosomes [42]. For example, in a thorough study regarding the delivery of antibody-drug conjugates it was demonstrated that the overall conditions in endocytic pathways are oxidizing and the reductive disulfide bond cleavage is very inefficient [43].

There seems to be a disulfide processing activity, however, in endosomes of some specific cell types, e.g. antigen presenting cells require gamma-interferon-inducible lysosomal thiol reductase (GILT) for correct presentation of the endocytosed antigens [41]. GILT is active at low pH and is regenerated by free cysteine instead of glutathione. Despite having an intrinsic function in antigen presenting cells, GILT-like endosomal thiol processing events might occur also in other cell types, as indicated by the cleavage of disulfide bond based FRET probes [44]. This cleavage occurs, however, with a half-life of 6 h which notably longer time than the course of our CPP uptake kinetics experiments. Furthermore, the half-life of luciferin–CPP conjugates was shown to be 11 h [18] which significantly lowers the possibility that the CPP conjugates could be actively degraded in the endolysosomal pathway and that the degradation products could diffuse through vesicle membranes, leading to false-positive readouts. Thus we hypothesize that the main signal in the used kinetic assay arises from the conjugates reaching cytoplasm. Indeed, the CPP half-lives in this investigation seem to be much longer compared to studies in which the internalization kinetics reflect also the endosomally accumulating peptide conjugates [17,32,34,39].

The quenched fluorescence based strategies are useful for assessing the CPP cytosolic uptake mechanisms but they are not free from limitations. For example, measurements are often restricted to suspension cells or complicated experimental setup is required for growing cells on thin films instead of tissue culture plates [28,39,40]. Also, because the fluorescence is never fully quenched it might be difficult in some cases to distinguish the high amount of endosomally entrapped conjugates that have not yet reached the cytoplasm from the low amount of cytosolic peptide when in the latter case the endosome-confined conjugate amount is small.

We are using luciferin-conjugated CPPs [18] to assess their cytosolic cargo delivery kinetics and mechanisms. Similarly to the quenched fluorescence assays, luciferin is released when the conjugates reach the cytoplasm (Fig. 1). In cytoplasm the expressed luciferase enzyme converts luciferin into oxyluciferin which is accompanied by the emission of luminescence. The issues arising from incomplete quenching are eliminated because neither the endosomally entrapped conjugates nor uncleaved luciferin molecules cannot be substrates for the enzyme. The luciferin assay allows to distinguish clearly different uptake kinetic profiles [17] and to describe the cytoplasmic cargo delivery kinetics in the presence of endocytosis inhibitors. Based on our hypothesis the rate limiting factor, and thus the origin of readout in this assay, is entry of luciferin–CPP conjugates into cytoplasm. Indeed, in case of fast-internalizing peptides the luminescence signal starts to increase without a lag period.

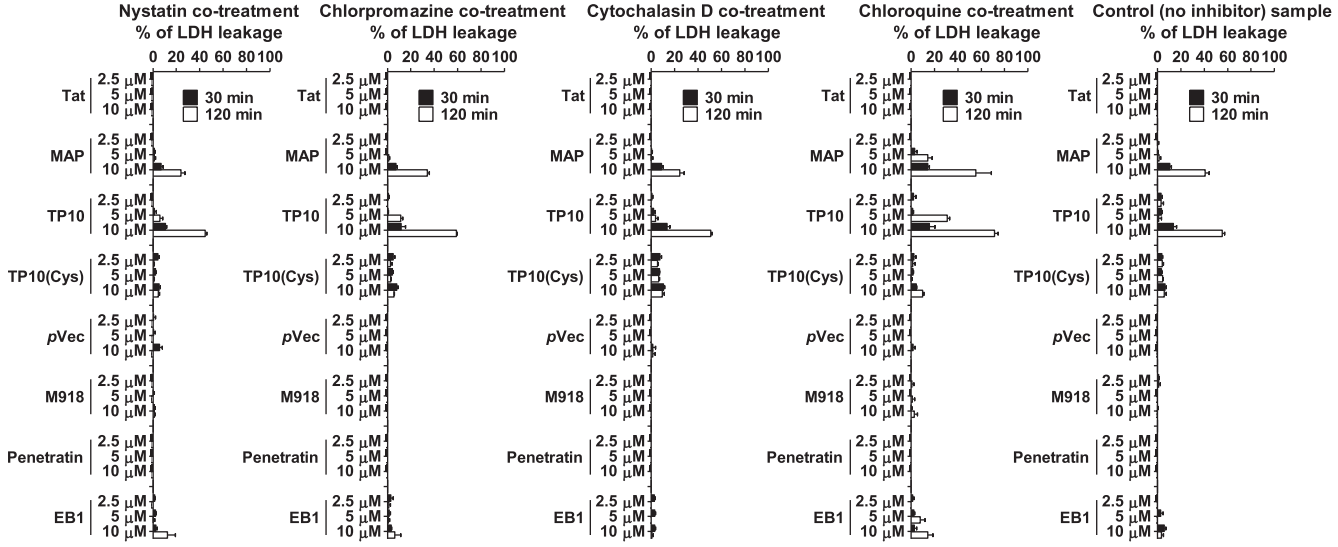

Fig. 4. CPP and inhibitor induced LDH leakage compared to the untreated samples. HeLa pLuc 705 cells were treated with various concentrations of luciferin–CPP conjugates in the presence/absence of endocytosis inhibitors and LDH leakage was measured using the CytoTox-One™ assay. The LDH leakage from untreated cells was regarded as 0% leakage and leakage from lysed cells as 100% leakage. The data represent two independent experiments, performed in triplicate, and the error bars describe standard error of mean (SEM).

The other factors, disulfide cleavage, linker cylization and luciferin-luciferase reaction, are not expected to be rate limiting. Of course, the disulfide cleavage reduction reaction is by far not trivial because in addition to glutathione different enzymes, such as PDI, participate in the process as well and the reaction involves potentially up to nine different steps [45]. However, it seems that when PDI catalyzes the glutathione-mediated cleavage then the disulfide reduction is expected to occur within minutes [45]. Upon disulfide cleavage, the linker self-cyclization is very fast, it is completed probably within seconds [18], and luciferin–luciferase reaction reaches its maximum rate in less than a second [46].

Based on the prior mentioned hypothesis, in the current study we used this kinetic assay to evaluate CPP uptake mechanisms. First we analyzed how the used endocytosis inhibitors Nys, Cpz, CyD and CQ (Table 2) affect the overall cytosolic delivery of the luciferin–CPP conjugates in different time points (Fig. 2, Table 3, Supplementary Fig. 1). According to this, CyD (an inhibitor of macropinocytosis) seems to be the most efficient inhibitor for all the tested peptides except Tat. Stronger inhibition is generally observed at longer incubation times. Macropinocytosis has been previously shown to be involved in CPP uptake especially in case of cargo-conjugated cationic peptides [3,15]. Our results are partly in line with a CPP mechanisms study based on a biological readout where the nuclear delivery of M918, penetratin and Tat was found to be strongly dependent on macropinocytosis [47]. This is not surprising because it has been suggested that macropinosomes might bypass the degrading lysosomal pathway [48] thus potentially enabling more efficient release of luciferin–CPP conjugates into the cytoplasm. However cytoplasmic entry of luciferin-TP10 conjugate was rather insensitive to the treatment with a CME inhibitor Cpz, contrasting to the previously mentioned study according to which the nuclear delivery of helical peptides depends strongly on CME [47]. The latter, however, does not seem to be a tendency in cytoplasmic entry, however cargo type dependent effects cannot be excluded.

In addition to macropinocytosis other pathways are also involved. For example, CME seems to be extensively used in the internalization of all the slow uptake group peptides, although in a considerably lesser extent (Fig. 2). While in case of TP10(Cys) the involvement of CME appears similar at every tested conjugate concentration, then in case of M918 and *p*Vec the CME is more involved at higher conjugate concentrations and in case of penetratin and EB1 the trend is reversed, according to our data. Nys co-treatment reveals that lipid raft/caveolae dependent endocytosis is involved at high luciferin–CPP concentrations, perhaps indicating the presence of compensatory transport mechanisms.

Interestingly, even the uptake of the fast internalizing CPP conjugates is affected by the endocytosis inhibitors (Fig. 2, Table 3), demonstrating a strong involvement of active transport in their uptake despite these curves closely resemble the behavior of membrane permeable naked luciferin as observed previously [17]. However this is not surprising because endocytosis can be very fast when participating for example in intrinsic receptor internalization tasks.

These results collectively indicate that the relative incorporation of different uptake routes may change depending on the peptide concentration and that more than one route could indeed be activated simultaneously. Additionally the data suggest that for cytoplasmic cargo delivery the most important and extensively used endocytosis route is macropinocytosis, even for the helical CPPs such as TP10. The involvement of CME and caveolae/lipid-raft mediated endocytosis depends on the CPP concentration (Table 4).

In the time frame of our experiments, the treatment with an endosomolytic agent CQ increases the cytoplasmic entry of only the fast uptake group peptides Tat, TP10 and MAP. In case of *p*Vec, M918 and EB1 the CQ tends to inhibit the overall uptake rather than increase it and the overall uptake of penetratin and TP10(Cys) is not affected by CQ at all. It seems that the endosomolytic effects of CQ are not revealed that clearly during the short course of our experiment. This is not surprising as similar effects have also been seen elsewhere where CQ treatment led to lowered short term CPP uptake while the long term biological effect was increased nevertheless [47]. CQ treatment reduces the overall endosomal trafficking rate, especially in early time points because it slows down the endosome acidification and overall endocytic machinery [49]. Many assays actually seem to benefit from this process as it gives more time for the internalized conjugates to enter cells before being recycled back to cell membrane. However, in case of certain peptides CQ induces certain biphasic change of the uptake rate kinetics profiles—uptake rate goes through a secondary increase later in the experiment (Fig. 2). The effect is present at every concentration of the TP10(Cys) and M918 conjugate, lower concentrations of the MAP and TP10 conjugates, and higher concentrations of *p*Vec and penetratin.

In case of the slow uptake group peptides the treatments do not induce any short term toxicity and the cell membrane remains intact. This suggests that the diffusion of luciferin–CPP conjugates into cells and the outflow of the reducing glutathione from cells is restrained, thus excluding potential artifacts. Co-treatment with endocytosis inhibitors does not change that, except in case of TP10(Cys) and EB1 where CQ tended to slightly compromise the membrane integrity.

High concentrations of the fast uptake group peptides tend to be toxic after 120 min incubation however it should be pointed out that the LDH leakage at the 30 min time point is considerably lower and most of the internalization of these peptides is observed in this time frame. It seems that the membrane active properties of MAP and TP10 are increased in the presence of all the used endocytosis inhibitors, but again this is restricted to high peptide concentrations and long incubation times when most of the uptake has already taken place. Thus the general analysis of the inhibitor-induced changes in uptake kinetics does not seem to be compromised by the toxicity. Different toxicity profile may explain to some extent why the uptake profiles of luciferin-TP10 and luciferin-TP10(Cys) conjugates are so different. On the other hand, the differences may also be related to different peptide structures as helical conformation of TP10 seems to be important for its membrane activity properties [50,51] and by conjugating luciferin cargo orthogonally to this peptide its helicity profile could be altered.

In conclusion, we have demonstrated that in the cytosolic delivery of luciferin–CPP conjugates the prevailing uptake route is macropinocytosis, even for the fast internalizing group peptides. To a smaller extent CME and caveolae/lipid-raft dependent endocytosis are involved too, being in accordance with literature. Furthermore, we have showed, based on uptake kinetic data analysis, that the involvement of different endocytosis sub-types may depend on luciferin–CPP concentration in case of certain CPPs but not in case of others. These are extremely important findings, in our opinion, as they may lead to yet another CPP-classification aspect. Also this exemplifies that CPP classification and characterization is by far not complete.

Table 4
Concentration dependent involvement of endocytosis pathways in luciferin–CPP uptake.

Endocytosis pathway	Involvement of the pathway is the most pronounced…		
	… at low conjugate concentration	… at high conjugate concentration	… at every conjugate concentration of no correlation
Clathrin mediated endocytosis	Penetratin, EB1	M918, pVec	Tat,MAP, TP10, TP10 (Cys)
Macropinocytosis	Tat, MAP, TP10	–	TP10(Cys), pVec, M918, penetratin, EB1
Caveolae/lipid raft dependent endocytosis	–	Penetratin, EB1, M918, pVec	Tat, MAP, TP10, TP10 (Cys)

The involvement of these pathways in the cytosolic luciferin–CPP delivery is reflected when comparing the overall total uptake in selected time points but they are more clearly revealed when analyzing the kinetic parameters or shapes of the uptake kinetics curves. This information is valuable for designing new experiments and novel peptide-based delivery vectors because the incorporated endocytosis route might define the fate of the internalized material and its subsequent intracellular trafficking.

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Conflicts of interest statement

None.

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