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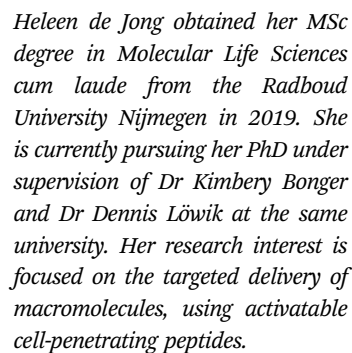
An important hurdle for the intracellular delivery of large cargo is the cellular membrane, which protects the cell from exogenous substances. Cell-penetrating peptides (CPPs) can cross this barrier but their use as drug delivery vehicles is hampered by their lack of cell type specificity. Over the past years, several approaches have been explored to control the activity of CPPs that can be primed for cellular uptake. Since the first report on such activatable CPPs (ACPPs) in 2004, various methods of activation have been developed. Here, we provide an overview of the different ACPPs strategies known to date and summarize the benefits, drawbacks, and future directions.

Cells have a complex and mostly impermeable cell membrane to ensure stable intracellular conditions and to protect the cell from harmful exogenous substances. This lipid bilayer membrane allows the diffusion of small molecules but excludes the entry of larger molecular entities. As a consequence, hydrophilic small

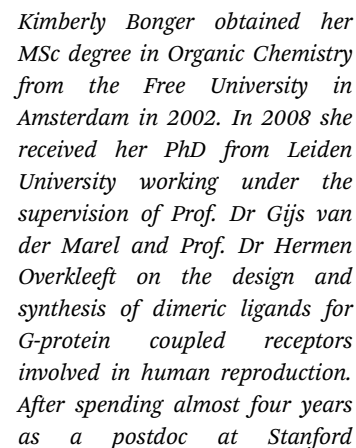
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molecules and protein- or nucleic acid-based therapeutics are also excluded and effectively transferring these over the cell membrane has been a challenge for many years.¹

A promising method for the intracellular delivery of membrane-impermeable therapeutics emerged with the discovery that certain peptides could transfer cargo across the cell membrane. In 1988, two separate research groups reported that human influenza virus (HIV) Tat trans-activator proteins enabled cellular uptake along with the transport of cargo.^{2,3} Green and Loewenstein also identified the primary sequence of the uptake region of Tat (RKRRRQRRR).² Since these first reports,



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University in the laboratory of Thomas Wandless working on molecular tools to control protein stability, she returned to the Netherlands as an assistant professor in Chemical Biology at the Radboud University in Nijmegen. Her research focusses on the development of novel bioorthogonal reactions and targeted drug delivery strategies as well as the fundamental understanding of cellular mechanisms involved in (auto)immunity.

The cellular uptake mechanisms of CPPs have been extensively studied, but are still poorly understood as different CPPs seem to enter cells through different pathways.^{11,12} Mechanisms of uptake can be classified in two categories: energy independent direct penetration and energy dependent endocytosis (Fig. 1). Direct penetration occurs in cases when a high concentration of peptide is available and involves a tight interaction between the CPPs and cell membrane. Direct cell penetration mechanisms include (1) pore formation, where the CPPs insert themselves in the membrane; (2) the carpet model, where CPPs position on the membrane as a carpet, thereby increasing membrane fluidity and passage of the CPPs; and (3) inverted micelle formation, where the phospholipid bilayer

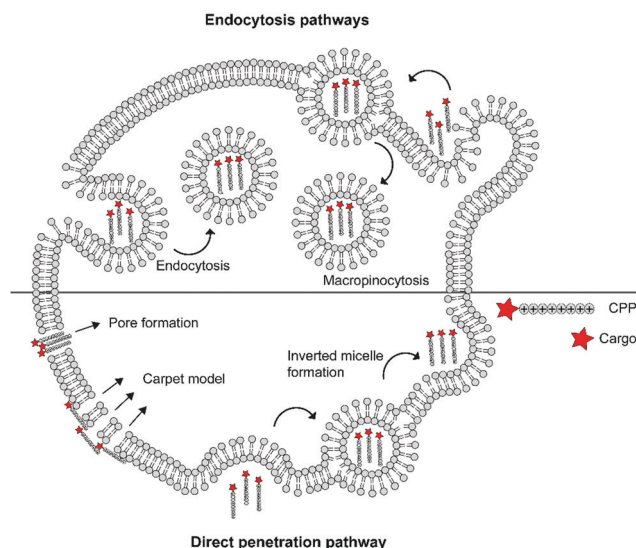


Fig. 1 Schematic summary of various mechanisms of cellular uptake of cationic cell-penetrating peptides (CPPs) via endocytic pathways or direct penetration.



Dennis W. P. M. Löwik

Nijmegen in the Bio-organic chemistry group of Prof. Jan van Hest. He was given an associate position in 2006 and his research revolves around peptide-based materials, operating at the interface of chemistry, biology and physics, in order to develop smart materials and targeted drug delivery systems. Current topics are cell penetrating peptides, peptide based hydrogels, cancer theranostics and peptide based vaccines.

Dennis Löwik completed his MSc in organic chemistry in 1994. In 1998 he received his PhD in medicinal chemistry under the supervision of Prof. Rob Liskamp at Utrecht University. This was followed by a post-doctoral position in Cambridge (UK) in the biotechnology group of Prof. Chris Lowe until 2000. After a year's stay in the supramolecular chemistry group of Prof. Bert Meijer, he was appointed in 2001 at the Radboud University

encapsulates the peptide by formation of inverted micelles. However, CPPs, especially those that carry cargo, internalize mainly through endocytic pathways.¹³ These include micropinocytosis, clathrin- or caveolin-mediated endocytosis, and clathrin/caveolin independent endocytosis.¹⁴ The path taken depends on the size and physicochemical properties of the cargo as well as the nature of the CPP and the target cell.¹⁵ For more detailed information on the uptake mechanisms of CPPs and the contributing factors we refer to some recent reviews on this topic.^{12,16}

Even though CPPs are potentially promising drug delivery vehicles, several issues hamper their use in practice. These include (1) their lack of cell type specificity resulting in uncontrolled uptake and potential adverse effects,¹⁷ (2) their toxicity at high concentrations, which is associated with membrane perturbation¹⁸ and (3) their fast blood clearance as was shown by a study where only 1% of the injected dose of ten different CPPs remained at the target site after 4 h in tumour bearing mice.¹⁷

To overcome these challenges, much research is focussed over the last years to control CPPs and to activate them only at the target site by use of an external trigger (Fig. 2). The Tsien

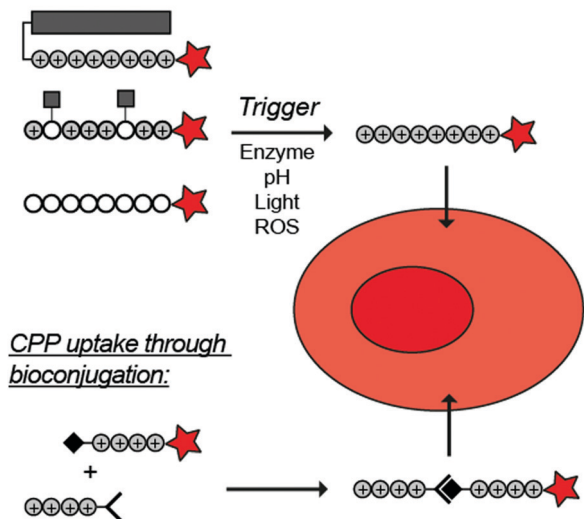
Triggered CPP uptake:

Fig. 2 Dormant cell-penetrating peptides (CPPs) cannot enter cells. Upon triggering they are activated and can be taken up by cells.

group first described such so-called Activatable CPPs (ACPPs) in 2004 where they fused a polycationic CPP to an inhibiting polyanionic domain *via* a protease cleavable linker.¹⁹ Since then, numerous advances in the development of ACPPs and their triggers have been reported. In this review, we provide an overview of the strategies in the design of ACPPs since their discovery more than 15 years ago.

CPP activation through removal of an inhibitory domain

Cationic CPPs interact with the negatively charged cell membrane via electrostatic attraction. Hence, masking the positive charge using an inhibitory domains restrains cellular uptake.^{15,19} Inhibitory domains that have been explored include poly(ethylene) glycol (PEG) blocks that sterically mask the CPP, or polyanionic domains that lower the isoelectric point of a cationic peptide. The inhibitory domains are conjugated to the CPP *via* a trigger responsive linker to control cellular uptake. Below and in Table 2, we summarize the various reported approaches in more detail.

Enzyme triggered removal

Compared to healthy tissue, diseased tissue may abundantly expresses particular proteolytic enzymes such as cathepsins, urokinases, caspases and matrix metalloproteases (MMPs).²⁰ The earliest example on ACPPs by the Tsien group exploited an MMP-sensitive PLGLAG linker to connect a polycationic CPP to a polyanionic inhibitory domain.¹⁹ Enzymatic cleavage of the linker resulted in dissociation of the inhibitory domain and specific CPP uptake in MMP rich fibrosarcoma cells with an 18-fold difference between activated and non-activated structures (Table 2, entry 1).¹⁹ In a follow-up study, similar structures exhibited reduced blood clearance, improved distribution to the

target site, and reduced toxicity compared to CPP controls.²¹ The Tsien group also showed that MMP-sensitive ACPPs are taken up in a variety of tumors *in vivo*.²²

Using a similar ACPP construct with the MMP-sensitive linker, the Zhang group delivered a protoporphyrin photosensitizer to fibrosarcoma cells *in vivo*.²³ Imaging experiments demonstrated efficient localization to tumours and a decrease in tumour size upon a 12-day treatment of injections once every three days. Alternatively, the group of Cao delivered doxorubicin (DOX) to human breast adeno-carcinoma cells, but not to MMP deficient human mammary endothelial cells, by coating DOX-loaded nanoparticles with MMP-triggered ACPPs.²⁴ The Kim group combined MMP-responsiveness with reactive oxygen species (ROS) triggers to additionally advance the control of drug delivery.²⁵ In this case, micelles were coated with ACPPs based on (poly(L-methionine-*block*-L-lysine)-PLGLAG-PEG) and loaded with DOX or IR-760 dye. MMP-assisted PLGLAG cleavage activated the polylysine-based ACPP to facilitate cellular uptake after which ROS oxidized the methionine thioether moieties to hydrophilic sulfoxide groups thereby destabilizing the micelle structures. High accumulation in tumours and a long retention time were observed, as well as efficient ROS triggered cargo release.²⁵

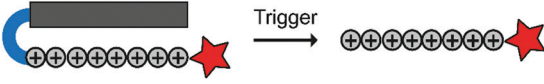
Chen and coworkers coated a cyclosporine A loaded nanocarrier with an ACPP connected via an alternative MMP sensitive linker (PVGLIG) and studied the localization in controlled cortical impact injury mice (Table 2, entry 2).²⁶ The structures crossed the blood brain barrier and internalized into primary astrocytes and neurons at the lesion site. Cyclosporine A inhibits the opening of the mitochondrial permeability transition pore thereby preventing apoptosis. Less cell death was observed around the lesion site in mice treated with the cyclosporine A-loaded and ACPP-coated nanocarriers compared to the cyclosporine A treated controls.

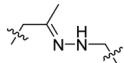
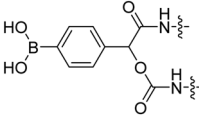
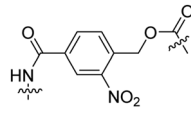
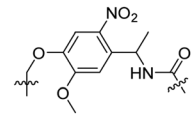
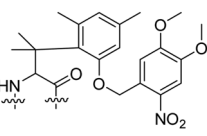
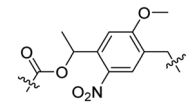
Besides MMPs, a cathepsin B cleavable sequence, PGFK, has been used to connect a polyanionic inhibitor to a Tat derived CPP that was conjugated to mesoporous silica quantum dot nanocarriers loaded with DOX (Table 2, entry 3).^{27,28} In the presence of endogenous cathepsin B levels, DOX was released and transported selectively to the nuclei of human adenocarcinoma cells while uptake was drastically decreased in the absence of cathepsin B.

Xiang and coworkers used the prostate-specific antigen (PSA), which is overexpressed in prostate cancer, as trigger to specifically deliver siRNA against the polo-like kinase 1 (PLK-1) transcript. PLK-1 induces apoptosis in cancer cells when depleted.^{29,30} The authors coated siRNA loaded liposomes with ACPPs containing a PSA-responsive HSSKYQ linker between a polycationic CPP and a polyanionic peptide inhibitor (Table 2, entry 4). Flow cytometry and imaging studies indicated increased uptake in PSA rich human prostate cancer 22Rv1 cells compared to PSA deficient PC-3 cells. Moreover, PLK-1 expression was decreased in the prostate cancer cell line treated with the liposomes, but not in the untreated control. In these experiments, increased apoptosis accompanied PLK-1 downregulation. *In vivo* studies with 22Rv1 xenograft tumours showed increased tumour accumulation for ACPP coated liposomes, compared to polycationic CPP coated liposomes.



Table 2 Activatable cell-penetrating peptides (ACPPs) based on linkage of an inhibitory domain. Amino acids are indicated via the single letter code, in which D-amino acids are noted in lower case. Cy5 = cyanine5 red dye; PpIX = protoporphyrin IX; Dox = doxorubicin; PLK-1 = polo-like kinase 1; PSA = prostate specific antigen; NE = neutrophil elastase; FITC = fluorescein; NA = not available; CPT = camptothecin; EGFR = epidermal growth factor; QDs = quantum dots

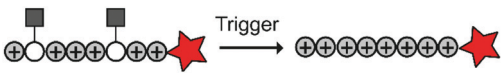


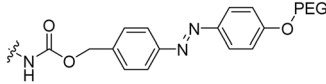
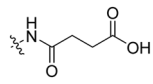
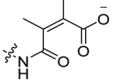
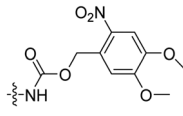
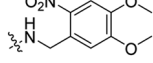
	Linker	CPP	Inhibiting domain	Cargo	Trigger	Ref.
Enzyme sensitive linkers						
1	PLGLAG	R ₉	e _{6,8}	Cy5	MMP-2 and -9	19 and 21
		R ₉	E ₈	PpIX		23
		R ₉	E ₉	Dox		24
		Poly(M-block-K)	PEG	Dox		25
2	PVGLIG	R ₉	(EGG) ₃	CsA-LMNC	MMP-9	26
3	PGFK	Tat	E ₆	Dox	Cathepsin B	28
4	HSSKYQ	R ₈	(DGG) ₄	PLK-1 siRNA	PSA	29
5	RLQLK(Ac)L	r ₉	e ₉	Cy5	NE	32
6	DPRSFL	r ₉	e ₈	Cy5 Rhodamine	Thrombin	33
pH sensitive linkers						
7		R ₈	(ehG) ₄	PLK-1 siRNA	pH < 6.8	37
ROS sensitive linkers						
8		R ₉	E ₉	FITC Cy5	H ₂ O ₂	43
Light sensitive linkers						
9		Tat	PEG	Liposomes loaded with Atto655	λ = 254 nm, 2 min	47
10		R ₇	E ₇	FITC CPT	λ = 365 nm, 10 min	48
11		Penetratin	E ₄ R ₄	EGFR siRNA	λ = 740 nm, 30 min and pH < 6.4	53
12		R ₇	E ₇	QDs	Two-photon, λ = 740 nm, 3 h	48

Neutrophil elastase is highly abundant in several cancers, such as human breast and lung cancer.³¹ The Tsien group included a neutrophil elastase sensitive RLQLK(Ac)L sequence in an ACPP design to visualize tumours with a Cy5 dye (Table 2, entry 5).³² The acetylated lysine improved specificity for the neutrophil elastase over other endogenously expressed elastases. Injection of ACPPs in nude mice with human breast cancer xenografts visualized the tumour 6 h after injection, while the structure was not observed in mice treated with control structures where the inhibiting domain was linked via a non-cleavable D-amino acid based sequence (rlqlkl).

The Tsien group further extended the ACPP concept using a thrombin-sensitive DPRSFL linker between a fluorescently labelled cationic CPP and an anionic inhibitory domain for imaging purposes (Table 2, entry 6).³³ Thrombin is active in blood coagulation and abundant in atherosclerotic plaques, which narrow arteries.^{34,35} Gel electrophoresis showed that purified thrombin could cleave the ACPP *in vitro*. The ACPP was injected in mice with induced atherosclerosis and 6 h after injection, a positive fluorescence correlation was observed with plaque burden for the ACPP but not for non-cleavable controls. Fluorescence distribution was also increased in slices of human

Table 3 Overview of activatable cell-penetrating peptides (ACPPs) based on side chain modifications. Amino acids are indicated via the single letter code, in which D-amino acids are noted in lower case. FITC = fluorescein; Luc PNA = luciferase peptide nucleic acid; Dox = doxorubicin; pen = penetratin; VB = vinorelbine bitartrate



		Number of R groups	CPP	Cargo	Trigger	Ref.
Enzyme sensitive side chain modifications						
1	 R = Ala or Gly-Pro	1 or 2	Tat	FITC	Aminopeptidase N dipeptidyl peptidase IV	55
2		2	M918	Luc PNA	Bacterial azoreductase	57
pH sensitive side chain modifications						
3		3	Tat	Dox	pH 5.0	58
4		6	CR ₈ G ₃ PK ₆	Dox	pH 6.8	59
Light sensitive side chain modifications						
5		3	Pen	(klaklak) ₂	$\lambda = 365 \text{ nm}$, 6 W, 10 min	60
6		3	Pen	VB	$\lambda = 740 \text{ nm}$, 30 min, $3.48 \times 10^{12} \text{ photons s}^{-1}$	61

via an azobenzene moiety (Table 3, entry 2).⁵⁷ As a proof of principle, a silencing peptide nucleic acid (PNA) specific for the firefly luciferase gene was conjugated to the C-terminus of the ACPP to monitor cell entry in luciferase-expressing colon adenocarcinoma cells. Incubation of the cells for two days resulted in azobenzene cleavage and 1,6-elimination followed by cell uptake of the CPP-PNA conjugate, as evidenced by a 40% decrease in luciferase activity compared to control cells.

pH triggered release

The Murdoch group reported on the control of CPP activity by using acid sensitive side chain modifications. Here, they conjugated succinyl moieties to the glutamine and both lysine residues of Tat and used these to coat PEG-based micelles loaded with Nile Red dye or DOX (Table 3, entry 3).⁵⁸ These ACPP-coated micelles were not taken up at physiological pH, while an 8 hour incubation at pH 5.0 resulted in cellular uptake comparable to that of micelles coated with unmodified CPPs. The ACPP-coated micelles displayed reduced blood clearance and increased localization to tumour cells *in vivo*, compared to unmodified CPP-coated controls. Furthermore, the tumour size in these mice was reduced upon treatment with DOX loaded structures.

Using a similar approach, Cheng *et al.* conjugated 2,3-dimethyl-maleic acid (DMA) to the lysine residues of a

cationic CPP peptide (Table 3, entry 4).⁵⁹ The DMA-linked lysine residues underwent electrostatic interactions with the arginine residues in the chain, thereby inactivating the ACPP. The labile amides hydrolysed at pH 6.8 and the DOX-linked ACPPs were efficiently taken up at this pH. Furthermore, they demonstrated that nonspecific cellular toxicity decreased, while repression of tumour growth resembled that of free DOX.

Light triggered release

In a light sensitive ACPP design, the group of David modified the lysine side-chains of Penetratin with an *o*-nitrobenzyl moiety and loaded these ACPPs onto FITC-labelled *N*-(2-hydroxypropyl)-methacrylamide (HPMA) based polymers along with a proapoptotic peptide known as klak (Table 3, entry 5).⁶⁰ Irradiation with UV light removed the photocage thereby allowing the CPP modified polymer to enter human prostate cancer cells, while polymers that were not illuminated did not show much cellular uptake.

Similarly, the Mei group conjugated *o*-nitrobenzyl photocages to the lysine residues of a Penetratin derived CPP and coated these onto liposomes loaded with anticancer agent Vinorelbine (VB, Table 3, entry 6).⁶¹ Two-photon NIR light activation ($\lambda = 740 \text{ nm}$, 30 min) of the ACPP-coated liposomes resulted in uptake into human fibrosarcoma cells accompanied by a decreased viability and indicating that cargo could be delivered effectively.



CPP activation through an induced conformational change

Changes in peptide conformation may affect the uptake properties of CPPs and have been explored to control CPP activity.⁶² Below we describe approaches where the conformation, and thus the activity of CPPs, can be tuned in response to triggers such as pH or light, without altering the primary peptide structure.

pH triggered conformational changes

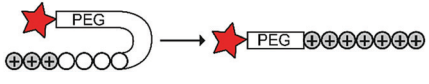
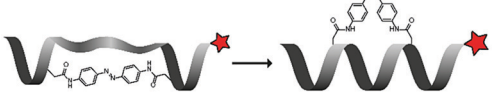
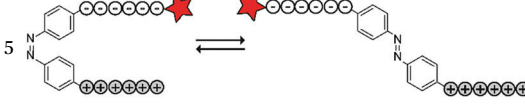
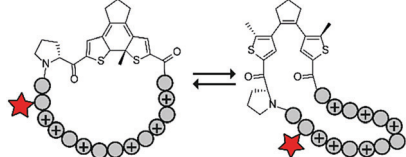
Histidines can serve as a pH trigger as their imidazole side chains have a pK_a around 6 and they are mostly unprotonated at physiological pH, while they can become protonated in an acidic environment. Lee and coworkers exploited this property and coated micelles with Tat-derived CPPs that were conjugated to long histidine repeats and PEG domains.⁶³ At physiological pH, the neutral histidine domains interacted with the micelles to bury Tat between the PEG domains. Upon acidification, the histidine residues became protonated, lost their hydrophobic interactions and exposed Tat (Table 4, entry 1). Acidification of the extracellular fluid from 7.4 to 7.0 or 6.8 was accompanied by a 30- or 70-fold increase in uptake in human breast cancer cells, respectively.

Both the Wang group and He group developed ACPPs by exchanging all lysine residues in two lysine-rich CPPs for histidines,

creating respectively TH (AGYLLGHINLHHLAHLXHHIL, with X = 2-aminoisobutyric acid)^{64,65} and LH (LHLLHHLHLLHH, Table 4, entry 2).⁶⁶ At physiological pH, neither peptide is able to enter cells, unlike their lysine rich counterparts (TK and LK, respectively). Acidification to pH 6 protonated the histidine residues and induced cellular uptake. With this system, the Wang group delivered anticancer drug camptothecin intracellularly with both peptides – TH for HeLa cells and LH for human breast adenocarcinoma cells.^{64,66} The He group coated TH onto liposomes loaded with either the drug paclitaxel or IR-probes.⁶⁵ Paclitaxel was delivered to tumorigenic tissue *in vitro*, as demonstrated by flow cytometry and cellular imaging, while NIR imaging showed *in vivo* delivery of IR-probes to the tumour sites.

The Kim group harnessed α -helical conformational requirements in their ACPP design. They introduced imidazole and carboxylic acid groups onto lysine residues to ensure pH-dependent conformational control of a synthetic, polylysine-based CPP (Table 4, entry 3).⁶⁷ At physiological pH, the charge distribution created an inactive, tightened helix, while in a slightly acidic environment (pH 6) the partial protonation gave rise to an intact helical structure that could enter cells. When the pH dropped below 4, however, additional protonation stretched the helix further through strong intramolecular electrostatic repulsion that led to inhibition of uptake. They demonstrated the potential of the approach by showing that the ACPPs were taken up in human lung carcinoma cells at pH 6, but not at pH 7.4.

Table 4 Overview of activatable cell-penetrating peptides (ACPPs) triggered through conformational changes. Amino acids are indicated *via* the single letter code. PEG = poly(ethylene) glycol; CPT = camptothecin; PTX = paclitaxel; probe = IR-probe; SC = side chain; LK = leucine and lysine rich CPP; *trans*-Ab = *trans*-azobenzene; Tamra = Tamra red dye; RhoB = rhodamineB dye; γ^{Ahx} = *O*-amino-hexylated γ -tyrosine; FITC = fluorescein

Premise	CPP	Inhibiting factor	Cargo	Trigger	Ref.
pH sensitive conformational changes					
1 	Tat	PEG	DOX loaded micelle	pH drop	63
2 	TH LH TH	H side chain charge	CPT CPT PTX probe	pH drop	64 66 65
3 	K ₉₇	SC modifications with imidazole or carboxylic acids		pH drop to 6.0	67
Light sensitive conformational changes					
4 	LK	<i>trans</i> -Ab	Tamra	365 nm, 5 min, 8 mW cm ⁻²	68
5 	R ₉	E ₉	RhoB	$\lambda = 488$, 1–1.5 μ W, ~ 60 ms μ m ⁻²	69
6 	RRF- γ^{Ahx} P-FRR	Circular structure	FITC	$\lambda = 590$ nm	70



	Premise	CPP	Cargo	Ref.
Fusion of two subunits				
1		R_8	FITC	71
2		R_8	FITC GFP	72
3		R_8	FITC HSA	75
Polymerization				
4		Poly Arg side chains	FITC or Tamra FITC DOX, avidin, BRD-4, CASP-3, BSA, Ab BSA, IgG QD MSNs	76 83 78 79 80 81
5		GSGSG KLAK DP > 5	KLAK	82

cellular uptake was observed through flow cytometry while the apoptotic properties of KLAK remained intact in a dose-dependent manner. It should be noted, however, that the structures were polymerized before administration and could not be activated on site. To evolve this strategy to its full potential with respect to ACPP design, the possibility of targeted on site polymerization is essential. Such a strategy may provide a potent approach for transportation of therapeutic peptides across the cell membrane.

Summary and outlook

CPPs can be temporarily inactivated by introducing interacting inhibitory domains or, when bulky groups are undesired, smaller side chain modifications on, for example, lysine residues to mask CPP activity. Masking groups can be removed by local triggers such as enzymes or changes in pH as well as

increased specificity of ACPPs over native, non-activatable CPPs greatly improves their possible use for other applications *in vivo*. Keeping in mind the variety of ACPPs and their activation triggers, we foresee promising clinical potential for the local cellular delivery of a variety of therapeutics using these structures.

Conflicts of interest

Acknowledgements

CPPs have entered clinical trials for treatments of several dysfunctions including hearing loss,⁸⁵ coronary artery disease,⁸⁶ macular degeneration,⁸⁷ solid tumours,⁸⁸ central nervous tumours,⁸⁹ scar prevention,⁹⁰ heart attack,⁹¹ Duchenne muscular dystrophy,⁹² and ocular inflammation.⁹³ However, to date, none have been approved for therapeutic use. CPP application in the clinic is challenged by the limited bio-distribution and accumulation of the structures in liver or kidney.¹⁷ In addition, the half-life of the structures varied from 1.2 to >72 hours, where short lived CPPs generally contained cationic residues likely making them more susceptible for proteolytic cleavage. Adopting ACPP strategies as described in here may improve the stability and bio-distribution of the constructs and therefor also possible clinical translation.

References

- To the best of our knowledge, one ACPP has been tested in a phase 1 clinical study and used for imaging purposes.⁹⁴ This ACPP was designed to visualize tumours during surgical procedures and resembled the ACPP designed by the Tsien group, containing an inhibitory domain and a protease-sensitive linker.^{19,95,96} In this construct, the CPP as well as the inhibitory domain each carried a fluorophore thereby inducing FRET. Proteolytic cleavage in tumorigenic tissue disabled FRET resulting in a measurable change of fluorescence intensity. The ACPP was administered via intravenous infusion for 30 minutes, 2 to 20 h before the surgery and allowed the discrimination between tumour-positive and tumour-negative tissue with limited adverse events. This positive application and the

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