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An opportunistic route to success: Towards a change of paradigm to fully exploit the potential of cell-penetrating peptides

Estel Collado Camps, Roland Brock *

Department of Biochemistry, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, Geert Grooteplein 28, 6525 GA Nijmegen, The Netherlands

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ABSTRACT

About 25 years ago it was demonstrated that certain peptides possess the ability to cross the plasma membrane. This led to the development of cell-penetrating peptides (CPPs) as vectors to mediate the cellular entry of (macro-)molecules that do not show cell entry by themselves. Nonetheless, in spite of an early bloom of promising pre-clinical studies, not a single CPP-based drug has been approved, yet. It is a paradigm in CPP research that the peptides are taken up by virtually all cells. In exploratory research and early preclinical development, this assumption guides the choice of the therapeutic target. However, while this indiscriminatory uptake may be the case for tissue culture experiments, in an organism this is clearly not the case. Biodistribution analyses demonstrate that CPPs only target a very limited number of cells and many tissues are hardly reached at all. Here, we review biodistribution analyses of CPPs and CPP-based drug delivery systems. Based on this analysis we propose a paradigm change towards a more opportunistic approach in CPP research. The application of CPPs should focus on those pathophysiologies for which the relevant target cells have been shown to be reached *in vivo*.

1. Introduction

Cell-penetrating peptides (CPPs), also named Protein Transduction Domains (PTDs),[1,2] are 5–30 amino acids long polypeptides that mediate the cellular uptake of (macro-)molecules that otherwise do not enter cells. Conjugation to cargo can either be through covalent bond formation or through non-covalent complexation.

The development of CPPs started in the mid-90s, with the demonstration of the cell-penetrating properties of penetratin, a fragment of the Drosophila antennapedia homeobox protein.[3] One of the first paradigms in CPP research was the receptor independence of import.[4] Instead, induction of uptake was related to general characteristics of the cell surface, namely, the charge distribution and amphiphilicity of the lipid bilayer and the glycocalyx, a dense layer of negatively charged oligosaccharides.[5] Consistent with the receptor independence CPPs show uptake in basically all dividing tissue culture cells, even though CPP-dependent differences in uptake efficiency certainly exist.[6] However, also *in vitro*, it has been shown that upon differentiation cells may completely loose their capacity for CPP uptake.[7]

The development of CPPs coincided with an explosion in knowledge on the pathophysiological role of intracellular molecular pathways, many of which involving networks of protein-protein interactions (PPI). PPIs, however, are notoriously difficult to target with small molecule inhibitors. CPPs created the perspective to address this target space by import of peptides and protein domains. In addition, siRNA emerged as a new therapeutic modality by mediating the down-regulation of target genes. Again, transfer to preclinical research and then to the clinic critically depended on the availability of an efficient import strategy.

In the delivery of PPI inhibitors and siRNA, CPPs contributed to preclinical success, and CPP-peptide conjugates also went into clinical trials. However, in spite of a rapid growth of the field,[8] so far no CPP-derived delivery vector has been successful in the clinical setting. In other words, the CPP field is very capable of producing innovative delivery approaches for proof-of-concept *in vitro*, but seems largely unsuccessful in translating this activity into efficacy in man. Therefore, we ask where the potential bottlenecks are and in which way research strategies should be changed.

Following a brief evaluation of the maturity of the CPP field in comparison to other delivery technologies we challenge the concept of cell-type independence as a critical misconception. Since CPPs are considered a generic solution to the delivery problem, *in vitro* preclinical work is exclusively target oriented. However, as we show through a review of literature on biodistribution, *in vivo*, strong preferences for specific organs and cell types exist.

A comparison of the biodistribution with the pathologies that are currently being targeted reveals a mismatch between the

* Corresponding author.
E-mail address: roland.brock@radboudumc.nl (R. Brock).

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current objectives and the *in vivo* potential of CPPs. As a consequence, we propose that the research strategy needs to be reversed: First, for a given delivery vector, target cells/organs should be determined through *in vivo* biodistribution studies. Only then should molecular targets related to pathophysiologies in these organs be selected.

2. CPP-based delivery – maturity of the field

We reasoned that for a given delivery technology, as it traverses from an early fundamental into a preclinical phase, the relative number of publications reporting on *in vivo* studies and investigating biodistribution would increase. Applying this line of reasoning, we therefore compared the number of pubmed-listed publications on penetrating peptides with those for polyethylenimine (PEI) and lipid-based nanoparticles (LNP) (Supplemental information 1). PEI has served as a reference in *in vitro* assays for years. However, for *in vivo* applications there are strong toxicity concerns.[9,10] Lipid-based nanoparticles have gained significance in oligonucleotide delivery with several ongoing clinical trials including phase III.[11,12]

Per year from 2000 to 2016 we extracted the total number of articles per field, the number of articles having "*in vivo*", and the number of articles having "*biodistribution*" in title or abstract or key words from pubmed (Fig. 1). We realized that a full text search on PMC National Library of Medicine produced significantly more hits than the pubmed search (for CPP-related research 996 instead of 53 for the search string specified in the Supplements). However, after a first inspection many turned out to be irrelevant. Therefore, we focused on the restricted search approach and extracted quantitative information as far as possible (see below).

Overall, for all three delivery systems, CPPs score the least publications. From 2000 to 2005 for LNPs similar numbers of publications were published as for CPPs, however, since then this field has taken off rapidly and in 2015 three times more publications appeared for LNPs than for CPPs. The fraction of publications reporting on *in vivo* data or on biodistribution over the years was constant for PEI reflecting the fact that this delivery polymer was established first but indicating as well, that this field has gained little momentum towards translation into the clinic. CPPs have been catching up but again LNPs took the lead. Overall, this analysis indicates that CPPs had a promising start but now are at a critical phase, in which initial momentum has been fading out while LNPs which had a later start are receiving more attention.

3. Biodistribution analysis – methodological approach

In 2010 Sarko et al. analysed the biodistribution and pharmacokinetics for a set of ten cationic CPPs conjugated to a ^{111}In-loaded DOTA chelator that were injected into tumour-bearing mice. Sequestration into the liver and kidneys was prominent. The brain received less than 0.1% of the total dose and also the tumours received less than 1% with only two exceptions. This biodistribution is in striking contrast to the perception of CPPs as a generic delivery strategy. Nevertheless, cationic CPPs have been repeatedly advertised as a means to cross the blood-brain-barrier.[13–15] CPPs are mostly used for the delivery of drugs for which the costs-of-goods are critical. Therefore, even if a relevant concentration could be reached, considering the minute fraction of total dose reaching the brain, a brain target may not be the appropriate application.

To further investigate whether the observations by Sarko et al. translated into a general pattern, we scanned the 53 publications retrieved from pubmed. Of these 53 entries, two were book entries, 8 were reviews, 3 did not perform a biodistribution study, 3 showed only semi-quantitative images,[16,17] two report on targeting peptides with no cell-penetrating capacity, and in one article the signal in the kidneys was so prominent that the scales of the graphs made it impossible to accurately extract quantities.[18] One article reported an oligoarginine CPP which, through addition of the three *N*-terminal amino acids NGH, acquired a strong propensity for prostate cancer and is therefore a borderline case of a tumour homing peptide.[19] Another interesting example of peptides that combine tumour-associated receptor targeting with cell penetration are the C-end rule (CendR) peptides that bind neuropilin-1 via an arginine-rich C-terminal motif (see Table 1 for an overview of the peptides).[20]

In total, 34 articles from the 53 included quantitative biodistribution data which we used for further analysis (Supplemental Table 1). Two more key CPP papers were manually included.[21,22] We extracted information about the delivery vector and cargo,

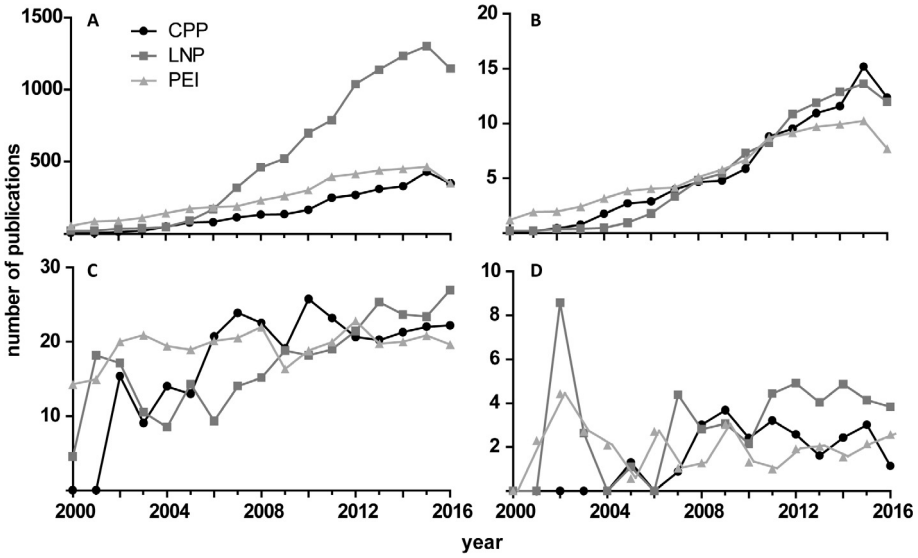

Fig. 1. Bibliometric analysis for different delivery vectors. Total number of publications for CPPs, lipid nanoparticles (LNP) and polyethyleneimine-based strategies (A), publications per year, normalised to the total number of publications for each delivery system to better visualize trends (B), fraction of publications addressing *in vivo* studies (C) and biodistribution (D). The number of publications per year was extracted from pubmed by searching in title, abstract and keywords (see Supplements).

Table 1
Peptides used in the delivery vehicles from the studies included in Supplementary Table 1.

Peptide	Sequence	References
Maurocalcine	YGDCLPHLKLCKENKDCCSKKCKRRGTNIEKRCR	90
sC18	GLRKRLRKFRNKIKEK	91
hCT	KKRKAPKKKRKFA-NH(C4)coupled to a K-FHTFPNTAIGLGAP	91
$(RXR)_4$	RXRRXRRXRRXR (where X is aminohexanoic acid)	51
R9 and other polyarginines	RRRRRRRRRR (or another number of R)	29, 32, 35, 37, 76, 78
VG-21	VTPHHVLVDEYTGEWVDSQFK	95
LMWP (and activatable versions)	CVSRRRRRRGGRRRR	44
BAC	RRIRPRPPRLPRPRPRPLPFPRP	43
TAT	GRKKRRQRRRPQ	30, 32, 33, 36, 50, 53, 56, 77, 92, 93
Penetratin	RQIKIWFQNRRMKWKK	40, 49
SPACE	ACTGSTQHQCG	83
tLyp-1	YGGNKRTR	47, 48
CIGB-552	Ac-HARIKpTFRRlKWKYKGKFW	38
PI	CASPSGALRSC	94
iNGR	GGGCRNGRGPDC	45
Pip6a	RXRRBRRXRYQFLIRXRBRXRB (where X is aminohexanoic acid)	52
$NHGR_{11}$	G-RRRRRRRRRR	19
SynB1	RGGRLSYSRRRFSTSTGR	77
SV40-derived sequence	PKKKRKV	39
MPG-8	βAFLGWLGAWGTMGWSPKKKRK-Cya	41
Pepfect6	(stearyl)-AGYLLGK(K(K(QN2)2)INLKALAALAKKIL	21
Pepfect14	(stearyl)-AGYLLGKLLOOLAAAALOOL (where O is ornithine)	22

the controls used in the study, the motivation of the study and translational application (if stated), the administration route, detection method, time-point, and any extra information relevant for the comparison to other vectors or to understand the study on its whole. Quantitative information was recovered from tables and graphs and further processed as described below.

All publications presented data on a CPP-containing carrier with the idea to enhance cellular uptake. From these, nine used a CPP directly coupled to an active molecule, nine used CPP-conjugated nanoparticles (including polymer-based nanoparticles), three used CPP-conjugated liposomes, three used CPP-conjugated proteins, five used CPP-conjugated nucleic acids, one a CPP conjugated to a small molecule (protoporphyrin), one used lipid nanoemulsions, and one used ethosomal carriers, a specific type of phospholipid, ethanol and water formulation. Two of the studies also incorporated a ligand for active targeting in their delivery vector.

According to the general paradigm that CPPs enter cells in a receptor independent manner, CPPs may be considered a passive targeting strategy, even though the triggering of uptake would qualify them as a special case of active targeting. In spite of the fact that in every case a CPP was present, this diverse set of molecular entities is expected to be subject to different mechanisms that control their biodistribution. For all macromolecular compounds and nanoparticles, passive targeting usually relies on the enhanced permeation and retention (EPR) effect, which is attributed to the disorganized and leaky tumour vasculature.[23,24] In active targeting, the delivery vector contains a ligand for a receptor that is specific for the targeted cells, for example the tumour cells. Another example of active targeting are functionalities that specifically react to the tumour microenvironment such as protease-activatable probes.[25]

In our attempt to compile the results into a comprehensive and quantitative picture, we encountered several obstacles. First, different methods to assess targeting efficiency were used such as fluorescent dyes or radiolabels, coumarin for detection of delivery by HPLC, mass spectrometry or fluorescence, and finally functional assays such as RNA knockdown. Clearly, the reporter can by itself influence the detected biodistribution.

For chelated radionuclides as for fluorescent dye conjugates partial degradation of the carrier and release of the reporter may disguise the actual biodistribution of the carrier. Furthermore, it is known that polymethine dyes are ligands for organic anion transporting polypeptides (OATP) transporters in hepatocytes. Such molecules have indeed been used to target siRNA specifically to hepatocytes.[26] Alexa Fluor 633 has been found to specifically bind elastin fibers in blood vessels.[27] Coumarin is a good fluorescent model compound for drug delivery using nanoparticulate drug carriers, however, must not leak out of the carrier. Finally, even though functional RNA knockdown typically is the application-relevant read-out, it may not correlate with the total quantity of oligonucleotide delivered.

To minimise the differences derived from the diverse approaches, we first normalised the extracted quantities to the sum of the values of all organs in each study. In this way, the numbers obtained indicate which fraction of the total measured activity was present in each organ. Then, to account for the fact that not the same organs were analysed in each article, the sum of the values for each organ was averaged for the number of individual values for that organ, i.e. the number of articles that had provided a measure for that organ.

Also, standalone CPP-cargo conjugates are less common than combinations of a nanoparticle with a CPP for which the dimensions of the carrier should also strongly affect the biodistribution. For tumours (models), where the (EPR) effect is present,[28] many biodistribution studies did not test control nanoparticles without CPP, thereby compromising an assessment of the effect of the CPP on the nanoparticle distribution.

The timepoint chosen for the biodistribution measurements is another potential source of variation. In general, accumulation in thoracic organs is very high in the first hours, but lower 24 h after administration. CPP-driven retention is more clearly observed after circulating compound is cleared, and therefore relative signal for tumours in respect to other organs may be maximised when a 24 h- or later-timepoint is used.[29,30]

The notable discrepancies between two articles providing biodistribution data on several commonly used CPPs, could thus be due to the different timepoints chosen (10 min, 1 h and 3 h versus 24 h).[31,32] The lack of overlap in the protocol is unfortunate, as explorative studies are highly valuable and cross-referencing would be desirable.

Liver, spleen and lung showed prominent retention (Fig. 2). Nevertheless, in some instances different studies report different biodistribution patterns for similar delivery vehicles. For example, for PEGylated and Tat-coupled cholesterol liposomes one study[33]

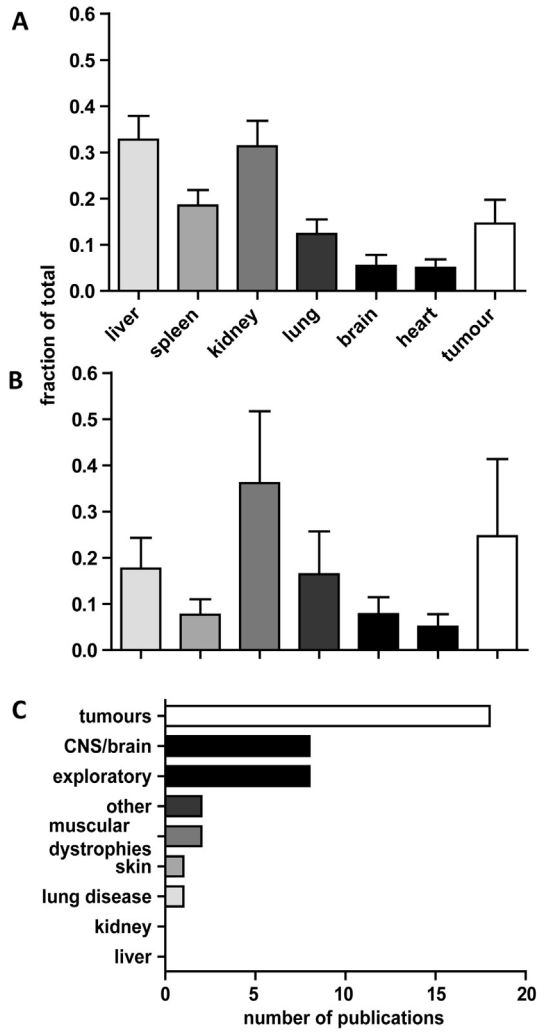

Fig. 2. Biodistribution and primary areas of application. (A) and (B) show the mean of the fraction of the total signal in every organ for data pooled from all articles. Inclusion criteria were: timepoint >3 h, only data for the best vector from an article in which several vectors had been tested, or data from the most common CPPs in case several had been used. To account for differences in the measurements, signal from all organs measured in each study was summed up and the signal for each organ in that study was normalised to the total value of the corresponding study. Error bars show SEM. A: data for non-targeted delivery vehicles; B: data for targeted delivery vehicles. (C) shows the objectives of all articles that are mentioned, also from those not fulfilling the criteria to be included in graphs A and B.

showed almost no lung retention over a 24 h time period while in another study from the same group retention in lung was higher than in liver,[34] even though administration was via the tail vein in both cases. Sharma et al. used liposomes decorated with poly-L-arginine and transferrin to target the brain.[35] In their study, liver and spleen showed more accumulation than the lung and the poly-L-arginine promoted brain delivery. Zheng et al. used transferrin and Tat-coated coumarin-loaded liposomes for glioblastoma delivery. However, coumarin concentration in this case was also higher in the lung than in liver and spleen.[36] A commonality between all these studies is that the desired target was the brain. However, in none of these studies, this organ showed prominent retention.

4. Biodistribution analysis

Overall, as mentioned, CPP-based delivery vehicles mostly accumulate in the liver, lung and spleen. Some studies also showed intestinal accumulation, which could be a consequence of biliary excretion. Accumulation in the lungs was less common but has been shown for some compounds. In contrast, accumulation in muscles and Central Nervous System (CNS) was modest to very modest. Strikingly, even when CPP-based vectors showed a relative superiority compared to the control in CNS or muscle targeting, absolute accumulation was a factor ten higher in the aforementioned organs.[35]

Fifteen of the considered articles aimed at tumour targeting for diagnosis and treatment. Cargoes included proteins, small-molecule drugs, dyes, radiolabelled compounds and small nucleic acids. Of these, three investigated the distribution of the CPP itself,[37,38,19] and a fourth one also attached a protoporphyrin for photodynamic therapy.[39] Two articles evaluated constructs that also included a ligand for specific targeting. In one of them, the ligand was a single-chain antibody,[40] in the other one transferrin attached to Tat-coated liposomes.[36] Two articles addressed CPP-based polyplexes,[41,42] one a CPP-conjugated dendrimer for polyplex formation,[30] and three CPP-conjugated nanoparticles (elastin-like peptide nanoparticles, PEG-PLGA, liposomes).[33,43,44]

In most cases orthotopic models were used to test whether the delivery vehicle improved retention in the tumour. Tumour accumulation was often only modest compared to accumulation in liver, kidney and spleen.

In some cases, vehicles showing increased tumour accumulation in comparison to the untargeted controls also showed higher background[32] or higher retention in the kidneys.[45] Interestingly, there are a few exceptions in which tumour accumulation showed a strong improvement in comparison to other organs.[38,40,30]

The study on the cell-penetrating anti-tumour peptide CIGB-552 illustrates the impact of time on biodistribution.[38] Following a rapid excretion via the kidney, over a time frame of 24 h about 5% of the injected dose, delivered by subcutaneous administration was retained in the tumour. In comparison to lung and heart, differences increased which may be due to retention by capture to the target as recently described for stapled peptides *in vitro*.[46] Liang et al., used a CendR peptide to direct nanoparticles to tumours,[47] two other articles for targeting of a radiolabel for diagnostic purposes.[48,45]

Seven publications aimed at reaching the brain. However, in two of the studies the liver showed a much higher accumulation,[49,44] one study showed the highest accumulation in the spleen, followed by the liver and the lungs,[35] and a fourth one more in the lung than in any other organ.[36] As mentioned above, in their studies on brain-targeted CPP-conjugated liposomes, Qin and colleagues published one article in which the accumulation in the lung was negligible, and another one where Tat- and R8-liposomes accumulated much more in the lung than in any other organ, with the only exception of control disordered Tat-liposomes.[33,34] Intranasally administered MPEG-PCL-Tat micelles yielded enhanced retention in brain and lung[50] in comparison to liver, heart, kidney and spleen. However, due to this different route of application, their biodistribution results are not comparable with those of intravenously injected vehicles.

CPP-based delivery of small nucleic acids for gene therapy of muscular dystrophies has also received considerable attention. For the delivery of charge-neutral phosphorodiamidate morpholino oligomers (PMO) CPPs are covalently attached in a co-linear manner. For negatively charged nucleic acids, a common approach is to harness their negative charge to form non-covalent nano-sized complexes with the positively charged CPPs. Of the reviewed articles, six studied the biodistribution of nucleic acid complexes: two[51,52] used PMOs and four used CPP/oligonucleotide complexes.[41,21,53,42]

Interestingly, the calcium condensed dTat/siRNA complexes of Baoum et al. showed more knockdown of the target enzyme

GAPDH in brain than in liver and lung, although the concentration of the siRNA itself was higher in the latter, particularly in the lung. However, knock-down varied between the two doses tested, and it cannot be ruled out that this effect was gene specific.

The CPPs PepFect 6 and PepFect 14 (PF6 and PF14) are representatives of the class of amphipathic CPPs.[21,22] In the first *in vivo* study on siRNA delivery with PF6 the strongest silencing was observed in liver, lung and kidney.[21] PF14 DNA complexes yielded by far the highest gene expression in the lungs with only little expression in tumours which could be redirected to tumours through introduction of an MMP-cleavable PEGylation.[42]

The covalent conjugation of Pip6a to a PMO enhanced retention in all organs and tissues tested with the biggest increase in the liver.[52] Even though the application was in muscle targeting, the concentrations in kidney and liver were two orders of magnitude higher than those in muscle. Surprisingly, brain accumulation strongly increased, even though the reasons for this observation are not clear. The same peptide also showed remarkable central nervous system activity in the delivery of a therapeutic oligonucleotide in a mouse model of spinal muscular atrophy. In this latter study only activity but not biodistribution was measured.[54] Also in the study by Amantana et al., conjugation of a PMO to the (RXR)4 peptide increased retention in all organs, especially in liver and spleen but also in the heart.[51] Skeletal muscle was not tested in this study.

To summarize, the addition of a CPP did not benefit the specific accumulation in only one organ, however, given the pronounced preference for liver, kidney and spleen, there is no basis to state that CPPs provide a generalized, systemic delivery approach. Very clearly, with a focus on the brain, tumours and muscle, the current research on CPP-based delivery strategies aims at organs or locations that are only poorly reached.

5. Opportunistic targets to improve effectivity/relevant pathologies

As follows from the analysis above, so far there has been a mismatch between the biodistribution of CPP-based vectors and the targeted pathologies. CPP-based delivery vehicles show a high accumulation in the liver, spleen and lungs compared to other organs. Thus, targeting pathologies in these organs seems a natural approach. In fact, outside the area of CPPs, delivery of oligonucleotides to the liver either through targeting of the asialoglycoprotein receptor or by making use of the propensity of lipid nanoparticles to reach this organ has shown activity, also in clinical development.[55]

CPP-based delivery vehicles for the treatment of hepatocarcinoma have already been explored.[56] The challenges to further develop this approach preclinically will be to use more realistic tumour models instead of xenografts. Animal models of hepatitis B virus infection provide a physiological setting to assess the liver tropism and effectiveness of a delivery vector. In this context, CPPs have shown activity in the delivery of peptide nucleic acids (PNAs).[57,58] Several groups are working on the targeting of Ito cells to prevent liver fibrosis.[59,60] Since the liver comprises of many different cell-types, involved in a variety of pathologies,[61] further research is needed to dissect delivery at the histological level.

As far as the kidney is concerned, it needs to be investigated to which degree kidney-associated signals reflect a transient accumulation associated with excretion or true delivery. Nevertheless, if the delivery vehicles are not degraded before reaching the urine, they could be used to target kidney diseases and malignancies of the urological tract as demonstrated for a tumour homing peptide targeting bladder cancer cells.[62]

Concerning the lung, cystic fibrosis and chronic obstructive pulmonary disease (COPD) are two major indications for which oligonucleotide delivery provides novel therapeutic means. In this case, however, delivery by inhalation may be the preferred route of application, thus circumventing the challenges of sequestration by other organs.[63]

The preferential uptake observed for organs of the reticuloendothelial system (liver and spleen) could be exploited in multiple ways. Imaging of macrophages has been explored as a means for monitoring macrophage accumulation in the sentinel lymph node,[64] and to image alveolar macrophages in a model of COPD.[65] Considering their central role in the tumour microenvironment and in (auto)inflammatory disease, imaging and functional modulation of macrophages is extremely valuable in these fields too.[66]

Even targeting of intracellular pathogens in macrophages could be pursued, for example by *in vivo* development of the targeting of macrophage-resident *Leishmania* parasites[67] and macrophage-resident bacteria.[68]

As far as the spleen is concerned, immune cells other than macrophages could also be targeted. The enhancement of presentation of peptide vaccines has been one of the first applications of CPPs.[69,70] Finally, CPPs have been used for injection-free transepithelial delivery of drugs that act systemically such as insulin.[71] Here, crossing of the barrier rather than mediating cellular delivery is the intended mode of action of the CPP.

6. Redirecting targeting

Very clearly, CPP-based delivery vectors are neither uniform enhancers of cellular uptake nor do they possess a specific cell-type selectivity. Not surprisingly, numerous attempts have been made to incorporate selectivity towards specific cell types. Several of the studies mentioned above included such targeting strategies, however, in most cases they only had moderate impact on biodistribution. Two general strategies for targeting can be discriminated: First, the combination with additional targeting ligands and second, the controlled exposure of the CPP functionality in response to environmental cues such as the presence of certain proteolytic activities[44,37] or a change in pH.[72]

An example for the first strategy is the aforementioned combination of liposome functionalization with transferrin and a CPP.[35] The most prominent and widely explored approach for environment-dependent CPP exposure are the activatable CPPs (ACPPs) that make use of the presence of proteases in the microenvironment of the target site. Here, a polyanionic peptide stretch is coupled to the CPP via a protease cleavage site. Folding back of the anionic stretch onto the polycationic CPP neutralizes the CPP activity.[25] Discrimination of the target site was enhanced, in both cancer and inflammation.[73,74] However, significant off-target distribution clearly pointed towards diagnostics rather than therapy as the preferred mode of application. Analysis of the time dependent biodistribution indicated an enhanced systemic circulation and avoidance of rapid liver clearance as a major mode of action of the internal masking[75] and questioned a simple mechanism according to the original rationale. This observation was substantiated by further studies which uncovered a role of vascular proteases in activation.[76,37]

For nanoparticles this strategy was implemented by linking the nanoparticle-coupled CPP via the protease cleavage site of a matrix metalloprotease to a PEG chain. For PF14 nanoparticles this strategy resulted in nearly exclusive redirection of targeting to the tumour.[42] Xia et al. used an activatable low molecular weight protamine (ALMWP) also activated by MMPs for nanoparticle delivery.[44] In their biodistribution studies, mice treated with ALMWP particles showed more accumulation in the tumour, but also in the liver. In this case, both plain particles and particles with regular LMWP were taken as controls.

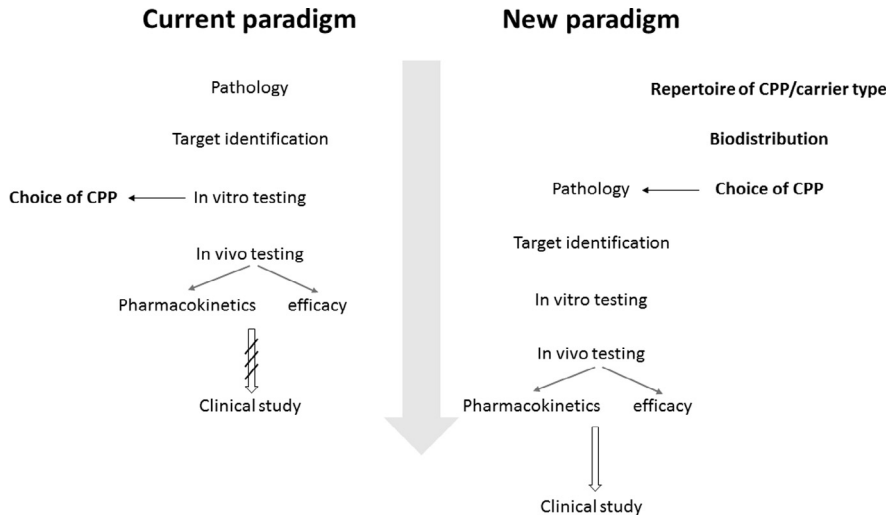

Fig. 3. Proposed paradigm change for CPP research. Based on the perception of CPPs as a generic solution to the delivery problem, at present, a pathology is the starting point for research. Instead, an inventory of biodistributions for a repertoire of CPPs and the respective carrier types should be generated. The biodistribution then determines the pathologies that can be addressed.

As a representative of receptor-targeted ligands with intrinsic CPP activity Liang et al. used a CendR peptide to direct nanoparticles to tumours.[47] However, along with enhanced tumour retention, delivery to the liver was increased to a similar degree. Studies on CendR-peptide conjugated radiolabels for diagnostics showed discordant results.[48,45] In both cases, distribution to the kidneys was dominating but in one case only little tumour targeting was achieved over a rather short time window.[48]

Alternatively, the route of application can be adjusted. Intranasal administration provides enhanced brain delivery in comparison to intravenous administration. For Tat-conjugated elastin-like polypeptide the lung was the only main off-target organ, possibly due to drainage, in comparison to the stomach and kidney after intravenous administration.[77] Kanazawa et al. compared accumulation levels for Tat-conjugated block-copolymer micelles after both administration routes. Again, brain was the main target organ, next to lung. Brain accumulation was 50% lower for intravenously administered micelles but the timepoints at which the animals were sacrificed were different (1 h for intranasal and 24 h for intravenous).[50] Also, direct delivery of CPP-based formulations into the lung has been explored, even though delivery was by intratracheal gavage and not by inhalation.[78]

A further route of delivery that has received little attention so far is intraperitoneal injection. Ovarian cancer is a highly metastatic malignancy with the great majority of cases restricted to the peritoneal cavity,[79] and intraperitoneal delivery of chemotherapeutics is often performed in combination with surgery.[80] Colon cancer can also metastasize into the peritoneum, and different modes of intraperitoneal chemotherapy are used to prevent the expansion of metastases.[81] Intraperitoneal injection of a CendR peptide afforded efficient delivery of dextran and doxorubicin to intraperitoneal metastases.[82]

Finally, topical application is a safe approach, which – in comparison to systemic delivery – reduces the demands on biodistribution. Our literature search produced one article on topical delivery of cyclosporine A for the treatment of psoriasis using a phage display derived peptide.[83] However, CPP-based topical delivery has been explored more widely.[84] Of particular note is the non-covalent complexation of botulinum toxin with an arginine and lysine rich CPP.[85] However, this formulation failed in a phase III clinical trial due to lack of efficacy in comparison to the current treatments. Finally, ocular drug delivery with CPPs has also been pursued.[86–89]

7. Conclusion and perspectives

Nearly 25 years after their discovery, still no CPP-based drug has entered the clinic. Here, we argue that this may be due to the fact that the CPP field started with too high expectations of being generic delivery vectors, which in turn had been a consequence of *in vitro* observations which were then extrapolated towards *in vivo* expectations.

Considering the rapid success of LNP-based delivery approaches which opportunistically exploit the liver tropism, we propose that also CPP-based delivery should follow a more opportunistic approach (Fig. 3). As a consequence, more efforts are needed to not only map biodistribution on an organ level but also on a cell-type specific level. In addition, it needs to be defined to which degree efficient uptake also leads to functional delivery. While for delivery of therapeutics this typically requires release into the cytoplasm, for diagnostic purposes, cellular uptake as such is sufficient. The further refinement of targeting approaches as well as a critical evaluation of the route of application should be further elements of this strategy.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmc.2017.11.004.

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