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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,

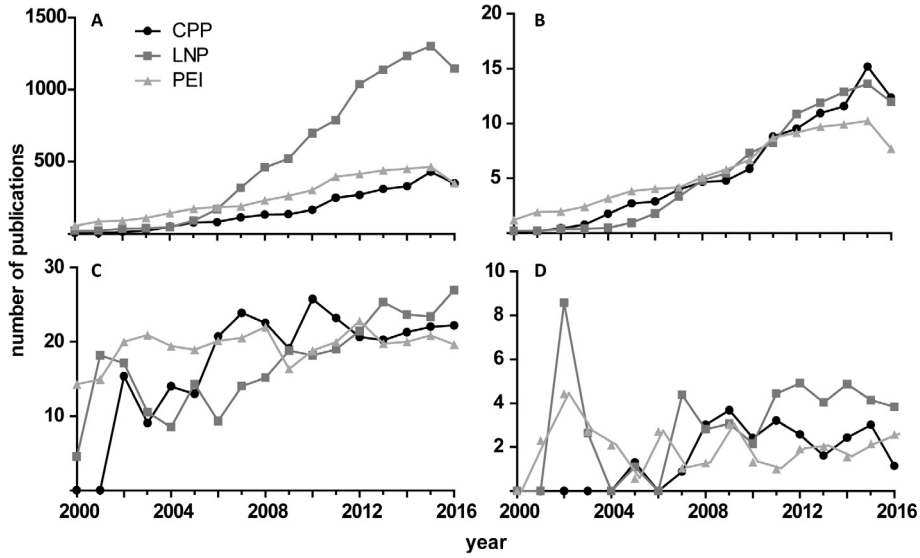

Fig. 1. Bibliometric analysis for different delivery vectors. Total number of publications for CPPs, lipid nanoparticles (LNP) and polyethyleneimine-based strategies (A), publications per year, normalised to the total number of publications for each delivery system to better visualize trends (B), fraction 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).