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Editorial

Happy birthday cell penetrating peptides: Already 20 years

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ABSTRACT

The recent discovery of new potent therapeutic molecules that do not reach the clinic due to poor delivery and low bioavailability has made of delivery a keystone in therapeutic development. Several technologies have been designed to improve cellular uptake of therapeutic molecules, including cell-penetrating peptides (CPPs). CPPs were discovered 20 years ago based on the potency of several proteins to enter cells. So far numerous CPPs have been described which can be grouped into two major classes, the first requiring chemical linkage with the drug for cellular internalization, the second involving formation of stable, non-covalent complexes with cargos. Nowadays, CPPs constitute as a very promising tool for non-invasive cellular import of cargos and have been successfully applied for *ex vivo* and *in vivo* delivery of therapeutic molecules varying from small chemical molecules, nucleic acids, proteins, peptides, liposomes to particles. This short introduction will highlight the major breakthroughs in the CPP history, which have driven these delivery agents to the clinic.

1. Delivery, a major piece of therapeutic puzzle

Although, small molecules remain the major drugs used in clinic, in numerous cases, their therapeutic impact has reached limitations such as insufficient capability to reach targets, lack of specificity, requirement for high doses leading to toxicity and major side effects. Over the past ten years, in order to circumvent limitations of small molecules and of gene-based therapies, we have witnessed a dramatic acceleration in the discovery of larger therapeutic molecules such as proteins, peptides and nucleic acids which present a high specificity for their target but do not follow Lipinski's rules. Pharmaceutical potency of these molecules remains restricted by their poor stability *in vivo* and by their low uptake in cells. Therefore, "delivery" has become a central piece of the therapeutic puzzle and new milestones have been established to validate delivery strategies: (a) lack of toxicity, (b) efficiency at low doses *in vivo*, (c) easy to handle for therapeutic applications (d) rapid endosomal release and (d) ability to reach the target [1–4]. Although, viral delivery strategies had given much hope for gene and cellular therapies, their clinical application has suffered of side- and toxicity- effects [3]. Researches were mainly focused on the development of non-viral strategies and different methods have been proposed including lipid, polycationic nanoparticles and peptide-based formulations, but only few of these technologies have been efficient *in vivo* and have reached the clinic. Cell penetrating peptides (CPP) are among of the most promising non-viral strategies. Although definition of CPPs is constantly evolving, they are generally described as short peptides of less than 30 amino acids either derived from proteins or from chimeric sequences. They are usually amphipathic and possesses a net positive charge [5,6]. CPPs are able to penetrate biological membranes, to trigger the movement of various biomolecules across cell membranes into the cytoplasm and to improve their intracellular routing thereby facilitating interactions with the target [5–7]. CPPs can be subdivided into two main classes, the first requiring chemical linkage with the cargo [8–11] and the second involving the formation of stable, non-covalent complexes [12–15]. CPPs from both strategies have been reported to favor the delivery of a large panel of cargos (plasmid DNA, oligonucleotide, siRNA, PNA, protein, peptide, liposome, nanoparticle…) into a wide variety of cell types and *in vivo* models [7].

2. Cell penetrating peptides: 20 years of history

Twenty years ago, the concept of protein transduction domain (PTD) was proposed based on the observation that some proteins, mainly transcription factors, could shuttle within cells and from one cell to another. The first observation was made in 1988, by Frankel and Pabo [16]. They showed that the transcription-transactivating (Tat) protein of HIV-1 could enter cells and translocate into the nucleus. In 1991, the group of Prochiantz reached the same conclusions with the Drosophila Antennapedia homeodomain and demonstrated that this

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domain was internalized by neuronal cells [17]. These works were at the origin of the discovery in 1994 of the first Protein Transduction Domain: a 16-mer peptide derived from the third helix of the homeodomain of Antennapedia named Penetratin [18]. In 1997, the group of Lebleu identified the minimal sequence of Tat required for cellular uptake [19] and the first proof-of-concept of the application of PTD *in vivo*, were reported by the group of Dowdy, for the delivery of small peptides and large proteins [20]. Historically, the notion of cell penetrating peptide (CPP) was introduced by the group of Langel, in 1998, with the design of the first chimeric peptide carrier, the Transportan, which derived from the N-terminal fragment of the neuropeptide galanin, linked to mastoparan, a wasp venom peptide, [5,6,21]. Transportan has been originally reported to improve the delivery of PNAs both in cultured cells and *in vivo* [21]. In 1997, the group of Heitz and Divita proposed a new strategy involving CPP in the formation of stable but non-covalent complexes with their cargo [22]. The strategy was first based on the short peptide carrier (MPG) consisting of two domains: a hydrophilic (polar) domain and a hydrophobic (apolar) domain. MPG was designed for the delivery of nucleic acids [22–24]. The primary amphipathic peptide Pep-1 was then proposed for non-covalent delivery of proteins and peptides [25]. Then the groups of Wender and of Futaki demonstrated that polyarginine sequences (Arg8) are sufficient to drive small and large molecules into cells and *in vivo* [26,27]. Ever since, many CPPs derived from natural or unnatural sequences have been identified and the list is constantly increasing [7]. Peptides have been derived from VP22 protein of Herpes Simplex Virus [28], from calcitonin [29], from antimicrobial or toxin peptides [30], from proteins involved in cell cycle regulation [31], as well as from polyproline-rich peptides [32].

3. Mechanism from “mystic” to reality

For long, mechanism of cellular uptake of CPP/PTD remained mysterious, being non-specific, independent of endocytosis and of specific receptor, and requiring no energy. But such mystery was not satisfactory for the cartesian mind of scientists and extensive researches have been performed in order to solve the code of CPP-mechanism. As such in 2003, the groups of Johansson and of Lebleu pointed out serious artifact in the earlier studies and proposed a revised cell uptake mechanism for CPPs, essentially associated with the endosomal pathway [33,34]. Ever since, the mechanism of many CPPs has been reexamined and was reported to be mediated by endocytosis [35–38]. However, this does not end the debate because the mechanism of uptake of many CPP-cargo remains controversial. For most CPPs, evidence for several routes of cell entry have been reported, some of which are independent of the endosomal pathway and involve a trans-membrane potential [39–41]. The evolution of technologies in cell biology, including live imaging for monitoring biological events, and the discovery of new cellular pathways have had major consequences on the understanding of CPP cellular uptake mechanisms [42,43]. There is a clear consensus on the fact that the first contact between a CPP and a cell surface occur through electrostatic interactions and implicate the extracellular matrix, the cell surface proteoglycans glucosaminoglycan platform (GAG) [43–46]. These first contacts are followed by a remodeling of the actin network and a selective activation of the small GTPase Rho A or Rac1. These signals constitute the ‘onset’ of the internalization mechanism and have a major impact on the fluidity of membranes promoting CPP entry in cell via macropinocytosis, clathrin-dependent endocytosis, or via membrane perturbation mechanisms [43–46]. In most cases, cells are loading CPP-cargos complexes but only 2% of the delivered cargos are biologically active, the rest remaining trapped in endosome compartments. The recent discovery that CPPs can trigger membrane repair processes has strengthened the hypothesis of a direct mechanism of uptake evoking the idea that the membrane response is due to a direct CPP binding and to damages associated with the direct CPP interaction [47]. Various parameters can affect CPP cellular uptake pathways, including (i) secondary structures of CPP, (ii) CPP ability to interact with cell surface and membrane lipids, (iii) the nature and the concentration of cargos, and finally (IV) the cell type and its membrane composition [39,40,48]. At low concentrations of CPP-cargo conjugates, cellular uptake mechanisms are essentially associated with an energy-dependent endosomal pathway; clathrin-, caveolin-mediated endocytosis, or macropinocytosis, depending on the cargo [7,47]. In contrast, at high concentrations, cell entries are partially associated to non-endosomal processes [7,12,13]. Secondary structure and structural polymorphism of CPPs play a major role in uptake of CPP/cargo complexes [40,48–50], and as for antimicrobial peptides in the balance between efficiency and toxicity. In contrast, when amphipathic peptides such as MPG, PEP and CADY [47,51] are forming stable non-covalent nanoparticles with their cargos, clustering of numerous peptides around the cargo induces a high local concentration of CPP at the cell membrane surface which favors uptake through a mechanism independent of endocytosis, even at low concentrations. In these conditions, cell entry should be controlled by the size of nanoparticles and by the peptide ability to interact directly with the lipid moiety of cell membranes [47,52].

4. Molecular modeling: New possibilities of approach of CPP mechanism?

Debates around the cellular uptake mechanism of CPP are mainly due to the complexity of biological processes underneath and to the diversity of CPP-cargo routes into cells. One could support that, in front of such diversities, conclusions from experimental approaches are somehow restricted by the conditions of assays required for significant signals. *In silico* modeling constitutes a very different approach of the problem and could thus be an interesting complement to understand CPP mechanism.

Molecular modeling of biological molecules started only 60 to 50 years ago. It initially aimed at simulating properties of molecules using equations describing movements of atoms and variations of energy between atoms. Hence modeling procedures required algorithms to describe atom movements and force fields to evaluate energy. The force fields included terms such as torsion axes potential and angle deformation potential to account for interactions between bound atoms, and terms referring to van der Waals and electrostatic energies to account for interactions between non-bound atoms. Calculations, initially made on big computers are now run on portable computers due to the fact that processor capacities have been doubling every 18 months during the last decades. When modeling studies started, computers were about 10^6 less powerful than they are now and this power increase has boosted the development of *in silico* approaches. If computing capacities increased, storage, graphics and video performances have also been improved. The first computers were black and white and the visual display of a small molecule lasted forever. It is only in the 80's that color screens appeared, helping in more performing identification of molecular system complexities. Finally, the first kinetics of molecule folding started in 1973 [53] and the burst off of computers capacities after the 90's opened modeling approaches to very realistic simulations of almost any molecular compound.

Algorithms to describe the movements of atoms were initially developed by physicists [54] who used consistent force fields based on few potentials and Newton's equation «f = ma» to derivate acceleration and to calculate movements, knowing the force (f) between partners and their masses. To give a direction to these movements, procedures of energy minimization such as simplex, conjugated gradients, Monte Carlo and others were developed. Calculations were aiming to find the 3D model of minimal energy because this model was expected to be the active molecule configuration. Another factor participated to the expansion of modeling approaches: initial

algorithms were written in FORTRAN, the classical language for scientific applications but more popular languages as C and C++ were more recently used. Numerous applications consequently appeared, some were widely diffused in the scientific community. Very popular applications are CHARMS [54], Modeler [55] and GROMACS [56]. One actual problem of modeling experiments is in the abundance of available procedures that somehow reflects our unclear vision of the rules of molecule folding and disperses assays onto too many possibilities of unequal pertinence. The CASP trial that is organized by the modeling community has been and is still very important to evaluate modeling procedures of proteins [57]. As early as 1981, Brasseur et al. developed a platform of modeling procedures mixing different types of molecules by using a unique semi empirical force field for all types of molecules (chemical drugs, lipids, peptides and proteins) and the Newton's mass law for describing 3D structures and their molecular and angular movements [58–63]. The platform is including different types of algorithms for docking molecules in membranes [61], but also for simulating processes of transfer across, insertion in and destabilization of membranes.

A glance at literature helps to classify modeling applications into two large categories; those aiming to produce 3D models of structures of either monomers or complexes using mainly homology, but also *ab initio* procedures in case of small molecules, and those describing mechanisms and properties of molecules down to atom movements and interactions. Modeling of peptides follows this partition: peptide 3D structures were looked for as complementary pieces of information to CD and FTIR data and *in silico* models were expected to correspond to Rx and NMR models [60]. Peptides were also used as short, thus convenient fragments of proteins for the analysis of amino acid interactions. However, if proteins and peptides are both linear chains of amino acids, their 3D folds show major differences. A hydrophobic core stabilizes the globular protein structure in water but peptides are too short to build up hydrophobic cores [64]. Hence, peptides are largely in contact with, and thus affected by the external environment. Modeling of peptides has long been polluted by the dogma of the unique low energy 3D structure. Acknowledging the existence and the importance of disorder has been a significant progress boosting developments of peptide modeling [61]. Indeed if molecules are more or less frozen in one or another conformation in Xray and NMR experiments, modeling computations do not have those restrictions and populations of 3D models can be and are now explored [60]. The main problem remains in the way our cartesian and somehow rigid approach of data, fast and easy on one to one molecule interactions will catch up the information when populations of models will be playing in the game.

Evolution of molecular modeling since its beginning has provided numerous and diverse tools and the next decennial should drive us much farther toward models for biological mechanisms explaining the rules for molecule selectivity and ubiquity. It will be fantastic to design the selective drug to cure a protein dysfunction simply by writing down a sequence because all the ways from the sequence to the function will be obvious basic knowledge.

5. CPP-applications: From bench to bedside

So far more than thousand applications of CPPs have been reported using either covalent or conjugate CPP-based strategies from *in vitro* to *in vivo* deliveries [7,14,15,30,65–68]. The interest for CPPs is mainly due to their low cytotoxicity and to the fact that there seems to be no limitation for the type of cargos. Although CPPs were shown to favor delivery of cargos that vary greatly in size and nature (small molecules, oligonucleotide, plasmid DNA, peptide, protein, nanoparticle, lipid-based formulation, virus, quantum dots...) most applications describe the delivery of oligopeptides/proteins [14,15,65,66] and nucleic acids or analogs [30,68].

The first, proof-of concept of the potentiality of CPPs, was for protein delivery and over the last two decades, CPPs have successfully delivered peptides, antibodies and proteins to target different diseases including cancer, asthma, apoptosis, ischemia, stimulating cytotoxic immunity and diabetes [7,65–67]. CPPs were also applied in a diagnostic purpose for the delivery of biosensors and probes for *ex vivo* and *in vivo* fluorescence imaging [69–72]. Although, most applications use covalently linked CPPs (Tat, Penetratin, poly-Arginine, VP22) more recently no covalent approaches, such as Pep-1 technology have been successful *ex vivo* and *in vivo* [66]. CPP strategy has been extended to the delivery of other uncharged and charged cargoes, including, replication-deficient viruses, PNA DNA mimics and semiconductor quantum dots [7,14,15,30,65–68]. Steric blocks small neutral oligonucleotides including peptide nucleic acids (PNA) and phosphorodiamidate morphorodiamidate morpholino oligomers (PMO) constitute potent molecules for either antisense application or mRNA splicing correction strategies. Several CPPs have been successfully applied for the delivery of uncharged PNA and PMO *ex vivo* and *in vivo* through covalent coupling [73,74] and some of them are currently evaluated in the clinic [75]. CPP-based strategies have also been developed to improve the delivery of oligonucleotides both *ex vivo* and *in vivo*. Delivery of oligonucleotides and siRNA is more challenging as multiple anionic charges of the nucleic acid interact with CPP moiety and inhibit uptakes by steric hindrance. Delivery of oligonucleotides was achieved using either peptide-based covalent and non-covalent [7,13,15], or PNA-hybridization strategies [30]. Non-covalent strategies appear to be more appropriate for siRNA delivery and yield significant associated biological response [15,51,76]. The first application for non-covalent CPP-based siRNA delivery in challenging cell lines and *in vivo*, has been reported with the primary amphipathic peptide MPG, which improves siRNA delivery into a large panel of cells [24]. Then the non-covalent approach was extended to other CPPs including the secondary amphipatic peptide, polyarginine, Penetratin, Tat and transportan-derived peptides [15,30].

Finally, numerous preclinical and clinical assays of CPP-based delivery approaches are currently under evaluation. The first clinical trial was initiated by Cell Gate Inc. for topical delivery of cyclosporine linked to polyarginine, and entered phase II trials in 2003. Ever since, several companies have been working on clinical development of CPPs, for topical and systemic administrations of different therapeutic molecules; Avi Biopharma for the *in vivo* steric block splicing correction using 6-aminohexanoic acid spaced oligoarginine ((R-Ahx-R)4). KAI Pharmaceutical expertise is in the selective modulation of intracellular protein:protein interactions via delivery of peptide drugs into the cell [77]. KAI is currently evaluating a Tat-protein kinase C inhibitor peptide modulator of protein kinase C for acute myocardial infarction and cerebral ischemia, which enter Phase 2b. Revance Therapeutics Inc. company has developed TransMTS™, a cell penetrating based platform technology which enables topical, needleless, delivery of botulinum toxin and other macromolecules across skin, which are evaluated in Phase 2. Other companies including Traversa Inc., for Tat-based non-covalent siRNA delivery and CEPEP-III, are currently evaluating CPP at pre-clinical and clinical trials.

6. Conclusions

In conclusion, 20 years after their discovery, CPPs are at the door of the clinic. The success reported on the preclinical and clinical evaluation of CPPs during the last decade, has revealed tremendous therapeutic potentials in clinic and in many preclinical disease models. Covalent strategies have been validated for protein and peptide delivery and the recent success of phases I and II clinical trials has open great hope in the used of CPPs for therapy. Moreover, the development of non-covalent strategies has provided innovative non-conventional delivery technology for siRNA that will have a major impact on the clinical application of these molecules.

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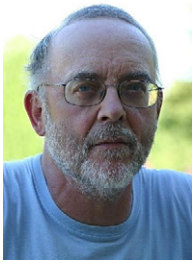

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