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Review

Cell-penetrating and cell-targeting peptides in drug delivery

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ABSTRACT

During the last decade, the potential of peptides for drug delivery into cells has been highlighted by the discovery of several cell-penetrating peptides (CPPs). CPPs are very efficient in delivering various molecules into cells. However, except in some specific cases, their lack of cell specificity remains the major drawback for their clinical development. At the same time, various peptides with specific binding activity for a given cell line (cell-targeting peptides) have also been reported in the literature. One of the goals of the next years will be to optimize the tissue and cell delivery of therapeutic molecules by means of peptides which combine both targeting and internalization advantages. In this review, we describe the main strategies that are currently in use or likely to be employed in the near future to associate both targeting and delivery properties.

Contents

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1. Cell-penetrating peptides (CPPs)

The efficient passage of drugs through the plasma membrane remains a major hurdle for drug delivery. Good cell uptake often requires the administration of high quantities of drugs in order to obtain the expected intracellular biological effect. Therefore, improving the translocation process across the plasma membrane will significantly reduce the quantity of drug to be administered, and the side effects on healthy tissues that are currently observed in most of the cases.

In the nineties, several proteins have been shown to translocate spontaneously through the plasma membrane when incubated in the extracellular medium. In particular, two of these molecules have been extensively studied in order to define the structural or sequence elements needed for the translocation: the Tat protein from the HIV-1 virus [1,2] and the *Drosophila melanogaster* Antennapedia homeodomain [3]. A study on the structure–activity relationship was carried out on these two small proteins of 101 and 60 amino acids, respectively, and the minimal domain needed for translocation was defined [4–6]. This corresponds to short sequences of 10 to 16 amino acids, thus opening the way to the chemical synthesis of different mutants and analogues that are called "cell-penetrating peptides" (CPPs) or "protein transduction domains" (PTDs). However, since "non-natural" peptide sequences that are quite different from the conventional PTD have also been used as cell translocating units for drug delivery [7,8], we prefer the acronym CPP for such family of peptides.

Both Tat and Antennapedia peptides contain several basic amino acids. The native Tat peptide is composed of several cationic amino acids, including 6 arginine and 2 lysine residues. Starting from this native composition, the potential of arginine homopeptides to promote cellular uptake has been rapidly realized [9,10], as it was demonstrated that the arginine-rich peptide is more efficient than the other cationic (i.e. poly-lysine, poly-histidine or poly-ornithine) homopolymers [11]. The strong impact of arginine residues has been described by Futaki's group [10,12] and further investigated by Rothbard and Wender, and others, who performed a systematic replacement of arginine residues with alanine residues [11,13]. Such substitutions induced a strong reduction of peptide uptake that was directly correlated with the number of substituted arginines. Therefore, Rothbard et al. proposed that a bidentate hydrogen-bonding interaction between the guanidinium group of arginine residues and phosphate groups in the membrane [14] is implicated in the mechanism of translocation.

Surprisingly, the role of cationic amino acids in the Antennapedia peptide has not been so extensively studied, whereas the influence of the tryptophane residues has been comprehensively investigated [4]. In line with this, a peptide made of arginine and tryptophane residues only and showing an efficient translocating potency has been recently designed [15].

Altogether, the studies on Tat and Antennapedia peptides represent more than 75% of the published work on CPPs (for reviews, see [16] and [17]), and in the last two years this percentage even increased with several publications being reported weekly in the literature. The Antennapedia peptide has been also marketed as "Penetratin". Under this commercial version, an activated group sensitive to nucleophilic attack by a sulfhydryl function conveniently allows the spontaneous formation of a disulfide bridge between any cargo molecule and "Penetratin". At the time of writing this review however, only 172 results appeared in Medline for the keyword "Penetratin". Most of them are fundamental studies mainly about the entry mechanism of CPPs or the biological evaluation of a coupled drug, whereas only a little number concerns clinical applications. It is noteworthy to consider that a stable covalent linkage has to be formed between CPP and cargo to allow translocation, at least for Tat, Antennapedia, or poly-Arg peptides, although a couple of publications also reported a surprising efficacy upon simple mixing with the cargo entities [18,19]. Similarly, another CPP, Pep-1, which has been marketed as "Chariot" [20], can induce internalization of a cargo molecule just by being mixed with it [21]. A very little number of references on "Chariot" can be however found in the literature despite its apparent ease of use. As for other CPPs, the debate about the mechanism of entry of "Chariot" is still ongoing. Although being described initially as energy-independent, further works have proposed different mechanisms, such as the association of helices [22] or the formation of discrete nanoparticles [23]. Controversies about the formation of pores through the membrane have also been reported [24,25].

1.1. CPPs and cell entry

As mentioned in the previous section, the entry mechanism of CPPs into cells is still a matter of some debate. Historically, two hypotheses were put forward to explain how these peptides could possibly deliver various kinds of molecules, and also much larger macromolecular structures, into the cell (for a review [16]). It was first proposed that CPPs, especially Tat and Antennapedia, but also others such as poly-Arg [10,26], Transportan [27], MPG [28] or Pep-1 [20], could pass through the plasma membrane via an energy-independent pathway. Some suggestions have been put forward to explain the translocation of these peptides, such as the formation of micromicelles at the membrane [5], or direct translocation through the lipid bilayer [29,30]. If conceivable for small CPPs, these models cannot explain the passage through the plasma membrane of CPPs-cargoes of very important size [31,32]. The hypothesis of a direct translocation through the plasma membrane became less popular when the entry mechanism for the Tat and the poly-arginine CPPs had to be re-evaluated following evidences of fixation artifacts during the preparation for samples for microscopic observation [33]. Indeed, fixation has been described to interfere with the sub-cellular localization of constructs with a high content in cationic residues, such as histones and the VP22 protein [34]. This redistribution upon fixation has been clearly demonstrated using fusion proteins made of Antennapedia, poly-Arg, or Tat peptides [35]. As a consequence, the majority of the new microscopic studies on CPP-cargoes localization have been conducted on living cells. As a result, during these last few years, numerous new works about the mechanism of entry of CPPs appeared in the literature, but the conclusions we can draw from these very elegant works could be summarized by: "the more we learn, the less we know". As a matter of fact, there has been a profusion of publications highlighting one or another entry route, sometimes with some obvious discrepancies. CPP-mediated transport has been shown, so far, to mainly follow a cellular endocytosis-mediated uptake [36–38].

According to this mechanism, CPPs, particularly those with a high content in cationic residues, are first simply adsorbed at the cell surface thanks to the numerous anionic moieties, such as heparan sulfate, sialic or phospholipidic acid [39–41]. Then CPP-mediated transport has been reported to happen through different endocytosis routes [33]: via caveolae [42], macropinocytosis [43,44], through a clathrin-dependent pathway [45], via a cholesterol-dependent clathrin-mediated pathway [46] or in the *trans*-Golgi network [47]. Some publications have provided convincing arguments against one or the other of these cellular pathways despite the use of rather similar experimental models. It has been suggested that these controversies might be due to the use of different peptide concentrations as they can trigger different endocytotic pathways [38,48]. Higher CPP concentration (>10 μM) could also lead to an energy-independent internalization [38,49]. A molecular mechanism for a direct translocation of the Tat peptide through the plasma membrane has been also recently described [50]. In conclusion, more work is needed to highlight unambiguously the precise mechanism(s) of entry of these peptides.

In addition, since no cellular pathway appears absolutely predominant or more convincing than another one, most of these pathways could be involved depending on yet unknown events such as the