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Therapeutic Delivery

Cell-penetrating peptides in vaccine delivery: facts, challenges and perspectives

Mariusz Skwarczynski*,[1] & Istvan Toth[1,2,3]

[1]School of Chemistry & Molecular Biosciences, The University of Queensland, St Lucia QLD 4072, Australia

[2]School of Pharmacy, The University of Queensland, Woolloongabba, QLD 4102, Australia

[3]Institute for Molecular Biosciences, The University of Queensland, St Lucia, QLD 4072, Australia

*Author for correspondence: m.skwarczynski@uq.edu.au

"The discovery of the cell-penetrating function of HIV TAT protein in 1988 commenced a new era in intracellular drug delivery"

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The discovery of the cell-penetrating function of HIV TAT protein in 1988 commenced a new era in intracellular drug delivery [1]. This discovery was especially important in overcoming the challenges of cellular delivery of peptides and proteins. The TAT peptide (GRKKRRQRRRPQ), a short cationic fragment of the TAT protein, quickly became a popular research tool used to enhance the transport of biomolecules into cells [2]. However, it should also be noted that the ability for highly cationic peptides to cross the plasma membrane of cells had also (separately) been under investigation since 1965 [3]. Today, a large number of cell-penetrating peptides (CPPs), both naturally derived and also synthetic/artificial, have been characterized and applied to deliver a variety of cargos into cells, including small molecule-based drugs, peptides, proteins, DNA/RNA, nanoparticles and liposomes [4]. In addition to highly cationic CPPs, such as TAT peptide, amphipathic and hydrophobic peptides possessing membrane-penetrating properties have also been discovered [5]. Several mechanisms of CPP internalization have been proposed; however, the process is still not fully understood. It is thought that internalization could proceed via a variety of pathways, even for the same CPP [6]. Moreover, the internalization mechanisms have been reported to be influenced by several factors, including the type of CPP cargo, the type of CPP itself, and also CPP concentration. However, there is broad agreement that CPPs can be internalized by both endocytic and nonendocytic pathways, to enter endosomes and cytoplasm, respectively. Naturally, CPP–cargo follows the fate of CPPs; however, the cargo can also enter the cytoplasm through the endocytic pathway.

The majority of vaccines currently undergoing development are designed based on the use of small antigens, such as proteins (or the DNA encoding them), peptides and carbohydrates. These subunit vaccines have improved safety profiles in comparison to traditional whole microorganism-based approaches, as a variety of the potentially toxic, allergic and autoimmune-triggering components of the pathogen are removed through this strategy [7]. The subunit approach is also the only choice currently available for the development of therapeutic anticancer vaccines. However, the removal of redundant components from vaccine formulations also removes most of the 'danger signals', which normally activate the immune system and trigger the protective immune responses against a pathogen. This is especially relevant when considering the level of antigen recognition and its uptake by antigen-presenting cells (APCs). Immune stimulants (adjuvants) and/or delivery systems are used in all modern vaccine designs to overcome this drawback [8].

To improve antigen uptake by APCs, CPPs can be incorporated into vaccine antigen or formulation. CPPs can deliver antigen directly to the APCs' cytoplasm following the nonendocytic pathway. Once an antigen enters cytoplasm, it is processed by proteasome into short peptide fragments, which are recognized by MHC I molecules, and thus vaccine antigen follows exactly the same processing/recognition pathway as natural viral and cancer antigens. Then, MHC I molecules present these short peptides (epitopes) to $CD8^+$ T lymphocytes triggering cellular immune responses. Moreover, as CPPs and their cargo can be taken up via endocytosis, the antigen can be

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processed into short peptides in the endo/lysosomal pathway in APCs and recognized by MHC II molecules. These molecules present these peptides to T-helper cells, which in-turn stimulates both cellular and humoral (antibody production) immune responses.

As TAT peptide is derived from the protein that is essential for cellular internalization and viral gene expression, it is not surprising that TAT-based constructs have been popular for gene delivery, including DNA-based vaccines [5]. Interestingly, TAT has also been used for the delivery of DNA that does not encode antigens, but instead acts as an adjuvant. For example, the fusion protein of human papillomavirus E7 oncoprotein, acting as an antigen, and TAT were self-assembled, based on opposite-charge interaction, into nanoparticles with *GM-CSF* DNA-based adjuvant [9]. These nanoparticles were able to eradicate established tumors in mice more efficiently than any other formulations examined, including polylysine-E7/pGM-CSF. Thus, the positive charge, alone, is not sufficient to improve the performance of a vaccine delivery system. This fact was further proven during development of liposomal formulation for a Group A Streptococcus vaccine. The incorporation of polylysine into then liposomes decreased both the activation of dendritic cells and humoral immune responses [10], demonstrating ability of polylysine to downregulate an immune response.

Interestingly, when TAT peptide has been used for the delivery of protein-based vaccines, it has usually been fused with a protein antigen – not self-assembled, like the DNA vaccines [5]. These fusion proteins were able to stimulate enhanced cellular and humoral immune responses. CPPs have also been incorporated into other delivery systems, especially nanoparticles [5,11,12]. For example, the TAT synthetic analog, polyarginine, was conjugated to *N*-vinylacetamide-co-acrylic acid (VA-AA) and administered to mice with influenza virus antigens [13]. The strongest immune responses were observed when a linker between polyarginine and VA-AA was introduced, which influenced grafting efficacy of VA-AA with polyarginine, ζ-potential of formed nanoparticles and presumably conformation of polyarginine unit, demonstrating that how CPP is incorporated into the delivery system is also important.

In addition, CPPs have been proven to have the ability to not only enhance the cellular uptake of an antigen, but also to help to pass other biological barriers. For example, the cationic CPP, penetratin, stimulated the uptake of an OVA peptide antigen through the skin [14], while TAT helped in the cross-mucosal vaginal delivery of a DNA vaccine [15]. While cationic peptides have been the most popular CPPs used for vaccine delivery, amphiphilic CPPs, such as VP22 [16], Pep-1 [17] and MPG [18], have also been used successfully, especially for the delivery of DNA vaccines.

Despite the fact that several promising vaccine delivery systems based on CPPs have been produced, this approach is still underdeveloped and requires further significant investigation. This is especially true, given [5]:

- Many studies on CPPs did not use positive controls and did not compare the CPP-based systems to the classical adjuvant-based approach;
- The use of CPPs was often not sufficient for vaccine efficacy and an additional adjuvant was required;
- The mechanism of CPP–cargo internalization remains unclear;
- A comprehensive study comparing different CPPs for the delivery of a single antigen has not been performed/reported;
- No clinical data are available to determine the efficacy of CPP-based vaccines in humans.

On the bright side, CPPs are inexpensive, easy to manufacture and usually nontoxic. Therefore, even off-target delivery of an antigen is not a considerable drawback. The incorporation of CPPs into vaccine delivery systems has triggered enhanced immune responses, especially when administered with an adjuvant; therefore, CPPs are promising co-stimulants. CPP-based drug delivery systems have reached clinical trials, where they showed the predicted efficacy [19]. Thus, the expectation for similar progress in the development of CPP-based vaccine formulations is not unreasonable.

In conclusion, CPPs are promising immune enhancers when incorporated into appropriate vaccine delivery systems. However, further extensive research is required before these peptides will be ready to advance to clinical application.

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