

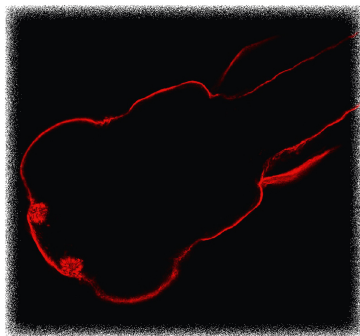


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**High-throughput screening and evaluation of
combinatorial cell penetrating peptoid libraries to
identify organelle- and organ-specific drug delivery
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Abstract

The global pharmaceutical drug market is rapidly expanding. However, for many diseases, such as neurological disorders, the treatment options are still poor. Therapeutics including proteins, DNA or RNA with promising *in vitro* results, fail in clinical trials due to insufficient bioavailability or loss of activity. To overcome this issue drug delivery systems can increase the pharmacokinetic and pharmacodynamic properties of various therapeutic agents. These systems can ensure a controlled release, improve the specificity of drugs to the target tissue, and therefore decrease side effects to the healthy surrounding tissue. Cell penetrating peptides have been investigated for drug delivery for several years, displaying many suitable properties but also disadvantages, such as fast degradation by enzymes. Poly-*N*-substituted glycines, so-called peptoids, mimic the structure of peptides by maintaining the ability to penetrate cells. In addition, they show improved stability and cellular uptake compared to peptides. In this work, several fluorescently labeled peptoid libraries were synthesized and investigated for cellular uptake, intracellular localization and cytotoxicity *in vitro* and *in vivo*. In order to elucidate a structure-function relationship and identification of organelle and organ targeting peptoids, differing in backbone length, side chain composition, hydrophilicity and fluorophore labeling, as well as cyclic peptoids, were investigated. A highly efficient automated evaluation approach was used to identify a new class of mitochondria penetrating peptoids. These peptoids are composed of lipophilic and aromatic side chains and can be subdivided, concerning their cytotoxicity in the low micromolar range. While peptoids with moderate lipophilicity display low cytotoxic effects and are suitable mitochondria specific transporter molecules, highly lipophilic peptoids show selective toxicity to cancer cells. Cytotoxic peptoids were analyzed as mitochondria-targeting anticancer agents with promising results in two-dimensional and three-dimensional cell culture. With regard to drug delivery, cyclization of peptoids could decrease their cytotoxicity and lead to differences in cellular uptake, compared to their linear counterparts. While the composition of the peptoid side chains had a strong impact on the intracellular localization, the fluorescent dye displayed little impact on cellular uptake and organelle specificity of the peptoid. To increase the complexity, the organ targeting of the peptoids was analyzed in *in vivo* studies by using the zebrafish as model organism. Phenotypic characterization revealed different organ-specific categories. The most interesting category was the colocalization with the olfactory neurons, which gave



Abstract

rise to the possibility for brain specific transport. Microinjection of an olfactory specific peptoid, rich in aromatic and nonpolar side chains, in the blood circulation of zebrafish embryos proved a high affinity of the peptoid to the brain tissue. Therefore, lead structures for brain specific transporters were identified, which allow further optimization and evaluation. Furthermore, fluorescent polymeric nanoparticles were investigated for cellular uptake and brain specific accumulation. High water-solubility, good cell penetration ability and low toxicity exhibited their biocompatibility. Both, nanoparticles and cell penetrating peptoids, have great potential as transporters for organelle- and organ-specific drug delivery, especially with respect to the brain as target tissue.



1. Introduction

1.1. Drug delivery

Over the past decades, the search for pharmacologically active compounds is growing and a tremendous progress in the treatment of various diseases has been made. A wide variety of small molecules, with a molecular weight less than 500 g/mol, peptides, proteins, nuclei acids and macromolecules were investigated. However, many active molecules with promising *in vitro* results fail in *in vivo* studies or clinical trials. Instead of specific accumulation of the drug in the desired tissue, they are evenly distributed in the body, leading to various side effects, e.g. cytotoxicity of drugs to the healthy surrounding tissue [1]. Adverse drug events lead to extended hospital stays and increased risk of mortality of patients and therefore to billions of health care costs per year [2]. Another major problem is the loss of *in vivo* activity, as biological barriers, such as cell membranes or tissue, are obstacles for therapeutic agents to reach their target. Furthermore, many drugs display poor pharmacokinetic properties, e.g. due to clearance by the kidney, fast enzymatic degradation of drugs, like peptides or proteins, or poor efficiency at physiological pH. Hence, most molecules with short half-lives are not suitable for *in vivo* applications or high invasive doses and continuous infusions, increasing the possibility for complications, are needed for successful treatment [3]. A further issue is the treatment of diseases of the central nervous system (CNS), such as Alzheimer's, Parkinson's disease or brain tumors, which are frequently occurring but their diagnosis is usually poor. Even though great effort in screening of potential drugs for CNS diseases is made the success rate is low and the global market for those drugs is very small. For most drugs overcoming the blood-brain barrier (BBB) is challenging, as only few, small and lipophilic molecules are able to cross this barrier [4-6]. The BBB is a selective, natural and essential barrier, separating the CNS from the peripheral blood [7]. Due to this boarder the brain is protected from circulating pathogens and thus brain infections are rare. The BBB is defined by an extremely tight blood vessel system in the brain. In contrast to normal capillaries, brain capillaries contain a tight endothelial cell layer surrounded by pericytes and astrocytes. These cells ensure the upregulation of tight junctions, neurons and a strong basal membrane [8-10]. Only lipophilic and small molecules can penetrate the membranes of endothelia cells and cross the barrier, while hydrophilic and charged molecules display no affinity to the lipophilic lipid membranes and are not able to cross membranes. In addition, even molecules which are predicted to cross the BBB can be



discharged by efflux transporters, e.g. P-glycoprotein, which are the gate keepers of the BBB by serving as a transport barrier. Presently, treatments of CNS diseases often include risky invasive surgery or local application of drugs. Thus, possibilities for enhanced delivery of drugs to the brain, giving them the capacity to cross the BBB, are critical.

In general, the applicability of a pharmacological active drug can be calculated by dint of the therapeutic index (TI). The TI is defined by the proportion of lethal dose (LD_{50}), causing increased toxicity or death, compared to effective dose (ED_{50}), inducing the desired therapeutic effect [11, 12]. As the TI of drugs is often low, new methods for enhanced delivery are strongly needed, decreasing their toxicity and improving the effectivity. Thus, not only the development of drugs is a growing research field but also drug targeting. Specific accumulation in the target tissue can be achieved with passive and active drug targeting. Passive targeting benefits of abnormal properties of the target tissue e.g. wide fenestrations of blood vessels in tumors. Enhanced permeability and retention (EPR) effect leads to increased uptake of macromolecules and nanoparticles in the tumor tissue [13]. Active targeting can be achieved with cell- or organ- specific ligands. For both, passive and active targeting, drug delivery systems (DDS) can be used. DDS enhance cellular uptake, increase the solubility of drugs and protect them from degradation by enzymes. Cargos can be transported across various biological barriers, even the BBB, to specific cellular organelles or target organs. Therefore, side effects to the healthy surrounding tissue can be reduced and similarly an improvement of dose-efficiency relationship is possible. Hence, the pharmacological properties and the therapeutic indices of drugs can be strongly improved.

1.2. Drug delivery systems

Enhanced cellular uptake of drugs or organ-specific delivery and controlled release can be achieved by attachment of drugs to various drug delivery systems (DDS). Over the last decades a wide range of DDS have been investigated for biocompatibility, cell penetration abilities, pharmacokinetic properties, controlled drug release and ability to transport cargos. Important properties for DDS are low toxicity, intense stability *in vivo*, with avoidance of long-term accumulation in the body, and the ability to cross insistent barriers, e.g. the BBB [14]. Carrier molecules can be for example nanoparticles, liposomes, antibodies, polymers, viruses, micelles or peptides. Each DDS has different advantages and disadvantages and therefore not all carrier systems might be suitable for all drugs or target organs. Liposomes for example are



prone to encapsulate drugs, to increase their solubility and decrease their cytotoxicity [15]. However, encapsulation is not suitable for neutral hydrophobic molecules as they are rapidly released [16]. Nanoparticles are especially interesting in the field of cancer therapy as they show increased accumulation in tumor tissue. Unfortunately, many nanoparticles display increased cytotoxicity. Cell penetrating peptides have been emphasised by the literature as they have the ability to cross different cell membranes and display low toxicity *in vivo* and *in vitro*.

1.3. Cell penetrating peptides

In 1988 Green and Loewenstein, as well as Frankel and Pabo, discovered that the trans-activator of transcription (Tat) protein of HIV-1 is able to cross the membrane of various cells and could even reach the nucleus [17, 18]. At that point in time this was a completely new finding, as only one year before Sternson et al. reported that proteins and peptides display poor cellular uptake and might therefore not be suitable as drug candidates [19]. The Tat protein consists of 86 amino acids and it could be shown that it is rapidly taken up by cells and stimulates HIV-LTR driven RNA synthesis [17]. Green and Loewenstein were also able to synthesize active mutant peptides, with 21 to 41 amino acids. In the following years the Tat protein was extensively analyzed and several mutant peptides have been investigated to identify the minimal sequence required for cellular uptake. Vivès *et al.* discovered in 1997, that the basic domain Tat-(48-60) is needed for cellular uptake and deletion or substitution of amino acids within this domain reduced the cellular uptake. This cationic area consists mainly of arginine and lysine side chains. Further investigations, to find the shortest structure needed for cellular uptake, showed that the peptide can be reduced to an eleven amino acids motif, Tat-(47-57), which is still able to translocate cells. In 1991 it was discovered, that the 60 amino acid polypeptide of the *Drosophila* Antennapedia homeodomain (pAntp) was able to penetrate cells as well [20]. Based on these results, in 1994, a 16-mer cell penetrating peptide, so called penetratin, derived from the third helix of Antennapedia was synthesized. As internalization of the peptide was also found at 4 °C, it was assumed that the peptide is taken up by an energy-independent mechanism [21]. In the following years many further cell-penetrating peptides, derived from protein transduction domains, were found, such as transportan, a synthetic peptide built from the neuropeptide galanin linked to mastoparan, or VP22, based on a sequence of herpes virus-type 1 virus [22, 23]. Additionally, it was discovered



that not only peptides rich in cationic amino acids are able to penetrate cells but also amphipathic peptides, consisting of a hydrophilic and a hydrophobic domain. Therefore, amphipathic peptides contain not only lysine and arginine but also hydrophobic amino acids like alanine, leucine, isoleucine and valine [24]. The class of amphipathic cell penetrating peptides can be subdivided in three groups: primary, secondary and proline-rich peptides. Primary amphipathic peptides are derived from a sequential assembly of hydrophobic and hydrophilic domains in their primary sequence, for example MPG or Pep-1 [25]. In contrast, secondary amphipathic peptides are received after folding in secondary structures, mostly α -helical structure, leading to hydrophobic domains opposite to hydrophilic domains, e.g. peptides in the MAP (model amphipathic peptide) family [26]. Last, but not least, hydrophobic peptides, containing mainly nonpolar amino acids, were found to have a high affinity to hydrophobic cellular membranes, giving them the ability to translocate the membrane spontaneously. As an example, C105Y, derived from α -1-antitrypsin, can be mentioned [27]. However, compared to cationic and amphipathic peptides, the amount of discovered hydrophobic peptides is low and therefore knowledge about them is small [28]. Table 1 gives an overview of different, well characterized cationic, amphipathic and hydrophobic cell penetrating peptides.

Table 1: Overview of eight well-studied cationic, amphipathic and hydrophobic CPPs. Sequences are shown in one letter code for each CPP [28]

Peptide	Sequence	Class
Tat-(47-57)	YGRKKRRQRRR	Cationic
Penetratin	RQIKIWFQNRRMKWKK	Cationic
Transportan	GWTLNSAGYLLGKINLKALAALAKKIL	Cationic
VP22	DAATATRGRSAASRPTERPRAPAR-SASRPRRPVD	Cationic
MPG	GALFLGFLGAAGSTMGAWSQPKKKRKV	Amphipathic
Pep-1	KETWWETWWTEWSQPKKKRKV	Amphipathic
MAP	KALAKALAKALA	Amphipathic
C105Y	CSIPPEVKFNKPFVYLI	Hydrophobic

As CPPs are highly charged, mostly due to cationic side chains, and accumulate not only in endosomes but also in cytoplasm and the cell nucleus, toxicity of CPPs was intensively studied. Low impacts on the viability of adherent and nonadherent cell lines, in low micromolar range,



was found for most CPPs. Only pAntp displays increased toxicity for concentrations above 50 μM , whereas it was shown for Tat(48-57) that even concentrations of 100 μM were harmless to various cell lines [29, 30]. These results were also confirmed in *in vivo* studies, finding no visible toxicity after injection of a fusion protein in mice [31].

1.3.1. Mechanisms of cellular uptake

So far, it is known that specific peptides are able to cross the membranes of several cell types, however the mechanism of cellular uptake of peptides has not been completely understood and it is assumed that different pathways are possible. There are probably various factors, such as peptide structure and size, cell type, temperature, concentration and incubation time, which play a crucial role for the route to enter the cell [32]. However, it can be differentiated between two entry mechanisms: the energy-dependent endocytosis and direct penetration of the membrane, which is energy-independent. Initially, it was believed that uptake is only taken place by direct penetration, however, these findings were artifacts, due to protocols involving fixation of cells [21, 33, 34]. Lately, it has been assumed, that the most frequent pathway to enter the cell for CPPs is endocytosis [35-37]. Possible endocytosis ways are phagocytosis, macropinocytosis, caveolin- or clathrin-mediated endocytosis and clathrin/caveolin-independent endocytosis [38]. Phagocytosis, an active pathway, which is possible only in specific cell types and involves several specific cell-surface receptors, usually takes place for clearance of large particles, such as pathogens or apoptotic cells and is therefore probably less important for CPPs [39]. More important for CPPs are pinocytosis pathways, occurring in all mammalian cell types. It was found, that CPPs are able to use more than one pathway simultaneously, for example for Tat macropinocytosis, clathrin-mediated as well as caveolin-mediated endocytosis was found [37]. For direct penetration there are also different possible ways: formation of pores, formation of inverted micelles and the carpet model. It is reported, that amphipathic CPPs are able form pores in the membrane, for example due to their α -helical structure. Hydrophobic side chains in the helix facing the membrane, while hydrophilic side chains are hidden inside the pore [40]. The carpet model suggests that peptides self-associate and lie parallel on the membrane, interacting with their cationic side chains with the negative charges of the phospholipid headgroups on the membrane surface. Thus, membrane organization is disturbed and peptides are able to cross the membrane border [41]. Furthermore, the inverted micelle model was found, initially for

pAntp. CPPs interacts with the membrane, leading to the formation of inverted micelles, encapsulating the peptides. Further interactions of CPPs with the membrane, in the inverted micelle, destabilizes the membrane and CPPs are released into the cell [21, 42, 43]. An overview of different possible pathways is shown in figure 1.

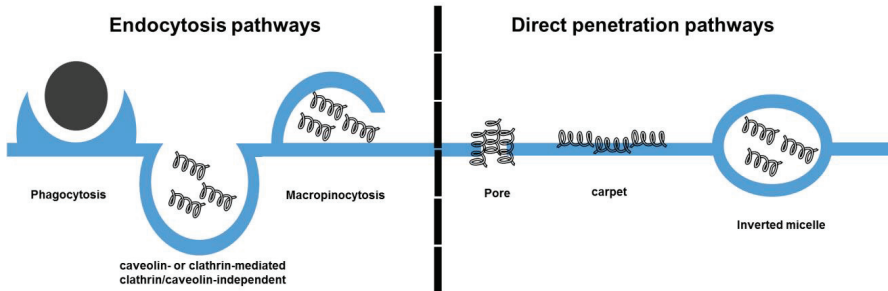


Figure 1: Different possible pathways for uptake of CPPs are shown. Left: three possible ways of endocytosis: phagocytosis, caveolin- or clatherin-mediated and caveolin/clatherin-independent pathway and micropinocytosis. Right: Three ways for direct penetration: formation of pores, carpet model and inverted micelles. Cell membrane is pictured by a blue barrier and CPPs are represented by short helices. Source: [28, 44]

1.3.2. Application of cell penetrating peptides

Since the discovery of cell penetrating abilities of peptides not only peptides themselves have been studied but also their capability to transport cargo into cells. For their application as transport vectors there is the possibility of covalent conjugation of the cargo to the peptide or complexation based on non-covalent interaction. Suitable covalent conjugation are for example amide bonds, disulfide bonds or thioester linkages as they give the possibility to release the cargo after penetration of the cell membrane [45-47]. Covalent binding of cargo has been shown to be suitable for the transport of various molecules, under reproducible conditions, but it might also inhibit the biological activity of the cargo [48]. Non-covalent conjugation can be achieved by simple mixing of cargo and CPP, due to hydrophobic or electrostatic interactions, depending on physiochemical properties of peptide and cargo. Non-covalent bindings are easier to achieve, however, resulting complexes are hard to control and variations in size and composition occur, whereas compositions for covalent bindings are defined [47]. In 1998 Nagahara *et al.* was able to conjugate Tat peptide covalently to proteins and could successfully show transport abilities of Tat *in vitro* [49]. In 2001, Morris *et al.* could demonstrate efficient delivery of peptides and proteins, non-covalently linked to Pep-1, into



mammalian cells. Pep-1 was highly recommended, due to low toxicity and good stability in the presence of physiological buffers [50]. Since then, various cargo could be transported into cells, including macromolecules, DNA, RNA and proteins [51-53]. Furthermore, CPPs were investigated for delivery across the BBB, finding promising results, as it could be shown, that Tat is able to overcome the barrier and deliver Bcl-xl, which plays a role in neuronal apoptosis, in the brain tissue and protecting it against ischemic brain injury [54]. Tat was also fused to β -galactosidase and after injection in mice β -Gal activity was not only found in different organs, including kidney, heart and lung, but also in the brain [55]. Rouselle et al. compared doxorubicin (dox) concentrations, a well-known chemotherapeutic agent, of free dox to dox coupled to CPPs in rat brains. It could be shown that CPPs significantly increased the dox concentration in the brain [56]. To sum up, many promising results have been found for CPPs as delivery vectors, including low toxicity, strong cellular uptake and the ability to transport cargo into cells.

1.3.3. Limitations of CPPs

Despite the numerous advantages of CPPs they also have disadvantages, limiting their usage as suitable transporter vehicles for drug delivery. For most CPPs endosomal uptake is found, and even though they internalize cells in this manner, they are still encapsulated by endosomal membranes, unable to deliver their cargo in cytoplasm or to the nuclei [57]. Endosomal escape is hard to achieve, as it requires destabilization of endosomal membranes, accomplishable only by adding cytotoxic auxiliary compounds. Major drawbacks can also be found regarding their application *in vivo*. As they are able to penetrate different cell lines, most CPPs are found to be not cell or tissue specific. Therefore, CPPs lack of specificity for delivery of cargo *in vivo* and distribution is found in various parts of the body in animal models [55, 58]. After fusion of CPPs to antibodies, cellular uptake of antibodies could be improved significantly, however, also unspecific enrichments were found in different non-targeted tissues [59, 60]. The major problem of peptides, however, is their limited usage *in vivo*. Due to their natural and peptidic structure rapid degradation takes place in the presence of serum or intestinal fluids [61]. Thus, CPPs display poor pharmacokinetic properties and high doses are needed for therapeutic effects.



1.4. Peptidomimetics

As the demand of peptides which are more stable *in vivo*, is growing, an important and promising research field are peptidomimetics. These non-natural molecules mimic natural peptides but due to modifications in the backbone or side chains they can exhibit increased stability against proteolysis. Due to modifications in the peptide structure improved cellular uptake and less nonspecific receptor binding can be achieved, leading to enhanced pharmacokinetic and pharmacodynamics properties [62]. Stability of peptides can already be improved by integration of non-natural side chains or changing the natural occurring L-amino acids to D-amino acids (*inverso*). Additionally, the order of amino acids can be reversed (*retro*), to keep stereochemistry to their peptide counterparts. Stability of *retro inverso* peptides is improved however, they also display increased toxicity to cells [63]. Another possible approach to increase pharmacological properties of peptides are for example β - or γ -peptides, consisting of β - or γ -amino acids, which have their amino group bound to β - or γ -carbon instead of the α -carbon. Shifting of the amino group leads to an extension of the peptide backbone and due to increased conformational flexibility to changes in secondary structures of those peptides. For β -peptides not only enhanced stability against several peptidases has been shown but also improved biological activity [64, 65]. In addition, it is possible to replace one or more amino groups in the backbone to an oxygen atom, so called depsipeptides. This change induces structural changes and more flexible structures, as hydrogen bonds are decreased after elimination of the amino group. Several depsipeptides have been synthesized or isolated from bacteria and studied for antimicrobial activity with promising results. Furthermore, cyclization of peptides can improve their bioavailability, as amide bonds are sheltered in the cycle, enhancing cellular uptake and decreasing enzymatic degradation. Structures of exemplary peptidomimetics are shown in figure 2.