Introduction: challenges in drug delivery

Over the past 10 years, in order to circumvent limitations of small molecule- and gene-based therapies, we have witnessed a dramatic acceleration in the production of new large therapeutic molecules, which do not follow Lipinski’s rules, such as proteins, peptides and nucleic acid therapeutics. However, their development is restricted by very specific issues including poor stability in vivo, lack of cellular uptake and insufficient capability to reach targets. This is associated with the complete loss of pharmaceutical potency or at least with the requirement for high doses and risk of major side effects. Therefore, delivery constitutes a major piece of the therapeutic puzzle, and there is a real demand for new and more efficient drug delivery systems. Major rules have to be satisfied, in particular: (i) delivery efficiency in different and challenging cell lines; (ii) rapid endosomal release; (iii) ability to reach the target; (iv) activity at low doses; (v) lack of toxicity; and (vi) facility of therapeutic application.

Substantial progress has been made in the design of new technologies to improve cellular uptake of therapeutic compounds (Opalinska and Gewirtz, 2002; Järver and Langel, 2004; Glover et al., 2005; Torchilin, 2005; De Fougerolles et al., 2007; Kong and Mooney, 2007). A number of non-viral strategies have been proposed including lipid, polycationic, nanoparticle and peptide-based formulations as reported in this special issue (Morris et al., 2000; Ogris and Wagner, 2002; Järver and Langel, 2004; Torchilin, 2005), but only a subset of these technologies are efficiently applied in vivo at either preclinical or clinical levels. Protein transduction domains (PTDs) or cell-penetrating peptides (CPPs) correspond to short 30 residue synthetic peptides and are part of the most promising strategy to overcome both extracellular and intracellular limitations of various biomolecules of including plasmid DNA,
oligonucleotide, siRNA, peptide-nucleic acid (PNA), proteins, peptides as well as liposomes. CPPs can trigger the movement of a cargo across the cell membrane into the cytoplasm of cells and improve its intracellular routing, thereby facilitating interactions with the target (Derossi et al., 1994; Fawell et al., 1994; Pooga et al., 1998; Wender et al., 2000; Deshayes et al., 2005; Meade and Dowdy, 2007; Morris et al., 2008).

**Cell-penetrating peptide families**

Twenty years ago, the notion of PTD was proposed based on the observation that some proteins, mainly transcription factors, could shuttle within the cell and from one cell to another. Historically, the first observation was made in 1988, by Frankel and Pabo, who showed that the transcription-transactivating (Tat) protein of HIV-1 could enter cells and translocate into the nucleus (Frankel and Pabo, 1988). In 1991, the group of Prochiantz demonstrated that Drosophila Antennapedia homeodomain could be internalized by neuronal cells (Joliot et al., 1991), which was at the origin of the discovery in 1994 of the first PTD or CPP: a 16-mer-peptide derived from the third helix of the homeodomain of Antennapedia termed penetratin (RQIKIYFQRRMKWKK) (Derossi et al., 1994). In 1998 the group of Lebleu identified the minimal peptide sequence of Tat required for cellular uptake (YGRKKRRQRRR) (Vives et al., 1997). In 1997, the first non-covalent CPP for delivery of nucleic acids MPG was designed by the group of Heitz and Divita (Morris et al., 1997) closely followed by development of Pep-1 for non-covalent delivery of proteins and peptides (Morris et al., 2001). The groups of Wender and of Futaki demonstrated that polycationine sequences (ArgX) were sufficient to drive molecules into cells and proposed that their uptake mechanism involves a bidentate hydrogen-bonding interaction between guanidinium group of arginine residues and phosphate group in the membrane (Wender et al., 2000; Futaki et al., 2001). A major breakthrough in the CPP field came from the first proofs-of-concept of their in vivo application, by the groups of Dowdy, for the delivery of small peptides and large proteins (Schwarze et al., 1999), and of Langel, for delivery of PNA using the chimeric peptide Transportan, derived form the N-terminal fragment of the neuropeptide galanin, linked to mastoparan, a wasp venom peptide (Pooga et al., 1998). Ever since many other CPPs able to trigger the movement of a cargo across the cell membrane into the cytoplasm have been designed (Järver and Langel, 2004; Joliot and Prochiantz, 2004; Deshayes et al., 2005; Snyder and Dowdy, 2005). CPPs are generally peptides of less than 30 amino acids, derived from natural or unnatural protein or chimeric sequences and can be subdivided into two main classes, the first requiring chemical linkage with the cargo and the second involving formation of stable, non-covalent complexes. CPPs can also be distinguished from a structural point of view, as either polycationic, essentially containing clusters of polycarboxylic in their primary sequence or amphipathic. The representative CPPs are reported in Table 1. Although this review mainly focuses on CPPs based on natural amino acids, recent concepts of CPPs containing unnatural and modified residues have been proposed in order to improve either the stability or the efficiency of the carrier (Farrera-Sinfreu et al., 2007).

### Table 1: Representative CPP sequences, applications, and major related references

| Peptides | Origin | Cargo types | References |
|----------|--------|-------------|------------|
| Peptides deriving from protein transduction domains | | | |
| Tat | HIV-Tat protein | Protein/peptide/siRNA/liposome | Snyder and Dowdy (2005); Futaki et al. (1999); Joliot and Prochiantz (2004) |
| Peptides deriving from protein transduction domains | | | |
| Antennapedia | Homeodomain | Protein/peptide | Derossi et al. (1994); Eilott et al. (1997) |
| Peptides deriving from protein transduction domains | | | |
| MAP | Oligoarginine | Protein/peptide | Morris et al. (2008); Gros et al. (2006) |
| MAP-Chimeric | MAP-peptide | Protein/peptide | Richer et al. (2005) |
| MAP-Chimeric | MAP-peptide | Protein/siRNA | Wendor et al. (2000); Futaki et al. (1998); Rousselle et al. (2000); Emperou et al. (2001) |
| MAP-Chimeric | MAP-peptide | Protein/siRNA | Wendor et al. (2000); Futaki et al. (1998); Rousselle et al. (2000); Emperou et al. (2001) |
| MAP-Chimeric | MAP-peptide | Protein/siRNA | Wendor et al. (2000); Futaki et al. (1998); Rousselle et al. (2000); Emperou et al. (2001) |
| MAP-Chimeric | MAP-peptide | Protein/siRNA | Wendor et al. (2000); Futaki et al. (1998); Rousselle et al. (2000); Emperou et al. (2001) |
| MAP-Chimeric | MAP-peptide | Protein/siRNA | Wendor et al. (2000); Futaki et al. (1998); Rousselle et al. (2000); Emperou et al. (2001) |
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| MAP-Chimeric | MAP-peptide | Protein/siRNA | Wendor et al. (2000); Futaki et al. (1998); Rousselle et al. (2000); Emperou et al. (2001) |
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| MAP-Chimeric | MAP-peptide | Protein/siRNA | Wendor et al. (2000); Futaki et al. (1998); Rousselle et al. (2000); Emperou et al. (2001) |
| MAP-Chimeric | MAP-peptide | Protein/siRNA | Wendor et al. (2000); Futaki et al. (1998); Rousselle et al. (2000); Emperou et al. (2001) |
| MAP-Chimeric | MAP-peptide | Protein/siRNA | Wendor et al. (2000); Futaki et al. (1998); Rousselle et al. (2000); Emperou et al. (2001) |
| MAP-Chimeric | MAP-peptide | Protein/siRNA | Wendor et al. (2000); Futaki et al. (1998); Rousselle et al. (2000); Emperou et al. (2001) |
| MAP-Chimeric | MAP-peptide | Protein/siRNA | Wendor et al. (2000); Futaki et al. (1998); Rousselle et al. (2000); Emperou et al. (2001) |
| MAP-Chimeric | MAP-peptide | Protein/siRNA | Wendor et al. (2000); Futaki et al. (1998); Rousselle et al. (2000); Emperou et al. (2001) |
| MAP-Chimeric | MAP-peptide | Protein/siRNA | Wendor et al. (2000); Futaki et al. (1998); Rousselle et al. (2000); Emperou et al. (2001) |
| MAP-Chimeric | MAP-peptide | Protein/siRNA | Wendor et al. (2000); Futaki et al. (1998); Rousselle et al. (2000); Emperou et al. (2001) |
| MAP-Chimeric | MAP-peptide | Protein/siRNA | Wendor et al. (2000); Futaki et al. (1998); Rousselle et al. (2000); Emperou et al. (2001) |
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| MAP-Chimeric | MAP-peptide | Protein/siRNA | Wendor et al. (2000); Futaki et al. (1998); Rousselle et al. (2000); Emperou et al. (2001) |
| MAP-Chimeric | MAP-peptide | Protein/siRNA | Wendor et al. (2000); Futaki et al. (1998); Rousselle et al. (2000); Emperou et al. (2001) |
| MAP-Chimeric | MAP-peptide | Protein/siRNA | Wendor et al. (2000); Futaki et al. (1998); Rousselle et al. (2000); Emperou et al. (2001) |
| MAP-Chimeric | MAP-peptide | Protein/siRNA | Wendor et al. (2000); Futaki et al. (1998); Rousselle et al. (2000); Emperou et al. (2001) |
| MAP-Chimeric | MAP-peptide | Protein/siRNA | Wendor et al. (2000); Futaki et al. (1998); Rousselle et al. (2000); Emperou et al. (2001) |
| MAP-Chimeric | MAP-peptide | Protein/siRNA | Wendor et al. (2000); Futaki et al. (1998); Rousselle et al. (2000); Emperou et al. (2001) |
| MAP-Chimeric | MAP-peptide | Protein/siRNA | Wendor et al. (2000); Futaki et al. (1998); Rousselle et al. (2000); Emperou et al. (2001) |
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**Covalent strategy**

Cell-penetrating peptide-based technologies described so far mainly involve the formation of a covalent conjugate between the cargo and the carrier peptide, which is achieved by chemical cross-linking or by cloning followed by expression of a CPP fusion protein (Nagahara et al., 1998; Gait, 2003; Moulton and Moulton, 2004; Zatsepin et al., 2005). Most of the work has been reported for peptides derived from Tat (Fawell et al., 1994; Vives et al., 1997; Franken and Pabo, 1988), penetratin (Derossi et al., 1994), polyarginine peptide Arg sequence (Wender et al., 2000; Futaki et al., 2001) and Transportan, (Pooga et al., 1998). Other protein-derived peptides such as VP22 protein from Herpes Simplex Virus (Elliott and O’Hare, 1997), pVec (Elmqquist et al., 2001), calctinin-derived peptides (Schmidt et al., 1998; Krauss et al., 2004), antimicrobial peptides Buforin I and SynB (Park et al., 1998; Park et al., 2000), as well as polyproline sweet arrow peptide (Pujals et al., 2006) have also been successfully used to improve the delivery of covalently linked cargos (Joliot and Prochiantz, 2004; El-Andaloussi et al., 2005; Murriel and Dowdy, 2006). More recently, new generations of CPPs, combining different transduction motifs (Abes et al., 2007) or transduction domains in tandem with protein or oligonucleotide-binding domains (Meade and Dowdy, 2007) have been proposed. Different chemistries have been proposed for stable or cleavable conjugation involving mainly disulfide or thio-esters linkages. According to the stability and efficiency of the cargo, several parameters need to be considered including the type of linkage chemistry, the nature of the spacer (Gait, 2003; Zatsepin et al., 2005). Covalent strategies have been mainly reported for the delivery of DNA mimic molecules or steric block oligonucleotides, including PNA (Koppelhus et al., 2002; Fabani et al., 2008), phosphorodiamidate morpholino-oligomer (PMO) (Abes et al., 2006; Lebleu et al., 2008; Moulton and Moulton, 2008), peptide and protein (Snyder and Dowdy, 2005). Conjugation methods offer several advantages for in vivo applications including rationalization, reproducibility of the procedure, together with the control of the stoichiometry of the CPP-cargo. However, the covalent CPP technology is limited from a chemical point of view and risks altering the biological activity of the cargo. This is particularly true, in the case of charged oligonucleotide or siRNA, for which CPP coupling has led to restricted biological activities (Juliano et al., 2008), non-covalent strategies thereby appearing more appropriate.

**Non-covalent strategy**

This strategy is mainly based on short amphipathic peptide carriers consisting of two domains: a hydrophilic (polar) domain and a hydrophobic (non-polar) domain (Table 1). The amphipathic character may arise from either the primary structure or the secondary structure. Primary amphipathic peptides can be defined as the sequential assembly of a domain of hydrophobic residues with a domain of hydrophilic residues. Secondary amphipathic peptides are generated by the conformational state that allows positioning of hydrophobic and hydrophilic residues on opposite sides of the molecule (Deshayes et al., 2005). Several CPPs have been reported to form non-covalent complexes with biomolecules and to improve their delivery into mammalian cells (Morris et al., 2008).

Non-covalent approach was originally developed for gene delivery; several peptides able to condense DNA associated with peptides that favour endosomal escape including fusion peptide of HA₂ subunit of influenza hemaglutinin have been described (Lear and Degrado, 1987; Parente et al., 1990). Synthetic peptides anologs GALA, KALA, JTS1 (Gottschalk et al., 1996; Wyman et al., 1997), PPTG1 (Rittner et al., 2002), MPG (Morris et al., 1999) and histidine-rich peptides (Midoux et al., 1998; Kichler et al., 2003) were also reported as potent gene delivery systems. In 2001, we demonstrated that the amphipathic peptide Pep-1 could be successfully applied to the delivery of small peptides and proteins in a non-covalent approach (Morris et al., 2001). In 2003, a non-covalent strategy based on MPG was shown to efficiently deliver siRNA into cultured cell lines (Simeoni et al., 2003). Pep-1 and MPG are primary amphipathic peptides containing a hydrophilic lysine-rich domain derived from the nuclear localization sequence (NLS) of SV40 large T antigen (KKKKKKK), and a variable N-terminal hydrophobic moiety derived formed the fusion sequence of the HIV protein gp41 (GALFLGFLAGGSTMGA) for MPG, and from a tryptophan-rich cluster (KETW-WETWWTEW) for Pep-1, separated by a linker domain, which improves the flexibility and the integrity of both the hydrophobic and hydrophilic domains (Morris et al., 1997; Morris et al., 1999; Simeoni et al., 2003). MPG and Pep-1 form stable complexes with their respective cargo (oligonucleotide or protein/peptide) through non-covalent electrostatic and hydrophobic interactions (Morris et al., 1997; 1999; 2001; Simeoni et al., 2003; Gros et al., 2006; Munoz-Morris et al., 2007). Non-covalent strategies for protein and oligonucleotide delivery have been recently been extended to other CPPs, including Tat (Meade and Dowdy, 2007), polyarginine (Kim et al., 2006; Kumar et al., 2007) and Transportan-derived peptides (Pooga et al., 2001; Lundberg et al., 2007).

**Cellular uptake mechanism of cell-penetrating peptides**

The cellular uptake mechanism of CPPs is an essential piece of the puzzle for the development and optimization of appropriate strategies for in vivo therapeutic applications. Although cellular internalization of CPPs was reported in a wide variety of cell types, their mechanism of internalization remained ‘mysterious’ for a long time, as being independent of endocytosis, of energy and of specific receptor. In the last 5 years, the CPP field has suffered and learnt from technical artifacts. As such, in 2003, Lebleu and colleagues, proposed a revised cellular uptake mechanism for CPPs, essentially associated with the endosomal pathway (Richard et al., 2003). Ever since, the mechanism of many CPPs has re-examined and reported to be mediated by endocytosis (Lundberg and Johansson, 2001; Nakase et al., 2004; Wadia et al., 2004; Fischer et al., 2005; Richard et al., 2005; Murriel and Dowdy, 2006). However, for most CPPs, the cellular uptake mechanism still needs to be confirmed and remains controversial, partly due to the fact that different methods, which are not comparable from one lab
to another, have been employed to this aim. Therefore, results should be taken with care as in most of the cases the visualization of CPPs inside the cell is based on fluorescein-labelled CPPs with the risk that fluorescent dyes may alter the uptake mechanism or trigger an unusual cell entry pathway, which does not reflect the biologically active fraction of the CPPs or of the cargo. Evidence for several routes of entry has been reported, some of which are independent of the endosomal pathway and involve the trans-membrane potential (Terrone et al., 2003; Thoren et al., 2003; Rothbard et al., 2004; Deshayes et al., 2005). Therefore, for therapeutic purposes the challenge remains in identifying the route yielding a biological response, which may not be the predominant one and to correlate the uptake pathway with a biological response associated with a specific cargo (Wadia et al., 2004; Gros et al., 2006). For that purpose, several approaches have been described, by using biological reporters (Wadia et al., 2004; Lebleu et al., 2008) or phenotypic (Morris et al., 2007a) assays enabling to follow shuttling and release of the cargo in real time in cultured cells (Lee et al., 2008) or in animal models (Wender et al., 2007).

Although it remains difficult to establish a general scheme for CPP uptake mechanism, there is a consensus that the first contacts between the CPPs and the cell surface take place through electrostatic interactions with proteoglycans, and that the cellular uptake pathway is driven by several parameters including: (i) the nature and secondary structure of the CPP; (ii) its ability to interact with cell surface and membrane lipid components; (iii) the nature, type and active concentration of the cargo; and (iv) the cell type and membrane composition (Figure 1).

**Role of proteoglycans**
Proteoglycans play an essential role in the regulation of cell surface microdomains, and evidence for direct relationships between cytoskeletal organization and activation of small GTPases has been clearly established (Conner and Schmid, 2003; Eitzen, 2003). Heparan sulfate proteoglycans and syndecans are the major components of the extracellular matrix, and their clustering triggers cytoskeletal remodelling upon activation of protein kinase C and Rho/Rac GTPases, which control the dynamics of cholesterol-rich ‘raft’ microdomains, and therefore ligand binding and cellular uptake pathways (Couchman, 2003; Beauvais and Rapraeger, 2004). The first contacts between the CPPs and the cell surface take place through electrostatic binding with cell surface proteoglycans GlucosAminoGlycan (GAG) platform, follow by a remodelling of the actin network and a selective activation of the

**Figure 1** Model of cellular uptake and intracellular trafficking of cell-penetrating peptides (CPPs). Cellular uptake of CPP by the covalent (CPP-CS) and non-covalent (CPP-NCS) strategies. (1) Binding of CPPs or CPP/cargo complexes to extracellular matrix via the cell surface proteoglycan platform, (2) clustering of GlucosAminoGlycan platform triggers selective activation of small GTPase and remodelling of the actin network, (3) increase of membrane fluidity or microdomain dynamic promotes the cell entry and release in the cytosol of CPP-NCS and of CPP-CS (at high concentrations) via membrane fusion or cellular uptake of CPP-CS/CPP-NCS via (4) endocytosis pathway (a: caveolin-dependent, b: clathrin-dependent, c: clathrin- and caveolin-independent) or (5) macropinocytosis. After endocytic capture, CPP-CS can escape from lysosomal degradation and enter the cytosol and the nucleus (6), remain in the early or late endosomes (7), or be delivered in the Golgi apparatus and the endoplasmic reticulum (8).
The gates of the cell: cell entry and trafficking pathways
Following binding to the GAG ‘platform’, which facilitates accumulation of the CPP and CPP-cargo complexes at the cell surface, different cell entry gates have been reported depending on the CPPs. Correlation of cellular uptake with a cargo-associated biological response is a major requirement to validate the efficiency of a CPP, as originally established for Tat (Wadia et al., 2004) and has been extended to series of well-known CPPs (Nakase et al., 2004; Padari et al., 2005). One of the major differences between CPPs resides in their mode of interaction with the cellular surface components. The interaction of peptides such as Tat (Console et al., 2003; Murriel and Dowdy, 2006) polyarginine and penetratin (Nakase et al., 2004; 2007) with the extracellular matrix has been reported to be primarily electrostatic and to trigger uptake through an energy-dependent endocytotic process (Rusnati et al., 1999; Murriel and Dowdy, 2006). Although, macropinocytosis has been reported as the major route of internalization of cationic CPPs (Wadia et al., 2004; Kaplan et al., 2005), other endocytotic pathways including clathrin- and caveolin-dependent endocytosis (Richard et al., 2005; Ziegler et al., 2005) and trans-Golgi network-mediated internalization (Fischer et al., 2005) have been described for CPPs. Moreover, different mechanisms of membrane translocation and endocytosis may concur simultaneously for most CPPs. This is especially true for amphipathic peptides, which tend to interact with lipids and adopt secondary structures within the membrane that modified membrane integrity. The secondary structure of CPPs and their dynamics constitute major factors in the mechanism of cellular uptake (Magzoub and Graslund, 2004; Deshayes et al., 2005; 2008; Esbjörner et al., 2007). Increasing the local concentration of CPPs at the cell surface favours uptake independently of endocytosis and leads to a more cytoplasmic distribution of CPPs. Indeed, the major route for cell entry of CPP-based nanoparticles, such as Pep-1 and MPG has been shown to be independent of the endosomal pathway. Cellular uptake is associated with the ability of MPG and Pep-1 to interact with membrane lipids, mainly through their hydrophobic domain, and to form transient trans-membrane helical or beta structures that temporarily affect membrane organization, thereby facilitating insertion into the membrane and initiation of the translocation process associated with membrane potential (Deshayes et al., 2004a,b). Cellular uptake of biologically active Pep-1 or MPG/cargo complexes is directly correlated with the structure of the nanoparticle that creates a local high concentration of peptides at the cell surface (Gros et al., 2006; Munoz-Morris et al., 2007).

In contrast, to cellular uptake that is well characterized for a subset of CPPs, very little is known about their cellular trafficking, which is important to allow the cargo to reach its target within the cell. Clearly, endosomal escape remains a major limitation and the rate-limiting step of CPP-mediated drug delivery. A small fraction of CPPs is able to escape from the endosome throughout either their endosomal breaker property or to the fact of the poor integrity of the macropinocytosis vesicles. Several studies report that CPPs can traffic through the endoplasmic reticulum and the Golgi network via a ‘retrograde pathway’ that involves cytosolic release (Fischer et al., 2005). Moreover, CPPs harbouring functional NLS motif can directly localize and trigger cargo in the nucleus (Cartier and Reszka, 2002; Simeoni et al., 2003).

Application of CPP strategies to the delivery of therapeutic molecules

The number of applications using CPPs is consciously increasing, and so far more than 300 studies using either covalent or non-covalent CPP-based strategies from in vitro to in vivo have been reported (Dietz and Bähr, 2004; Gros et al., 2006; Moschos et al., 2007; Patel et al., 2007; Foerg and Merkle, 2008). The interest for CPPs is mainly due to their low cytotoxicity and to the fact that there is no limitation for the type of cargo. Although CPPs have been used to improve delivery of cargo that varies greatly in size and nature (small molecules, oligonucleotide, plasmid DNA, peptide, protein, nanoparticle, lipid-based formulation, virus, quantum dots) most of the applications describe the delivery of oligopeptide/protein (Dietz and Bähr, 2004; Gros et al., 2006; Patel et al., 2007) and nucleic acids or analogs (Juliano et al., 2008) (Table 1).

CPP-based strategies for gene delivery

The poor permeability of the plasma membrane of eukaryotic cells to DNA together with the low efficiency of DNA or oligonucleotides to reach their target within cells constitutes the two major barriers for the development of these therapeutic molecules. In the last decade, a number of peptide carriers that combine DNA binding, mainly electrostatic domain (polylysine and polycarginine) and membrane-destabilizing properties have been developed to facilitate gene transfer into cultured cells and living animals (Niidome and Huang, 2002; Glover et al., 2005; Morris et al., 2008). Amphipathic peptides with pH-dependent fusogenic and endosomolytic activities such as the fusion peptide of HA2 subunit of influenza hemaglutinin, or synthetic analogs GALA, KALA, JTS1, and histidine-rich peptides have been shown to increase transfection efficiency when associated with poly-L-lysine/DNA, condensing peptide/DNA, cationic lipids, poly-ethylenimine or polyamidoamine cascade polymers (for review: Morris et al., 2008). Single peptide chains able to condense DNA and to favour endosomal escape (PPTG1) (Rittner et al., 2002) or prevent endosomal uptake (MPG: Morris et al., 1999) have also been used for gene delivery in cultured cells. However, only a few CPPs have been validated in vivo for gene delivery and so far, the secondary amphipathic peptide PPTG1 constitutes one of the only examples reporting a significant in vivo gene expression response following intravenous injection (Rittner et al., 2002). Tat, Transportan and polyarginine CPPs have

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small GTPase Rho A or Rac1 (Duchardt et al., 2007; Ziegler, 2008). GTPase activation and actin remodelling constitute the ‘onset’ of the internalization mechanism and have a major impact on membrane fluidity, thereby promoting cell entry of Arg9, penetratin and Tat via macropinocytosis (Nakase et al., 2007) or clathrin-dependent endocytosis (Richard et al., 2005), of MPG or Pep-1 particles via membrane perturbation mechanism (Gerbal-Chaloin et al., 2007).
been associated with other lipid-based non-viral gene delivery methods, including liposomes, PEI or nanostructures (Branden et al., 1999; Tung et al., 2002; Ignatovich et al., 2003; Rudolph et al., 2003; Kilk et al., 2005). The association of Tat and octa-arginine to pharmaceutical nano-carriers, described as non-viral delivery systems based on new packing concepts ‘Programmed packaging’ Multifunctional Envelope-type NanoDevice (MEND) (Kogure et al., 2004; MacKay et al., 2008), has been shown to improve gene delivery and to offer the advantage of combining delivery, packaging and targeting motifs within the same particle (Torchilin, 2008; Vivès et al., 2008).

The second major barrier of non-viral gene delivery systems is their poor nuclear translocation, which is however essential for transfection of non-dividing cells and gene therapy. In order to improve nuclear delivery of DNA-plasmids, synthetic peptides containing NLS have been extensively applied (Cartier and Reszka, 2002; Escriou et al., 2003). Most of these studies were performed with the sequence derived from SV40 large T antigen NLS (PKKKRRK). This sequence was associated with either membrane-penetrating or cationic peptides, but also directly linked to cargoes or combined with other transfection methods to facilitate delivery into the nucleus. Moreover, NLS sequences have been associated with different hydrophobic CPPs in order to favour nuclear targeting as well as DNA binding and compaction. The NLS domain of MPG has been shown to improve the nuclear translocation of nucleic acids without requiring nuclear membrane breakdown during mitosis. MPG technology has been applied to both plasmid DNA and oligonucleotide delivery with high efficiency into a large number of adherent and suspension cell lines (Simeoni et al., 2003; Morris et al., 2007b).

CPP-based strategies for oligonucleotide analog delivery
Steric block small neutral oligonucleotide including 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 in vitro and in vivo through covalent coupling (Gait, 2003; Moulton and Moulton, 2004; Zatsipin et al., 2005; Juliano et al., 2008). Originally reported with Transportan for in vivo delivery of an antisense PNA targeting galanine receptors and modifying pain transmission (Pooga et al., 1998), the use of CPPs for steric block oligonucleotide delivery has been extended to Tat, penetratin, TP10 (a short version of Transportan) and arginine-rich peptides. Several CPP-based covalent approaches have been reported for the delivery of antisense PNA (Koppelhus and Nielsen, 2003). However only a few have been used in vivo, and until recently none of them were reported to be active at submicromolar concentrations (Gait, 2003; Abes et al., 2007). A detailed study of CPP-mediated PNA delivery has reported that the major limitation is due to their endosomal sequestration (Koppelhus and Nielsen, 2003), and more recently new CPPs have been described by Lebleu and Gait groups including R6-penetratin and 6-aminohexanoic acid spaced oligoarginine (R-Ahx-R6), which exhibit limited endosomal sequestration and lead to submicromolar antisense or splicing correction response (Abes et al., 2006; 2007). These CPPs have been validated in vivo for splicing correction on two therapeutic models: Duchenne’s muscular dystrophy (Fletcher et al., 2007) and coronavirus replication in mice (Burrer et al., 2007). Non-covalent strategies have also been applied to the delivery of PNA and DNA mimic molecules (Nan et al., 2005). Pep-3 peptide, a variant of Pep-1, was successfully applied to the delivery of PNA and analogs targeting the cell cycle regulatory protein cyclin B1 in vitro and in vivo (Morris et al., 2004b; 2007b). Interestingly, the nanoparticle organization of Pep-3/PNA complex allows functionalization of the surface layer of the particle, and PEGylation of the carrier significantly improves the efficacy of the response by stabilizing the complexes. This study shows that such a modification improves Pep-3 for systemic administration into mice, thereby allowing for a significant reduction of the dose required to induce a specific and robust biological response, which consequently limits non-specific cytotoxic effects described upon treatment with high concentrations of CPP-PNA conjugate or non-covalently complexes (Morris et al., 2007b).

Oligonucleotide and siRNA delivery
Decoy oligonucleotides and short interfering RNAs (siRNA) constitute powerful biomedical tools to control protein activation and/or gene expression post-transcriptionally. (Elbashir et al., 2001; Hannon, 2002). However, the major limitation of siRNA applications, like most antisense or nucleic acid-based strategies remains their poor cellular uptake associated with the poor permeability of the cell membrane to nucleic acids. Several viral and non-viral strategies have been proposed to improve the delivery of either siRNA-expressing vectors or synthetic siRNAs both in cultured cells and in vivo (De Fougerolles et al., 2007; Juliano et al., 2008). CPP-based strategies have been developed to improve the delivery of oligonucleotides both in vitro and in vivo. Delivery of charged oligonucleotide 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 charged oligonucleotide was achieved by using either peptide-based non-covalent or PNA-hybridization strategies. In the latter, CPPs are covalently linked to a PNA that is able to hybridize with a double-stranded decoy oligonucleotide containing on one strand a flanking sequence complementary to the PNA. Strategies have been applied with Transportan and TP1O CPP for the delivery of decoy oligonucleotide interacting with NFkB or Myc (Fisher et al., 2004; El-Andaloussi et al., 2005). The MPG peptide-based delivery system has been successfully applied for the delivery of various type of nucleic acid, including phosphodiester-oligonucleotide targeting the protein phosphatase cdc25C (Morris et al., 1999), phosphorothioate-oligonucleotides targeting MDR-1 promoter in human CEM leukaemia cells (Marthinet et al., 2000) and thio-phosphoramide telomerase template antagonists in cancer cells (Asai et al., 2003; Gryaznov et al., 2003). Several CPP-based strategies have been used for the delivery of siRNA into cultured cells. siRNA covalently linked to Transportan (Muratovska and Eccles, 2004) and penetratin (Davidson et al., 2004) have been associated with a silencing response. Nevertheless, non-covalent strategies appear to be more appropriate for siRNA delivery and yield significant associated
biological response (Simeoni et al., 2003; Kim et al., 2006; Veldhoen et al., 2006; Crombez et al., 2007; Kumar et al., 2007; Lundberg et al., 2007; Meade and Dowdy, 2007). MPG peptide has been reported to improve siRNA delivery into a large panel of cell lines including adherent cell lines, cells in suspension, cancer and challenging primary cell lines (Simeoni et al., 2003; Morris et al., 2004a; Nguyen et al., 2006). MPG has been applied for in vivo delivery of siRNA targeting OCT-4 into mouse blastocytes (Zeineldine et al., 2006) and of siRNA targeting an essential cell cycle protein, cyclin B1; intravenous injection of MPG/cyclin B1 siRNA particles has been shown to efficiently block tumour growth (Crombez et al., 2007). A variant of MPG (MPG-alpha) harbouring five mutations in the hydrophobic domain, in order to favour helical conformation of the peptide, has also been shown to improve siRNA delivery (Veldhoen et al., 2006). However, such modifications of MPG increase toxicity and favour endosomal cellular uptake (Deshayes et al., 2004c; Veldhoen et al., 2006). This non-covalent approach has been extended to other CPPs including polyarginine- (Kim et al., 2006; Kumar et al., 2007), penetratin- (Lundberg et al., 2007) and Tat- (Meade and Dowdy, 2007) derived peptides. Tat peptide associated with an RNA-binding motif has been reported to block in vivo epidermal growth factor (EGF) factor, cholesterol-Arg9 has been shown to enhance siRNA delivery in vivo against vascular endothelial growth factors (Kim et al., 2006) and more recently, a small peptide derived from rabies virus glycoprotein associated with an RNA-binding motif has been reported to block proliferation/cancer, asthma, apoptosis, ischaemia, stimulating cytotoxic immunity and diabetes (Dietz and Bähr, 2004; Snyder and Dowdy, 2005; Gros et al., 2006). Most of these applications use CPPs (Tat, penetratin, polyarginine, VP22) covalently linked to peptides or as fusion proteins. More recently, in vivo applications of Pep-1 technology have been described including intravenous, intra-tumoural and intracheal injections, as well as transduction into oocytes, sprays for nasal delivery or direct penetration through the skin (Gros et al., 2006; Morris et al., 2008). One of the principal applications of CPPs involves the delivery of peptides and proteins for cancer and anti-proliferation treatments. The tumour suppressor p53 constitutes a choice target, and different p53-derived peptides covalently linked to CPPs have been demonstrated to restore p53 functions in cancer cells. Tat-mediated delivery of a peptide derived form the C-terminus of p53 reduces tumour growth upon intra-peritoneal injection into mice with β-cell lymphoma (Snyder et al., 2004; Tang et al., 2007). Similarly, PNC-28, a peptide derived from the MDM-2-binding domain of p53 linked to penetratin has been described to block tumour growth (Michl et al., 2006; Bowne et al., 2007). Another successful anti-proliferation application has been reported using a peptide derived from the N-terminus of the Smac protein, which inactivates the inhibitor of apoptosis protein (Kim et al., 1999). Smac peptide associated to CPPs sensitizes cells to pro-apoptotic stimuli and a synergetic effect of Smac peptide and TNF-related apoptosis inducing ligand has been shown on intracranial glioblastoma xenografted mice (Fulda et al., 2002). Peptides and protein domains derived from natural protein inhibitors (p16INK, p21, p15 or p27kip) of cyclin-dependent kinases involved in cell cycle progression have been used to block cancer cell proliferation. A tumour suppressor function in vivo was reported by using p27kip tumour suppressor protein genetically coupled to Tat (Nagahara et al., 1998; Snyder et al., 2004), as well as a p16ink-derived peptide associated to penetratin (Hosotani et al., 2002). Small peptide inhibitors of cyclin-dependent kinase activation have been delivered by using the non-covalent Pep-1-based approach and shown to block cancer cell proliferation (Gondeau et al., 2005). CPPs have also been used to target B-cell lymphoma oncogene. A peptide for EBV ‘Epstein Barr Virus’ associated to Tat blocks proliferation (Knight et al., 2006). Tat-mediated BCL6 peptide inhibitor delivery has been reported to modulate B-cell phenotype (Polo et al., 2004; Melnick, 2007). Pep-1 strategy was also applied to the evaluation of the antitumoural activity of peptide inhibitors of protein kinases or to repair a defective step in a cellular signalling pathway in vivo (Gros et al., 2006; Morris et al., 2008).

Deregulation of apoptosis has been directly or indirectly associated with many pathologies. Several successful applications of CPP-assisted delivery of proteins or peptides regulating apoptosis have been reported. Tat-FLIP (caspase 8 inhibitor) fusion peptide interferes with the activation of FAS inducing signalling complex, thereby preventing apoptosis in vivo (Krautwald et al., 2004). A peptide issued from the Bcl2 homology domain 4 (BH4) or Bcl2/BclXl protein associated with Tat can regulate apoptosis and induce cytoprotection in vivo (Sugioka et al., 2003). Survivin mutants associated with Tat facilitate apoptosis in cancer cells (Wadia et al., 2004). Pep-1 strategy has been applied in vivo to the delivery of proteins into the lungs of mice to produce alveolar wall apoptosis or to correct defects in protein kinase A function (Aoshiba et al., 2003; Maron et al., 2005).

The ability of CPPs to cross the blood brain barrier and to favour the delivery of proteins in the brain has been used to improve the outcome of ischaemic events. Death of neuronal cells following cerebral ischaemia is associated with apoptosis, and Tat-BclXl protein can be delivered into mouse brain to decrease neuronal cell death in the area of ischaemic damages (Cao et al., 2002). Reduction of cerebral ischaemia and protection of ischaemia in brain injury has been reported with Tat-cJNK peptide, Tat-NMR2 and Tat-Bclx protein (Cao et al., 2002). Tat-δV-1 peptide, a selective inhibitor of PKCγ has been...

**CPP-based strategies for in vivo delivery of proteins and peptides**

In order to circumvent the technological problems of gene delivery an increasing interest has been taken in designing novel strategies to enable delivery of peptides and full-length proteins into a large numbers of cells. The first proof of concept of the in vivo potency of CPPs was provided by Dowdy and colleagues in 1999, showing that Tat-β-galactosidase fusion protein can be delivered into almost all tissues including the brain, following intra-peritoneal injection into mice (Schwarze et al., 1999). Over the last decade, CPP-based delivery has been successfully used to deliver peptides and proteins to target different diseases including cell proliferation/cancer, asthma, apoptosis, ischaemia, stimulating cytotoxic immunity and diabetes (Dietz and Bähr, 2004; Snyder and Dowdy, 2005; Gros et al., 2006). Most of these applications use CPPs (Tat, penetratin, polyarginine, VP22) covalently linked to peptides or as fusion proteins. More recently, in vivo applications of Pep-1 technology have been described including intravenous, intra-tumoural and intracheal injections, as well as transduction into oocytes, sprays for nasal delivery or direct penetration through the skin (Gros et al., 2006; Morris et al., 2008). One of the principal applications of CPPs involves the delivery of peptides and proteins for cancer and anti-proliferation treatments. The tumour suppressor p53 constitutes a choice target, and different p53-derived peptides covalently linked to CPPs have been demonstrated to restore p53 functions in cancer cells. Tat-mediated delivery of a peptide derived form the C-terminus of p53 reduces tumour growth upon intra-peritoneal injection into mice with β-cell lymphoma (Snyder et al., 2004; Tang et al., 2007). Similarly, PNC-28, a peptide derived from the MDM-2-binding domain of p53 linked to penetratin has been described to block tumour growth (Michl et al., 2006; Bowne et al., 2007). Another successful anti-proliferation application has been reported using a peptide derived from the N-terminus of the Smac protein, which inactivates the inhibitor of apoptosis protein (Kim et al., 1999). Smac peptide associated to CPPs sensitizes cells to pro-apoptotic stimuli and a synergetic effect of Smac peptide and TNF-related apoptosis inducing ligand has been shown on intracranial glioblastoma xenografted mice (Fulda et al., 2002). Peptides and protein domains derived from natural protein inhibitors (p16INK, p21, p15 or p27kip) of cyclin-dependent kinases involved in cell cycle progression have been used to block cancer cell proliferation. A tumour suppressor function in vivo was reported by using p27kip tumour suppressor protein genetically coupled to Tat (Nagahara et al., 1998; Snyder et al., 2004), as well as a p16ink-derived peptide associated to penetratin (Hosotani et al., 2002). Small peptide inhibitors of cyclin-dependent kinase activation have been delivered by using the non-covalent Pep-1-based approach and shown to block cancer cell proliferation (Gondeau et al., 2005). CPPs have also been used to target B-cell lymphoma oncogene. A peptide for EBV ‘Epstein Barr Virus’ associated to Tat blocks proliferation (Knight et al., 2006). Tat-mediated BCL6 peptide inhibitor delivery has been reported to modulate B-cell phenotype (Polo et al., 2004; Melnick, 2007). Pep-1 strategy was also applied to the evaluation of the antitumoural activity of peptide inhibitors of protein kinases or to repair a defective step in a cellular signalling pathway in vivo (Gros et al., 2006; Morris et al., 2008).

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reported to attenuate heart ischaemia (Bright et al., 2004). CPPs have also been used for the delivery of small molecules through the blood brain barrier, as such D-penetratin or SynB1 have been reported to significantly increase uptake of doxorubicine into the brain (Rousselle et al., 2001). Pep-1 technology has also been demonstrated to be a potent strategy to deliver therapeutic proteins in vivo and across the blood brain barrier (Gallo et al., 2002; Aoshiba et al., 2003; Gallo, 2003; Maron et al., 2005; Gros et al., 2006).

Cell-penetrating peptides have also been used for the treatment of asthma, by using dominant negative forms of Ras or phosphoinositol 3 kinase (PI3K) fused to Tat to inhibit the airway inflammatory response by cytokine blockage in a mouse (Myou et al., 2003). Different proteins and peptides coupled with Tat and penetratin have been used for immunization against specific infectious diseases. Tat peptide has also been used for the delivery of modular antigen molecules useful for treatment of allergy and vaccine production (Rhyner et al., 2007). Superoxide dismutase (SOD) fused to Tat or to Pep-1 has been shown to protect pancreatic beta cells against oxidative stress (Eum et al., 2004).

Clinical evaluation of CPP-based delivery strategies

Numerous preclinical and clinical evaluations of CPP-based delivery approaches are currently under evaluation. The first, CPP clinical trial was initiated a few years ago by Cell Gate Inc. for topical delivery of cyclosporine linked to polyarginine and entered phase II trials in 2003 (Rothbard et al., 2004). Ever since, several companies are working on clinical development of CPPs, for topical and systemic administration of different therapeutic molecules. Avi Biopharma for the in vivo steric block splicing correction using 6-aminohexanoic acid spaced oligoarginine [(R-Ahx-R),] (Lebleu et al., 2008; Moulton and Moulton, 2008). Kai Pharmaceutical (Chen and Harrison, 2007) is currently evaluating a Tat protein kinase C inhibitor peptide modulator of protein kinase C for acute myocardial infarction and cerebral ischaemia, which entered phase II in 2007. Other companies including Traversa Inc., for Tat-based non-covalent siRNA delivery, Panomics Inc., for secondary amphipathic peptide-based non-covalent delivery of siRNA are currently evaluating CPP at preclinical and clinical trials.

Conclusions

The dramatic acceleration in the discovery of new and highly potent therapeutic molecules, which do however not make it to the clinic due to poor delivery, low bioavailability and lack of rational targeting has made it clear that delivery was a key stone in therapeutic development (Kong and Mooney, 2007). Accordingly, carrier peptides represent a new and innovative concept to bypass problems of bioavailability associated with certain drugs such as peptides, proteins and nucleic acids, which are currently rarely considered as therapeutics due to the above-mentioned limitations. Such peptide-based strategies present several advantages, including rapid delivery of cargoes into cells with very high efficiency, stability in physiological buffers, lack of toxicity and of sensitivity to serum. Twenty years after their discovery, CPPs are at the door of the clinic. The success reported on the preclinical evaluation of CPPs during the last decade has revealed a tremendous potential of clinical treatment. Covalent strategy has 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 introduction of the CPP-based non-covalent strategy has allowed the introduction of oligonucleotide and siRNA on preclinical states. The lack of prerequisites for covalent coupling upon formation of carrier/macromolecule particles favours the intracellular routing of the cargo and enables its controlled release into the target cellular compartment. Whatever the nature of the delivery system, a major attention should be paid to the targeting of the carrier/drug in order to mediate drug delivery into specific cell types and to limit its dispersion in the whole body.

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Conflict of interest

None.

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