Peptide Delivery to the Brain via Adsorptive-Mediated Endocytosis: Advances With SynB Vectors

Submitted: June 6, 2002; Accepted: August 20, 2002; Published: October 1, 2002

Guillaume Drin¹, Christophe Rousselle¹, Jean-Michel Scherrmann², Anthony R Rees¹ and Jamal Tamsamani¹

¹ Synt:em, Parc Scientifique Georges Besse; 30000 Nîmes, France

² Université René Descartes Paris 5 and INSERM U26, Hôpital Fernand Widal, 200 Rue du Faubourg Saint-Denis; 75475 Paris Cedex 10, France

ABSTRACT Biological membranes normally restrict the passage of hydrophilic molecules. This impairs the use of a wide variety of drugs for biomedical applications. To overcome this problem, researchers have developed strategies that involve conjugating the molecule of interest to one of a number of peptide entities that are efficiently transported across the cell membranes. In the past decade, a number of different peptide families with the ability to cross the cell membranes have been identified. Certain of these families enter the cells by a receptor-independent mechanism, are short (10-27 amino acid residues), and can deliver successfully various cargoes across the cell membrane into the cytoplasm or nucleus. Surprisingly, some of these vectors, the SynB vectors, have also shown the ability to deliver hydrophilic molecules across the blood-brain barrier, one of the major obstacles to the development of drugs to combat diseases affecting the CNS.

KEYWORDS: intracellular delivery, peptide vector, blood-brain barrier, multidrug resistance.

INTRODUCTION A large number of hydrophilic molecules such as peptides, proteins, and oligonucleotides are poorly taken up by cells, since they do not efficiently cross the lipid bilayer of the plasma membrane. This is considered to be a major limitation for their use as therapeutic agents in biomedical research and in the pharmaceutical industry. In particular, it has been widely accepted that peptide neuromodulators fail to significantly affect their target cells within the brain when administered peripherally. This is likely due to the existence of the blood-brain barrier (BBB), a complex biological interface that prevents transport of most drugs from the vasculature into the brain parenchyma. The endothelium of the central nervous system (CNS) vasculature has a structure somewhat different than that of most other organs. Between the endothelial cells are tight junctions that block the entry of water-soluble agents into the internal environment of the brain via aqueous paracellular pathways.¹ While a wide variety of neuropharmaceutical drugs are presently available, few possess the physicochemical properties that would

render them ideal for the treatment of CNS disease.

The most important factors determining the extent to which a molecule will be delivered from the blood into the CNS are lipid solubility, molecular mass, and charge. Therefore, based simply on lipid solubility and molecular mass, any peptide-based or oligonucleotide-based

neuropharmaceutical will almost certainly be impeded by the BBB.

To overcome the limited access of drugs to the brain, at least 3 strategies have been developed that achieve BBB penetration²⁻⁵:

1. Neurosurgery-based strategies, which bypass the BBB by means of intraventricular drug infusion, intracerebral infusion, or disruption of the BBB;
2. Pharmacology-based strategies, some examples of which employ lipidation, or chemical modification of the drug to improve its ability to diffuse across the BBB; and
3. Physiology-based strategies, which take advantage of BBB nutrient carriers or specific receptors, mediating transport via these transporter systems. One example has been to conjugate the therapeutic drug with a protein or a monoclonal antibody that gains access to the brain by either receptor - or adsorptive-mediated transcytosis (eg, transferrin receptor-mediated transfer).²

However, problems have been encountered with many of these approaches: for example, increasing the lipophilicity of a peptide also decreases its solubility in serum, whereas direct injections into the brain could result in toxic manifestations. Thus, pharmacological disruption of the BBB may not always be desirable as a general approach, even though the barrier can be opened during periods of CNS trauma, allowing for acute delivery of drugs (as in, for example, brain cancer or stroke). Our view is, therefore, that new, noninvasive methods of administration are urgently needed.

During the past decade, several peptides have been described—such as penetratin,^{6,7} a basic segment of the transcription-activating factor (Tat),^{6,7} and SynB vectors⁵—that allow the intracellular delivery of polar, biologically active compounds in vitro and in vivo. As reported in **Table 1**, the peptides, belonging to various families, are heterogeneous in size (10-27 amino acids) and sequence. However, all these peptides possess multiple positive charges, and some of them share

Correspondence to:

Jamal Tamsamani

E-mail: jtemsamani@nimes.syntem.com

Table 1. Principal Cell-Penetrating Peptides*

Cell-Penetrating Peptides	Sequence [†]	Origin	Ref
Amphipathic sequences			
MAP	KLALKLALKALKAALKLA		8
pAntp ₄₃₋₅₅ (or penetratin)	RQIKWFWQNRRMKWKK	AntpHD	9
Transportan	GWTLSAGYLLGKINLKALAALA KKIL	Galanin and mastoparan	10
SBP	MGLGLHLLVLAALQGAWSQPK KKRKV	Caiman crocodylus Ig(v) light chain/NLS (from SV40 T antigen)	11
FBP	GALFLGWLGAAAGSTMGAWSQP KKRKV	gp41 fusion peptide/NLS (from SV40 T antigen)	12
Cationic sequences			
Tat ₄₈₋₆₀	GRKKRRQRRRPPQ	HIV Tat	13
SynB1	RGGRLSYSRRRFSTSTGR	Protegrin I	14
SynB3	RRLSYSRRRF	Protegrin I	15

*AntpHD indicates Antennapedia homeodomain; MAP, model amphipathic peptide; SBP, sequence signal-based peptide; FBP, fusion sequence-based peptide; NLS, nuclear localization signal; SV40 T antigen, Simian virus 40 large antigen; HIV, human immunodeficiency virus; Tat, transcription-activating factor.

[†]The sequence indicated in this table corresponds to the original sequence. Various analogues were described in the literature.

Table 2. Principal Features of the Cell-Penetrating Peptides*

Peptide Name	n [†]	<μH> [‡]	<H> [‡]	Net Charge	Affinity With Lipid Bilayer		Structure of the Lipid-Bound Form [‡]	Lytic Activity		Refs
					Neutral	Negatively Charged		Cells	Model Membranes	
MAP	18	10.59	-11.21	5+	Yes	Yes	α	Yes	Yes	8, 16, 17
pAntp ₄₃₋₅₅	16	8.29	-13.18	8+	No	Yes	α or β	No	No	18-20
Transportan	26	11.22	-7.58	4+	Yes	Yes	α	Yes	—	20, 21
FBP	27	8.95	-15.03	6+	Yes	Yes	β	—	—	12
SBP	27	6.51	-14.71	6+	Yes	Yes	β	—	—	11
Tat ₄₈₋₆₀	12	1.36	-22.25	8+	ND	ND	—	No	—	13,20
SynB1 [§]	18	4.19	-15.94	6+	—	Yes	—	No	No	
SynB3 [§]	10	4.98	-9.26	6+	—	Yes	—	No	—	

*MAP indicates model amphipathic peptide; FBP, fusion sequence-based peptide; SBP, sequence signal-based peptide; ND (not determined); Tat, transcription-activating factor.

[†]The n value designates the amino acid length of the peptides. The <μH> and <H> reflects respectively the helical amphipathicity and the hydrophobicity of a peptide. These values have been calculated using the software MPEX (<http://blanco.biomol.uci.edu/mpex/>). The hydrophobicity value corresponds to the free energy ΔG (in kcal/mol) of the octanol-to-water transfer of the considered peptide.

[‡]We have indicated in this column the preferential secondary structure adopted by the peptides upon binding to a lipid bilayer (α-helix or β-sheet). The conformation of pAntp₄₃₋₅₅ is versatile and depends on the peptide concentration.

[§]Data concerning the lipid-binding affinity and cell lysis activity of SynB peptides are unpublished ([Drin et al, unpublished data, 1Apr. 2002]).

common features, such as important theoretical hydrophobicity and helical moment (reflecting the peptide amphipathicity), the ability to interact with a lipid membrane and to adopt a significant secondary structure upon binding to lipids (**Table 2**). Since these peptides penetrate into cells by a receptor-independent nonendocytotic process (see later), attempts have been made to demonstrate that interaction of some of them with the lipid

matrix of the plasma membrane could play a key role in their cell uptake (for a detailed overview, see Langel ⁷). Although the detailed mechanism by which these peptides enter cells is poorly understood, the facility with which they cross the membrane into the cytoplasm even when carrying hydrophilic molecules has provided a new and powerful tool in biomedical research.

This review will address the use and mechanism of transport of cell-penetrating peptides for the delivery of molecules into cells and across complex physiological barriers, with a focus on their potential use for the transport of drugs across the BBB.

CELL-PENETRATING PEPTIDES

SynB vectors

SynB vectors are a new family of vectors derived from the antimicrobial peptide protegrin 1 (PG-1), an 18-amino-acid peptide originally isolated from porcine leukocytes.²² The peptide has a β -hairpin structure in which 2 antiparallel strands linked by a β -turn are stabilized by 2 disulfide bridges.²⁴ As previously reported, the PG-1 peptide interacts with, and forms pores in, the lipid matrix of bacterial membranes.²⁴⁻²⁵ Since it has been shown that the pore formation capability of PG-1 depends on its cyclization,²⁴ researchers designed various linear analogues of PG-1 that lack the cysteine residues. These linear peptides (SynB vectors) are able to interact with the cell surface and cross the plasma membrane without their membrane-disrupting activity (**Table 2**). Furthermore, the internalization of these peptide vectors into cells does not appear to depend on a chiral receptor, since the D-enantio form penetrates as efficiently as the parent peptide (L-form), and retro-inverso sequences exhibit identical penetrating activity. These linear protegrin analogues were the starting point for developing a new potent strategy for drug delivery into complex biological membranes such as the BBB (see later).

Penetratin

It has been shown that the homeodomain of the Antennapedia protein. (AntpHD), a *Drosophila* homeoprotein, is internalized by cells in culture and is conveyed to the nucleus, where it binds specifically to its DNA cognate site.^{26,27} The sequence responsible for this translocation has been mapped to a region comprising the third helix of AntpHD.⁹ Furthermore, it was established that a short peptide segment, pAntp₄₃₋₅₈ (penetratin), corresponding to the helix itself is able to penetrate into primary neuronal cultures.⁹ An original feature of penetratin is that it can be recovered within the cells after incubation at 4°C. Additional studies showed that analogues of penetratin corresponding to its enantio- (43-58 all D) or retro-inverso form (58-43) penetrated as efficiently as the parent peptide,^{28,29} suggesting that pAntp₄₃₋₅₈ translocates through cell membranes without binding to a stereospecific receptor and by a nonendocytotic pathway. Moreover, the uptake of peptide is not saturable and appears to be independent of cell type since it has been shown to enter into various cell lines, such as lymphocytes or endothelial cells.^{8,30} The unusual cellular import of the pAntp₄₃₋₅₈ peptide could depend on its capacity to interact with the lipid matrix of plasma membrane,^{18,19} although the exact mechanism is still unresolved.

Tat peptide

As found for the homeoproteins, the transcription factor Tat, involved in the replication cycle of human immunodeficiency virus (HIV), was demonstrated to penetrate into cells.³¹ In addition, a 35-amino-acid peptide corresponding to fragment 37-72 of the HIV Tat protein has been shown to promote the intracellular delivery of covalently bound proteins.³² Vivès et al¹³ showed that several fragments (Tat37-60, Tat43-60, Tat48-60) derived from the most basic region of the protein penetrate into HeLa cells at 37°C as well as 4°C. In addition, it was observed that neither endocytosis nor potocytosis seemed to be involved in this process.^{13,33} One of the shortest peptides, Tat48-60, which contains a nuclear localization signal (NLS), was defined as the minimal translocating fragment, although the deletion of 3 nonbasic residues within this sequence did not affect its cell-penetrating ability.¹³ This observation underlines the role of basic residues in the translocating ability of Tat-derived peptides. In addition, it has been reported recently that arginine-rich peptides deriving from DNA- or RNA-binding domain are also imported into eukaryotic cells.³³

Other cell-penetrating peptides

A number of other cell-penetrating peptides that derive not from natural proteins but from the engineering of various short peptides have been described, such as, for example, transportan,^{10,20} the model amphipathic peptide (MAP),^{16,17} and various signal sequence-based peptides,^{11,12} and homoarginine vectors³⁴ (see **Tables 1** and **2**). Transportan is a 27-amino-acid chimeric peptide composed of the neuropeptide galanin and mastoparan-X linked by a central lysine. The cell uptake of this peptide is rapid, occurs at 4°C, and is unaffected by the presence of inhibitors of endocytosis.¹⁰ Likewise, it has been reported that MAP and some of the other sequence-based peptides seemingly enter into cells via a nonendocytotic pathway.^{11,12,16} The sequence-based peptides resulted from the fusion of a hydrophobic peptide (eg, a signal sequence, a fusion peptide) with the NLS motif, whereas the MAP peptide is a complete canonical amphipathic helix. As observed with pAntp₄₃₋₅₈, the unconventional internalization of these peptides seems to be neither saturable nor dependent on the cell type^{8,10} and could be related to their lipid-binding capacity.^{11,12,17,21} As far as the homoarginine peptides are concerned, they have also been described as entering into cells by an energy-dependent nonendocytic process.³⁴

INTRACELLULAR DELIVERY

One of the first applications of these peptide vectors was the design of cell-permeable constructs that are able to enhance the cell uptake of cargo molecules. For instance, it has been demonstrated that phosphopeptides linked in tandem with pAntp₄₃₋₅₈ can stimulate a mitogenic response by activating a regulatory protein involved upstream of this process.³⁵ Similarly, it has been shown that such constructs can specifically inhibit

ligand-dependent transduction pathways in various cell lines.³⁶⁻³⁹ Several studies have also focused on the use of the pAntp₄₃₋₅₈ peptide to promote the delivery of fragments of proteins able to inhibit cyclin-dependent kinase (Cdk), involved in the regulation of the cell cycle.⁴⁰⁻⁴² These cell-penetrating constructs have been proposed to have an anticancer potential since it was shown that they block, in vitro, the phosphorylation activity of Cdk and arrest cell proliferation.

Peptide vectors are also able to translocate large proteins into cells. For example, proteins linked to the Tat-translocating domain are able to enter into a wide variety of human and murine cell lines.⁴³⁻⁴⁵

Similarly, peptide vectors have been used successfully to deliver oligonucleotides.⁴⁶⁻⁴⁸ For example, it has been reported that the AntpHD-derived peptide and transportan were able to transport into melanoma cells a 21-mer Peptide Nucleic Acid (PNA) that was unable to cross the plasma membrane by itself. Once in the cytoplasm, the oligonucleotide blocked the expression of the galanin type I receptor by interacting with the messenger RNA (mRNA) coding for this protein. In this instance, the oligonucleotide was linked to the peptide vector via a disulfide bridge. It is likely that this bridge was cleaved within the cell, thereby releasing the oligonucleotide for interaction with its target RNA in the cytoplasm or pre-mRNA in the nucleus.⁴⁷

BRAIN DELIVERY

Enhancement of brain uptake

As described earlier, the BBB poses a formidable obstacle to drug therapy for the CNS. The fact that a peptide vector is internalized inside the cell does not guarantee that it will cross the BBB. The BBB is more complex than a simple cell layer; it comprises a specialized endothelium (compared with that of other blood vessels) associated with pericytes and astrocyte foot processes, which together elaborate a cellular barrier containing an efficient system of tight junctions. Despite this complexity, we have demonstrated that SynB peptide vectors can enhance the delivery of many different types of drugs across the BBB.

In one study, we assessed the efficacy of SynB vectors to enhance the brain uptake of the anticancer agent doxorubicin. Doxorubicin was conjugated to SynB vectors via a chemical linker (succinate), and its ability to cross the BBB was studied using in situ cerebral perfusion in rats and mice.^{14,15} This "ectorization" of doxorubicin to SynB vectors significantly enhanced its brain uptake in all the grey areas assessed—without compromising BBB integrity. The amount of vectorized doxorubicin that was delivered to the brain parenchyma (shown after applying the capillary depletion method) was about 20- to 50-fold higher than for free doxorubicin, depending on the vector used. Interestingly, we also observed that SynB vectorized doxorubicin bypasses the P-glycoprotein (P-gp) that has been shown to be present in the luminal membrane of the BBB endothelial cells.⁴⁹

This 170-kDa ATP-dependent efflux pump, because of its unidirectional orientation from brain to blood, restricts the brain entrance, or increases the brain clearance, of a broad number of therapeutic compounds, including cytotoxic drugs.^{50,51} Other experiments, carried out in vitro using resistant cells, have confirmed that vectorized doxorubicin bypasses the P-gp and enhances its potency in those cells.⁴⁹

As a comparison, we have also conjugated doxorubicin to D-penetratin and assessed its brain uptake by in situ brain perfusion. Although a 5- to 7-fold enhancement in brain uptake was observed, the brain vascular volumes were 2-fold larger than those observed with the SynB vectors, suggesting an opening of the tight junctions.¹⁴ Bolton et al have shown that pAntp₄₃₋₅₈ (penetratin L-form) does not pass the BBB after an intravenous injection.⁵²

The ability of SynB vectors to enhance the brain uptake of doxorubicin was also assessed after intravenous injection of vectorized doxorubicin into mice. The tissue and plasma distribution of doxorubicin were dramatically modified when the drug was vectorized. The brain concentrations were higher for vectorized doxorubicin than for free doxorubicin.¹⁴ Interestingly, vectorized doxorubicin shows significantly lower levels in the heart, strongly suggesting that cardiotoxicity—the main side effect of doxorubicin—could be reduced using this strategy.

To assess the broader potential of this approach, we have investigated the transport of other small molecules, peptides, and even proteins. We have shown that conjugation of the antibiotic benzylpenicillin (B-Pc) to SynB vectors improves its penetration across the BBB in a significant manner and that the vectorized B-Pc is distributed in all grey areas.⁵³ Antibiotics must cross the BBB to be suitable for use in the treatment of severe cerebral infections such as bacterial meningitis.

The use of SynB vectors has also been successfully applied to brain delivery of druglike peptide molecules. In a pharmacological application focused on pain management, the brain uptake of an enkephalin analogue was enhanced several hundredfold after vectorization. Importantly, systemic administration of the vectorized enkephalin led to a dramatic enhancement of its analgesic effect in mice compared to that of free enkephalin (Rousselle et al, unpublished results, Jan 2002). In a further application, we have shown that attachment of SynB vectors to the protein streptavidin (via a SynB vector-biotin construct) results in enhanced uptake of streptavidin into the brain (Rousselle et al, unpublished results, Feb 2002).

Similar results have also been seen with the Tat peptide. Schwarze et al,⁵⁴ after fusion of this peptide to β -galactosidase, assessed the tissue distribution after intraperitoneal injection in mice. They observed strong β -Gal activity in all the tissues analyzed, including liver, kidney, heart muscle, lung, and brain. This suggested that the protein had crossed the BBB and, in addition, had passed into most other biological tissues.

Mechanism of brain uptake

We have shown by in situ perfusion studies, a technique allowing a first-pass exposure, that the internalization of Dox-SynB is a saturable process.¹⁵ The measured K_m , which was in the range of 4 to 9 μM , compares well with the values observed for substrates reported to be taken up by adsorptive-mediated endocytosis. Furthermore, no difference in brain uptake was seen between doxorubicin linked to L-SynB or D-SynB vectors, indicating that a stereospecific receptor is not a requirement for its brain transport. In addition, we have reported that the passage of peptides can be inhibited in a competitive manner by polycationic molecules such as poly(L)lysine or protamine, which act as endocytosis inhibitors. These observations suggest that the crossing of BBB by SynB vectors is via an energy-dependent adsorptive-mediated endocytosis mechanism.¹⁵

It is known that at physiological pH values the luminal surface of the brain endothelium presents an overall negative charge (due in part to significant sialylation) and thus creates an environment more selective to positively charged substances.^{55,56} The SynB peptides are positively charged. This net positive charge is likely to play a key role in the adsorptive-mediated endocytosis process, wherein electrostatic interactions of the peptide vector with the surface of endothelial cells may mediate surface binding and subsequent internalization of the peptide vectors into the brain capillaries.

For the transcytosis of peptides through the BBB, 3 steps have been proposed: (1) binding and internalization at the luminal side of endothelial cell membrane, (2) diffusion through the cytoplasm of endothelial cells, and (3) externalization at the basolateral side of endothelial cells.⁵⁷ The main components of the basal membrane are type IV collagen, fibronectin, laminins, chondroitin, and heparan sulfate from glycosaminoglycans. The most abundant component, type IV collagen, polymerizes with laminin and fibronectin proteins via protein-binding domains such as integrin and lectin receptors.⁵⁸ These components not only provide a mechanical supporting structure for the capillary wall, they are also important as a negatively charged barrier arising from the chondroitin and heparan sulfate residues, in addition to the anionic properties of the luminal and abluminal membranes of the endothelial cells.⁵⁹ Our results suggest that adsorptive-mediated endocytosis occurs at least at the luminal side of brain capillaries. The similarity in behavior observed for the peptide vectors studied suggests that the externalization at the abluminal side of endothelial cells may also be via a receptor-independent mechanism. However, since endocytosis inhibitors have been tested at only the luminal side of the endothelial cells, it is possible that a different mechanism may be involved in the externalization step.

Conclusion Biological membranes pose a formidable obstacle to drug therapy where the drugs are large or hydrophilic. As new drugs for neurological and other disorders are discovered, new delivery techniques will have to be developed in concert to overcome this transport

obstacle. As rapid advances in cell and molecular biology lead to a proliferation of potent molecules that cannot be effectively delivered to cells and brain by conventional means, continuing refinement of the new delivery methods will be essential to realizing the potential of these molecular drugs. The use of peptide vectors as a delivery system represents a novel and promising approach. The recent results obtained both in vitro and in vivo demonstrate that these vectors can successfully transport drugs across the membranes of many different cell types, and furthermore can traverse the more demanding BBB. The knowledge already acquired about the various peptide sequences will result in the design of more efficient peptide vectors. The optimization of these vectors will also be aided by a greater understanding of the transport mechanisms operating at the cell membranes and the BBB.

ACKNOWLEDGMENTS We are thankful to our colleagues at Syntem and at INSERM U26 for helpful discussions.

REFERENCES

- Brightman MW. Morphology of blood-brain interfaces. *Exp Eye Res.* 1977;25:1-25.
- Pardridge WM. New approaches to drug delivery through the blood-brain barrier. *Trends Biotechnol.* 1994;12:239-245.
- Pardridge WM. *Brain Drug Targeting: The Future of Brain Drug Development.* Cambridge, UK: Cambridge University Press; 2001.
- Joliet-Riant P, Tillement JP. Drug transfer across the blood-brain barrier and improvement of brain delivery. *Fundam Clin Pharmacol.* 1999;13:16-26.
- Temsamani J, Rousselle C, Rees AR, Scherrmann JM. Vector-mediated drug delivery to the brain. *Expert Opin Biol Ther.* 2001;1:773-782.
- Lindgren M, Hällbrink M, Prochiantz A, Langel U. Cell-penetrating peptides. *Trends Pharmacol Sci.* 2000;21:99-103.
- Langel U. *Cell Penetrating Peptides: Processes and Applications.* New York, NY: CRC Press; 2002.
- Oehlke J, Scheller A, Wiesner B, et al. Cellular uptake of an alpha-helical amphipathic model peptide with the potential to deliver polar compounds into the cell interior non-endocytically. *Biochim Biophys Acta.* 1998;1414:127-139.
- Derossi D, Joliet AH, Chassaing G, Prochiantz A. The third helix of the Antennapedia homeodomain translocates through biological membrane. *J Biol Chem.* 1994;269:10444-10450.
- Pooga M, Hällbrink M, Zorko M, Langel U. Cell penetration by transportan. *FASEB J.* 1998;12:67-77.
- Chaloin L, Vidal P, Heitz A, et al. Conformations of primary amphipathic carrier peptides in membrane mimicking environments. *Biochemistry.* 1997;36:11179-11187.
- Vidal P, Chaloin L, Heitz A, et al. Interactions of primary amphipathic vector peptides with membranes: conformational consequences and influence on cellular localization. *J Membr Biol.* 1998;162:259-264.
- Vivès E, Brodin P, Lebleu B. A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. *J Biol Chem.* 1997;272:16010-16077.

14. Rousselle C, Clair P, Lefauconnier JM, Kaczorek M, Scherrmann JM, Tamsamani J. New advances in the transport of doxorubicin through the blood-brain barrier by a peptide vector-mediated strategy. *Mol Pharmacol*. 2000;57:679-686.
15. Rousselle C, Smirnova M, Clair P, et al. Enhanced delivery of doxorubicin into the brain via a peptide-vector-mediated strategy: saturation kinetics and specificity. *J Pharmacol Exp Ther*. 2001;296:124-131.
16. Scheller A, Oehlke J, Wiesner B, et al. Structural requirements for cellular uptake of alpha-helical amphipathic peptides. *J Pept Sci*. 1999;5:185-194.
17. Dathe M, Schumann M, Wieprecht T, et al. Peptide helicity and membrane surface charge modulate the balance of electrostatic and hydrophobic interactions with lipid bilayers and biological membranes. *Biochemistry*. 1996;35:12612-12622.
18. Drin G, Dmn H, Tamsamani J, Brasseur R. Translocation of the pAntp peptide and its amphipathic analogue AP-2AL. *Biochemistry*. 2001;40:1824-1834.
19. Persson D, Thorn PE, Nordn B. Penetratin-induced aggregation and subsequent dissociation of negatively charged phospholipid vesicles. *FEBS Lett*. 2001;505:307-312.
20. Hllbrink M, Florn A, Elmquist A, Pooga M, Bartfai T, Langel U. Cargo delivery kinetics of cell-penetrating peptides. *Biochim Biophys Acta*. 2001;1515:101-109.
21. Magzoub M, Kilk K, Eriksson LE, Langel U, Grslund A. Interaction and structure induction of cell-penetrating peptides in the presence of phospholipid vesicles. *Biochim Biophys Acta*. 2001;1512:77-89.
22. Kokryakov VN, Harwig SS, Panyutich EA, et al. Protegrins: leukocyte antimicrobial peptides that combine features of corticostatic defensins and tachyplesins. *FEBS Lett*. 1993;327:231-236.
23. Aumelas A, Mangoni M, Roumestand C, et al. Synthesis and solution structure of the antimicrobial peptide protegrin-1. *Eur J Biochem*. 1996;237:575-583.
24. Mangoni ME, Aumelas A, Charnet P, et al. Change in membrane permeability induced by protegrin 1: implication of disulphide bridges for pore formation. *FEBS Lett*. 1996;383:93-98.
25. Sokolov Y, Mirzabekov T, Martin DW, Lehrer RI, Kagan BL. Membrane channel formation by antimicrobial protegrins. *Biochim Biophys Acta*. 1999;1420:23-29.
26. Joliot A, Pernelle C, Deagostini-Bazin H, Prochiantz A. Antennapedia homeobox peptide regulates neural morphogenesis. *Proc Natl Acad Sci U S A*. 1991;88:1864-1868.
27. Le Roux I, Joliot A, Bloch-Gallego E, Prochiantz A, Volovitch M. Neurotrophic activity of the Antennapedia homeodomain depends on its specific DNA-binding properties. *Proc Natl Acad Sci U S A*. 1993;90:9120-9124.
28. Derossi D, Calvet S, Trembleau A, Brunissen A, Chassaing G, Prochiantz A. Cell internalization of the third-helix of the antennapedia homeodomain is receptor-independent. *J Biol Chem*. 1996;271:18188-18193.
29. Brudigou J, Legrand C, Mry J, Rabi A. The retro-inverso form of a homeobox-derived short peptide is rapidly internalised by cultured neurones: a new basis for an efficient intracellular delivery system. *Biochem Biophys Res Commun*. 1995;214:685-693.
30. Fenton M, Bone N, Sinclair A. The efficient and rapid import of a peptide into primary B and T lymphocytes and a lymphoblastoid cell line. *J Immunol Methods*. 1998;212:41-48.
31. Frankel AD, Pabo CO. Cellular uptake of the tat protein from human immunodeficiency virus. *Cell*. 1988;55:1189-1193.
32. Fawell S, Seery J, Daikh Y, et al. Tat-mediated delivery of heterologous proteins into cells. *Proc Natl Acad Sci U S A*. 1994;91:664-668.
33. Suzuki T, Futaki S, Niwa M, Tanaka S, Ueda K, Sugiura Y. Possible existence of common internalization mechanisms among arginine-rich peptides. *J Biol Chem*. 2001;277:2437-2443.
34. Mitchell DJ, Kim DT, Steinman L, Fathman CG, Rothbard JB. Polyarginine enters cells more efficiently than other polycationic homopolymers. *J Peptide Res*. 2000;56:318-325.
35. Derossi D, Williams EJ, Green PJ, Dunican DJ, Doherty P. Stimulation of mitogenesis by a cell-permeable PI 3-kinase binding peptide. *Biochem Biophys Res Commun*. 1998;251:148-152.
36. Hall H, Williams EJ, Moore SE, Walsh FS, Prochiantz A, Doherty P. Inhibition of FGF-stimulated phosphatidylinositol hydrolysis and neurite outgrowth by a cell-membrane permeable phosphopeptide. *Curr Biol*. 1996;6:580-587.
37. Williams EJ, Dunican DJ, Green PJ, et al. Selective inhibition of growth factor-stimulated mitogenesis by a cell-permeable Grb2-binding peptide. *J Biol Chem*. 1997;272:22349-22354.
38. Bardelli A, Longati P, Williams TA, Benvenuti S, Comoglio PM. A peptide representing the carboxyl-terminal tail of the met receptor inhibits kinase activity and invasive growth. *J Biol Chem*. 1999;274:29274-29281.
39. Cussac D, Vidal M, Leprince C, et al. A Sos-derived peptidimer blocks the Ras signaling pathway by binding both Grb2 SH3 domains and displays antiproliferative activity. *FASEB J*. 1999;13:31-38.
40. Mutoh M, Lung FD, Long YQ, Roller PP, Sikorski RS, O'Connor PM. A p21(Waf1/Cip1)carboxyl-terminal peptide exhibited cyclin-dependent kinase-inhibitory activity and cytotoxicity when introduced into human cells. *Cancer Res*. 1999;59:3480-3488.
41. Bonfanti M, Taverna S, Salmona M, D'Incalci M, Broggini M. p21WAF1-derived peptides linked to an internalization peptide inhibit human cancer cell growth. *Cancer Res*. 1997;57:1442-1446.
42. Fhraeus R, Paramio JM, Ball KL, Lain S, Lane DP. Inhibition of pRb phosphorylation and cell-cycle progression by a 20-residue peptide derived from p16CDKN2/INK4A. *Curr Biol*. 1996;6:84-91.
43. Ezhevsky SA, Ho A, Becker-Hapak M, Davis PK, Dowdy SF. Differential regulation of retinoblastoma tumor suppressor protein by G(1) cyclin-dependent kinase complexes in vivo. *Mol Cell Biol*. 2001;21:4773-4784.
44. Caron NJ, Torrente Y, Camirnd G, et al. Intracellular delivery of a Tat-eGFP fusion protein into muscle cells. *Mol Ther*. 2001;3:310-318.
45. Embury J, Klein D, Pileggi A, et al. Proteins linked to a protein transduction domain efficiently transduce pancreatic islets. *Diabetes*. 2001;50:1706-1713.
46. Troy CM, Derossi D, Prochiantz A, Greene LA, Shelanski ML. Downregulation of Cu/Zn superoxide dismutase leads to cell death via the nitric oxide-peroxynitrite pathway. *J Neurosci*. 1996;16:253-261.
47. Pooga M, Soomets U, Hllbrink M, et al. Cell penetrating PNA constructs regulate galanin receptor levels and modify pain transmission in vivo. *Nat Biotechnol*. 1998;16:857-861.
48. Morris MC, Vidal P, Chaloin L, Heitz F, Divita G. A new peptide vector for efficient delivery of oligonucleotides into mammalian cells. *Nucleic Acids Res*. 1997;25:2730-2736.
49. Mazel M, Clair P, Rousselle C, et al. Doxorubicin-peptide conjugates overcome multidrug resistance. *Anticancer Drugs*. 2001;12:107-116.
50. Gottesman MM, Pastan I. Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu Rev Biochem*. 1993;62:385-427.
51. Tsuji A. P-glycoprotein-mediated efflux transport of anticancer drugs at the blood-brain barrier. *Ther Drug Monit*. 1998;20:588-590.
52. Bolton SJ, Jones DN, Darker JG, Eggleston DS, Hunter AJ, Walsh FS. Cellular uptake and spread of the cell-permeable peptide penetratin in adult rat brain. *Eur J Neurosci*. 2000;12:2847-2855.

53. Rousselle C, Clair P, Temsamani J, Scherrmann JM. Improved brain delivery of benzylpenicillin with a peptide-vector-mediated strategy. *J Drug Target.* 2002;10:309-315.
54. Schwarze SR, Ho A, VoceroAkbani A, Dowdy SF. In vivo protein transduction of a biologically active protein in the mouse. *Science.* 1999;285:1569-1572.
55. Hardebo JE, Kahrstrom J. Endothelial negative surface charge areas, and blood-brain barrier function. *Acta Physiol Scand.* 1985;125:495-499.
56. Vordbrodt AW. Ultrastructural cytochemistry of blood-brain barrier endothelia. *Prog Histochem Cytochem.* 1988;18:1-99.
57. Bar RS, DeRose A, Sandra A, Peacock ML, Owen WG. Insulin binding to microvascular endothelium of intact heart: a kinetic and morphometric analysis. *Am J Physiol.* 1983;244:477-482.
58. Virgintino D, Monaghan P, Robertson D, et al. An immunohistochemical and morphometric study on astrocytes and microvasculature in the human cerebral cortex. *Histochem J.* 1997;29:655-660.
59. Bertossi M, Virgintino D, Maiorano E, Occhiogrosso M, Roncali L. Ultrastructural and morphometric investigation of human brain capillaries in normal and peritumoral tissues. *Ultrastruct Pathol.* 1997;21:41-49.