Expert Opinion

1. Introduction
2. Transactivating transcriptional activator
3. Role of arginine residues in translocation
4. Mechanisms of internalisation by translocation
5. Transactivating transcriptional activator as a transduction vector for hydrophilic cargoes
6. Expert opinion

Transactivating transcriptional activator-mediated drug delivery

Bhawna Gupta & Vladimir P Torchilin†
†*Department of Pharmaceutical Sciences, Northeastern University, Boston, MA 02115, USA*

Cell-penetrating peptides (CPPs) are peptide vectors that can traverse through the plasma membrane barrier without breaching the integrity of the cell, and deliver various cargoes inside cell. The range of cargoes that can be delivered intracellularly by CPPs encompasses a broad variety of hydrophilic molecules, such as peptides, proteins, antibodies, imaging agents, DNA and even nanosized entities, including polymer-based systems, solid nanoparticles and liposomes. Multiple studies have focused on CPPs such as transactivating transcriptional activator peptide (TATp), penetratin, VP22, transportan and synthetic oligoarginines because of their high inherent potential as intracellular delivery vectors. However, the TATp remains the most popular CPP used for a variety of purposes. This review article attempts to bring together the available data on TAT-mediated intracellular uptake of a broad range of molecules and nanoparticles. It also considers potential practical applications of this approach.

Keywords: cell-penetrating peptides, drug delivery, gene delivery, liposomes, nanoparticles, proteins, TAT

Expert Opin. Drug Deliv. (2006) 3(2):177-190

1. Introduction

Cell-penetrating peptides (CPPs) are a group of peptides, which usually contain a cluster of basic residues that have been recognised as promising drug delivery vectors over the last decade. The CPPs are gaining increasing attention as they possess the remarkable property of translocating across the hydrophobic cell membrane, which forms a formidable barrier to the entry of hydrophilic and high molecular-weight drugs. As a result, different therapeutic moieties, which are mostly hydrophilic in nature and/or are of high molecular weight, can be tagged to CPPs and transported across the cell membrane to exert their pharmacological actions at the subcellular level. This process of traversing across the biological barrier is called protein transduction and is confined to a domain of < 20 amino acids, termed as protein transduction domain (PTD) or CPP. CPPs are rich in basic residues and are either derived from corresponding transducing proteins or are synthesised. The original concept of protein transduction was formed in 1988 when Green [1] and Frankel [2] independently demonstrated that the transactivating transcriptional activator (TAT) protein encoded by HIV-1 was efficiently internalised by cells *in vitro*, resulting in transactivation of the viral promoter. Such transactivation was observed at nanomolar concentrations in the presence of lysosomotropic agents, and TAT was subsequently detected in the nuclei. It was then speculated that TAT may serve as a delivery vector, ferrying various biological molecules within the cells. Subsequently, several other proteins and peptides were found to display the translocation activity, which encompasses: penetratin [3]; VP22 [4]; transportan [5]; model amphipathic peptide (MAP) [6]; signal sequence-based peptides [7]; and synthetic arginine-enriched sequences. Examples of the latter include: polyarginines [8] and arginine-substituted CPPs of R_9-TAT type; RNA-binding peptides, such as HIV-1 Rev (34 – 50) peptide and flock house virus coat (35 – 49) peptide; and

Ashley Publications
www.ashley-pub.com

DNA-binding peptides, such as c-Fos (139 – 164), c-Jun (252 – 279) and yeast GCN4 (231 – 252).

Penetratin is a CPP derived from the homeodomain of Antennapedia (*Drosophila* homeoprotein). Homeoproteins are transcription factors that comprise a stretch of 60 amino acids (called the homeodomain), which is involved in the DNA binding. The homeodomain was shown to traverse through the mammalian nerve cells and accumulate in their nuclei [9]. More specifically, the translocation ability was narrowed down to a 16-mer peptide, termed as penetratin (Antp PTD; 43 – 58 residues, RQIKIWFQNRRMKWKK), present in the third helix of the homeodomain [3]. VP22 is a herpes virus type 1 protein associated with the transport between cells, where it also ends up in the nuclei [4]. Transportan is a chimeric CPP composed of galanin and mastoparan. Inside the cells, transportan is taken up into the nuclei and concentrates in subnuclear structures, probably in the nucleoli [5]. MAP is a synthetic 18-mer peptide capable of transporting various cargoes across the cellular membrane. It has presented the highest uptake and cargo delivery efficiency among other CPPs [10]. Signal sequence-based peptides comprise membrane translocating sequences that accurately direct the preprotein towards the respective intracellular organelles. Such sequences, when coupled to nuclear localisation signals, can be directed to accumulate in the nuclei of the cells [11]. To identify the specific regions of the TAT peptide (TATp) that are responsible for translocation, synthetic peptides rich in arginines were prepared, such as arginine-substituted TAT (R_9-TAT), and D-amino acid substituted TAT (D-TAT) [8]. Such peptides were internalised with similar efficiencies as the TATp. In addition, various arginine-rich peptides (such as RNA-binding peptides derived from proteins HIV-1 Rev, flock house virus coat, and DNA-binding peptides from c-Fos, c-Jun and yeast GCN4) displayed translocation properties. Nonetheless, TATp remains the most frequently used CPP for drug delivery purposes, and will be the main subject of this review.

2. Transactivating transcriptional activator

HIV-1 is a retrovirus that encodes for the TAT protein [12]. TAT is a small nuclear polypeptide of 101 amino acids, encoded by two exons, with the first 1 – 72 residues encoded by the first exon and the residues 73 – 101 by the second. The frequently used 86-amino acid TAT is a truncated version of the naturally occurring full-length (101 amino acids) protein, generated as a result of tissue-culture passaging. The 101-amino acid TAT is divided into five domains: domains 1 – 4 encoded by the first exon are responsible for the complete transactivation. Domain 1 (amino acids 1 – 20) is an N-terminal acidic domain; domain 2 (amino acids 21 – 40) is rich in cysteine residues essential for transactivation; domain 3 (amino acids 41 – 48) includes the Arg- Lys-Gly- Leu-Gly-Ile motif; and domain 4 (amino acids 49 – 72) is an important domain with the basic Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg motif, which is associated with the direct RNA-binding [13] and nuclear localisation of the protein [14]. The RNA-binding property is specifically related to arginine residues. The arginine-rich region binds to the three-nucleotide bulge in transactivation responsive RNA [15], where the side chains of the arginine residues form hydrogen bonds with the phosphates near the RNA loops and bulges, and stimulates the transcription [16]. TAT-mediated transactivation occurs primarily due to the enhancement of the transcriptional elongation step [17].

2.1 TAT transduction domain

With the introduction of the 86-mer TAT as a translocating protein, studies were performed to exploit its potential as a delivery vector for intracellular delivery of a variety of cargo molecules [18]. Different shorter sequences of TAT protein were investigated for their transduction properties using circular dichroism and energy minimisation, which elucidated that the TAT(38 – 45) domain can form a α-helical structure, possesses amphipathic characteristics, and is responsible for the cellular uptake [19]. In contrast, the nuclear localisation signal is present in the basic cluster extending from the residues 49 – 57, which does not overlap with the helical structure. To gain an insight regarding the specific determinants responsible for the translocation and transactivation, synthetic peptides were prepared with deletions in the α-helical domain and the basic cluster domain, and investigated for their translocation ability [20]. Surprisingly, it was found that the α-helical domain is not required for the intracellular delivery, whereas the whole basic cluster of residues 48 – 60 is responsible for membrane translocation. Any deletion or substitution of basic residues in TAT(48 – 60) reduced the cellular uptake property. The translocation was as rapid as 5 min at concentrations of 100 nm. In another study, in an attempt to determine the minimum length required for transduction, the TAT(48 – 57) region was studied by deletions at the N and C termini [21]. The deletion of Gly48 did not affect the transduction efficiency; however, the deletions of Lys50,51, Arg55 – 57 and Gln54 markedly reduced transduction efficiencies. The minimal transduction domain was thus assigned to TAT(49 – 57). This domain is highly rich in positively charged residues. These authors have found that the substitution of this domain with polylysine (9-lysine residues) or polyarginine (9-arginine) did not affect the transduction ability, and both polylysine polyarginine domains transduced to cytosol and nuclei to the similar extent as TAT(49 – 57). Therefore, the positive charge in the transduction domain contributes to the transduction ability of TAT protein. The commonly studied transduction domain of TAT (TAT PTD) extends from residues 47 – 57: YGRKKRRQRRR, which contains six arginine and two lysine residues [22].

3. Role of arginine residues in translocation

As the CPPs such as TAT(49 – 57) and penetratin(43 – 58) are rich in arginine residues, the effect of arginine substitution and chain length of arginine residues on the transduction efficiency was examined by synthesising analogues of the

TAT(48 – 60) peptide with D-amino acids (D-TAT), or by substituting (49 – 57) residues with 9-arginines (R_9-TAT) [8]. Both D-TAT and R_9-TAT were internalised and localised into the cytosol and nucleus with efficiencies similar to the TAT(48 – 60) peptide. In contrast, and opposite, to what was found in [21], these authors discovered that lysine-rich peptides were poor transducing peptides. In any case, these data suggested that the arginine residues are important in the translocation process. To determine the optimum number of arginine residues required for efficient transduction, synthetic peptides with 4 – 16 arginine residues (R_4 – R_{16}) were examined. Synthetic peptide R_4 showed very little transduction, R_6 and R_8 showed the maximum internalisation and accumulation in the nuclei, whereas R_{16} showed reduced transduction compared with R_8. Thus, oligoarginine with eight arginine residues seem to be the optimal vector for efficient translocation [8].

On the similar lines, Mitchell *et al.* [23] studied homopolymers rich in cationic amino acids, such as arginine, lysine, ornithine and histidine, for their cellular uptake ability. It was found that the polymers of arginine were more effective than homopolymers of lysine, ornithine and histidine at the same chain length, suggesting the importance of namely arginine residues for translocation. Similarly to the findings in [8], changing the chain length within arginine homopolymers affected the transduction ability. Again, arginine oligomers with six or more residues were more effective than those with less than five residues, with the translocation efficiency increasing up to 15 amino acids. In addition, the chirality of amino acid did not affect the cellular uptake as both D- and L-arginine polymers entered the cells effectively. This suggested that the peptide backbone does not participate in hydrogen bonding with the receptor during the transport. Further exploration of the structural components required for the cellular uptake revealed that the guanidinium headgroup of arginine is a critical component for translocation. Citrulline, an isostere of arginine, differs from arginine in having a urea rather than guanidine on its side chain. The heptamers of citrulline showed very poor staining of the cells compared with the heptamers of arginine, emphasising the role of guanidinium in translocation. The high pK_a value of the guanidine group imparts a high charge to arginine moieties at physiological pH. The arginine residues can then strongly interact with the plasma membrane, forming very stable bidentate hydrogen bonds with the anions as phosphate and sulfate on the plasma membranes.

The importance of guanidinium groups of TAT(49 – 57) has also been highlighted in [24]. The authors of this study showed that the truncated and alanine-substituted derivatives of TAT(49 – 57) are less efficient at internalisation than TAT(49 – 57); thereby suggesting that all the cationic residues of TAT(49 – 57) are required for the efficient translocation. Nevertheless, charge alone is not important for translocation. The oligomers of arginine, 9-mer of L- and D-arginine, were more efficient than TAT(49 – 57) at cellular uptake, again emphasising that the guanidinium groups of TAT(49 – 57) play an important role in enhancing cellular uptake than either the charge or backbone structure. Based on these studies, these authors attempted to design polyguanidine peptoid derivatives that are superior to TAT(49 – 57), resistant to proteolysis, and convenient to prepare. The peptoid derivative, with a six-methylene spacer between the guanidine head group and backbone, displayed significantly enhanced uptake compared with TAT(49 – 57) and D-arginine oligomer). The study suggested that the proper spacing between guanidine moieties is essential for the interaction with the plasma membrane and, hence, cellular uptake. This hypothesis was confirmed in [25], where these authors prepared a series of decamers containing seven arginines and three non-arginine residues. The decamers with spacer groups within were found to be better in cellular uptake than hepta-arginine itself.

4. Mechanisms of internalisation by translocation

There has been no unified perspective regarding the internalisation mechanisms of CPPs. Over the years, different mechanisms have been suggested by different authors. Earlier studies proposed that the TAT protein was internalised by adsorptive endocytosis [26]. The TAT protein was shown to bind nonspecifically to the cell surface, with > 10^7 sites per cell. It showed punctuated staining on the cell surface, with subsequent localisation to the cytoplasm and nuclei. The binding did not involve any specific receptor; however, it was blocked in the presence of heparin or dextran sulfate and at low temperature. A few years later, the rate of translocation of CPPs was found to be independent of temperature [5,20,27]. The TAT(48 – 60) peptide, penetratin (43 – 58 of Antennapedia), and transportan were efficiently taken up at 4 and 37°C, suggesting that endocytosis is not involved in the internalisation. The studies suggested a model for the internalisation of the TATp and penetratin, according to where direct ionic interaction between the basic residues of the CPPs and the negative phospholipid headgroups of the plasma membrane occurs. This leads to the local invagination of the plasma membrane, followed by the formation of inverted micelles and, ultimately, the release of the peptide inside the cell. Similarly, arginine-rich peptides were also found to be internalised at 4°C [8]. The internalisation of the TAT(48 – 60) peptide and other arginine-rich peptides was found to be dose-dependent, saturable and was inhibited in the presence of the excess amount of the peptide [28]. The cellular uptake of these peptides was not affected by endocytosis and metabolic inhibitors; however, the uptake was inhibited in the presence of heparan sulfate, suggesting the electrostatic interaction between the CPPs and negative charges of heparan sulfate are involved for translocation. Collectively, it was suggested that the arginine-rich peptides share a common internalisation mechanism, which is independent of classical endocytosis.

Later, the receptor-independent mechanism was indicated to be the result of the artifacts due to the fixation of the cells [29]. The study described that the cell-fixation procedure produces altered images due to the artifactual redistribution of the peptides into the nuclei. The peptides adhere strongly to the negatively charged cell membrane through their dense positive charges, and cannot be removed by routine washings unless trypsinised. The authors of this study, therefore, observed the uptake of peptides in live unfixed cells, and found that the cellular uptake of TAT(48 – 60) and R_9 peptides was inhibited by low temperature or by the depletion of the cellular pool of ATP. Fluorescence microscopy studies on live cells demonstrated the punctuate distribution of the peptides, and suggested endocytosis as a major mechanism for peptide uptake by the cells.

There are two types of the endocytic uptake of the CPPs that have been proposed: the classical clathrin-mediated endocytosis and the lipid-raft mediated caveolae endocytosis. Clathrin-mediated endocytosis involves the formation of clathrin-coated membrane pits that pinch off the membrane to form vesicles for subsequent processing [30]. This type of endocytosis was suggested in [29], where the TATp showed the colocalisation with the classical endocytic marker, transferrin. This was substantiated further in [31], where TAT PTD and Antp PTD showed uptake only at 37°C. In addition, the internalisation required the expression of negatively charged glycosaminoglycans on the cell surface for interaction with CPPs, which was followed by endocytosis. Studies also suggested that the PTDs do not provoke a 'real translocation', but are only responsible for cell surface adherence, which subsequently results in their endocytosis and accumulation in endosomes [32-34]. In fact, direct electrostatic interaction between the positive residues of CPPs and the negative residues of the cell-surface proteoglycans or glycosaminoglycans (e.g., heparan sulfate and heparin) is required in internalisation, regardless of the mechanism of cellular uptake [31,35-37]. Contradictory studies suggested the lack of correlation between proteoglycans and the transduction process [38].

Another proposed mechanism for internalisation is caveolae-mediated clathrin-independent uptake. Caveolae uptake involves the formation of flask-like uncoated invaginations (50 – 70 nm), principally composed of a subclass of detergent-resistant membrane domains enriched in cholesterol and sphingolipids, called lipid rafts [39]. This type of uptake was suggested in [40,41]. Unlike the rapid uptake of transferrin, a marker for clathrin-mediated endocytosis, the internalisation of TAT-cargo was very slow, reaching the plateau after several hours; and the colocalisation of TATp with the markers of caveolar uptake. The cellular uptake was not inhibited by nonionic detergents, a feature of lipid rafts, but was affected in the presence of drugs that either disrupt lipid rafts or alter caveolar trafficking.

A different mechanism has been proposed for the transport of guanidinium-rich CPPs conjugated to small molecules (molecular weight of < 3000 Da) [42,43]. The guanidinium groups of the CPPs form bidentate hydrogen bonds with the negative residues on the cell surface; the resulting ion pairs then translocate across the cell membrane under the influence of the membrane potential. The ion pair dissociates on the inner side of the membrane, releasing the CPPs into the cytosol. Nevertheless, the number of guanidinium groups is critical for translocation, with approximately eight groups being the optimum number for the efficient translocation.

A recent mechanism proposed for CPP conjugated to large cargoes (molecular weight of > 30,000 Da) is nonclathrin, noncaveolar endocytosis, called macropinocytosis. Macropinocytosis is a nonspecific form of cellular uptake, caused by large vesicles, known as macropinosomes, which are generated from actin filaments [44]. It has been shown in [45] that TAT–Cre fusion proteins were internalised by the lipid-raft dependent macropinocytosis. As a majority of fusion proteins remained entrapped in macropinosomes, the TAT transduction domain was conjugated to a fusogenic peptide (the N-terminus domain of the influenza virus haemagglutinin protein HA2), to trigger the release of the TAT fusion protein from endosomes, and thereby enhance their nuclear transport. A very recent study also demonstrates the macropinocytosis mechanism for small PTD peptides (1000 – 5000 Da) [46,47].

Therefore, it seems that more than one mechanism works for CPP-mediated intracellular delivery of small and large molecules. Individual CPPs, or CPP conjugated to small molecules, are internalised into cells via electrostatic interactions and hydrogen bonding, while CPP-conjugated to large molecules occur via the energy-dependent macropinocytosis. However, in both cases, the direct contact between the CPPs and negative residues on the cell surface is a requisite for successful transduction.

As the majority of the studies believe in endocytosis as a major mechanism for transduction, an important moment is the escape of CPPs from the endosomes and their translocation to the nuclei. Studies suggest that endosomal acidification prior to the disruption is required for CPP escape [48]. Another study suggested that the TAT fusion proteins enter cells via the endosomal pathway, circumvent lysosomal degradation and then sequester in the periphery of the nuclei [49]. Overall, the efficiency of the nuclear translocation process is limited.

5. Transactivating transcriptional activator as a transduction vector for hydrophilic cargoes

Ever since the discovery of the TAT and its ability to traverse across the plasma membrane, different studies have exploited TAT to deliver various cargo molecules intracellularly, both *in vitro* and *in vivo* for therapeutic applications. The plasma membrane poses a strong hydrophobic barrier for the transport of molecules > 500 Da. As a result, many therapeutic drugs cannot reach their intracellular targets unless they enter cells via the endocytic pathway associated with intensive drug degradation inside the lysosomes. Such drugs, when conjugated with CPPs, may overcome the plasma membrane barrier and

be transported intracellularly. CPPs such as TAT, penetratin, VP22 and transportan have been used for intracellular delivery of proteins, genes and even nanoparticles such as liposomes [50-54]. As the TATp remains the most popular and frequently used CPP for intracellular drug delivery, various examples of certain TAT-driven delivery systems, which have revealed potential in different fields of cancer therapy, gene therapy, nanomedicine and diagnostic imaging will be considered in this Section.

5.1 Protein delivery

As previously mentioned, full-size TAT protein was found to enter the cells when present in the surrounding tissue culture media [1,2]. The ability of the TAT protein to deliver the cargoes within the cells was first examined in 1994 [18]. TATp (residues 1 – 72 or 37 – 72) were conjugated to different proteins, such as β-galactosidase, horseradish peroxidase, RNase A and domain III of *Pseudomonas* exotoxin A. TATp was able to deliver the cargo molecules within different types of cells *in vitro*. When tested *in vivo* with the TAT(37 – 72)-conjugate with β-galactosidase, the protein was delivered to different tissues such as the heart, liver, spleen, lungs and skeletal muscle. Thus, it was confirmed that TATp can deliver different sized molecules inside the cells. In addition, the truncated TATp (37 – 72), which carried the basic domain, was capable of transducing molecules across the cell membrane. Subsequently, Nagahara *et al.* developed full-length TAT fusion proteins from a bacterial expression vector, pTAT-HA, which has an N-terminal 6-histidine leader followed by 11-mer TAT PTD, a haemagglutinin (HA) tag and a polylinker [55]. TAT fusion proteins ranging in size from 15 to 115 kDa were able to transduce mammalian cells *in vitro* with ~ 100% efficiency. The transduction was observed quickly, within 10 min, and was concentration dependent. The TAT fusion protein technology could deliver a broad range of proteins that regulate apoptosis, cell cycle progression and differentiation [56,57]. To assess this technology for *in vivo* transduction of small peptides, the 11-mer TAT-PTD was labelled with fluorescein isothiocyanate (FITC) and injected intraperitoneally into mice [58,59]. The TAT–FITC peptide transduced all blood and splenic cells with high efficiency. Interestingly, the peptide was also able to cross the formidable blood–brain barrier and reach cells within the brain. The *in vivo* delivery of large proteins was also examined by the intraperitoneal injection of FITC-labelled TAT-β-galactosidase fusion protein. Strong β-galactosidase activity was found to be present in the liver, kidneys, lungs and heart. Similar to the TAT–FITC peptide, TAT–β-galactosidase fusion protein also transduced brain cells through the intact blood–brain barrier. The study thus suggested that it is possible to transduce mammalian cells with a much larger cargo molecule (β-galactosidase), ~ 200-times larger than the molecules that normally cross the plasma membranes.

Among many other applications, proteins are administered to generate cytotoxic T lymphocytes to degrade pathogen-derived peptides. However, exogenous proteins cannot traverse through the membranes of antigen-presenting cells for MHC class I presentation. Again, proteins can be introduced intracellularly using the TATp. A conjugate of TATp (49 – 57) with the model protein ovalbumin resulted in the production of ovalbumin-specific cytotoxic T lymphocytes, killing the target cells [60,61]. TAT-mediated antigenic protein delivery is useful for therapeutic or prophylactic vaccine design.

TAT-mediated protein delivery is also useful as a therapeutic approach in disorders such as oxidative stress, inflammatory conditions and ischaemia. TATp has allowed for the delivery of therapeutic enzymes required during oxidative stress. The fusion proteins of TAT and superoxide dismutase (TAT–SOD) and catalase (TAT–CAT) transduced HeLa cells, which were under the oxidative stress. The TAT fusion proteins replenished the antioxidant enzymes SOD and CAT, increasing the viability of the cells [62,63]. The transduction efficiency of SOD was further augmented when it was fused to TAT PTD at its N and C termini [64]. Another potential therapeutic application of TAT-mediated delivery is to regulate the inflammation in conditions such as rheumatoid arthritis. Chronic inflammation is the result of the activation of the transcription factor NF-κB via cytokines. Methods that regulate the cytokines or NF-κB can help pacify the inflammation. The fusion protein of TAT PTD-super-repressor IκBα resulted in the delivery of super-repressor IκBα within the cells, where it binds to NF-κB and inhibits its activation mediated by cytokines [65]. This study therefore provided a novel way of regulating pathological conditions.

Another area where TAT-transduction has shown potential is in the prevention of cell death associated with disorders such as infarction and stroke. Timely delivery of protein therapeutics provides cytoprotection, preventing apoptosis during ischaemia. The administration of the TAT–ARC (apoptosis repressor with caspase recruitment domain) fusion protein in the isolated heart model before the global ischaemia and reperfusion, decreased the infarct size after hypoxia [66]. It also prevented cell death arising from oxidative stress. Similarly, apoptosis of neurons during the cerebral ischaemia was prevented by the delivery of antiapoptotic protein, Bcl-xL fused with TAT PTD, to neurons [67]. The fusion protein decreased the cerebral infarction in a dose-dependent fashion. The study provided the first proof of transducing a neuroprotectant in experimental cerebral ischaemia.

The intracellular delivery of proteins mediated by TATp is not organelle specific. The proteins can stay in the cytosol or can be delivered to nuclei. In contrast to other findings, the authors in [68,69] showed that TAT fusion protein can be transduced into the mitochondria. However, for the fusion protein to stay inside the mitochondria for a longer duration for mitochondrial therapeutics, the TAT fusion protein was appended to the mitochondrial targeting sequence. Thus, the protein delivery can be made organelle specific by attaching a signal sequence to TAT fusion proteins, thus enabling the modulation at subcellular levels. It has recently been suggested that

by appending the mitochondrial targeting signal to the N terminus of the TAT–exonuclease fusion protein, the protein delivery can be directed into the mitochondria of breast cancer cells [70]. The protein delivery inside the mitochondria of the cancer cells then helps to regulate the mitochondrial function and, thus, facilitating cancer cell death.

Another growing application of the transduction technology is in the field of cancer therapy, where the transduction methodology seems to circumvent the problems encountered with the conventional chemotherapeutic regimens, such as nonspecificity and exclusion of drugs by efflux transporters in multi-drug resistant cells. The TAT transduction domains of Antennapedia and TAT have been linked to the tumour suppressor peptide p53, which enhanced the accumulation of p53 in the tumour cells and activated the apoptotic genes for the selective killing of tumour cells both *in vitro* and *in vivo* [71-73]. The TATp has also been used to deliver proteins that modulate the cell cycle and cause the arrest of tumour growth [55,74]. Another approach to selectively kill tumour cells is to transduce dendritic cells with tumour antigens to generate cytotoxic lymphocytes that eradicate tumours. The TAT transduction domain was used to transduce dendritic cells with ovalbumin, a recombinant model tumour-associated antigen. The transduced dendritic cells generated cytotoxic lymphocytes against tumours [75]; immunisation with transduced dendritic cells imparted an antitumour immunity and inhibited lung metastases in a 3-day tumour model [76].

5.2 Antibody delivery

The intracellular delivery of antibodies by conventional methods of electroporation or microinjection disrupts the cellular membrane, and so decreasing the cell viability [77,78]. In contrast, CPPs have shown enhanced delivery of antibodies within cells without compromising the cellular structures. The poorly internalised antitumour antibody Fab fragments (after conjugation to the peptide analogues of the 37 – 62 sequence region of the HIV TAT protein) displayed an enhanced cell surface binding, followed by internalisation of the Fab fragments [79]. TAT(37 – 72) peptide conjugated to antibodies was also used to provide neuroprotection against toxins. Because of the very slow degradation of the tetanus toxin (TET) in nerve cells, anti-TET antibodies are required for the neutralisation of TET. The conventional method of electroporation for the delivery of antibodies cannot be used in a clinical situation. However, the conjugate of TAT(37 – 72) peptide and antitetanus $F(ab')_2$ fragments was readily taken up by chromaffin cells, leading to the neutralisation of TET [80].

A novel method for the intracellular delivery of antibodies comprises the use of genetically engineered TAT fusion protein. The TAT fusion protein was conjugated to the Fc fragment of IgG, and the conjugate was studied for the cellular uptake by flow cytometry and confocal microscopy [81]. The TAT–antibody conjugate delivered the antibody into the cytosol of the cells with no delivery to the nuclei. The intracellular delivery of the antibodies proceeded in a time- and dose-dependent fashion.

In another study, the TAT–antibody conjugate was explored for its ability to specifically target the tumours in *in vivo* conditions [82]. TATp with D- and L-amino acids showed similar transduction *in vitro*. However, when the biodistribution studies on D-amino acid-containing TAT(44 – 57) peptide–antibody conjugates were performed in tumour-bearing mice, the conjugate displayed markedly reduced tumour-targeting activity compared with the unmodified antibody. The study raised some concerns about the potential of CPPs for the delivery of drugs *in vivo*.

5.3 Gene delivery

Gene therapy is a promising field for treating human diseases by delivering a functional copy of the desired gene into the target area. Viral vectors are very efficient in gene delivery into cells; however, their use is restricted due to the problems of the immune response and insertional mutagenesis [83,84]. The alternatives include nonviral vectors such as cationic lipids and polycation-based delivery vectors, which are comparatively safer than the viral vectors, despite their low efficiency for gene delivery [85-87]. In addition, nonviral vectors lack the endosomal-escape ability and nuclear-translocation property. Because PTDs direct the cargoes to the nuclei, they were investigated for their potential as gene-delivery vectors [88]. TATp, when attached to the surface of phage (TAT–phage), efficiently delivered the gene into the cells with no toxicity. The process of gene transport does not occur by classical endocytic route [89]. Historically, this study has opened the possibility of TAT-mediated gene delivery *in vivo*. The transfection efficiency (gene-delivery efficiency) with TATp–DNA complexes was superior compared with the standard nonviral gene-delivery vectors [90]. Further studies revealed that TATp (14 mer) delivered the polyanions, such as DNA and glycosaminoglycans, to the nuclei of mammalian cells [36]. The transfection was time, concentration and temperature dependent. TATp was assumed to interact electrostatically with the cell-surface proteoglycans for traversing across the plasma membrane. However, in another study investigating the mechanism of TAT-mediated gene delivery, the authors have found that TATp interacts with DNA electrostatically to form complexes, and these complexes enter the mammalian cells by endocytosis [91].

One of the approaches used in gene delivery targets the suicide gene therapy with the 'bystander effect', which involves killing the cells adjacent to the target area, and so producing an additive cytotoxic effect. TAT PTD efficiently transfected the cells *in vitro* with thymidine kinase, enhancing their sensitivity to the acyclovir or ganciclovir drug [92,93]. The bystander effect was also augmented.

5.4 Nanoparticulate delivery

The applications of TAT-mediated delivery extend to nanoparticle delivery inside cells. The concept of TAT-mediated nanoparticle delivery was first realised in 1999 when dextran-coated superparamagnetic-iron oxide nanoparticles (size

~ 40 nm), conjugated to TATp (48 – 57), showed significantly higher uptake in lymphocytes *in vitro* than in the control TAT-free particles [94]. The technique showed potential for magnetically labelling cells in order to allow for their magnetic separation or MRI. *In vivo*, TATp effectively transduced iron nanoparticles into haematopoietic and neural progenitor cells for stem cell analysis [95], and into T cells for MRI [96]. Contrary to nuclear translocation of TATp, particulate TATp–iron conjugates were observed in the cytoplasm, and not in the nuclei [97]. As superparamagnetic nanoparticles were shown to be useful magnetic resonance contrast agents for imaging, and also useful for cell labelling and cell tracking, the conjugation of such nanoparticles with TATp provides a better signal from the treated cells for MRI [98].

Similarly, gold nanoparticles were also modified with TATp [99]. As with iron conjugates, these particles did not reach the cell nuclei in experiments with NIH3T3 and HepG2 cells. The uptake of the particles was found to proceed by endocytosis. TATp (48 – 57) was also conjugated to FITC-doped silica nanoparticles for bioimaging purposes [100]. This type of conjugate showed efficient transduction both *in vitro* and *in vivo*, as well as an attractive ability to deliver therapeutic agents to the brain without compromising the blood–brain barrier.

A new application of TAT is in the labelling of cells with quantum dots. Quantum dots are gaining interest over standard fluorophores as they do not suffer from bleaching problems, and they also allow concurrent imaging of the multiple species *in vivo*. Recently, quantum dots were encapsulated in PEG–PE micelles, tagged in turn with a TAT-PEG-PE linker [101]. TATHlabelled quantum dots were readily taken up by mouse endothelial cells *in vitro* and subsequently intensively labelled them. *In vivo*, it became possible to monitor the behaviour of bone marrow-derived progenitor cells loaded with quantum dot-loaded TAT micelles within the tumour vasculature.

Lipid-based nanoparticles, when modified with dimeric TATp, showed an enhanced gene delivery to cells *in vitro* and *in vivo*, as compared with a standard polyethylenimine carrier [102]. Shell crosslinked (SCK) nanoparticles (nanocaged structures) derivatised with TAT PTD displayed a surface pronounced binding and transduction in CHO and HeLa cells [103]. To improve the transduction efficiency of SCK, different ratios of TAT PTD:SCK were investigated [104]. The constructs were then examined *in vitro* and *in vivo* for biocompatibility estimation [105]. Although the constructs resulted in inflammatory responses *in vitro*, *in vivo* evaluation proved their application to be quite safe. Such TAT PTD-functionalised SCKs can, therefore, be used as scaffolds for antigens used for vaccination.

TAT-mediated nanoparticulate delivery is also becoming established in the vaccination field to elicit a better immune response. When TAT(1 – 72)-coated anionic nanoparticles were used to immunise mice, it generated antibodies and T helper type-1 immune response to TAT [106]. In another study, TAT microspheres were used for vaccination [107]. Anionic microspheres of different compositions, size and surface charge density were prepared, and all of them adsorbed biologically active TAT protein in a reversible mode. The microspheres were delivered by TAT intracellularly and were not toxic, both *in vitro* and *in vivo*.

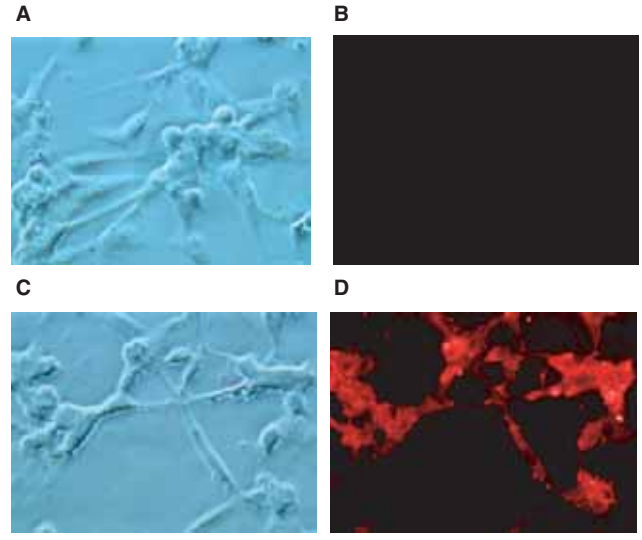

Figure 1. The uptake of TAT-modified rhodamine-labelled liposomes by cells in culture. H9C2 cells (murine myoblasts) were incubated in the presence of various liposomal preparations for 1 h at 37°C. **A, B)** Plain (TAT-free) liposomes. **C, D)** TATp-modified liposomes. **A, C)** Bright field microscopy. **B, D)** Fluorescence microscopy with rhodamine filter. The dramatic enhancement of TAT-liposome uptake can be easily seen (red fluorescence inside cells in **D**).
TAT: Transactivating transcriptional activator; TATp: Transactivating transcriptional activator peptide.

TATp has also been exploited for the intracellular delivery of lipid-based pharmaceutical nanocarriers, such as liposomes. It was shown that relatively large 200-nm plain and pegylated liposomes could be efficiently delivered by surface-attached TAT PTD into different cells, including mouse Lewis lung carcinoma cells, human breast tumour BT20 cells and rat cardiac myocyte H9C2 cells [108]. Cells treated with liposomes, in which the TATp–cell interaction was hindered either by direct attachment of TATp to the liposomes surface or by the presence of long-chain polymer grafts on the liposome surface shielding the TAT moiety, did not show internalisation of the liposomes. However, TAT-liposome preparations allowing for the direct contact of the TATp with the cell surface displayed a significantly enhanced uptake of TAT-liposomes (Figure 1). The translocation of TATp–liposomes into cells thus requires a direct unhindered interaction of TAT PTD with the cell surface or its certain specific components (e.g., proteoglycans).

Intracellular trafficking studies with TATp–liposomes revealed that they remained intact in the cell cytoplasm for some time following the translocation; within 2 h they migrated into the perinuclear zone (Figure 2) and gradually

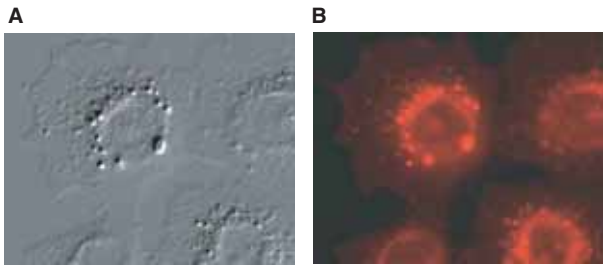

Figure 2. The association of TAT-modified liposomes with the perinuclear zone. BT20 cells (human breast carcinoma) were incubated with rhodamine-labelled TAT-liposomes for 1 h at 37°C. The microscopy was carried out 2 h after the incubation was completed. **A)** Bright field microscopy.
B) Fluorescence microscopy with a rhodamine filter. Liposome accumulation within the perinuclear zone is clearly observed.
TAT: Transactivating transcriptional activator.

disintegrated there with time [109]. TATp-liposomes have also been investigated for their gene delivery ability. TATp-liposomes were prepared containing a small fraction (≤ 10% mol) of a cationic lipid (DOTAP) and incubated with DNA. The liposomes formed firm noncovalent complexes with DNA. Such TATp–liposome–DNA complexes provided an efficient transfection both *in vitro* (Figure 3) and *in vivo* with lower cytotoxicity than the commonly used Lipofectin® (Invitrogen Life Technologies).

The translocation of liposomes by TATp seemed to be proportional to the number of peptide molecules attached to the liposomal surface. The uptake was found to be peptide and cell-type dependent, and as few as five TATp moieties on the liposome surface were sufficient to enhance the intracellular delivery of liposomes [54].

In another study, TAT PTD was coupled to small unilamellar liposomes, and these conjugates showed a higher uptake in tumour cells and dendritic cells than the unmodified control liposomes [110]. The uptake was time and concentration dependent, and ≥ 100 PTD molecules per small unilamellar liposome were required for efficient translocation of liposomes into the cells. The cell uptake was inhibited by the preincubation of liposomes with heparin. This confirms that heparan sulfate proteoglycans may be involved in TAT PTD-mediated uptake. In a different study, in order to enhance the biocompatibility of the cationic lipoplexes, thiocholesterol-based cationic lipids were used in the formation of nanolipoparticles (NLPs), which were then sequentially modified with TATp. TAT-modified NLPs with a zwitterionic surface resulted in a higher transfection than the cationic NLPs [111].

Studies on the mechanisms of TAT-liposomes have suggested endocytosis to be the main mechanism of cell uptake. Conjugation of TATp to the lipoplexes enhanced the gene transfection within primary cell cultures by endocytosis [112]. The coupling

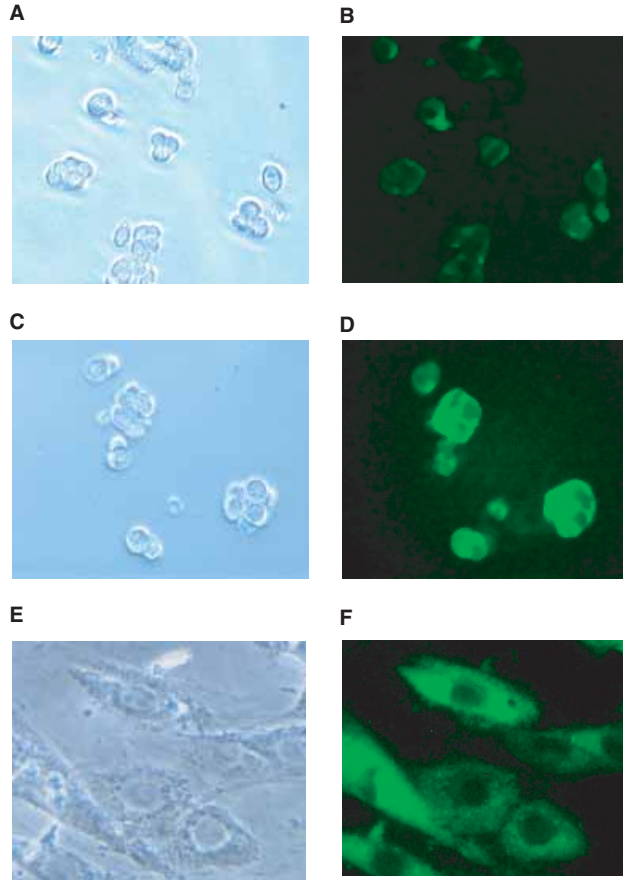

Figure 3. The enhancement of the liposome-mediated transfection by TATp. HCC1500 cells (**A – D**: human breast carcinoma) or H9C2 cells (**E, F**: murine myoblasts) were incubated with the presence of liposome/pEGFP-N1 plasmid (encoding for the GFP) complexes (DNA 10 µg/100,000 cells) for 4 h at 37°C. The transfection efficiency (the appearance of the green fluorescence of the GFP inside the cells) was detected after 72 h. **A, B)** Cells were incubated with control plain (TAT-free) plasmid-bearing liposomes. **C – F)** Cells were incubated with plasmid-bearing TAT-liposomes of the same composition. **A**, **C, E)** Bright field light microscopy. **B, D, F)** Fluorescent microscopy with FITC filter. Background transfection can only be seen with the controls, whereas the introduction of TATp into the preparation provides a dramatic enhancement of the GFP expression (i.e., increases the transfection efficiency).
FITC: Fluorescein isothiocyanate; GFP: Green fluorescent protein;
TATp: Transactivating transcriptional activator peptide.

of TATp to the outer surface of liposomes resulted in an enhanced binding and endocytosis of the liposomes in ovarian carcinoma cells [113]. The binding was inhibited in the presence of heparin or dextran sulfate; again confirming the involvement of proteoglycans expressed on the cell surface.

5.5 Miscellaneous delivery

CPPs have also been useful in the delivery of imaging agents, toxins and polymer-bound drug. The imaging agents, similar

Table 1. Partial data on intracellular delivery of various pharmaceutical cargoes by conjugation with TAT.

Cargoes delivered by TAT	Cells (*in vitro*/*in vivo*)	Ref.
β-Galactosidase	Mouse *in vivo*	[18,58]
Fluorescein isothiocyanate	Mouse *in vivo*	[58]
Ovalbumin	Mouse *in vivo*	[61]
Superoxide dismutase	HeLa cells *in vitro*	[62]
Catalase	HeLa cells *in vitro*	[63]
Ovalbumin	Dendritic cells *in vitro*	[75]
TRP2 peptide	Dendritic cells *in vitro*	[76]
Super-repressor rIκBα	Jurkat T cells *in vitro*	[65]
β-Galactosidase	H9C2 cells *in vitro*	[66]
ARC	H9C2 cells *in vitro*, isolated rat heart	[66]
Bcl-xL	Primary neurons *in vitro*, mice brain *in vivo*	[67]
Phage	COS-1 *in vitro*	[89]
Antitetanus $F(ab')_2$	Rev-2-T-6, NG108-15 cells *in vitro*	[80]
DNA	CHO cells *in vitro*	[36]
pEGFP	H9C2, NIH3T3 cells *in vitro*, mouse *in vivo*	[109]
Iron oxide nanoparticles	Mouse lymphocytes *in vitro*	[94]
Iron oxide nanoparticles	Haematopoietic and neural progentor cells *in vivo*	[95]
Gold nanoparticles	HeLa cells, NIH 3T3 cells, HepG2 cells *in vitro*	[99]
Quantum dots	Mouse endothelial cells *in vitro*, mouse *in vivo*	[101]
Liposomes	LLC, BT20, H9C2 cells *in vitro*	[108]
Liposomes	HTB-9, A431 cells *in vitro*, C26 *in vivo*	[54]
HPMA copolymer	A2780 *in vitro*	[118,119]

ARC: Apoptosis repressor with caspase recruitment domain; CHO: Chinese hamster ovary; HPMA: *N*-(2-Hydroxypropyl)methacrylamide; TAT: Transactivating transcriptional activator; TRP: Tyrosinase-related protein.

to many other substances, cannot cross the cellular barrier by themselves. Linking them to CPPs brings the imaging agents in sufficient concentrations inside cells. Oxotechnetium(V) and oxorhenium(V), commonly used in imaging and radiotherapy, were conjugated to the TAT(48 – 57) peptide [114]. The conjugates were rapidly internalised by Jurkat cells and showed significantly enhanced accumulation inside the cytoplasm and nuclei. In another study, paramagnetically labelled DOTA chelator was attached to a 13-mer TATp, and this conjugate was efficiently taken up by mammalian cells in sufficiently high concentrations, allowing for the detection by MRI [115]. Similarly, superparamagnetic-iron oxide particles conjugated with TATp and fluorescein isothiocyanate were rapidly internalised by T cells, B cells and macrophages, with subsequent localisation in the cytoplasm that could be followed by the MRI [116].

The fusion of diphtheria toxin A-fragment with the TAT transduction domain or VP22 resulted in a protein with enhanced cell-surface binding; however, the conjugate was unable to effectively deliver an enzymatically active diphtheria toxin A-fragment to the cytosol [117]. However, the coupling with TAT increased the toxicity to the corresponding holotoxin.

Another application of CPPs encompasses the modification of polymer-based drug delivery systems to bypass endocytosis, for a better drug accumulation in the cell cytoplasm. The TAT transduction domain was attached to a water-soluble synthetic *N*-(2-hydroxypropyl)methacrylamide copolymer, and the intracellular localisation of the conjugate was investigated in human ovarian carcinoma cells by confocal microscopy and subcellular fractionation [118-120]. It was found that TATp could deliver the polymer-bound drug to both the cytoplasm and nuclei via a nonendocytic process; in contrast, the conjugates without TAT were internalised only inside endocytic vesicles. The TATp-modified polymeric carrier can, therefore, be used for the cytoplasmic delivery of therapeutic drugs.

To summarise, Table 1 presents a partial list of various cargo molecules that have been intracellularly delivered by TATp both *in vitro* and *in vivo*.

6. Expert opinion

CPPs can clearly serve as versatile delivery vectors for intracellular drug delivery. They can deliver inside cells a wide range of cargoes of different sizes, from small molecules to relatively

large nanoparticles. Of different CPPs available, TATp remains the most frequently used for this purpose in experimental cancer therapy, gene therapy, immunotherapy and intracellular delivery of particulates, such as nanoparticles, liposomes and polymers. However, the detailed comparative analysis for different natural and synthetic CPPs is missing. There are also some other unanswered questions that require investigation before the transduction technology can be applied in the clinical setting. The TATp (and other CPPs) nonspecifically transduces almost every cell and organ when injected systemically. This does not allow for the specific targeting to a desired area, and the application of TAT-based systems is, therefore, limited to local administration, or requires an additional introduction of various specific-targeting ligands. Regardless of this, the mystery about the exact internalisation mechanism and its individual fine details needs to be unveiled for a better understanding of the translocation process and the ways to control it. The kinetics of the interaction of the CPPs with the cell surface and their migration inside cells should be studied in more detail to allow for the preparation with the predictable intracellular fate. As the rate of translocation of CPPs is influenced by the cargo size, the kinetics for differently sized cargoes also requires an optimisation. So far, the detailed kinetics of the intracellular delivery of medium to large cargoes tethered to CPPs remain unavailable. The precise intracellular end point for the preparations modified by CPPs is uncertain as different studies suggest the cytoplasm, perinuclear zone or mitochondria as such an end point. In addition, the side effects and the toxicity profile of the positively charged CPPs in the physiological situation need consideration. Last, but not least, the way that some cargo proteins should be attached to CPPs, by chemical modification or by making fused constructs, should also be analysed. In addition, more synthetic analogues should be made available as alternatives to CPPs. With all these issues being resolved, the transduction technology should certainly find its way to clinical settings, making it possible to deliver therapeutic agents directly to the required compartments inside cells for the treatment of various diseases.

Bibliography

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. GREEN M, LOEWENSTEIN PM: Autonomous functional domains of chemically synthesized human immunodeficiency virus TAT trans-activator protein. *Cell* (1988) 55:1179-1188.
• One of the key early papers to introduce TAT-mediated transduction.
2. FRANKEL AD, PABO CO: Cellular uptake of the TAT protein from human immunodeficiency virus. *Cell* (1988) 55:1189-1193.
• Another one of the key early papers to introduce TAT-mediated transduction.
3. DEROSSI D, JOLIOT AH, CHASSAING G, PROCHIANTZ A: The third helix of the Antennapedia homeodomain translocates through biological membranes. *J. Biol. Chem.* (1994) 269:10444-10450.
4. ELLIOTT G, O'HARE P: Intercellular trafficking and protein delivery by a herpesvirus structural protein. *Cell* (1997) 88:223-233.
5. POOGA M, HALLBRINK M, ZORKO M, LANGEL U: Cell penetration by transportan. *FASEB J.* (1998) 12:67-77.
6. 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.
7. LINDGREN M, HALLBRINK M, PROCHIANTZ A, LANGEL U: Cell-penetrating peptides. *Trends Pharmacol. Sci.* (2000) 21:99-103.
8. FUTAKI S, SUZUKI T, OHASHI W *et al.*: Arginine-rich peptides. An abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery. *J. Biol. Chem.* (2001) 276:5836-5840.
9. JOLIOT A, PERNELLE C, DEAGOSTINI-BAZIN H, PROCHIANTZ A: Antennapedia homeobox peptide regulates neural morphogenesis. *Proc. Natl. Acad. Sci. USA* (1991) 88:1864-1868.
10. HALLBRINK M, FLOREN A, ELMQUIST A, POOGA M, BARTFAI T, LANGEL U: Cargo delivery kinetics of cell-penetrating peptides. *Biochim. Biophys. Acta* (2001) 1515:101-109.
11. LIN YZ, YAO SY, VEACH RA, TORGERSON TR, HAWIGER J: Inhibition of nuclear translocation of transcription factor NF-kappa B by a synthetic peptide containing a cell membrane-permeable motif and nuclear localization sequence. *J. Biol. Chem.* (1995) 270:14255-14258.
12. JEANG KT, XIAO H, RICH EA: Multifaceted activities of the HIV-1 transactivator of transcription, TAT. *J. Biol. Chem.* (1999) 274:28837-28840.
13. WEEKS KM, AMPE C, SCHULTZ SC, STEITZ TA, CROTHERS DM: Fragments of the HIV-1 TAT protein specifically bind TAR RNA. *Science* (1990) 249:1281-1285.
14. RUBEN S, PERKINS A, PURCELL R *et al.*: Structural and functional characterization of human immunodeficiency virus TAT protein. *J. Virol.* (1989) 63:1-8.
15. CALNAN BJ, BIANCALANA S, HUDSON D, FRANKEL AD: Analysis of arginine-rich peptides from the HIV TAT protein reveals unusual features of RNA-protein recognition. *Genes Dev.* (1991) 5:201-210.
16. CALNAN BJ, TIDOR B, BIANCALANA S, HUDSON D, FRANKEL AD: Arginine-mediated RNA recognition: the arginine fork. *Science* (1991) 252:1167-1171.
17. FEINBERG MB, BALTIMORE D, FRANKEL AD: The role of TAT in the human immunodeficiency virus life cycle indicates a primary effect on transcriptional elongation. *Proc. Natl. Acad. Sci. USA* (1991) 88:4045-4049.
18. FAWELL S, SEERY J, DAIKH Y *et al.*: TAT-mediated delivery of heterologous proteins into cells. *Proc. Natl. Acad. Sci. USA* (1994) 91:664-668.
• The first paper to prove the concept of TAT-mediated transduction of different proteins.
19. LORET EP, VIVES E, HO PS, ROCHAT H, VAN RIETSCHOTEN J,

JOHNSON WC Jr: Activating region of HIV-1 TAT protein: vacuum UV circular dichroism and energy minimization. *Biochemistry* (1991) 30:6013-6023.

20. VIVES 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) 272:16010-16017.
- The first paper to introduce shorter domains of the TAT protein required for transduction.

21. PARK J, RYU J, KIM KA *et al.*: Mutational analysis of a human immunodeficiency virus type 1 TAT protein transduction domain which is required for delivery of an exogenous protein into mammalian cells. *J. Gen. Virol.* (2002) 83:1173-1181.

22. SCHWARZE SR, HRUSKA KA, DOWDY SF: Protein transduction: unrestricted delivery into all cells? *Trends Cell Biol.* (2000) 10:290-295.

23. MITCHELL DJ, KIM DT, STEINMAN L, FATHMAN CG, ROTHBARD JB: Polyarginine enters cells more efficiently than other polycationic homopolymers. *J. Pept. Res.* (2000) 56:318-325.

24. WENDER PA, MITCHELL DJ, PATTABIRAMAN K, PELKEY ET, STEINMAN L, ROTHBARD JB: The design, synthesis, and evaluation of molecules that enable or enhance cellular uptake: peptoid molecular transporters. *Proc. Natl. Acad. Sci. USA* (2000) 97:13003-13008.

25. ROTHBARD JB, KREIDER E, VANDEUSEN CL, WRIGHT L, WYLIE BL, WENDER PA: Arginine-rich molecular transporters for drug delivery: role of backbone spacing in cellular uptake. *J. Med. Chem.* (2002) 45:3612-3618.

26. MANN DA, FRANKEL AD: Endocytosis and targeting of exogenous HIV-1 TAT protein. *EMBO J.* (1991) 10:1733-1739.

27. 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.

28. 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.* (2002) 277:2437-2443.

29. RICHARD JP, MELIKOV K, VIVES E *et al.*: Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake. *J. Biol. Chem.* (2003) 278:585-590.
- An important paper to reveal the concept of artifacts on fixed cells.

30. SIECZKARSKI SB, WHITTAKER GR: Dissecting virus entry via endocytosis. *J. Gen. Virol.* (2002) 83:1535-1545.

31. CONSOLE S, MARTY C, GARCIA-ECHEVERRIA C, SCHWENDENER R, BALLMER-HOFER K: Antennapedia and HIV transactivator of transcription (TAT) 'protein transduction domains' promote endocytosis of high molecular weight cargo upon binding to cell surface glycosaminoglycans. *J. Biol. Chem.* (2003) 278:35109-35114.

32. LUNDBERG M, WIKSTROM S, JOHANSSON M: Cell surface adherence and endocytosis of protein transduction domains. *Mol. Ther.* (2003) 8:143-150.

33. LEIFERT JA, HARKINS S, WHITTON JL: Full-length proteins attached to the HIV TAT protein transduction domain are neither transduced between cells, nor exhibit enhanced immunogenicity. *Gene Ther.* (2002) 9:1422-1428.

34. VIVES E, RICHARD JP, RISPAL C, LEBLEU B: TAT peptide internalization: seeking the mechanism of entry. *Curr. Protein Pept. Sci.* (2003) 4:125-132.

35. TYAGI M, RUSNATI M, PRESTA M, GIACCA M: Internalization of HIV-1 TAT requires cell surface heparan sulfate proteoglycans. *J. Biol. Chem.* (2001) 276:3254-3261.

36. SANDGREN S, CHENG F, BELTING M: Nuclear targeting of macromolecular polyanions by an HIV-TAT derived peptide. Role for cell-surface proteoglycans. *J. Biol. Chem.* (2002) 277:38877-38883.

37. MAI JC, SHEN H, WATKINS SC, CHENG T, ROBBINS PD: Efficiency of protein transduction is cell type-dependent and is enhanced by dextran sulfate. *J. Biol. Chem.* (2002) 277:30208-30218.

38. VIOLINI S, SHARMA V, PRIOR JL, DYSZLEWSKI M, PIWNICA-WORMS D: Evidence for a plasma membrane-mediated permeability barrier to TAT basic domain in well-differentiated epithelial cells: lack of correlation with heparan sulfate. *Biochemistry* (2002) 41:12652-12661.

39. ANDERSON RG: The caveolae membrane system. *Ann. Rev. Biochem.* (1998) 67:199-225.

40. FITTIPALDI A, FERRARI A, ZOPPE M *et al.*: Cell membrane lipid rafts mediate caveolar endocytosis of HIV-1 TAT fusion proteins. *J. Biol. Chem.* (2003) 278:34141-34149.
- An important paper to mention the caveolar endocytosis mechanism for transduction.

41. FERRARI A, PELLEGRINI V, ARCANGELI C, FITTIPALDI A, GIACCA M, BELTRAM F: Caveolae-mediated internalization of extracellular HIV-1 TAT fusion proteins visualized in real time. *Mol. Ther.* (2003) 8:284-294.

42. ROTHBARD JB, JESSOP TC, LEWIS RS, MURRAY BA, WENDER PA: Role of membrane potential and hydrogen bonding in the mechanism of translocation of guanidinium-rich peptides into cells. *J. Am. Chem. Soc.* (2004) 126:9506-9507.
- One of the key papers to discuss the mechanism of uptake of guanidinium-rich peptides.

43. ROTHBARD JB, JESSOP TC, WENDER PA: Adaptive translocation: the role of hydrogen bonding and membrane potential in the uptake of guanidinium-rich transporters into cells. *Adv. Drug Deliv. Rev.* (2005) 57:495-504.

44. SWANSON JA, WATTS C: Macropinocytosis. *Trends Cell Biol.* (1995) 5:424-428.

45. WADIA JS, STAN RV, DOWDY SF: Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. *Nat. Med.* (2004) 10:310-315.
- The key paper to introduce macropinocytosis as a mechanism for the transduction of large molecules.

46. KAPLAN IM, WADIA JS, DOWDY SF: Cationic TAT peptide transduction domain enters cells by macropinocytosis. *J. Control. Release* (2005) 102:247-253.

47. NAKASE I, NIWA M, TAKEUCHI T *et al.*: Cellular uptake of arginine-rich peptides: roles for macropinocytosis and actin rearrangement. *Mol. Ther.* (2004) 10:1011-1022.

48. POTOCKY TB, MENON AK, GELLMAN SH: Cytoplasmic and nuclear delivery of a TAT-derived peptide and a

beta-peptide after endocytic uptake into HeLa cells. *J. Biol. Chem.* (2003) 278:50188-50194.

49. CARON NJ, QUENNEVILLE SP, TREMBLAY JP: Endosome disruption enhances the functional nuclear delivery of TAT-fusion proteins. *Biochem. Biophys. Res. Commun.* (2004) 319:12-20.
50. ROEDER GE, PARISH JL, STERN PL, GASTON K: Herpes simplex virus VP22-human papillomavirus E2 fusion proteins produced in mammalian or bacterial cells enter mammalian cells and induce apoptotic cell death. *Biotechnol. Appl. Biochem.* (2004) 40:157-165.
51. ZAVAGLIA D, NORMAND N, BREWIS N, O'HARE P, FAVROT MC, COLL JL: VP22-mediated and light-activated delivery of an anti-c-raf1 antisense oligonucleotide improves its activity after intratumoral injection in nude mice. *Mol. Ther.* (2003) 8:840-845.
52. ZAVAGLIA D, FAVROT MC, EYMIN B, TENAUD C, COLL JL: Intercellular trafficking and enhanced *in vivo* antitumour activity of a non-virally delivered P27-VP22 fusion protein. *Gene Ther.* (2003) 10:314-325.
53. POOGA M, SOOMETS U, HALLBRINK M *et al.*: Cell penetrating PNA constructs regulate galanin receptor levels and modify pain transmission *in vivo*. *Nat. Biotechnol.* (1998) 16:857-861.
54. TSENG YL, LIU JJ, HONG RL: Translocation of liposomes into cancer cells by cell-penetrating peptides penetratin and TAT: a kinetic and efficacy study. *Mol. Pharmacol.* (2002) 62:864-872.
55. NAGAHARA H, VOCERO-AKBANI AM, SNYDER EL *et al.*: Transduction of full-length TAT fusion proteins into mammalian cells: TAT-p27Kip1 induces cell migration. *Nat. Med.* (1998) 4:1449-1452.
56. VOCERO-AKBANI A, LISSY NA, DOWDY SF: Transduction of full-length TAT fusion proteins directly into mammalian cells: analysis of T cell receptor activation-induced cell death. *Methods Enzymol.* (2000) 322:508-521.
57. BECKER-HAPAK M, MCALLISTER SS, DOWDY SF: TAT-mediated protein transduction into mammalian cells. *Methods* (2001) 24:247-256.
58. SCHWARZE SR, HO A, VOCERO-AKBANI A, DOWDY SF: *In vivo* protein transduction: delivery of a biologically active protein into the mouse. *Science* (1999) 285:1569-1572.
59. SCHWARZE SR, DOWDY SF: *In vivo* protein transduction: intracellular delivery of biologically active proteins, compounds and DNA. *Trends Pharmacol. Sci.* (2000) 21:45-48.
60. MOY P, DAIKH Y, PEPINSKY B, THOMAS D, FAWELL S, BARSOUM J: TAT-mediated protein delivery can facilitate MHC class I presentation of antigens. *Mol. Biotechnol.* (1996) 6:105-113.
61. KIM DT, MITCHELL DJ, BROCKSTEDT DG *et al.*: Introduction of soluble proteins into the MHC class I pathway by conjugation to an HIV TAT peptide. *J. Immunol.* (1997) 159:1666-1668.
62. KWON HY, EUM WS, JANG HW *et al.*: Transduction of Cu,Zn-superoxide dismutase mediated by an HIV-1 TAT protein basic domain into mammalian cells. *FEBS Lett.* (2000) 485:163-167.
63. JIN LH, BAHN JH, EUM WS *et al.*: Transduction of human catalase mediated by an HIV-1 TAT protein basic domain and arginine-rich peptides into mammalian cells. *Free Radic. Biol. Med.* (2001) 31:1509-1519.
64. EUM WS, JANG SH, KIM DW *et al.*: Enhanced transduction of Cu,Zn-superoxide dismutase with HIV-1 TAT protein transduction domains at both termini. *Mol. Cells* (2005) 19:191-197.
65. KABOURIDIS PS, HASAN M, NEWSON J, GILROY DW, LAWRENCE T: Inhibition of NF-kappa B activity by a membrane-transducing mutant of I kappa B alpha. *J. Immunol.* (2002) 169:2587-2593.
66. GUSTAFSSON AB, SAYEN MR, WILLIAMS SD, CROW MT, GOTTLIEB RA: TAT protein transduction into isolated perfused hearts: TAT-apoptosis repressor with caspase recruitment domain is cardioprotective. *Circulation* (2002) 106:735-739.
67. CAO G, PEI W, GE H *et al.*: *In vivo* delivery of a Bcl-xL fusion protein containing the TAT protein transduction domain protects against ischemic brain injury and neuronal apoptosis. *J. Neurosci.* (2002) 22:5423-5431.
68. DEL GAIZO V, PAYNE RM: A novel TAT-mitochondrial signal sequence fusion protein is processed, stays in mitochondria, and crosses the placenta. *Mol. Ther.* (2003) 7:720-730.

• One of several papers to point at the mitochondrial localisation of TAT-bearing conjugates.

69. DEL GAIZO V, MACKENZIE JA, PAYNE RM: Targeting proteins to mitochondria using TAT. *Mol. Genet. Metab.* (2003) 80:170-180.
70. SHOKOLENKO IN, ALEXEYEV MF, LEDOUX SP, WILSON GL: TAT-mediated protein transduction and targeted delivery of fusion proteins into mitochondria of breast cancer cells. *DNA Repair (Amst.).* (2005) 4:511-518.
71. SELIVANOVA G, IOTSOVA V, OKAN I *et al.*: Restoration of the growth suppression function of mutant p53 by a synthetic peptide derived from the p53 C-terminal domain. *Nat. Med.* (1997) 3:632-638.
72. HARBOUR JW, WORLEY L, MA D, COHEN M: Transducible peptide therapy for uveal melanoma and retinoblastoma. *Arch. Ophthalmol.* (2002) 120:1341-1346.
73. SNYDER EL, MEADE BR, SAENZ CC, DOWDY SF: Treatment of terminal peritoneal carcinomatosis by a transducible p53-activating peptide. *PLoS Biol.* (2004) 2:E36.
74. PARADA Y, BANERJI L, GLASSFORD J *et al.*: BCR-ABL and interleukin 3 promote haematopoietic cell proliferation and survival through modulation of cyclin D2 and p27Kip1 expression. *J. Biol. Chem.* (2001) 276:23572-23580.
75. SHIBAGAKI N, UDEY MC: Dendritic cells transduced with protein antigens induce cytotoxic lymphocytes and elicit antitumor immunity. *J. Immunol.* (2002) 168:2393-2401.
76. WANG HY, FU T, WANG G *et al.*: Induction of CD4(+) T cell-dependent antitumor immunity by TAT-mediated tumor antigen delivery into dendritic cells. *J. Clin. Invest.* (2002) 109:1463-1470.
77. CHAKRABARTI R, WYLIE DE, SCHUSTER SM: Transfer of monoclonal antibodies into mammalian cells by electroporation. *J. Biol. Chem.* (1989) 264:15494-15500.
78. ARNHEITER H, HALLER O: Antiviral state against influenza virus neutralized by microinjection of antibodies to interferon-induced Mx proteins. *EMBO J.* (1988) 7:1315-1320.
79. ANDERSON DC, NICHOLS E, MANGER R, WOODLE D, BARRY M,

FRITZBERG AR: Tumor cell retention of antibody Fab fragments is enhanced by an attached HIV TAT protein-derived peptide. *Biochem. Biophys. Res. Commun.* (1993) 194:876-884.

80. STEIN S, WEISS A, ADERMANN K, LAZAROVICI P, HOCHMAN J, WELLHONER H: A disulfide conjugate between anti-tetanus antibodies and HIV (37-72)TAT neutralizes tetanus toxin inside chromaffin cells. *FEBS Lett.* (1999) 458:383-386.

81. MIE M, TAKAHASHI F, FUNABASHI H, YANAGIDA Y, AIZAWA M, KOBATAKE E: Intracellular delivery of antibodies using TAT fusion protein A. *Biochem. Biophys. Res. Commun.* (2003) 310:730-734.

82. NIESNER U, HALIN C, LOZZI L *et al.*: Quantitation of the tumor-targeting properties of antibody fragments conjugated to cell-permeating HIV-1 TAT peptides. *Bioconjug. Chem.* (2002) 13:729-736.

83. KAISER J: Gene therapy. Side effects sideline hemophilia trial. *Science* (2004) 304:1423-1425.

84. CHECK E: Gene therapy put on hold as third child develops cancer. *Nature* (2005) 433:561.

85. TEMPLETON NS: Cationic liposome-mediated gene delivery *in vivo*. *Biosci. Rep.* (2002) 22:283-295.

86. BROWN MD, SCHATZLEIN AG, UCHEGBU IF: Gene delivery with synthetic (non viral) carriers. *Int. J.Pharm.* (2001) 229:1-21.

87. KIRCHEIS R, WIGHTMAN L, WAGNER E: Design and gene delivery activity of modified polyethylenimines. *Adv. Drug. Deliv. Rev.* (2001) 53:341-358.

88. SNYDER EL, DOWDY SF: Protein/peptide transduction domains: potential to deliver large DNA molecules into cells. *Curr. Opin. Mol. Ther.* (2001) 3:147-152.

89. EGUCHI A, AKUTA T, OKUYAMA H *et al.*: Protein transduction domain of HIV-1 TAT protein promotes efficient delivery of DNA into mammalian cells. *J. Biol. Chem.* (2001) 276:26204-26210.

90. RUDOLPH C, PLANK C, LAUSIER J, SCHILLINGER U, MULLER RH, ROSENECKER J: Oligomers of the arginine-rich motif of the HIV-1 TAT protein are capable of transferring plasmid DNA into cells. *J. Biol. Chem.* (2003) 278:11411-11418.

91. IGNATOVICH IA, DIZHE EB, PAVLOTSKAYA AV *et al.*: Complexes of plasmid DNA with basic domain 47-57 of the HIV-1 TAT protein are transferred to mammalian cells by endocytosis-mediated pathways. *J. Biol. Chem.* (2003) 278:42625-42636.

92. TASCIOTTI E, ZOPPE M, GIACCA M: Transcellular transfer of active HSV-1 thymidine kinase mediated by an 11-amino-acid peptide from HIV-1 TAT. *Cancer Gene Ther.* (2003) 10:64-74.

93. MERILAINEN O, HAKKARAINEN T, WAHLFORS T, PELLINEN R, WAHLFORS J: HIV-1 TAT protein transduction domain mediates enhancement of enzyme prodrug cancer gene therapy *in vitro*: a study with TAT-TK-GFP triple fusion construct. *Int. J. Oncol.* (2005) 27:203-208.

94. JOSEPHSON L, TUNG CH, MOORE A, WEISSLEDER R: High-efficiency intracellular magnetic labeling with novel superparamagnetic-TAT peptide conjugates. *Bioconjug. Chem.* (1999) 10:186-191.

• An early paper on intracellular delivery of nanoparticles by TAT.

95. LEWIN M, CARLESSO N, TUNG CH *et al.*: TAT peptide-derivatized magnetic nanoparticles allow *in vivo* tracking and recovery of progenitor cells. *Nat. Biotechnol.* (2000) 18:410-414.

96. DODD CH, HSU HC, CHU WJ *et al.*: Normal T-cell response and *in vivo* magnetic resonance imaging of T cells loaded with HIV transactivator-peptide-derived superparamagnetic nanoparticles. *J. Immunol. Methods.* (2001) 256:89-105.

97. KAUFMAN CL, WILLIAMS M, RYLE LM, SMITH TL, TANNER M, HO C: Superparamagnetic iron oxide particles transactivator protein-fluorescein isothiocyanate particle labeling for *in vivo* magnetic resonance imaging detection of cell migration: uptake and durability. *Transplantation* (2003) 76:1043-1046.

98. ZHAO M, KIRCHER MF, JOSEPHSON L, WEISSLEDER R: Differential conjugation of TAT peptide to superparamagnetic nanoparticles and its effect on cellular uptake. *Bioconjug. Chem.* (2002) 13:840-844.

99. TKACHENKO AG, XIE H, LIU Y *et al.*: Cellular trajectories of peptide-modified gold particle complexes: comparison of nuclear localization signals and peptide transduction domains. *Bioconjug. Chem.* (2004) 15:482-490.

100. SANTRA S, YANG H, DUTTA D *et al.*: TAT conjugated, FITC doped silica nanoparticles for bioimaging applications. *Chem. Commun. (Camb.)* (2004) 24:2810-2811.

101. STROH M, ZIMMER JP, DUDA DG *et al.*: Quantum dots spectrally distinguish multiple species within the tumor milieu *in vivo*. *Nat. Med.* (2005) 11:678-682.

• An interesting example of using TAT-modified quantum dots for cell tracking.

102. RUDOLPH C, SCHILLINGER U, ORTIZ A *et al.*: Application of novel solid lipid nanoparticle (SLN)-gene vector formulations based on a dimeric HIV-1 TAT-peptide i*in vitro* and *in vivo*. *Pharm. Res.* (2004) 21:1662-1669.

103. LIU J, ZHANG Q, REMSEN EE, WOOLEY KL: Nanostructured materials designed for cell binding and transduction. *Biomacromolecules* (2001) 2:362-368.

104. BECKER ML, REMSEN EE, PAN D, WOOLEY KL: Peptide-derivatized shell-cross-linked nanoparticles. 1. Synthesis and characterization. *Bioconjug. Chem.* (2004) 15:699-709.

105. BECKER ML, BAILEY LO, WOOLEY KL: Peptide-derivatized shell-cross-linked nanoparticles. 2. Biocompatibility evaluation. *Bioconjug. Chem.* (2004) 15:710-717.

106. CUI Z, PATEL J, TUZOVA M *et al.*: Strong T cell type-1 immune responses to HIV-1 TAT (1-72) protein-coated nanoparticles. *Vaccine* (2004) 22:2631-2640.

107. CAPUTO A, BROCCA-COFANO E, CASTALDELLO A *et al.*: Novel biocompatible anionic polymeric microspheres for the delivery of the HIV-1 TAT protein for vaccine application. *Vaccine* (2004) 22:2910-2924.

108. TORCHILIN VP, RAMMOHAN R, WEISSIG V, LEVCHENKO TS: TAT peptide on the surface of liposomes affords their efficient intracellular delivery even at low temperature and in the presence of metabolic inhibitors. *Proc. Natl. Acad. Sci. USA* (2001) 98:8786-8791.

• The first paper to discuss TAT-mediated internalisation of liposomes.

109. TORCHILIN VP, LEVCHENKO TS, RAMMOHAN R, VOLODINA N, PAPAHADJOPOULOS-STERNBERG B, D'SOUZA GG: Cell transfection i*in vitro* and *in vivo* with nontoxic TAT peptide-

liposome-DNA complexes. *Proc. Natl. Acad. Sci. USA* (2003) 100:1972-1977.

110. MARTY C, MEYLAN C, SCHOTT H, BALLMER-HOFER K, SCHWENDENER RA: Enhanced heparan sulfate proteoglycan-mediated uptake of cell-penetrating peptide-modified liposomes. *Cell. Mol. Life Sci.* (2004) 61:1785-1794.

111. HUANG Z, LI W, MACKAY JA, SZOKA FC Jr: Thiocholesterol-based lipids for ordered assembly of bioresponsive gene carriers. *Mol. Ther.* (2005) 11:409-417.

112. HYNDMAN L, LEMOINE JL, HUANG L, PORTEOUS DJ, BOYD AC, NAN X: HIV-1 TAT protein transduction domain peptide facilitates gene transfer in combination with cationic liposomes. *J. Control. Release* (2004) 99:435-444.

113. FRETZ MM, KONING GA, MASTROBATTISTA E, JISKOOT W, STORM G: OVCAR-3 cells internalize TAT-peptide modified liposomes by endocytosis. *Biochim. Biophys. Acta* (2004) 1665:48-56.

114. POLYAKOV V, SHARMA V, DAHLHEIMER JL, PICA CM, LUKER GD, PIWNICA-WORMS D: Novel TAT-peptide chelates for direct transduction of technetium-99m and rhenium into human cells for imaging and radiotherapy. *Bioconjug. Chem.* (2000) 11:762-771.

115. BHORADE R, WEISSLEDER R, NAKAKOSHI T, MOORE A, TUNG CH: Macrocyclic chelators with paramagnetic cations are internalized into mammalian cells via a HIV-TAT derived membrane translocation peptide. *Bioconjug. Chem.* (2000) 11:301-315.

116. KAUFMAN CL, WILLIAMS M, RYLE LM, SMITH TL, TANNER M, HO C: Superparamagnetic iron oxide particles transactivator protein-fluorescein isothiocyanate particle labeling for *in vivo* magnetic resonance imaging detection of cell migration: uptake and durability. *Transplantation* (2003) 76:1043-1046.

117. FALNES PO, WESCHE J, OLSNES S: Ability of the TAT basic domain and VP22 to mediate cell binding, but not membrane translocation of the diphtheria toxin A-fragment. *Biochemistry* (2001) 40:4349-4358.

118. NORI A, JENSEN KD, TIJERINA M, KOPECKOVA P, KOPECEK J: TAT-conjugated synthetic macromolecules facilitate cytoplasmic drug delivery to human ovarian carcinoma cells. *Bioconjug. Chem.* (2003) 14:44-50.

119. NORI A, JENSEN KD, TIJERINA M, KOPECKOVA P, KOPECEK J: Subcellular trafficking of HPMA copolymer-TAT conjugates in human ovarian carcinoma cells. *J. Control. Release* (2003) 91:53-59.

120. NORI A, KOPECEK J: Intracellular targeting of polymer-bound drugs for cancer chemotherapy. *Adv. Drug Deliv. Rev.* (2005) 57:609-636.

Affiliation

Bhawna Gupta[1] MS &
Vladimir P Torchilin[†2] PhD, DSc
[†]Author for correspondence
[1]PhD Student, Department of Pharmaceutical Sciences, Northeastern University, Boston, MA 02115, USA
[2]Professor and Chairman, Department of Pharmaceutical Sciences, Northeastern University, Boston, MA 02115, USA
Tel: +1 617 373 3206; Fax: +1 617 373 8886;
E-mail: v.torchilin@neu.edu