Expert Opinion

TAT-based drug delivery system – new directions in protein delivery for new hopes?

Matan Rapoport & Haya Lorberboum-Galski†
Hebrew University, Department of Cellular Biochemistry and Human Genetics, Jerusalem, Israel

There has been great progress in the use of TAT-based drug delivery systems for the delivery of different macromolecules into cells *in vitro* and *in vivo*, thus circumventing the bioavailability barrier that is a problem for so many drugs. There are many advantages to using this system, such as the ability to deliver these cargoes into all types of cells in culture and into all organs *in vivo*. This system can even deliver cargoes into the brain across the blood–brain barrier. In addition, the ability to target specific intracellular sub-localizations such as the nuclei, the mitochondria and lysosomes further expands the possibilities of this drug delivery system to the development of sub-cellular organelle-targeted therapy. The therapeutic applications seem almost unlimited, and the use of the TAT-based delivery system has extended from proteins to a large variety of cargoes such as oligonucleotides, imaging agents, low molecular mass drugs, nanoparticles, micelles and liposomes. In this review the most recent advances in the use of the TAT-based drug delivery system will be described, mainly discussing TAT-mediated protein delivery and the use of the TAT system for enzyme replacement therapy.

Keywords: cell-penetrating peptides, drug delivery, enzyme replacement therapy, fusion proteins, protein transduction domains, TAT

Expert Opin. Drug Deliv. (2009) 6(5):453-463

1. Introduction

In 1988 the original concept of protein transduction was described by Vives [1], Green [2] and Frankel [3]. They separately reported that the transcriptional activator of transcription (Tat) protein from the human immunodeficiency virus-1 (HIV-1) is able to enter cells in culture when added to the medium. Later, the domain that is responsible for this translocation was identified as the short region of residues 47 – 57, and was named TAT peptide [1-3].

Since then there has been great progress in the field of protein transduction domains (termed PTDs or cell penetrating peptides, CPPs) and more peptides possessing this ability have been described. These include Antennapedia (Antp), VP22, transportan, synthetic oligoarginines (such as R_9) and model amphipathic peptide MAP [4-7]. Most of the original PTDs are short peptides (8 – 16 amino acids) rich in basic residues, arginine and/or lysine, and are therefore highly cationic, however more recently other PTDs have been described [6,8,9]. Among the numerous PTDs, the TAT peptide remains the best known, investigated and tested. TAT is an 11 amino acid portion of the HIV-1 Tat protein that activates transcription of the viral genome. TAT is rich with arginine and lysine, thus highly charged, hydrophilic and basic [2,3,8]. It is well established that proteins that are fused to the TAT peptide are rapidly and efficiently introduced into cultured cells and into live tissues when injected into mice, while retaining their biological activity [10-15].

informa
healthcare

10.1517/17425240902887029 © 2009 Informa UK Ltd ISSN 1742-5247

1.1 TAT delivery mechanism

It seems that there is little consensus regarding the exact mechanism of TAT transfer through biological membranes. This has been the subject of ongoing controversy for the past decade and the accurate mechanisms of translocation are far from being firmly identified. Data published from many different research groups are difficult to compare because of the diversity of the cell type used, the attached or not cargo, the chemical characteristics of this cargo and the different experimental protocols utilized [8,16-21]. Nevertheless, most studies agree on the importance of a direct contact between the TAT and the negative residues on the cell surface as a preliminary requirement for successful transduction to occur.

In contrast, a variety of mechanisms for the translocation itself have been suggested. Among these are two types of endocytic uptake: clathrin-mediated and lipid raft-mediated through the formation of caveolae; and a non-clathrin, non-caveolar endocytosis called macropinocytosis [5-7,22,23]. Macropinocytosis is described as a fluid phase endocytosis that occurs in all cell types, allowing them to take up large extracellular particles from the surrounding medium. Lately, the macropinocytosis model for TAT-mediated protein transduction has become more acceptable and involves numerous steps. First, TAT binds to the cell surface. There is still some uncertainty regarding the identity of the molecules that TAT binds to on the cell surface to facilitate its uptake. There is strong evidence that binding to cell surface polyanionic glycan sugar chains like heparan sulfate has a critical role in the transduction of TAT into the cell. According to this model, this binding promotes macropinocytosis of TAT and cargo into macropinosomes [5-7,22-24]. The last step is evasion from the macropinosomes into the cytoplasm. This is believed to be the rate-limiting step in the efficiency of the transduction.

Many studies have harnessed PTD technology in order to develop therapeutic applications for the delivery of different macromolecules into cells *in vitro* and *in vivo*, thus circumventing the bioavailability barrier that is a problem for so many drugs.

There are many advantages to using a TAT-based delivery system for the introduction of a variety of cargoes into cells and organs. First, it seems that TAT is able to deliver these cargoes into all types of cells in culture and into all organs *in vivo*, even into the brain when injected to mice [6,10,13,14,23,25,26]. This ability of TAT to cross the blood–brain barrier (BBB) makes the TAT-based delivery system a very promising strategy in the development of novel therapeutic approaches to many diseases with CNS involvement. Another important advantage is that this is a non-viral therapy, and thus does not require the integration of foreign nucleic acid sequences into the genome [23]. In addition, it has also been shown that TAT-based delivery systems can be targeted to specific intracellular sub-localizations such as the nuclei, the mitochondria and lysosomes, thus expanding even more the possibilities of this drug delivery system in developing sub-cellular organelle-targeted therapy [27-31].

However, the TAT-mediated delivery system still has major drawbacks. First, this system is used primarily for non-specific targeting, not allowing delivery to a desired cell/organ, thus limiting the clinical applications of this system. In addition, TAT-delivered molecules may cause toxicity at high concentrations or upon prolonged treatment [32]. There are limited toxicity studies on TAT-delivered molecules mainly *in vivo* and more studies are required to address this major concern. Moreover, the ability of the TAT to move out of the cells by the same delivery mechanism can cause lowering of the cargoes' intra-cellular concentrations, thus limiting their desired effect. This may influence the amounts of TAT-delivered molecules administered in order to achieve physiological benefit. Most important is the possible immunogenicity of the TAT delivery system. It was speculated that the TAT, especially through repeated dosing, would produce a significant immunogenic response, thus limiting its clinical applications. This major issue remains to be further examined.

At this point, the therapeutic applications seem almost unlimited, and the use of the TAT-based delivery system has extended from proteins to a large variety of cargoes such as oligonucleotides, imaging agents, low molecular mass drugs, nanoparticles, micelles and liposomes [5,6,19,21-23].

In this review recent advances in the use of the TAT-based drug delivery system will be described, mainly focusing on protein delivery, with an emphasis on the use of TAT for enzyme replacement therapies (ERT).

2. TAT-mediated nucleic acid delivery

Efficient and safe delivery of nucleic acids is necessary for successful gene therapy in humans. Recently, with the discovery of small interference RNA (siRNA) and its ability to specifically and efficiently downregulate gene expression, the delivery of nucleic acids into cells became a major therapeutic goal for many diseases. Numerous gene delivery systems have been developed over the years; however they exhibit major drawbacks which have limited their clinical application. The viral carriers are highly efficient but can present problems of high immunogenicity and insertional mutagenesis. The non-viral carriers such as cationic polymers have low efficiency and high toxicity [33,34]. This has led researchers to explore the possibility of using the TAT-based system for the delivery of oligonucleotides. This includes the delivery of peptide nucleic acid (PNA), phosphorodiamidate morpholino oligomers (PMO) and siRNA [19,35]. However, TAT delivery of conjugated PNA or PMO has been shown to be of relatively poor efficacy [35]. Linking TAT to oligonucleotides can be achieved in several ways: non-covalent packaging, covalent packaging by the formation of a disulfide bond (chemical conjugation), construction of TAT fusion proteins that bind nucleic acids or through the production of nanoparticles that contain the nucleic acids [33,34,36,37]. The latter will be discussed in the section on 'TAT-mediated nanoparticle delivery'.

An interesting strategy for the delivery of PNAs with TAT was presented in a paper by Folini *et al* [38]. The authors used a combined TAT delivery system with a photochemical internalization (PCI) technique that relies on the properties of photosensitive molecules (photosensitizers) to allow light-induced permeabilization of endocytic vesicles. This leads to the release of endocytozed macromolecules into the cytoplasm, thus overcoming one of the obstacles of delivering nucleic acids into cells, which is their entrapment in the endocytic vesicules and degradation upon lysosomic fusion. This has been shown to be one of the major rate-limiting steps of the intracellular delivery of nucleic acids. PNAs are DNA mimics in which a pseudopeptide backbone composed of *N*-(2-aminoethyl) glycine units replaces the phosphate backbone. PNAs have excellent hybridization properties and are extremely stable in biological systems, due to their resistance to nucleases and peptidases. Cell-penetrating PNA conjugates have been generated by coupling PNAs with different PTDs. In this study, the authors conjugated a 15-mer PNA targeting human telomerase reverse transcriptase (hTERT) with the TAT and combined PCI of the conjugates to deliver them more efficiently into DU145 human prostate cancer cells (**Figure 1**).

hTERT is the catalytic retrotranscriptase subunit of the telomerase, an RNA-dependent DNA polymerase that is able to maintain telomere length. It has been recently reported that hTERT plays a role in protection of genome stability by contributing to telomere capping and chromatin resetting during DNA replication. In addition, hTERT was found to be important for maintaining tumor cell survival and proliferation. Thus targeting the telomerase has been proposed as a promising anticancer therapy, and a variety of inhibitory strategies have been successfully developed including those relying on the use of conventional or modified antisense oligonucleotides such as PNAs. The authors showed that the combined strategy is more efficient for the delivery of PNAs than simple conjugation to an internalizing peptide or the use of PCI with unconjugated PNAs. Photochemical internalization of hTERT-PNA-TAT led to almost complete inhibition of telomerase activity and cell growth, whereas photochemical internalization of the hTERT-PNA alone resulted in only a 60% reduction of telomerase activity and a 55% reduction in cell growth. Reduction in telomerase activity by hTERT-PNA-TAT correlated with a marked reduction in telomerase expression levels, acute DNA damage response and induction of caspase-mediated apoptosis in the cells [38].

Another interesting approach to the delivery of nucleic acids using the TAT delivery system is the use of designed multi-domain proteins [37,39]. This approach is based on the combination of various peptide motifs to create a molecule that is more efficient in both DNA binding and transfer into cells and their nuclei. The novel fusion protein contains TAT fused to an adenoviral protein Mu, which is rich in basic amino acids residues and has the potential to bind DNA and also enhance the cationic lipid-mediated transfection [39]. Mu is a 19 amino acid peptide that is associated with the adenoviral core complex. This peptide possesses a DNA-condensing ability, thus improving the passage of the DNA through biologic membranes. The authors combined the TAT and Mu moieties believing that this fusion protein would benefit from the DNA condensation ability of the Mu, the transduction through cellular membrane ability of the TAT and its ability to localize into the nucleus in order to efficiently deliver DNA through the plasma and nuclear membranes. Indeed, TAT-Mu was able to bind DNA and mediate its transfer into CHOK-1, MCF-7 and COS cells [39]. The researchers continued their work by generating a newly designed three-domain cationic fusion protein that contained, in addition, a nuclear localization signal (NLS) to facilitate the transfection efficiency [37]. This TAT-NLS-Mu (TNM) contains three epitopes, each of them contributing to the transfection efficacy – the Mu is the DNA binding domain, the TAT is the transduction domain and the NLS aids in the nuclear entry of the protein carrying the sequences. The results demonstrated the ability of TNM to condense DNA efficiently and successfully transfect a variety of cells. However, the authors showed in addition that conjugation of the fusion protein with cationic lipid nanoparticles improved the transfection efficiency considerably [37].

3. TAT-mediated nanoparticle delivery

The TAT delivery system for the transduction of many therapeutic molecules, including nucleic acids, has been widely used recently in combination with known nanocarriers such as solid lipid nanoparticles (SLN), polymeric nanoparticles (complexes of polyethyleneiminine and DNA), thiocholesterol-based cationic lipids (TLC), lipoplexes and liposomes. Conjugating TAT with these nanocarriers, often named TAT-modified nanoparticles, combines the advantages of these two delivery systems and improves their effectiveness both *in vitro* and *in vivo* [6,7,22]. The nanocarriers are used to increase the stability of the administered therapeutic molecules, improve their efficacy and decrease undesired side effects. Among the most investigated drug carriers are liposomes (mainly for the delivery of water soluble drugs) and micelles (for the delivery of poorly soluble drugs). Liposomes are artificial phospholipid vesicles with a size of 50 – 1000 nm, which can be loaded with a variety of drugs. Micelles are colloidal dispersions with a particle size within the 5 – 100 nm range, which have the ability to increase the solubility and bioavailability of poorly soluble pharmaceuticals [22]. It has been widely shown that the addition of TAT on the surface of nanoparticles enhances their ability to be delivered across cellular membranes and also across the BBB. In two recently published papers [40,41] the authors tested the ability of TAT-modified micelles to deliver antibiotics across the BBB for the treatment of brain inflammation caused by acute bacterial infection. They used self-assembled polymeric micelles from cholesterol-conjugated polyethylene glycol (PEG) conjugated with TAT named TAT-PEG-b-Chol. The authors demonstrated the successful encapsulation of

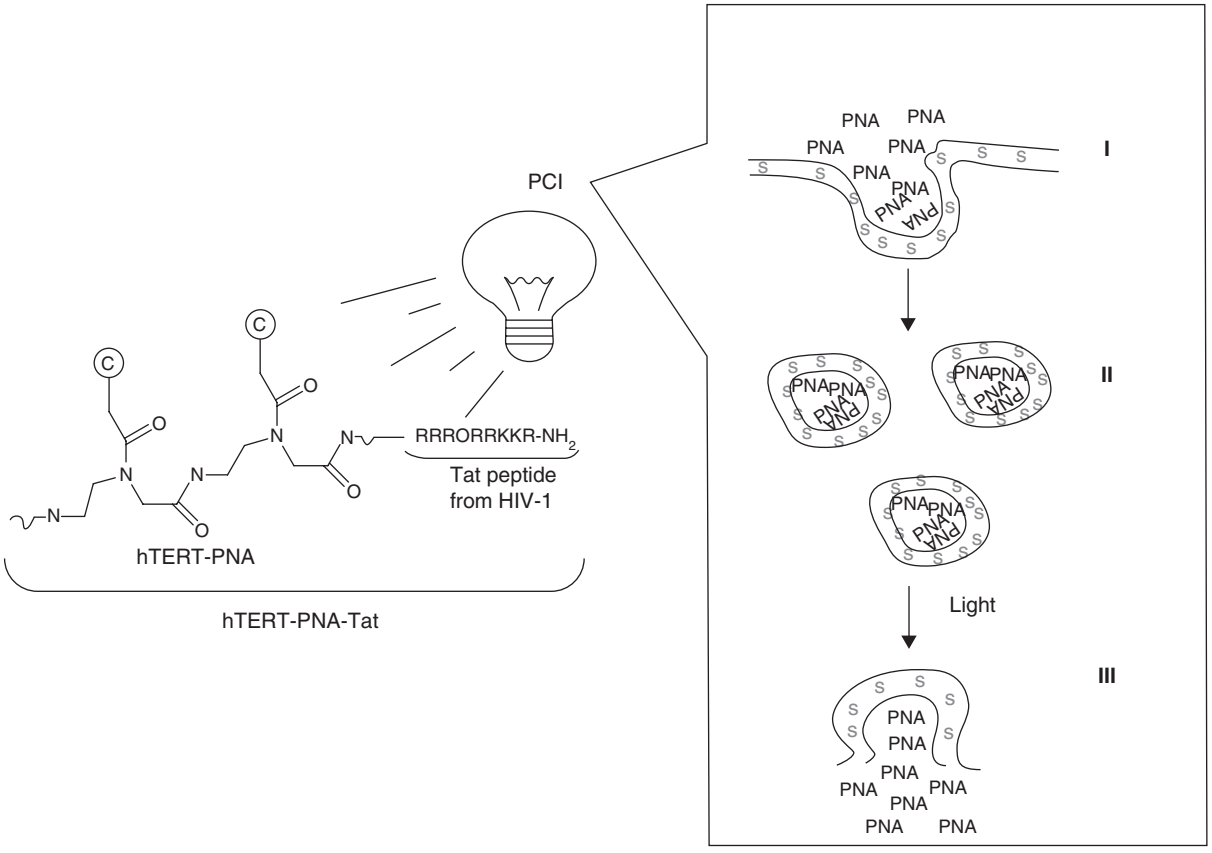

Figure 1. Schematic representation of hTERT-PNA-TAT and the photochemical internalization approach. Representation of the combined approach employed to deliver PNA-targeting hTERT into DU145 cells. The schematic structure of hTERT-PNA conjugated to the TAT-internalizing peptide along with a draft of the photochemical internalization (PCI) technique is depicted. The right panel summarizes the principle of PCI: (**I**) endocytosis of the photosensitizer (S) and the therapeutic molecule (e.g., PNA); (**II**) localization of the photosensitizer and the therapeutic molecule in the same endocytic vesicles; (**III**) rupture of the endosomal membrane upon light exposure and subsequent release of the therapeutic molecule into the cytosol.
Taken from [38].

ciprofloxacin with these micelles. They showed that when loaded with FITC, the TAT-PEG-b-Chol micelles were able to enter human astrocytes in culture faster and more efficiently than FITC-loaded PEG-b-Chol micelles lacking the TAT moiety, because the TAT promotes the cellular uptake of the micelles. Most importantly, i.v. administration of the FITC-loaded TAT-PEG-b-Chol micelles into rats resulted in FITC signaling in hippocampus brain sections after 2 – 4 h, thus demonstrating the ability of these micelles to cross the BBB [40,41].

In a different study, the authors used TAT-conjugated nanoparticles (NPs) for the delivery of anti-HIV drugs into the central nervous system (CNS) [42]. The HIV-1 enters the CNS at an early stage of infection, making the CNS one of the principal reservoirs for the replicating virus, which reinfects the peripheral tissues, thus causing continuous reactivation of the infection. In addition, the HIV-1 virus causes neurological symptoms such as progressive dementia, memory loss and encephalopathy [42]. Some of the anti-retroviral drugs that are currently used in the treatment of HIV are protease inhibitors (PIs) that block the protease enzyme required by the virus to replicate. These PIs have poor bioavailability in the CNS because they are substrates of the human multidrug resistance transporter P-glycoprotein (MDR-1), which prevents their passage through the BBB. The authors show that TAT-conjugated NPs loaded with ritonavir (a model PI) are delivered into both MDCK cells and MDR1-expressing MDCK cells more efficiently than NPs that are not conjugated to TAT. Furthermore, when ritonavir levels in the brains of mice were assessed 2 weeks after administration, the level of ritonavir was 800-fold higher in the TAT-conjugated NPs group compared to that administered with ritonavir in solution. This demonstrated the greater efficacy of TAT-conjugated NPs to transport the ritonavir across the BBB and sustain its level at a therapeutically significant range over a long period. The authors discuss the localization of the NPs in the brain ventricles, suggesting that NPs are retained in these cavities initially, then diffuse to the cerebral cortex through secretion into cerebrospinal fluids (CSF). The CSF is one of the chief reservoirs for HIV-1 in the CNS,

and a high concentration of PIs within this compartment is essential for their therapeutic efficacy. The authors suggest that the combined effect of sustained release properties and the ability to overcome the cellular and tissue barriers make TAT-conjugated NPs a promising strategy that could improve the therapeutic efficacy of PIs and other anti-HIV drugs [42].

Another use of the TAT delivery system with nanoparticles is enhancing the transmembrane permeability of semiconductor quantum dots (QDs) [43-47]. QDs are a novel type of fluorescence probes that have recently shown great potential for biomolecular labeling, cellular imaging and cellular tracking. They possess many advantages such as their nanometer size, broad excitation but narrow emission, bright photoluminescence and high stability, however their ability to cross cellular membranes is insufficient for research purposes [47]. The conjugation of QDs with TAT allows their efficient delivery into cells and live tissues. One of the uses of TAT-QDs is to further characterize the mechanisms underlying TAT cellular uptake [45,47]. Other uses include labeling cells in rat brains [44] by administration of TAT-CdS:Mn/ZnS QDs that cross the BBB, or labeling mesenchymal stem cells using TAT-conjugated PEG-encapsulated CdS/ZnS QDs and following their tissue distribution upon administration into NOD/SCID beta2 M null mice [46].

4. TAT-mediated protein delivery

The exogenous delivery of proteins into cells has many possible therapeutic prospects. These include influencing various signaling cascades; affecting cell cycle progression; restoring deficiencies in enzymes; driving cells into apoptosis or saving them from that fate; and manipulation of cellular function and differentiation. In the late 1990s the first use of TAT as a delivery moiety for introducing proteins into cells *in vitro* [48,49] and in mice *in vivo* was suggested [14]. Dowdy and colleagues demonstrated the delivery of biologically active β-galactosidase (which is a very large protein – 120kDa) fused with TAT into many tissues in mice, including the brain, by intraperitoneal injection [14].

Since then numerous studies have reported on the use of TAT for the delivery of various proteins into cells in the form of TAT-fusion proteins or TAT–protein conjugates. There are many different examples for the delivery of proteins into cells via TAT, and the variety of the purposes of this delivery is vast. This review will describe the different use of TAT-fusion proteins.

4.1 TAT-fusion proteins in cancer therapy

One of the therapeutic fields in which TAT-fusion proteins are widely used is cancer therapy. Many different possible therapeutic strategies involving protein delivery have been proposed and tested as part of the fight against cancer. Among these strategies are restoring the tumor suppressor function and pro-apoptotic activity of p53 [9] and blocking the cell proliferation signals transmitted by the insulin-like growth factor-I receptor (IGF-IR), which a variety of cancer cells are dependent on for their growth [9].

Another approach is making chemotherapy- or radiotherapy-resistant cancer cells more prone to apoptosis by delivering the pro-apoptotic protein Smac [9]. Smac is normally released from the mitochondria into the cytosol in response to apoptotic stimuli and antagonizes inhibitors of apoptosis proteins (IAPs). In many malignant tumors IAPs are over-expressed and inhibit caspase activity, thus making the cells resistant to anticancer therapies. The delivery of TAT-Smac into these cells should upregulate its activity, making the cells more responsive to apoptosis signals [9].

A very recent study employed TAT-NPMΔC fusion protein in an inflammation-associated leukemia model in mice to treat cancer [50]. The authors investigated the therapeutic value of targeting the over-expression of nucleophosmin (NPM) in cancer cells. NPM is a multifunctional protein that plays important roles in the regulation of cell proliferation and apoptosis, and in cancer it is often found to be over-expressed. NPM has been identified as an NF-κB coactivator. The authors fused the N-terminus of the NPM (NPMΔC) with TAT peptide and found that it was delivered into pre-leukemic cells in culture and into tissues of leukemic mice. They used an inflammation-associated leukemogenic model and reported that TAT-NPMΔC treatment delayed leukemic development in mice. They showed that this effect may be attributed to the fact that in leukemic cells the fusion protein specifically interfered with NF-κB function by forming complexes with the endogenous NPM and NF-κB, thus inactivating them both. This interference may lead to the repression of NF-κB transactivation of numerous inflammatory and anti-apoptotic genes, thus resulting in a beneficial effect on leukemic development in mice. The positive effect may be also contributed to the fact that the over-expressed NPM was trapped as biologically nonfunctional complexes with the TAT-NPMΔC. The authors suggested that TAT-NPMΔC could be used to treat inflammation-associated tumors that require NF-κB signaling for survival [50].

4.2 TAT-fusion protein delivery in the treatment of various diseases with an emphasis on those with CNS involvement

As previously mentioned, the possibilities when using TAT-fusion proteins are almost endless. Moreover, the ability of the TAT-fusion proteins to cross the BBB encouraged many researchers to use this system in developing therapies for diseases with CNS manifestations.

In a recent report, Kubo and his co-workers used TAT-peroxiredoxin 6 (PRDX6) to delay cataractogenesis [51]. PRDX6 is an antioxidant enzyme that detoxifies reactive oxygen species (ROS), thus providing cells with protection against internal or external oxidative stress. PRDX6 is highly expressed and active in the lens, and the authors had previously shown that a decrease in its activity in the lens, due to environmental stress or aging, is one of the causes of cataract progression in a rat model. The authors evaluated the therapeutic value of TAT–PRDX6 in postponing the onset of

cataractogenesis in Prdx6 knockout mice. They showed that TAT–PRDX6 can be introduced into cells or whole lenses *in vitro* and *in vivo* and protect them from oxidative stress by delaying the formation of lens opacity [51].

Two recent papers deal with the development of a novel therapy for cerebral injuries. The authors set out to prevent apoptosis of injured neurons through blocking the signals that prevent myelin regeneration and axonal growth, which are crucial for recovery from strokes and traumatic brain and spinal cord injuries [52,53]. They fused TAT with the Nogo extracellular peptide 1 – 40 (NEP1 – 40) in order to deliver it through the BBB into the brain. NEP1 – 40 is an antagonist of Nogo-66 receptor (NgR). Nogo-66 has a central role in limiting axonal regeneration after CNS injuries. It has been shown that the blockage of Nogo-66's interaction with NgR using NEP1 – 40 increases functional recovery and axonal growth regeneration after spinal cord injury. TAT-NEP1 – 40 fusion protein was shown to be delivered into PC12 cells *in vitro* (using the oxygen glucose deprivation [OGD]-induced PC12 cells injury model) and through the BBB into rat brains *in vivo* when injected intraperitoneally. In PC12 cells that were exposed to OGD, treatment with TAT–NEP1 – 40 in the culture medium decreased cell death, upregulated the BCL-2/Bax ratio and promoted neurite outgrowth. This suggests that the TAT–NEP1 – 40 fusion protein has a protective effect against the lack of oxygen and glucose which mimics, for example, the initial phase of a stroke. Moreover, given the fact that it can cross the BBB, it could be a potential treatment for CNS injury [52,53].

4.3 TAT-fusion protein delivery in enzyme replacement therapy

The ability to deliver an active enzyme into cells has raised the possibility of developing many ERTs using the TAT-based delivery system. ERT is a therapeutic approach to metabolic disorders whereby the deficient or absent enzyme is artificially manufactured, purified and given to patients on a regular basis. At present, ERT is the successful treatment of choice for metabolic lysosomal storage diseases [54]. However, the inability of the administered enzymes to penetrate the BBB severely limits this approach from being implemented in the treatment of other metabolic disorders involving the CNS [54-56]. Hence, the ability of TAT to deliver active enzymes across the cellular membrane and especially across the BBB makes this delivery system very promising for the development of therapies in this field.

It should be emphasized that usually in these types of genetic disorders the administration of the deficient enzyme would be on a regular basis, raising problems regarding the immune response to the delivery system.

In a recent report of a potential epilepsy treatment, TAT was used to deliver creatine kinase (CK) into PC12 cells in order to restore its activity there [57]. CK is a member of the phosphagen kinase enzyme family and plays a key role in energy metabolism in cells with high energy demands, such as neurons. It has been shown that CK expression and activity is significantly reduced in some neurodegenerative diseases including epilepsy. In this study the authors designed the TAT–CK protein and tested its ability to raise CK activity in PC12 cells. They showed that the CK activity in PC12 cells treated with TAT-CK increased in a time- and dose-dependant manner. The intracellular CK activity levels increased sixfold after only 1 h incubation with the fusion protein [57].

Another example of the augmentation of the activity of an enzyme using the TAT delivery system was described in a report on the restoration of the activity of purine nucleoside phosphorylase (PNP) *in vivo* in PNP-deficient mice by the administration of TAT–PNP [26]. PNP is a ubiquitous cytoplasmic enzyme essential for purine degradation and salvage. Deficiency in PNP activity results in nucleoside imbalance, leading to T-cell immunodeficiency. TAT–PNP fusion protein was shown to be rapidly and efficiently delivered into many tissues of $PNP^{-/-}$ mice including the brain. The delivered PNP was active within the various tissues, not toxic and protected from neutralizing antibodies. Most importantly, the authors showed that the administration of TAT–PNP over a period of 24 weeks corrected the metabolic abnormalities and immunodeficiency resulting in normal thymus weight, maturation of thymocytes and T lymphocyte number and function. Moreover, 77.3% of $PNP^{-/-}$ mice treated with TAT–PNP were still surviving after 24 weeks, compared with no survival in the control PBS-treated mice, demonstrating the prolonged benefit of intracellular delivery of PNP into the deficient mice [26].

These last two examples of restoring the activity of deficient enzymes lead us to two interesting projects that were conducted in our laboratory. We have been working on developing ERT for different metabolic disorders using the TAT delivery system. One of these disorders is phenylketonuria (PKU), which results from a mutation in the liver enzyme phenylalanine hydroxylase (PAH). PAH is a key enzyme in the metabolic pathway of phenylalanine (Phe). To restore PAH activity in the liver of PKU patients, we constructed the TAT–PAH fusion protein. We demonstrated that TAT–PAH is delivered into a variety of human liver cell lines and PAH activity is retained after internalization. We also showed that plasma phenylalanine levels were dramatically lowered in mice treated with TAT–PAH after i.v. administration. Plasma Phe levels started to decrease 15 min after i.v. TAT–PAH injection and had decreased dramatically by 30 min. Phe levels remained < 20% of the levels in control untreated mice for as long as 6 h. It is important to point out that this decrease in Phe levels was seen in healthy C57BL mice. This effect would probably be stronger in the PKU mouse model because plasma Phe levels in this mouse are much higher, and the main objective in treatment is lowering and maintaining a normal Phe concentration (**Figure 2**) [58].

Another type of metabolic disorder for which we have been trying to develop ERT using the TAT delivery system is mitochondrial disorders. We have been working on lipoamide dehydrogenase (LAD) deficiency. LAD is the E3 subunit of the

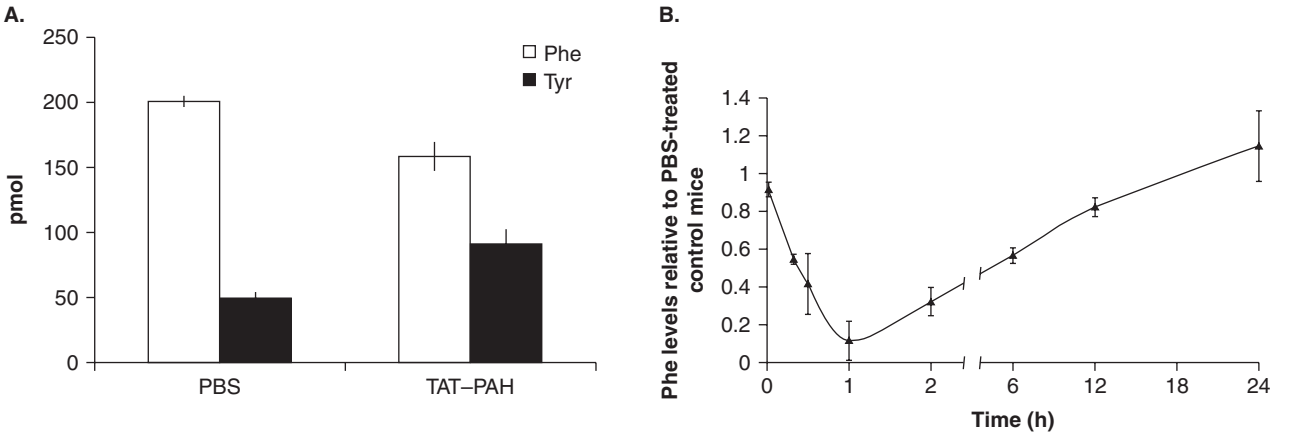

Figure 2. Effect of TAT–PAH on mouse primary cell lines and *in vivo*.

(**A**) Phenylalanine hydroxylase (PAH) activity in extracts of mouse primary hepatocytes treated with PBS and TAT–PAH (20 µg/ml final concentration) for 3 h. Results are expressed as mean ± SEM of three independent repeats. (**B**) Plasma Phe levels relative to PBS-treated control mice. Plasma Phe concentrations were measured by high performance liquid chromtography (HPLC) after i.v. injection of TAT–PAH (20 µg) (as described [58]). Three C57Bl mice were used for each time point. Results are expressed as means ± SEM (taken from [58]).

three α-ketoacid dehydrogenase complexes in the mitochondrial matrix, which are crucial for the metabolism of carbohydrates and amino acids. These are the pyruvate dehydrogenase complex (PDHC), the α-ketoglutarate dehydrogenase complex (KGDHC) and the branched chain ketoacid dehydrogenase complex (BCKDHC). Modern medicine offers no cure for patients with LAD deficiency. Therefore, we set out to test the plausibility of ERT in the treatment of LAD deficiency as a proof-of-concept for the treatment of mitochondrial disorders.

Repair of mitochondrial disorders is necessarily more complex than replacement of a cytosolic gene product and must take into account not only the need to target and cross multiple membranes in mitochondria, but also the fact that many enzymes in mitochondria are components of massive enzymatic complexes and need to be processed naturally in order to integrate properly. Additionally, many of the mitochondrial gene defects cause severe neurologic symptoms as the primary, or most prominent, phenotype, and, as mentioned above, drug delivery across the BBB is difficult.

Specifically, in the case of LAD deficiency, the replacing enzyme needs to be delivered into the cells and reach the mitochondria, be processed there and be incorporated in three multi-component enzymatic complexes.

We constructed and highly purified the TAT–LAD fusion protein. We used the natural pre-cursor sequence of the human LAD containing the N-terminus 35 amino acid mitochondrial targeting sequence (MTS) to facilitate the natural processing of the TAT–LAD upon delivery into the mitochondria, thus allowing the incorporation of the delivered LAD into the α-ketoacid dehydrogenase complexes. We demonstrated that TAT–LAD enters patients' cells rapidly and efficiently, reaching the mitochondria. Inside the mitochondria, TAT–LAD is naturally processed and restores LAD activity there. Most importantly, we showed that TAT–LAD is able to restore the activity of the PDHC within treated patients' cells almost back to normal levels [29]. PDHC is a 9.5×10^6 Da macromolecular machine whose multipart structure assembly process involves numerous different subunits: a pentagonal core of 60 units of the E2 component (dihydrolipoamide), attached to 30 tetramers of the E1 component ($\alpha_2\beta_2$) (pyruvate decarboxylase), 12 dimers of the E3 (LAD, dihydrolipoamide) component and 12 units of the E3 binding protein. The structure of all α-ketoacid dehydrogenase complexes is similar to that of PDHC [29]. The complexity of this structure emphasizes our achievement in showing for the first time that TAT-mediated replacement of one mutated component restores the activity of an essential mitochondrial multi-component enzymatic complex in cells of enzyme-deficient patients (**Figure 3**) [29].

In addition, we took advantage of the native MTS of LAD and showed that it was necessary for maximal restoration of LAD enzymatic function. Deleting the MTS restored a significantly smaller amount of LAD activity within the mitochondria [29]. This is an important point to consider as TAT can move both ways across a membrane and thus pull the therapeutic cargo out of the mitochondria. With the MTS included, the matrix processing peptidases recognize the sequence and clip it, and the cargo (in this case the mature LAD) is left in the matrix while the TAT peptide can transduce out of the mitochondrion. Repeated dosing should therefore result in accumulating amounts of cargo in the mitochondria over time.

Another important point is that in these types of metabolic and especially mitochondrial disorders there is no need to restore enzyme activity back to 100%, but rather raise it above a certain energetic threshold which can vary from patient to patient depending on basal enzymatic activity. So the delivery of sufficient amounts of the deficient enzyme can be surprisingly easy to achieve, even into the CNS.

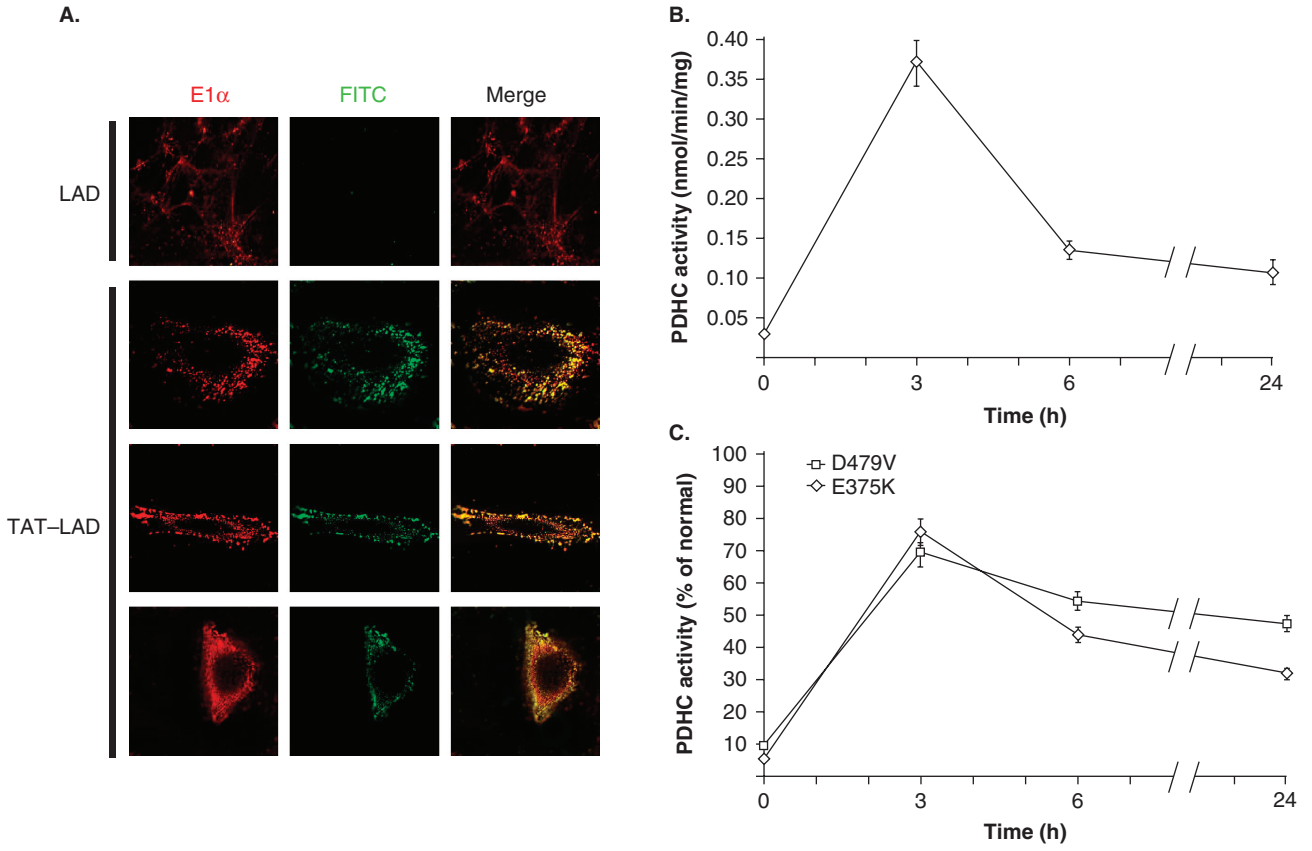

Figure 3. Pyruvate dehydrogenase complex (PDHC) co-localization and enzymatic activity in TAT–LAD treated patients' cells. (**A**) D479V cells were treated with FITC-labeled TAT–LAD or LAD (green), washed, fixed, permeabilized and incubated with anti-E1α antibody. Cells were then washed and incubated with anti-mouse Cy5 antibody (red). Cells were analyzed for co-localization by confocal microscopy (merge, yellow). Original magnifications: × 60 (LAD) and × 100 (TAT–LAD). (**B**–**C**) Cells were incubated with TAT–LAD (0.1 µg/µl, final concentration) for 3, 6 and 24 hrs. PDHC activity assays were performed as described in [29]. (**B**) PDHC activity in treated E375K patients' cells. Activity values are presented as nmol/min/mg protein. (**C**) PDHC activity in treated E375K and D479V patients' cells. Activity values are presented as percentage of normal PDHC activity measured in healthy fibroblasts in the same experiments. Activity assays were repeated three times. The values (in b and c) are presented as the mean ± SD.
Taken from[29].

We are now investigating TAT–LAD treatment in a mouse model of LAD deficiency with very encouraging results (unpublished data).

5. Expert opinion

With progress in molecular biology, completion of the human genome project and our advanced understanding of a variety of biological fields, many long-standing dreams in the realm of medicine regarding the delivery of various molecules to cells and even to their intra-cellular organelles/compartments are coming close to fruition. This has been achieved mainly by the discovery of PTDs which enable the rapid and efficient introduction of proteins, fused to them, into cultured cells and live tissues in a whole organism.

Among these PTDs, the TAT peptide is the most investigated and used domain. TAT was first used for the delivery of proteins into cells. Now, however, the research capabilities and the therapeutic applications seem almost unlimited, and the use of the unique TAT-based delivery system has extended from proteins to a large variety of cargoes such as oligonucleotides, imaging agents, low molecular mass drugs, nanoparticles, micelles and liposomes.

However, the main use of the TAT delivery system remains for the delivery of proteins. TAT-fusion proteins have been tested in a large variety of pathological conditions including cancer, diseases with CNS involvement and more recently metabolic disorders, in which inborn mutations are carried in 'housekeeping enzymes'. In the latter case ERT is being used as part of the effort to develop novel modalities for such disorders. Huge progress in this direction has been made in recent years and, as reported by our group, TAT has also been shown to be a very promising treatment strategy for mitochondrial disorders, which are

considered to be a very complicated set of diseases. We demonstrated for the first time that TAT-mediated replacement of a single component of a multi-enzymatic complex can actually restore the activity of a whole machine, even a very complex one, such as the Pyruvate Dehydrogenase Complex (PDHC), thus making this approach very promising. Moreover, using the native organelles' targeting sequences, such as the mitochondrial targeting sequence (MTS) or the nuclear localization sequence (NLS), fused to TAT and the desired molecule ensures that the desired molecule stays in the target compartment, where it is cleaved off, thus also staying in its natural form. This allows for the delivered proteins or enzymes to be readily integrated into multi-component complexes, if needed.

This approach could be applied to the many other known mitochondrial and metabolic disorders in which the damaged enzymes/proteins involved have been identified and cloned. We believe that this approach will revolutionize the management of these types of disorders in modern medical practice.

The TAT delivery system still has some limitations. As mentioned, this system is primarily non-specific, making delivery to a desired organ more complicated. In addition, the ability of the TAT to move out of the cells can cause lowering in the cargoes' intra-cellular concentrations, thus limiting their desired effect. Administering adequate amounts of the TAT delivery system to overcome this limitation can cause toxicity side effects. Most important is the immunogenic issue of the TAT delivery system. It was speculated that TAT-delivered molecules, especially through repeated dosing, would produce a significant immunogenic response, thus preventing clinical applications of this promising technology. These major drawbacks remain to be further studied in detail.

However, although PTD/TAT delivery systems are still basic tools, it is virtually certain that they will advance in the very near future to novel treatment modalities for a variety of human diseases.

Declaration of interest

The authors state no conflict of interests and have received no payment in the preparation of this manuscript.

Bibliography

1. 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(25):16010-7
2. Green M, Loewenstein PM. Autonomous functional domains of chemically synthesized human immunodeficiency virus TAT trans-activator protein. Cell 1988;55(6):1179-88
3. Frankel AD, Pabo CO. Cellular uptake of the TAT protein from human immunodeficiency virus. Cell 1988;55(6):1189-93
4. Torchilin VP. TATp-mediated intracellular delivery of pharmaceutical nanocarriers. Biochem Soc Trans 2007;35(Pt 4):816-20
5. Torchilin VP. TAT peptide-mediated intracellular delivery of pharmaceutical nanocarriers. Adv Drug Deliv Rev 2008;60(4-5):548-58
6. Gupta B, Torchilin VP. Transactivating transcriptional activator-mediated drug delivery. Expert Opin Drug Deliv 2006;3(2):177-90
7. Murriel CL, Dowdy SF. Influence of protein transduction domains on intracellular delivery of macromolecules. Expert Opin Drug Deliv 2006;3(6):739-46
8. Herce HD, Garcia AE. Molecular dynamics simulations suggest a mechanism for translocation of the HIV-1 TAT peptide across lipid membranes. Proc Natl Acad Sci USA 2007;104(52):20805-10
9. Harada H, Kizaka-Kondoh S, Hiraoka M. Antitumor protein therapy; application of the protein transduction domain to the development of a protein drug for cancer treatment. Breast Cancer 2006;13(1):16-26
10. Cai SR, Xu G, Becker-Hapak M, et al. The kinetics and tissue distribution of protein transduction in mice. Eur J Pharm Sci 2006;27(4):311-9
11. 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(8):5836-40
12. Luft FC. Transducing proteins to manipulate intracellular targets. J Mol Med 2003;81(9):521-3
13. Schwarze SR, Dowdy SF. In vivo protein transduction: intracellular delivery of biologically active proteins, compounds and DNA. Trends Pharmacol Sci 2000;21(2):45-8
14. 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(5433):1569-72
15. Schwarze SR, Hruska KA, Dowdy SF. Protein transduction: unrestricted delivery into all cells? Trends Cell Biol 2000;10(7):290-5
16. Jones AT. Gateways and tools for drug delivery: endocytic pathways and the cellular dynamics of cell penetrating peptides. Int J Pharm 2008;354(1-2):34-8
17. Lundin P, Johansson H, Guterstam P, et al. Distinct uptake routes of cell-penetrating peptide conjugates. Bioconjug Chem 2008;19(12):2535-42
18. Brooks H, Lebleu B, Vives E. TAT peptide-mediated cellular delivery: back to basics. Adv Drug Deliv Rev 2005;57(4):559-77
19. Howl J, Nicholl ID, Jones S. The many futures for cell-penetrating peptides: how soon is now? Biochem Soc Trans 2007;35(Pt 4):767-9
20. Vives E. Present and future of cell-penetrating peptide mediated delivery systems: 'is the Trojan horse too wild to go only to Troy?'. J Control Release 2005;109(1-3):77-85
21. Chauhan A, Tikoo A, Kapur AK, Singh M. The taming of the cell penetrating domain of the HIV TAT: myths and realities. J Control Release 2007;117(2):148-62
22. Torchilin VP. Cell penetrating peptide-modified pharmaceutical nanocarriers for intracellular drug and gene delivery. Biopolymers 2008;90(5):604-10

23. Gump JM, Dowdy SF. TAT transduction: the molecular mechanism and therapeutic prospects. Trends Mol Med 2007;13(10):443-8
24. Amand HL, Fant K, Norden B, Esbjorner EK. Stimulated endocytosis in penetratin uptake: effect of arginine and lysine. Biochem Biophys Res Commun 2008;371(4):621-5
25. Becker-Hapak M, McAllister SS, Dowdy SF. TAT-mediated protein transduction into mammalian cells. Methods 2001;24(3):247-56
26. Toro A, Grunebaum E. TAT-mediated intracellular delivery of purine nucleoside phosphorylase corrects its deficiency in mice. J Clin Invest 2006;116(10):2717-26
27. Del Gaizo V, MacKenzie JA, Payne RM. Targeting proteins to mitochondria using TAT. Mol Genet Metab 2003;80(1-2):170-80
28. 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(6):720-30
29. Rapoport M, Saada A, Elpeleg O, Lorberboum-Galski H. TAT-mediated delivery of LAD restores pyruvate dehydrogenase complex activity in the mitochondria of patients with LAD deficiency. Mol Ther 2008;16(4):691-7
30. Zhang XY, Dinh A, Cronin J, et al. Cellular uptake and lysosomal delivery of galactocerebrosidase tagged with the HIV TAT protein transduction domain. J Neurochem 2008;104(4):1055-64
31. Yoshikawa T, Sugita T, Mukai Y, et al. Organelle-targeted delivery of biological macromolecules using the protein transduction domain: potential applications for Peptide aptamer delivery into the nucleus. J Mol Biol 2008;380(5):777-82
32. Jones SW, Christison R, Bundell K, et al. Characterisation of cell-penetrating peptide-mediated peptide delivery. Br J Pharmacol 2005;145(8):1093-102
33. Kim K, Han JS, Kim HA, Lee M. Expression, purification and characterization of TAT high mobility group box-1A peptide as a carrier of nucleic acids. Biotechnol Lett 2008;30(8):1331-7
34. Meade BR, Dowdy SF. Enhancing the cellular uptake of siRNA duplexes following noncovalent packaging with protein transduction domain peptides. Adv Drug Deliv Rev 2008;60(4-5):530-6
35. Abes R, Arzumanov AA, Moulton HM, et al. Cell-penetrating peptide-based delivery of oligonucleotides: an overview. Biochem Soc Trans 2007;35(Pt 4):775-9
36. Turner JJ, Jones S, Fabani MM, et al. RNA targeting with peptide conjugates of oligonucleotides, siRNA and PNA. Blood Cells Mol Dis 2007;38(1):1-7
37. Xavier J, Singh S, Dean DA, et al. Designed multi-domain protein as a carrier of nucleic acids into cells. J Control Release 2009;133(2):154-60
38. Folini M, Bandiera R, Millo E, et al. Photochemically enhanced delivery of a cell-penetrating peptide nucleic acid conjugate targeting human telomerase reverse transcriptase: effects on telomere status and proliferative potential of human prostate cancer cells. Cell Prolif 2007;40(6):905-20
39. Rajagopalan R, Xavier J, Rangaraj N, et al. Recombinant fusion proteins TAT-Mu, Mu and Mu-Mu mediate efficient non-viral gene delivery. J Gene Med 2007;9(4):275-86
40. Liu L, Venkatraman SS, Yang YY, et al. Polymeric micelles anchored with TAT for delivery of antibiotics across the blood–brain barrier. Biopolymers 2008;90(5):617-23
41. Liu L, Guo K, Lu J, et al. Biologically active core/shell nanoparticles self-assembled from cholesterol-terminated PEG-TAT for drug delivery across the blood–brain barrier. Biomaterials 2008;29(10):1509-17
42. Rao KS, Reddy MK, Horning JL, Labhasetwar V. TAT-conjugated nanoparticles for the CNS delivery of anti-HIV drugs. Biomaterials 2008;29(33):4429-38
43. Xue FL, Chen JY, Guo J, et al. Enhancement of intracellular delivery of CdTe quantum dots (QDs) to living cells by TAT conjugation. J Fluoresc 2007;17(2):149-54
44. Santra S, Yang H, Stanley JT, et al. Rapid and effective labeling of brain tissue using TAT-conjugated CdS:Mn/ZnS quantum dots. Chem Commun (Camb) 2005;7(25):3144-6
45. Ruan G, Agrawal A, Marcus AI, Nie S. Imaging and tracking of TAT peptide-conjugated quantum dots in living cells: new insights into nanoparticle uptake, intracellular transport, and vesicle shedding. J Am Chem Soc 2007;129(47):14759-66
46. Lei Y, Tang H, Yao L, et al. Applications of mesenchymal stem cells labeled with Tat peptide conjugated quantum dots to cell tracking in mouse body. Bioconjug Chem 2008;19(2):421-7
47. Chen B, Liu Q, Zhang Y, et al. Transmembrane delivery of the cell-penetrating peptide conjugated semiconductor quantum dots. Langmuir 2008;24(20):11866-71
48. Moy P, Daikh Y, Pepinsky B, et al. TAT-mediated protein delivery can facilitate MHC class I presentation of antigens. Mol Biotechnol 1996;6(2):105-13
49. 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(12):1449-52
50. Zhou Y, Du W, Koretsky T, et al. TAT-mediated intracellular delivery of NPM-derived peptide induces apoptosis in leukemic cells and suppresses leukemogenesis in mice. Blood 2008;112(6):2474-83
51. Kubo E, Fatma N, Akagi Y, et al. TAT-mediated PRDX6 protein transduction protects against eye lens epithelial cell death and delays lens opacity. Am J Physiol Cell Physiol 2008;294(3):C842-55
52. Wang Q, Gou X, Jin W, et al. TAT-mediated protein transduction of nogo extracellular peptide 1-40 and its biological activity. Cell Mol Neurobiol 2009;29(1):97-108
53. Wang Q, Gou X, Xiong L, et al. Trans-activator of transcription-mediated delivery of NEP1-40 protein into brain has a neuroprotective effect against focal cerebral ischemic injury via inhibition of neuronal apoptosis. Anesthesiology 2008;108(6):1071-80
54. Brady RO, Schiffmann R. Enzyme-replacement therapy for metabolic storage disorders. Lancet Neurol 2004;3(12):752-6
55. Wang D, Bonten EJ, Yogalingam G, et al. Short-term, high dose enzyme replacement therapy in sialidosis mice. Mol Genet Metab 2005;85(3):181-9

56. Brady RO. Enzyme replacement for lysosomal diseases*. Annu Rev Med 2006;57:283-96

57. Jeong MS, Kim DW, Lee MJ, et al. HIV-1 TAT-mediated protein transduction of human brain creatine kinase into PC12 cells. BMB Rep 2008;41(7):537-41

58. Eavri R, Lorberboum-Galski H. A novel approach for enzyme replacement therapy. The use of phenylalanine hydroxylase-based fusion proteins for the treatment of phenylketonuria. J Biol Chem 2007;282(32):23402-9

Affiliation

Matan Rapoport & Haya Lorberboum-Galski†
†Author for correspondence
Faculty of Medicine
Hebrew University,
Department of Cellular Biochemistry and Human Genetics,
Jerusalem, Israel
Tel: +972 2675 7465; Fax: +972 2641 5848;
E-mail: hayag@ekmd.huji.ac.il