

TAT and TAT-Like Peptides for Protein Transduction and Intracellular Drug Delivery

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1. Introduction

Advances in biomedical research have significantly enhanced our capability to identify drug targets for medical intervention. At same time, major technological breakthroughs such as combinatorial chemistry, computer-assisted drug design, robotic screening, and molecular cloning have made thousands of small compounds and macromolecules such as proteins and nucleic acids available for therapeutic consideration. Despite this progress, two major obstacles need to be overcome before we are able to move these products from basic research into clinical application. The first of these obstacles is the lack of specificity of drug compounds toward target cells. Although exposure of intended cellular targets to a drug results in a therapeutic effect, exposure of unintended nontarget cells often results in undesirable toxic effects. The other obstacle is the inability of most drugs, especially those that are hydrophilic and those of high molecular weight, to pass through the cell membrane and enter cells. The cell membrane imposes a significant physical barrier to the entrance of drug molecules into cells. The current strategy for overcoming the first obstacle is to develop drugs that are cell specific, or pro-drugs that remain inactive until they are inside the intended target cells. A common approach to resolve the second limitation is to employ receptor-mediated endocytosis, pinocytosis, or phagocytosis to enhance drug internalization. Despite being extensively studied and well-documented, these approaches have nevertheless had limited success and have met with serious obstacles, including the lack of proper receptors, insufficient endosomal release or avoidance of lysosomal degradation, and low delivery efficiency. At present, only a handful of delivery systems have the potential to overcome these obstacles and none are capable of high-efficiency delivery of hydrophilic molecules, especially macromolecules. Therefore, development of a highly effective carrier for intracellular drug delivery has been the goal of much research effort in both the pharmaceutical industry and academic institutions.

Recently, application of a short peptide derived from the trans-activating transcriptional activator (TAT) of the human immunodeficient virus (HIV), to the delivery of bioactive molecules into a variety of cell types has drawn significant interest from the scientific community, particularly in the area of drug delivery (1). By covalently link-

ing this peptide (commonly called the TAT peptide) to a variety of molecular cargoes such as fluorescent probes (2,3), peptides (4), enzymes (2,5), nucleic acids (6,7), nanoparticles (8), or liposomes (9), delivery of these attached cargoes into cells in culture (2–9) and in animal tissues (2) was accomplished. TAT-mediated intracellular delivery is unprecedented in efficiency and potency that cannot be easily duplicated by any of the known cell-entry mechanisms, including receptor-mediated endocytosis. Although the mechanism of TAT-mediated internalization remains unclear, evidence accumulated so far appears to suggest that it occurs in a nonspecific fashion without involvement of receptors or transporters (10–12).

The apparent efficiency with which TAT is able to enter cells has raised the possibility of its utilization to mediate the delivery of various molecules into cells for basic research and for drug therapy. In this chapter, we will detail major aspects of the TAT-based delivery system and provide readers with our perspectives for future development.

2. TAT and TAT-Like Peptides

TAT is an essential protein involved in transcriptional regulation in HIV replication. The size of the protein varies between 86 and 102 amino acid residues depending on the origin of viral strain. The molecule contains three functional domains: the N-terminal acidic domain involved in *trans*-activation; a cysteine-rich DNA binding domain; and a basic domain that is thought to be the domain for nuclear localization (13). Green and Loewenstein (14) and Frankel and Pabo (15) independently discovered that a full length of TAT protein could cross the cell membrane and transactivate the long terminal repeat promoter of HIV. Subsequent to this discovery, several other proteins with membrane-penetrating capability were also identified (for review, *see ref. 16*), including the *Drosophila* antennapedia transcription factor ANTP (also called penetratin-1) (17), and the herpes simplex virus type-1 (HSV-1) VP22 transcription factor (18). However, interest in using these transcription factors for intracellular delivery of biologically active compounds did not become widespread until 1994 when Fawell and colleagues (5) discovered that heterogeneous proteins chemically cross-linked to several short peptides derived from TAT protein were able to enter cells. Additional studies by Vives and colleagues (19) led to the identification of an 11 amino acid long peptide spanning the region from amino acid residue 47 to 57 in the TAT protein as the functional domain for protein translocation (19). Structure–function relationship studies on this TAT peptide revealed that a cluster of six or more basic amino acid residues is critical for its membrane penetrating activity. Deletion or substitution of the basic amino acid residues of the TAT peptide impaired membrane translocation activity. The nuclear localization signal (NLS) of TAT, although also enriched with basic amino acid residues and essential for HIV *trans*-activation, does not have transduction activity by itself (19). Other NLS sequences such as the NLS from Simian Virus 40 (SV40) large T-antigen exhibited no translocation activity (5). Interestingly, it has been reported that NLS sequence containing TAT peptides can penetrate into the nucleus through the nuclear pores, whereas those without an NLS remain in the cytosol after internalization (20).

The domains responsible for protein translocation in ANTP and VP22 have also been identified. The minimal amino acid residues from 267 to 300 of VP22 (18) and those from 43 to 58 in the third alpha helix of ANTP homeodomain are required for

protein transduction (17). Encouraged by the success of TAT-mediated protein delivery, the transduction activity of various homopolymers of lysine, arginine, or histidine has also been examined. Various arginine rich peptides including arginine substitutes in TAT (3,21), the RNA binding peptides derived from virus proteins, and the DNA binding segment of the leucine zipper also exhibited translocation activity at levels similar to or even better than that of TAT peptides (22). Among the polyarginine peptides examined, Arg₈ exhibited optimal translocation efficiency. Futaki attributed the activity of polyarginine peptides to their ability to form hydrogen bonds between guanidine group of the arginine residues and phosphate or sulfate groups on the cell surface. Using diphtheria toxin A chain as a model, Branke and coworkers (23) demonstrated that fusion proteins of the A chain with 6 lysine residues (Lys₆) at the N-terminal was approx 100-fold more active than similar fusion proteins with Arg₆ or His₆. The translocation efficiency appeared to depend on peptide chain length: Lys₈ > Lys₆ > Lys₃. In agreement with this work, Mai and colleagues have recently demonstrated that the transduction efficiency of homeolysine-based peptides consistently exceeded that of previously identified TAT-derived peptides and polyarginines (24). Table 1 provides a list of important TAT-like peptides that have been used for intracellular delivery. Although varying in amino acid composition, peptide sequence, and length, all of these peptides contain a cluster of basic amino acid residues. Although the possibility exists, the secondary structure of these cationic peptides does not seem to be critical for their activity. To date, fusion proteins containing TAT peptides have demonstrated better transduction activity than similar fusion proteins containing the protein-transduction domains of ANTP or VP22, although the recently devised peptide sequence of the retro-inverse form of TAT(57-47), and polyarginines appear to increase cellular uptake several-fold (3,25). D-amino acid-substituted TAT peptides were equally active (26). Using biotinylated peptides and a biotin-streptavidin-based bridge for attachment of the cargo molecules, Mai and colleagues demonstrated that the minimum number of basic amino-acid residues needed for an intracellular delivery is 6 (24).

3. Linking the Cargo to TAT or TAT-Like Peptides

There are many choices available for the attachment of a TAT peptide to its cargo, depending on the size and chemical properties of a particular cargo. For example, when the cargo is a peptide of a moderate size, one can simply design and then synthesize a TAT-cargo fusion peptide by solid-phase peptide synthesis. For larger proteins, a TAT-fusion protein can be made using recombinant DNA techniques and appropriate expression systems.

Physical association between cationic TAT peptides and anionic molecules through electrostatic interaction may also be used to deliver the complexes into cells. Connection through a biotin-avidin-biotin sandwich structure has also been utilized, where biotinylated peptides and cargo molecules are connected by avidin (24).

For those cargoes that do not fall within the aforementioned categories, chemical conjugation approaches can be used (Fig. 1). Depending on reaction type and available functional groups on the cargo, these can be achieved through the formation of amide (a, b), carbamate (c), isourea (d), disulfide (e), thioether (f), or thiazolidine (g) bonds, as well as Schiff base formation (h-j) (28-31).

Table 1
Most Commonly Used TAT and TAT-Like Peptides

Peptide name		Amino acid sequence	Reference
TAT		TGRKKRRQRRR	(19)
D-TAT (D-amino acids in italics)		GRKKRRQRRPPQ	(26)
Retro-inverso form of TAT		RRRQRRKKRGT	(25)
VP22		DAATATRGRSAASRPTRPR- APARSASRPRRPVE	(18)
ANTP		RQIKIWFQNRRMKWKK	(17)
Polyarginine	R6	RRRRRR-GC	(3)
	R8	RRRRRRRR-GC	
	R10	RRRRRRRRRR-GC	
	R12	RRRRRRRRRRRR-GC	
	R16	RRRRRRRRRRRRRRRR-GC	
Poly Lys	6K	KKKKKK	(24)
	8K	KKKKKKKK	
	10K	KKKKKKKKKK	
	12K	KKKKKKKKKKKK	
LMWP		VSRRRRRGRRR	(27)

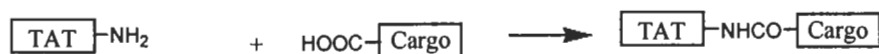
4. TAT-Mediated Intracellular Delivery

Since the first report by Fawell and colleagues (5) on the use of the TAT peptide for intracellular protein delivery, many reports have appeared in the literature documenting its activity in intracellular delivery of variety of molecules. Table 2 lists some representative studies in this area. For more information, readers are referred to a special issue of *Current Protein and Peptide Science* (34a), focusing solely on TAT-related topics. The following three subsections were prepared to provide a brief discussion on issues that are most relevant to the theme of this book.

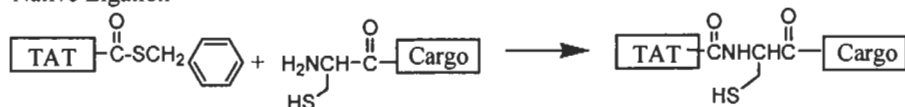
4.1. TAT-Mediated Protein Delivery

TAT-mediated intracellular delivery of proteins is commonly called protein transduction. This process is found to be most efficient when the proteins are covalently linked to TAT peptides. Becker and colleagues have developed a bacterial-expression system that permits in-frame insertion of the gene sequence for a fusion protein containing the 11 amino acid transduction domain at the N-terminal of the fusion protein (35). Sequence coding for 6xHis was also included into a gene-expression cassette that produces His₆-TAT-X fusion proteins in *Escherichia coli* (X representing the cargo protein). Inclusion of 6xHis motif provides a convenient means for purification of the expressed protein using metal-affinity chromatography and commercially available kits. Using this and other protein-expression systems, a large number of fusion proteins with biological activity have been produced and effectively delivered into cells (for a recent review, see ref. 36). For example, Vocero-Akbani and colleagues engineered a TAT-fusion protein with apoptosis-promoting caspase-3 protein (TAT-Casp3) that contains an HIV proteolytic cleavage site (37). In HIV-infected cells, TAT-Casp3 is pro-

a. Amide



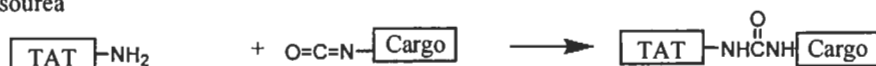
b. "Native Ligation"



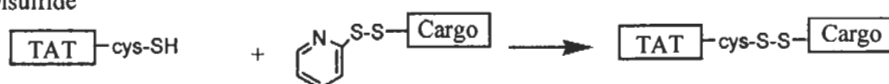
c. Carbamate



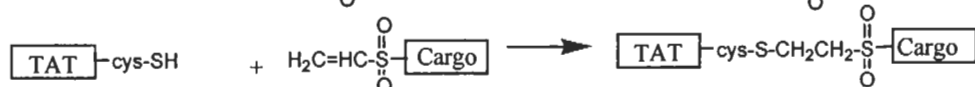
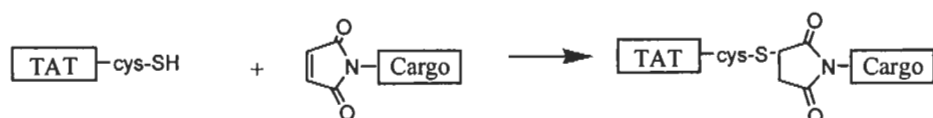
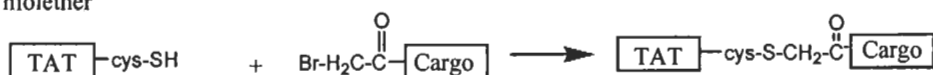
d. Isourea



e. Disulfide



f. Thioether



g. Thiazoline



h. Reductive amination



i. Aminoxy



j. Hydrazone

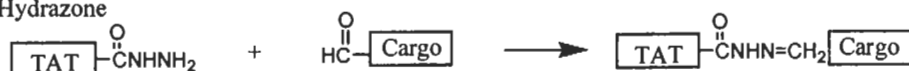


Fig. 1. Conjugation chemistry for linking TAT peptide to its cargo.

cessed into an active form of Casp3 by HIV protease, with resulting apoptosis of the infected cells, whereas in uninfected cells it remained in inactive zymogen form. Nagahara et al. have reported that direct transduction of the denatured TAT fusion protein, TAT-p27^{Kip1}, could regulate intracellular processes in experimental systems by arresting the cell cycle in the G₁ phase (38). Additionally, ovalbumin (OVA) conju-

Table 2
Intracellular Delivery of Various Molecules by TAT or TAT-Like Peptides

Cargo molecules delivered	Peptides	Linkage	References
Proteins	TAT, PolyR, PolyK, ANTP	Chemical conjugation Genetic, biotin-avidin bridge	(19) (24)
Synthetic peptides	TAT, ANTP, PolyR, PolyK	Peptide bond, Disulfide	(32) (4)
Pharmaceutical drug	TAT, PolyR PolyK	Disulfide	(33)
Protein nucleic acid (PNA)	ANTP	Chemical conjugation	(34)
Fluorescence molecules	TAT	Chemical conjugation	(2,3)
Antisense oligo nucleotide	ANTP, TAT	Disulfide	(6,7)
Radioisotopes	TAT	Chelate	
Magnetic beads	TAT	Chemical conjugation	(8)
Liposomes	TAT	Chemical conjugation	(9)

gated with the TAT peptide was found to be an effective antigen for major histocompatibility complex (MHC) class I-restricted cytotoxic T lymphocytes (CTL) responses and could be used to stimulate CD4 positive T cells, whereas polylysine-conjugated OVA induced no stimulation (39). In this study, antigen-specific CTL immunity was also induced in vivo by treating mice with histocompatible dendritic cells (DCs) that had been exposed to TAT conjugates.

Interestingly, Schwarze and colleagues reported that it is much more efficient for TAT-proteins to enter cells when they are in the denatured form than in their native form (2). Whereas TAT fusion protein failed to induce significant transduction, the same protein misfolded by urea denaturation transduced approx 100% cells in a rapid, concentration-dependent manner. One explanation for the successful delivery of denatured proteins by TAT is that the misfolded proteins may have reduced structural constraints and become more flexible. It remains to be seen, however, whether this is true for other proteins. In general, in-frame fusion proteins generated from genetically engineered TAT expression vectors showed better transduction efficiency, compared with chemically cross-linked TAT peptides (2,38). Apparently, more work is needed to understand the mechanisms by which TAT peptides are able to deliver proteins with various chemical and physical properties.

4.2. Intracellular Delivery of Small Molecules

TAT-mediated intracellular delivery is not limited only to proteins. The utility of this delivery system has been explored with various oligonucleotides and other pharmaceutically interesting molecules. A good example of intracellular delivery of small molecules is the delivery of cyclosporin A, a drug that is ineffective on topical application because of its poor percutaneous adsorption. In a study published by Rothbard and colleagues (33), cyclosporin A was conjugated to a heptamer of arginine through a linker designed to release the active cyclosporin A at physiologic pH. Topical application of the TAT-cyclosporin A conjugates resulted in an efficient transfer into cells in mouse and human skin. More importantly, cyclosporin A reached dermal T lympho-

cytes and inhibited cutaneous inflammation. Considering that the topical approach is the simplest and most convenient route for drug administration and that skin is a formidable barrier to drug adsorption, the use of TAT or TAT-like peptides to enhance drug bioavailability provides a potential new clinical approach not only to the treatment of skin disorders but also to the treatment of systemic diseases. It will be interesting to see whether the same approach is effective in enhancing the penetration of therapeutic agents across the intestinal epithelium. Enhanced permeability of drug molecules across the intestinal epithelium will have significant impact on the pharmaceutical industry.

Application of TAT peptide to the delivery of nucleic acids into cells has also been explored. In the studies published by Astriab-Fisher and colleagues (6,7), TAT peptide was conjugated to oligonucleotides through disulfide bonds and tested for an enhanced intracellular delivery. Both phosphodiester and phosphorothioate antisense oligonucleotides were evaluated. The results of these studies showed that peptide-antisense oligonucleotides conjugates, but not mismatched control conjugates, were able to inhibit the expression of MDR1, the target gene for the antisense oligonucleotides tested in the study. However, the rate of intracellular accumulation of the peptide-oligonucleotide conjugates was considerably slower than that of the peptide alone or that of oligonucleotides complexed with cationic liposomes, presumably owing to the charge shielding effect of the highly anionic oligonucleotide moiety. These results suggest that conjugation of TAT or TAT-like peptide to antisense oligonucleotides may offer a promising means for gene-function studies and for disease treatment.

4.3. Delivery of Drug Carriers Into Cells

The utility of TAT peptides for intracellular delivery of nanoparticles was initially evaluated by Lewin and coworkers (8). In an effort to monitor the distribution and differentiation of progenitor and stem cells *in vivo*, TAT peptides were conjugated to the surface of a superparamagnetic iron oxide core that was sterically shielded by cross-linked dextran. The overall diameter of the particles was approx 50 nm. Addition of these particles to hematopoietic and neural progenitor cells resulted in intracellular accumulation of up to 10–30 pg of the superparamagnetic ion per cell. The labeled cells retained their ability to differentiate or proliferate. In a series of studies, Torchilin and colleagues (9) explored the possibility of intracellular delivery of liposomes by TAT peptides. It was shown that liposomes as large as 200 nm in diameter could be delivered into cells in a manner identical to those of TAT fusion proteins. They also demonstrated that direct contact of TAT peptides with the cell surface is critical. Steric hindrance to TAT peptide-cell interaction induced by attaching polyethylene glycol (PEG) to the liposome surface abolished liposome internalization, confirming the notion that direct binding of TAT peptides to the cell surface is essential for TAT-mediated internalization. More recent work by the same group showed that TAT-containing liposomes are capable of delivering plasmid DNA into cells (40).

5. Mechanism of TAT-Mediated Intracellular Delivery

By genetic or chemical conjugation with a variety of cargo molecules such as hydrophilic fluorescence probes (2), metal chelators (41), oligonucleotides (6,7), a large number of proteins (for recent review, *see ref. 36*), magnetic iron oxide nanoparticles (8), and even liposomes as large as 200 nm in diameter (9), TAT peptide was shown to be

able to transport these cargoes into cells. The mechanism by which TAT brings the cargo molecules into the cells is not fully understood. It was originally believed that cellular attachment to TAT protein might be mediated through a previously unknown receptor, a 90-kDa cell surface protein that binds to the transduction domain of TAT proteins (42,43). Histological analysis from TAT- β -galactosidase transduction experiments disclosed punctate surface-associated staining at early time points, suggesting the involvement of the endocytosis pathway. However, more recent studies demonstrated that the translocation activity of TAT or TAT-like peptides is not crucially inhibited even at 4°C or in the presence of metabolic inhibitors, as in case of ANTP homeoprotein (3,9,19). A D-amino acid substituted analog of TAT was internalized into cells as efficiently as the corresponding peptide sequences of TAT (3,26) and TAT-like peptides, suggesting no structure-activity relationship. Moreover, the fact that certain cationic polypeptides (polyarginines or polylysines) exhibited similar ability in intracellular delivery suggests that typical endocytosis does not play a critical role. Dowdy and colleagues demonstrated enzyme activity in many internal organs in mice injected intraperitoneally with TAT-galactosidase fusion protein (2). This lack of selectivity of protein transduction *in vivo* serves as additional evidence for a receptor-independent mechanism.

On the other hand, TAT-mediated intracellular delivery appears to require adsorption of the cationic TAT peptides to the negatively charged cell surface, most likely through binding to anionic heparin sulfate on cell surface, because the uptake of TAT was significantly inhibited by heparin sulfate or chondroitin sulfate A, B, and C (10). In addition, treatment of cells with anti-heparin sulfate antibody or heparinase III clearly reduced the degree of translocation mediated by TAT or TAT-like peptides. These results suggest that although TAT-mediated intracellular delivery may not involve receptor-mediated endocytosis, the electrostatic adsorption of TAT onto cell surface is essential.

A hypothesis was put forward by Prochiantz to explain the mechanism underlying TAT-mediated translocation (12). Figure 2 is a schematic presentation of TAT-based intracellular delivery based on Prochiantz's model. The TAT-based delivery system consists of two parts, the TAT peptide and the cargo, which are connected by various covalent or noncovalent bonds. Upon addition of cargo-carrying TAT peptide to cells, the positively charged basic amino acid residues of the TAT peptides electrostatically bind to the anionic cell surface and induce membrane rearrangement and the formation of an inverted micelle through a regional change of the lipid bilayer into a hexagonal phase. The central micellar structure within the membrane accommodates the peptide-cargo complex in its hydrophilic cavity. Consequently, the TAT-cargo complex enters the cell and reaches the cytosol. Depending on the sequence of the peptide, the conjugates could reach the nucleus through the activity of NLS or simply stay in the cytoplasm. In principle, proteolytic degradation of TAT peptide or breakdown of the linkage bond between the peptide and the cargo will result in cargo release.

Despite mounting evidence supporting the proposed model, it must be pointed out that the conclusive evidence for a ubiquitous internalization mechanism has not yet been found. Cellular translocation of a TAT fusion protein likely is dependent not only on the cationic characteristics of the TAT peptide, but also the cargo molecule carried. For instance, a recent study showed that the cellular accumulation of TAT-oligonucleotides was temperature-dependent and that the oligonucleotides seemed to accumulate

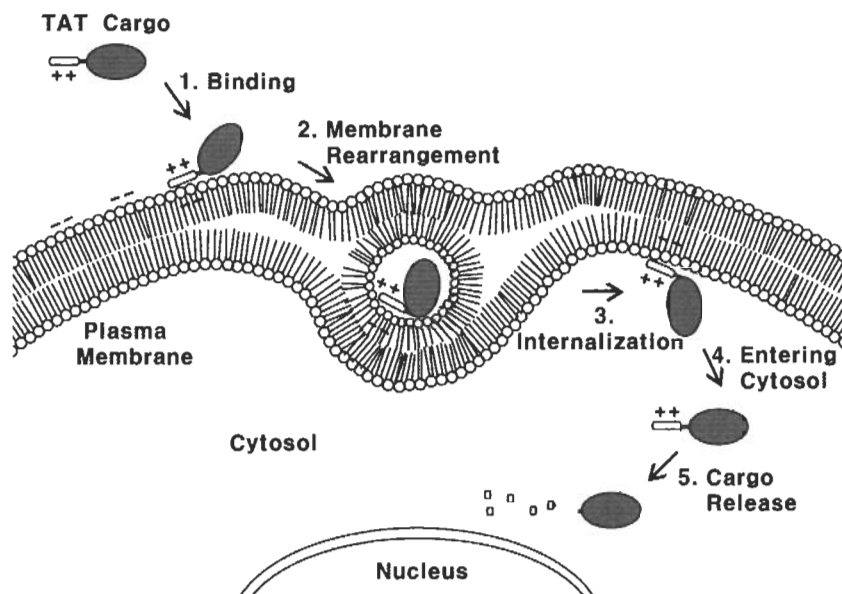


Fig. 2. Proposed mechanism for TAT-mediated intracellular delivery.

in endosomal compartments before entering the cytoplasm (6), which was in contrast to the delivery of TAT-conjugated proteins.

6. Summary and Perspectives

Intracellular delivery mediated by TAT peptides is so efficient that it can hardly be matched by any of the existing entry mechanisms, including the thoroughly studied receptor-mediated endocytosis or transporters. Early work by Fawell and colleagues demonstrated that a biological response could be detected at a concentration of 1 nM and that approx 10^7 TAT molecules were able to enter a single cell (15). Such ability of TAT peptides to deliver molecules across the cell membrane makes the TAT-peptides ideal vehicles for the intracellular delivery of hydrophilic molecules, especially macromolecules such as proteins. The unique characteristics of TAT peptides including their high efficiency, low toxicity, ease of manipulation, minimal susceptibility to the serum, and ability to cross both blood-brain barrier and the cutaneous barrier, provide an invaluable tool for pharmaceutical applications as well as biomedical research.

TAT-mediated intracellular delivery is not limited by cell type or physiological conditions. The same delivery efficiency was seen in cells in primary culture, established cell lines and normal cells in animals. Although it has been reported that TAT protein stimulates both HIV-1 gene expression and the development of Kaposi's sarcoma lesions (44), no significant toxicity has been detected from various *in vitro* and *in vivo* studies with TAT peptide carriers. Moreover, several *in vivo* experiments showed that TAT peptides are able to deliver their conjugates to all tissues in mice including the liver, kidney, lung, heart, spleen, and even the brain, by either intravenous or intraperitoneal administration (2). In most cases, the concentration of TAT-conjugates in transduced cells reached maximal in a few minutes.

The therapeutic potential of the TAT system encompasses both *ex vivo* and *in vivo* applications. The *ex vivo* approach is obvious because much work has already been performed in cell-culture systems. Experimental conditions for loading TAT-conjugates into cells have already been established and can be applied to therapeutic and cell-marking studies. For *in vivo* applications, although more practical and desirable, possible side effects may hamper clinical development. The primary concern is that although it is an advantage that the TAT-based system is capable of delivering its cargo to any cell type, the lack of selectivity in delivery may lead to unwanted exposure of normal cells to drug. However, the application of TAT to therapies where local administration can be employed would be an advantage. To achieve selective entry of TAT-drug conjugates without diminishing their high cell-entering activity, one may need to hide the cationic groups so that they will become available only at the surface of target cells. Reversibly shielding the positive charges of the TAT for this purpose may prove to be a valuable approach for future development. In its current form, the most appropriate therapeutic application of the TAT-based system is in the area of topical application to enhance the bioavailability of drugs with poor adsorption rates. From the technological standpoint, protein transduction by TAT-mediated protein delivery may prove extremely useful in studying gene or protein function in cells.

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