EXPERT OPINION



# Intracellular transduction and potential of Tat PTD and its analogs: from basic drug delivery mechanism to application

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***Introduction:*** It has been 20 years since the discovery of the membrane-translocating property of the HIV-1 transactivator of transcription (Tat) protein. The Tat protein transduction domain (Tat PTD) is a very promising tool for non-invasive cellular import of cargos and has been successfully applied to *in vitro* and *in vivo* delivery of different therapeutic agents for the treatment of many diseases. A growing number of reports on Tat PTD-mediated delivery have extensively revealed the mechanisms involved. Yet, due to the varied conditions used, the reports on the internalization mode of Tat PTD-cargo chimera are often varied.

***Areas covered:*** This article reviews the possible intracellular trafficking mechanisms of Tat PTD including its binding, cellular entry process, and the roles of participants of the cell membrane. The therapeutic applications via local administration, such as those for the treatment of skin, ocular, cardiac and cerebral diseases, are also reviewed. In addition, some novel systems built by different groups are elucidated, which are utilized to overcome the poor targeting efficiency of Tat PTD for the treatment of CNS diseases, cancer and other diseases via systemic administration.

***Expert opinion:*** With the development of targeting factors, such as antibodies, some cell targeting peptides and novel polymers, Tat PTD is expected to play a more efficient and/or better tolerated therapeutic role in the drug delivery field.

**Keywords:** mechanism, systemic delivery, targeted systems, Tat PTD, topical delivery

*Expert Opin. Drug Deliv. (2012) 9(4):457-472*

## 1. Introduction

In the past two decades, a wide variety of short peptides with cell membrane translocation properties have been identified. They are named cell penetrating peptides (CPPs) or protein transduction domains (PTDs) [1,2]. Typically, they are a class of short (<20 amino acids) cationic peptides derived from either proteins or chimeric sequences [3,4]. Until now, a variety of CPPs have been discovered such as transactivator of transcription (Tat) PTD, transportan, polyarginine sequences (Arg8), MPG, penetratin and so on, and the list is increasing all the time [5]. They are usually amphipathic and possess a net positive charge [6]. It is noted that Tat PTD and polyarginine are not amphipathic. However, in line with other CPPs, they are also capable of transversing the biological membrane, triggering the movement of various molecules across cell membranes into the cytoplasm and improving their intracellular routing, thereby facilitating interactions with the target [7]. Thus they have been used to favor the delivery of a large panel of cargos (proteins, peptides, plasmid





10.1517/17425247.2012.663351 © 2012 Informa UK, Ltd. ISSN 1742-5247

**Article highlights.**

- Lipids and glycosaminoglycans (GAGs) play important roles in the binding process of transactivator of transcription protein transduction domain (Tat PTD) as they both provide negative charges which can cohere to Tat PTD.
- Hydrogen bonding and specific interactions also have functions in the binding process of Tat PTD.
- Tat PTD or Tat PTD-cargo might translocate into cells directly or through endocytosis. The entry mechanism might be involved in different endocytosis pathways simultaneously. Following internalization of Tat PTD or Tat PTD chimera via endocytosis, it must escape from the endosome so that the molecule can be released to the cytoplasm and take further action in the cytoplasm or the nucleus.
- Tat PTD shows great promise to cure diseases such as skin, ocular, cardiac and cerebral diseases via local administration.
- Via appropriate choice of delivered agents, complement, modification or assistance of some targeting materials, even or through some novel systems, Tat PTD can also be used as a potential systemic drug delivery carrier.

This box summarizes key points contained in the article.



DNA, oligonucleotides, siRNA, peptide nucleic acid, liposomes, nanoparticles etc.) into multiple cell types and *in vivo* models [8-11].

Tat PTD is the predominant CPP used in the delivery of small molecules (such as peptides and oligonucleotides) and large molecules (such as full-length proteins and nanocarriers), based on its distinguished ability to pass through biological membranes with different cargoes. It is derived from the Tat protein of HIV-1. Tat PTD has been paid great attention since Frankel and Pabo [12] found in 1988 that Tat protein of HIV-1 could enter cells and translocate into the nucleus. Massive studies of CPP concerning the characteristics of passing through the cell membrane followed. The first evidence of the possible scientific and therapeutic significance offered by Tat-mediated protein delivery was shown by Fawell *et al.* in 1994 when they demonstrated that large proteins such as β-galactosidase, horseradish peroxidase and RNase A could be transduced into cells by chemically cross-linking them to peptides corresponding to amino acids 1-72 or 37-72 of Tat [13]. In these experiments, the large protein (amino acid residues 1-72 of Tat) or longer peptides (amino acid residues 37-72 of Tat) were used to investigate the mediation of cargos instead of a short peptide (amino acid residues 49-57). Soon after, Vives *et al*. identified a small sequence of Tat required for cellular uptake [14]. The first proof-of-concept of the application of PTD *in vivo* was reported by Dowdy's group, for the delivery of small peptides and large proteins [15]. Tat PTD, composed of 9 amino acid residues (RKKRRQRRR, 49-57 sequence of Tat) is generally considered as the minimal sequence for its transduction [16,17]. The sequences of 47-57 and 48-60 of Tat were also investigated to explore their different transduction properties [15,18,19]. With an apparent characteristic difference from other CPPs, such as MPG(a peptide vector), Tat PTD is a non-amphipathic peptide owing to the existence of six arginines and two lysines (both are hydrophilic, basic amino acids). The composition endows high cationic charges onto Tat PTD and all these properties contribute to its extraordinary intracellular trafficking ability as an efficient delivery strategy. Tat PTD is also called the "Trojan horse peptide" owing to its ability to deliver different cargoes into cells [20]. Its non-amphipathic properties also determine its distinct cellular entry mechanism, which is a continuous hot topic [21-23].

## 2. The entry mechanism of Tat PTD

Despite a large number of studies on Tat PTD being available, there is no consensus on its precise processes of binding to and crossing the plasma membrane, and the release of its cargo to cellular compartments [24-26]. It might be attributed to multiple elements, which could have an impact on its uptake process. Due to experimental variations, contradictory results are often obtained. The high diversity of attached cargoes and the wide variety of cell lines, even the different protocols applied to investigate the entry mechanism, might influence the internalization behavior of Tat PTD or its chimera [16]. Different opinions about the process of membrane binding and the passage to the cell membrane are reviewed in the following text.

### 2.1 Binding to the cell surface

The first step in the internalization of Tat PTD is its binding to the cell surface [23] and it generally occurs at any temperature, including 4°C. Non-specific interactions, specific interactions or both might be involved in the binding process.

#### *2.1.1 Electrostatic interactions*

Owing to the high cationic charges of Tat PTD and the anionic characteristic of cell surfaces, electrostatic interactions are considered as the primary effects when Tat PTD associates with the cell surface, although it appears to be unspecific and weak. There are a large panel of cellular polyanions involved in the process of binding, such as anionic lipids, glycosaminoglycans (GAGs), DNA, RNA, actin, tubulin and so on [5,16,27]. Anionic lipids and GAGs are regarded as the most important electrostatic binding participants for Tat PTD-mediated delivery into cells.

##### *2.1.1.1 Lipids*

The percentage of anionic lipids is crucial for the membrane affinity of Tat PTD. Different cell types have varied membrane lipid compositions, so the affinity of Tat PTD to different cell types is not the same. As a non-amphipathic CPP, Tat PTD might have a low or no affinity to membranes with anionic lipid contents of <30% at low micromolar concentrations [28]. Additionally, owing to its lack of the amphipathic

characteristics, Tat PTD was found to be adsorbed only superficially, instead of being buried in the hydrophobic head group region of the membrane, which means it does not experience structural changes upon lipid binding [29].

#### 2.1.1.2 GAGs

Owing to the positive charge of Tat PTD, it can associate electrostatically with various other cellular anions at the cell surface such as inositol phospholipids, negatively charged proteins (e.g., nucleolin), N-linked GAGs (e.g., sialic acid) and O-linked GAGs (e.g., heparan sulfate), all of which are involved in endocytic events and cell signaling [5]. However, only a few of these molecules have been substantiated so far in the context of Tat PTD uptake. Heparan sulfate proteoglycans (HSPGs) with a high number of sulfate and carboxylic groups on their molecules have been the focus of investigations. The investigations are based on two points: heparan sulfate provides the highest charge density to these molecules and, thus has the highest potential for electrostatic interactions; and heparan sulfate has important functions in endocytic pathways [30,31]. The involvement of heparan sulfate in the Tat PTD uptake has been demonstrated by *in vivo* studies where the genetic or enzymatic removal of GAGs from the surface of living cells reduced the uptake [32-34]. Yet, in early studies it was observed that there was no correlation between heparan sulfate and the permeation barrier in Madin–Darby canine kidney (MDCK) and Caco-2 cells both of which expressed HSPG [35]. The contradictory results might be attributed to the fact that proteoglycans might not be the sole participant involved in the binding process.

### 2.1.2 Hydrogen bonding

Besides electrostatic forces, hydrogen bonding also plays important roles in the binding process of Tat PTD to cell membranes. Hydrogen bonding is the attractive interaction of a hydrogen atom with an electronegative atom and is weaker than electrostatic interaction. The carboxylate and sulfate groups of GAGs were shown to provide a bidentate hydrogen-binding pattern for the guanidinium cation of arginine so that the binding was stabilized by both ion pairing and hydrogen bonding [36]. In a more recent study, Tat(48-60) adhering to the membrane–water interface was stabilized not only by electrostatic attraction to the anionic lipids, but also by intermolecular hydrogen bonding with the lipid phosphates and water by solid-state NMR experiments, which might take the role of intramolecular hydrogen bonds in canonical secondary structures [37].

### 2.1.3 Specific interactions/mediation of receptors

Although the physical and unspecific functions, such as electrostatic interactions and hydrogen bonding, play dominating roles in the process of binding, specific interactions of polycationic peptides and phosphocholine head groups with electroneutral nature can also be involved. Some receptors on the cell surface are reported to be involved in the attachment of Tat PTD, such as HSPGs [38-40]. It has also been widely suggested that HSPGs serve as an initial receptor for the adherence and subsequent internalization of Tat PTD. HSPGs on the cell surface are grouped into two types: the glycosylphosphatidylinositol anchored glypicans and the integral transmembrane protein syndecans (SDCs), with the second type more ubiquitous [41]. A recent study [31] provided additional evidence to receptor-mediated uptake of Tat PTD. A series of experiments were explored to prove that the uptake of Tat PTD being mediated by SCDs, and the most impressive finding in this literature was that Tat PTD entered cells together with SDC4, rather than just penetrating alone as ligands bound to SDC4, as indicated by co-localization studies. Although, the uptake of Tat PTD by K562 cells devoid of SDCs was still detectable, the uptake was less than SDC transfected cells, suggesting that the SDC4-dependent uptake might be the primary, but not the only mechanism, responsible for Tat PTD translocation. Despite acknowledgment of SDC/HSPG-mediated delivery, the affinity of HSPG (SDCs) to Tat PTD might not be totally specific, as electrostatic interactions should not be ignored (based on the high negative charges of HSPGs and high positive charges of CPPs).

In conclusion, different interactions might be simultaneously involved in the cellular binding of Tat PTD. It is most likely that one interaction plays a predominant role and others have subsidiary effects. Consequently, different interactions might result in different mechanisms for the cellular entry process. For example, a receptor-mediated delivery might not directly translocate Tat PTD into cells; in this situation Tat PTD might penetrate the membrane via endocytosis and the following endosomal pathway.

## 2.2 Passage through the membrane

After binding to the cell surface, how is Tat PTD translocated into the cytoplasm? There are various, even contradicting, points of view in terms of the kinetics of Tat PTD. Endocytosis is the most popular view; however, there are other arguments with convincing evidence, such as direct translocation [42].

### 2.2.1 Independent-endocytosis/direct translocation

Although Tat PTD is not thought to directly translocate into the cell membrane owing to its hydrophilic properties [5,43], several studies have revealed that direct translocation might be a possible internalization pathway into cells [44]. Translocation of free Tat peptide was considered to involve direct cell penetration, not endocytosis, as the peptide was frequently internalized at low temperatures into genetically modified cells lacking certain endocytic pathways [42]. In addition, based on microscopic observations and flow cytometry experiments, there seemed to be a certain concentration threshold above which direct translocation would be favored [45,46]. Using a releasable luciferin assay, the kinetic behavior of cytosolic entry of luciferin–CPP conjugate was investigated in real

time by Eiríksdóttir *et al.* [43]. It was found that Tat PTD and higher concentrations of MAP and TP10 conjugates displayed fast internalization profiles that resembled the membrane-permeable free luciferin kinetic profile. The authors speculated that the results might be induced by the increase of CPP concentration, which led to endocytosis and a direct translocation transition. Membrane potential was necessary for the entry of CPPs when they entered cells directly [47,48]

### 2.2.2 Endocytosis

In terms of kinetics, the entry process of Tat PTD appears more in agreement with the endocytic route. In many studies, Tat PTD behaved in an energy-dependent manner requiring temperatures above 4°C and ATP; and its cellular uptake was sensitive to low temperature incubation, indicating that a very important proportion of the peptide entered cells through the active endocytic cellular machinery [20]. Endocytosis is an important pathway of membrane mobile transport based on the membrane fluidity, by which cells absorb substances by engulfing them. Endocytosis pathways can be subdivided into four categories: clathrin-mediated endocytosis, caveolae, macropinocytosis and phagocytosis. Clathrin-mediated endocytosis, caveolae and macropinocytosis are all considered as the potential pathways of Tat PTD entry into cells.

#### 2.2.2.1 Clathrin-mediated endocytosis

Clathrin-mediated endocytosis is a process by which cells internalize molecules by the inward budding of plasma membrane vesicles containing proteins that are mainly associated with the cytosolic protein clathrin, with receptor sites specific to the molecules being internalized. Clathrin-coated vesicles are found in virtually all cells and form domains of the plasma membrane termed clathrin-coated pits. Coated pits can concentrate large extracellular molecules that have different receptors responsible for the receptor-mediated endocytosis of ligands. In early studies, Tat PTD protein chimera was indicated to translocate following clathrin-mediated endocytosis [49]. A recent study further demonstrated that Tat PTD passed through cells via this pathway [47]. In this study, fluorescently labeled RI-CKTat9 was shown to enter HeLa cells in a concentration- and energy-dependent manner and to co-localize with labeled transferrin in a punctate structure, suggesting that the peptide entered HeLa cells by clathrin-dependent endocytosis. In addition, any way of blocking clathrin-mediated endocytosis (e.g., RNAi, MDC, cell energy-depletion, 0°C, sucrose or mitotic phase of the cell cycle) eliminated or greatly decreased labeled RI-CKTat9 entry into topological compartments.

It is generally believed that a small portion of adsorbed molecules on the cell surface directly follows the clathrin-mediated pathway [50,51]. Most positively charged CPP molecules may first bind to the mammalian cell surface with nonspecific, low affinity receptors, such as the negatively charged proteoglycans. This binding is followed by further clustering (i.e., avidity-enhanced concentration) into the coated pits and clathrin-mediated endocytosis ensues [38].

#### 2.2.2.2 Caveolae

Caveolae are the most commonly reported non-clathrin-coated plasma membrane buds, which exist on the surface of many, but not all, cell types. They consist of cholesterol-binding protein caveolin (Vip21) with a bilayer enriched in cholesterol and glycolipids. Caveolae are small (~50 nm in diameter) flask-shaped pits in the membrane that resemble the shape of a cave (hence the name caveolae). Uptake of extracellular molecules is also believed to be specifically mediated via receptors in caveolae. Tat PTD was proposed to internalize into cells via caveolae-mediated endocytosis following elaborative investigations [49]. GST-Tat-GFP entered cells via the caveolae pathway (the same as GFP-cargo). It was assumed that the GFP protein had to be trapped in a neutral environment to maintain its fluorescent property. No reduction of the fluorescence activity was recorded in the intracellular trafficking process, therefore, indirectly confirming that the GFP cargo was taken up by cells through the caveolae pathway; which was not acidified during the course of intracellular trafficking [52]. In another study, a rhodamine-tagged Tat PTD fused to a cargo peptide co-localized with cholera toxin (a lipid raft marker) and not with transferrin [53]. The removal of cholesterol from the plasma membrane abolished the internalization of the fusion peptide, suggesting a lipid raft-dependent endocytic mechanism. As we know, caveolae are a special type of lipid raft, therefore, this result was exactly in agreement with the study on GST-Tat-GFP that implicated the caveolar endocytosis pathway [54].

#### 2.2.2.3 Macropinocytosis

Macropinocytosis, which usually occurs from highly ruffled regions of the plasma membrane, is the invagination of the cell membrane to form a pocket, which then pinches off into the cell to form a vesicle (0.5 – 5 μm in diameter) filled with a large volume of extracellular fluid and molecules within it (equivalent to ~100 clathrin-coated vesicles). The filling of the pocket occurs in a non-specific manner. Macropinocytosis has already been suggested as the major uptake mechanism of arginine-rich CPPs [55]. Tat PTD was reported to follow the macropinocytosis pathway in some studies [31,56]. In the assays reported by Richard *et al.* amiloride, a potent inhibitor of macropinocytosis, significantly decreased Tat PTD cellular uptake in selected cell lines. Additionally, Tat-Cre was also reported to cross the membrane by macropinocytosis. In this study, biological response was strongly increased upon co-incubation with a Tat peptide fused to the fusogenic sequence derived from the influenza hemagglutinin protein, which is known to promote membrane fusion once exposed to an acidic environment [57]. This observation indicated that the Tat-Cre fusion protein was taken up by a pathway undergoing acidification. These findings seemed conflicting compared

with the GST-Tat-GFP construct which was taken up through a mechanism of entry with a stable neutral pH environment [49]. In fact, these contradictory results might be due to the use of different Tat PTD chimeras or experiment protocols.

### 2.3 Endosomal escape

Following internalization of Tat PTD or Tat PTD chimera by endocytosis, it must escape from the endosome so that the molecule can be released to the cytoplasm and go on to further function in the cytoplasm or nucleus [24]. Many CPPs, including Tat PTD, were reported to adopt an α-helical structure at endosomal pH leading to hydrophobic and hydrophilic faces that can interact with the endosomal membrane to cause disruption and pore formation. Some studies proved that Tat PTD was taken up by cells through the formation of endosomes. In an experiment, prototypic HIV-1 Tat peptide did not interact with liposomes mimicking the outer leaflet of the plasma membrane, but it did induce lipid mixing and membrane leakage as it translocated into liposomes mimicking the lipid composition of late endosomes. Both lipid mixing and membrane leakage, which relied on the bis(monoacylglycero) phosphate (BMP) content, were promoted at acidic pH, which is a feature of late endosomes. Additionally, membrane leakage and peptide translocation were both modulated by inhibitors of lipid mixing, further confirming the hypothesis that Tat peptide crosses BMP-enriched membranes by inducing leaky fusion [58]. In a recent study, the cellular entry mechanism of Tat PTD was systematically investigated [47]. Fluorescently labeled RI-CKTat9 was indicated to enter HeLa cells via an endocytic pathway demonstrating both a diffuse and punctate (vesicular) appearance inside the cells. Incubation of cells with an isotonic/high $K^+$ buffer (KPBS) or an $NH_4Cl$ solution abolished the diffuse appearance, but not the punctate fluorescence, suggesting that membrane potential plays a critical role in endosomal escape. This result also suggests that the flux originates from the endosome, not the extracellular space, and relies on the acidity of the endosome. As cells in the mitotic phase shut down endocytosis, but maintain plasma membrane potential, this property was used to further confirm the endocytic mechanism. HeLa cells in mitotic phase did not display any vesicular nor diffuse fluorescence of labeled RI-CKTat9, providing compelling evidence for the sequential steps of endocytosis and endosomal escape.

### 2.4 An assumption on the process of endocytosis

Regarding the different potential mechanisms of Tat PTD, clathrin-mediated encytosis and the following endosomal escape have the most evidence. A relative, systematic, comprehensive and reasonable assumption about this pathway for the entry of Tat PTD into the cell has been referred to in a study [47] with convincing reports. First, Tat PTD or Tat PTD-cargo binds to the cell surface using electrostatic interactions between the positively charged Tat PTD and negatively charged lipids or nonspecific receptors (HSPGs); followed by endosome formation (i.e., pinching off). At this moment, the two binding entities remain in nM to μM concentrations and Tat PTD associates with substances on the surface. The binding is followed by further clustering on the cell surface into the coated pits and clathrin-mediated endocytosis ensues. Even without clustering, a small portion of molecules may follow the clathrin-mediated pathway. Thus, a formed endosome concentrates Tat PTD more than a 1000-fold from the extracellular fluid, as evidenced by the bright punctate fluorescence under microscopy. As soon as an endosome is pinched off, clathrin husking and V-ATPase activation occur. On the early endosome, the V-ATPase quickly lowers the luminal pH to 6.2, at which point most of the bound Tat PTD molecules are released, inducing a free luminal peptide concentration higher than 0.1 mM. At this high concentration, charge–charge repulsion becomes the driving force to push the peptide molecules across the endosomal membrane. Of course, this hypothesis is not perfect; for instance, a path of endocytosis into the later endosome and lysosome is not involved, in which the pH is about 5.3 and 5.0, respectively. However, based on this assumption, many experimental results can be reasonably explained.

### 2.5 There is not one single pathway involved in the translocation of Tat PTD

The complexity of experimental conditions makes it difficult to understand the concrete entry process of Tat PTD. Different groups choose different Tat sequences, varied cargoes, varied cell lines and different Tat PTD concentrations in their experiments. Tat PTD seems to enter cells via different pathways (**Figure 1**); however, one of the pathways might play a dominant role. In Zhang's study [47], fluorescently labeled RI-CKTat9 was indicated to follow clathrin-mediated endocytosis, yet its internalization could still be detected at <0°C, which demonstrated clathrin-mediated encytosis is a dominating, if not a single, route of RI-CKTat9.

As mentioned in the previous section, the debate about the mechanism of entry of Tat PTD will be ongoing for a long time. The precise identification of mechanism into cells of this chariot will certainly be helpful for its application, especially for novel drug delivery systems. Thus, magnitude lot of hard work still needs to be accomplished and more research needs to be carried out.

## 3. Tat PTD-mediated topical delivery

By taking advantage of the excellent ability of Tat PTD to translocate into cells *in vitro*, a large number of cargoes which are not capable of permeating cell membranes have been identified that can be delivered into different cell types with the assistance of Tat PTD. Tat PTD has great potential in topical therapeutic applications and examples are reviewed in the following section.

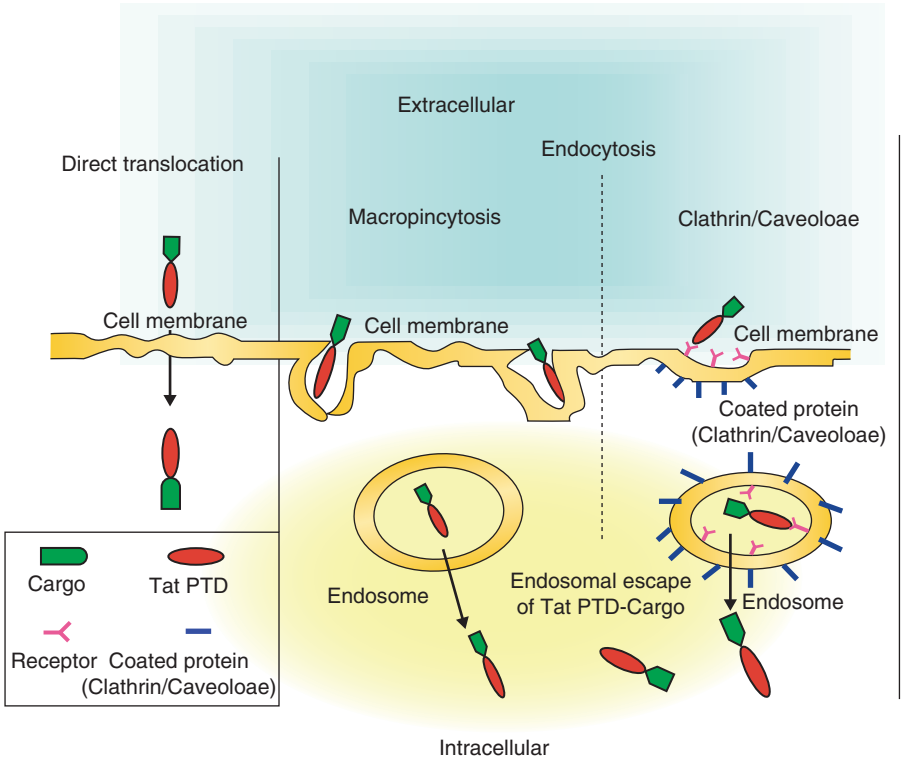


**Figure 1. The internalization of transactivator of transcription protein transduction domain (Tat-PTD)- cargo into cells based on different mechanisms (direct translocation, endocytosis dependent on macropinocytosis, clathrin- or caveolae-mediated endocytosis and subsequent endosomal escape).**

### 3.1 Tat PTD and ployarginine (an analog to Tat PTD)-mediated transdermal delivery

As a potential delivery tool, Tat PTD was paid great attention in the treatment of skin diseases. It was indicated to increase the penetration of a rather ill-defined, radio-iodinated mixture of botulinum toxin and other proteins (Neuronox®, Medy-Tox, Inc., Seoul, South Korea) across human skin membranes via a non-covalent complex in a patent reviewed in Howl's article [59]. Ployarginine was used to deliver cargoes across the skin with great success. PsorBan® (CellGate, Inc.), a topical product for psoriasis, is a cyclosporine A–polyarginine conjugate and it entered a Phase II clinical trial in 2003. CellGate, Inc. chemically transformed cyclosporine A via linkage to polyarginine to increase absorption by tissues and cells when applied locally [60]. With the help of polyarginine, cyclosporine A was absorbed into the skin, and penetration of the stratum corneum and diffusion into the epidermis was observed. This shows that a great development has been obtained in Tat PTD-mediated transdermal delivery.

### 3.2 Tat PTD-mediated ocular delivery

In addition to its application for transdermal delivery, Tat PTD performs well as an ocular peptide delivery system [61]. FITC-labeled D-Tat was shown to be effective not only in trafficking photoreceptor cells in culture, but also when injected into the vitreous or subretinal space. D-Tat PTD-FITC was clearly visible in the retina after 14 days of injection. Intraocular injection of anti-apoptotic fusion protein Tat PTD-Bcl-x(L) resulted in a survival of more than twice as many retinal ganglion cells 14 days post-surgery as when control protein was injected [62]. A more recent study might bring new hope to the treatment of retinal disease via non-invasive ocular Tat PTD-mediated delivery [63]. In this study, Tat PTD-aFGF-His exhibited efficient penetration into the retina following topical administration on the ocular surface (Tat PTD-aFGF-His used as eye drops). Tat PTD-aFGF-His protein was readily observed in the retina at 30 min and remained detectable for at least 8 h after local administration. Furthermore, after ischemia–reperfusion injury, the retina of rats, after the use of Tat PTD-aFGF-His eye drops, showed a better-maintained inner retinal layer structure, reduced apoptosis of retinal ganglion cells and improved retinal function compared with rats treated with aFGF-His or phosphate buffered saline (PBS). These results indicated that the conjugation of Tat PTD to aFGF-His could noticeably ameliorate the ability of aFGF-His to cross the ocular barrier without damage to its biological function. Thus, Tat PTD provides a potential vehicle for efficient drug delivery in the treatment of retinal diseases.

### 3.3 Tat PTD-mediated delivery for the treatment of cardiac and cerebral diseases

Tat PTD also has enormous potential in the treatment of cardiac and cerebral diseases such as ischemia and stroke. It works via intracoronary or cerebrovascular injection, which is regarded as local administration in this literature [64,65]. Intracoronary injections of a Tat-delta protein kinase C inhibitor chimera (KAI-9803) have also been used for the treatment of acute myocardial infarction (AMI) [66]. KAI-9803 is currently under clinical development for cardioprotection from ischemia–reperfusion damage in ST-elevation AMI and a local administration to the coronary artery of ST-elevation AMI patient leads to the improvement of key biomarkers of myocardial damage [67,68].

There are a wide variety of successful examples of Tat PTD-mediated local delivery to the brain. D-JNKI1, a conjugated peptide of Tat PTD and a protease-resistant peptide selectively inhibiting the c-Jun-N-terminal kinase (JNK), showed that intra-cerebrovascular administration of D-JNKI1 resulted in a reduction of neuronal damage [69,70]. Tat PTD-Bcl-x(L) was effectively transferred into the spinal cord neurons and motor neurons of a familial amyotrophic lateral sclerosis (ALS) mouse model by intrathecal infusion [71]. Administration of Tat PTD-Bcl-x(L) delayed the disease onset, prolonged the survival and improved motor performance, which indicate that Tat PTD-fused protein is an effective clinical tool for the treatment of ALS. Although these studies have indicated the potential for drug development with Tat PTD-conjugated compounds, most of these reports do not demonstrate efficacy using clinically relevant, simple routes of administration; therefore, more research needs to be carried out in this area.

## 4. Tat PTD-mediated systemic delivery

Due to its excellent ability to penetrate cell membranes, Tat PTD displays no specificity and selectivity to cells and tissues, which limits the application of Tat PTD for systemic administration. However, through appropriate choice of delivered agents, transient shielding through negative substances, targeting by an antibody and delivery by a novel system, such as liposomes and micells, Tat PTD can also be used as a potential systemic drug delivery carrier. There are some successful examples of its systemic administration, mainly in the treatment of cerebral diseases and cancers. Delivery of Tat PTD chimera via systemic administration is reviewed next.

### 4.1 Delivery of Tat PTD conjugates for the treatment of cardiac ischemia

Some proteins coupled with Tat PTD were shown to play a superior cardioprotective effect *in vivo* via intravenous (i.v.) injection in different mouse models of myocardial ischemia–reperfusion injury. KAI-9803 could also be regarded as a pre-eminent drug in this area, as it commenced a Phase 1 clinical study for the treatment of AMI in March 2007 [26]. There are also other successful applications. For example, a single low dose (1 mg/kg) i.v. injection of Tat-BH4 anti-apoptotic protein (BH4 derived from the BH4 domain of the Bcl-x(L)) 5 min before reperfusion was able to strikingly decrease infarct volumes (~47%) and to suppress apoptosis (~60%) in the left ventricle of treated mice [72].

### 4.2 Delivery of Tat PTD conjugates for the treatment of CNS diseases

Due to impermeability, a large number of molecules cannot cross the blood–brain barrier (BBB), which makes it difficult for molecules to arrive in the brain via systemic administration. Tat PTD was proved to deliver large, enzymatically active proteins throughout the body, including the brain [15]. This discovery opened up the application of Tat PTD in CNS diseases, and a massive variety of molecules have been demonstrated to cross the BBB and have positive effects in their target site via systemic administration by being linked to Tat PTD [73]. Neuroglobin (Ngb), an agent to protect against brain hypoxic–ischemic injury, cannot penetrate BBB, thus, it is not used in brain injury via systemic administration. Recently, Tat PTD was shown to successfully deliver Ngb into the brain in mice by i.v. injection [74]. Compared with the Ngb- and saline-treated groups, the group with Tat PTD-Ngb, treated 2 h before intraluminal middle cerebral artery occlusion (MCAO) showed a significantly less brain infarct volume and better neurologic outcomes. Furthermore, a Tat PTD-Ngb injection following a 30 min MCAO treatment significantly increased neuronal survival in the striatum. The results demonstrated that Tat PTD-Ngb could be efficiently transduced into neurons in the mouse and protect the brain from mild or moderate ischemic injury. Tat PTD-Bcl-x(L) was also observed to protect hippocampal neurogenesis after cerebral ischemia in mice via i.v. injection [75].

Tat PTD also showed good ability in the delivery of chaperones. The delivery of heat shock proteins (Hsp) using Tat PTD has been applied in different models, such as stroke and neurodegenerative diseases. For a review of Hsp on CPP-mediated delivery please refer to Dietz's literature [76]. A recent study demonstrated that the i.v. injection of Tat PTD-Hsp70 in a mouse model for transient focal cerebral ischemia resulted in a decreased infarct size and an obvious improvement as assessed by the rota rod, tight rope and water maze tests compared with Tat PTD and a saline treated control group [77]. Tat PTD-Hsp was also validated in a Parkinson's disease model and displayed protective effects on primary dopamine midbrain neurons against MPTP toxicity after systemic protein application [78].

Although the examples above showed promise for Tat PTD as a brain delivery vehicle, it should be noted that ischemic injury causes some damage to the BBB, which might cause leaky endothelial barriers and increase the penetration of Tat PTD into the brain. A recent study substantiated this point [79]

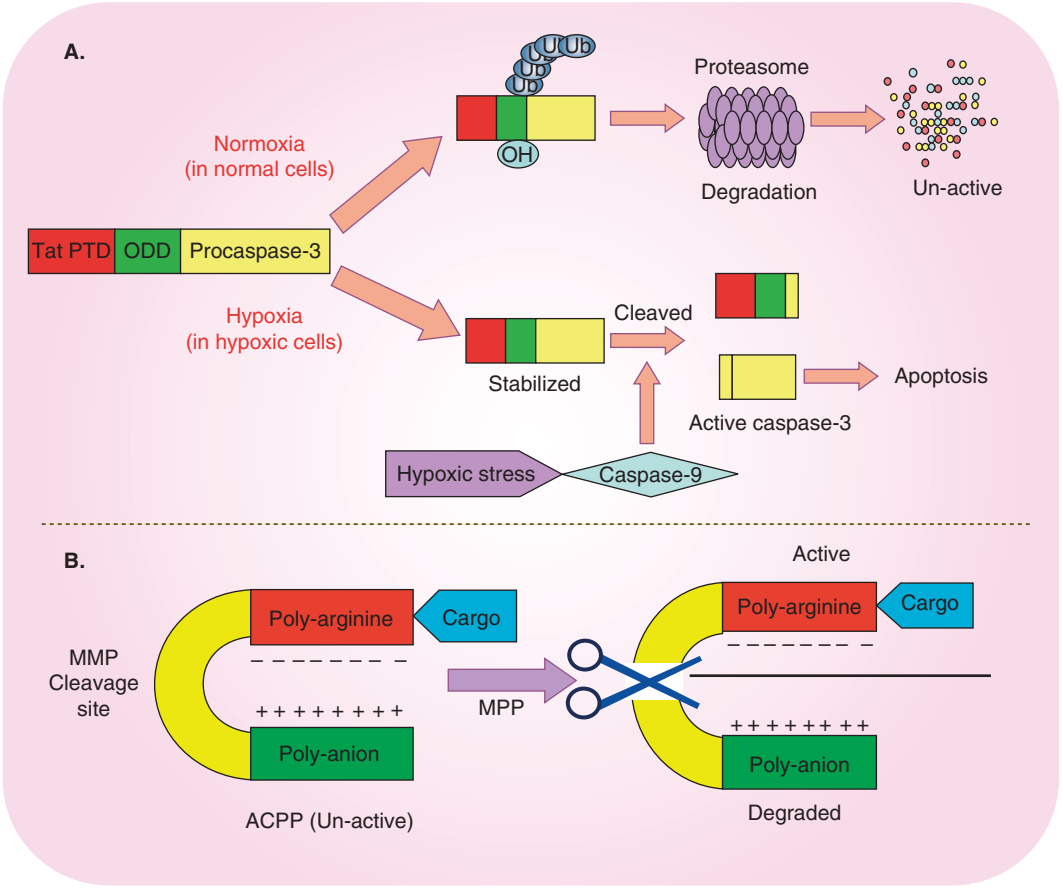


**Figure 2. Schematic diagram of TOP3 and activatable cell penetrating peptides (ACPPs). A.** Hypoxia-dependent pro-apoptotic function of TOP3. TOP3 was degraded through the ubiquitin–proteasome system under normoxic conditions, but stabilized under hypoxic conditions. As caspases-9 could be activated by hypoxic stress, TOP3 was cleaved to generate an active caspase-3, resulting in the enhancement of apoptotic cell death. **B.** Cellular uptake of ACPPs was blocked by a short stretch of acidic residues attached by a cleavable linker. Once the linker was cleaved by matrix metalloproteinase (MPP), the acidic inhibitory domain drifted away and the cationic CPP was free to carry its cargo into cells.

and demonstrated ischemic injury increased the transport of GFP-Tat PTD across an endothelial monolayer (comprised of bEnd.3 cells) *in vitro* and consequently, increased the TAT-mediated delivery into astrocytes on the other side. Moreover, the group also found that Tat PTD was not capable of transcellular delivery across an intact bEnd.3 endothelial monolayer *in vitro* and concluded that Tat PTD-mediated delivery might not be an effective method for trans-BBB delivery into the brain parenchyma when the BBB was integral [80]. This viewpoint is contradictory to some earlier studies. For instance, Tat β-galactosidase was detectable in all tissues in the mouse, including the brain via i.p. injection, which implicated that Tat PTD could deliver the cargo across the BBB. These contradictory conclusions might result from different administration methods and cargoes. However, Tat PTD might be useful as a solution for delivering therapeutics to the brain parenchyma in regions where the BBB has been compromised due to injury or diseases.

### 4.3 Delivery of Tat PTD conjugates for the treatment of carcinoma

Specific distribution to tumors is required for the delivery of a tumor-targeted drug, which restricts the application of Tat PTD in cancer therapy [81,82]. Although Tat PTD has no specific affinity to a tumor, with some special approaches such as use of a pro-drug and the guidance of a receptor, it is feasible to apply Tat PTD to improve the permeability and targeting efficiency of antitumor drugs [83]. These approaches are generally based on the particular environment of a tumor tissue such as hypoxia, high concentration of a certain enzyme and low pH [84-86].

#### *4.3.1 Dependence on hypoxia of tumor tissues*

Based on the hypoxia condition of tumor tissues, Tat PTD oxygen-dependent degradation domain (ODD) (Tat PTD-ODD) was developed [81,87,88]. Relying on ODD, the chimera can be degraded in normal tissues (normoxia), while it is stabilized in tumor tissues (hypoxia). A conjugate of

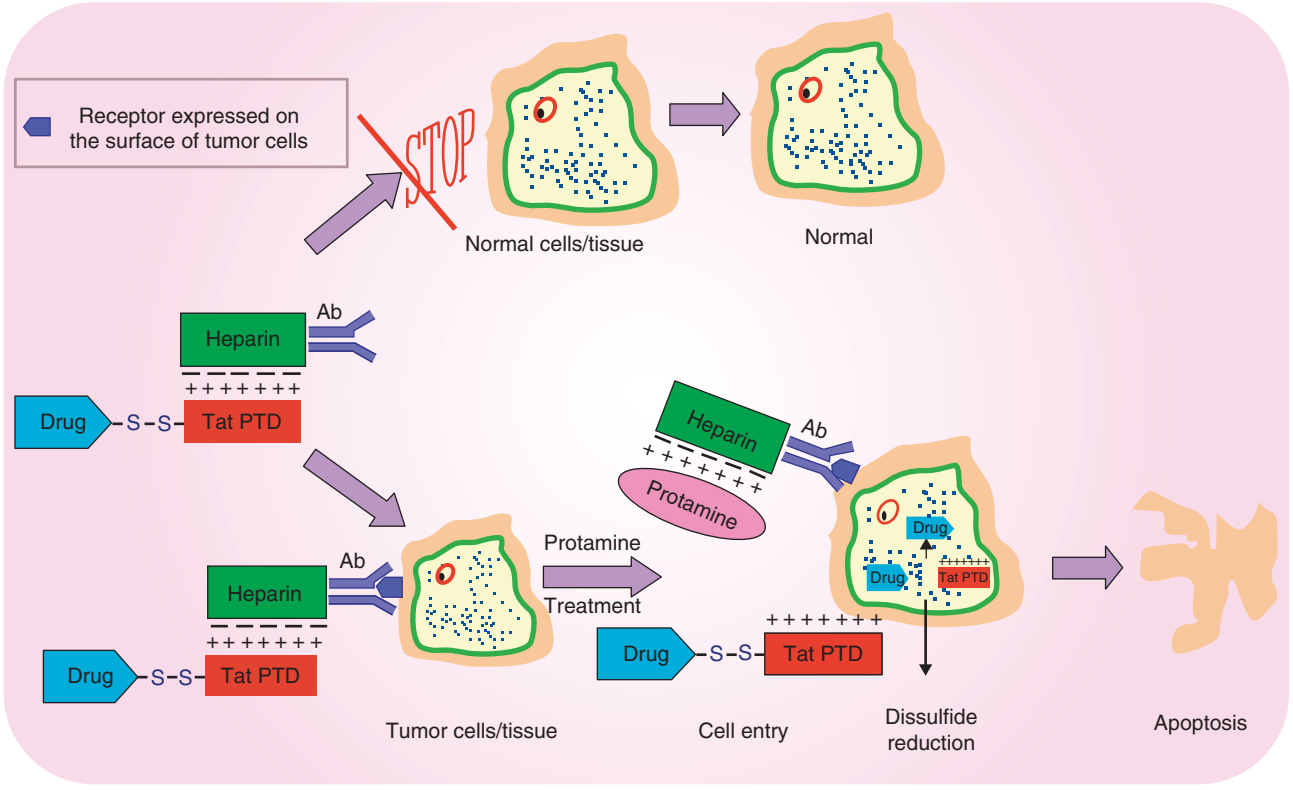


**Figure 3. The "ATTEMPTS" system based on the functions of transactivator of transcription protein transduction domain (Tat PTD), heparin, protamine and antibody.** Tat PTD-drug/heparin-antibody complexes could be directly delivered to the tumor cells, but not normal cells with the guidance of targeting antibody. After the complexes reached tumor cells, protamine sulfate, which can bind to heparin, was then given as a competing agent to separate the Tat PTD-drug chimera from the heparin-antibody counterpart. Once heparin inhibition was lost by protamine, Tat PTD-drug conjugates penetrated to the targeted tumor cells were to take effect.

Tat-ODD and procaspase-3, termed TOP3, was constructed (**Figure 2A**) [81]. As upstream caspases (e.g., caspase-9) can be activated by hypoxic stress, TOP3 is cleaved to generate an active caspase-3, resulting in apoptotic cell death. The *in vivo* study has just proved the feasibility of TOP3. First, TOP3 reduced the tumor size as well as inhibited the tumor growth without any obvious side effects in tumor-bearing mice via i.p. injection. The hypoxia targeting effect was also examined by a rat ascites model and a significant rise in the lifespan of rats with the malignant ascites was seen; furthermore, 60% of the treated animals were cured without the recurrence of ascites.

### 4.3.2 *Dependence on matrix metalloproteases*

In 2004, Tsien *et al.* first proposed, devised and tested a novel targeting system called activatable CPPs (ACPPs) that could selectively deliver molecules to tumor cells depending on the high concentrations of matrix metalloproteinase (MMP) around the tumor [84]. ACPPS consisted of three integrants: a polyarginine (a synthetic CPP similar to Tat PTD); a polyanion to neutralize the cationic charges of polyarginine and to prevent it dispersing into other tissues before arrival at tumors; and a linker of the two parts, a MMP cleavable sequence. ACPPs are stable in the bloodstream due to the insufficient level of MMPs, but when they arrive at tumors which secrete MMP2 and 9, the linker is cleaved by MMP. Subsequently, ACPPs are activated and enter into tumor cells with the exposed polyarginine (**Figure 2B**). Moreover, Tsien's group systematically studied the characteristics of ACPPs with Cy5 labels, including *in vivo* distribution and targeting activity in cancer. This indicates that ACPPs may become a more efficient tool to deliver imaging and therapeutic agents to the sites of invasion, tumor-promoting inflammation and metastasis than CPPs [89,90]. Although Tat PTD is not used in this study, the similarity between Tat PTD and polyarginine provides a new idea for the future application of Tat PTD in carcinomas.

### 4.3.3 *Dependence on receptors of tumor cells*

In order to effectively deliver agents into tumor cells and to decrease toxicity to other tissues, Yang's group [85,91,92] developed an "ATTEMPTS"(antibody targeted, triggered, electrically modified prodrug-type strategy) approach, which integrated Tat PTD into a heparin/protamine-regulated

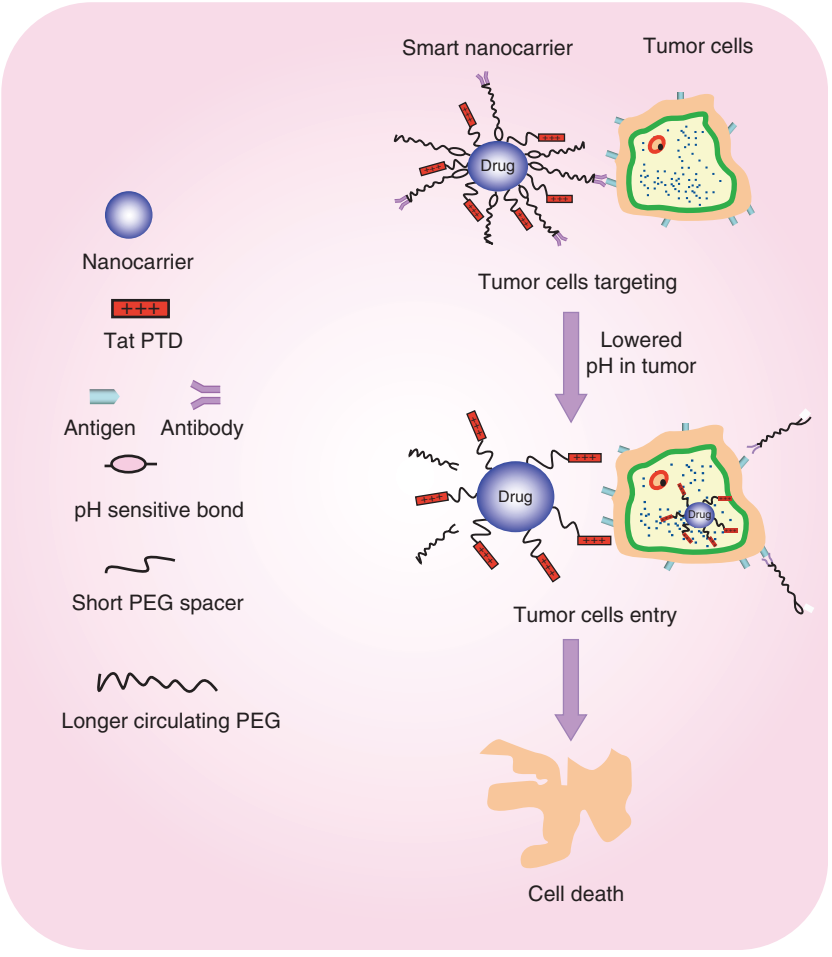


**Figure 4. "Smart" nanocarriers dependent on the acidic pH surrounding tumors built by Torchilin's group.** After reaching the targeted cells via protecting longer polymer (PEG) and antibody guidance, the pH-sensitive bond would detach under the low pH of tumors and Tat PTD would be exposed to induce the subsequent delivery of the carrier into cells.

delivery system, as shown in **Figure 3**. This system consisted of two components: a Tat PTD-drug chimera and the targeting component, the latter including a specific targeting factor, which could be a peptide ligand or an antibody (Ab). The targeting factor is coupled to anionic heparin (Hep) which associates with cationic Tat PTD via electrostatic interactions. Such interactions temporarily inhibit the cell-penetrating function of Tat PTD and prevent its diffusion to the whole body. Following administration, due to the existence of heparin and the targeting factor, the Tat PTD-Drug/Hep-Ab complexes could be directly delivered to the tumor cells and not normal tissues, thus diminishing drug-associated adverse effects. After the complexes reached the target with guidance of the targeting factor protamine sulfate, a clinical heparin antidote that attached to heparin with higher affinity than Tat PTD, could then be medicated as a competing agent to separate Tat PTD-drug chimera from the Hep-Ab counterpart. Once heparin inhibition was lost by protamine, the cell-penetrating activity of the exposed Tat PTD could be restored and the Tat PTD-drug conjugates were allowed to penetrate the targeted tumor cells to induce their apoptosis. Asparaginase (ASNase), an enzymatic antitumor agent, was applied to evaluate the characteristics of the "ATTEMPTS" system as a model drug [92]. An *in vitro* study showed that Tat PTD-ASNase conjugates were not only able to enter the MOLT-4 cells and induce the cytotoxic effects, but also the conjugate uptake could be adjusted (with on/off control) by the addition of heparin and protamine. In the following *in vivo* study, due to the lack of an available antibody for targeting, an alternative strategy was adopted in order to simulate the process of targeting to a tumor [93]. The researchers validated the *in vivo* applicability of this system in animals harboring ASNase-encapsulated L5178Y lymphoma cells. Preliminary results demonstrated that animals inoculated with L5178Y cells containing Tat PTD-ASNase exhibited an extended survival rate of 13% over those harboring L5178Y cells without the encapsulation of ASNase. Furthermore, the Tat PTD-ASNase-treated mice also displayed a significantly

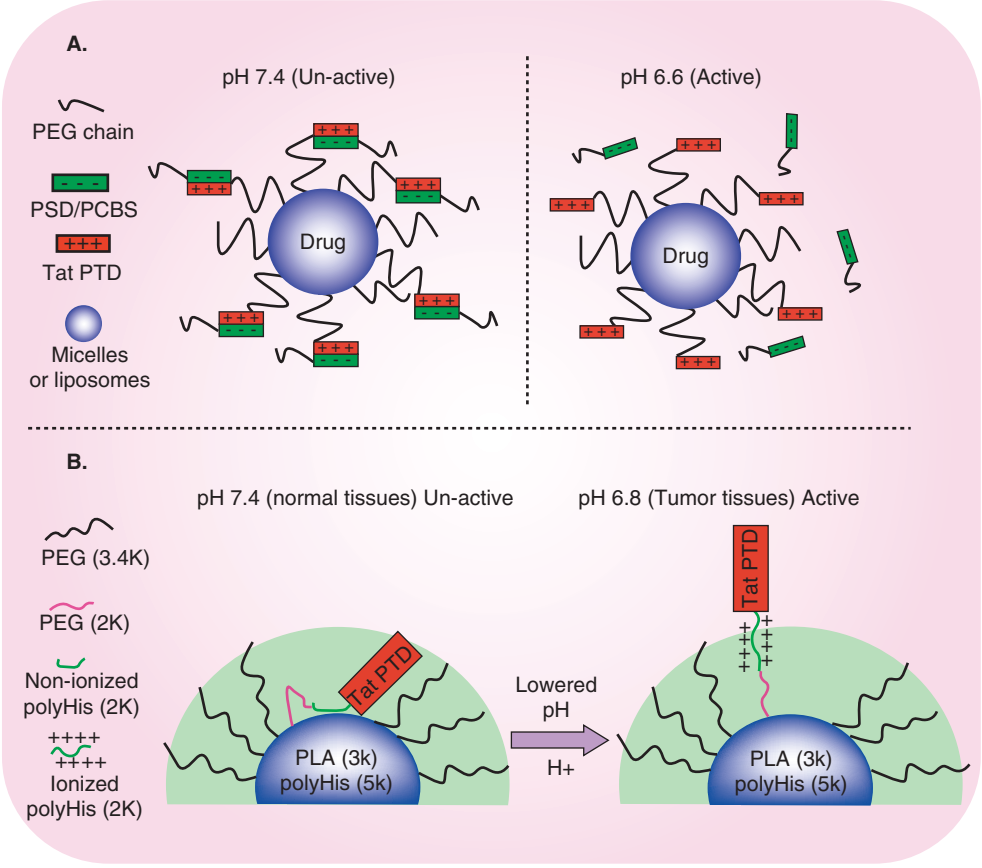


**Figure 5. Targeted systems that are dependent on pH designed by Bae's group. A.** PSD-b-PEG and PCBS-b-PEG were both pH-sensitive polymers, which could shield the cationic charges of transactivator of transcription protein transduction domain (Tat PTD) via ionic interactions in normal pH. When a slight acidic microenvironment caused the protonation of PSD or PCBS, they detached from Tat PTD and the system with exposed Tat PTD became active. **B.** At pH 7.4 Tat PTD was masked within the hydrophilic PEG corona shell by hydrophobic interactions between non-ionized polyHis (2 kDa) and the PLA micelle core. At an acidic pH the micelles would expose Tat PTD on their surface when polyHis (2 kDa) was ionized and could efficiently be delivered into tumor cells.

improved hematologic and liver histologic status compared with the control groups. This was a landmark discovery of a novel delivery system for the treatment of tumors. Once an appropriate antibody is selected, the intact animal evaluation of the entire "ATTEMPTS" system for targeted tumor therapy will be accomplished and this system will progress [86].

#### 4.3.4 *Dependence on acidic pH surrounding tumors*

In order to improve the selectivity of Tat PTD to tumors, "smart" nanocarriers were built by Torchilin's group resorting to some pH-sensitive polymers [94,95]. Smart nanocarriers were long-circulating generally modified, targeted PEGylated liposomes and PEG–phosphatidylethanolamine (PEG–PE)-based micelles. There were two important points: first, these liposomes or micelles were additionally modified with Tat PTD on the surface of the nanocarrier by using TAT-short PEG–PE derivatives; second, a non-specific Tat PTD function was masked by attaching the monoclonal antibody (cancer-specific anti-nucleosome antibody 2C5) to the nanocarriers' surface via a pH-dependent degradable para-nitrophenyl (pNP)–PEG–PE moiety. On reaching the target via protecting polymer or antibody guidance, the pH-sensitive bond would detach under the low pH conditions of tumors, and Tat PTD would be exposed to induce the subsequent delivery of the carrier into cells (**Figure 4**) [96]. The *in vitro* results demonstrated that after brief incubation (15 – 30 min) at lower pH values (pH 5.0 – 6.0), nanocarriers lost their protective PEG shell and Tat PTD-modified nanocarriers were effectively translocated into cells. There is little data to report the *in vivo* characteristics of this smart system with the guidance of an antibody; nevertheless, there are some *in vivo* studies about the systems with long PEG chains that could shield the Tat PTD. In order to evaluate the preliminary *in vivo* properties of this system, liposomes loaded with DNA (plasmid encoding for GFP, pGFP) and additionally modified with Tat PTD and PEG, with PEG binding to

the liposomal surface via both pH-sensitive hydrazone and non-pH-sensitive bonds, were constructed [97]. The results demonstrated that after intratumoral administration in tumor-bearing mice, liposomes with the pH-sensitive hydrazone bonds resulted in highly efficient transfection, as the disruption of PEG under the low intratumoral pH led to the exposure of the liposome-attached Tat PTD. This enhanced the translocation of the liposomes into tumor cells and resulted in a more effective intracellular delivery of pGFP, while liposomes with non-sensitive bonds displayed minimal translocation of tumor cells. These results illustrated the feasibility of smart systems for cancer treatment via intratumoral administration.

Recently, a novel drug targeting system for acidic solid tumors was developed based on an ultra pH-sensitive polymer and Tat PTD by Bae's group (**Figure 5A**) [98,99]. This system generally depended on some nanocarriers, such as micelles and liposomes, which consisted of two components: a hydrophobic core that could entrap antitumor agents and an ultra pH-sensitive diblock copolymer of poly(methacryloyl sulfadimethoxine) (PSD) and PEG (PSD-b-PEG), which could shield the cationic charges of Tat PTD via ionic interactions during delivery and avoid the uptake by non-target cells until it reached the tumors, where the slightly acidic microenvironment induced the protonation of PSD. The following ionic separation of PSD and Tat PTD induced the subsequent exposure of the Tat PTD, which allowed the preferential translocation of the drug-loaded micelles into the surrounding tumor cells. The results indicated that as the pH decreased (from pH 6.6 to 6.0), the detachment of Tat PTD-micelles and PSD-b-PEG occurred immediately and Tat PTD-micelles translocated into MCF-7 cells. However, as PSD-b-PEG was non-biodegradable, which might cause toxic effects if its accumulation was higher than a critical point, Bae's group designed a new biodegradable-TAT shield, that is, an ultra pH-sensitive smart block copolymer PCBS-b-PEG (poly(L-cystine bisamide-g-sulfadiazine)) instead of PSD-b-PEG [99]. The detached PCBS-b-PEG could be degraded rapidly by the native glutathione. Anticancer drug doxorubicin was encapsulated into this micelles and the *in vitro* cytotoxicity at different pHs was evaluated. The system was able to distinguish pHs 7.2 and 7.0 in terms of cytotoxicity. However, a further appreciation of the delivery system, in terms of *in vivo* properties, needs to be consummated.

Bae's group also constructed another novel system "TAT pop-up pH-sensitive micelles" ($PHSM^{pop\text{-}upTat}$) encapsulating doxorubicin (an antitumor agent) [100]. This micelle was composed of a hydrophobic core (PLA (3 kDa) and polyHis (5 kDa) blocks), a hydrophilic shell (PEG (2 kDa) and PEG (3.4 kDa) blocks), and Tat PTD linked to PEG (2kDa) via a pH-sensitive molecular chain actuator, a short polyHis (2kDa). At pH 7.4 Tat PTD was masked within the hydrophilic PEG corona shell by hydrophobic interactions between a non-ionized polyHis (2 kDa) and a PLA micelle core (**Figure 5B**). However, the micelles would expose Tat PTD on their surface when polyHis (2 kDa) was ionized at acidic pH and could efficiently be delivered into tumor cells, which resulted in the increase of the intracellular agent. The *in vitro* and *in vivo* studies substantiated the feasibility of this system. When tested with the xenograft tumors of different drug-resistant cells in a nude mouse model, all tumors significantly regressed in size after three bolus injections of doxorubicin encapsulated $PHSM^{pop\text{-}upTat}$ at 3-day intervals, while a minimum weight loss was observed compared with the control group. Thus, this approach might replace the need for cell-specific antibodies or targeting ligands, thereby providing a general strategy for solid tumor targeting.

## 5. Conclusion

Different mechanisms are involved in the entry of cells by Tat PTD and they may occur independently or simultaneously. The differences depend on the cargo, cell types and even the concentration of chimera. No matter in what way the Tat PTD or its chimera penetrates cells, there is no doubt about its distinguished ability to translocate the cargo into cells. Accordingly, it is used to carry different cargoes with poor penetrating abilities to treat various diseases such as psoriasis, stroke, cancer and so on. It has been shown that with the assistance of Tat PTD, drugs might become more efficient in certain diseases. It is necessary for systematic research to clarify its process in the cells and the factors that influence this. A clearer knowledge of its mechanism of action will better instruct its application in clinical treatment.

## 6. Expert opinion

Thanks to its outstanding ability to cross cell membranes with cargo, Tat PTD can work to cure diseases via local administration of drugs which allows them to reach the action site as soon as possible. For instance, effective agents could enter an infarct position rapidly with the guidance of Tat PTD in the treatment of AMI via intracoronary injection. In this situation, the targeting efficiency of Tat PTD is not important as the agents would play effective roles close to the administration site.

Yet, owing to the high risk and inconvenience of some local administrations, such as intracoronary, intracerebral and intravitreal injections, systemic administration such as i.v. and intraperitoneal injection (i.p.) is still the leading way to medically treat various diseases at present. However, this route of administration puts a much higher demand on the drugs to achieve efficiency. The broad unspecific distribution of Tat PTD limits its application to a large extent; however, the ability of Tat PTD to cross the BBB makes it plausible to apply Tat PTD to drug delivery systems for the CNS. As shown above, Tat PTD has been used to deliver a quantity of drugs to the brain for the treatment of CNS diseases. Though a variety of studies revealed the superiority of Tat PTD chimera

compared with free drugs for the treatment of CNS diseases, there are still many problems to overcome. When the agents penetrate the BBB they also disperse to other non-target tissues, which might result in side effects. Consequently, agents which only have actions at target sites, such as prodrugs, should be used to study Tat PTD-mediated delivery to the CNS. The treatment for cancer with mediation of Tat PTD is similar to that for CNS diseases. Thanks to newly developed technologies such as antibodies, nanocarriers and novel polymers, it is plausible for efficient treatment of cancer with Tat PTD. Although some novel systems with Tat PTD could selectively deliver the agents into tumor cells or tissues in *in vitro* or preliminary *in vivo* studies, it is difficult to achieve clinical application of Tat PTD for the treatment of cancer owing to excessive issues such as the choice of appropriate antibody, antitumor agents, the transient shield of Tat PTD and so on. Unless a transient mask is present before being exposed at a target site, the application of Tat PTD-mediated drug delivery into a specific cell type or tissue will not become common in pharmacology. Despite some drawbacks with the development of antibodies, novel carriers and CPPs, Tat PTD is expected to play a more efficient and/or more tolerated therapeutic role in drug delivery.

## Declaration of interest

The authors state no conflict of interest. This work was funded by Graduate Independent Innovation Foundation of Shandong University.



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