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Tat-Mediated Peptide Intervention in Analgesia and Anesthesia

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Abstract

Membrane-permeable peptide carriers are attractive drug delivery tools. Among such carriers, the protein transduction domain (PTD) of the human immunodeficiency virus-type 1 Tat protein is most frequently used and has been successfully shown to deliver a large variety of cargoes. The Tat PTD can facilitate the uptake of large, biologically active molecules into mammalian cells, and recent studies have shown that it can mediate the delivery of different cargoes into tissues throughout a living organism. Given that the Tat PTD-mediated delivery is size-independent, this technology could make previously non-applicable large molecules usable to modulate biological function *in vivo* and treat human diseases. It is likely that the peptide carrier-mediated intracellular delivery process encompasses multiple mechanisms, but endocytic pathways are the predominant internalization routes. Tat PTD has been successfully used in preclinical models for the study of cancer, ischemia, inflammation, analgesia, and anesthesia. Our recent studies have shown that intraperitoneally injected fusion Tat peptide Tat-PSD-95 PDZ2 can be delivered into the spinal cord to dose-dependently disrupt protein-protein interactions between PSD-95 and NMDA receptors. This peptide significantly inhibits chronic inflammatory pain and reduces the threshold for halothane anesthesia. The ability of the Tat PTD to target any cell is advantageous in some respects. However, the drug delivery system will be more attractive if we can modify the Tat PTD to deliver cargo only into desired organs to avoid possible side effects.

Keywords

protein transduction domain; pain modulation; minimum alveolar anesthetic concentration; protein-protein interaction

INTRODUCTION

The development of new drugs, including analgesics and anesthetics, is necessary to improve efficacy and reduce side effects. However, in the process of drug development, drug discovery is only the first step. The active ingredient of the drug must reach the right place in order to have the desired therapeutic effects. Many drug candidates show promising *in vitro* activity but fail to proceed to clinical trial because of their poor absorption, distribution, metabolism, or excretion (ADME) profiles. Moreover, many drugs have limited administrative routes for clinical use. Data indicate that only about 1 out of 10 new drug candidates survives after entering clinical trials and that more than 30% of the failures are attributable to inadequate ADME profiles in early clinical phase [Kola and Landis 2004]. In fact, many would-be drugs that are proteins, peptides, or DNA cannot be properly delivered to the desired organs by available delivery systems. In addition, many small-molecule drugs are not utilized to their full therapeutic potential because of poor aqueous solubility and/or

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inadequate delivery tools. Thus, a novel drug delivery technology could substantially improve the efficacy of some new drugs. In 2000, annual sales of drug delivery systems in the United States were estimated to be more than $20 billion [Langer, 2001]. Thus, substantial scientific and technical challenges and tremendous commercial opportunity remain in the arena of new drug delivery systems.

Over the past 10 years, membrane-permeable peptide carriers have been developed for use as intracellular delivery devices for a range of bioactive, membrane-impermeable molecules. [Chung et al., 2008; Gump and Dowdy, 2007; Nakase et al., 2008; Rapoport and Lorberboum-Galski, 2009; Snyder and Dowdy, 2005; Wadia and Dowdy, 2002].) This technological advance could make it possible to add to the therapeutic arsenal agents that include peptides, proteins, nucleic acids and their derivatives, liposomes, nanoparticles, and synthetic polymers [Joliot and Prochiantz, 2004; Futaki, 2006; Gupta and Torchilin, 2006; Goun et al., 2006; Snyder and Dowdy, 2005; Jarver and Langel, 2006; Gupta et al., 2005]. Among membrane-permeable peptide carriers, the protein transduction domain (PTD) of the human immunodeficiency virus-type 1 (HIV-1) Tat protein (residues 47–57 of HIV-1 Tat) [Becker-Hapak et al., 2001] and oligoarginines [Rothbard et al., 2000; Futaki et al., 2001] are the most frequently used and have been proven to be effective peptide carriers. These peptide carriers are rich in arginine residues. Several lines of evidence have suggested that hydrogen-bond formation between arginine guanidinium groups and phosphates, sulfates, and carboxylates on cellular components is crucial for achieving intracellular delivery [Rothbard et al., 2004; Sakai et al., 2005; Nakase et al., 2008].

PTD-mediated *in vivo* delivery of biologically active peptides represents a novel and promising strategy to treat central nervous system (CNS) diseases. It has been demonstrated that fusion peptides containing the PTD sequence derived from HIV-1 Tat protein can be transduced into the CNS after systemic administration [Denicourt and Dowdy, 2003]. Previous work also has shown that the PTD can be used efficiently to transduce a biologically active neuroprotectant in experimental cerebral ischemia [Cao et al., 2002]. In our recent studies, fusion Tat peptide that was injected ip into mice, entered the spinal cord and dose-dependently disrupted protein-protein interactions [Tao et al., 2008; Tao and Johns, 2008]. This peptide significantly inhibited chronic inflammatory pain [Tao et al., 2008] and reduced the threshold for halothane anesthesia [Tao and Johns, 2008].

MEMBRANE-PERMEABLE PEPTIDE CARRIERS

The original concept of protein transduction was described in 1988 when it was reported that HIV-1 Tat was able to enter cells in culture when added to the medium [Green and Loewenstein, 1988; Frankel and Pabo, 1988]. The domain that is responsible for this intracellular delivery was then identified as the short region of Tat residues 47–57 [Green and Loewenstein, 1988; Frankel and Pabo, 1988]. Since then, great progress has been made in this research field, and other PTDs possessing this ability have been found. These PTDs include Antennapedia (Antp, a *Drosophila* homeoprotein), Herpes simplex virus type 1 protein VP22, chimeric PTD transportan, synthetic oligoarginines (such as 9-mer of arginine, R9), nuclear localization signal sequences (NLS), and model amphipathic peptide [Gupta and Torchilin, 2006; Torchilin, 2008b; Murriel and Dowdy, 2006; Ragin et al., 2002]. Most of these PTDs are short peptides (8–16mers) rich in basic residues (arginine and/or lysine) and are therefore highly cationic. Among these PTDs, the Tat PTD remains the best known and investigated. The Tat PTD is an 11mer (residues 47–57) sequence of the HIV-1 Tat protein that activates transcription of the viral genome. Like other basic PTDs, the Tat PTD is rich in arginine and lysine, and thus, highly positively charged and hydrophilic [Green and Loewenstein, 1988; Frankel and Pabo, 1988; Herce and Garcia, 2007]. It has been demonstrated that peptides or proteins that are fused to the Tat PTD are

rapidly and efficiently delivered into cultured cells and into live tissues when injected into mice, while retaining their biological activity [Futaki et al., 2001; Tao et al., 2008; Tao and Johns, 2008; Cai et al., 2006; Luft, 2003; Schwarze, et al. 1999; Schwarze and Dowdy, 2000].

In addition to PTDs, membrane-permeable peptide carriers are also known as cell-penetrating peptides (CPPs), Trojan peptides, or membrane translocating sequences (MTS). They may be classified into three groups: basic peptides, such as Tat PTD; basic/amphiphilic peptides, such as Antp; and hydrophobic peptides, such as NLS [Futaki et al., 2003; Chung et al., 2008]. The peptide carriers vary greatly in size, amino acid sequence, and charge type, but share the common ability to rapidly cross the plasma membrane and deliver cargo to the cytoplasm or nucleus [Lindgren et al., 2000]. It has been demonstrated that the peptide carriers can deliver a wide range of bioactive molecules to a variety cell types and to different cellular compartments [Jarver and Langel, 2006; Gupta et al., 2005]. Peptide-mediated delivery of bioactive molecules is superior to commonly used delivery systems in many aspects. Advantages such as high delivery yield, low toxicity, and the possibility to add diverse modifications to the peptide backbone make peptide carriers excellent candidates for future drug delivery platforms.

As described above, Tat PTD is rich in the basic or cationic amino acid arginine. Various other arginine-rich viral and synthetic peptides have been determined to have intracellular delivery ability similar to that of the Tat PTD [Futaki et al., 2001]. These peptides differ from each other in primary or secondary structure, but they all have several arginine residues in their sequences. By using arginine oligomers of different lengths, previous study has shown that the optimum number of arginine residue is around eight or nine for efficient delivery [Futaki et al., 2001]. R9 was 20 times more efficient than the Tat PTD [Wender et al., 2000]. However, positive charge of these peptides is not sufficient for cargo transport because oligomers of several other cationic amino acids (such as histidine, lysine, and ornithine) were less effective than the Tat PTD [Wender et al., 2000]. These results suggest that the guanidinium groups of the arginine residues play an essential role in the peptide carrier-mediated cargo translocation [Chung et al., 2008].

MECHANISM OF TAT PTD-MEDIATED TRANSDUCTION INTO CELLS

The cellular uptake of the Tat PTD and other arginine-rich peptides once was thought to occur via temperature- and energy-independent mechanisms rather than through endocytic pathways [Vives et al., 1997]. However, subsequent studies showed that cell fixation caused membranes of endosomes loaded with arginine peptides to become highly leaky [Richard et al., 2003; Lundberg and Johansson, 2002]. When cells were treated with arginine-rich peptides at low temperature (e.g., 4°C) where endocytosis should not occur, perturbation of plasma membranes by fixation procedures induced peptides adsorbed on the cell surface to leak into the cytosol and nucleus. Consequently, the peptides became distributed diffusely in these regions. However, careful reinvestigation with intact living cells has shown that endocytic pathways are the predominant translocation routes for these peptides under defined conditions.

There are two types of endocytic uptake. The first is clathrin-mediated and lipid raft-mediated uptake through the formation of caveolae, and the second is non-clathrin, non-caveolar endocytosis called macropinocytosis [Gupta and Torchilin, 2006; Murriel and Dowdy, 2006; Torchilin, 2008a,b; Gump and Dowdy, 2007]. Tat PTD-mediated protein translocation occurs via the latter. Macropinocytosis is a fluid phase endocytosis by which cells can take up large extracellular molecules from the surrounding medium [Rapoport and Lorberboum-Galski, 2009]. It is also a non-selective endocytosis for efficient uptake of

solute macromolecules. The first step of the process appears to involve a charge-charge interaction of the basic Tat PTD with acidic motifs on the cellular membrane. It has been reported that membrane-associated proteoglycans including heparan sulfate play crucial roles in the uptake of peptides by this type of endocytosis [Richard et al., 2005; Suzuki et al., 2002; Console et al., 2003; Ziegler and Seelig, 2004; Goncalves et al., 2005; Fuchs and Raines, 2004; Nakase et al., 2004; Nakase et al., 2007; Wadia et al., 2004; Kaplan et al., 2005; Fretz et al., 2006; Futaki et al., 2007]. Considerable evidence has shown that Tat PTD binding to polyanionic glycan sugar chains like heparan sulfate on the cell surface promotes macropinocytosis of Tat PTD and cargo into macropinosomes [Torchilin, 2008a,b; Murriel and Dowdy, 2006; Gupta and Torchilin, 2006; Gump and Dowdy, 2007; Amand et al., 2008]. The rate-limiting step in this process is the last step in which Tat PTD and its cargo emerge from the macropinosome into the cytoplasm.

A range of mechanistic pathways has been proposed for peptide carriers. It is very likely that different PTDs utilize different uptake mechanisms that depend not only on the nature of the peptide carrier, but also on the size and nature of the cargo to which the carriers are conjugated, the membrane composition, and the physiological state of the target cells [Joliot and Prochiantz, 2004; Tunnemann et al., 2006].

TAT PTD-MEDIATED TRANSDUCTION IN ANALGESIA AND ANESTHESIA

The ability of the Tat PTD-fusion peptides/proteins to cross the blood-brain barrier (BBB) has encouraged many researchers to use this system in developing therapies for CNS diseases. The exogenous delivery of peptides and proteins into cells has many possible therapeutic prospects. These include influencing various signaling pathways; disrupting protein-protein interactions; affecting cell cycle progression; restoring deficiencies in enzymes; changing cell fate by driving cells into apoptosis or saving them from cell death; and manipulation of cellular function and differentiation. In the late 1990s, the first use of Tat PTD as an intracellular delivery tool was reported for introducing proteins into cells *in vitro* [Moy et al., 1996; Nagahara et al., 1998] and into mice *in vivo* [Schwarze et al., 1999]. Since then, numerous investigators have used Tat PTD to deliver various peptides/proteins into cells in the form of Tat PTD-fusion peptides/proteins [Gump and Dowdy, 2007; Rapoport and Lorberboum-Galski, 2009]. So far, Tat PTD has been successfully used in the treatment of cancer, ischemia, and inflammation as well as to investigate mechanisms of analgesia and anesthesia [Aarts et al., 2002; Cao et al., 2002; Tao et al., 2008; Tao and Johns, 2008; Harada et al., 2006; Zhou et al., 2008; Liu et al., 2008; Myou et al., 2003a,b]. Below we describe the recent use of Tat PTD-fusion peptides in analgesia and anesthesia.

Chronic pain stemming from a variety of health conditions is the primary reason people seek medical care. Considerable evidence indicates that the development of central hyperexcitability and persistent pain involves the activation of *N*-methyl-D-aspartate receptors (NMDARs), which play an important role in the processing of nociceptive information [Garry et al., 2000; Mao et al., 1992; Ren et al., 1992; Wei et al., 2001]. However, directly blocking the function of NMDARs is therapeutically impractical because doing so would also impede other vital synaptic transmissions in the CNS. Recently, Liu et al. [2008] created a Tat PTD-fusion peptide consisting of amino acids 40–49 of the protein tyrosine kinase Src (a key enhancer of NMDAR function) fused to Tat PTD (Src40–49Tat) to inhibit the interaction between NMDARs and Src without blocking NMDARs. They found that this Tat PTD-fusion peptide prevented pain behaviors in rodents induced by intraplantar formalin injection and reversed pain hypersensitivity produced by intraplantar injection of complete Freund's adjuvant (CFA) or peripheral nerve injury [Liu et al., 2008]. They also showed that the fusion peptide Src40–49Tat had no effect on basal sensory thresholds, acute nociceptive responses, or cardiovascular, respiratory, locomotor, or

cognitive functions [Liu et al., 2008]. Thus, they were able to suppress chronic pain through targeting of Src-mediated enhancement of NMDARs, without causing the deleterious consequences of blocking NMDARs directly. This type of approach might be adaptable for broad application in clinical management of chronic pain.

In recent studies [Tao et al., 2008; Tao and Johns, 2008], we demonstrated the ability of another Tat PTD-fusion peptide to inhibit CFA-induced chronic inflammatory pain and reduce the threshold for inhaled anesthetic halothane anesthesia. Postsynaptic density protein-95 (PSD-95), a scaffolding protein, has been identified to attach NMDARs to internal signaling molecules at neuronal synapses of the CNS [Christopherson et al., 1999; Kornau et al., 1995]. This function suggests that PSD-95 might be involved in physiological and pathophysiological actions triggered via the activation of NMDARs in the CNS. Therefore, targeting PSD-95 protein represents a potential therapeutic approach for diseases that involve NMDAR signaling. NMDAR–PSD-95 protein interactions are mediated by a PDZ domain (a term derived from the names of the first three proteins identified to contain the domain: PSD-95, Dlg, and ZO-1). PSD-95 possesses three PDZ domains. The second (PSD-95 PDZ2) interacts with NMDAR NR2 subunits at a seven-amino acid, COOH-terminal domain that contains a terminal tSXV motif (where S is serine, X is any amino acid, and V is valine) [Kornau et al., 1995]. Our previous studies have shown that the expression of spinal PSD-95 is critical for NMDAR-mediated thermal hyperalgesia [Tao et al., 2000] and that the knockdown of spinal PSD-95 by intrathecal injection of PSD-95 antisense oligodeoxynucleotide delays the onset of neuropathic pain and diminishes the maintenance of pain behaviors [Tao et al., 2001; Tao et al., 2003]. To define further the role of PDZ domain–mediated NMDAR–PSD-95 protein interactions in inflammatory sensitization of nociceptive behaviors, we constructed a peptide comprising the PSD-95 PDZ2 and rendered it cell permeable by fusing it to Tat PTD to obtain the fusion peptide Tat-PSD-95 PDZ2. We injected mice intraperitoneally (systemically) or intrathecally (locally) with this fusion peptide and then assessed their behavioral responses to intraplantar injection of CFA [Tao et al., 2008]. Importantly, we showed that Tat-PSD-95 PDZ2 was delivered into the spinal cord after ip administration. Furthermore, the fusion peptide dose-dependently disrupted the protein-protein interactions between NMDAR NR2 subunits and PSD-95 and significantly inhibited inflammatory sensitization of the behavioral responses induced by intraplantar injection of CFA [Tao et al., 2008]. These results suggest that PDZ domain–mediated protein interactions at spinal synapses might play an important role in the molecular mechanisms of inflammatory pain behaviors. Our study provides novel insight into the molecular mechanisms that underlie chronic inflammatory pain states and a new approach for chronic inflammatory pain therapy. We also investigated the effect of disrupting NMDAR–PSD-95 protein interactions with the fusion peptide Tat-PSD-95 PDZ2 on the threshold for halothane anesthesia in mice [Tao and Johns, 2008]. We found that the Tat fusion peptide significantly reduced the minimum alveolar anesthetic concentration and righting reflex EC_{50} value for inhaled anesthetic halothane [Tao and Johns, 2008], suggesting that PDZ domain–mediated protein interactions at synapses in the CNS contribute to the central mechanisms of inhalational anesthesia.

CONCLUSIONS AND PERSPECTIVES

Over the past 10 years, the progress toward using a Tat PTD-based delivery system for the introduction of a variety of cargoes into cells and organs has been exciting and encouraging. This Tat PTD-based delivery system has many advantages. First, Tat PTD is able to deliver different cargoes into all types of cells in culture and into all organs *in vivo* [Schwarze et al., 1999; Schwarze and Dowdy, 2000; Gupta and Torchilin, 2006; Gump and Dowdy, 2007; Cai et al., 2006; Becker-Hapak et al., 2001]. In addition, the ability of Tat PTD to cross the BBB makes this delivery system a promising technique in the development of novel

therapeutic approaches for the treatment of CNS diseases. Second, translocation of cargo by the Tat PTD-based delivery system is size-independent; constituents can range in size from small peptide molecules to massive structures such as liposomes [Torchilin et al., 2003; Torchilin et al., 2001]. Third, the Tat PTD-based delivery system is a non-viral system and thus eliminates the possibility of integration of foreign nucleic acid sequences into the genome [Gump and Dowdy, 2007]. Finally, the Tat PTD-based delivery system can be used for specific subcellular delivery (such as to nuclei, mitochondria, and lysosomes); therefore this delivery system potentially can be developed as a subcellular organelle-targeted therapy [Del et al., 2003; Del and Payne, 2003; Rapoport et al., 2008; Zhang et al., 2008; Yoshikawa et al., 2008].

As with any new technology, the Tat PTD-based delivery system also has some pitfalls. The most important drawback is that it does not allow for specific cell or organ targeting. In addition, high concentrations or prolonged use could lead to toxicity [Jones et al., 2005] and possible immunogenicity, especially after repeated use. These shortcomings will limit its clinical application. In the future, it will be necessary to modify the Tat PTD in order to make its delivery more specific and thereby widen its therapeutic potential. The ability to specifically target Tat PTD could theoretically reduce the side effects produced by the delivery of cargo to undesired organs and reduce the total amount of Tat PTD-fusion peptide required to achieve a therapeutic effect.

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