Expert Opinion





***Ashley Publications***
***www.ashley-pub.com***

# Recent advances in the use of protein transduction domains for the delivery of peptides, proteins and nucleic acids *in vivo*

Eric L Snyder & Steven F Dowdy†
*†UCSD School of Medicine, Howard Hughes Medical Institute and Department of Cellular & Molecular Medicine, 9500 Gilman Drive, La Jolla, CA 92093-0686, USA*

Protein transduction domains (PTDs) are small cationic peptides that can facilitate the uptake of large, biologically active molecules into mammalian cells. Recent reports have shown that PTDs can mediate the delivery of cargo to tissues throughout a living organism. Such technology could eliminate the size restrictions on usable drugs, so enabling previously unavailable large molecules to modulate *in vivo* biology and alleviate disease. This article will review the evidence that PTDs can be used both to deliver active molecules to pathological tissue *in vivo* and to treat models of disease such as cancer, ischaemia and inflammation.

**Keywords:** cell-penetrating peptide, CPP, protein transduction, PTD, TAT

*Expert Opin. Drug Deliv. (2005) 2(1):43-51*

## 1. Introduction

The terms 'protein transduction domain' (PTD) and 'cell-penetrating peptide' (CPP) are typically used to refer to a class of small (< 20 amino acid) cationic peptides that can traverse the plasma membrane of many, if not most, mammalian cells (for recent reviews see [1,2]). The importance of these peptides for drug delivery lies in their ability to uniformly transport large, biologically active molecules (such as proteins or oligonucleotides) into ~ 100% of a population of mammalian cells growing under standard culture conditions. Further interest in these peptides was stimulated by the observation that PTDs can also facilitate systemic delivery of recombinant proteins to a large number of tissues in a living mouse [3]. These results raised the possibility that large intracellular proteins linked to PTDs might be used therapeutically just as extracellular proteins (i.e., insulin, monoclonal antibodies) are employed in clinical practice today. This review will focus on recent studies that have demonstrated the ability of PTD conjugates to modulate the biology of living organisms.

## 2. Protein transduction domains

Among the most frequently utilised PTDs are the TAT, Antennapedia (Antp), and poly-arginine peptides. Protein transduction was first observed when the full-length HIV TAT protein was found to be capable of entering mammalian cells and activating transcription from an HIV long-terminal repeat promoter construct [4,5]. Subsequent studies defined the specific region of the protein necessary for cellular uptake [6]. In a similar fashion, the Antp peptide was derived from a *Drosophila* homeodomain protein [7]. Both of these peptides are rich in lysines and arginines; the basic nature of which appears to be essential for their cell-permeation properties. Indeed, independently derived PTDs often consist largely of positively charged amino

acids [8], and even short peptides consisting entirely of arginines [9] or lysines [8] deliver cargo to the cellular interior.

Although both TAT and Antp have been used extensively as carriers for relatively small cargo such as peptides and oligonucleotides, TAT has been the predominant PTD used in the delivery of large molecules such as full-length proteins [10]. Since their initial description [11], many in-frame TAT fusion proteins produced in bacteria have been shown to enter mammalian cells and carry out intracellular functions ranging from cytoskeletal reorganisation to recombination of genomic DNA (for a recently compiled list of transducible TAT fusion proteins, see [10]). TAT peptides have even been used to affect the intracellular entry of much larger molecules such as 45 nm iron beads [12], lambda phage [13], and liposomes complexed with plasmid DNA [14,15].

Recent experiments have demonstrated that TAT proteins [16] and peptides (Kaplan *et al.*, unpublished observations) enter mammalian cells by macropinocytosis, a lipid-raft-dependent, receptor-independent type of endocytosis. These studies showed that uptake occurs via endocytosis but is independent of caveolae or clathrin. In contrast, uptake does require the presence of lipid rafts in the plasma membrane and is blocked by inhibitors of macropinocytosis. These observations are consistent with older reports that neither sequence inversion nor synthesis with D-amino acids ablates the function of TAT and Antp, indicating that they do not enter the cell by interaction with a chiral receptor [9,17]. These observations also extend more recent re-evaluations of the protein transduction literature, which suggested that early claims of endocytosis-independent uptake of TAT were based on artifactual redistribution of peptide after cellular fixation [18].

Knowledge of the mechanism of protein transduction should facilitate efforts to make intracellular delivery more efficient. The influenza virus synthesises a protein (HA2) that affects endosomal escape via lipid membrane destabilisation. TAT–HA2, a transducible peptide derived from this protein, significantly enhanced intracellular DNA recombination by a TAT–Cre fusion protein in living cells [16]. It is likely that such enhancers of transduction, either in the form of peptides or small molecules, will also be used to facilitate protein transduction in living organisms.

## 3. *In vivo* applications of protein transduction domains

PTDs have proven their ability to manipulate the biology of cultured mammalian cells by delivering cargo such as peptides and recombinant proteins (**Figure 1**) [19]. However, the ability to deliver large, biologically active molecules to the interior of cells in a living organism would also be of tremendous benefit. To determine whether PTDs might facilitate such delivery, a TAT–α-galactosidase fusion protein was delivered to mice by intraperitoneal injection [3]. Analysis of tissue sections revealed delivery of the fusion protein to many, if not most, tissues of the mouse, including the brain. Importantly, the X-gal assay used in this study demonstrated that the TAT–α-galactosidase, a 120 kDa protein enzyme, retained its activity *in vivo*. These observations stimulated a number of groups to examine the ability of PTD peptides and proteins to modulate the biology of cells and tissues *in vivo*. In the past 5 years, PTDs have been successfully used to treat mouse models of cancer, ischaemia, inflammation and other diseases.

### 3.1 Cancer

A number of studies have examined the ability of transducible peptides and proteins to inhibit tumour growth *in vivo*. Given the lack of specificity of current cancer therapy and the limitations of gene therapy, PTDs appear to be an attractive means by which to introduce tumour suppressors or other pro-apoptotic proteins directly into the cancer cells that make up tumours *in vivo*.

Mutation of genes in the p53 pathway is thought to be nearly universal in human cancer [20], so restoration of p53 function in cancer cells would be a useful application of *in vivo* protein transduction. The authors' laboratory attempted to do this by linking TAT to a peptide derived from the C terminus of p53 [21]. This p53C′ peptide was previously shown to activate wild-type and certain p53 mutants in cancer cells, leading to apoptosis [22-24]. One potential pitfall of using peptides in living organisms is the short half-life of many peptides secondary to degradation. To address this, the group synthesised a retro-inverso version of the TAT–p53C′ peptide by inverting the peptide sequence and using D-amino acids. This double inversion of peptide structure often leaves the surface topology of the side chains intact, and has been used extensively to stabilise biologically active peptides for *in vivo* applications [25].

After confirming that the RI–TATp53C′ peptide retained the p53-activating function of its parental peptide, its efficacy in three different models of human cancer was tested. In the first model it was found that intraperitoneal injection led to delivery of peptide to subcutaneous tumours and caused significant inhibition of their growth. In a second model, peptide administration resulted in a sevenfold increase in lifespan in a model of terminal peritoneal carcinomatosis. Finally, peptide-treated mice harbouring a model of B-cell lymphoma achieved a 50% long-term survival (> 200 days), whereas control mice died an average of 35 days after lymphoma cell inoculation [21]. Notably, two of these models utilised immune-competent mice, showing that the immune system is not an absolute barrier, at least in the short-term, to successful *in vivo* protein transduction. The fact that these studies utilised models of terminal malignancy are also relevant because anticancer therapeutics are defined as clinically successful by their ability to alleviate pathology and extend survival, and not simply by their ability to reduce tumour size.

Another highly successful anticancer protein transduction strategy has involved the use of a peptide derived from the Smac protein. Multiple groups have found that the N-terminus of Smac can be linked to either TAT or Antp to facilitate

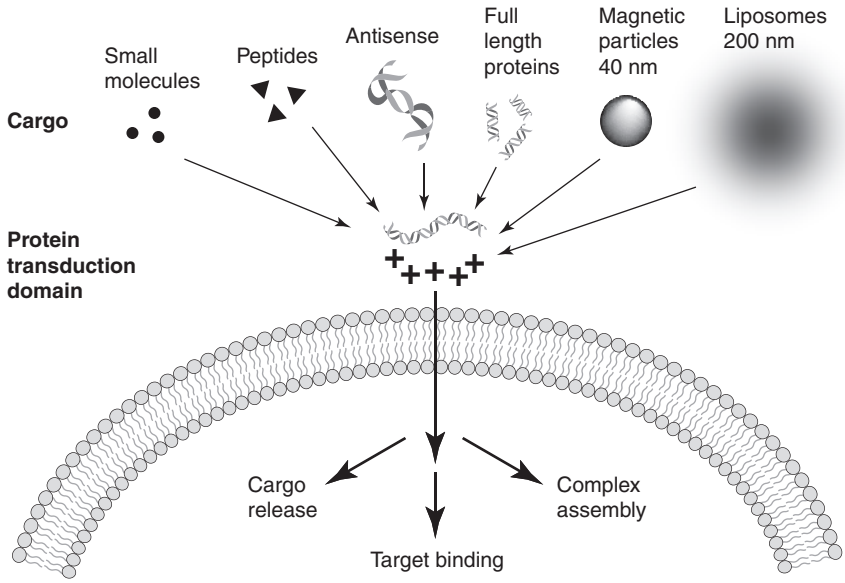


**Figure 1. Protein transduction into cells.** Cationic protein transduction domains carry cargo into cells by a receptor-independent, fluid phase macropinocytosis: a specialised form of endocytosis. Delivered classes of cargo molecules represent a wide range of sizes and biophysical properties from small molecules to peptides to proteins to peptide nucleic acid to DNA to phage particles to magnetic nanoparticles and liposomes.

cellular uptake [26-28]. Smac is a mitochondrial protein that can inactivate the inhibitor of apoptosis (IAP) proteins, and all three studies found that Smac–TAT or Smac–Antp sensitised cells to pro-apoptotic stimuli. One group took these results a step further and tested the function of the Smac–TAT peptide in an intracranial glioblastoma xenograft model [27]. The authors first showed that local administration of TNF-related apoptosis-inducing ligand (TRAIL), a death receptor ligand with specificity for tumour cells, reduced tumour volume and moderately extended the life of nude mice bearing established intracranial U87MG tumours. By contrast, local treatment with Smac–TAT alone had no effect on tumour growth. When TRAIL and Smac–TAT were co-administered, however, there was a synergistic effect on tumour volume and mouse survival. Whereas control mice all died of tumour burden by 35 days after tumour cell injection, mice treated with Smac–TAT and the highest doses of TRAIL survived beyond 70 days after the start of the experiment. Furthermore, histological analysis revealed no evidence of remaining tumour in the brain. The general applicability of this approach was later demonstrated by an independent group who used nude mice harbouring subcutaneous lung cancer xenografts to show that intratumoural injection of a transducible Smac peptide sensitised the cancer cells to systemic cisplatin therapy [29].

Some groups have sought to take advantage of the tumour microenvironment when designing anticancer protein transduction strategies. In a clever series of experiments, Harada *et al.* fused the oxygen-dependent degradation domain (ODD) of hypoxia-inducible factor (HIF)-1α to the TAT–α-gal and the TAT–caspase-3 fusion proteins [30]. The ODD domain stimulates degradation of HIF-1α under normoxic, but not hypoxic, conditions. Thus, the authors hypothesised that a TAT–ODD fusion protein would be stable in the hypoxic core of tumours, but would be degraded and non-functional in normal tissue. Indeed, after intraperitoneal injection of TAT–ODD–α-gal into tumour-bearing nude mice, only the hypoxic regions of the tumours showed evidence of TAT–ODD–α-gal protein. By contrast, TAT–α-galactosidase protein could be detected throughout tumours after intraperitoneal delivery. Furthermore, TAT–ODD–α-gal was undetectable in mouse liver after intraperitoneal injection, again unlike the parental TAT-α-gal protein. Next, tumour-bearing mice were given intraperitoneal injections of a TAT–ODD–caspase-3 protein. TAT–ODD–caspase-3 reduced tumour growth without causing the toxic side effects that would be expected from delivering active caspase-3 to an entire mouse. This group recently extended their results by testing the TAT–ODD–caspase-3 protein in a rodent model of malignant ascites in which cancer cells grow in the hypoxic environment of the peritoneum [31]. They found that administration of TAT–ODD–caspase-3 induced a 60% cure rate in an otherwise lethal model of cancer. Similarly, Willam *et al.* [32] used TAT delivery of ODD peptides to modulate

angiogenesis *in vivo*. These studies show that functional domains such as ODD can be used to modulate the type of tissue in which TAT fusion proteins are active and in this way increase their specificity for cancer cells.

Several other reports have shown that PTDs can be used to attack cancer cells *in vivo* by targeting a number of the signal transduction pathways known to be dysregulated during tumorigenesis. Local administration of CPPs that block Cdk2/cyclin A activity [33], sequester mdm2 [34] or induce apoptosis by mitochondrial disruption [35] has been shown to reduce tumour burden in living animals. Other examples of systemic delivery of CPPs include the fusion of TAT to a peptide derived from the von Hippel-Lindau (VHL) tumour suppressor that inhibits IGF-I receptor signalling in renal cell carcinomas [36]. Intraperitoneal administration of TAT–VHL peptide slowed the growth of subcutaneous renal cell carcinoma tumours in nude mice, primarily through inhibition of cell proliferation rather than by induction of apoptosis. Peptide treatment appeared to reduce tumour invasion into the underlying tissue as well. This study also provides strong immunohistochemical evidence that the TAT–VHL peptide is homogeneously delivered to the tumours after intraperitoneal injection. Another report found that intraperitoneal delivery of an Antp–p16 fusion peptide moderately inhibited the growth of pancreatic cancer cells growing as intraperitoneal and as subcutaneous tumours in nude mice [36]. Although p16 functions primarily as an inhibitor of cell-cycle progression, the authors found that Antp–p16 slowed tumour growth by inducing apoptosis of cancer cells *in vivo*. In neither study was any toxicity to normal tissue observed [36,37], indicating that cancer cells may be much more sensitive to the effects of transducible tumour suppressor peptides than non-transformed cells. If this proves to be a general rule, CPPs may prove to be a powerful tool in the design of anticancer agents that target tumours while sparing normal tissue.

### 3.2 Ischaemia

Cerebral and cardiac ischaemia remain some of the most common causes of morbidity and mortality. In particular, only one medical therapy for cerebral ischaemia (stroke) has been approved (recombinant tissue plasminogen activator), and it must be administered within 3 h of the onset of ischaemia to be effective [38] (this is a time point at which most patients have not obtained medical care). Cerebral ischaemia and reperfusion lead to neuronal necrosis and apoptosis by a variety of mechanisms, including energy depletion, peri-infarct depolarisation and excitotoxicity secondary to the *N*-methyl-D-aspartate (NMDA) receptor activation.

Protein transduction has been used to target multiple levels of the neuronal response to ischaemia in order to block cell death and preserve neurological function after stroke. Most recently, inhibition of the c-Jun N-terminal kinase (JNK) has been shown to block neuronal cell death after ischaemia *in vivo* [38,39]. Previous data had shown that JNK activity increased after NMDA receptor (NMDAR) activation, and was at least partly responsible for inducing neuronal cell death through phosphorylation of its multiple effectors. The authors, therefore, linked TAT to a 20 amino acid peptide derived from JNK-interacting-protein in order to competitively block interactions between JNK and its substrates. The authors also generated a stable, retro-inverso version of this peptide, which they termed D-JNKI-1. They found that D-JNKI-1 inhibited JNK activity *in vitro* and *in vivo*. They also showed that administration of the peptide was effective in reducing infarct size in rodent models of both transient and permanent cerebral artery occlusion. For example, intraventricular injection of D-JNKI-1 up to 6 h after transient middle cerebral artery occlusion led to a ~ 90% reduction in infarct size at time points up to 14 days post-occlusion. Peptide-treated ischaemic mice also displayed no decline in locomotor performance at up to 14 days post-occlusion when compared with ischaemic mice given no treatment. This is an important experimental end point because new medications for stroke victims will ultimately be judged on their ability to improve neurological outcomes rather than to reduce absolute infarct size.

Another report used TAT peptides to target a different level of the cell death pathway that lies downstream of NMDAR activation [40]. During ischaemia, NMDARs interact with the intracellular protein PSD-95, which leads to nitric oxide production and cell death. Aarts *et al.* found that an NMDAR-derived peptide could be linked to TAT and delivered to cultured neurons. This TAT–NMR2 peptide then sequestered intracellular PSD-95, thereby blocking NMDAR-mediated apoptosis. Intravenous administration of TAT–NMR2 1 h after cerebral artery occlusion also reduced cerebral infarction volume and led to better neurological scores in rats. Importantly, this peptide had no effect on NMDAR-mediated currents, as *in vivo* NMDAR blockade is too deleterious to neurons to be used as a stroke therapy.

More examples of the therapeutic potential of TAT fusion proteins have been provided by studies using a recombinant TAT–Bcl-xL protein. A member of the Bcl-2 family of proteins, Bcl-xL can act at the mitochondria to suppress apoptosis in multiple cell types. In one recent study [41], TAT–Bcl-xL (but not Bcl-xL) was shown to inhibit apoptosis in cultured neurons at concentrations (30 – 100 nM) much lower than those used with traditional small-molecule apoptosis inhibitors. The authors also demonstrated the *in vivo* efficacy of TAT–Bcl-xL by using a murine model of stroke in which cerebral artery occlusion leads to focal ischaemia followed by neuronal apoptosis. The authors found that intraperitoneal administration of 9 mg/kg TAT–Bcl-xL protein could significantly decrease the size of the cerebral infarction (> 60%) and reduce neuronal caspase-3 activity. The protein was effective even if it was administered 45 min after the ischaemic episode. In contrast, non-transducible Bcl-xL protein had no effect on neuronal apoptosis *in vivo*. These results were corroborated by another report in which intravenous TAT–Bcl-xL administration reduced neuronal apoptosis and infarct

volume in a murine stroke model [42]. In a separate paper this group also showed that TAT–Bcl-xL was able to suppress axotomy-induced apoptosis in retinal ganglion cells after local delivery into the vitreous space of the eye [43]. Furthermore, the TAT–Bcl-xL protein could still be detected in cultured cerebellar granule cells at 10 days after protein addition, indicating that not all proteins are so short-lived as to be poor candidates for therapeutics.

Other reports have provided more evidence for the therapeutic utility of TAT–Bcl-xL. In one example, a gain of function Bcl-xL mutant (termed FNK, which refers to the amino acid substitutions Y22F, Q26N and R165K) was linked to the TAT PTD [44]. The TAT–FNK protein was cytoprotective in cultured neurons and was detected in the brain after intraperitoneal administration to mice. Furthermore, intraperitoneal administration of TAT–FNK to gerbils (5 mg/kg) protected hippocampal CA1 neurons from cell death after transient global ischaemia.

Protein kinase C δ (δPKC) has been shown to be upregulated at the mRNA and protein levels after cerebral ischaemia and reperfusion [45]. A transducible inhibitor of δPKC, termed δV1-1, was tested in a rat model of cerebral ischaemia/reperfusion injury. Systemic delivery of the peptide up to 6 h after transient middle cerebral artery occlusion significantly reduced both infarct size and neurological deficits in rats 24 h after ischaemia. The authors also detected decreased levels of apoptosis, increased phospho-Akt and decreased levels of membrane-associated Bad in neurons of peptide-treated rats.

Damage to the heart due to ischaemia and reperfusion is also a major cause of morbidity and mortality. The δV1-1 peptide has also been shown to ameliorate ischaemia/reperfusion injury in both *ex vivo* [46] and *in vivo* models of acute myocardial infarction [47]. In the latter report, the mid-left anterior descending coronary artery was occluded for 30 min in pigs. During the last minute of occlusion, δV1-1 peptide was administered locally through the occluding balloon catheter. The authors found that peptide administration reduced infarct size by 80%, troponin release by 85% (a marker of cardiac muscle damage) and myocardial cell apoptosis by 67% (all assessed at 4 h post-reperfusion). Unlike untreated animals, peptide-treated animals experienced a complete recovery of their hearts' ejection fraction after 5 days. The authors used the same balloon catheters to deliver peptide as those used in angioplasty in humans. Therefore, they suggest that peptide administration via the balloon catheter during angioplasty-induced reperfusion might facilitate recovery of heart function after acute myocardial infarctions. In addition, another group delivered a protein (TAT–ARC) that can bind and inhibit caspases-2 and -8 [48]. In this case, *ex vivo* perfusion with TAT–ARC reduced the amount of cell death in the heart following experimentally induced ischaemia.

### 3.3 Inflammation

Pathological levels of inflammation underlie many common human disease states, including arthritis and asthma. Although existing anti-inflammatory agents, such as corticosteroids and non-steroidals (NSAIDS), are effective in many patients, they can also produce significant side effects that threaten the health of the patient. Consequently, more specific inhibitors of the inflammatory response are needed.

One group has shown that TAT-mediated blockade of Ras or PI3-kinase activity is effective in treating a mouse model of asthma. Asthma presents as a combination of Th2 cytokine-mediated airway inflammation and bronchial hyper-responsiveness. Systemic administration of either TAT–DN-H-Ras [49] or TAT–DN-E85 (derived from class IA PI3K regulatory subunit p85α) [50] reduced airway infiltration by eosinophils and lymphocytes after challenge with antigen or IL-5. Both proteins also reduced airway expression of the Th2 cytokines IL-4 and IL-5 after antigen stimulation. One specific clinical manifestation of asthma is airway hyper-responsiveness to methacholine, and both TAT–DN-H-Ras and TAT–DN-E85 proteins were able to block this hyper-responsiveness in antigen-sensitised mice. Moreover, the authors detected a specific decrease in phospho-Akt levels in lung tissue of TAT–DN-E85-treated mice, indicating that the protein was modulating an established PI3K target [50].

The transcription factor NF-κB is thought to be a central regulator of inflammation [51]. Two different groups have used protein transduction to inhibit NF-κB in rodent models of inflammation. In the more recent case, Clohisy *et al.* designed stable mutants of IκB, an inhibitor of NF-κB, and linked them to TAT [51]. They then tested the ability of these TAT–IκB proteins to inhibit inflammation in a mouse model of rheumatoid arthritis. In this model NF-κB is thought to mediate both the secretion of pro-inflammatory cytokines and the activation of osteclasts, which degrade bone in the arthritic joint. The authors found that TAT–IκB proteins localised to the joint space after systemic delivery and were able to block joint swelling and erythema. Osteoclast recruitment to the joint and consequent bone erosion were reduced as well. At the molecular level, TAT–IκB protein administration lowered the level of active NF-κB in the joint space and inhibited production of pro-inflammatory cytokines TNF-α and receptor activator of NF-κB ligand (RANKL). The authors noted no adverse side effects of systemic delivery of TAT–IκB protein for 7 days.

Using a different strategy to inhibit NF-κB, May *et al.* designed a peptide (termed NBD for NEMO binding domain) that could block the interaction of NEMO (NF-κB essential modifier) with the IKK (inhibitor of κB-kinase) complex [52]. This interaction is necessary for the activation of NF-κB in response to pro-inflammatory stimuli. Thus, they were able to use an Antp–NBD peptide to block the response of cultured cells to inflammatory stimuli such as TNF-α. Furthermore, they demonstrated that administration of Antp–NBD reduced the inflammatory response *in vivo* in ear oedema and in peritonitis models of inflammation. At least in these models, the Antp–NBD peptide was as efficacious as the established anti-inflammatory drug dexamethasone.

Finally, Bucci *et al.* fused a peptide from the endothelial nitric oxide synthase (eNOS) binding domain of caveolin-1 to Antp [53]. The Antp–Cav peptide blocked the production of nitric oxide in cultured endothelial cells by inhibiting eNOS. Intraperitoneal administration of Antp–Cav reduced vascular leakage and interstitial oedema in two mouse models of inflammation.

### 3.4 Other examples

Not all applications of *in vivo* protein transduction can be so neatly classified into three major categories. For example, one recent study showed that systemic delivery of a transducible caspase-8 inhibitor FLIP (TAT–FLIP) was able to reduce apoptosis and prolong survival in mice treated with a lethal dose of Fas-activating antibody [54]. Another recent paper shows that intraperitoneal injection of a transducible superoxide dismutase (TAT–SOD) results in delivery to the pancreas, where TAT–SOD subsequently protects α-cells from oxidative stress [55]. Intriguingly, one group has used protein transduction to modulate memory in living animals. Phospholipase Cγ1 (PLCγ1) has been implicated in long-term potentiation (a possible surrogate for memory formation) in hippocampal neurons [56]. The role of PLCγ1 in the acquisition and retention of spatial memory *in vivo* was tested by infusing a transducible peptide inhibitor of PLCγ1 into the hippocampi of rats during training for a water maze task. Peptide infusion did not block the acquisition of training but did attentuate long-term retention of training when mice were tested 48 h after the lasting training episode [56]. Importantly, a negative control peptide, which differed from the active peptide by only two phosphate groups, had no effect on memory retention. The authors conclude from this study that neuronal PLCγ1 plays a role in the memory storage. They also provide a striking illustration of the power of protein transduction to manipulate higher order brain function such as memory formation, and dissect the intracellular signalling pathways that regulate it. Lastly, Sugioka *et al.* showed that TAT–BH4 (anti-apoptotic domain of Bcl-XL) exerts several anti-apoptotic activities *in vivo*, including inhibition of apoptosis induced by X-Ray in small intestine of mice, and partial inhibition of Fas-mediated fulminant hepatitis [57].

## 4. Delivery of DNA by protein transduction domains *in vivo*

The authors are aware of only one demonstration of the ability of PTDs to facilitate delivery of DNA in living organisms. In this study, liposomes complexed with TAT peptide and an enhanced green fluorescent protein (eGFP) expression plasmid were injected into tumours grown on the flanks of mice [15]. The authors of this study observed expression of eGFP in the tumours 3 days after injection. In contrast, injection of liposomes that were complexed with an eGFP expression plasmid, but lacked TAT, resulted in no eGFP expression *in vivo*. These promising early results will need to be extended by the delivery of plasmids encoding functional antitumour genes. Nevertheless, they provide a proof of principle that TAT can facilitate gene delivery *in vivo*. It remains to be seen whether the optimal strategy for DNA delivery will involve the use of TAT liposomes [15], TAT-poly-lysine peptides [58], or an as yet undeveloped methodology.

## 5. Conclusion

Numerous studies have revealed the ability of PTDs to modulate the biology of living organisms. These studies have often taken place in the context of animal models of cancer, stroke and other diseases that are responsible for a substantial amount of morbidity and mortality. Importantly, many of these reports have demonstrated not only the modulation of intracellular biology *in vivo*, but also an improvement in relevant clinical end points (e.g., survival for cancer, neurological function for stroke).

The pharmacokinetics and potential immunogenicity of PTD-linked molecules need to be examined in more detail. Such studies may enable the design of 'next-generation' PTDs that have even more favourable biodistribution or immunogenic properties than current PTDs that were designed primarily for tissue culture experiments. However, the intrinsic heterogeneity of polypeptides may make it difficult to generalise the results of pharmacokinetic and immunogenicity studies from one TAT fusion protein to another. In addition, the results reviewed here already show that at least some TAT fusions have adequate biodistribution to ameliorate models of disease, and that the immune system is not an absolute barrier to *in vivo* efficacy in these cases.

## 6. Expert opinion

The quantity and quality of reports of successful *in vivo* transduction during the past 5 years has been exciting and encouraging. Although there is still preclinical work to be done, we expect that the time is drawing near for the design of Phase I clinical trials using some of the most efficacious peptides and full-length proteins linked to PTDs thus far. In the field of medical oncology, PTDs have tremendous potential for translating what has been learned about the molecular basis of cancer into viable clinical treatments. Cancer is a disease of multiple genetic alterations, and so it is unlikely that any one TAT fusion protein will yield a 'cure' of any particular class of cancer. However, many types of cancer are so refractory to treatment that agents that provide even modest gains in survival are welcomed with open arms. Even more importantly, we envision a near future in which molecular analysis will reveal the specific genes and pathways that are mutated for each patient's tumour. In such a case, a combination of TAT fusion proteins could be used to restore tumour suppressor pathways that are missing and block the oncogenic pathways that are upregulated in that particular cancer.

In addition to the initiation of clinical trials of PTDs, there remains much preclinical work to be done in the field of protein transduction. For example, it is clear that delivery of TAT fusion proteins to the cellular interior can be enhanced [16], but the enhancing agents described so far are likely to represent only the tip of the iceberg. More enhancers of transduction must be sought, both by rational, mechanism-driven approaches and by screens of large chemical libraries. After discovery and characterisation in cultured cells, these molecules must then be tested for their ability to enhance protein transduction in living animals with a minimum of toxicity. Finally, the relative lack of specificity of protein transduction must be addressed. The ability to target theoretically any cell is advantageous in some respects. However, it also may in some cases hinder the delivery of adequate levels of PTDs to the tissue of interest without delivering so much to other tissues that side effects ensue. The ability to target PTDs to specific cells or receptors has the potential to widen their therapeutic index. Targeted PTDs could theoretically reduce the side effects of delivery to undesired tissue and lower the total amount of administered polypeptide needed to achieve a therapeutic effect.

Therapeutic use of PTDs is unlikely to be a panacea for any human disease. However, the preclinical studies published in the last 5 years have shown that they have tremendous potential to become the basis of an entirely new class of therapeutic agents: the intracellular biologicals.

## Bibliography

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


1. JOLIOT A, PROCHIANTZ A: Transduction peptides: from technology to physiology. *Nat. Cell Biol. (*2004) **6**:189-196.
2. TREHIN R, MERKLE HP: Chances and pitfalls of cell penetrating peptides for cellular drug delivery. *Eur. J. Pharm. and Biopharm.* (2004) **58**:209-223.
3. SCHWARZE SR, HO A, VOCERO-AKBANI A, DOWDY SF: *In vivo* protein transduction: delivery of a biologically active protein into the mouse. *Science* (1999) **285**(5433):1569-1572.

•• ***In vivo* systemic delivery of enzymatically active TAT-fusion protein.**

4. FRANKEL AD, PABO CO: Cellular uptake of the tat protein from human immunodeficiency virus. *Cell (*1988) **55**(6):1189-1193.
5. GREEN M, LOEWENSTEIN PM: Autonomous functional domains of chemically synthesized human immunodeficiency virus tat trans-activator protein. *Cell* (1988) **55**(6):1179-1188.

•• **These two papers ([4] and [5]) provided the first observations of protein transduction by the full-length TAT protein.**

6. VIVES E, BRODIN P, LEBLEU B: A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. *J.Biol. Chem.* (1997) **272**(25):16010-16017.
7. DEROSSI D, JOLIOT AH, CHASSAING G, PROCHIANTZ A: The third helix of the Antennapedia homeodomain translocates through biological membranes. *J.Biol. Chem.* (1994) **269**(14): 10444-10450.
8. MAI JC, SHEN H, WATKINS SC, CHENG T, ROBBINS PD: Efficiency of protein transduction is cell type-dependent and is enhanced by dextran sulfate. *J.Biol. Chem.* (2002) **277**:30208-30218.
9. WENDER PA, MITCHELL DJ, PATTABIRAMAN KP, PELKEY ET, STEINMAN L, ROTHBARD JB: The design, synthesis, and evaluation of molecules that enable or enhance cellular uptake: peptoid molecular transporters. *Proc. Natl. Acad. Sci. USA* (2000) **97**:13003-13008.
10. WADIA JS, DOWDY SF: Modulation of cellular function by TAT mediated transduction of full length proteins. *Curr. Prot. Pep. Sci.* (2003) **4**:97-104.
11. NAGAHARA H, VOCERO-AKBANI AM, SNYDER EL *et al.*: Transduction of full-length TAT fusion proteins into mammalian cells: TAT-p27Kip1 induces cell migration. *Nat. Med.* (1998) **4**(12):1449-1452.

• **First description of the use of TAT-fusion proteins to modulate the intracellular biology of cultured mammalian cells.**

12. LEWIN M, CARLESSO N, TUNG C-H *et al.*: Tat peptide-derivatized magnetic nanoparticles allow *in vivo* tracking and recovery of progenitor cells. *Nat. Biotechnol.* (2000) **18**:410-414.
13. EGUCHI A, AKUTA T, OKUYAMA H *et al.*: Protein transduction domain of HIV-1 Tat protein promotes efficient delivery of DNA into mammalian cells. *J.Biol. Chem.* (2001) **276**:26204-26210.
14. TORCHILIN VP, RAMMOHAN R, WEISSIG V, LEVCHENKO TS: TAT peptide on the surface of liposomes affords their efficient intracellular delivery even at low temperature and in the presence of metabolic inhibitors. *Proc. Natl. Acad. Sci. USA* (2001) **98**:8786-8791.
15. TORCHILIN VP, LEVCHENKO TS, RAMMOHAN R, VOLODINA N, PAPAHADJOPOULOS-STERNBERG B, D'SOUZA GGM: Cell transfection *in vitro* and *in vivo* with nontoxic TAT peptide-liposome-DNA complexes. *Proc. Natl. Acad. Sci. USA* (2003) **100**:1972-1977.

• **TAT-mediated delivery of GFP-encoding plasmid DNA to tumours *in vivo*.**

16. WADIA JS, STAN RV, DOWDY SF: Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. *Nat. Med.* (2004) **10**:310-315.

•• **This study demonstrates that cellular uptake of TAT-fusion proteins occurs by macropinocytosis, a specific form of endocytosis. Based on this knowledge of mechanism, the authors also describe a strategy for enhancing delivery of TAT-fusion proteins to the cellular interior.**

17. DEROSSI D, CALVET S, TREMBLEAU A, BRUNISSEN A, CHASSAING G, PROCHIANTZ A: Cell internalization of the third helix of the Antennapedia homeodomain is receptor-independent. *J. Biol. Chem.* (1996) **271**(30): 18188-18193.
18. RICHARD JP, MELIKOV K, VIVES E *et al.*: Cell-penetrating peptides: a re-evaluation of the mechanism of cellular uptake. *J. Biol. Chem.* (2002) **278**:585-590.
19. LINDSAY MA: Peptide-mediated cell delivery: application in protein target

validation. *Curr. Opin. Pharmacol.* (2002) **2**:587-594.

20. VOUSDEN KH, LU X: Live or let die: the cell's response to p53. *Nat. Rev. Cancer* (2002) **2**:594-604.
21. SNYDER EL, MEADE BR, SAENZ CC, DOWDY SF: Treatment of a terminal peritoneal carcinomatosis by a transducible p53-activating peptide. *PLoS Biology* (2004) **2**:186-193.

•• **Describes the use of a transducible p53-activating peptide to effectively treat three different models of cancer, resulting in long-term disease-free survival in one model. This peptide was stabilised by synthesis of a retro-inverso isoform, thus increasing its potency *in vivo*.**

22. SELIVANOVA G, RYABCHENKO L, JANSSON E, IOTSOVA V, WIMAN KG: Reactivation of mutant p53 through interaction of a C-terminal peptide with the core domain. *Mol. Cell. Biol.* (1998) **19**:3395-3402.
23. SELIVANOVA G, IOTSOVA V, OKAN I *et al.*: Restoration of the growth suppression function of mutant p53 by a synthetic peptide derived from the p53 C-terminal domain. *Nat. Med.* (1997) **3**:632-638.
24. KIM AL, RAFFO AJ, BRANDT-RAUF PW *et al.*: Conformational and molecular basis for induction of apoptosis by a p53 C-terminal peptide in human cancer cells. *J. Biol. Chem.* (1999) **274**:34924-34931.
25. CHOREV M, GOODMAN M: A dozen years of retro-inverso peptidomimetics. *Acc. Chem. Res.* (1993) **26**:266-273.
26. AARNT CR, CHIOREAN MV, HELDEBRANT MP, GORES GJ, KAUFMAN S: Synthetic Smac/DIABLO peptides enhance the effects of chemotherapeutic agents by binding XIAP and cIAP1 in situ. *J. Biol. Chem.* (2002) **277**:44236-44243.
27. FULDA S, WICK W, WELLER M, DEBATIN K-M: Smac agonists sensitize for Apo2L/TRAIL- or anticancer drug-induced apoptosis and induce regression of malignant glioma *in vivo*. *Nat. Med.* (2002) **8**:808-815.

• **Another report of long-term survival in a mouse model of cancer; in this case after co-administration of a transducible peptide and an extracellular anticancer agent.**

28. VUCIC D, DESHAYES K, ACKERLY H *et al.*: SMAC negatively regulates the anti-apoptotic activity of melanoma inhibitor of apoptosis (ML-IAP). *J.Biol. Chem.* (2002) **277**:12275-12279.
29. YANG L, MASHIMA T, SATO S *et al.*: Predominant suppression of apoptosome by inhibitor of apoptosis protein in non-small cell lung cancer H460 cells: therapeutic effect of a novel polyarginine-conjugated smac peptide. *Cancer Res.* (2003) **63**(4):831-837.
30. HARADA H, HIRAOKA M, KIZAKA-KONDOH S: Antitumor effect of TAT-oxygen-dependent degradation-caspase-3 fusion protein specifcally stabilized and activated in hypoxic tumor cells. *Cancer Res.* (2002) **62**:2013-2018.

• **The first demonstration that specific domains can be added to TAT-fusion proteins in order to control the cells in which they are active.**

31. INOUE M, MUKAI M, HAMANAKA Y, TATSUTA M, HIRAOKA M, KIZAKA-KONDOH S: Targeting hypoxic cells with a protein prodrug is effective in experiment malignant ascites. *Int. J. Onc.* (2004) **25**:713-720.
32. WILLAM C, MASSON N, TIAN Y *et al.*: Peptide blockade of HIF-alpha degradation modulates cellular metabolism and angiogenesis. *Proc. Natl. Acad. Sci. USA* (2002) **99**(16):10423-10428.
33. MENDOZA N, FONG S, MARSTERS J, KOEPPEN H, SCHWALL R, WICKRAMASINGHE D: Selective cyclin-dependent kinase 2/cyclin a antagonists that differ from ATP site inhibitors block tumor growth. *Cancer Res.* (2003) **63**(5): 1020-1024.
34. HARBOUR JW, WORLEY L, MA D, COHEN M: Transducible peptide therapy for uveal melanoma and retinoblastoma. *Arch. Opthalmmol.* (2002) **120**:1341-1346.
35. MAI JC, MI Z, KIM S-H, NG B, ROBBINS PD: A proapoptotic peptide for the treatment of solid tumors. *Cancer Res.* (2001) **61**:7709-7712.
36. DATTA K, SUNDBERG C, KARUMANCHI SA, MUKHOPADHYAY D: The 104-123 amino acid sequence of the beta-domain of von Hippel-Lindau gene product is sufficient to inhibit renal tumor growth and invasion. *Cancer Res.* (2001) **61**:1768-1775.
37. HOSOTANI R, MIYAMOTO Y, FUJIMOTO K *et al.*: Trojan p16 peptide suppresses pancreatic cancer growth and prolongs survival in mice. *Clin. Cancer Res.* (2002) **8**:1271-1276.
38. BORSELLO T, CLARKE PGH, HIRT L *et al.*: A peptide inhibitor of c-Jun N terminal kinase protects against excitoxicity and cerebral ischemia. *Nat. Med.* (2003) **9**:1180-1186.

• **One recent example of the many papers showing that transducible peptides and proteins are effective in the treatment of rodent stroke models. Also notable for the use of a stabilised retro-inverso peptide.**

39. HIRT L, BADUAT J, THEVENET J *et al.*: D-JNK1, a cell-penetrating c-Jun-N-terminal kinase inhibitor, protects against cell death in severe cerebral ischemia. *Stroke* (2004) **35**:1738-1743.
40. AARTS M, LIU Y, LIU L *et al.*: Treatment of ischemic brain damage by perturbing NMDA receptor-PSD-95 protein interactions. *Science* (2002) **298**:846-850.
41. CAO G, PEI W, GE H *et al.*: *In vivo* delivery of a Bcl-xL fusion protein containing the TAT protein transduction domain protects against ischemic brain injury and neuronal apoptosis. *J. Neurosci.* (2002) **22**:5423-5431.
42. KILIC E, DIETZ GPH, HERMANN DM, BAHR M: Intravenous TAT-Bcl-xL is protective after middle cerbral artery occlusion in mice. *Ann. Neurol.* (2002) **52**:617-622.
43. DIETZ GPH, KILIC E, BAHR M: Inhibition of neuronal apoptosis *in vitro* and *in vivo* using TAT-mediated protein transduction. *Mol. Cell. Neurosci.* (2002) **21**:29-37.
44. ASOH S, OHSAWA I, MORI T *et al.*: Protection against ischemic brain injury by protein therapeutics. *Proc. Natl. Acad. Sci. USA* (2002) **99**:17107-17112.
45. BRIGHT R, RAVAL AP, DEMBER JM *et al.*: Protein Kinase C delta mediates cerebral reperfusion injury *in vivo*. *J. Neurosci.* (2004) **24**(31):6880-6888.
46. CHEN L, HAHN H, WU G *et al.*: Opposing cardioprotective actions and parallel hypertrophic effects of deltaPKC and epsilonPKC. *Proc. Natl. Acad. Sci. USA* (2001) **98**(20):11114-11119.
47. INAGAKI K, CHEN L, IKENO F *et al.*: Inhibition of {δ}-protein kinase c protects against reperfusion injury of the ischemic heart *in vivo*. *Circulation* (2003) **108**(19):2304-2307.
48. GUSTAFSSON AB, SAYEN MR, WILLIAMS SD, CROW MT, GOTTLIEB RA: TAT protein transduction into isolated perfused hearts. *Circulation* (2002) **106**:735-739.
49. MYOU S, ZHU X, MYO S *et al.*: Blockade of airway inflammation and

hyperresponsiveness by HIV-TAT-dominant negative Ras *J. Immunol.* (2003) **171**(8):4379-4384.

50. MYOU S, LEFF AR, MYO S *et al.*: Blockade of inflammation and airway hyperresponsiveness in immune-sensitized mice by dominant-negative phosphoinositide 3-kinase-TAT. *J. Exp. Med.* (2003) **198**(10):1573-1582.
51. CLOHISY JC, ROY BC, BIONDO C *et al.*: Direct inhibition of NF-{κ}B blocks bone erosion associated with inflammatory arthritis. *J. Immunol.* (2003) **171**(10):5547-5553.
52. MAY MJ, D-ACQUISITO F, MADGE LA, GLOCKNER J, POBER JS, GHOSH S: Selective inhibition of NF-κB activation by a peptide that blocks the interaction of NEMO with the IκB kinase complex. *Science* (2000) **289**:1550-1554.
53. BUCCI M, GRATTON J-P, RUDIC RD *et al.*: *In vivo* delivery of the caveolin-1 scaffolding domain inhibits nitric oxide synthesis and reduces inflammation. *Nat. Med.* (2000) **6**:1362-1367.
54. KRAUTWALD S, ZIEGLER E, TIEDE K, PUST R, KUNZENDORF U: Transduction of TAT-FLIP fusion protein results in transient resistance to Fas-induced apoptosis *in vivo. J. Biol. Chem.* (2004):M401327200.
55. EUM WS, CHOUNG IS, LI MZ *et al.*: HIV-1 TAT-mediated protein transduction of Cu,Zn-superoxide dismutase into pancreatic beta cells *in vitro* and *in vivo. Free Radic. Biol. Med.* (2004) **37**:339-349.
56. BLUM S, DASH PK: A cell-permeable Phospholipase Cγ1-binding peptide transduces neurons and impairs long-term spatial memory. *Learning Memory* (2004) **11**:239-243.
    - **Transducible peptides can be used to modulate and study higher cognitive functions such as memory retention.**
57. SUGIOKA R, SHIMIZU S, FUNATSU T *et al.*: BH4-domain peptide from Bcl-xL exerts anti-apoptotic activity *in vivo*. *Oncogene* (2003) **22**(52):8432-8440.
58. HASHIDA H, MIYAMOTO M, CHO Y *et al.*: Fusion of HIV-1 Tat protein transduction domain to poly-lysine as a new DNA delivery tool. *Br. J. Cancer* (2004) **90**:1252-1258.

## Affiliation

Dr Eric L Snyder[1,2] & Dr Steven F Dowdy[†1]
[†]Author for correspondence
[1]UCSD School of Medicine, Howard Hughes Medical Institute and Department of Cellular & Molecular Medicine, 9500 Gilman Drive, La Jolla, CA 92093-0686, USA
E-mail: sdowdy@ucsd.edu
[2]Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, MO 63110, USA