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INVESTIGATION OF A RECOMBINANT SMN PROTEIN DELIVERY SYSTEM TO TREAT SPINAL MUSCULAR ATROPHY

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Abstract

Spinal muscular atrophy (SMA), the most common genetic cause of infant death, is a neurodegenerative disorder affecting motor neurons. SMA results from a loss in full-length survival of motor neuron (SMN) protein due to deletions/mutations in the *SMN1* gene. In this study, we assessed the ability of cell-penetrating peptides (CPP) to deliver recombinant SMN protein to cultured neurons as a prelude for a potential therapeutic to treat SMA. Firstly, we confirmed that *E. coli* produced recombinant GFP protein fused to TAT (YGRKKRRQRRR; TAT-GFP) transduced rat cortical neurons in a concentration dependent manner. However, due to low yields of recombinant TAT-SMN protein obtainable from *E. coli*, we investigated the potential of a modified TAT (TATκ: YARKAARQARA) or R9 (RRRRRRRRR) peptide downstream of the fibronectin (FIB) secretory signal peptide to generate recombinant CPP-fused SMN protein. While U251 cells transduced with an adenoviral vector expressing CMV-FIB-TATκ-SMN secreted recombinant TATκ-SMN protein, we did not detect TATκ-SMN protein transduction of cortical neurons. Further, purified TATκ-SMN was unable to transduce SH-SY5Y cells, nor block apoptosis following LY294002 treatment of these cells. Our findings indicate that TATκ is not a suitable CPP to deliver SMN protein to neurons. Nonetheless, we have developed a novel method to generate full-length recombinant SMN protein using a mammalian expression system, which can be used to explore the application of other CPPs to deliver SMN protein as a treatment for SMA.

Keywords

• Spinal muscular atrophy • Recombinant SMN protein • Cell penetrating peptides • TAT • Adenoviral vectors

List of abbreviations

BBB	- Blood-brain barrier
CPP	- Cell penetrating peptide
DIV	- On day *in vitro*
DMEM	- Dulbecco's Modified Eagle Medium
FCS	- Foetal calf serum
FIB	- Fibronectin
GFP	- Enhanced Green Fluorescent Protein
MOI	- Multiplicity of infection
PBS	- Phosphate buffered saline
PI3	- Phosphatidylinositol-3 kinase
SDS-PAGE	- Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SMA	- Spinal muscular atrophy
SMN1	- Survival of Motor Neuron 1 gene
SMN	- Survival of motor neuron protein

Introduction

Spinal muscular atrophy (SMA), an often fatal autosomal recessive genetic disease, is characterised by the selective degeneration of spinal cord motor neurons, which causes muscle weakness and atrophy. With an incidence of one in 6,000 live births, SMA is the most common genetic cause of infant mortality. Clinically, SMA is characterised by profound muscle weakness, hypotonia and trunk paralysis, and depending on disease severity and age of onset [1], is classified into 3 main phenotypic subtypes (I-III). To date, there is no cure for SMA, making the development of potential therapies a high priority.

SMA is a monogenic disorder in which the *survival of motor neuron* (*SMN1*) gene is mutated or absent in more than 95% of cases [2]. During human evolution a 500kb inverted duplication of the *SMN1* locus has given rise to a second copy of the gene, termed *SMN2*. The *SMN2* gene is essentially identical to *SMN1* but a translationally silent nucleotide change (C→T) in exon 7 causes the exons' exclusion from *SMN2* transcripts [3,4]. Consequently, only about 10% of the survival motor neuron 2 (SMN2) protein is functional, while the other 90% is truncated and unstable [5]. The occurrence of multiple *SMN2* gene copies in patient groups correlates with increased full-length SMN transcripts, greater amounts of functional protein, and reduced disease severity [6,7]. Therefore, increasing SMN protein levels in SMA patients presents a feasible treatment approach, and has previously improved the phenotype and survival rates in SMA animal models [8].

While several candidate therapeutic strategies targeting SMN levels have been developed to treat SMA, they have been of limited success. For example, histone deacetylase (HDAC) inhibitors, antisense oligonucleotides, direct viral delivery, neuroprotective factors and exercise have all showed promise in preclinical trials [8-13]. In addition, several HDAC inhibitors, phenylbutyrate and valproic acid have failed to show any significant clinical benefits in SMA patients (reviewed in [14]). Therefore, there is a need to develop and assess new approaches to treat SMA.

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In an earlier study, recombinant SMN was fused to the neuronal targeting diphtheria toxin fragment (TTC-DTx) and evaluated for cell transduction in primary neuronal cultures [15]. Whilst this approach proved unsuccessful, and cells failed to take up exogenous SMN protein, it highlighted a concept whereby exogenous SMN protein could be delivered to neurons if a suitable cell targeting peptide was available. In this respect, the 11 amino acid TAT peptide, which is derived from the HIV-1 TAT protein can mediate the efficient cellular transduction of protein cargos *in vitro* [16]. Moreover, TAT-fused peptides (and related peptides; e.g. Arginine-9; R9) can cross the blood brain barrier (BBB) and impart therapeutic benefits in animal models of disease [17-19].

Therefore, in this study, we investigated the feasibility of related TAT peptides (TATκ and R9) to deliver recombinant SMN protein to neurons. In doing so, we developed a mammalian adenoviral vector secretory expression system to generate SMN protein and then assessed the delivery potential of SMN fused to TATκ to enter neurons.

Experimental procedures

Rat primary cortical neuronal cell cultures

All animal procedures were approved by the University of Western Australia Animal Ethics Committee. Establishment of cortical cultures was as previously described [20]. Briefly, cortical tissue from E18 to E19 Sprague-Dawley rats were dissociated in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Melbourne, Australia) supplemented with 1.3 mM L-cysteine, 0.9 mM $NaHCO_3$, 10 U/ml papain (Sigma, St. Louis, MO, USA) and 50 U/ml DNaseI (Sigma) and washed in cold DMEM/10% horse serum. Before seeding, wells (96 well microtitre plate; Nalge Nunc International, Thermo Fisher Scientific, Waltham, MA, USA) were coated with poly-D-lysine overnight (50 ml/well: 50 µg/ml; 70 - 150K, Sigma). Excess poly-D-lysine solution was then removed and replaced with Neurobasal (containing 2% B27 supplement; 4% foetal bovine serum; 1% horse serum; 62.5 mM glutamate; 25 mM 2-mercaptoethanol; 10 µg/ml streptomycin and 10 mg/ml penicillin). Neurons were plated to obtain approximately 10,000 viable neurons in each well on day *in vitro* 12. Neuronal cultures were maintained in a CO_2 incubator (5% CO_2, 95% air balance, 98% humidity) at 37°C. On day *in vitro* 4, one third of the culture medium was removed and replaced with fresh Neurobasal/2% B27 containing the mitotic inhibitor cytosine arabinofuranoside (1 µM final concentration; Sigma). On day *in vitro* 8, one half of the culture medium was replaced with Neurobasal/2% B27.

Generation of fibronectin fused constructs

Relevant DNA sequences for each of the construct components are shown in Table 1. Briefly, the rat fibronectin sequence was fused to either a TATκ-HA or R9-HA sequence. Constructs containing an SMN or GFP sequence were generated with a 6xHIS sequence for protein purification and as an epitope for detection by western blot analysis.

Cell maintenance, differentiation and transfection

HEK293, U251 and SH-SY5Y cells were maintained in DMEM containing penicillin (10 µg/ml), streptomycin (10 µg/ml) and foetal calf serum (FCS; 5-10%; heat-inactivated), and incubated at 37°C in a 5% CO_2 incubator. The differentiation of SH-SY5Y cells using retinoic acid (15 µM; 5 days) has previously been described [21]. HEK293 cells (150,000) seeded in 24 well plates were transfected using Lipofectamine 2000® in OPTI-MEM serum-free media (Invitrogen, Melbourne, Australia) according to the manufacturers recommendations.

Adenoviral vectors and transduction of cells in culture

Recombinant adenoviruses were prepared according to the method of He *et al.* 1998 [22], with some modifications [23]. The FIB-TATκ-SMN and FIB-TATκ-GFP cDNA fragments were released by restriction enzyme digestion and ligated into a modified pShuttle plasmid (pShuttle-CMV/WPRE) [23]. Briefly, pShuttle plasmid DNA was linearized by *PmeI* digestion and electroporated (Gene Pulser II, Bio-Rad Laboratories, Hercules, CA, USA) into the *Escherichia coli* strain BJ5183 carrying the pAdEasy plasmid [24]. HEK293 cells were transfected with 3 mg of *PacI* linearized recombinant plasmid DNA using Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA). Following the appearance of viral plaques (5-10 days), culture lysates were used for viral amplification in HEK293 cells. Adenoviral particles (AdCMV-FIB-TATκ-SMN or AdCMV-FIB-TATκ-GFP) were purified and concentrated from HEK293 cell lysates using the Adeno-X kit (BD Biosciences, San Jose, CA, USA). Adenoviral vectors expressing SMN (AdSMN) and a control vector (AdEmpty) have been described previously [21]. For adenoviral transduction of cells, adenovirus was diluted in serum free DMEM media for use at an appropriate multiplicity of infection (MOI) ratio (50-300) prior to addition to cells in cultures.

Generation of recombinant GFP and TAT-GFP proteins in *E. coli*

GFP or TAT-GFP cDNA flanked by *Kpn1* (5' end) and *XhoI* (3'end) restriction enzyme sites was inserted into a variant of the *E. coli* pET28a expression vector (Novagen, Merck KGaA, Darmstadt, Germany) containing compatible sites. The growth of KRX cells carrying this

Table 1. Sequences used in the construction of the TAT and R9 secretory constructs.

Fragment	Sequence
FIB	CTCAGGGGTCCGGGACCCGGGCGGCTGCTGCTGCTAGCAGTCCTGTGCCTGGGGACATCGGTGCGCTGCACCGAAACCGGGAAGAGCAAGAGGGCCGCCGCC
TATκ	TACGCTCGTAAAGCAGCTCGTCAGGCTCGTGCT
R9	CGTCGTCGTCGTCGTCGTCGTCGTCGT
HA	TATCCTTACGACGTTCCAGATGTAGCA

plasmid and induction of CYPA protein was essentially as per the manufacturer's recommendation (KRX cells, Promega, Madison, WI, USA). In brief, *E. coli* KRX cells (containing pET28a:GFP or pET28a:TAT-GFP) were grown in Terrific Broth medium (with 50 µg/ml kanamycin) at 37°C (shaking at 225 rpm) to an optical density (OD_{600}) of 0.8-1.0, then at room temperature (RT; shaking at 225 rpm) to an OD_{600} of 1.2-1.5. Induction of protein expression in *E. coli* was achieved following the addition of of isopropyl-β-D-thiogalactopyranoside (IPTG, 1 mM; Promega) and Rhamnose (0.1%; Sigma) at RT for 16 hours. Cells were re-suspended in lysis buffer (20 mM Tris-HCl; 300mM NaCl; 20 mM imidazole and 1% NP-40) containing EDTA-free protease inhibitor (Sigma) and DNAse (2 Units/ml), lysed using a French press (SLM-Aminco Instruments, Horiba Instruments Inc., Irvine, CA, USA).

Recombinant proteins were purified using Ni^{2+} nitrilotriacetic acid (Ni-NTA) superflow cartridges (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. Purified proteins were dialysed against a 200x volume of dialysis buffer (20 mM Tris-HCl, pH 7; 200 mM NaCl; 10% w/v glycerol). Protein purity and concentrations were determined by SDS-PAGE electrophoresis followed by Coomassie staining and Bradford assays (Bio-Rad Laboratories, Hercules, CA, USA), respectively.

Generation and purification of recombinant TAT-GFP and TAT-SMN proteins from mammalian cells

Human U251 glioma cells cultured in DMEM containing 5% FCS were transduced with AdCMV-FIB-TATκ-SMN or AdCMV-FIB-TATκ-GFP (MOI of 30) and incubated for 48 hours prior to cell lysis and protein extraction. Cell supernatants were harvested, combined with lysis buffer (20 mM Tris- HCl; 300 mM NaCl; 20 mM imidazole and 1% NP-40) containing EDTA-free protease inhibitor (Sigma). The recovery and purification of recombinant proteins was achieved as described above.

Transduction of recombinant proteins in culture

Recombinant proteins (0.5 µM to 5 µM) generated from bacterial or mammalian cells were tested for transduction activity in the following manner. Media was removed and proteins diluted in 100 µl of serum free DMEM media were added to cells in a 96 well plate for 1 hour. The media was removed and the cultures were washed 3 times with balanced salts solution (BSS; 116 mM NaCl mM, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 1 mM NaH_2PO_4; pH 7) prior to the analysis by fluorescence microscopy, cell viability assessment or protein lysate preparation.

PI3-kinase inhibition apoptotic model

Assessment of the anti-apoptotic function of SMN using the phosphatidylinositol-3 kinase (PI3-kinase) inhibitor, LY294002 (Sigma), has previously been described [21]. Briefly, LY294002 was diluted in serum free media to a final concentration of 25 µM prior to addition to differentiated SH-SY5Y cells.

Cell viability assessment

Cell viability was assessed using the MTS assay (Promega, Madison, WI, USA). The MTS assay measures the cellular conversion of tetrazolium to the water-soluble formazan product, which is detected spectrophotometrically at 495 nm.

Protein extraction and western blotting

Proteins were extracted from cultured cells using RIPA lysis buffer (1% NP-40, 0.1% SDS, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate and 1 mM EDTA). Western blotting has been described previously [21]. Briefly, membranes were blocked in PBS-Tween 20 (0.1%) containing ovalbumin (1 mg/ml) for 1 hour and incubated overnight with SMN (1:3000; Santa Cruz, Santa Cruz, CA, USA), HIS (1:2000; Santa Cruz) or β-tubulin (1:10000; Santa Cruz) primary antibodies diluted in PBS-T (0.1%) plus ovalbumin (1 mg/ml). Proteins were detected using a HRP complexed secondary antibody (1:15000-1:35000 donkey anti-rabbit/sheep anti-mouse; GE Healthcare, Piscataway, NJ, USA), and visualised using ECL plus detection reagent (GE Healthcare). Quantification and band densitometry of western blots was undertaken using ImageJ (NIH, Bethesda, MD, USA) software.

Immunohistochemistry

U251 glioma cells were grown in 24 well plates and transduced with adenoviral vectors for 48 hours. Cells were fixed in 10% formaldehyde, rinsed in PBS, blocked with 10% normal goat serum for 10 minutes, and exposed to anti-SMN antibody (1:200; Santa Cruz) diluted in PBS with 1% goat serum and incubated at 4°C overnight. Cells were washed in PBS prior to the addition of the secondary antibody (Alexa Fluor 594 goat anti-mouse, Invitrogen; 1:400) and incubated for 1 hour at 4°C. Fluorescence microscopy (Olympus IX70; Olympus DP70 digital camera) was used to analyse adenoviral vector protein expression.

Fluorescence microscopy

GFP reporter expression and secretion was assessed using fluorescence microscopy (Olympus IX70; Olympus DP70 digital camera). Briefly, cells were fixed in 2% (w/vol) paraformaldehyde in PBS for 30 minutes, washed three times in PBS, permeabilized with Triton X-100 (0.2%) in PBS and washed three times in PBS.

Statistics

Statistical differences between experimental groups were determined by ANOVA followed by post-hoc Fischer's protected least significant difference (PLSD) test. $P < 0.05$ was considered statistically significant. Unless otherwise stated, all experiments were conducted at least three times.

Results

Recombinant TAT-GFP is capable of transducing rat cortical neurons

Fluorescence microscopy and western blot analysis confirmed that *E. coli*-generated recombinant TAT-GFP, but not GFP, was capable of transducing cultured neurons in a concentration dependent manner (Figure 1A and 1B). Transduction of TAT-GFP was highly effective (60%-80% of cells were GFP positive) at the two highest concentrations (2 and 5 µM) tested. By contrast, transduction of the native GFP was not detected at any concentration.

The expression of TAT-SMN and TAT-SMN-GFP in *E. coli*

Next, we set out to generate a recombinant TAT-SMN protein in *E. coli*. Whilst recombinant, TAT-SMN protein was not evident by SDS-PAGE (Figure 2A), presumably due to low yields, western blot analysis revealed a protein band, consistent with the predicted molecular weight of TAT-SMN protein (Figure 2B).

Given the high recombinant TAT-GFP protein recovery rates we obtained from *E. coli*, we postulated that GFP may enhance SMN protein recovery. Therefore, we generated and expressed the SMN protein as a larger fusion protein with GFP (TAT-SMN-GFP). However, although TAT-SMN-GFP fusion protein yields were significantly increased as revealed by SDS-PAGE analysis, two distinct protein bands were detected (Figure 2C). Western blot analysis using a specific anti-SMN antibody detected multiple bands similar to and larger than the molecular weight of the SMN protein (Figure 2D). These results suggest that the TAT-SMN-GFP fusion protein may be subject to cleavage, degradation or incomplete translation in *E. coli*.

Generation of recombinant SMN fused to the fibronectin signal peptide

The development of a plasmid vector capable of secreting TAT-fused GFP from HEK293 cells has been previously described [25]. The study determined that efficient secretion of intact TAT-fused proteins requires the furin proteolytic sites within TAT to be mutated. Hence, the original TAT sequence YGRKKRRQRRR was modified to YARKAARQARA (referred to as TATκ; [25]). Importantly, TATκ was shown to retain protein transduction efficiency. In our system, we used the adenoviral pShuttle plasmid expression vector, which incorporates the woodchuck post-transcriptional regulatory element (WPRE) for high-level expression [26]. GFP or SMN protein was fused to the rat fibronectin (FIB) signal peptide sequence and either the TATκ or R9 CPP sequence (Figure 3A).

FIB-CPP-GFP transfected HEK293 cells show a secretory phenotype

To determine if FIB-TATκ-GFP and FIB-R9-GFP secretory constructs induce the characteristic appearance of a secretory protein, HEK293 cells

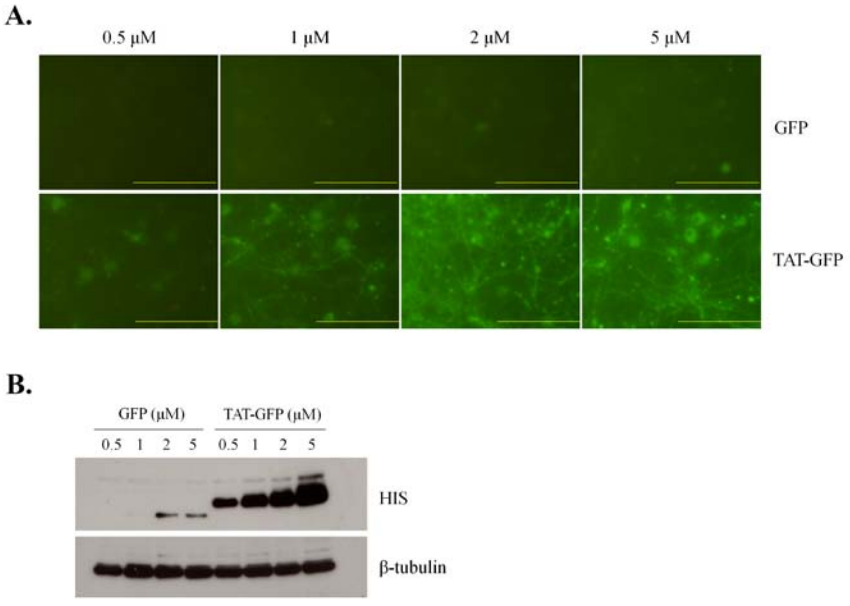

Figure 1. TAT-GFP protein transduces cortical neurons in a dose dependent manner. (A) Fluorescence microscopy of rat cortical neurons following 1 hour incubation with GFP or TAT-GFP recombinant proteins at 0.5 µM, 1 µM, 2 µM and 5 µM concentrations (Scale bar = 200 µm). (B) Western blot analysis of cell lysates harvested after 1 hour incubation with GFP or TAT-GFP recombinant proteins.

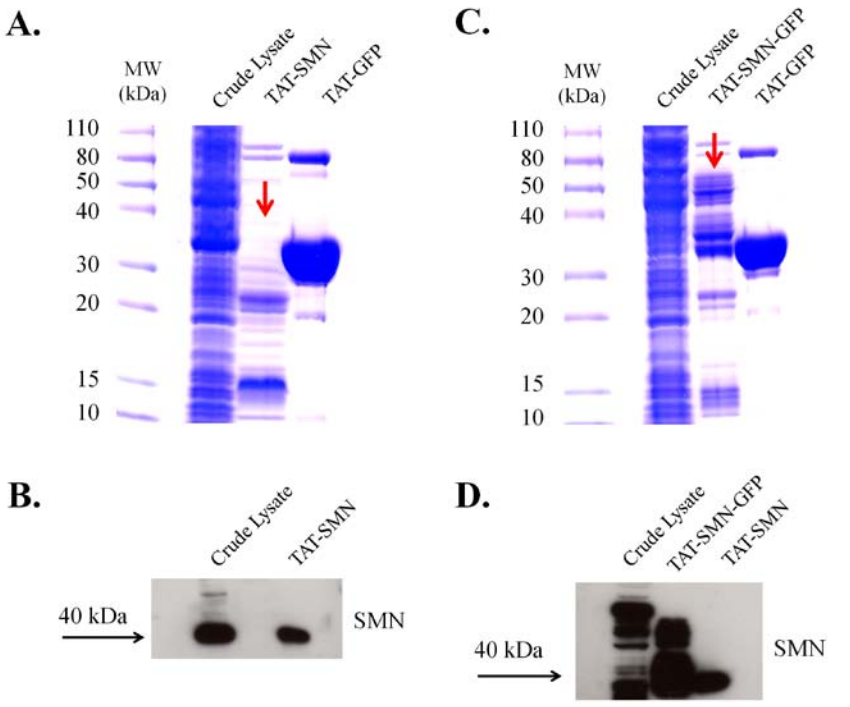

Figure 2. Generation of TAT-SMN and TAT-SMN-GFP recombinant proteins. (A) Coomassie staining of TAT-SMN crude lysate and purified TAT-SMN recombinant protein. (B) Western blot analysis of recombinant TAT-SMN protein. (C) Coomassie staining of crude lysate and purified TAT-SMN-GFP protein. (D) Western blot analysis of recombinant TAT-SMN-GFP protein.

were transfected and assessed via fluorescence microscopy. Consistent with previous findings [25,27], HEK293 cells transfected with the pShuttle FIB-TATκ-GFP plasmid exhibited a weak intracellular GFP signal (Figure 3B), in which GFP fluorescence appeared to be localised to the cell membrane. By contrast, HEK293 cells transfected with the FIB-R9-GFP plasmid showed strong punctate cytoplasmic fluorescence (Figure 3B).

Confirmation of adenovirual vector expression of FIB-TATκ-GFP and FIB-TATκ-SMN proteins in U251 cells

Using U251 cells, we assessed the efficacy of adenoviral (MOI 30) mediated expression of FIB-TATκ-GFP by fluorescence microscopy, and FIB-TATκ-SMN by immuno-fluorescence (Figure 4A). Cells transduced with AdCMV-FIB-TATκ-GFP exhibited membrane localised GFP fluorescence consistent with GFP secretion (Figure 4A). Immuno-fluorescence staining for SMN of AdCMV-FIB-TATκ-SMN transduced cells was cytoplasmic and diffuse (Figure 4A). By contrast, Immuno-fluorescence staining for SMN in cells transduced with the AdCMV-SMN control vector was cytoplasmic, but punctate in appearance.

Using GFP, SMN and HIS antibodies, western blot analysis of lysates from U251 cells transduced with the different adenoviral vectors confirmed the expression of TATκ-GFP and TATκ-SMN proteins (Figure 4B). To confirm that TATκ-GFP and TATκ-SMN were secreted, cell culture supernatants from transduced U251 cells were analysed by western blot analysis. Both the TATκ-GFP and TATκ-SMN proteins were detected in culture supernatants using the HIS antibody and their respective protein specific antibodies (Figure 4C).

Recombinant TAT fused proteins can be purified from transduced U251 cell lysates and culture supernatants

Recombinant SMN protein derived from human cells, such as U251 cells, are likely to be correctly folded and post-translationally modified, which reportedly is required for full functional activity [28]. Recombinant TATκ-GFP and TATκ-SMN proteins were isolated and purified from culture medium, and from U251 cell lysates, using the 6xHIS tag and Ni^{2+} affinity

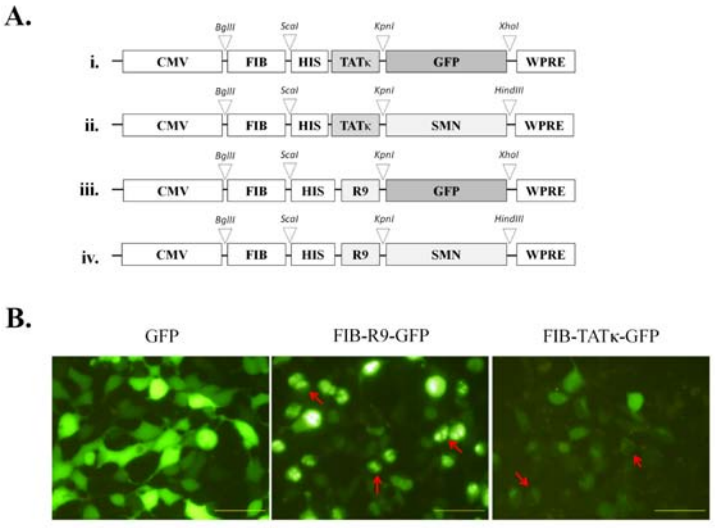

Figure 3. Construction of CPP secretory vectors. (A) Diagrammatic representation of the modified pShuttle plasmid vectors used in this study. Four vectors containing either a TATκ or R9 CPP fused to a GFP or SMN sequence were constructed. (B) The expression phenotype of GFP fused vectors was assessed via GFP fluorescence of FIB-TATκ-GFP and FIB-R9-GFP transfected HEK293 cells (Scale bar = 100 μm).

chromatography (Figure 5A). Following SDS-PAGE analysis protein bands consistent with the predicted molecular weights for TATκ-GFP and TATκ-SMN proteins were only detected from U251 cell lysate preparations at very low yields (estimated at 0.1 mg/ml; Figure 5B). However, following Ni^{2+} affinity chromatography of adenovirally transduced U251 cell culture supernatants and lysates, western blot analysis detected purified forms of the TATκ-GFP and TATκ-SMN proteins from both preparations (Figure 5C).

Purified TATκ-SMN and TATκ-GFP proteins do not transduce cortical neurons

To determine if recombinant TATκ proteins purified from supernatants and cell lysates could transduce cortical neurons, we added TATκ-GFP or TATκ-SMN proteins (0.5-1μM) to cultures. However, western blot analysis of lysates failed to detect any transduced TATκ-SMN or TATκ-GFP protein (Figure 6A). Similarly, recombinant TATκ-fused proteins purified from supernatants of U251 cultures infected with the FIB-TATκ-GFP and FIB-TATκ-SMN expressing adenoviral vectors were also unable to transduce neurons when compared to controls (Figure 6B).

TAT-SMN does not rescue SH-SY5Y cells from apoptosis

While protein transduction of TATκ-SMN was not detectable by western blot analysis (Figure 6A and 6B), we hypothesised that even undetectable levels of TATκ-SMN was potentially functional and neuroprotective. Thus, to assess the functionality of transduced U251 cell supernatant and purified TATκ-SMN protein, we used an apoptotic cell death model that we had developed previously, in which exogenous SMN over-expression protects SH-SY5Y cells from LY294002 mediated PI3-kinase/Akt inhibition [21]. Treatment of differentiated SH-SY5Y cells with supernatant from transduced U251 cells was ineffective at reducing LY294002 induced cell death compared to controls (Figure 6C). In addition, purified TATκ-SMN protein (~0.5 μM) from U251 transduced cultures was ineffective at reducing LY294002 induced SH-SY5Y cell death (Figure 6D).

Discussion

The most promising therapies currently being developed to treat SMA are aimed at increasing SMN protein levels, indirectly, by redirecting *SMN2* splicing or directly, by using viral delivery

of an SMN transgene [29,30]. However, a number of SMA therapies, developed on the basis of promising animal and *in vitro* experimentation, have either failed to provide therapeutic benefits or remain in preclinical testing [31,32]. Thus, it is imperative to explore and develop novel therapeutic approaches. One possibility involves the delivery of SMN protein directly to the central nervous system using CPPs, such as TAT, which are capable of transporting their cargo across the blood-brain barrier (BBB) and into cells of the central nervous system [33].

In the present study, we first confirmed that the TAT peptide can promote the transduction of the GFP protein across neuronal membranes. Consistent with previous studies [33-35] we observed a dose dependent increase in the transduction of a TAT fused GFP. To determine if TAT could facilitate the transduction of SMN into cells, we generated an *E. coli* expression vector to express a TAT-SMN recombinant protein. However, only small amounts of TAT-SMN protein were recovered, suggesting that protein was retained within inclusion bodies. To overcome this hurdle, we generated a fusion TAT-SMN-GFP protein to improve solubility and increase SMN protein yield. However, while total protein yield was increased, western blot analysis showed that the SMN fusion protein was proteolytically cleaved and/or inefficiently translated in the *E. coli* expression system. Thus it would appear that the future success in generating an SMN CPP fusion protein will require an improved host expression system, redesign of the expression construct, including the elimination of any putative protease cleavage sites, and improvements to the protein purification methodology.

In lieu of resolving the problems outlined above, we opted for an alternative to the bacterial expression of TAT-SMN protein. In this instance we developed an adenoviral vector to secrete CPP-fused proteins from mammalian cells. This approach was also adopted because it broadened the applicability of CPPs for *in situ* therapeutic protein delivery. The secretion of proteins from mammalian cells is well characterised [36], and has shown potential in the treatment of neurodegenerative disorders

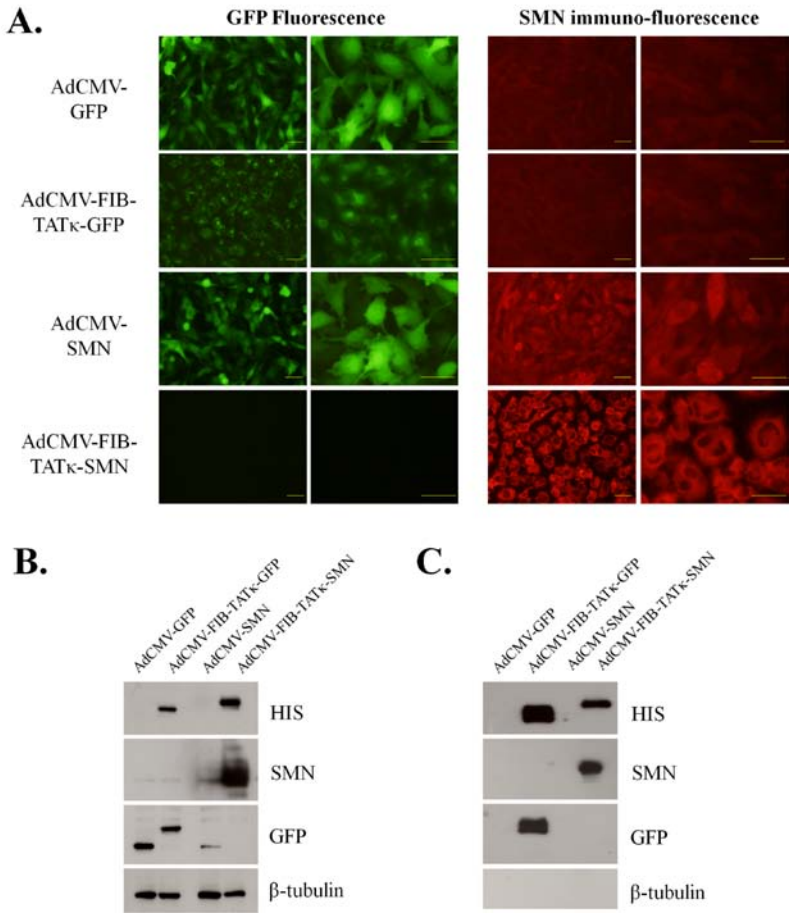

Figure 4. Adenoviral mediated protein expression in U251 cells. (A) Adenoviral constructs expressing CMV-FIB-TATκ-GFP and CMV-FIB-TATκ-SMN were generated and assessed in U251 cells using fluorescence and immuno-fluorescence alongside control Empty and SMN adenoviruses (Scale bar = 100 μm). (B) Confirmation of adenoviral expression was observed using western blot analysis of U251 cellular extracts, and (C) supernatants of transduced U251 cells.

[37-39]. Fusion of a therapeutic protein to the fibronectin signal peptide, an endoplasmic reticulum targeting motif, triggers its secretion via the constitutive pathway. Secretion is initiated following cleavage of the signal peptide and is completed by molecular trafficking events, which deliver the protein to the plasma membrane [40]. In deciding a CPP to adopt for the secretary system, we chose to compare the action of a modified TAT peptide (TATκ) and R9. While the TATκ peptide is not cleaved by the convertase enzyme furin, R9 is cleaved by this enzyme [41]. Transient transfection of pShuttle plasmids expressing FIB-TATκ-GFP in HEK293 cells appeared to confirm that TATκ fused GFP was secreted, as only weak cytoplasm expression was observed. In contrast, HEK293 cells transfected with the pShuttle plasmid expressing FIB-R9-GFP revealed GFP to accumulate as aggregates within the cytoplasm, as would be expected if the fusion protein was being cleaved by furin.

To achieve protein expression in multiple cell lines, we constructed adenoviral vectors expressing FIB-TATκ-SMN and the control protein FIB-TATκ-GFP. A similar approach using an adeno-associated viral vector to express FIB-galanin demonstrated that galanin was efficiently secreted and capable of targeting NMDA receptors [37]. Immuno-detection/

fluorescence and western blot analysis of AdCMV-FIB-TATκ-SMN and AdCMV-FIB-TATκ-GFP transduced U251 cells confirmed secretion of the proteins. In addition, molecular weight estimates based on western blot analysis confirmed that the FIB signal peptide was removed, consistent with secretion via the constitutive pathway. Further, immuno-fluorescence staining of AdCMV-FIB-TATκ-SMN and AdCMV-FIB-TATκ-GFP transduced U251 cells confirmed the characteristic low level cytoplasmic localisation of the respective proteins. Together, these results provided confirmation of the methodological basis for using this system to produce secretable CPP-fused recombinant proteins.

To purify secreted recombinant proteins from our mammalian expression system, we incorporated a 6xHIS tag to the N-terminal of each protein. Next, we confirmed that TATκ-SMN and TATκ-GFP could be purified and concentrated using the HIS tag from both the cell culture supernatant and from cell lysate. In addition, western blot analysis of the purified TATκ-SMN and TATκ-GFP using an anti-HIS tag antibody confirmed that the proteins retained their N-termini, indicating the presence of an intact TATκ peptide sequence. While recombinant proteins were successfully purified, the yields were low. Due to limitations in the culturing conditions such as low overall cell numbers and reduced culture volumes compared to bacterial systems, low recombinant protein yields are commonly encountered when using small scale eukaryotic expression systems. Nonetheless, proteins purified from eukaryotic cell lysates are free of potential toxic bacterial cell products and are more likely to possess the necessary post-translational modifications. Finally, it should be noted that the SMN secretary system could easily be scaled-up to produce enough TATκ-SMN protein for assessment in SMA mouse models.

We observed that purified TATκ-GFP or TATκ-SMN protein from transduced U251 cells was unable to transduce cortical neurons. To exclude the possibility that the purification process may have affected TATκ transduction activity, culture medium from adenoviral transduced CMV-FIB-TATκ-GFP and CMV-

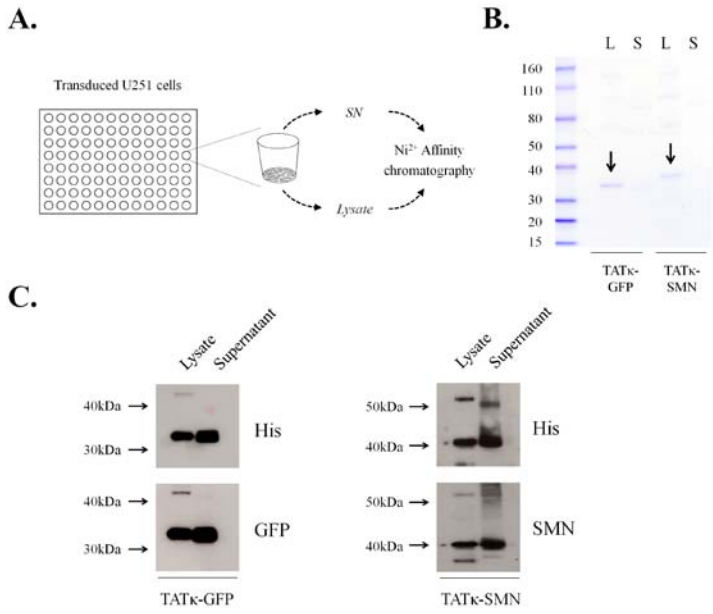

Figure 5. Purification and assessment of TATκ-GFP and TATκ-SMN recombinant proteins from mammalian U251 cells. (A) Schematic depicting recombinant protein purification methodology. (B) Coomassie staining of recombinant TATκ-fused proteins purified from U251 cellular extracts and supernatants by 6xHis tag affinity purification. (C) Western blot analysis of TATκ-fused proteins purified from U251 cellular extracts and supernatants by 6xHis tag affinity purification.

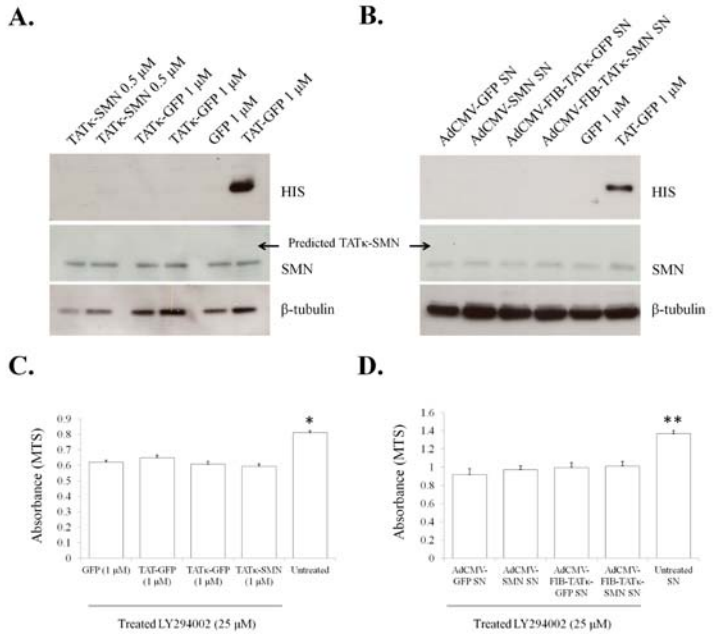

Figure 6. Assessment of TATκ fused recombinant proteins and transduced U251 supernatants. (A) Western blot analysis of cortical neurons treated for 1 hour with TATκ-GFP (1 μM) or TATκ-SMN (1 μM) recombinant proteins. (B) Western blot analysis of neuronal lysates treated for 1 hour with transduced U251 cell supernatants containing recombinant TATκ-GFP or TATκ-SMN proteins. (C) Assessment of SH-SY5Y cell viability following incubation with purified TATκ-GFP (1 μM) or TATκ-SMN (1 μM) recombinant proteins or (D) culture supernatant containing recombinant TATκ-GFP or TATκ-SMN protein prior to 25 μM LY294002 treatment (*P < 0.05, **P < 0.01).

FIB-TATκ-SMN cells was added directly to neuronal cultures. Similar to the purified TATκ-GFP or TATκ-SMN proteins, culture medium containing these proteins did not result in any detectable neuronal transduction. Our results contradicted those of a previous study using the TATκ peptide, which demonstrated TATκ-GFP secretion from a pSecTag2-TATκ-GFP transfected stable cell line, and subsequent transduction of the protein in H357 and Saos-2 cultures [25]. However, it should be noted that the contrasting results could be due to primary neuronal cultures being used in the present study, while established non-neuronal cell lines were used in the [25] study.

We previously demonstrated that SMN or Bcl-xL protein over-expression can reduce SH-SY5Y cell death and caspase-3 activity following PI3-kinase inhibition-induced apoptosis [21,42]. In the present study, we aimed to replicate the neuroprotective function of SMN by transducing cells with purified TATκ-SMN protein and culture medium containing TATκ-SMN protein. Treatment of SH-SY5Y cells with TATκ-SMN showed no improvement in cell viability compared to controls. The inability of TATκ-SMN protein to reduce PI3-kinase inhibition-induced apoptosis could be related to the inability of the SMN fusion protein to transduce SH-SY5Y cells and reach therapeutic intracellular levels, or interference of the TATκ peptide with SMN activity.

This study has developed an adenoviral expression vector capable of secreting and facilitating the purification of recombinant proteins expressed in mammalian cells. Although purified TATκ-SMN protein did not show functionality in transduction or viability assays, this may be due to low protein yields. Nonetheless, the methodology described in this study could be applied in other situations where secreted recombinant proteins with or without a fusion to a CPP may have a clinical benefit.

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