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The Design and Delivery of a Thermally Responsive Peptide to Inhibit S100B Mediated Neurodegeneration

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Abstract

S100B, a glial secreted protein is believed to play a major role in neurodegeneration in Alzheimer's disease, Down syndrome, traumatic brain injury and spinocerebellar ataxia type 1 (SCA1). SCA1 is a trinucleotide repeat disorder in which the expanded polyglutamine mutation in the protein ataxin-1 primarily targets Purkinje cells (PCs) of the cerebellum. Currently, the exact mechanism of S100B mediated PC damage in SCA1 is not clear. However, here we show that S100B may act via the activation of the RAGE signaling pathway resulting in oxidative stress mediated injury to mutant ataxin-1 expressing neurons. To combat S100B mediated neurodegeneration, we have designed a selective thermally responsive S100B inhibitory peptide, Synb1-ELP-TRTK. Our therapeutic polypeptide was developed using three key elements: (1) the elastin-like polypeptide (ELP), a thermally responsive polypeptide, (2) the TRTK12 peptide, a known S100B inhibitory peptide and (3) a cell penetrating peptide, Synb1, to enhance intracellular delivery. Binding studies revealed that our peptide, Synb1-ELP-TRTK, interacts with its molecular target S100B and maintains a high S100B binding affinity as comparable with the TRTK12 peptide alone. In addition, *in vitro* studies revealed that Synb1-ELP-TRTK treatment reduces S100B uptake in SHSY5Y cells. Furthermore, the Synb1-ELP-TRTK peptide decreased S100B induced oxidative damage to mutant ataxin-1 expressing neurons. To test the delivery capabilities of ELP based therapeutic peptides to the cerebellum; we treated mice with fluorescently labeled Synb1-ELP and observed that thermal targeting enhanced peptide delivery to the cerebellum. Here, we have laid the framework for thermal based therapeutic targeting to regions of the brain, particularly the cerebellum. Overall, our data suggests that thermal targeting of ELP based therapeutic peptides to the cerebellum is a novel treatment strategy for cerebellar neurodegenerative disorders.

Keywords

Purkinje cells; S100B; ataxin-1; Spinocerebellar ataxia type 1; cerebellum; neurodegeneration; Bergmann glia; elastin-like-polypeptide; thermal targeting

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Introduction

Spinocerebellar ataxia type 1 (SCA1) belongs to a group of CAG repeat neurodegenerative diseases. In SCA1, the expanded poly-glutamine containing protein ataxin-1 causes progressive ataxia resulting from the loss of cerebellar Purkinje cells (PCs) and neurons in the brainstem (Koeppen, 2005, Orr and Zoghbi, 2007, Matilla-Duenas et al., 2008). Overexpression of the human mutant ataxin-1 gene in PCs of SCA1 transgenic (Tg) mice results in a phenotype very similar to SCA1 patients (Burright et al., 1995). In SCA1 Tg mice, the earliest morphologic change is the appearance of cytoplasmic PC vacuoles containing the Bergmann glial (BG) protein S100B (Skinner et al., 2001, Vig et al., 2006, Vig et al., 2009). Recently, we reported that these PC vacuoles develop both in SCA1 Tg mice and SCA1 patients, where the vacuolar formation is associated with abnormal PC morphology (Skinner et al., 2001, Vig et al., 2006, Vig et al., 2009). The S100B protein belongs to the EF-hand family of calcium binding proteins and acts as a neurotrophic or neurotoxic signaling molecule for neighboring neurons (Winningham-Major et al., 1989, Donato, 1991, Reeves et al., 1994, Barger et al., 1995, Whitaker-Azmitia et al., 1997, Donato, 1999, Huttunen et al., 2000, Donato, 2001, Rothermundt et al., 2003, Zimmer et al., 2005, Donato et al., 2009, Sorci et al., 2010). Many studies suggest that increased levels of S100B in the disease prone regions are a major causative factor for neurodegeneration in a variety of neurological disorders (Griffin et al., 1989, Kato et al., 1990, Rothermundt et al., 2003, Vig et al., 2011). S100B has been shown to interact with a large array of protein targets including myo-inositol-monophosphatase-1, receptor for advanced glycation end products (RAGE), protein kinase C, neuromodulin, p53, and nuclear Dbf2-related kinases (Donato, 1999, McClintock and Shaw, 2000, Wilder et al., 2006, Vig et al., 2009). S100B is also a known RAGE ligand, and stimulation of S100B-RAGE signaling increases the production of reactive oxygen species (ROS), causing oxidative damage to neurons (Adami et al., 2004, Donato et al., 2009, Sorci et al., 2010). Currently, it is not clear how S100B mediates the SCA1 pathology; however, here we define a neuro-damaging role for S100B along an oxidative damage pathway in the SCA1 disease. To combat the neuro-damaging effects of S100B in the SCA1 pathology, we have designed a therapeutic polypeptide using three key elements: (1) the elastin-like polypeptide (ELP), a thermally responsive polypeptide, (2) the TRTK12 peptide, a known S100B inhibitory peptide, and (3) a cell penetrating peptide, Synb1, to enhance intracellular delivery. ELP is a thermally responsive polypeptide derived from mammalian elastin and is composed of multiple Val-Pro-Gly-X-Gly pentapeptide repeats, where X is any amino acid except Proline (Li et al., 2001, Bidwell and Raucher, 2005, Bidwell et al., 2007, Dreher et al., 2007, Bidwell and Raucher, 2009, Raucher et al., 2009). ELP based polypeptides are designed to be soluble at temperatures below 38°C, and to aggregate and concentrate at a transition temperature between 39-42°C (Li et al., 2001, Bidwell and Raucher, 2005, Bidwell et al., 2007, Dreher et al., 2007, Bidwell and Raucher, 2009, Raucher et al., 2009). The ELP based delivery mechanism works by active thermal targeting using externally focused hyperthermia directed at tissues with a large vasculature structure, such as cancerous tumors (Dreher et al., 2007). The Synb1 peptide is derived from naturally occurring protegrin peptides and mediates the delivery of peptides or compounds across the blood brain barrier (Rousselle et al., 2000, Sarantseva et al., 2009). On the C-terminus, our therapeutic peptide is fused to the S100B inhibitory peptide, TRTK12. The peptide TRTK12 has been shown to have a high binding affinity for S100B with the capability to block S100B interaction with S100B target proteins (Ivanenkov et al., 1995, Bianchi et al., 1996, McClintock and Shaw, 2000, Frizzo et al., 2004, Charpentier et al., 2010). Previously, we have shown that the TRTK12 peptide is therapeutic to SCA1 mice, where animals given TRTK12 displayed a significant improvement in ataxic behavior (Vig et al., 2011). Here, we demonstrate that the ELP based technology has a great potential to thermally target therapeutics to the cerebellum and

possibly other areas of the brain to combat multiple CNS disorders, especially the cerebellar ataxias.

Materials and Methods

Materials

Fugene 6 transfection reagent and green fluorescent protein (GFP) antibody were purchased from Roche (Indianapolis, IN, USA). The S100B antibody was purchased from Abcam (Cambridge, MA, USA). S100B protein was purchased from Sigma-Aldrich. RAGE and Synaptophysin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The TRTK12 peptide was purchased from AnaSpec (San Jose, CA, USA) as previously described (Vig et al., 2011). Fetal bovine serum (FBS) was purchased from HyClone (Logan, UT, USA).

Polypeptide Production

ELP was modified by the addition of the Synb1 peptide (RGGRLSYRRRFSTSTGR) and TRTK12 peptide (TRTKIDWNKILS) or the GGC peptide to produce Synb1-ELP-TRTK or Synb1-ELP-GGC by molecular cloning in a pET25b+ expression vector by recursive directional ligation and selected by ampicillin resistance as previously described (Meyer and Chilkoti, 1999). Constructs were verified by DNA sequencing. CPP-ELP-GGC polypeptides are commonly used as CPP-ELP control peptides (Massodi et al., 2009, Moktan et al., 2010), where the GGC stands for the last three amino acids (glycine, glycine and cysteine) of the CPP-ELP; also CPP-ELP-GGC polypeptides are commonly labeled on the C terminal cysteine residue with a fluorescent dye to monitor the impact of the CPP on fluorescent ELP localization in cell culture experiments (Massodi et al., 2009, Moktan et al., 2010). ELP constructs were expressed using the pET25 expression system and purified by inverse transition cycling as described previously (Meyer and Chilkoti, 1999, Bidwell and Raucher, 2005). A turbidity assay was used to determine the transition temperature (T_t), where concentrations of Synb1-ELP-TRTK and Synb1-ELP-GGC from 5 μ M to 40 μ M were placed in DMEM media containing 10% fetal bovine serum as described previously (Bidwell and Raucher, 2005, Bidwell et al., 2010). The polypeptide solutions were heated at a rate of 1 $^{\circ}$ C/min in a multi-cell holder in a UV-VIS spectrophotometer (Cary 100, Varian instrument). Turbidity was monitored by measuring the absorbance change at 350 nm every minute. This absorbance data was converted to a percentage of maximum absorbance as described previously (Bidwell and Raucher, 2005). The T_t was defined as 50% of the maximum of polypeptide aggregation.

Thermal Pull-down Assay

Solutions containing 60 μ M Synb1-ELP-TRTK or Synb1-ELP-GGC were mixed with or without 1 μ M S100B in PBS for 30 minutes at 37 $^{\circ}$ C. The ELP peptides were then purified using thermal cycling and washing with PBS. Thermal cycling consists of heating an ELP solution to 42 $^{\circ}$ C followed by centrifugation at 13,000 rpm (13,800 RCF) for 2 minutes using an Eppendorf table top centrifuge (model 5415C, Eppendorf, Hamburg, Germany). The resulting ELP pellet was then resuspended in cold PBS. The thermal cycling procedure was repeated for a total of five times to remove unbound S100B. To assess any ELP peptide S100B interactions, the purified ELP pellets were subjected to western blotting using an S100B antibody.

Binding Studies

The tryptophan fluorescence of 5.0 μ M Synb1-ELP-TRTK, 5.0 μ M Synb1-ELP-GGC and 5.0 μ M TRTK12 were measured using an Aminco Bowman Series 2 fluorescent

spectrophotometer with an Excitation wavelength of 295 nm and an Emission wavelength of 350 nm in 25 mM Tris HCL and 0.1 mM CaCl₂ at 12 °C (to avoid aggregation of ELP) or in a solution containing 25 mM Tris HCL, 0.1 mM CaCl₂ and 1 mM EGTA at 12 °C. S100B was titrated from 100 nM up to 3.0 μM into the various peptide solutions and changes in tryptophan fluorescence were recorded into an Excel file and graphs produced. Δ Fluorescence % was plotted against free S100B concentration using GraphPad Prism software. Δ Fluorescence % was calculated as follows: [(Maximum fluorescence – Observed fluorescence)/(Maximum fluorescence-Minimum Fluorescence)] X 100%. Where the maximum fluorescence is the fluorescence of 5.0 μM Synb1-ELP-TRTK or 5.0 μM TRTK12 before titration of S100B and the minimum fluorescence is the fluorescence of 5.0 μM Synb1-ELP-TRTK or 5.0 μM TRTK12 fully saturated with bound S100B. GraphPad Prism software was used to calculate K_d, R² values and B_{max} for the binding studies.

Cell Culture Studies

SHSY5Y cells (Invitrogen, Carlsbad, CA, USA) were maintained in DMEM/F12 media (Fisher, Houston, TX, USA) supplemented with 15% FBS, penicillin-streptomycin (Sigma), and grown in an incubator at 37 °C in the presence of 5% CO₂. S100B was labeled with Alexa 488 labeling kit (Invitrogen) according to the manufacture's protocol to produce 488-S100B. The Alexa 488 labeling kit uses an *Alexa Fluor*® 488 carboxylic acid, tetrafluorophenyl ester that reacts with primary amines of proteins mainly on exposed lysine residues, to form stable dye-protein conjugates. WT SHSY5Y cells were placed on 2-well slides or 12-well plates (Fisher) at concentration of 100,000 cells per well. Next, the cells were pretreated with either 50 μM Synb1-ELP-TRTK or Synb1-ELP-GGC for 1hr at 37°C. After Synb1-ELP pretreatment, cells were washed then treated with 1 μM 488-S100B for 3 hrs and S100B uptake was analyzed by Epi-fluorescence or by Beckman Cytometric FC 500 flow cytometer as previously described (Bidwell et al., 2009). For flow cytometry experiments, 488-S100B uptake was measured by quenching extracellular fluorescence by coating the cells with Trypan Blue (Fisher) as described previously (Bidwell et al., 2009). Experiments were repeated at least three times and statistics taken. SHSY5Y stable cell lines were produced by transfecting with either GFP-ATXN1[82Q] or GFP-ATXN1[30Q] vectors (described previously (Hearst et al., 2010)) using Fugene 6 transfection reagent and selected by G418 (G418 sulfate 600 μg/ml, Sigma) resistance as previously described (Hearst et al., 2010). Cells were grown in 15% FBS in F-12/DMEM media and plated on 2 well Lab-Tek CC2 chamber slides (Fisher). Cells were subjected to treatment with 25 nM TPA (Sigma) for 3 days to produce differentiated SHSY5Y cell lines as described previously (Presgraves et al., 2004, Cheung et al., 2009). Next, slides were fixed and probed with the primary GFP, RAGE and Synaptophysin antibodies and fluorescent secondary antibodies Alexa 488 and Alexa 546 (Invitrogen) followed by DAPI (Invitrogen) staining as previously described (Hearst et al., 2010). Cells were observed on an Olympus BX60 epifluorescence microscope. Changes in neurite length after S100B treatment were measured using Image J software. Statistical significance was calculated using the student's t-test, where n=100 per group, where P<0.05 is considered significant. Experiments were repeated for a total of three times to gather statistical significance.

Oxyblot Experiments

GFP-ATXN1[82Q] or GFP-ATXN1[30Q] stable SHSY5Y cells were placed on 12-well plates at a concentration of 60,000 cells per well. Cells were treated with 25 nM TPA for 3 days to produce differentiated SHSY5Y neuronal cell. After TPA treatment, the cells were then pretreated with 50 μM Synb1-ELP-TRTK for 1hr then treated with 5 μM S100B for 2 hrs or treated with 150 μM H₂O₂ (Fisher) for 2 hrs, where control cells were treated with serum free media for 3hrs. (It is important to note that cell culture studies were performed by 1st pretreating the cells with Synb1-ELP-TRTK or Synb1-ELP-GGC for an allotted time,

followed by S100B treatments to test the ability of our ELP peptides to block the S100B induced effects.) Then the cells were lysed in 1% BME (Sigma) and RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% sodium deoxycholate and 1% NP-40, Sigma). Cell lysates were analyzed with the Oxyblot kit according to manufacturer's protocol (Roche) and with a β -tubulin antibody (Sigma), where 5 μ gs of protein was loaded in each well of 4–20% acrylamide gel (Bio-Rad Laboratories, Hercules, CA, USA). Optical densities of the protein bands were taken using ImageJ software. Graphs were produced in Excel and show the optical density ratio of oxidized protein/ β -tubulin normalized to the control. Experiments were repeated for a total of three times to gather statistical significance, $n=3$. Statistics were taken using the student's t-test, where $P<0.05$ is considered significant.

In Vivo Studies

Wild-type (WT, FVB) mice were obtained from Jackson Labs, Bar Harbor, ME, USA. All animal protocols were approved by Institutional Animal Care and Use Committee (IACUC) at the University of Mississippi Medical Center. The 3 week old animals were anesthetized with isoflurane (Butler Animal Health Supply, Dublin, OH, USA) and a temperature probe was gently inserted into the cerebellum and the frontal lobe to monitor temperature changes under the thermal cycling procedure. The thermal cycling procedure consisted of 30 minutes of heating and 5 minutes of cooling followed by two additional cycles of 20 minutes of heating and 5 minutes of cooling. The cerebellum was heated with the Mettler Laser-System 540® Laser System using the 785 nm Single AlGaAs Diode Class 3B laser applicator with an elliptical beam area of 9.2 mm² (Mettler Electronics, Anaheim, CA, USA). Synb1-ELP-GGC protein was labeled on the C-terminal cysteine residue with an Alexa 750 maleimide labeling kit (Invitrogen) to produce fluorescent labeled Synb1-ELP-GGC. After anesthesia with isoflurane, 3 week old FVB mice were injected intraperitoneal (IP) with 100 mg/kg fluorescent labeled protein or 200 μ L saline as the control. 2 hrs post heating, the animals were euthanized by CO₂ inhalation and their brain, heart, spleen, right lung, right kidney and liver removed. The fluorescent distribution of the brain and other organs were visualized with IVIS Live Animal Imager (Caliper Life Sciences, Hopkinton, MA, USA; courtesy of the UMMC Cancer Institute and the UMMC Laboratory Animal Facility) using an Excitation wavelength of 745 nm and an Emission wavelength of 800 nm. Radiant Efficiency is a common unit used to measure Epi-fluorescence changes *in vivo* (Shcherbo et al., 2010). The average Radiant Efficiency of the cerebellum $\pm$ SE was calculated by taking an ROI of the cerebellum and using the Living Image software (Caliper). The average Radiant Efficiency of the brain, heart, spleen, right lung, right kidney and liver $\pm$ SE was calculated by taking an ROI of the organ and using the IVIS software. The experiments were repeated three times with a total of three animals per treatment group, $n=3$. Statistics were taken using student's t-test, where $P<0.05$ is considered significant.

Results

The Design of an ELP Based S100B Inhibitory Peptide

Peptide based drugs are gaining popularity as promising new therapeutics due to the ease of peptide design and the high specificity of peptides for their molecular targets (Bidwell and Raucher, 2005, Bidwell et al., 2007, Dreher et al., 2007, Bidwell and Raucher, 2009, Raucher et al., 2009, Bidwell and Raucher, 2010). In this study, we have designed an S100B inhibitory polypeptide, Synb1-ELP-TRTK. Our therapeutic peptide was designed to take advantage of the unique properties of each of its three fused peptides, (1) the ELP, a thermally responsive polypeptide, (2) the TRTK12 peptide, a known S100B inhibitory peptide, and (3) a cell penetrating peptide, Synb1, to enhance intracellular delivery (Fig. 1a). In previous studies, the delivery of ELP conjugated therapeutic peptides has been

successfully demonstrated (Bidwell and Raucher, 2005, Bidwell et al., 2007, Dreher et al., 2007, Bidwell and Raucher, 2009, Raucher et al., 2009, Bidwell and Raucher, 2010). The ELP thermally responsive element allows for simple peptide purification by inverse transition cycling, where ELP conjugated polypeptides aggregate and precipitate at a transition temperature between 39°C to 42°C (Fig. 1b and c) (Li et al., 2001, Bidwell and Raucher, 2005, Bidwell et al., 2007, Dreher et al., 2007, Bidwell and Raucher, 2009, MacKay et al., 2009, Raucher et al., 2009, Bidwell and Raucher, 2010). Taking advantage of the ELP thermal responsive element of our therapeutic peptide, we developed the first thermal pull-down assay to test the interaction between Synb1-ELP-TRTK and the S100B protein (Fig. 1d). We found that when precipitated by thermal cycling, Synb1-ELP-TRTK bound both S100B monomers and dimers (Fig. 1e). The dimeric S100B protein has been previously shown not to disassociate under standard SDS-PAGE conditions and is detectable on western blots (Donato, 1999). However, our control peptide, Synb1-ELP-GGC, which lacks the TRTK12 peptide, showed little to no interaction with the S100B protein (Fig. 1e). These data suggest that our therapeutic peptide Synb1-ELP-TRTK directly interacts with its molecular target, the S100B protein. Furthermore, our pull-down technique can be used as an alternative to standard pull-down assays using Pro-A or Pro-G beads or further modified by the addition of ELP to accommodate other proteins of interest.

Synb1-ELP-TRTK S100B Binding Affinity

Alterations in the binding affinity of a peptide due to its fusion to ELP have never been studied previously. Since our therapeutic peptide Synb1-ELP-TRTK interacted with the S100B protein, by using fluorescent spectroscopic techniques, we further investigated if the binding affinity of Synb1-ELP-TRTK for S100B differed from that of the native TRTK12. Fluorescent spectroscopy is a sensitive tool used to monitor changes in the local environment of tryptophan residues that occur during a protein-ligand binding event; furthermore, changes in tryptophan fluorescence can be used to calculate binding affinity (Weljie and Vogel, 2000, Bocedi et al., 2004). Previous work has shown that S100B binding reduces tryptophan fluorescence of TRTK12 and like peptides (Ivanenkov et al., 1995). The tryptophan fluorescence of 5.0 μ M Synb1-ELP-TRTK and 5.0 μ M Synb1-ELP-GGC were measured using a fluorescent spectrophotometer with an Excitation wavelength of 295 nm and an Emission wavelength of 350 nm in 25 mM TrisHCL and 0.1 mM CaCl₂ buffer at 12°C. At 295 nm, tryptophan emission spectrum is dominant over the weaker tyrosine and phenylalanine fluorescence (Vivian and Callis, 2001). Synb1-ELP-TRTK contains two tryptophan residues (W755 and W767), while Synb1-ELP-GGC contains only one tryptophan residue (W755). The W767 residue of Synb1-ELP-TRTK corresponds to W7, the major binding residue of the S100B binding peptide TRTK12 (Ivanenkov et al., 1995). In Synb1-ELP-GGC the W755 residue has very little fluorescence at 350 nm which may be due to the 150 neighboring VPGXG repeats of ELP (Fig. 2a). In Synb1-ELP-TRTK, W767, in combination with W755 shows a large fluorescence emission at 350 nm (Fig. 2a). A decrease in the tryptophan fluorescence intensity of Synb1-ELP-TRTK was observed upon the addition of S100B from 100 nM to 3.0 μ M (Fig. 2b). This may be due to changes induced by the S100B interaction leading to less exposure of the tryptophan residues to the solvent solution and burial of W767 into the hydrophobic S100B binding site. S100B is a dimeric protein with two TRTK12 binding sites, where the binding is calcium dependent (Zimmer and Weber, 2010). Titration of S100B into a solution containing 25 mM Tris HCL, 0.1 mM CaCl₂ and 1 mM EGTA displayed no effect in quenching the tryptophan fluorescence of Synb1-ELP-TRTK at 350 nm (Fig. 2c). Fluorescent quenching can be used to study protein-ligand binding when the formation of the protein-ligand complex leads to a change in fluorescence of the complex (Weljie and Vogel, 2000, Bocedi et al., 2004). Using this fluorescence quenching technique, we assume that protein-ligand complex formation is relative to the change in fluorescence. Therefore, Synb1-ELP-TRTK S100B binding was

monitored by the percent change in tryptophan fluorescence (Δ Fluorescence %) plotted against free S100B concentration (Fig. 2d) with a K_d of $0.51 \pm 0.08 \mu\text{M}$ and an R^2 of 0.98. Next, we repeated the same fluorescence techniques to acquire the binding affinity of the TRTK12 peptide alone to S100B. As expected, the major binding residue W7 of the TRTK12 peptide, displayed strong tryptophan fluorescence intensity at 350 nm (Fig. 3a). Titration of S100B into a solution containing $5.0 \mu\text{M}$ TRTK12 showed a decrease in the tryptophan fluorescence intensity of TRTK12 upon addition of S100B from 100 nM to $2.5 \mu\text{M}$ (Fig. 3b). Also, depletion of calcium inhibited this S100B binding process and had little effect on the tryptophan fluorescence intensity of TRTK12 at 350 nm (Fig. 3c). TRTK12-S100B binding was monitored by the Δ Fluorescence % plotted against free S100B concentration (Fig. 3d) with a K_d of $0.21 \pm 0.03 \mu\text{M}$ and an R^2 of 0.98. Our results are supported by earlier reports that found that the TRTK12 peptide had an S100B binding affinity between 200-300 nM (Wright et al., 2009). Overall, our binding data indicated that the addition of Synb1-ELP to the TRTK12 peptide, to create Synb1-ELP-TRTK, had a 2 fold impact on S100B binding affinity compared to the TRTK12 peptide alone; where Synb1-ELP-TRTK maintains a high S100B binding affinity.

Synb1-ELP-TRTK Blocks S100B Uptake in SHSY5Y Cells

S100B is a known RAGE ligand, where S100B uptake is mediated through RAGE receptor binding (Leclerc et al., 2007, Perrone et al., 2008). The neuroblastoma, SHSY5Y cell line, is a well known RAGE expressing cell line and has been previously used to study S100B-RAGE signaling (Leclerc et al., 2007). Fluorescent labeled S100B has been used previously to monitor S100B localization *in vitro* (Vig et al., 2006). Treatment of SHSY5Y cells with fluorescent labeled S100B, 488-S100B, displayed a localization of S100B in both the cytoplasm and the nucleus (Fig. 4a). We tested the hypothesis that Synb1-ELP-TRTK could block S100B-RAGE signaling and reduce S100B uptake in SHSY5Y cells by pretreating SHSY5Y cells with Synb1-ELP-GGC or Synb1-ELP-TRTK for 1 hr followed by 488-S100B treatment. Synb1-ELP-GGC pretreatment had no effect on 488-S100B uptake, however, Synb1-ELP-TRTK pretreatment reduced 488-S100B cellular uptake and localized 488-S100B to large intracellular aggregates (Fig. 4a). These results reconfirmed earlier reports that Synb1-ELP based peptides show both extracellular and intracellular localization in cultured cells (Bidwell et al., 2010). Using flow cytometry techniques under the same experimental conditions, we further validated our Epi-fluorescence results. Flow cytometric analysis revealed that Synb1-ELP-TRTK pretreatment significantly reduced 488-S100B uptake in SHSY5Y cells compared to Control and Synb1-ELP-GGC pretreated cells (Fig. 4b). This data suggest that our therapeutic peptide Synb1-ELP-TRTK can be used to block S100B-RAGE signaling.

S100B Causes Damaged to Neurons Expressing Mutant Ataxin-1

S100B acts both as a neurotrophic and a neurotoxic signaling molecule, where its toxicity is based on its concentration in the extracellular environment (Donato, 1991, 1999, 2001, Donato et al., 2009). High levels of S100B through S100B-RAGE signaling causes the production of ROS and downstream oxidative stress (Rothermundt et al., 2003, Donato et al., 2009, Sorci et al., 2010). S100B-RAGE signaling has also been shown to produce ROS in SHSY5Y cells (Leclerc et al., 2007). We set out to determine if SHSY5Y cells expressing the mutant form of ataxin-1, ATXN1[82Q], would be more susceptible to oxidative stress through S100B-RAGE signaling compare to SHSY5Y cells expressing normal ataxin-1, ATXN1[30Q]. A recent report found that the super oxide dismutase (SOD) enzyme is sequestered to mutant ataxin-1 inclusion bodies and suggests that the polyglutamine-expanded ataxin-1 increases the levels of reactive oxygen species by reducing the functional activity of SOD (Kim et al., 2003b). Loss of SOD function in mutant ataxin-1 expressing cells is further supported by reports demonstrating that ATXN1[82Q] expressing cells are

more susceptible to oxidative damaged compared to ATXN1[30Q] expressing cells (Kim et al., 2003a, Kim et al., 2003b, Ryu et al., 2010). We produced stable expressing GFP-ATXN1[82Q] and GFP-ATXN1[30Q] SHSY5Y cell lines by G418 selection. Once established, we further differentiated our GFP-ATXN1[82Q] and GFP-ATXN1[30Q] SHSY5Y cell lines into neurons by TPA treatment. TPA has been used to differentiate SHSY5Y cells into neurons, where differentiated SHSY5Y cells grow long neuronal processes or neurites and express the synaptic vesicle protein, synaptophysin (Presgraves et al., 2004, Cheung et al., 2009). After TPA treatment both GFP-ATXN1[82Q] (Fig. 5a) and GFP-ATXN1[30Q] (not shown) SHSY5Y cell lines expressed the synaptophysin protein and grew long neuronal processes. Also, both GFP-ATXN1[82Q] (Fig. 5b) and GFP-ATXN1[30Q] (not shown) SHSY5Y cell lines expressed the RAGE receptor after TPA treatment. Knowing that both differentiated GFP-ATXN1[82Q] and GFP-ATXN1[30Q] SHSY5Y cell expressed RAGE, we tested the impact of S100B on cells with neurites. We found that after S100B treatment, ATXN1[82Q] expressing neurons showed a significant reduction in the percentage of cells with neurites compared to untreated ATXN1[82Q] expressing neurons (Fig. 5c). Further, S100B treatment significantly reduced neurite length in ATXN1[82Q] expressing neurons compared to untreated ATXN1[82Q] expressing neurons (Fig. 5d). However, S100B treatment had no significant effect on ATXN1[30Q] expressing cells compared to untreated cells (Fig. 5c and d). The loss of SOD function due to ATXN1[82Q] expression and the ROS production activated by S100B-RAGE signaling may cause increased oxidative damage in ATXN1[82Q] expressing neurons, activating neurodegenerative processes. The Oxyblot technique has been used to detect the level of oxidized protein in cultured SHSY5Y cell resulting from ROS production (Jiang et al., 2004, Takano et al., 2007). Using this technique, we treated TPA differentiated GFP-ATXN1[82Q] and GFP-ATXN1[30Q] SHSY5Y cell with S100B and analyzed S100B's impact on the level of oxidized proteins compared to the level of β -tubulin protein. We found that differentiated GFP-ATXN1[82Q] cells displayed a greater level of oxidized proteins when treated with S100B compared to GFP-ATXN1[30Q] cells (Fig. 6a and b). Furthermore, Synb1-ELP-TRTK pretreatment significantly blocked S100B's impact on the level of oxidized proteins in ATXN1[82Q] cells (Fig. 6a). We used H₂O₂ induced oxidative damage as a positive control to ensure the Oxyblot technique was working properly. We found that mutant ataxin-1 expressing cells are more sensitive to H₂O₂ compared to normal ataxin-1 expressing cells; this confirmed earlier reports that ATXN1[82Q] expressing cells display higher oxidative damage compared to ATXN1[30Q] cells when exposed to H₂O₂ (Fig. 6a) (Kim et al., 2003a, Kim et al., 2003b, Ryu et al., 2010). Previously, we have shown that TRTK12 peptide improves SCA1 PC morphology in cerebellar slice cultures (Vig et al., 2011). The fact that RAGE is highly expressed in PCs of the cerebellum, along with our findings that ATXN1[82Q] expressing neurons are more sensitive to S100B-RAGE signaling, as well as the beneficial effects of TRTK12 on SCA1 PCs in slice culture, defines a role for S100B as a toxic signaling protein in the SCA1 neurodegenerative pathway. Therefore, using therapeutic peptides, such as Synb1-ELP-TRTK to block S100B induced neurotoxic effects is a novel SCA1 treatment strategy.

Thermal Cycling Increases Localization of Fluorescent Labeled Synb1-ELP to the Cerebellum

Research has shown that hyperthermic targeting of the ELP biopolymer to tumor vasculature is a very effective methodology to concentrate the macromolecular drug carrier, ELP, to specific biological locations (Meyer et al., 2001, Liu et al., 2006, Dreher et al., 2007). Furthermore, thermal cycling has been shown to be more efficient than continuous heating to increase the accumulation and delivery of ELP to tumors in mice models (Dreher et al., 2007). To test the feasibility of heating the mouse cerebellum without drastically heating other areas of the brain, we inserted a temperature probe into the cerebellum as well as the

frontal lobe and monitored the temperature changes under a thermal cycling procedure (Fig 7a). Our thermal cycling procedure consisted of 30 minutes of heating and 5 minutes of cooling followed by two additional cycles of 20 minutes of heating and 5 minutes of cooling; where a 785 nm laser pointed at the cerebellum was used as the heating source. During the thermal cycling procedure, the temperature of the cerebellum reached a maximum temperature of 40°C remaining 2 degrees above the frontal lobe temperature as well as the body temperature (Fig 7a). Next, we monitored the localization and accumulation of Synb1-ELP-GGC (the control peptide, lacking the TRTK12 modification) in the brain under heated and unheated conditions. The Synb1-ELP-GGC peptide contains only one cysteine residue found at the end of the C-terminus. Synb1-ELP-GGC was labeled on the C-terminal cysteine residue with Alexa 750 fluorescent dye according to the manufacture's protocol. Three, 3 week old FVB WT mice were anesthetized with isoflurane and given an IP injection of 100 mg/kg fluorescent labeled Synb1-ELP-GGC suspended in a saline solution or saline alone. One hour post injection, the cerebellum of one animal was externally heated (using the thermal cycling procedure above) while the other animals were left unheated. 2 hrs post heating, the animals were euthanized by CO₂ inhalation and their brains and organs removed. The brains were then lined up and photographed with the IVIS Animal Imager with an Excitation wavelength of 745 nm and an Emission wavelength of 800 nm (Fig. 7b). Shown is the image displaying a photograph of the brains and the fluorescent uptake of labeled Synb1-ELP-GGC. The Overlay image shows that thermal cycling is an effective targeting method to increase localization of Synb1-ELP-GGC to the cerebellum. The Epi-fluorescent color scale measures Radiant Efficiency, where blue coloration is low fluorescence, red is mid level fluorescence and green is high fluorescence (Fig. 7b). Next, we used the IVIS software to measure the change in Radiant Efficiency of the cerebellum after thermal targeting of Synb1-ELP-GGC to the cerebellum compared to unheated and saline treated animals. We found a significant increase in the Radiant Efficiency of the cerebellum after a thermal cycling procedure compared to the unheated and the control cerebellums (Fig. 7c). Next, we looked at the bio-distribution of Synb1-ELP-GGC in the brain, heart, spleen (Fig. 7d), lung, kidney and liver (Fig. 7e) from control animals, unheated animals and animals where the cerebellum was heated by thermal cycling. We found that heating the brain had no significant effect on Synb1-ELP-GGC levels in the various organs analyzed. However, there appears to be an increase in the total brain uptake in animals where the cerebellum was heated compared to unheated animals; however the increase was not significant (Fig. 7d). The increase in brain uptake is most likely due to the enhanced localization of Synb1-ELP-GGC to the cerebellum induced by the focused hyperthermia. Our results are a unique finding, as ELP peptide transport into the brain has never been studied. Overall, we found that thermal targeting by applying local focused hyperthermia significantly increased Synb1-ELP-GGC localization to the cerebellum. This is the first evidence demonstrating that ELP based therapeutics can be thermally targeted to a particular region of the brain to combat neurodegenerative diseases.

Discussion

We have recently reported that the S100B inhibitory peptide, TRTK12, modulates mutant ataxin-1 mediated PC morphology in slice cultures and improves behavioral abnormalities in SCA1 mice (Vig et al., 2011). The TRTK12 peptide was developed from the high specificity, S100B binding protein CapZ (Charpentier et al., 2010). The possibility of TRTK12 non-specific interactions is limited by the extensive biophysical characterization of the TRTK12-S100B interaction using solution NMR and X-ray crystallography techniques (Inman et al., 2002, Charpentier et al., 2010). To further amplify the therapeutic benefits and enhance TRTK12 delivery to the cerebellum, we designed the first thermally responsive S100B inhibitory peptide, Synb1-ELP-TRTK. Our peptide interacts with its molecular target S100B (Fig. 1). Though the S100B binding affinities of both Synb1-ELP-TRTK and

TRTK12 peptide (alone) are in the nanomolar range (Fig. 2 and 3), we believe that our Synb1-ELP-TRTK polypeptide has an added advantage over TRTK12 alone, due to its enhanced intracellular accumulation from the CPP, Synb1, and from the thermal response of ELP. To support the argument that the TRTK12 peptide of the Synb1-ELP-TRTK polypeptide maintains its biological function, we showed that Synb1-ELP-TRTK reduces S100B-RAGE mediated uptake (Fig. 4) as well as S100B induced oxidative damage in mutant ataxin-1 expressing cells (Fig. 6). Furthermore, we show that thermal targeting of Synb1-ELP-GGC by applying local hyperthermia to the cerebellum after systemic administration of the polypeptide is a novel treatment strategy to increase therapeutic peptide uptake and delivery to the cerebellum (Fig. 7).

In this study, we further characterize the role of BG in the SCA1 pathogenesis and show that S100B-RAGE signaling directly influences degeneration of mutant ataxin-1 expressing neurons by an oxidative stress mechanism (Fig. 5, 6 & 8). The RAGE receptor is highly expressed in PCs of the cerebellum; and due to the loss of SOD function, mutant ataxin-1 expressing neurons are more susceptible to oxidative damage through S100B-RAGE signaling (Kim et al., 2003a, Kim et al., 2003b, Vig et al., 2006, Ryu et al., 2010). Thus, signaling mechanisms that stimulate ROS production in SCA1 PCs are major contributing factors in oxidative stress mediated neurodegeneration. Reports indicate that S100B transport is mediated by a RAGE dependent uptake mechanism both *in vitro* and *in vivo* (Vig et al., 2006, Perrone et al., 2008). The presence of S100B vacuoles in degenerating SCA1 PCs along with the improvements in SCA1 mice by TRTK12 treatment is striking evidence indicating the neurotoxic effects of S100B signaling (Vig et al., 2006, Vig et al., 2009, Vig et al., 2011). S100B is a damage associated protein, where S100B-RAGE signaling causes the production of ROS and oxidative stress (Rothermundt et al., 2003, Donato et al., 2009, Sorci et al., 2010). Furthermore, S100B has been reported to play a neurodegenerative role in brain injury, Alzheimer's disease as well as Down syndrome (Griffin et al., 1989, Kato et al., 1990, Rothermundt et al., 2003, Mori et al., 2010). S100B has been shown to increase tau protein hyper-phosphorylation and exacerbates cerebral amyloidosis in the Alzheimer's disease mouse model (Esposito et al., 2008, Mori et al., 2010). Interestingly, S100B ablation has been shown to improve Alzheimer's disease pathology in Tg mice (Roltsch et al., 2010). Since S100B-specific inhibitors are not currently available, the use of S100B peptide based inhibitors is a novel treatment strategy for reducing S100B mediated effects in a number of neurodegenerative diseases where high S100B levels contribute to pathology.

This work also presents the novel strategy of utilizing the thermally responsive Elastin-like polypeptide for thermally targeted delivery of agents to the cerebellum to treat neurological disorders. Thermal targeting of ELPs to tumors has shown promising results (Meyer et al., 2001, Liu et al., 2006, Dreher et al., 2007), and a thermal cycling protocol similar to that used in this work has been shown to be beneficial compared to long sessions of constant hyperthermia (Dreher et al., 2007). These previous studies have demonstrated that ELP has characteristics, including a long plasma half-life, resistance to proteolysis, tumor targeting (Liu et al., 2006), and the ability to escape the vasculature and enter tumor cells (Bidwell and Raucher, 2010), that are desirable in a drug delivery agent. However, there has been no demonstration of the use of ELP for thermally targeted delivery to the brain. The results shown here indicate that brain delivery is feasible, and the technology necessary to induce hyperthermia to the brain currently exists in the clinical setting in the form of MRI – guided high intensity focused ultrasound (Ram et al., 2006, Martin et al., 2009, Hertzberg et al., 2010). Future work will examine the ability of ELP to deliver the TRTK12 peptide to PCs *in vivo*, and will define the efficacy of this approach for treatment of SCA1 using a transgenic mouse model. If efficacious, this approach can be taken beyond TRTK12 peptide delivery and SCA1. The ELP delivery system can be modified to accommodate a large repertoire of

cargo, including peptides, drugs, or DNA to treat a multitude of CNS diseases making a huge impact on the neurodegenerative field (Bidwell and Raucher, 2005, Bidwell et al., 2007, Chen et al., 2008, Bidwell and Raucher, 2009, MacKay et al., 2009, Raucher et al., 2009).

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Highlights

- Design of the first thermally responsive S100B inhibitory peptide
- This polypeptide, Synb1-ELP-TRTK, interacts with its target S100B
- Show thermally responsive targeted delivery of agents to the cerebellum.
- Thermally responsive TRTK12 is a potential candidate for treating SCA1

Figure 1.

(a) Displayed is a diagram of the therapeutic polypeptide Synb1-ELP-TRTK. Displayed are the three key elements: (1) ELP, a thermally responsive peptide, (2) TRTK12 peptide, a known S100B inhibitory peptide, and (3) a cell penetrating peptide, Synb1, to enhance intracellular delivery. Shown are the turbidity assay results of various concentrations of (b) Synb1-ELP-GGC and (c) Synb1-ELP-TRTK heated from 25°C to 75°C. The peptides Synb1-ELP-GGC and Synb1-ELP-TRTK, show a transition temperature between 39°C to 42°C. (d) Shown is the thermal pull-down assay procedure, where a solution containing S100B and Synb1-ELP-TRTK protein are allowed to interact at 37°C in a microcentrifuge tube. Next, the solution is heated to 42°C, where the ELP peptide undergoes a heat induced conformational change causing the ELP peptides to aggregate and precipitate. Finally, these ELP precipitates are spun at high speed in a centrifuge to form a pellet containing the ELP peptide and its interacting partners, in this case Synb1-ELP-TRTK and S100B. Once the supernatant is removed the protein pellet can be analyzed by western blotting techniques to reveal protein-protein interactions. (e) Shown are the results from our thermal pull-down assay. Solutions containing 60 μM Synb1-ELP-TRTK or Synb1-ELP-GGC (control peptide) were mixed with or without 1 μM S100B and subjected to thermal pull-down assay procedure and analyzed by western blotting using an S100B antibody. Synb1-ELP-TRTK peptide showed a direct interaction with S100B while Synb1-ELP-GGC showed little to no interaction with S100B.

Figure 2.

(a) Shown is the tryptophan fluorescence of 5.0 μM Synb1-ELP-TRTK containing W755 and W767 and the tryptophan fluorescence of 5.0 μM Synb1-ELP-GGC containing only W755. Both peptides displayed maximum fluorescence at 350 nm. (b) Shown is the effect on tryptophan fluorescence of 5.0 μM Synb1-ELP-TRTK observed upon the addition of S100B from 100 nM to 3.0 μM in the presence to CaCl_2 . (c) Shown is the effect on tryptophan fluorescence of 5.0 μM Synb1-ELP-TRTK observed upon the addition of S100B from 100 nM to 3.0 μM in the presence to CaCl_2 and EGTA. (d) Shown is Δ Fluorescence % of Synb1-ELP-TRTK plotted against free S100B concentration with a K_D of 0.51 ± 0.08 μM , an R^2 of 0.98 and a B_{max} of 100.

Figure 3.

(a) The graph shows tryptophan fluorescence of 5.0 μM TRTK12 containing W7 with a maximum fluorescence at 350 nm. (b) Shown is the effect on tryptophan fluorescence of 5.0 μM TRTK12 observed upon the addition of S100B from 100 nM to 2.5 μM in the presence to CaCl₂. (c) Shown is the effect on tryptophan fluorescence of 5.0 μM TRTK12 observed upon the addition of S100B from 100 nM to 2.5 μM in the presence to CaCl₂ and EGTA. (d) Shown is Δ Fluorescence % of TRTK12 plotted against free S100B concentration with a K_d of 0.21 ± 0.03 μM, an R² of 0.98 and a B_{max} of 100.

Figure 4.

(a) WT SHSY5Y cells were placed on chamber slides and pretreated with 50 μ M Synb1-ELP-TRTK or Synb1-ELP-GGC for 1 hr at 37°C. Next, cells were washed then treated with 1 μ M 488-S100B for 3 hrs followed by washing, fixation and DAPI staining. Shown is DAPI nuclear stain in blue and 488-S100B in green. Scale bar: 10 μ m. (b) SHSY5Y cells were placed on 6-well plates and pretreated with 50 μ M Synb1-ELP-TRTK12 or Synb1-ELP-GGC for 1 hr at 37°C then treated with 1 μ M 488-S100B for 3 hrs, then cells were removed and 488-S100B uptake was measured using flow cytometry. Data is shown as MEAN $\pm$ STDEV of the % Uptake relative to the control. Statistics were taken using student's t-test. *P<0.05.

Figure 5. GFP-ATXN1[82Q] SHSY5Y stable cells were placed on 2-well slides and subjected to treatment with 25 nM TPA for 3 days to produce differentiated SHSY5Y cells. Next, the slides were fixed and probed with (a & b) GFP, (a) Synaptophysin and (b) RAGE antibodies and fluorescent secondary antibodies Alexa 488 and Alexa 546 followed by DAPI staining. Scale Bars: 10 μm. Differentiated SHSY5Y cells were treated with 3.0 μM S100B for 48hrs and neurite changes were visualized under the microscope. (c) Data is shown as MEAN ± STDEV of the percentage of cells with neurites, n=100. (d) Data is shown as MEAN ± S E of neurite length, n=100. Statistics were taken using student's t-test. *P<0.05.

Figure 6.

(a)GFP-ATXN1[82Q] and (b)GFP-ATXN1[30Q] SHSY5Y stable cells were placed on 12-well plates and subjected to treatment with 25 nM TPA for 3 days to produce differentiated SHSY5Y cell lines. Next, the cells were pretreated with 50 μ M Synb1-ELP-TRTK for 1 hr then treated with 5 μ M S100B for 2hrs or treated with 150 μ M H₂O₂ for 2hrs, where control cells were treated with serum free media for 3hrs. Then the cells were lysed and analyzed with the Oxyblot kit and a β -tubulin antibody. Shown is the resulting oxyblot/western blot data. (c) Optical densities of the protein bands were taken using ImageJ software. Graphs shown the ratio of oxidized protein/ β -tubulin protein normalized to the control. Statistics were taken using the student's t-test. * GFP-ATXN1[82Q]: S100B vs Synb1-ELP-TRTK +S100B, $P < 0.05$. ψ GFP-ATXN1[82Q]: H₂O₂ vs GFP-ATXN1[30Q]: H₂O₂, $P < 0.05$.

Figure 7.

(a) The graph shows the temperature of the cerebellum during Cooling Cycles in blue and Heating Cycles in red. Shown as the black dotted line, is the body temperature taken with a rectal thermometer over the course of the 90 minute experiment. The Frontal Lobe temperature taken over the course of the 90 minute experiment is shown by the gray line. (b) Shown is the scanned IVIS image displaying a photograph of the brains and the fluorescent uptake of fluorescent labeled Synb1-ELP-GGC taken from a control animal, an unheated animal and an animal where the cerebellum was heated by thermal cycling. Scale bar: 4.0 mm. (c) The graph shows the Synb1-ELP-GGC Average Radiant Efficiency of the cerebellum $\pm$ SE taken from a control animal, an unheated animal and an animal where the cerebellum was heated by thermal cycling. Statistics were taken using the student's t-test. *No Heat vs Heat, $P < 0.05$, $n = 3$. (d) The graph shows the Synb1-ELP-GGC Average Radiant Efficiency of the brain, heart, spleen, (e) lungs, kidney and liver $\pm$ SE taken from a control animal, an unheated animal and an animal where the cerebellum was heated by thermal cycling, $n = 3$.

Figure 8.

The diagram depicts the S100B signaling pathway in SCA1. S100B activates the RAGE receptor producing ROS. Under normal conditions SOD inhibits high levels of ROS. However, due to expression of the mutant ataxin-1 protein, SOD function is reduced allowing ROS to accumulate, causing neurodegeneration in SCA1. This S100B induced oxidative damage pathway can be inhibited by TRTK12, Synb1-ELP-TRTK or like peptides.