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A dose–response study of thymosin β4 for the treatment of acute stroke

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ABSTRACT

Background: Thymosin β4 (Tβ4) is a 5 K actin binding peptide. Tβ4 improves neurological outcome in a rat model of embolic stroke and research is now focused on optimizing its dose for clinical trials. The purpose of this study was to perform a dose–response study of Tβ4 to determine the optimal dose of neurological improvement in a rat model of embolic stroke.

Methods: Male Wistar rats were subjected to embolic middle cerebral artery occlusion (MCAo). Rats were divided into 4 groups of 10 animals/group: control, 2, 12 and 18 mg/kg. Tβ4 was administered intraperitoneally 24 h after MCAo and then every 3 days for 4 additional doses in a randomized controlled fashion. Neurological tests were performed after MCAo and before treatment and up to 8 weeks after treatment. The rats were sacrificed 56 days after MCAo and lesion volumes measured. Generalized estimating equation was used to compare the treatment effect on long term functional recovery at day 56. A quartic regression model was used for an optimal dose determination.

Results: Tβ4 significantly improved neurological outcome at dose of 2 and 12 mg/kg at day 14 and extended to day 56 (p-values <0.05). The higher dose of 18 mg/kg did not show significant improvement. The estimated optimal dose of 3.75 mg/kg would provide optimal neurological improvement.

Conclusions: This study shown that Tβ4 significantly improved the long term neurological functional recovery at day 56 after MCAo with an optimal dose of 3.75 mg/kg. These results provide preclinical data for human clinical trials.

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1. Introduction

Thymosin β4 (Tβ4) is a developmentally expressed 43-amino acid peptide that sequesters G-actin monomers. Tβ4 has multiple additional biological functions, including inhibiting inflammation and promoting angiogenesis as demonstrated in both dermal and cardiac injury models [1,13,20]. Administration of Tβ4 improves functional neurological outcome in a rat model of embolic stroke, a mouse model of multiple sclerosis (EAE, experimental autoimmune encephalomyelitis) and a rat model of traumatic brain injury [15,22,23]. A common observation in these neurological disease models is that Tβ4 promotes axonal repair and myelination by promoting differentiation of oligodendrocyte progenitor cells (OPCs) in the subventricular zone (SVZ) and in the intact white matter providing evidence to support the hypothesis that Tβ4 is a neurorestorative agent. Neurorestorative agents act on existing intact parenchymal cells to promote axonal remodeling and neurite outgrowth in the injured brain which bolsters improvement in neurological functional outcome [27]. Administration of neurorestorative agents involves treatment at subacute (>24 h) time points resulting in greater availability for clinical treatment.

Tβ4 improves neurological outcome in a rat model of embolic stroke when administered 24 h after middle cerebral artery occlusion (MCAo) at a dose of 6 mg/kg [15]. Improvement in functional outcome was observed as early as 14 days after stroke and extended to the completion of the study period of 56 days. In this study, a significant increase of mature oligodendrocytes (OL) at the ischemic boundary as

Abbreviations: Tβ4, thymosin beta 4; EAE, experimental autoimmune encephalomyelitis; OPCs, oligodendrocyte progenitor cells; SVZ, subventricular zone; MCAo, middle cerebral artery occlusion; OL, mature oligodendrocytes; rt-PA, recombinant tissue plasminogen activator; CCA, common carotid artery; ECA, external carotid artery; ICA, internal carotid artery; IP, intraperitoneally; BrdU, 5-bromo-2′-deoxyuridine; ART, adhesive-removal test; mNSS, modified Neurological Severity Score; FFT, foot fault test; MCID, microcomputer imaging device; NG-2, chondroitin sulfate proteoglycan; APC (CC1), adenomatous polyposis coli; MBP, myelin basic protein; GEEs, generalized estimation equations; NOEL, no observed effect level.

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well as an observed increase of OPCs in the SVZ, striatum and corpus callosum was detected suggesting that an underlying mechanism of axonal remodeling and oligodendrogenesis contributes to neurological improvement. OLs are highly vulnerable to focal cerebral ischemia and mature OLs are unable to migrate or divide [8,17]. New OLs, however, can be generated by OPCs that are present in the SVZ and white matter of adult rodent brain [9,16,24]. Therefore, these observations support the hypothesis that Tβ4 acts as a neurorestorative agent.

Further testing and exploration of Tβ4 after stroke are warranted. Toxicology studies in animal and human models demonstrate that Tβ4 is a safe drug. Since Tβ4 showed neurological improvement after stroke at a dose of 6 mg/kg, testing of the drug at different doses needs to be performed to determine the optimal dose of Tβ4, as well as the ceiling effect of the drug. Determining these important parameters is vital for translation to human clinical trials. In this study, we hypothesized that Tβ4 would dose dependently improve neurological outcome in a rat model of embolic stroke and that an optimal dose of Tβ4 could be derived for use in clinical trials.

2. Material and methods

All experimental procedures were approved by the Institutional Animals Care and Use Committee of Henry Ford Hospital.

2.1. Embolic stroke rat model

The middle cerebral artery (MCA) of male Wistar rats (320 to 380 g, $n = 50$) was occluded by placement of an embolus at the origin of the MCA, as previously described [26]. Briefly, under an operating microscope (Carl Zeiss, Inc., Thornwood, NY, USA), the right common carotid artery (CCA), the right external carotid artery (ECA) and the internal carotid artery (ICA) were isolated via a 3 cm ventral neck midline incision. A 6-0 silk suture was loosely tied at the origin of the ECA and ligated at the distal end of the ECA. The right CCA and ICA were temporarily clamped using a curved microvascular clip (Codman & Shurtleff, Inc., Randolph, MA, USA). A modified PE-50 catheter filled with a fibrin rich clot from a donor rat, was attached to a 100-μl Hamilton syringe, and introduced into the ECA lumen through a small puncture. The suture around the origin of the ECA was tightened around the intraluminal catheter to prevent bleeding, and the microvascular clip was removed. The catheter was then gently advanced from the ECA into the lumen of the ICA to reach the origin of the MCA. The clot along with 5 μl of saline in the catheter is injected into the ICA over 10 s. The catheter was withdrawn from the right ECA 5 min after injection and ligated. Buprenex was administered intraperitoneally (IP) post-surgery at a dose of 0.01 mg/kg if the rat showed signs of distress.

2.2. Experimental design

Rats subjected to MCAo were randomized into one of 4 groups of 10 animals/group: control, 2, 12 and 18 mg/kg. Tβ4 (RegeneRx Biopharmaceuticals Inc.) was administered IP 24 h after MCAo and then every 3 days for 4 additional doses. Randomization was performed by placing labeled tabs (control, 2, 12 or 18 mg/kg) in a large envelope. A research associate then placed his/her hand in the envelope to randomly choose a labeled tab. The selected rat was then assigned this dose. We determined a sample size of ten rats in each group were required for analysis. Since this study was designed to observe functional outcome, we only included those rats which completed the functional tests until time of sacrifice (56 days). These doses were based on previous toxicology studies performed in rodent, dog and human models [5]. Moreover, these doses were chosen because it was likely that this range of dosing will be used in human clinical studies. For labeling proliferating cells, 5-bromo-2′-deoxyuridine (BrdU, 100 mg/kg; Sigma, St. Louis, MO) was injected IP into rats 24 h after MCAo and then daily for 7 days. Rats were sacrificed 56 days after MCAo to investigate the long term effect of Tβ4 on brain remodeling and functional outcome.

2.3. Neurological functional tests

In the embolic stroke rat model, a battery of behavioral tests was performed at 1, 7, 14, 21, 28, 35, 42, 49 and 56 days after MCAo by an investigator who was blinded to the experimental groups. The battery of tests consisted of the adhesive-removal test (ART), the modified Neurological Severity Score (mNSS) and foot fault test (FFT) [3,4,25]. Briefly, the ART involves placing two small pieces of adhesive-backed paper dots on the wrist of each forelimb (of equal size, 113.1 mm^2) to act as bilateral tactile stimuli occupying the distal-radial region. The time required for the rat to remove both stimuli from each limb was recorded in 5 trials per day. Each animal received 5 trials on all testing days after MCA occlusion and the mean time required to remove both stimuli from limbs was recorded. In order to increase the sensitivity of the test, the adhesive-backed paper dots were reduced in size by one-half at day 35. The mNSS test is a composite score in which motor, sensory, balance and reflex measures are used to calculate a value ranging from 1 to 18, with the higher score implying greater neurological injury. Points are awarded for the inability to perform the tasks or for the lack of a tested reflex. (normal score 1, maximal deficit score 18). Rats with a mNSS less than 8 were excluded from the study. The FFT measures the ability of the rat to walk along an elevated hexagonal wire grid and the number of times the paw slips (foot fault) between the wires. The total number of foot faults is divided by the total number of steps the rat uses to cross the grid. The average number of foot faults of a normal rat is 2–3%. As anticipated, rats died due to MCAo procedure (at day 1 or 2 before and after randomization) and were excluded from the analysis.

2.4. Histological and immunohistochemical assessment

Rat brains were fixed by transcardial perfusion with saline, followed by perfusion and immersion in 4% paraformaldehyde, and the brains were embedded in paraffin. The cerebral tissues were cut into seven equally spaced (2 mm) coronal blocks. A series of adjacent 6 μm-thick sections was cut from each block in the coronal plane and was stained with hematoxylin and eosin. Seven brain sections were traced using a microcomputer imaging device (MCID) image analysis system (Imaging Research, St. Catharines, Canada) by laboratory associates who were blinded to the dose and functional testing results. The indirect lesion area, in which the intact area of the ipsilateral hemisphere was subtracted from the area of the contralateral hemisphere, was calculated. Lesion volume is represented as a volume percentage of the lesion compared with the contralateral hemisphere.

Standard paraffin blocks were obtained from the center of the lesion, corresponding to coronal coordinates for bregma -1-1 mm. A series of 6 μm thick sections at various levels (100 μm interval) were cut. Immunostaining was performed on these sections. Antibodies used for identification of OPCs and OLs were NG-2 (chondroitin sulfate proteoglycan) (1:800, polyclonal rabbit, incubated overnight at 4 °C, Chemicon, CA) and APC (CC1) (adenomatous polyposis coli) (1:20, monoclonal mouse, incubated 60 min at room temperature, Genway, CA), respectively. To identify proliferating OPCs and OLs, double immunostaining of NG2/BrdU and APC/BrdU was performed, respectively. Double immunolabeling was visualized by secondary antibodies conjugated to FITC and Cy3 (Jackson Immuno.). Myelin basic protein (MBP) antibody (1:1000, monoclonal mouse, incubated 60 minutes at room temperature, Covance, CA) was used to identify myelinated axons. Following sequential incubation with biotin-conjugated anti mouse or goat IgG (dilution 1:200, Vector laboratories, INC), the sections were treated with an ABC kit (Vector laboratories, INC). Diaminobenzidine was then used as a sensitive chromogen for light microscopy. Isotype matched non-immune IgG controls were performed for each antibody.

2.5. Image acquisition and quantification

Coronal sections were digitized using a 40× objective using a three-CCD color video camera (Sony DXC-970MD) interfaced with a MCID system. Laboratory associates performing the quantitative analysis were blinded to the dose and functional testing results. For quantitative analysis of proliferating OPCs, the numbers of double labeled NG2 and BrdU immunoreactive cells were counted throughout the ipsilateral SVZ and the peri-infarct area at the corpus callosum and striatum. The number of positive cells for three coronal sections per rat was averaged to obtain a mean number of cells. A similar analysis of proliferating mature OL was performed using double labeling of APC and BrdU cells in the peri-infarct area at the corpus callosum and striatum. Data are presented as the density of immunoreactive cells relative to the area of the peri-infarct corpus callosum and striatum.

For quantification of myelinated axons, MBP immunoreactive area within the peri-infarct corpus callosum and striatum and the contralateral homologous area were digitized. Three coronal sections (100 μm interval) from the center of the ischemic core (bregma 0.2 mm) per animal with eight fields of view in each hemisphere were analyzed. Data are presented as the percentage of increase in density at the peri-infarct corpus callosum and striatum compared with the contralateral homologous region on the same section.

2.6. Data analysis and statistical procedures

Data transformations were considered if data were not normal. As a result, the ranked ART and mNSS were used for the comparison because the functional data were not normally distributed. As the data illustration, mean ± SE was presented at each time point. To mimic analysis employed in clinical stroke recovery studies [12], a global test using generalized estimation equations (GEEs), PROC GENMOD in SAS [19], was performed to evaluate the effect of Tβ4 on functional recovery measured from three neurological behavior tests at day 56, as the primary outcome compared to controls. The GEE approach was also used to study the Tβ4 effect on the functional recovery at the other time points (secondary endpoints), as well as the histological evaluation (remyelination, the number of proliferating OPCs, and mature OLs) and lesion volume measure at day 56, as exploratory endpoints. A quartic regression model, PROC RSREG in SAS, was used to explore the optimal dose of Tβ4 which optimizes functional outcome [2].

3. Theory

Our initial single dose study demonstrating that Tβ4 improved functional outcome after stroke suggested that Tβ4 warranted further preclinical testing. Currently, treatment of stroke is limited to a time-constrained 4.5 hour window of administration of rt-PA from symptom onset which severely limits the number of patients that can be treated. It may be more efficacious, however, to treat stroke patients in the sub-acute phase (>24 h) of stroke rather the acute phase. Projections on the percentage of patients who present within the treatment window of rt-PA treatment option are low even with optimal public education [14]. As the population ages, the number of stroke victims will increase dramatically necessitating a comprehensive treatment strategy. Treatment in the sub-acute phase will allow more patients to be treated as well as provide physicians time to gather pertinent historical and physical information that is often not available in the acute phase of stroke.

4. Results

Tβ4 was administered at three doses (2, 12, 18 mg/kg) 24 h after MCA occlusion and then every 3 days for four additional doses. A total of fifty rats were randomized into the study; however, ten rats died one to two days after MCAo. Of them, 4 died in the control group, 4 rats died in the 2 mg/kg, 1 rat died in the 12 mg/kg group, and 1 rat died in the 18 mg/kg group. The remaining rats survived until sacrifice.

4.1. Neurological functional outcome

To determine if Tβ4 dose-dependently improves functional outcome after stroke, we performed behavioral tests prior to and one day after MCAo and then weekly for 56 days at dosages of 2, 12 and 18 mg/kg. Figs. 1–3 demonstrate the time course of improvement on the ART, mNSS and FFT at each specific dose and the dose effect compared to the controls (p-value) using global test is listed in Table 1. At day 56, compared to the control group, the 2 and 12 mg/kg groups both showed an improvement ($p < 0.05$) while the higher dose, 18 mg/kg, showed no improvement ($p > 0.05$). Improvement was observed at day 14 and continued until the rats were sacrificed. The 2 mg/kg dose demonstrated a 24.2, 32.1, and 29.6% relative global improvement in the ART, mNSS and FFT, respectively, while the 12 mg/kg showed a similar improvement of 33.0, 26.0, and 35.5% when compared to controls ($p < 0.05$). The sample distribution of lesion volumes for the control, 2, 12 and 18 mg/kg groups was 33 ± 8.7%, 26 ± 10.1%, 24 ± 9.9% and 33% ± 12.6%, respectively. There were no significant differences in lesion volume between the control and each of 2, 12 and 18 mg/kg groups ($p = 0.278, 0.085, 0.957$) (Fig. 4). Based on the results of the neurological tests at each individual dose, the quartic response surface model was used to calculate the optimal dose of Tβ4 which optimizes functional outcome. Statistical curve fitting determined that a dose of 3.75 mg/kg showed optimal neurological improvement.

4.2. Tβ4 promotes myelination after stroke

To determine if Tβ4 treatment promotes myelination after stroke, brain sections were stained using MBP. The 12 mg/kg dose was chosen as this dose provided the greatest significant cumulative improvement in the neurological tests. Fig. 5 demonstrates significant increases in MBP immunoreactivity in the striatal ischemic boundary in the Tβ4 treatment (12 mg/kg) group (92.95 ± 3.12%) when compared to the control group (69.62 ± 3.75%) ($p < 0.05$) (mean ± S.E.). No differences were observed in the corpus callosum.

4.3. Tβ4 increases proliferating oligodendrocyte progenitor cells and mature oligodendrocytes

To determine if Tβ4 treatment increases the number of proliferating OPCs and OLs, NG-2, a marker of OPCs and APC, a marker of OL were

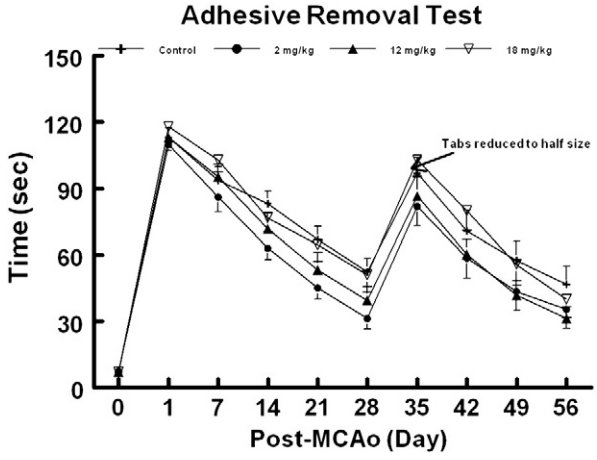


Fig. 1. The adhesive removal test (ART) of embolic stroke rats treated with control 2, 12 and 18 mg/kg ($n = 40$, 10 per group). For each functional outcome, generalized estimation equations were used to study the Tβ4 effect at each time point indicated (mean ± SE). Adhesive-backed paper dots were reduced in size by one-half at day 35 (arrow).

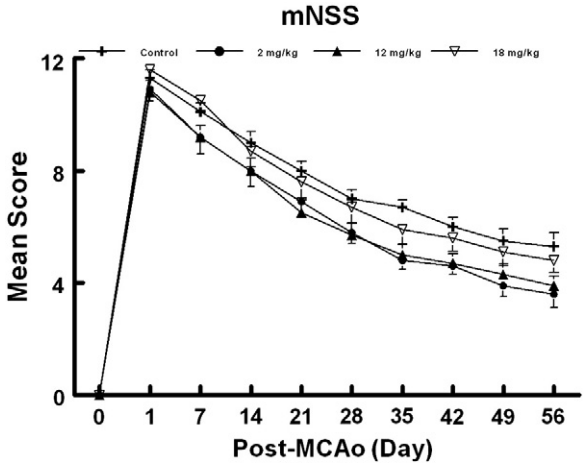


Fig. 2. The modified Neurological Severity Score (mNSS) of embolic stroke rats treated with control 2, 12 and 18 mg/kg ($n = 40$, 10 per group). For each functional outcome, generalized estimation equations were used to study the Tβ4 effect at each time point indicated (mean ± SE).

measured in BrdU positive cells, respectively. Figs. 6 and 7 demonstrate NG2 + BrdU (OPC) and APC + BrdU (OL) positive cells, respectively. Fig. 6 demonstrates a significant increase in OPC density (cells/mm^2) in the SVZ (0.31 ± 1.49 vs 0.03 ± 0.43), striatal ischemic boundary (4.44 ± 0.81 vs 3.38 ± 0.69) and corpus callosum (3.53 ± 0.81 vs 1.14 ± 0.41) when compared to the control ($p < 0.05$) (mean ± S.E.). Fig. 7 demonstrates a significant increase in OL density in the corpus callosum (31.33 ± 6.24) when compared to the control (9.34 ± 1.75) group ($p < 0.05$) (mean ± S.E.). No differences were observed in the striatal ischemic boundary.

5. Discussion

This study demonstrates a dose–response relationship of Tβ4 and improvement of neurological function in a widely employed rat model of embolic stroke. Our results show that both the 2 mg/kg and 12 mg/kg treatment groups showed improvement in neurological functional outcome, with a calculated optimal dose of 3.75 mg/kg using the quartic surface response model. Moreover, we demonstrate a ceiling effect of Tβ4 as the 18 mg/kg group showed no improvement in functional outcome. In addition, we also show that Tβ4 treatment increased proliferating OPCs and mature OLs in agreement with previous animal models of multiple sclerosis and traumatic brain injury [22,23].

The results of this study are in good agreement with our previous study demonstrating efficacy at 6 mg/kg [15]; as in the present study, we found a significant overall improvement in the ART and the mNSS when compared to controls. The percentages of improvement of both the ART and mNSS at 6 mg/kg were in good agreement with the results of this manuscript. The improvement of neurological outcome shown in these two separately performed studies using different dosages further supports the hypothesis that Tβ4 improves neurological recovery after stroke. This present study provides a calculated optimal dose that may be translated into clinical trials.

The doses chosen for this study, 2, 12 and 18 mg/kg were based on previous toxicology studies performed in rodent, dog and human models [5]. Sprague–Dawley rats were administered Tβ4 at doses of 0.6, 6.0 and 60 mg/kg to assess the potential toxicity associated with the IV administration of Tβ4 once daily for 28 days as well as to determine the reversibility of any adverse findings. These doses were selected because it was likely that this range of dosing would be used in human clinical studies. The no observed effect level (NOEL) of Tβ4, based on in-life observations/data, clinical pathology, macroscopic organ weights and microscopic findings was equal to or greater than 60 mg/kg in male and female animals. Two toxicology studies were subsequently performed with IV administration in a nonrodent species, beagle dogs. The dose range was 0.6, 6, 9, 12, 18, 25 and 60 mg/kg administered daily for 28 days. Although there were no macroscopic, microscopic, or clinical laboratory findings, the dogs in the higher dose groups (25 and 60 mg/kg) appeared to salivate post dose for a short period of time, whereupon the salivation ceased. Although this

Table 1
Summary of global test *p*-values calculated.

GLOBAL TEST *p*-value				
Days after MCAo	Control vs. 2 mg/kg	Control vs. 12 mg/kg	Control vs. 18 mg/kg	Group
1	0.387	0.385	0.316	
7	0.235	0.335	0.264	
14	0.016	0.045	0.713	*
21	0.001	0.015	0.595	*
28	0.006	0.013	0.749	*
35	0.002	0.015	0.830	*
42	0.022	0.031	0.900	*
49	0.009	0.041	0.686	*
56[a]	0.029	0.045	0.475	

[a] Primary endpoint.
* Significant: $p < 0.05$.

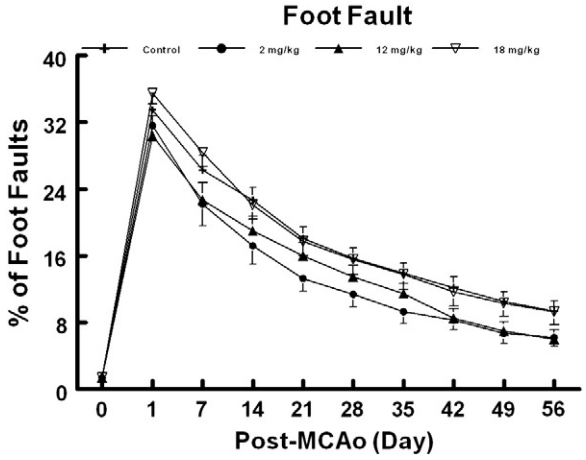


Fig. 3. The foot fault test of embolic stroke rats treated with control 2, 12, 18 mg/kg ($n = 40$, 10 per group). For each functional outcome, generalized estimation equations were used to study the Tβ4 effect at each time point indicated (mean ± SE).

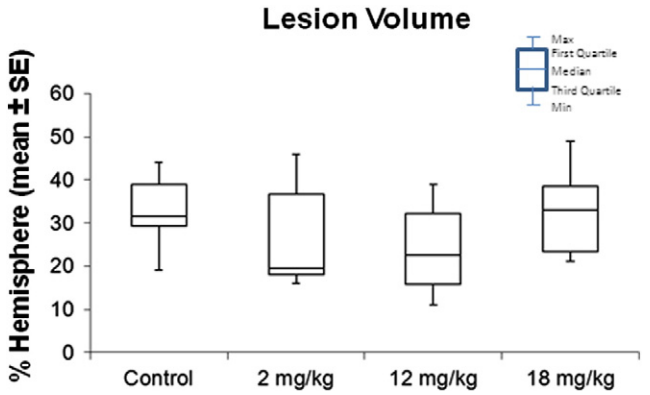


Fig. 4. Lesion volumes of embolic stroke rats treated with control 2, 12 and 18 mg/kg ($n = 40$, 10 per group). There were no significant differences in lesion volumes among the control, 2, 12 and 18 mg/kg groups ($p = 0.278$, 0.085, 0.957).

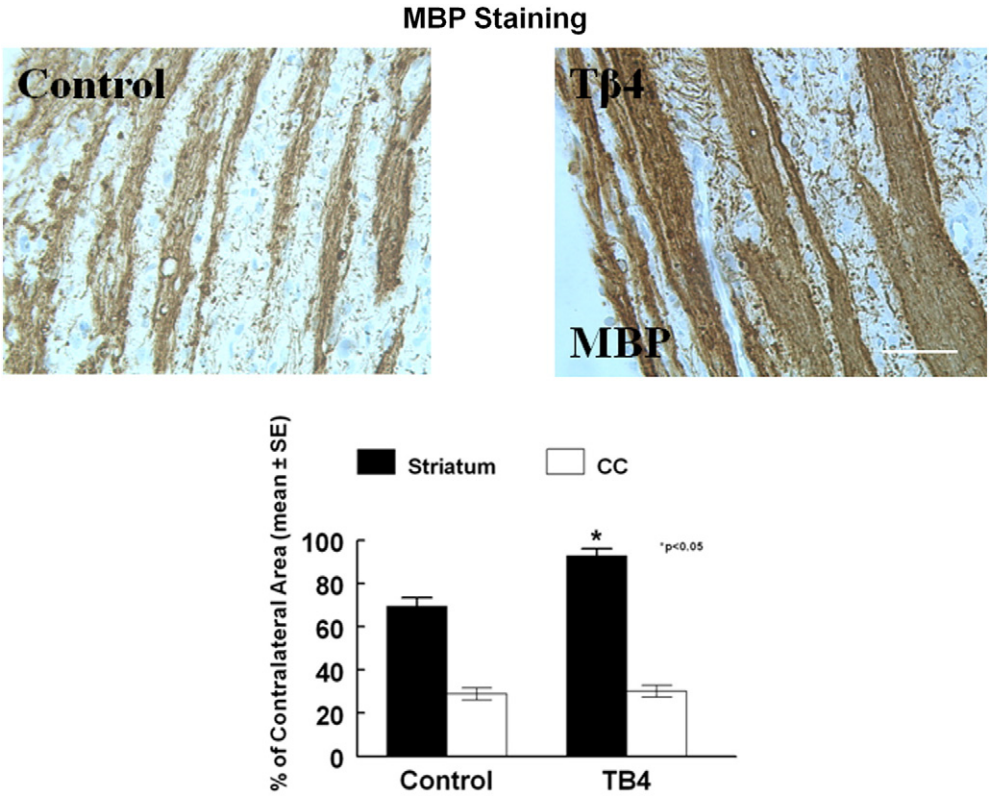


Fig. 5. Photograph shows MBP staining in the white matter bundles of the ipsilateral striatal ischemic boundary of control ($n = 10$) and Tβ4 (12 mg/kg) ($n = 10$) treated rats. There is an increased density in the Tβ4 treated rats compared to the demyelination of the control. Generalized estimation equations were used to analyze the Tβ4 effect. Quantitative data show significantly increased staining in the striatum of Tβ4 treated rats compared to the control ($p < 0.05$) (mean ± SE). Bar = 50 μm.

observation could not be attributed to the drug acting directly on the salivary glands or the brain or whether it was a conditioned response in some of the higher dose group animals, the NOEL was determined to be 18 mg/kg. Although the rat studies showed no effect at the 60 mg/kg dose regimen, given the findings in dogs, the 18 mg/kg dose was the most likely highest dose that would be administered to

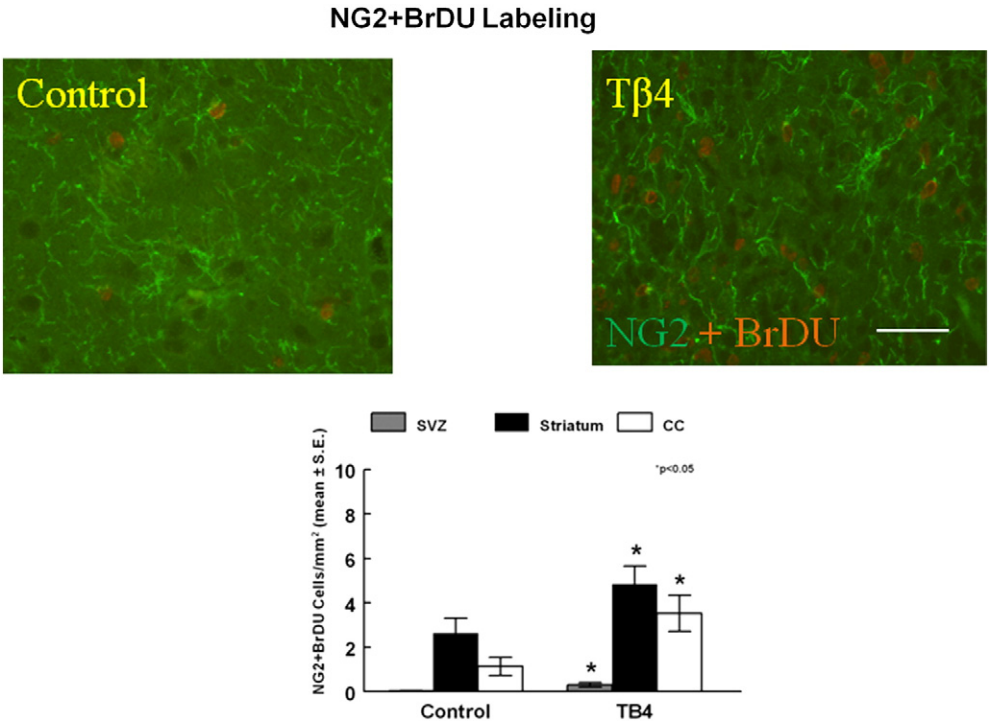


Fig. 6. Photograph shows NG-2 + BrdU double-labeled cells in the ipsilateral striatum. NG-2 + BrdU double-labeled cells is increased in the ipsilateral SVZ, striatum and corpus callosum of Tβ4 (12 mg/kg) ($n = 10$) treated rats when compared to control ($n = 10$). Generalized estimation equations were used to analyze the Tβ4 effect. Quantitative data show significantly increased double-labeled cells in these areas in the Tβ4 treated rats compared to the control ($p < 0.05$) (mean ± SE). Bar = 50 μm.

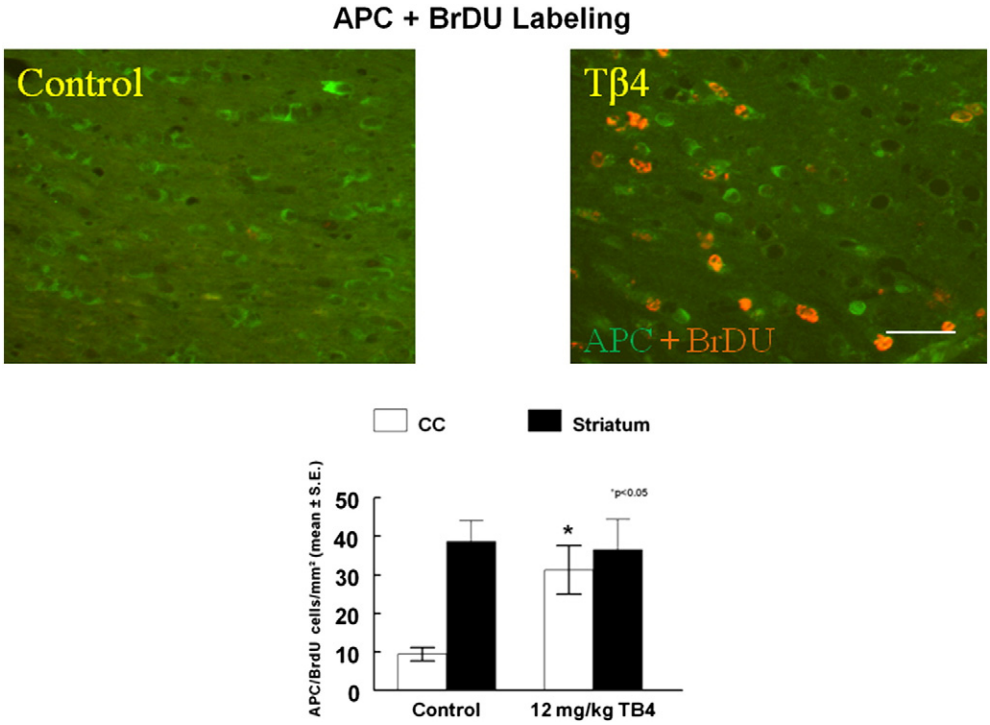


Fig. 7. Photograph shows APC + BrdU double labeled cells in the corpus callosum. APC + BrdU double-labeled cells are increased in the ipsilateral corpus callosum of Tβ4 (12 mg/kg) ($n = 10$) treated rats when compared to saline control ($n = 10$). Generalized estimation equations were used to analyze the Tβ4 effect. Quantitative data show significantly increased double-labeled cells in the corpus callosum of Tβ4 treated rats compared to the control ($p < 0.05$) (mean ± SE). Bar = 50 μm.

humans. The lower doses of 2 and 12 mg/kg were chosen based on the results of our previous study which showed functional improvement at 6 mg/kg IP.

Following these animal studies, Tβ4 was tested in a randomized, double-blind, placebo-controlled, dose–response phase 1A and 1B study of the safety and tolerability of the intravenous administration of Tβ4 and its pharmacokinetics after single doses in healthy volunteers [18]. Forty healthy volunteers were administered IV Tβ4 in single doses of 42, 140, 420 and 1260 mg (18 mg/kg for average 70 kg adult) and in the phase 1B trial were given the same doses but daily for 14 days. All subjects were confined in a phase 1 pharmacology unit for the entire dosing period. There were no dose limiting or serious adverse events through the dosing period. Multiple ascending dose safety data revealed the most frequent adverse events to be headache and upper respiratory infection. Based on the safety demonstrated in this phase 1 study in healthy volunteers, our results demonstrating efficacy of Tβ4 24 h after onset of experimental embolic stroke provide preclinical data for randomized clinical trials.

Stroke is a major cause of morbidity and disability, and functional recovery is slow and uncertain [6]. The only FDA approved treatment for stroke is administration of rt-PA within 4.5 h of symptom onset [10,21]. Use of rt-PA has been limited because of its narrow time-dependent treatment window as most stroke patients present to the Emergency Department well beyond 6 h of symptom onset. The restriction on time and potential adverse effects have confined the use of rt-PA to approximately 3% of stroke patients [11]. Our results that show efficacious Tβ4 treatment of stroke 24 h after symptom onset has profound clinical implications. Rather than treating a small minority of patients Tβ4 may be employed to treat most stroke patients.

Our histology data show that Tβ4 promotes oligodendrogenesis. The observations that Tβ4 increases proliferating OPCs in the striatal ischemic boundary as well as the SVZ and corpus callosum support the hypothesis that Tβ4 is a neurorestorative agent which acts on intact parenchymal cells and on progenitor cells to promote neurogenesis, oligodendrogenesis, synaptogenesis and angiogenesis. In addition, the observation that the increased proliferating OPCs are observed to 7 weeks after administration of the drug is suggestive of its potential robust neurorestorative effects.

Tβ4's stimulation of OPCs in the SVZ and intact white matter may contribute to the observed recovery. Remyelination occurs only from OPCs and not from surviving OLs or from mature surviving OLs adjacent to the injured axons [7–9,16]. Mature OLs are for the most part, unable to migrate or divide and therefore, new OLs must originate from OPCs. Mature, mitotically active OLs were observed in the corpus callosum, suggesting that OPCs were actively differentiating to mature OL. Moreover, significant myelination was observed in the vulnerable striatal ischemic boundary area. OLs are highly vulnerable to focal cerebral ischemia and our data suggest that Tβ4 increases differentiation of OPCs to mature myelin-secreting OLs [17].

Although our results suggest that Tβ4 acts as a neurorestorative agent, we cannot rule out other molecular mechanisms of recovery such as, neuroprotection. The lesion volumes for the 2 and 12 mg/kg groups showed a trend toward a reduction in lesion volume. Tβ4 has been shown to upregulate the survival kinase Akt in postnatal cardiomyocytes [1] and investigating this potential mechanism may be of value; however, because Tβ4 was administered 24 h after MCAo, a neuroprotective mechanism seems unlikely.

In summary, our data demonstrate that Tβ4 improves functional outcome in the rat stroke embolic model when administered 24 h after MCAo at 2 and 12 mg/kg but not at a dose of 18 mg/kg. The optimal dose calculated was 3.75 mg/kg. Tβ4 increases proliferating OPCs in the SVZ and in the white matter of the corpus callosum, as well as promotes myelination in the ischemic vulnerable striatal boundary. These preclinical results provide practical and robust data for initiation of human clinical trials testing Tβ4 in the treatment of stroke.

Conflict of interests statement

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of

Health. HFHS has a Material Transfer Agreement with RegeneRx Biopharmaceuticals Inc, Rockville, MD. A US Provisional Patent 61/163,556 has been filed for use of Tβ4 in neurological injury.

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