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T3D-959: A Multi-Faceted Disease Remedial Drug Candidate for the Treatment of Alzheimer's Disease

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Abstract

Background—T3D-959, a dual PPAR- δ /PPAR γ nuclear receptor agonist and former diabetes drug candidate, has been repositioned as an Alzheimer's disease (AD)-modifying therapy.

Objective—This study examines the effectiveness and mechanisms of T3D-959's therapeutic effects using *in vivo* and *ex vivo* rat models of sporadic AD.

Methods—A sporadic AD model was generated by intracerebral (i.c.) administration of streptozotocin (STZ). Control and i.c. STZ treated rats were gavaged with saline or T3D-959 (0.3 to 3.0 mg/kg/day) for 28 days. Spatial learning and memory were evaluated using the Morris water maze test. Frontal lobe slice cultures generated 24 hours after i.c. STZ or vehicle were used to study early effects of T3D-959 (0.5–1.0 μ M) on viability and molecular markers of AD.

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Results—T3D-959 significantly improved spatial learning and memory in i.c STZ-treated rats. Mechanistically, T3D-959 significantly improved culture viability and brain morphology, reduced levels of oxidative stress and A β , and normalized expression of phosphotau, choline acetyltransferase, and myelin-associated glycoprotein. Protective effects occurred even at the lowest tested dose of T3D-959.

Conclusions—Pre-clinical proof of concept has been demonstrated that T3D-959 can improve multiple pathologies of AD resulting in significant improvements in cognitive function and molecular and biochemical indices of neurodegeneration. These results support the theses that (1) effective disease modification in AD can be achieved by targeting relevant nuclear receptors, and (2) treating AD as a metabolic disease has the potential to be disease remedial. A Phase 2a trial of T3D-959 in mild-to-moderate AD patients has been initiated ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT02560753) identifier NCT02560753).

Keywords

Alzheimer's disease; amyloid; PPAR agonist; slice culture; spatial learning and memory; Streptozotocin; T3D-959; Type 3 diabetes

INTRODUCTION

There is a critical need for effective disease remedial therapy for the treatment of Alzheimer's disease (AD). AD treatment strategies remain dominated by therapies that target symptoms, e.g., acetylcholinesterase inhibitors like Aricept[®], or modify the actions of excitatory neurotransmission, e.g., the NMDA receptor antagonists, Namenda[®]. Acetylcholinesterase inhibitors and NMDA receptor antagonists each address a single neurotransmitter deficit and have only modest short-term benefits on cognition and function. Furthermore, these treatments do not slow, halt or reverse the course of disease.

Over the course of several decades of research, evidence has emerged that AD is quite complex and associated with a multitude of cellular, biochemical and molecular abnormalities, including loss of neurons, deposition of amyloid- β , accumulation of phosphotau-containing neuronal cytoskeletal lesions such as neurofibrillary tangles and dystrophic neurites, activation of cell death cascades, deficits in energy metabolism, mitochondrial dysfunction, increased inflammation, DNA damage, and oxidative stress. Mechanistically, what can explain all of these phenomena? Since 2005 a large body of evidence is pointing to insulin resistance as the 'trigger' for the above abnormalities that cause the neurodegeneration leading to the progressive and severe cognitive and functional impairments in AD. In fact, AD is now being called a form of diabetes that selectively or predominantly affects the brain, so-called 'Type-3 Diabetes' [1,2]. The earliest abnormalities that occur at, or before, the onset of cognitive impairment are those involving sugar (glucose) utilization and energy metabolism [3–5].

Unlike other parts of the body the brain is totally dependent on glucose as an energy source. Reduction of brain glucose metabolism is a hallmark of the disease and is the best predictor of cognitive decline; superior to amyloid plaque or tau bundle load. Clinical symptoms of AD almost never occur without decreases in glucose metabolism [6–9]. Insulin is the key

hormone responsible for the efficient utilization of glucose by brain cells. Insulin signaling controls major biological responses including: cell growth, survival, plasticity, energy production, acetylcholine production, inhibition of oxidative stress, and inhibition of programmed cell death (apoptosis). Reduction of brain glucose metabolism results from neurons becoming resistant to the actions of insulin (insulin resistance). Without the ability to use insulin to access glucose as an energy source neurons die; the result is cognitive and functional decline as occur in AD. Insulin resistance can account for the majority of molecular, biochemical, and histopathological lesions in AD and mediate cognitive impairment [2, 10–18]. The brain requires integral insulin signaling for metabolic homeostasis and neuronal plasticity. Insulin resistance disrupts energy balance and signaling networks needed for a broad range of functions. Impaired insulin signaling in neurons enhances apoptosis [19,20], promotes oxidative cell death induced by A β _{1–42} [21], increases secretion of A β _{1–42} [22], blocks removal of extracellular A β -oligomers [23], and increases plaque loads [24].

A growing body of evidence suggests that brain insulin resistance promotes or possibly triggers key defects in AD [2, 3, 11, 25–34] and is supported by observed changes in levels of insulin signaling molecules in AD forebrains and associated changes in memory [2, 11, 12, 27, 32, 34–36]. In addition to the aforementioned role of brain insulin resistance in AD, “insulin resistance in peripheral tissues (as seen in Type 2 diabetes) could contribute to brain insulin resistance by reducing brain insulin uptake and by raising brain levels of A β , Tau phosphorylation, oxidative stress, pro-inflammatory cytokines, advanced glycation end products, dyslipidemia, and apoptosis” [12,13, 37–41]. AD and Type 2 Diabetes (T2D) have many shared pathophysiological features including insulin resistance, peripheral oxidative stress and inflammation, amyloid aggregation and deposition, neurodegeneration, and decline in cognition [25,42]. An association of elevated blood sugar with memory problems and low hippocampal volume has been reported [43]. Insulin resistance is a key determinant of blood-sugar levels and is the one shared factor in these two diseases that can cause these shared pathophysiologies [26, 37, 44].

A critical barrier to progress in developing disease modifying drug therapies for AD is that current approaches largely target only one defect, in particular A β accumulation. However, the most advanced compounds targeting A β , bapineuzumab, avagacestat, solanezumab, LY2886721, and gammagard have all failed to achieve their primary cognitive and functional endpoints in the late stage clinical testing. Given the spectrum of abnormalities in AD, a more promising approach for disease remediation may require treating multiple defects (pathologies) at once [45].

Peroxisome proliferator-activated receptors (PPARs) are nuclear hormone receptors that function as transcription factors and regulate gene expression [46–49]. PPAR- α is abundantly expressed in liver, adipose tissue, muscle, and kidney. PPAR- β/δ is highly expressed in brain, and PPAR- γ is widely expressed throughout the body. Binding to PPARs causes them to heterodimerize with retinoid x receptors [46–49]. The resulting complex regulates target genes by binding to peroxisome proliferator hormone response elements of DNA promoters [46–49]. PPAR signaling regulates energy metabolism, cell growth, and differentiation, and inhibits inflammation and oxidative stress [50–54]. Therefore, PPARs

are attractive as potential therapeutic targets for AD. Correspondingly, previous studies demonstrated PPAR- δ agonists to have therapeutic utility in experimental models of AD, improving cognition in the STZ [10] and 5XFAD [55] models of AD. The relevance of PPAR- δ as a logical target of drug discovery in AD is further supported by findings of increased cerebral cortex levels of phosphorylated tau, 2-fold induction in BACE1, increased IL-6 and RAGE, and an increased Cdk5 and ERK1/2 in PPAR- δ null mice [56]. PPAR- γ agonists have also been shown to have therapeutic utility in AD, improving cognition in the Tg2576 [57] and 3xTg-AD [58] models. Since PPAR- δ is abundantly expressed in the brain, and both PPAR- δ and PPAR- γ agonists exhibit therapeutic efficacy, dual activation of both receptors could enhance therapeutic coverage by supporting functions differentially subserved by either receptor.

T3D-959 is a small molecule dual nuclear receptor agonist. PPAR- δ is the primary target of T3D-959. T3D-959 is 15-times more potent for PPAR- δ (human ED_{50} = 19 nM) than for the secondary target of the drug, PPAR- γ (human ED_{50} = 297 nM) [59, 60]. PPAR- δ is highly expressed throughout the central nervous system (CNS) and enriched in areas of the brain involved in energy homeostasis, e.g. mediobasal hypothalamus. PPAR- γ is more restricted in its brain expression.

MATERIALS AND METHODS

Intracerebral Streptozotocin (STZ)

The experimental model is depicted in Fig. 1 Intracerebral (i.c.) treatment with STZ was used to cause brain metabolic dysfunction and insulin resistance as occurs in human sporadic AD [3, 16], with minimal or no systemic side-effects. Long Evans 4-week old male and female rats (8–12/group) were anesthetized by intraperitoneal (i.p.) injection of ketamine (100 mg/kg) plus xylazine (10 mg/kg). After shaving and cleaning (betadine x2 followed by 70% ethanol) the scalps, the rats were positioned in a stereotaxic frame and a midline incision was made. With subcutaneous tissue and skeletal muscle separated and retracted with small surgical hooks, the bregma and lambda areas were cleaned with 3% H_2O_2 soaked cotton swabs. A Burr hole was made over the right cerebral hemisphere corresponding to the position of the lateral ventricle (1mm caudal, 2 mm lateral to the bregma) using a hand-held electric drill and a 1 mm dental bit [61]. Drilling was stopped leaving a thin layer of translucent bone, which was punctured with a 27 G needle. After removing the disc removed with fine forceps, a Hamilton syringe with a permanently attached 30-gauge needle was mounted in the micro-manipulator attached to the stereotaxic frame, and used to slowly (50–75 nl/min) deliver STZ (0.9 mg/kg) or saline to the lateral ventricle (1–2 μ l). Sham surgery controls were included in the study. However, since their results were similar to those obtained for the control+vehicle treated group, they are not further discussed. A 2-min waiting period prior to withdrawing the needle prevented brain swelling. The injection site was cleaned with sterile saline using cotton swabs. The incision was closed with resorbable sutures and covered triple antibiotic ointment, and the peri-incision area was injected with 2% Lidocaine for analgesia. Rats were administered 10 ml/kg of subcutaneous sterile saline to avoid post-operative dehydration. Rats were kept warm using a temperature-controlled heated blanket or heating lamps (rectal temperature

monitoring). Close post-operative monitoring ensured that all animals remained in good health and continued to thrive throughout the experiment.

Formulation and administration

T3D-959 was formulated as a solution in 0.9% NaCl at a concentration of 4.44 mg/ml and prepared fresh daily. Rats were treated with 0.3, 0.7, 1.0, or 3.0 mg/kg T3D-959 or normal saline vehicle, once daily from 24 h or 7 days after the i.c. STZ or saline vehicle treatments. Oral gavage was performed using a ball ended feeding needle. The distance that the needle needed to be inserted into the rat from the nose to the first rib was estimated and marked on the needle. Rats were restrained by extension in a straight line to facilitate introduction of the gavage needle. The needle was inserted into the space between the left incisors and molars, and gently directed caudally toward the right ramus of the mandible. The rats were able to swallow as the feeding tube approached the pharynx, facilitating entry into the esophagus. Once the desired position was attained, drug or vehicle was injected (250 µl) and the syringe was withdrawn. Rats were monitored closely for 1 h post-gavage.

Morris water maze (MWM)

Damage done to the hippocampus and temporal lobe by AD is responsible for the deficits in learning and memory. These effects are routinely assessed in experimental animals using the MWM test of spatial learning and memory [16]. In brief, on Trial day 1, rats were oriented to the MWM and taught how to locate and land on the platform in the center of the maze. On the subsequent 3 days, the rats were progressively challenged by introducing them into the MWM at different spatial points and with the platform submerged. The rats were allowed to swim until they located and landed on the platform. However the maximum duration of each trial was 120 s, after which the rats were guided to the platform. The rats were given 3 trials on each day of testing, with 5–10 min rests between trials. The rats were tested over 4 consecutive days. All trials were video-recorded for path length, path complexity, velocity, latency, and errors using EthoVision 8.5 (Noldus, Leesburg VA). However, since the results showed that control and STZ-treated rats swam at the same average speeds and that their longer latencies were associated with more errors and increased path complexity and length, we elected to present data corresponding to latency. Inter-group latencies on each trial day were compared based upon area under the curve calculations for the 3 trials in each rat. The area under the curve is an integrated measurement of an effect. In these studies, the calculated data correspond to cumulative measurements of performance over the 3 trials each day.

At the conclusion of each trial, the rats were dried with a towel and kept warm to maintain comfort. At the end of each day the rats were returned to their cages. Twenty-four hours after MWM studies, the rats were sacrificed by isoflurane inhalation. The temporal lobes with hippocampi were divided for snap freezing and later molecular and biochemical studies, or immersion fixation in 4% neutral buffered formaldehyde, i.e., 10% formalin for histological studies.

Frontal lobe slice cultures

Postnatal day 3 (P3) Long Evans rat pups were used in these experiments because adult brains are not suitable for long-term slice cultures. This entire procedure was performed in a laminar flow tissue culture hood using standard sterile technique. P3 pups were given i.c. STZ or saline, and 24 h later, the rats were sacrificed to harvest brains for *ex vivo* experiments. Freshly micro-dissected frontal lobes were washed twice in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Hank's balanced salt solution (HBSS) pre-chilled to 4°C, placed briefly on sterile filter paper to absorb excess HBSS, and then transferred to the center of the plastic mounting tray of a McIlwain Tissue Chopper (Ted Pella, Inc., Redding, CA). The brain tissue was positioned to the left of the blade and just beneath the edge of the fully raised blade. Optimum slicing was achieved by positioning the brain regions in the coronal plane. Systematic study revealed that the optimum slice thickness to be 250 μm thinner slices had poor tissue integrity, while slices thicker were highly variable with respect to viability. In addition, a slow-to-moderate chopping speed allowed the chilled brains to remain set in place and maintain their orientation without being crushed. After slicing through the entire specimen, the tray holding the sliced tissue was removed from the apparatus and held over a 100-mm² Petri dish containing ice cold full medium (Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% glutamine, 1% non-essential amino acids, and 1.2% penicillin and streptomycin solution (penicillin 10,000 IU/mL, streptomycin 10,000 $\mu\text{g}/\text{mL}$). Using a Pasteur pipette, culture medium was gently flushed around the base of the tissue to dislodge and transfer the slices to the dish. Under a dissecting microscope, the slices were completely separated and transferred to 12-well culture plates (3 slices/well) containing 300 μL of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 10 mM non-essential amino acids, 25 mM KCl, and 120 U/mL penicillin/streptomycin. Cultures were maintained at 37°C in a standard humidified CO₂ incubator for 72 h.

The cultures were treated with T3D-959 (0.5 μM) or vehicle by addition to the media which was changed every 24 h. At the conclusion of the experiment, culture medium was harvested for cytotoxicity assays, and the tissue slices were snap frozen and stored at -80°C for protein studies or fixed in formalin, embedded in paraffin, and used to generate Hematoxylin and Eosin stained histological sections (5 μm thick) for light microscopy.

Cytotoxicity assay

Cytotoxicity was measured with the Vybrant Cytotoxicity Assay Kit (Molecular Probes, Eugene, OR), which quantifies the release of glucose 6-phosphate dehydrogenase (G6PD) into the culture medium. The assays were performed according the manufacturer's protocol. In brief, 50 μL of culture supernatant were transferred to a white OptiPlate (PerkinElmer, Waltham, MA) and incubated (30 min at 37°C) with reaction mixture containing 4 mM resazurin. Fluorescence intensity was measured in a SpectraMax M5 microplate reader (Molecular Devices Corp., Sunnyvale, CA; Ex/Em: 530/590 nm). Results were normalized to tissue protein concentration in the well.

Enzyme-Linked Immunosorbent Assay (ELISA)

Tissue homogenates were prepared in NP-40 lysis buffer supplemented with protease (1 mM PMSF, 0.1 mM TPCK, 1 mg/ml aprotinin, 1 mg/ml pepstatin A, 0.5 mg/ml leupeptin, 1 mM NaF, 1 mM $\text{Na}_4\text{P}_2\text{O}_7$) and phosphatase (2 mM Na_3VO_4) inhibitors. Protein concentration was measured using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). For direct binding ELISAs, 100 ng protein in 50 μl bicarbonate buffer were adsorbed to the bottoms of MaxiSorp plates (Nunc, Rochester, NY) by overnight incubation at 4°C. After rinsing in TBS, the wells were blocked for 3 h with 250 μl /well of 2% BSA in TBS. The proteins were then incubated with primary antibody (0.1–0.5 $\mu\text{g}/\text{ml}$) for 1 h at 37°C temperature. Immunoreactivity was detected with horseradish peroxidase (HRP)-conjugated secondary antibody (1:10000; Pierce, Rockford, IL) and Amplex Ultra-Red soluble fluorophore (Molecular Probes, Eugene, OR). Amplex Red fluorescence was measured (Ex 530/Em 590) in an M-5 machine (fluorescence light units; FLU). Subsequently, the samples were incubated with biotin-conjugated antibodies to large ribosomal protein (RPLPO), and immunoreactivity was detected with streptavidin-conjugated alkaline phosphatase (1:1000; Vector, Burlingame, CA) and the 4-Methylumbelliferyl phosphate (4-MUP) fluorophore (Molecular Probes, Eugene, OR). Fluorescence (Ex360/Em450) intensity was measured in a SpectraMax M5 microplate reader (Molecular Devices Corp., Sunnyvale, CA). Binding specificity was determined from parallel negative control incubations in which the primary or secondary antibody was omitted. The ratio of specific protein/RPLPO immunoreactivities were calculated and used for inter-group statistical comparisons.

Statistics

Box plots depict the means (horizontal bars), 95% confidence interval limits (upper and lower boundaries of the boxes), and range (stems). Inter-group comparisons were made using one-way or two-way row means, or ordinary two-way analysis of variance (ANOVA) with the Holm-Sidak multiple comparisons *post hoc* test (GraphPad Prism 6, San Diego, CA). Significant post-test differences ($p < 0.05$) and trends ($0.05 < p < 0.10$) are shown in the graphs and tables.

Sources of reagents

Antibodies to tau (ab64193), phospho S396-tau (ab109390), phospho T205-tau (ab4841), ubiquitin (Ubi-1), myelin-associated glycoprotein (MAG-1; ab89780), glial fibrillary acidic protein (GFAP; ab7260), and A β PP-A β 1→42 (ab10148) were purchased from Abcam (Cambridge, MA). Rabbit polyclonal antibody to RPLPO (RPL23 16086-1-AP) was purchased from Proteintech Inc (Chicago, IL). Amplex UltraRed soluble fluorophore HRP-substrate, and 4-MUP alkaline phosphatase substrate were purchased from Invitrogen (Carlsbad, CA). T3D-959 was provided by T3D Therapeutics, Inc. (Research Triangle Park, NC).

RESULTS

Effects of STZ and T3D treatment doses and schedules on brain weight, body weight, and blood glucose

These experiments included 98 female and male rats that were treated by i.c. STZ or vehicle followed by daily oral gavage with T3D-959 or normal saline (vehicle). The T3D-959 treatments (0.3, 0.7, 1.0, or 3 mg/kg) were initiated either early (1 day) or late (7 days) after i.c. administration of STZ or vehicle, and continued to the 28th day (endpoint) of the experiment. Since there were no gender differences in treatment responses, the data for males and females were pooled. One-way ANOVA tests demonstrated significant alterations in brain weight ($F = 2.34$; $p = 0.02$) and blood glucose ($F = 4.36$; $p = 0.0002$), but not body weight ($F = 0.61$) among the groups. *Post hoc* tests were used to determine which groups differed significantly from control. The i.c. STZ+vehicle treatment significantly reduced brain weight ($p = 0.0006$) and elevated blood glucose ($p = 0.013$), but had no significant effect on body weight relative to control (Table 1), consistent with our previously reported findings [16, 52]. Although body weight increase is a known side-effect of chronic PPAR- γ agonist treatments [62], such responses to T3D-959 were not observed. Instead, trend effects ($0.05 < p < 0.10$) for reduced body weight were detected in rats administered i.c. STZ and treated early with 0.3 or 1.0 mg/kg/day or late with 1 mg/kg/day T3D-959 (Table 1). Otherwise, mean body weight was not altered by i.c. T3D-959 treatment.

T3D-959 abrogated the i.c. STZ-associated reduction in brain weight in all groups except the ones treated with the early ($p = 0.0007$) or late ($p = 0.004$) 1 mg/kg dose. T3D-959 normalized non-fasting early AM blood glucose levels in the early 1 mg/kg/day, and late 0.7, 1.0, and 3 mg/kg/day intervention groups. Hyperglycemia persisted in the early 0.3 ($p = 0.006$), 0.7 ($p = 0.018$), and 3.0 ($p = 0.008$) mg/kg/day and late 0.3 ($p = 0.0002$) mg/kg/day T3D-959 treatment groups.

T3D-959 interventions restore i.c. STZ-mediated impairments of spatial learning and memory

MWM tests were used to assess effects of i.c. STZ and T3D-959 treatments on spatial learning and memory. The MWM tests were run on Experimental Days 24–27, with 3 trials per day over 4 consecutive days (see Materials and Methods). Area-under-curve calculations corresponding to cumulative latencies over the 3 daily trials were used for inter-group comparisons. Results were analyzed to compare the STZ $\pm$ T3D-959 with controls, and STZ +T3D-959 to STZ to assess the degree to which T3D-959 “normalized” performance in the STZ group, and rendered performance significantly better than in the ic-STZ+vehicle group. Comparisons between the control and i.c. STZ groups demonstrated progressively shorter mean latencies over the 4 days of testing such that Trial Day (time) had a main effect on performance ($F = 62.75$; $p < 0.0001$). The i.c. STZ treatments resulted in longer cumulative mean latencies to locate and land on the platform, which corresponds with the significant STZ effect demonstrated by ANOVA ($F = 21.73$; $p = 0.0002$). *Post hoc* tests revealed significant inter-group differences on Trial Days 1, 2, and 4, and a trend effect on Trial Day 3 (Figs. 1 and 2).

Effects of early (1 day delayed) versus late (7 days delayed) T3D-959 administration on spatial learning and memory in i.c. STZ-treated rats were examined relative to control and STZ groups (Fig. 2). Inter-group statistical comparisons made by one-way ANOVA revealed significant inter-group differences for each of the 4 trial days (Fig. 2E). On Day 1, mean latencies were significantly longer in the i.c. STZ, but significantly shorter in the early treatment groups receiving 0.3 or 1.0 mg/kg/day (both $p < 0.05$), and the late treatment group given 0.3 mg/kg/day ($p < 0.01$) relative to control (Fig. 2A). In addition, all STZ +T3D-959 treated groups had significantly shorter latencies compared with i.c. STZ+vehicle ($p < 0.001$ – 0.0001).

On Trial Days 2–4, with few exceptions, the early and late treatment STZ+T3D groups (0.3 and 1 mg/kg/day) had significantly reduced mean performance latencies compared with STZ +vehicle, but they did not differ from control (Figs. 2B – D). Exceptions were as follows: 1) on Day 3, the early 0.3 mg/kg/day T3D-959 treatment group had significantly prolonged mean performance latency relative to control but exhibited similar results compared with STZ+vehicle; 2) On Day 4, the early 0.3 mg/kg/day group had significantly shorter mean latencies compared with control and STZ+vehicle; and 3) on Trial Day 4, the early 1.0 mg/kg/day group's performance was not significantly different from either control or i.c. STZ+vehicle.

Early versus late T3D-959 treatment normalizing effects on spatial learning and memory

The full set of MWM data were graphed to determine the degree to which early versus delayed T3D-959 treatment initiation and dose effectively normalized performance relative to controls. The graphs corresponding to performance over the 4 trials were analyzed by two-way row-means ANOVA to compare the control versus STZ+T3D groups on the same Trial days (Table 2). Irrespective of dose and early versus delayed treatment, main effects on performance were modulated by Trial day (all $p < 0.0001$). STZ+T3D or interactive effects of Trial Day x STZ+T3D. In addition, within the early treatment group, main effects on performance were due to T3D-959 or interaction between T3D-959 and Trial Day at the lower doses (Table 2). In contrast, within the delayed treatment group, main effects of T3D-959 and interactions with Trial Day primarily occurred at the lowest (0.3 mg/kg/day) and highest (3 mg/kg/day) doses. From the graphs and *post hoc* test, it is evident that in the early treatment groups, performance latencies were largely normalized relative to control at all but the 0.7 mg/kg/day T3D-959 dose (Figs. 3A–D). At the highest T3D-959 dose, all significant differences between control and i.c. STZ treated rats were abolished (Fig. 3D). Delayed intervention with 0.3, 0.7, or 1 mg/kg/day of T3D-959 also effectively normalized or improved performance in i.c. STZ treated rats (Figs. 3E–G). However, at the highest dose (3 mg/kg/day), performance on each trial day was significantly worse than control and comparable to the vehicle treated i.c. STZ group (Fig. 3H).

T3D-959 prevents STZ-induced neurotoxicity

Frontal lobe slice cultures generated from P3 rat pups administered i.c. STZ or vehicle were treated with vehicle or 0.5 μ M T3D-959 for 72 h. G6PD release into the culture supernatants was used to examine effects of STZ and T3D-959 on culture cytotoxicity. Results were analyzed by two-way ANOVA which demonstrated significant effects of T3D-959 and

interactive effects of STZ x T3D-959 (Table 3). *Post-hoc* Tukey tests demonstrated that the G6PD levels were significantly elevated in cultures generated from i.c. STZ treated rats, and that T3D-959 abrogated those effects, reducing G6PD supernatant levels to control (Fig. 4A). Control cultures had similar levels of G6PD in the presence or absence of T3D-959 treatment. H&E stained histological section demonstrated the control frontal cortex slice cultures to be abundantly populated with cells (Fig. 4B), and markedly reduced cell densities in the i.c. STZ exposed cultures (Fig. 4C). Treatment with T3D-959 resulted in similar cortical cell densities in the control and i.c. STZ groups (Figs. 4D, E). In addition, the cortical cells in both groups appeared more basophilic (metabolically active) and plump compared with vehicle treatment. Since the process of generating slice cultures represents a form of trauma (tissue chopping), these findings suggest that T3D-959 is also protective against non-specific injury.

T3D-959 Neuroprotection in the STZ model of sporadic AD

AD is associated with neuronal loss, white matter atrophy, gliosis, and deficits in cholinergic function, all of which can be linked to impairments in insulin/IGF signaling combined with oxidative stress and neuro-inflammation [1, 30, 45, 63]. Our main focus was to further examine the early mediators of T3D-959's therapeutic effects. Therefore, with the tissue samples from slice cultures, we used duplex ELISAs to measure immunoreactivity to myelin-associated glycoprotein-1 (MAG-1), a marker of mature myelin-producing oligodendrocytes, glial fibrillary acidic protein (GFAP), reflecting astrocyte function, choline acetyltransferase (ChAT), which is needed for acetylcholine synthesis, and acetylcholinesterase (AChE), which mediates acetylcholine turnover. Results were normalized to ribonuclear protein expression measured in the same wells.

Two-way ANOVA tests demonstrated significant effects of i.c. STZ on GFAP, MAG-1, and ChAT, significant effects of T3D-959 on AChE, and significant STZ x T3D-959 effects on MAG-1 (Table 3). The *post hoc* Tukey multiple comparisons tests demonstrated that the i.c. STZ treatments significantly reduced frontal lobe expression of MAG-1 (Fig. 5A) and ChAT (Fig. 5C), but not GFAP (Fig. 5B) or AChE (Fig. 5D). The inhibitory effects of STZ on MAG-1 were partly abrogated by T3D-959, rendering the expression levels similar to T3D-959 treated control cultures (Fig. 5A). However, MAG-1 expression was reduced in control cultures treated with T3D-959 relative to vehicle. In contrast, the T3D-959 did not significantly alter ChAT expression in either the control or STZ cultures. GFAP expression was similar in all four culture groups, although due to reduced variance, the STZ cultures treated with T3D-959 had significantly reduced levels of GFAP immunoreactivity relative to vehicle-treated control cultures (Fig. 5B). Finally, AChE expression was similar in the control and STZ slice cultures, and T3D-959 significantly and similarly increased the levels in both groups, rendering the differences from vehicle-treated cultures statistically significant (Fig. 5D).

Effects of T3D-959 on biomarkers of neurodegeneration

Duplex ELISAs were used to measure Tau, pTau (ST), amyloid- β peptide (1 $\rightarrow$ 42) of the amyloid- β protein precursor (A β PP-A β), and ubiquitin in the frontal lobe slice cultures. Two-way ANOVA tests demonstrated significant effects of STZ treatment on A β PP-A β and

ubiquitin, significant effects of T3D-959 on Tau, A β PP-A β , and ubiquitin, significant STZ x T3D-959 effects on Tau, A β PP-A β , and ubiquitin, and a trend effect on pTau (Table 3). STZ significantly reduced the levels of pTau (Fig. 6B), and increased A β PP-A β (Fig. 6C) and ubiquitin (Fig. 6D) relative to control. The T3D-959 treatments increased Tau expression in both control and STZ cultures, although the differences were only significant for the STZ +T3D-959 group relative to both vehicle-treated groups (Fig. 6A). The mean levels of pTau in the STZ cultures were slightly increased and consequently normalized relative to control by the T3D-959 (Fig. 6B). T3D-959 had striking effects on A β PP-A β expression in the STZ cultures, reducing the levels to those measured in control cultures (Fig. 6C). Similarly, T3D-959 significantly reduced ubiquitin immunoreactivity in the STZ cultures, although the differences relative to control remained significant (Fig. 6D). It is noteworthy that the T3D-959 treatments had no significant effect on Tau, pTau, A β PP-A β , or ubiquitin expression in control cultures, suggesting the responses were specific to the early stages of neurodegeneration.

DISCUSSION

AD, like diabetes mellitus, is associated with insulin resistance, except brain rather than skeletal muscle is the principal target organ. In the brain, insulin is a key regulator of glucose utilization and signal transduction networks that mediate cell growth, plasticity, metabolism, neuronal survival, myelin maintenance, and acetylcholine biosynthesis, and it inhibits oxidative stress and apoptosis [1, 14, 15, 30, 45, 64]. Proof of principle for this concept has been provided by experiments in which i.c. administration of STZ, a pro-diabetes toxin, was shown to impair spatial learning and memory and cause brain atrophy due to neurodegeneration with many AD-associated histopathological, molecular, and biochemical abnormalities [16]. Furthermore, the neurodegenerative effects of i.c. STZ were abrogated by early treatment with PPAR agonists [10]. The PPAR- δ agonist proved to be considerably more neuroprotective in preserving cognitive function and hippocampal/temporal lobe structure compared with the PPAR- γ agonist [10], corresponding with the greater abundance of δ versus γ receptor expression in brain [55].

PPARs are a family of nuclear hormone receptors that function as transcription factors each with distinctive functions. PPAR- δ and PPAR- γ isoforms promote many of insulin's actions and reduce the effects of insulin resistance. A major advantage of PPAR agonists is that they mediate their therapeutic effects within the nucleus, circumventing impairments in insulin signaling caused by reductions in surface receptor binding and receptor tyrosine kinase activation, which occur in AD [2,11]. In pursuing this approach to treatment, it is important to recognize that the neuroprotective effects of PPAR- δ and PPAR- γ agonists overlap but are non-identical with respect to downstream insulin-responsive targets [46–49].

Correspondingly, exploratory experiments demonstrated that dual treatment with a PPAR- δ and PPAR- γ agonist was more neuroprotective against i.c. STZ than either drug alone. However, extension of this approach to the treatment of AD is limited because of concerns about efficiency of blood-brain barrier penetrance, the need to administer the compounds by injection, and uncertainty regarding delivery of both agonists to all cells. T3D-959 circumvents these problems because this small molecule is a hybrid PPAR- δ/γ agonist that can be administered as a once daily oral dose and exhibits a high degree of CNS penetrance.

The preclinical studies reported herein assessed the neurobehavioral effects of T3D-959 in an established model of sporadic AD in which adult Long Evans rats were treated by i.c. STZ which is known to cause deficits in spatial learning and memory, neurodegeneration, impairments in brain insulin and insulin-like growth factor (IGF) signaling, and increased oxidative stress [10,16]. In addition to assessing T3D-959's efficacy, we examined the effects of different doses and compared responses to early versus late onset of therapeutic intervention. The dose range was selected based on exploratory *in vivo* and *in vitro* experiments. The delayed time of initiating treatment addresses the fact that many patients first draw attention about their dementia in the intermediate rather than early stages of disease. At the same time, it was important to assess responses to early intervention because as diagnostic tools improve, early treatment protocols will become feasible. Our findings did not demonstrate a therapeutic advantage to treatment at an earlier stage of disease with T3D-959. The potential for future clinical benefit of T3D-959 treatment in patients advancing to a moderate stage of disease is supported by these studies.

The present finding of i.c. STZ-induced brain atrophy confirms our previously published results [10, 16]. The contrasting result of mild hyperglycemia in adult rats compared with hypoglycemia in the younger rats [10, 16] suggests that deficiencies in brain insulin signaling differentially impact systemic metabolism depending on age, causing peripheral insulin resistance in adults and possibly insulin hypersensitivity at earlier stages of life. However, it is noteworthy that T3D-959, which was developed as a drug for treating diabetes mellitus, normalized blood glucose levels when administered at 0.7 mg/kg/day or higher, particularly in the group that was treated with a 7-day delay post i.c.-STZ.

As anticipated, i.c. STZ impaired spatial learning and memory as assessed using the MWM test. Although both control and i.c. STZ groups improved over time, significant functional impairments persisted and were detected at nearly all time points. T3D-959 improved MWM performance, resulting in overlap with control results at nearly all doses, whether administered after 1 or 7 days delay. For the 1-day delay groups, complete overlap with controls was achieved with the highest dose (3 mg/kg/day), whereas in the 7-day delay groups complete overlap was achieved at the 0.7 and 1 mg/kg/day doses. Only the 3 mg/kg/day 7-day delayed treatment group failed to improve relative to untreated i.c. STZ, despite normalization of blood glucose and brain weight. These divergent therapeutic effects of T3D-959 may be due to differences in receptor sensitivity and responsiveness at early versus intermediate stages of neurodegeneration. While T3D-959 is more potent on human PPAR- δ than PPAR- γ (15-fold), potency on rat PPAR- δ is ca. 3-fold less than rat PPAR- γ . The lower doses (0.3 or 0.7 mg/kg/day) of T3D-959 were less consistently effective than higher doses (1.0 or 3.0 mg/kg/day) for normalizing MWM performance in the early treatment group, suggesting important contributions of PPAR- δ agonism for restoring brain function in the early stages of neurodegeneration. With regard to responses in the delayed treatment group, the consistent improvements in performance detected with even the lowest doses of T3D-959 suggest that intermediate stages of neurodegeneration may be highly responsive to both PPAR- δ and PPAR- γ agonism. Thus, the unique PPAR selectivity profile of T3D-959 may provide the potential to treat a broad range of disease severity.

To examine the mechanistic effects of T3D-959, we utilized short-term frontal lobe slice cultures generated from control and i.c. STZ exposed rats. The studies demonstrated that T3D-959 significantly reduced cytotoxicity (G6PD release) in the i.c. STZ cultures, rendering the levels similar to control. Correspondingly, tissue and cellular morphology were better preserved by T3D-959. An unexpected finding was that control cultures also exhibited better tissue preservation and cellular morphology following T3D-959 treatment, suggesting neuroprotective effects in the absence of a potent neurodegenerative/neurotoxic agent. Future studies will examine the potential therapeutic application of T3D-959 as a neuroprotective agent in circumstances associated with increased oxidative stress.

It was of further interest to examine the effects of T3D-959 on neuroglial markers. MAG-1, a protein expressed by mature myelin-producing oligodendrocytes, was significantly reduced by i.c. STZ. Oligodendrocyte viability and function are regulated through insulin and IGF-1 signaling networks [65–67]. Although T3D-959 increased MAG-1 expression, the mean levels remained lower than control. ChAT expression was also modestly improved by T3D-959. One consideration is that these effects mark early trends that would continue over time and correlate with improvements in brain weight and spatial learning and memory. AChE expression was not affected by the i.c. STZ, but similarly increased by T3D-959 in both control and i.c. STZ groups. Although high levels of AChE could impair net cholinergic function, in fact, AChE is inhibited by oxidative stress [68–70]. Therefore, the increased expression of AChE in the T3D-959 treated cultures could reflect an adaptive response to reduced levels of stress as shown by the G6PD release assays and histological appearances of the cultures.

The T3D-959-associated increase in Tau in the i.c. STZ group could represent a positive effect on the neuronal cytoskeleton and reduced collapse associated with neurodegeneration. In contrast, T3D-959 had no significant effect on p-Tau (S396+T205) in the i.c. STZ group, although the levels were normalized relative to control. However, the slight increases in S396+T205pTau could be explained by the larger relative increase in Tau expression rather than an early AD neurodegenerative effect [71]. Most interesting was the finding that T3D-959 normalized the elevated levels of A β PP-A β in the i.c. STZ group. As A β PP-A β accumulation occurs early in the course of AD neurodegeneration and is linked to neuroinflammation and stress [11, 63], most likely the anti-oxidant and insulin-sensitizing properties afforded by T3D-959 and PPAR agonists in general are responsible for this anti-AD effect. Finally, the i.c. STZ-associated increases in brain ubiquitin immunoreactivity were significantly reduced by T3D-959. Although the i.c. STZ+T3D-959 mean brain ubiquitin level was still higher than control, the downward trend from untreated cultures indicates at least partial amelioration of the unfolded protein response which is pivotal to neurodegeneration.

In summary, this study demonstrates that T3D-959 has clear therapeutic and neuroprotective effects in an established model of sporadic AD. The main effects were associated with improved (normalized) spatial learning and memory, prevention of brain atrophy, preservation of brain structural protein expression, and reduced indices of neurodegeneration including cytotoxicity, A β PP-A β levels, and ubiquitin immunoreactivity. Importantly, therapeutic effects occurred *in vivo* after a delay in treatment with T3D-959, suggesting that

individuals with mild or moderate AD would benefit from this highly effective small molecule drug that has the benefit of delivery as a once-daily oral dose.

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Source of Variation	F-Ratio	P value
Trial x STZ	0.27	0.8496
Trial Day	62.75	< 0.0001
STZ Treatment	21.73	0.0002

Fig. 1.

Intracerebral (i.c.) streptozotocin (STZ) impairs spatial learning and memory. Long Evans rats were administered i.c. STZ or normal saline (Control). On 4 consecutive days (Days 24–27 post-treatment), rats were subjected to MWM tests with 3 trials per day (see Materials and Methods). Latency to reach and land on the platform in the maze was measured. Graphs depict calculated area-under-curve results. Inter-group comparisons were made using ANOVA with the Tukey *post hoc* test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; § $0.05 < p < 0.10$ (Trend effect).

Fig. 2.

T3D-959 rescue of i.c. STZ-mediated impairment of spatial learning and memory. Long Evans rats were administered i.c. STZ or normal saline (Control) and gavaged with a single daily dose of T3D-959. MWM tests were performed over 4 consecutive days with 3 trials per day. Area-under-curve calculations were used as measures of cumulative performance over the 3 daily trials. Graphs depict (A–D) Day 1–Day 4 results from control (i.c. saline), i.c. STZ, and i.c. STZ+0.3 or 1 mg/kg/day T3D-959 with interventions begun 1 or 7 days after the i.c. STZ administration. E) Results of one-way ANOVA tests for inter-group

comparisons on each trial day. F) Symbols for significant differences from control+Vehicle or i.c. STV+Vehicle.

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Fig. 3.

T3D-959 dose and time delay post i.c. STZ effects of spatial learning and memory demonstrated using the MWM test. Long Evans rats were administered i.c. STZ or normal saline (Control) and gavaged with a single daily dose of (A,E) 0.3, (B,F) 0.7, (C,G) 1.0, or (D,H) 3.0 mg/kg of T3D-959 beginning (A–D) 1 day or (E–H) 7 days after the i.c. injections. On 4 consecutive days (Days 24–27 post-treatment), rats were subjected to MWM tests with 3 trials per day (see Materials and Methods). Latencies to reach and land on the platform in the maze were measured with video-tracking. Graphs depict mean $\pm$ S.E.M. corresponding to the area-under-curve results (cumulative performance). Inter-group

comparisons were made using Two-Way Row Means (Trial day) ANOVA tests (see Table 2) with the Holm-Sidak *post hoc* test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

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Fig. 4. T3D-959 Prevents neurodegeneration. Frontal lobe slice cultures were generated from rats i.c. injected with (B, D) normal saline (control) or (C, E) STZ 24 h earlier. Cultures were treated with (B,C) vehicle or (D,E) 0.5 μ M T3D-959 for 72 h from the same day of culture. A) Cytotoxicity was evaluated using the G6PD release assay (higher levels indicate increased cytotoxicity). B–E) Representative slice cultures were formalin-fixed paraffin embedded to generate H&E stained histological sections. Note reduced cell density in the frontal lobe culture section from the vehicle-treated i.c. STZ group (C) relative to the corresponding control (B) (encircled regions). T3D-959 co-treatment improved tissue

integrity and cellular morphology (nuclei are more basophilic, larger and better organized in D and E relative to B and C) in both control and STZ cultures. T3D-959 mediated increased cell survival is clearly evident in the STZ groups (compare encircled regions in B and E).

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Fig. 5.

Effects of T3D-959 on neuronal and glial cell protein expression in i.c. STZ treated rats: Frontal lobe slice cultures were generated from control or i.c. STZ-treated rats. The cultures were treated with vehicle (normal saline) or 0.5 μ M T3D-959 for 72 h. Protein homogenates of the cultures were used to measure immunoreactivity to (A) myelin-associated glycoprotein 1 (MAG-1), (B) glial fibrillary acidic protein (GFAP), (C) choline acetyltransferase (ChAT), and (D) acetylcholinesterase (AChE) by duplex ELISA with results normalized to RPLPO (see Materials and Methods). Results were analyzed by two-way ANOVA (Table 2). *Post hoc* significance tests determined the specific inter-group differences as shown in the panels. * $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$.

Fig. 6.

Effects of T3D-959 on AD biochemical markers: Frontal lobe slice cultures were generated from control or i.c. STZ-treated rats. The cultures were treated with vehicle (normal saline) or 0.5 μ M T3D-959 for 72 h. Protein homogenates of the cultures were used to measure immunoreactivity to (A) Tau, (B) $S^{396+T205}$ -pTau, (C) AβPP-Aβ, and (D) ubiquitin were measured by duplex ELISA with results normalized to RPLPO (see Materials and Methods). Results were analyzed by two-way ANOVA (Table 2). *Post hoc* significance tests determined the specific inter-group differences as shown in the panels. * $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$.

Table 1
Effects of STZ and T3D Treatment Doses and Schedules on Body Weight, Brain Weight, and Blood Glucose

Group	Drug	Dose mg/kg/d	Treatment Initiated	# Rats	Female/Male	Body Weight (grams)	Brain Weight (grams)	Blood Glucose (mg/dL)
Control	Vehicle		1 day	11	4F/7M	315.4 ± 83.0	2.05 ± 0.11	117.50 ± 15.49
Control	T3D	0.714	1 day	11	5F/6M	288.6 ± 94.9	1.99 ± 0.12	122.41 ± 14.66
STZ	Vehicle		1 day	12	4F/8M	292.0 ± 89.4	1.89 ± 0.10 ^{***}	136.40 ± 9.15 [*]
STZ	T3D	0.3	1 day	8	4F/4M	263.1 ± 56.4	1.96 ± 0.10	140.10 ± 18.98 ^{**}
STZ	T3D	0.714	1 day	8	4F/4M	271.3 ± 12.6	1.96 ± 0.03	140.00 ± 12.02 ^{**}
STZ	T3D	1.0	1 day	8	4F/4M	260.1 ± 69.9	1.88 ± 0.10 ^{***}	120.90 ± 21.01
STZ	T3D	3.0	1 day	8	4F/4M	283.3 ± 5.9	1.97 ± 0.01	144.70 ± 15.33 ^{**}
STZ	T3D	0.3	7 days	8	4F/4M	274.6 ± 78.9	1.98 ± 0.12	149.30 ± 17.03 ^{***}
STZ	T3D	0.714	7 days	8	4F/4M	267.0 ± 13.2	1.95 ± 0.09	121.00 ± 18.57
STZ	T3D	1.0	7 days	8	4F/4M	257.3 ± 65.6	1.91 ± 0.12 ^{**}	111.50 ± 20.93
STZ	T3D	3.0	7 days	8	4F/4M	278.8 ± 26.7	1.97 ± 0.04	122.00 ± 18.49

98 Long Evans male and female rats were administered i.c. STZ or vehicle (sterile saline) at 4 weeks of age, and then treated by daily gavage of T3D-959 (0.3, 0.71, 1.0, or 3.0 mg/kg) or vehicle beginning 1 or 7 days later and continuing through Experimental Day 28. Tabulated data were obtained at sacrifice. Body weight, brain weight, and blood glucose data correspond to group means ± S.D.s and were analyzed by ANOVA with *post hoc* comparisons to control+vehicle using the Holm-Sidak test:

* $p < 0.05$;

** $p < 0.01$;

*** $p < 0.001$.

Two-way ANOVA Table of T3D-959's Dose and Time Point Initiation Effects on Morris Water Maze Performance in i.c. STZ+T3D-959 Treated Relative to Control Rats

Table 2

Panel	T3D Start	T3D (mg/kg/d)	Trial Day		STZ+T3D		Trial x STZ-T3D	
			F-Ratio	p-Value	F-Ratio	p-Value	F-Ratio	p-Value
A	1 Day	0.3	27.92	<0.0001	3.75	0.07	6.64	0.0008
B	1 Day	0.7	91.96	<0.0001	26.04	0.0003	8.14	0.0003
C	1 Day	1.0	33.89	<0.0001	1.23	NS	9.28	<0.0001
D	1 Day	3.0	41.85	<0.0001	0.81	NS	0.16	NS
E	7 Days	0.3	38.19	<0.0001	8.29	0.01	7.9	0.0002
F	7 Days	0.7	69.16	<0.0001	0.33	NS	0.88	NS
G	7 Days	1.0	59.28	<0.0001	4.67	0.046	0.94	NS
H	7 Days	3.0	42.46	<0.0001	42.59	<0.0001	4.47	0.009

Long Evans rats were treated with 0.3 to 3 mg/kg/day T3D-959 (T3D) by gavage beginning 1 or 7 days after the i.c. STZ injections. Morris water maze tests were performed over 4 consecutive days with 3 trials per day. Curves corresponding to calculated area-under-curves for latencies to reach and land on the platform were compared with control results using two-way row-means ANOVA tests. The table depicts significant effects of Trial Day throughout, reflecting performance improvement over time. In addition, significant independent effects of T3D-959 and/or interactive effects of Trial x T3D-959 were observed for 5 of the 8 experimental conditions. Corresponding graphs are shown in Fig. 1.

Effects of STZ and T3D-959 on Culture Cytotoxicity and Expression of Neuronal, Glial, and AD Biomarkers (2-Way ANOVA Results)

Table 3

Protein	STZ Factor		T3D-959 Factor		STZ x T3D959 Interaction	
	F-Ratio	p-value	F-Ratio	p-value	F-Ratio	p-value
G6PD Release	0.72	N.S.	11.03	0.006	6.74	0.023
GFAP	5.20	0.03	0.65	N.S.	0.03	N.S.
MAG-1	32.23	<0.0001	0.22	N.S.	23.37	<0.0001
Tau	0.40	N.S.	10.16	0.003	4.83	0.036
pTau	1.87	N.S.	0.22	N.S.	3.45	0.07
A β PP-A β	29.65	<0.0001	9.73	0.004	20.04	<0.0001
ChAT	69.12	<0.0001	1.08	N.S.	0.37	N.S.
AChE	2.28	N.S.	32.24	<0.0001	0.12	N.S.
Ubiquitin	122.1	<0.0001	9.09	0.005	26.34	<0.0001

Frontal lobe slice cultures were generated from P3 rat pups that had been treated 24 h earlier by i.c. STZ or vehicle. Cultures were treated with vehicle or T3D-959 and maintained for 72 h. G6PD release into the culture supernatant and normalized to H33342 fluorescence, served as a measure of cytotoxicity. Immunoreactivity was measured by duplex ELISAs in which results were normalized to large ribosomal protein measured in the same wells. Data were analyzed by 2-way ANOVA and the Tukey multiple comparisons post-test. F-ratios and *p*-values reflect variances with respect to i.c. STZ, T3D-959 *in vitro* treatments, and interactions between STZ and T3D-959. Results are graphed in Figs. 3–5.