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COMPARATIVE EFFECTS OF TOLFENAMIC ACID AND DONEPEZIL ON BEHAVIOR AND TAU PATHOLOGY BIOMARKER LEVELS

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COMPARATIVE EFFECTS OF TOLFENAMIC ACID AND DONEPEZIL ON
BEHAVIOR AND TAU PATHOLOGY BIOMARKER LEVELS

BY

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ABSTRACT

Alzheimer's disease (AD) is a progressive neurodegenerative disease and is considered the predominant cause of dementia worldwide. Pathologically, the disease has been attributed to excessive accumulation of amyloid- β peptide and hyperphosphorylated tau protein leading to the formation of amyloid plaques and tau tangles, respectively. Currently, management of AD is extensively based on cholinesterase inhibitors, such as donepezil (DPZ), which provide symptomatic improvement. While there is no disease-modifying treatment, our lab has shown that tolfenamic acid (TA), a non-steroidal anti-inflammatory drug (NSAID), has potential disease-modifying properties independent of its anti-inflammatory effects. Thus, here we intended to compare it with DPZ, the most commonly prescribed treatment for AD, on behavior and tau pathology biomarker levels using an hTau transgenic mouse model and an in vitro model. A prototypical NSAID, ibuprofen (IBP), was used to further confirm that TA's effects are independent of its anti-inflammatory properties. Our findings show that total tau and its hyperphosphorylation levels were reduced in TA and DPZ treated mice, and IBP had no such effects on these markers. Treatments that reduced tau levels provided consistent improvement of cognitive performance as measured by the Morris Water Maze (MWM). Further we demonstrated TA effects on neuroblastoma cell line in which both TA and DPZ reduced total tau protein level, but not ibuprofen.

Keywords: Tolfenamic Acid, Donepezil, Tau Pathology, Cognitive Performance, Alzheimer's Disease, Comparative Study.

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List of Abbreviations

Acetylcholinesterase Inhibitors

(AChEIs)

Alzheimer's Disease

(AD)

Carrier Control

(CC)

Cyclin-Dependent Kinase 5

(CDK5)

Cyclooxygenase

(COX)

Donepezil

(DPZ)

Ibuprofen

(IBP)

Morris Water Maze

(MWM)

Non-steroidal Anti-inflammatory Drugs

(NSAIDs)

Serine

Ser

Specificity Protein 1

(Sp1)

Threonine

Thr

Tolfenamic Acid

(TA)

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INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are known as COX-inhibitors and are used to treat a variety of diseases. Due to their anti-inflammatory properties, epidemiological studies strongly proposed NSAIDs for the management of Alzheimer's disease (AD); however, their potential benefits were not substantiated in clinical trials (reviewed in Imbimbo et al., 2010). Cyclooxygenase inhibition is a well characterized anti-inflammatory mechanism by which NSAIDs prevent the formation of eicosanoids and ultimately reduce formation of prostaglandins (Rao and Knaus 2008), Figure 1. In addition to their canonical molecular target, studies have suggested a polyvalent effect for NSAIDs in AD underlying pathogenesis. Studies have indicated that NSAIDs lower amyloid β 42, activate peroxisome proliferator-activated γ , prevent NF- κ B translocation and affect other molecular targets (Lleo et al., 2007).

In addition to non-indicative features such as neuroinflammation, AD is characterized by presence of extracellular amyloid β plaques and intracellular neurofibrillary tangles. These pathologic aggregates are composed mainly of amyloid β and hyperphosphorylated tau protein and are considered diagnostic biomarkers that distinguish AD from other neurodegenerative diseases (Sperling et al., 2014). Further, studies have shown that amyloid pathology is an upstream event that occurs years before symptoms become apparent (Bloom 2014). On the other hand, tau pathology is a late event and correlates best with cognitive impairments (Nelson et al., 2013). The underlying mechanism of tau toxicity is not resolved; however, tau protein is abnormally hyperphosphorylated in AD, the main

component of tau tangles (Stoothoff and Johnson 2005). Numerous pathogenic sites have been identified in abnormally hyperphosphorylated protein tau indicating that phosphorylation of certain sites, such as pSer-262, pSer-356, pSer-214 and pThr-231, reduces binding affinity of tau to microtubules (Hasegawa et al., 1992; Schneider et al., 1999; Cho and Johnson 2003; Hanger et al., 2010). Further, different phosphorylation events have been shown to be associated with different stages of tau pathology; pThr-153, pThr-231, and pSer-262 epitopes were associated with pre-tangle, non-fibrillar tau; while, pSer-404/pSer-396, pSer-202/pSer-199, and pThr-205 epitopes were associated with filamentous tau (Augustinack et al., 2002; Kimura et al., 1996). In addition, some reports indicated involvement of Specificity protein 1 (Sp1), a transcription factor, in regulating tau gene expression (Heicklen-Klein and Ginzburg 2000; Santpere et al., 2006).

Cholinergic pathways play an important role in memory and learning (Hasselmo 2009). In AD, the source of cortical cholinergic innervation, nucleus basalis of Meynert in the basal forebrain, undergoes severe neurodegeneration (Mesulam M. 1976; Whitehouse et al., 1918). This discovery and others have introduced acetylcholinesterase inhibitors (AChEIs) to be an integral part in the management of AD (recently reviewed in Hampel et al., 2018). Thus, currently donepezil (DPZ), tacrine, and rivastigmine are approved AChEIs for the management of AD (Hansen et al., 2008). DPZ is the most commonly used for treatment of AD due to good oral bioavailability and relatively fewer side effects compared with other AChEIs (Prickaerts et al., 2017). However, these treatments

do not prevent the disease progression and provide only palliative effects (Takeda et al., 2006).

Previously, we have shown that tolfenamic acid (TA), although an NSAID, ameliorates AD related biomarkers by mechanisms independent of its anti-inflammatory properties (Adwan et al., 2011; Subaiea et al., 2013; Adwan et al., 2014; Adwan et al., 2015; Subaiea et al., 2015), Figure 2. Thus, in this study we intended to compare the effects of TA with donepezil (DPZ) on tau pathology biomarkers and memory performance using a hTau transgenic mouse model. Ibuprofen (IBP), a prototypical NSAID, was used here to further demonstrate that TA works through COX-independent pathways. In addition, we further demonstrated effects of aforementioned treatments in a lead-exposed in vitro model using SH-SY5Y neuroblastoma cell line.

MATERIALS AND METHODS

Animals and treatments:

Transgenic mice strain of B6.Cg-Mapt^{tm1(EGFP)Klt} Tg(MAPT)8cPdav/J were obtained from Jackson lab (Bar Harbor, ME, USA). These mice express exclusively human tau isoforms (3R and 4R). The transgene includes: coding sequencing, intronic regions, and regulatory elements. These mice develop hyperphosphorylated tau and accumulation of tau tangles around three months of age. While, cognitive impairment has been characterized in this strain around six months of age. These mice carrying the transgene were bred and genotyped at the animal facility at the University of Rhode Island. At age of 5-8 months, mice

were assigned randomly to four treatment groups with n of 10 in each group as follows: (1) carrier control (CC) administered corn oil as a vehicle, (2) carrier treated with TA 5 mg/Kg, (3) carrier treated with DPZ of 1mg/Kg, and (4) carrier treated with IBP 62.5 mg/Kg. All treatments were purchased from Sigma and administered via oral gavage prior and during behavioral testing for 34 days as shown in Figure 3. TA dose was chosen based on a previous work; while, IBP and DPZ doses were chosen from literature, cited in the discussion section. Animals were kept on a cycle of 12:12 light-dark and room temperature was maintained at 22 ± 2 °C. Food and water were made available, *ad-libitum*, and body weight was assessed every 3-4 days. Animals were euthanized by exposure to CO₂ inhalation 24 h after the last day of treatment administration. Brain tissues were extracted and stored at -80 °C. All procedures and protocols for this study were approved by the URI Institutional Animal Care and Use Committee (IACUC). Animals during this study were also under continuous supervision by the URI animal facility staff.

Behavioral studies:

Morris Water Maze (MWM) was performed following a published method to assess spatial learning and memory performance on the 15th day of treatments administration (Morris et al., 1982). A plastic tank with 48 inches diameter and 30 inches height was used. The tank was made opaque with non-toxic paint (Crayola, New York City, NY) and filled with water to a depth of 14 inches. Along the tank's wall, visual cues were placed high up on the tank, and the tank was visually divided into four quadrants (SW, SE, NW, and NE). An escape platform (10 cm²) was submerged 1 cm below the surface of the water in one of the quadrants, known as

the platform zone (PFZ), and the position was fixed during all trials. For each trial, the mice were placed facing the wall and starting points were randomly assigned. Water temperature was maintained at 25 ± 2 °C during behavior testing. A computerized video-tracking system (ObjectScan, Clever Sys. Inc., Reston, VA) was used to analyze the path of each mouse during each trial of the behavior testing.

Acclimation-Day 0, before the beginning of memory training, acclimation was performed for one day. In this acclimation trial with no platform placed, each mouse was allowed to swim for 60s. Acquisition trials-Day1-Day7, commenced on the 15th day of drug administration: During these trials, a platform was placed as described above with a fixed position and each mouse received three daily training sessions with 20 min inter-trial interval. The starting points were randomly assigned, and mice were given 60s period of swimming to find the platform. If they failed to find it in the given time, mice were guided towards it, and allowed to sit for 30s; latency time was recorded and analyzed. Showing a preference towards the quadrant that had the platform, would be an indication that mice had developed a memory of the correct quadrant.

Probe trials-commenced on the 24th and 34th days of drug administration: during probe trials, the platform was removed, and mice allowed to swim for 60s. Developing a preference to the location of the platform, correct quadrant, would be an indication that mice have developed a memory of the correct place. Percentage of number of crossing of the correct quadrant was calculated for treatment groups and compared with their control counterpart.

Protein Extraction and Western Blotting:

Using radio-immunoprecipitation lysis buffer (RIPA) (Sigma Aldrich, MO) supplemented with 0.1% protease inhibitor cocktail and 5 μ L phosphatase inhibitor, cerebral cortices samples were homogenized and incubated on ice for 30 min. Then for 5 min, homogenates were sonicated and vortexed. After that, at 10,000 $\times$ g, homogenates were centrifuged for 20 min and supernatant were collected and stored at -80°C . Micro BCA kit (Thermo Scientific, Waltham, MA) was used to determine total protein concentration. Twelve per cent SDS-Page gels were prepared, and 30 μ g total protein is loaded followed by transferring to Polyvinylidene Fluoride (PVDF) membranes (GE, Piscataway, NJ). Following transferring, membranes were blocked for 1 h at room temperature with either 5% bovine serum albumin (BSA) or 5% non-dry milk in Tris buffered saline + 0.1% Tween 20 (TBST). Then at dilution of 1:1000, membranes were incubated overnight with primary antibodies of interest: Rabbit Anti-CDK5, Rabbit Anti-GAPDH, Mouse Anti-Tau-46, Rabbit Anti-Ser404, and Mouse Anti-Sp1 (Cell Signaling Technology, Danvers, MA). On the following day, membranes were washed and exposed to either IRDye[®] 680LT infrared dye or IRDye[®]800LT Infrared Dye (LI-COR Biotechnology, NE), goat anti-mouse/goat anti-rabbit diluted at 1:10000 for 1 h. Finally, Odyssey[®] Infrared Imaging System (Li-Cor, NE) was used to detect and quantify infrared signal of Western Blot bands.

RNA synthesis, cDNA, and Real Time PCR:

TRIzol[®] Reagent method was used (Invitrogen, Carlsbad, CA) for RNA isolation from cerebral cortex tissues followed by Nanodrop (Thermo Scientific, Wilmington, DE) to check integrity. Following manufacturer's instructions (Bio-Rad, Hercules, CA), cDNA was synthesized using iScript[™] Select cDNA synthesis kit in which samples were incubated for 90 min at 42 °C; and then to terminate the reaction, samples were further incubated for 5 min at 85 °C using MJ Research MiniCycle[™] (Bio-Rad, Hercules, CA). Primers for Tau and GAPDH were obtained from Invitrogen (Carlsbad, CA) as follows: Tau sense: 5'-AGTAAAGGGC AGCGGACCAGCGGCG-3' and antisense: 5'-GGCGCGCGCGGCGAGA GGGC GCGTT-3'; GAPDH sense: 5'-TGGTGAAGCAGGCATCTGAG-3' and anti-sense: 5'-TGCTGTTGAAGTCGCAGGAG-3'. Real time PCR reaction mix was prepared using 1.5 µM of cDNA, 1 µL of each primer, 9 µL of nuclease free water, and 12.5 µL of SYBR[®] Green PCR Master Mix (Applied Biosystems, Foster City, CA). 7500 Real Time PCR System (Applied Biosystems, Foster City, CA) was used for RT-PCR experiments following the standard protocols: samples were incubated at 5 °C for 2 min, followed by 95 °C for 10 min and 40 cycles of 95 °C for 15 sec; then 60 °C for 1 min. GAPDH was used as endogenous control with data was analyzed with relative quantifications.

Cell Culture and Western blotting:

To further investigate TA and DPZ effects on a lead (Pb)-exposed model from our laboratory to induce certain markers such as Specificity protein 1 (Sp1), human

neuroblastoma cells (SH-SY5Y) were obtained from American Type Culture Collection (ATCC, Manassas, VA). DMEM/F12 medium (Sigma-Aldrich, MO) supplemented with 10% fetal bovine serum (FBS), penicillin (10 IU/mL), streptomycin (10 mg/mL), and glutamine 2 mmol/L were used for culturing and maintaining the cells. Following published methods (Adwan, et al., 2014), cells were sub-cultured at 10^5 cells/mL in six well plate and upon 80% confluency cells were differentiated for one week using 10mM all-trans retinoic acid (Sigma Aldrich, St. Louis, MO) in DMEM/F12 medium supplemented with 1% FBS, penicillin (10 IU/mL), streptomycin (10 mg/mL), and glutamine 2 mmol/L. Medium was changed every 48 h and neurite outgrowth was examined every two days, as described before (Adwan et al., 2014; Jamsa et al., 2004). After differentiation, cells sequentially were exposed to lead (Pb) acetate 10 mM for two days to induce certain markers, cells were then exposed to TA, DPZ, and IBP diluted in DMSO for 72 h with the following concentrations: 5 μ M, and 25 μ M. DMSO of 0.05% is used and maintained for controls and treatments. Cells then harvested and protein extraction performed according to manufacturer's protocol (Bio-Rad, Hercules, CA). Western blot experiments were performed as stated previously.

Statistical analysis:

Proper statistical tests were used including Student's t-test, or ANOVA analysis of variance as indicated. Dunnett's test for multiple comparisons was further used if necessary. Graph pad prism 7.0 computer software (La Jolla, CA, USA) was used and $p < 0.05$ were considered to be statistically significant. Data were represented as mean $\pm$ SEM.

RESULTS

TA and DPZ treated mice showed consistent improvement in cognitive performance

Spatial learning and memory performance were assessed using MWM, in which mice have to navigate through and use visual cues to locate a hidden escape platform. Reference memory is measured by preference developed to the escape platform position. Behavioral assessment commenced for acquisition trials on the 15th day of treatments administration for 8 days shows that TA-treated mice showed a sustained and consistent improvement in cognitive performance as well as DPZ-treated mice to lesser degree during the acquisition trials, Figure 4 (a-c); while, IBP treated mice showed fluctuated performance compared with the CC group. However, all treatment groups showed a non-significant trend of improved memory performance on probe trials on the 24th and 34th days of treatments administration, Figure 4 (d, and e).

TA reduces total tau and p-tau protein levels, but not cyclin-dependent kinase 5 (CDK5) protein levels

Following administration of TA for 34 days, total tau and p-tau (Ser404) expression levels were reduced in cerebral cortex compared with the CC group determined by western blot analysis as shown in Figure 5, $p= 0.0011$ and $p= 0.0017$ respectively determined by two-tailed unpaired t-test. Western blot analysis indicates that TA had no effect on CDK5 expression levels, Figure 6.

DPZ reduces total tau and p-tau protein levels, but not CDK5

Next, we investigated whether DPZ had an impact on tau related biomarkers. Western blot analysis shows reduced total tau and p-tau (Ser404) protein levels in cerebral cortices of DPZ-treated mice compared with the CC group, $p=0.0020$ and $p=0.0021$, respectively determined by two-tailed unpaired t-test, Figure 7. We did not observe effects on CDK5 expression levels in DPZ-treated mice compared with the CC group, Figure 8.

IBP doesn't lower total tau, p-tau, or CDK5 protein levels

Further, we used IBP here as a negative control, prototypical NSAID, to demonstrate whether effects of TA on tau pathology are related to its anti-inflammatory properties. IBP had no effect on total tau and p-tau (Ser404) after 34 days of drug administration in this model analyzed using two-tailed unpaired t-test, Figure 9. In addition, we did not observe changes in CDK5 protein levels, Figure 10.

Tau mRNA levels in cerebral cortices of mice treated with TA, DPZ, and IBP

Following exposure for 34 days, we observed a non-significant trend of lowering effects on tau mRNA in cerebral cortices of mice treated with aforementioned treatments, Figure 11. Data were analyzed using two-tailed unpaired t-test.

TA and DPZ lower Sp1 and Total tau protein levels in vitro, but not IBP

After exposing SH-SY5Y cells to Pb for 48 h as described in the method section, treating cells with TA and DPZ of a 5 μ M and 25 μ M for 72 h resulted in a

significant reduction of Sp1 protein level. In addition, DPZ reduced total tau level significantly; and to some extent TA. However, we didn't observe such effects with IBP, Figure 12. Data were analyzed using one-way ANOVA.

DISCUSSION

Tau pathology is a key contributor to many neurodegenerative diseases including AD and given the heterogenicity of AD, targeting multiple points of the pathogenesis might be more efficacious than a single node (Iqbal et al., 2005). Previously in wild type mice, we have shown the ability of TA to lower AD related A β pathology via a transcriptional pathway (Adwan et al., 2011). These findings were further confirmed in APP transgenic mice in which we reported a reduction in amyloid-related pathology biomarkers with improved cognitive functions (Subaiea et al., 2013; Adwan et al., 2014; Adwan et al., 2015; Subaiea et al., 2015). In addition, we showed the ability of TA to reduce beta site APP cleaving enzyme 1 (BACE1) enzyme activity and gene expression as well as Sp1 expressions in APP mice (Adwan et al., 2014). Furthermore, we demonstrated recently the efficacy of TA in improving cognitive function in hTau transgenic mice accompanied with reduced total tau and p-tau levels, pThr181 and pThr-231, in the frontal cortex and cerebellum demonstrated by Western blot and immunohistochemistry analysis (Chang et al., 2018).

In this study we compared the behavioral performance of TA and DPZ treated groups. The MWM analysis showed that TA provided sustained and consistent improvement in memory performance as well as DPZ to a lesser degree during the acquisition and probe trials; while, IBP-treated mice showed fluctuation in their

daily performance, Figure 4. Previously, we showed that TA doesn't affect swim speed or velocity ruling out the possibility that such improvements might be due to improved overall well-being (Change et al., 2018; Leso et al., 2019). Further, a previous report on DPZ also showed that DPZ had no effect on swim speed compared with their untreated counterparts in APP transgenic model (Easton et al., 2013).

Western blot analysis showed a reduction in total tau and p-tau (Ser404) in cerebral cortices of mice treated with TA, Figure 5. These results further demonstrate the ability of TA to ameliorate tau pathology biomarkers and confirm previous work by our lab, cited above, and that it is comparable for DPZ effects, Figure 7. It's well-known that DPZ enhances cognitive function and has been used for a long time in AD patients (Rogers et al., 2000). Furthermore, Other studies also indicated that DPZ does not solely work at the Ach level, but in fact might have disease modifying effects through affecting the amyloid pathology (reviewed in Sabbagh et al., 2006). In addition, it has been reported that DPZ affects neuroinflammation, tau pathology and neurodegeneration in FTD-17 mouse model, line PS19 (Yoshiyama et al., 2010).

However, Western blot analysis of mice brains treated with IBP didn't show reduction in either total tau or its phosphorylation state, Figure 9. In addition, a previous report on IBP showed that IBP didn't provide significant improvement in memory performance tasks using APP23 transgenic mice (Dam et al., 2010). By contrast, McKee et al reported different findings that chronic administration of IBP for six months ameliorates amyloid and tau pathology in triple transgenic AD mice

and improves learning and memory performance (McKee et al., 2008). However, these effects on tau pathology might be secondary to the reduction of amyloid β accumulation as shown previously (Oddo et al., 2006). In addition, to mention here the difference in the duration of treatments and the model we used here as that might explain such differences in findings. It's also worth noting here also previous animal studies showed inconsistent results of IBP's effects on amyloid pathology related biomarkers (reviewed in Gasparini et al., 2004).

While we observed a significant reduction in the tau protein levels with TA and DPZ, mRNA finding suggests these treatments did not affect significantly tau gene expression, Figure 11. It has been shown that NSAIDs including TA are treatments that have polyvalent effects, of which activating proteasomal-dependent degradation pathways inducing degradation of certain targets (Maen and Safe 2005; Maen et al., 2006). Thus, it is plausible that both TA and DPZ induced tau protein degradation through activating cellular degradation pathways without affecting its gene expression and that warrants further investigation.

Although this mouse model exhibits tau pathology related biomarkers with manifested cognitive impairment, to our knowledge the inflammatory response has not been characterized in this model. We couldn't detect brain COX2, a typical target of NSAIDs, using Western blot analysis. It is possible that COX2 expression as an inflammatory mediator is not induced at this age. However, we have previously shown in the same model at 18 months old that TA reduces COX2 expression in presence and absence of tau mouse model (Leso et al., 2019). In

addition, using IBP as a control might further support the hypothesis that TA ameliorates AD related biomarkers independent of its anti-inflammatory properties.

Previous work has shown the involvement of Sp1 in the underlying AD pathogenesis including regulation of APP and BACE1 gene expression (Brock et al., 2008; Christensen et al., 2004; Citron et al., 2008). The in vitro work here shows that treating SH-SY5Y cells with TA and DPZ reduced the SP1 protein level; total tau level was also reduced significantly with DPZ, and to some degree with TA. However, with IBP-treated cells, we didn't observe any such effects on these markers which might further confirms the IBP in vivo findings as shown in Figure 12. However, including more dose concentrations for TA and IBP might be warranted to observe such potential reducing effects on tau expressions.

It's worth noting here that this study has several limitations. It's also possible that increasing the treatment doses are needed for the cell culture work to further recapitulate concentration-effects for the aforementioned treatments particularly for IBP, if any, on examined markers. In addition, adding more NSAIDs with similar structure, such as Mefenamic acid, as positive controls will further strengthen our hypothesis as that these compounds might show similar effects as TA on the AD markers. It's also possible that the reduction effects observed on tau phosphorylation might be related to reduced total tau protein levels rather than affecting related potential kinases expression levels/ or activity. Thus, having kinase enzyme activity assays and wild type controls will further allow us to examine and compare related potential kinase activity/expression and that requires further investigation.

CONCLUSION

In summary, this work suggests that TA works through a COX-independent pathway ameliorating AD-related biomarkers and that it is comparable with DPZ. In addition, treatments that reduced tau pathology biomarkers, namely TA and DPZ, provided consistent improvement in the behavioral assessment.

Figure 1. Representative of biosynthesis of prostanoids and main mechanism of action of non-steroidal anti-inflammatory drugs (NSAIDs). A well characterized mechanism of action for NSAIDs is through the inhibition of cyclooxygenase enzymes (COX 1 & 2), interfering with the formation of inflammatory mediators (prostaglandins and thromboxanes).

Figure 2. Proposed mechanism of action for TA independent of COX-pathways. TA reduces Sp1 protein level (Maen et al., 2006; Adwan et al., 2011; Subaiea et al., 2013) down-regulating tau gene expression. It's also possible that TA interferes with the binding of Sp1 to its target genes including tau, reducing their gene expressions and that warrants further investigation.

Figure 3. Timeline of treatment administration and behavioral assessment.

Mice were administered TA, DPZ, IBP, or corn oil for 34 days. Behavioral assessment commenced on day 15 beginning with habituation and acquisition trials for 8 days. Then, two probe trials were conducted on day 24 and day 34 and mice were euthanized on the day 35 and brain tissues were dissected.

Figure 4. Spatial learning of hTau carrier mice treated with TA, DPZ, and IBP compared to Carrier Controls in the MWM. The escape latency in seconds and percentage of number of crossing of the correct quadrant were assessed during acquisition trials and probe trials, commenced on the 15th-23rd, 24th, and 34th of treatments administration respectively (a-e). Each group contained 8-10 mice and each mouse received 3 trial/day during the acquisition trial and MWM experiments were conducted as mentioned in section 2.2. *Asterisks* indicate significant difference (two-tailed unpaired t-test and one-way ANOVA tests were used- * $p < 0.05$, ** $p < 0.01$; results were represented as mean $\pm$ SEM).

a.

b.

c.

d.

e.

f.

Figure 5. Total tau and p-tau (Ser404) protein levels in cerebral cortices of mice treated with TA. hTau carrier mice (5-8 months) treated with TA 5 mg/Kg for 34 days were compared with the CC group with *n* of 5 in each group. (a) Total Tau (b) Ser404. Asterisks indicate significant difference (two-tailed unpaired t-test- **p*<0.05, ***p*<0.01; results were represented as mean ± SEM).

a.

b.

Figure 6. CDK5 protein levels in cerebral cortices of mice treated with TA.

hTau carrier mice (5-8 months) treated with TA 5 mg/Kg for 34 days were compared with the CC group with *n* of 5 in each group. Western blot analysis doesn't indicate significant difference between the two groups (two-tailed unpaired t-test; results were represented as mean $\pm$ SEM).

Figure 7. Total tau and p-tau (Ser404) protein levels in cerebral cortices from mice treated with DPZ. hTau carrier mice (5-8 months) treated with DPZ 1 mg/Kg for 34 days were compared with the CC group with *n* of 5 in each group. (a) Total Tau (b) Ser404. Asterisks indicate significant difference (two-tailed unpaired t-test- **p*<0.05, ***p*<0.01; results were represented as mean ± SEM).

Figure 8. CDK5 protein levels in cerebral cortices of mice treated with DPZ.

hTau carrier mice (5-8 months) treated with DPZ 1 mg/Kg for 34 days were compared with the CC group with *n* of 5 in each group. Western blot analysis doesn't indicate significant difference between the two groups (two-tailed unpaired t-test; results were represented as mean $\pm$ SEM).

Figure 9. Total tau and p-tau (Ser404) protein levels in cerebral cortices of mice treated with IBP. hTau carrier mice (5-8 months) treated with IBP 62.5 mg/Kg for 34 days were compared with the CC group with *n* of 5 in each group. (a) Total Tau (b) Ser404. Western blot analysis doesn't indicate significant difference between the two groups (two-tailed unpaired t-test; results were represented as mean $\pm$ SEM).

Figure 10. CDK5 protein levels in cerebral cortices of mice treated with IBP.

hTau carrier mice (5-8 months) treated with IBP 65.2 mg/Kg for 34 days were compared with the CC group with *n* of 5 in each group. Western blot analysis doesn't indicate significant difference between the two groups (two-tailed unpaired t-test; results were represented as mean $\pm$ SEM).

Figure 11. Tau mRNA levels in cerebral cortices of mice treated with TA, DPZ, and IBP. Real time-PCR results of tau mRNA levels in cerebral cortices of mice (5-8 months) treated with aforementioned treatments for 34 days (a-c), respectively. GAPDH was used as an endogenous control and data were analyzed using two tailed unpaired t-test. Results were represented as mean $\pm$ SEM.

a.

b.

c.

Figure 12. Sp1 and total tau proteins levels in differentiated SH-SY5Y cells.

SH-SY5Y cells differentiated and treated with Pb for 48 h followed by 72 h of media containing TA, DPZ, or IBP as stated in the method section. Sp1 and tau protein levels were normalized to endogenous GAPDH level. Data were analyzed using one-way ANOVA with Dunnett's test for multiple comparisons. Results were represented as mean $\pm$ SEM.

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