Tolfenamic Acid: A Potential Modifier of Tau Protein in Alzheimer's Disease

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TOLFENAMIC ACID: A POTENTIAL MODIFIER OF TAU PROTEIN IN ALZHEIMER'S DISEASE

BY

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ABSTRACT

Alzheimer’s disease (AD) is the most common form of dementia. It is a progressive neurodegenerative disease that is characterized by the increased abundance of amyloid beta (Aβ) plaques, and neurofibrillary tangles composed of hyperphosphorylated tau. Only a handful of medications have been approved by the Food and Drug Administration (FDA) since AD’s discovery more than 100 years ago. These drugs only treat the symptoms and are best prescribed during the early stages of the disease. In addition, the drugs do not restore the cognitive loss. It is critical to discover a novel drug to treat and prevent this disease. We have chosen to repurpose tolfenamic acid (TA), a drug approved in Europe and parts of Asia, as a treatment for taupathies.

Data from our lab have shown that C57BL/6 mice treated with TA have lowered amyloid precursor protein (APP) and Aβ levels (Adwan et al., 2011). Our lab found that treating APP transgenic mice (line R1.40) with TA led to improved cognitive function and working memory assessed by the Morris water maze and Y-maze, respectively, and reductions in Aβ plaque burden (Subaiea et al., 2013; 2015). The lab has also shown that R1.40 mice treated with TA showed a reduction in total tau, phospho-tau (p-tau) Ser235, Thr181, and CDK5 levels (Adwan et al., 2014). Sp1 is a common transcription factor of both APP and tau that regulates their gene expression. Thus, we examined the ability of TA to alter tau and its phosphorylation levels, and its effects in improving learning and memory profile in an htau transgenic mouse model. These transgenic mice are hemizygous for the htau gene expressing all six isoforms of the human microtubule associated protein tau (MAPT), and they are
homozygous knockout for the murine tau. We administered mice a corn oil vehicle or TA at 5 or 50 mg/kg for 34 consecutive days. Memory and learning were assessed by MWM and Probe Trial I and II.

The protein was isolated from the cortices of the mice. Total tau, p-tau Ser396 and Thr231 protein levels were measured by Western blot. Administration of TA showed a decrease in protein levels of both 5 and 50 mg/kg treatment groups in contrast to the control group. Immunohistochemistry staining for p-tau Thr231 showed reduced p-tau present in the frontal cortex and striatum. In the MWM, the control group showed poor spatial learning and memory deficits that were attenuated in both the 5 and 50 mg/kg TA groups. In addition, both TA treatment groups showed improved memory retention in Probe Trial I and II compared to the control. These results show TA as a novel drug for AD as it lowered AD biomarker protein levels and improved cognitive function within the htau mouse model.

Keywords: Alzheimer's disease, tau, tolfenamic acid, htau mouse model
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I thank my parents for all they have done for me. I would not be here if it wasn't for their hard work and sacrifices. Their love has motivated me every day. Lastly, I would like to dedicate my work to my grandmother who has Alzheimer's disease. Her diagnosis has inspired me to pursue this research field. I thank her for raising me and for all the love that she has given me.
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CHAPTER 1
INTRODUCTION

Alzheimer’s disease (AD) affects over 5 million Americans and it is the sixth leading cause of death in the US (Alzheimer’s Association, 2015). It is a neurodegenerative disease and it is the most common form of dementia. Dementia is characterized by the progressive loss of memory and decline in everyday activities. Neurons become damaged in demented patients and eventually die, leading to changes in memory, decision making, and behavior. People with AD often have difficulty completing familiar tasks and remembering friends and family. Eventually, patients lose their independence and require family members or friends for care. In 2013, an estimated 15.5 million caregivers provided 17.7 billion hours of unpaid care to those with AD, a value estimated at $220 billion. Based on these numbers, the cost of care is expected to increase to $1.2 trillion in 50 years (Alzheimer’s Association, 2015).

Two pathophysiologic hallmarks of the disease are the excess amount of amyloid beta (Aβ) plaques from amyloid precursor protein (APP) and neurofibrillary tangles (NFTs) formed by hyperphosphorylated tau protein. Specificity protein 1 (Sp1) is a transcription factor associated with AD pathology and it is the common transcription factor for APP and tau. Tau is a microtubule-associated protein (MAPT) that binds to tubulin, promoting the assembly and stabilization of microtubules. Normally, tau is phosphorylated by the main kinases cyclin-dependent kinase 5 (CDK5) and glycogen synthase kinase 3 beta (GSK-3β). When tau becomes hyperphosphorylated at phosphorylation sites, such as Threonine (Thr) 231 or Serine
(Ser) 396, it can no longer bind to the microtubules. As a result, tau begins to aggregate and first form paired helical filaments (PHF) and then NFT.

The biggest risk factor for developing AD is age (Vinters, 2014). Currently, 1 in 9 Americans over the age of 65 have AD and 1 in 3 people over 85 have the disease (Alzheimer’s Association, 2015). The risk of developing AD is expected to increase at an alarming rate over the next 50 years. This is because of the longer life expectancy and aging baby-boomers will account for 51% of the people diagnosed with AD ages 65 and older (Alzheimer's Association, 2015). Without a cure, the number of people diagnosed is expected to rise from 5 million to 16 million by 2050 (Alzheimer’s Association, 2015, Brookmeyer et al., 1998; Nelson et al., 2011).

The costs associated with creating a new drug are on average of one billion dollars, and require up to 12 years of research and development (Ng, 2011, PhRMA, 2013). Each phase of drug development progresses with risk and has a failure rate of over 95% (Hartung, 2013). As a result, many compounds or drugs are left abandoned in the pipeline with the potential to be repurposed. Repurposing a drug involves studying a pre-existing compound or biologic that is currently in use to treat a disease and determining if it can be used for the treatment of another disease (Ng, 2011). Furthermore, it is useful to study a drug that has already been approved and released to the market. This is because of the pharmacokinetic (PK) and pharmacodynamic (PD) parameters that have been established, saving research investment.

Since the identification of AD more than 100 years ago no drug has successfully targeted the pathological features of AD to alleviate and restore memory and cognition. However, drugs are available to treat symptoms of the disease such as
cholinesterase inhibitors (e.g. donepezil) and N-methyl-D-aspartate antagonists (e.g. memantine). Cholinesterase inhibitors prevent the breakdown of acetylcholine (Ach), a neurotransmitter involved in memory, learning, and thinking. Patients with AD have lower levels of Ach and thus it is believed to benefit someone with impaired memory (Fayed et al., 2011). Drugs such as donepezil (Aricept) are best prescribed during the early stages of AD. However, the approved drugs only delay the worsening of cognitive deficits for 6-12 months on average and at least half of the patients do not respond to them (Alzheimer’s Association, 2015). In addition, the neuronal damage caused by the disease cannot be reversed by these drugs (Mancuso et al., 2014, Ozudogru et al., 2012). Another cholinesterase inhibitor (e.g. tacrine) is no longer prescribed because liver damage was a serious side effect (Qizilbash et al., 2000).

Developing a safe drug that not only targets the symptoms but also reverses the cognitive deficits is a critical step in fighting AD. Instead of emulating the currently approved FDA medications which cannot cure the disease, we propose the drug, tolfenamic acid (TA), possess a unique mechanism of action different from what exists on the market.

TA is a non-steroidal anti-inflammatory drug (NSAID) currently approved for the treatment of migraines and rheumatoid arthritis in Europe. TA has been available for over 35 years and the PK and PD parameters are well cited. Unlike many NSAIDs that may cause gastric irritation, TA is a well-tolerated and safe drug (Alhava, 1994; Eskerod, 1994; Hendel, 1994; Isomaki et al., 1984). In addition, long-term studies reported minimal or no adverse effects with TA in gastrointestinal histology, renal
function, and liver function (Khwanjai 2012; Sankpal 2013). This makes TA a superior candidate to repurpose for AD research.

The distinct mechanism of TA targets the transcription factor Sp1, a zinc-finger DNA binding protein. It preferentially binds to GC-rich promoter regions and regulates the expression of housekeeping genes (Beishline and Azizkhan-Clifford, 2014 and Li et al., 2004). Sp1 is also studied in cancer research for its regulation of genes involved with cell growth, differentiation, apoptosis (Li et al., 2004; Kaczynski and Urrutia, 2003; Tan and Khachigian 2009). It has been noted that high Sp1 levels correlates with decreased survival for cancer patients (Li et al., 2004). Multiple cancer studies have shown TA’s ability to inhibit Sp1 levels (Abdelrahim et al., 2006; Basha et al., 2005, 2011; Colon, 2009; Eslin et al., 2013; Maliakhal et al., 2012; Sankpal et al., 2013). In fact, when compared to many of the available NSAIDs, TA was able to significantly decrease Sp1 (Abdelrahim et al., 2006). This was a strong indicator to select TA as a novel mechanism for AD.

Sp1 co-activates the transcription of APP and tau (Christensen et al., 2004; Docagne et al., 2004, Heicklen-Klein and Ginzburg, 2000). As a result, we have chosen to study this drug as a modifier of an upstream target for AD. It was shown that TA crosses the blood brain barrier within mice brains (Subaiea et al., 2011). In a follow-up study, wild-type C57BL/6 mice were dosed with TA and it was found that the gene expression of APP was lowered and protein levels of Sp1, APP, and its product Aβ were reduced relative to controls (Adwan et al., 2011).

Next, the transgenic mouse line (R1.40), hemizygous for the human APP gene, and SH-SY5Y cell line were used to investigate TA. It was reported that Sp1, APP,
and Aβ were reduced in both models (Adwan et al., 2013). More evidence was provided by dosing the same mouse model with TA for 34 days and finding a decrease in Sp1 and APP protein levels and Aβ, while Sp1 mRNA remained unchanged (Subaiea et al., 2013). Moreover, cognitive deficits and memory were recovered in behavioral tests, including the Morris water maze (MWM) and Y-maze.

Since Sp1 regulates the gene expression of both APP and tau, further research on tau was performed. Dosing R1.40 mice with TA for 34 days showed that tau mRNA and protein levels were lowered (Adwan et al., 2014). In addition, levels of phospho- tau (p-tau) at sites Thr 181 and Ser 235 were reduced. Based on the evidence that TA reduces tau in a human APP mouse model, we chose a new tau transgenic mouse model (htau) to study the effects of TA and tau proposed in Figure 1. It was important to select a mouse model that contained the human tau gene with a non-mutated promoter region. htau mice develop tau pathology slower when compared to some mouse models, which have accelerated tau accumulation and earlier impaired memory. This way, the human AD pathology is resembled more accurately.

To study whether TA is effective in treating htau mice, total tau, p-tau Thr231 and Ser396 were analyzed by Western blot. In addition, the gene expression of tau was measured by quantitative polymerase chain reaction (qPCR). Finally, the MWM and Probe Trial I and II behavior studies were performed to assess improvement in cognitive function.
CHAPTER 2
MATERIALS AND METHODS

Animal model

The htau mouse model, strain B6.Cg-Mapt<sup>tm1(EGFP)Klt</sup> Tg(MAPT)8cPdav/J, was obtained from Jackson Laboratory (Bar Harbor, ME). The animals are hemizygous for the human tau gene, expressing all six isoforms of the human tau (3R and 4R). The transgene contains the coding sequence, intronic regions, and regulatory elements of the endogenous human promoter region and they are homozygous for the knock-out murine tau. These mice aggregate paired helical filaments made of insoluble tau as early as 2 months and hyperphosphorylated tau is detected at 3 months of age. Visuospatial and learning deficits occur as early as 6 months assessed by the MWM. Mice were bred in-house in the designated animal facility at the University of Rhode Island with a 12:12 hour light-dark cycle (light on at 6:00 AM; light off at 6:00 pm). Food and water were provided ad libitum.

Genotyping of htau mice

Presence or absence of the human tau gene was determined through genotyping by standard polymerase chain reaction (PCR) using MJ Research MiniCycler PTC- 150 (Bio-Rad, Hercules, CA). Following the literature, DNA was isolated from 2 mm mouse tail snips (Turuet et al., 2000). Tail snips were placed in an Eppendorf tube and 75 µl of 25 mM NaOH/ 0.2 mM EDTA was added and placed in a thermocycler at 98 °C for 1 h. After, the tail snips were mechanically ground with a pipette tip and then the temperature was reduced to 15 °C for 30 min. Next, 75 µl of
40 mM Tris HCl (pH 5.5) was added and centrifuged at 4,000 rpm for 3 min and supernatants were collected. DNA integrity was checked using NanoDrop 2000 Micro- Volume UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, DE).

All the following reagents in this section were obtained from Invitrogen (Carlsbad, CA), unless otherwise stated. Standard PCR was used with the following primers: sense 5'-CGAAGTGATGGAAGATCACG-3' and antisense 5'-GTCTTGGTGCATGGTGTAGC-3'. Each reaction consisted of 44 µl Platinum PCR SuperMix, 2 µl of each primer, 1 µl 10X BlueJuice loading dye, and 1 µl DNA with the reaction cycle as follows: 95 °C for 2 min, 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 15 s for 35 cycles. Next, bands were separated on a 2% UltraPure agarose gel containing ethidium bromide by gel electrophoresis and imaged using Typhoon™ 9410 Variable Mode Imager (GE Healthcare, Piscataway, NJ).

Mice exposure to tolfenamic acid (TA)

The previous mouse model used by the lab (APP YAC, line R1.40) showed TA affected the amyloid and tau pathology. Our lab has shown that TA has the ability to decrease levels of APP, Aβ, tau, CDK5, β- APP cleaving enzyme 1 (BACE1), and Sp1 in this mouse model (Adwan et al., 2014; Subaeia et al., 2011, 2013, 2015). In addition, we have shown that memory and cognitive function were attenuated in these mice by administering TA.

TA was obtained from Sigma- Aldrich (St. Louis, MO). Since tau begins to aggregate at 3 months, male and female htau mice from different cohorts, aged 3-4 and 5-8 months, were used to examine TA's ability to lower tau pathology. Each
cohort was separated into 4 groups by similar age variation and gender and dosed with TA or corn oil vehicle by oral gavage for 34 consecutive days. On Day 15 of dosing, behavior studies began and concluded on Day 35 where mice were sacrificed and brain tissues were collected and stored at -80 °C until further use. Mice (aged 3-4 months) were divided into two control groups as carrier of the human tau (n= 10) and non-carrier of the human tau (n= 10). The third and fourth groups were given TA at a low dose of 5 mg/kg (n= 9) or high dose of 50 mg/kg (n= 10). Mice (aged 5-8 months) followed the same dosing scenario by oral gavage: control carrier (n= 5), control non-carrier (n= 12), and TA at 5 mg/kg (n= 8) or 50 mg/kg (n= 7). The timeline of behavior studies are shown in Figure 2. Female mice (aged 13 months) were also dosed with 0 (n= 3), 5 mg/kg (n= 3), or 50 mg/kg (n= 4) TA for 34 days for immunohistochemistry. All experiments were performed in accordance with the standard guidelines and protocol approved by the University of Rhode Island Institutional Animal Care and Use Committee with supervision of the university’s veterinarian.

**Morris water maze**

In the hidden version of the MWM, mice had to locate the hidden platform by learning multiple spatial cues by associating the platform location with the distal extramaze cues (Gulinello et al., 2009; Laczo et al., 2009; Vorhees and Williams, 2006). The maze apparatus consisted of a white pool (48" diameter, 30" height) filled with water to a depth of 14". White, non-toxic paint (Crayola, New York City, NY) was used to make the water opaque. Distinct visual cues were placed along the sides of the pool for the animals to navigate to the escape platform. A clear Plexiglas
platform 10 cm$^2$ was submerged 0.5 cm below the surface of the water and temperature of the water was maintained at 25 ± 2 °C during all procedures.

A habituation trial allowed the mice to swim freely for 60 s to acclimate to the experiment, which commenced on Day 15 of drug administration. Mice received 3 daily training sessions for a total of 8 days. The starting position was randomly assigned between four possible positions while the platform remained fixed for each trial. Each animal had a maximum duration of 60 s to swim to find the immersed, hidden platform. If a mouse failed to locate the platform, it would be guided to sit on the platform for 30 s. If a mouse was successful, it could sit on the platform for a maximum of 10 s. Following the completion of 8 training day sessions, probe trials were performed up to 60 s on Day 24 and Day 34 to assess long-term memory retention and the mice's preference for the correct quadrant where the hidden platform was previously located. The swim paths and latencies were videotaped and analyzed with a computerized video-tracking system (ObjectScan, Clever Sys. Inc., Reston, VA, USA).

**Protein extraction and Western blot analyses**

Brain cortices were homogenized with radio-immunoprecipitation assay (RIPA) lysis buffer (10 mM Tris- HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 1 mM ethylenediaminetetraacetic acid, 0.1% protease inhibitor cocktail, and 10 μl phosphatase inhibitor (Sigma- Aldrich). The homogenates were centrifuged at 8,000 rpm for 10 min at 4 °C and supernatants were collected. Protein concentration was determined by Pierce bicinchoninic assay (BCA) kit
(Thermo Scientific, Waltham, MA). Twenty- forty microgram of total protein were separated onto 10- 12% polyacrylamide gel at 100 V for 2 hours and then transferred to polyvinylidene difluoride (PVDF) membranes (GE- Healthcare). Membranes were blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline containing 0.5% Tween-20 (TBST) for 1 hour. Membranes were incubated with appropriate dilution of specific antibody in TBST overnight at 4 °C. The following antibodies were used: 1: 5,000 dilution of total tau (ab32057, Abcam, Cambridge, MA); 1: 500 Ser396 (PHF13) (9632, Cell Signaling, Beverly, MA); 1: 500 Thr231 (710126, Thermo Scientific); 1: 1000 GAPDH (Sigma-Aldrich). Membranes were washed with TBST 3 times and then incubated with the respective anti-mouse or anti-rabbit IRDye 680 (Li-Cor, Lincoln, NE) at dilution 1: 5,000- 10,000 for 1 h at room temperature. After washing the membranes with TBST 3 times and TBS 2 times, images were taken with Odyssey Infrared Imaging System (Li-Cor). Membranes were reprobed for GAPDH to obtain protein/GAPDH ratio. The intensities of the Western blot bands were obtained using Odyssey V1.2 Software (Li-Cor).

**RNA isolation, cDNA synthesis, and quantitative real-time PCR**

RNA was isolated from cortical tissue following the TRIZol Reagent method (Invitrogen), checked for integrity using NanoDrop 2000 Micro-Volume UV-Vis Spectrophotometer (Thermo Scientific) and reverse transcribed to cDNA using iScript Select cDNA Synthesis Kit using the manufacturer's instructions (Bio-Rad). About 1000 ng of RNA was diluted to 20 µl with nuclease-free water, 4 µl 5x iScript select reaction mix, and 1 µl of reverse transcriptase added. Samples were incubated at 42 °C
for 90 min and then at 85 °C for 5 min to terminate the reaction. All incubations were conducted using MJ Research MiniCycler PTC- 150 (Bio-Rad). Primer pairs for human tau and GAPDH were obtained from Invitrogen as follows: tau (exon 2-3) sense 5’-TGAACCAGGATGGCTGAGC-3’ and antisense 5’-
TTGTCATCGCTTCCAGTCCRT-3’ and GAPDH sense 5’-
AGGTGCGTGTGAACGGATTTG-3’ and antisense 5’-
TGTAGACCATGTAGTTGAGGTCA-3’. Each real-time PCR reaction mix contained 1 µl cDNA, 0.5 µl of each sense and antisense primer, 4.25 µl nuclease-free water, and 6.25 µl SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) following the standard protocol: 50 °C for 2 min followed by 95 °C for 10 min then 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. The reaction was performed and analyzed using ViiA7 RUO Software (Applied Biosystems) and expression data were reported relative to GAPDH mRNA using the 2-ΔΔCt method.

Perfusion Preparation and Immunohistochemistry

After 34 days of TA treatment, htau mice (aged 13 months) were prepared for immunohistochemistry on Day 35. Mice were deeply anesthetized with an intraperitoneal injection of 0.1 ml/10 g of xylazine- ketamine mixture (100 mg/ml- 10 mg/ml) and perfused transcardially with 100 ml of perfusion wash consisting of 0.8% sodium chloride, 0.8% sucrose, 0.4% dextrose, 0.034% anhydrous sodium cacodylate, and 0.023% calcium chloride. After that, mice were perfused with 100 ml of perfusion fix that consisted of 4% paraformaldehyde, 4% sucrose, and 1.07% anhydrous sodium cacodylate (pH 7.2- 7.4), and their brains were removed. The extracted brains were
post-fixed in the perfusion fix solution overnight and then they were cryopreserved in 30% sucrose solution. Fixed brains were sent to NeuroScience Associates (Knoxville, TN) for staining and coronal sectioning for p-tau Thr231 at a dilution of 1: 2,500. The frontal cortex and striatum were sectioned and the cell bodies and apical dendrites and axon tracts were stained, respectively.

**Statistical Analysis**

Data were represented as the mean ± standard error of the mean (SEM). Assessment of performance in MWM daily training sessions was determined using one-way ANOVA with Tukey-Kramer multiple comparison post-test, using GraphPad InStat 3 software (GraphPad Software, La Jolla, CA). Statistical analysis of western blot bands was determined by one-way ANOVA and Holm-Sidak comparisons with SigmaStat 3.5 software (Systat Software, Inc., San Jose, CA). Results with a p-value of <0.05 were considered statistically significant and were marked with asterisks accordingly.
CHAPTER 3
RESULTS

Body weight was monitored during the study with no significant change and no overt toxic effects of TA administration were observed when compared to the non-treated mice (Table 1). Standard PCR determined which mice contained the human tau transgene (Fig. 3). The presence of a band distinguished which mice were carriers of the human tau gene and which mice were non-carriers of the gene. In addition, Western blot confirmed that the carriers contained the human tau gene, shown by p-tau Ser396, while the non-carriers were absent of the band (Fig. 4). GAPDH was re-probed on the same membrane to confirm the presence of the protein. The effects of TA on tau are reported below.

Evaluation of cognitive function in htau mice

Visuospatial and learning memories were assessed by the MWM in animals aged 3-4 months. To ensure carrier mice of the human tau gene were an appropriate control group with impaired memory at this age, they were compared to the non-carrier htau mice. After the second day of training, the control carriers exhibited slower learning when compared to the control non-carrier group (Fig. 5). After Day 2, the non-carriers consistently had faster escape latencies between the two control groups.

Administration of tolfenamic acid restores cognitive function in htau mice

It was previously shown that mice in the control carrier group have impaired cognitive function and memory deficit in the MWM. To further study if TA improves
the memory deficits in the behavior study, 5 and 50 mg/kg TA was administered to animals (aged 3-4 months). Over the training course of 8 days, both the memory in the 5 and 50 mg/kg TA were attenuated with drug treatment when compared to the control group (Fig. 6). Both TA treatment groups had improved learning acquisition with faster escape latencies than the control carrier. The 5 mg/kg TA group had the fastest escape latency between the groups and learning memory was significantly improved in Days 3, 4, and 7, measured by one-way ANOVA (p < 0.05). In addition, probe trials, which assessed the percent time spent in the correct quadrant containing the hidden platform, demonstrated that the control carriers had learning deficits compared to the control non-carrier, 5 and 50 mg/kg TA in both Day 24 and Day 34 (Fig. 7). This further illustrated that not only do the non-carriers retain the long-term memory of the platform's location, but that animals dosed with TA recovered their cognitive function as shown by significantly spending more time in the correct quadrant with the hidden platform measured by one-way ANOVA (p < 0.05).

**Tolfenamic acid decreases tau protein levels and gene expression**

Following 34 days of TA administration, total tau protein levels were significantly decreased in both the 5 and 50 mg/kg TA treated groups when compared to the control carriers as measured by Western blot (Fig. 8a). Tau protein levels were quantified as a ratio to housekeeping protein GAPDH (Fig. 8b). Statistics was determined by one-way ANOVA (p = 0.032) and significance by Holm-Sidak post-test (p < 0.05). In addition, tau gene expression was lowered with TA treatment. Tau
mRNA were reduced by 12% and 23% in the 5 and 50 mg/kg, respectively, compared to the control carriers (Fig. 9).

**Phosphorylated tau is lowered with tolfenamic acid treatment**

Hyperphosphorylated tau loses its ability to bind and stabilize microtubules so levels of p-tau were examined with TA. p-tau Ser396 protein levels were lowered in both 5 and 50 mg/kg TA groups analyzed by Western blot (Fig. 10a). Band intensities were measured (Fig. 10b). Protein levels of p-tau Thr231 were also reduced after dosing with TA. Thr231 levels were decreased with drug treatment measured by Western blot (Fig. 11a). Thr231 protein was quantified with GAPDH levels and was reduced (Fig. 11b) as determined by one-way ANOVA (p = 0.007) with Holm-Sidak post-test (p < 0.05).

**Immunohistochemistry**

As previously described, htau mice begin aggregating hyperphosphorylated tau at 3 months of age. After treating mice with 0, 5, and 50 mg/kg TA, mice (aged 13 months) brains were sectioned and stained for p-tau Thr231 (Fig. 12; 5 mg/kg TA not shown). The frontal cortex showed decreased Thr231 staining in the cell bodies and dendrites with the TA treatment compared to the control group. Dosing with TA also reduced the Thr231 intensity staining of the axon tracts in the striatum.
CHAPTER 4
DISCUSSION

TA is a novel drug for AD treatment. It has been used for the management of migraines and rheumatoid arthritis for over 35 years in Europe. This makes TA a well-established and promising drug for repurposing in AD. Although the anti-inflammatory benefits of NSAIDs have been studied in AD (Weggan et al., 2001; Wilkinson et al., 2012), it is TA's unique mechanism to chelate Sp1 that makes it an outstanding candidate for drug development in AD (Abdelrahim et al., 2006; Adwan et al., 2011; unpublished data). Many cancer studies have reported TA inhibition of Sp1 (Basha et al., 2006, 2011; Colon et al., 2011; Eslin et al., 2013; Maliakal et al., 2012; Sankpal et al., 2012; Sutphin et al., 2014). Sp1 was shown to be elevated in the frontal cortex of AD brains and in the brains of AD mouse models (Citron et al., 2008; Sanpere et al., 2006). Targeting this transcription factor is not likely to produce detrimental effects because Sp1 binds to CG-rich promoter regions which are present in <10% of genes (Levotsky and Dynan, 1989). In addition, Sp1 is essential during early embryonic development but not necessary for cell growth and differentiation later in life (Marin et al., 1997). Although TA can affect both APP and tau through Sp1, other studies suggest targeting tau may be more effective than the APP pathway.

The amyloid hypothesis has driven the development of many drugs that target the Aβ pathway and thus far, no drug has been successfully approved. For example, the hopeful anti-amyloid immunotherapy drugs, which use antibodies against Aβ (e.g. bapineuzumab and solanezumab) made it to Phase 3 clinical trials. However both drugs failed in that stage, showing no significant difference between the treatment
groups and control (Salloway et al., 2014). Semagacestat, a drug that inhibits the cleaving process of APP, has also failed in Phase 3. Moreover, 65.6% of drugs targeting the Aβ pathway from 2002-2012 have failed, showing no efficacy against this target (Cummings et al., 2014). As a result, we have shifted our focus on tau since it is more associated with cognitive failure than Aβ (Medina, 2011; Terry et al., 1964; Thal et al., 2004). Reducing tau and Aβ levels did improve cognition in mouse models that express tangles and plaques; however, lowering Aβ alone did not improve cognitive function (Iqbal et al., 2009). Additionally, AD is the most common tauopathy and by focusing on tau, it may help treat other diseases.

Tauopathies are a class of over 20 neurodegenerative diseases characterized by hyperphosphorylated tau and impaired memory with no known cure (Brunden et al., 2009; Lee et al., 2001; Santpere et al., 2006). Here, tau and cognitive deficits exist without the presence of Aβ (Iqbal et al., 2010). In fact, tau pathology is more correlated with dementia in humans than β-amyloidosis (Terry et al., 1964; Thal et al., 2004). By targeting tau and the cognitive deficits associated with it, TA could be effective in such tauopathies like frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17), progressive supranuclear palsy, and Pick's disease.

TA's distinct mechanism of action is a promising, upstream intervention that is able to target tau and other AD pathological features. Furthermore, we have previously shown that administering TA in mice lowers Sp1 levels, reducing the subsequent AD biomarkers such as APP, Aβ, BACE1, CDK5, p-tau Ser235 and Thr181 (Adwan et al., 2011, 2014; Subaeia 2013, 2015). p-tau Thr231 and Ser396 are almost fully phosphorylated in PHF tau (Morishima-Kawashima et al., 1995). In addition, Thr231
phosphorylation inhibits tau's microtubule-binding capability by 26% (Sengupta et al., 1998). TA has the ability to decrease tau and its phosphorylated state as shown in Figure 8, 10, and 11. In the MWM, the low dose of 5 mg/kg TA showed better improvement in learning and memory compared to the 50 mg/kg TA. This reflects the human equivalent dose more accurately, where it is recommended to take a dose of 200 mg, up to 3 times a day, during a migraine attack (EMEA, 1997).

We have proposed a novel drug that can not only recover cognitive function in mice that contain the human tau gene, but also demonstrated that tau can be reduced in older mice with tau aggregation. The current AD drugs are not disease-modifying, as they only mask the symptoms and the memory is not attenuated. TA has the potential to treat AD by targeting crucial biomarkers involved in this neurodegenerative disease.
Figure 1. Proposed mechanism of tolfenamic acid (TA) acting on transcription factor Sp1. TA prevents Sp1 from binding to the promoter region of the tau gene, reducing the amount of tau protein and potential NFT, resulting in lower AD pathology.
Figure 2. Timeline of dosing mice daily with TA for 34 days. Morris water maze (MWM) started on day 15 and continued for eight consecutive days. Following MWM conclusion, Probe Trial I was performed on Day 24 and Probe Trial II on Day 34, to assess long-term memory retention. Mice were sacrificed on Day 35 and tissues were collected.
Figure 3. Genotyping of htau mice analyzed by standard PCR. Bands represent human tau transgene while blank lanes represent mice without the human tau gene.
Figure 4. The presence or absence of the human tau gene in htau mice analyzed by Western blot. Using p-tau Ser396-specific antibodies, cortical p-tau Ser396 was not found in non-carrier (NC) mice and while carrier (C) mice had Ser396 protein present. Housekeeping protein GAPDH was re-probed on the same membrane.
Figure 5. Impaired memory in the carrier animals were seen when compared to the non-carrier group in the Morris water maze. Learning deficits were seen in the carriers at Day 2 of training and continued to have a longer escape latency compared to the non-carrier. Statistics were analyzed by one way ANOVA with Tukey-Kramer post hoc test (p < 0.05), control non-carrier n= 10, control carrier n= 10.
Figure 6. Administration of tolfenamic acid attenuates memory observed in the Morris water maze. "*" indicates significantly different from control, determined by one-way analysis of variance with Tukey-Kramer post hoc test to compare all the columns (*p < 0.05, **p < 0.01), obtained using GraphPad Instat 3 software. Control n= 10, 5 mg/kg = 9, 50 mg/kg = 10.
Figure 7. Long-term memory assessment of htau mice in probe trials after dosing with tolfenamic acid. The time spent in correct quadrant after acquisition trial-training trials was measured (A) Probe Trial I (Day 24). (B) Probe Trial II (Day 34). "*" indicates significantly different from the other groups measured by one way ANOVA with Tukey-Kramer post hoc test (p < 0.05) using GraphPad InStat3 software. Control non-carrier n = 10, control carrier n = 10, 5 mg/kg = 9, 50 mg/kg = 10.
Figure 8. Cortical tau levels after administration of tolfenamic acid. (A) Total tau protein levels measured by Western blot. (B) Quantitative total tau protein levels normalized to GAPDH levels. Values shown are for the mean ± SEM, n= 4. "*" indicates significantly lower than the control determined by one way ANOVA (p = 0.032) and p < 0.05 according to Holm- Sidak post-test.
Figure 9. Tau gene expression decreased with tolfenamic acid administration. Tau mRNA was measured in cortical tissue by real-time PCR (qPCR) with GAPDH as endogenous control. Values are shown as ±SEM, control n= 4; 5 mg/kg TA n= 5; 50 mg/kg TA n= 5.
Figure 10. Levels of phosphorylated tau at Ser396 after dosing with tolfenamic acid. (A) p-tau Ser396 protein represented by Western blot. (B) Protein levels were quantified as protein-to-ratio of housekeeper GAPDH. Values shown are for the mean ± SEM, n=4).
Figure 11. Phosphorylated levels of tau at site Thr231 after tolfenamic acid administration. (A) p-tau Thr231 protein levels determined by Western blot analysis. (B) Protein quantification was normalized to GAPDH protein levels. Values are shown for the mean ± SEM, n=4. "*" denotes statistically different from control measured by one-way ANOVA (p = 0.07) and p < 0.05 according to Holm-Sidak post-test.
Phosphorylated tau staining was reduced following treatment with tolfenamic acid. Cell bodies, dendrites and axonal tracts in the frontal cortex and striatum were stained for p-tau Thr231 in the control and 50 mg/kg tolfenamic acid group.
Table 1. Average weight of each treatment group over administration of either control corn oil or tolfenamic acid showed no serious change in weight or toxic effects of tolfenamic acid during the 34 days of dosing, followed by sacrifice on Day 35.

<table>
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<tr>
<th>Groups</th>
<th>Day 1 (g)</th>
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<tr>
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<tr>
<td>Control Non-carrier</td>
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BIBLIOGRAPHY


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