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Article in *Journal of Neurochemistry* · October 2014

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ORIGINAL
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implications for dementia and tauopathies

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Abstract

Tau and its aggregates are linked to the pathology of Alzheimer's disease (AD) and other tauopathies and, therefore, are explored as therapeutic targets for such disorders. Tau belongs to a family of microtubule-associated proteins that promote microtubule assembly. When hyperphosphorylated, tau becomes prone to forming aggregates. Increased brain levels of hyperphosphorylated tau correlate with dementia. Specificity protein 1 (Sp1), a transcription factor elevated in AD, is responsible for the transcription of AD-related proteins including the amyloid precursor protein, tau, and its cyclin-dependent kinase-5 (CDK5) activators. Tolfenamic acid promotes the degradation of Sp1, our previous studies demonstrated its ability to down-regulate transcriptional

targets of Sp1 like amyloid precursor protein and reduce amyloid beta (A β), the main component of AD plaques. In this study, we administered tolfenamic acid daily to hemizygous R1.40 transgenic mice for 34 days, and examined tau and CDK5 gene and protein expression within the brain. Our results demonstrate that tolfenamic acid lowers tau mRNA and protein, as well as the levels of its phosphorylated form and CDK5. Thus, we present a drug candidate that inhibits the transcription of multiple major intermediates in AD pathology, thereby helping uncover a new mechanism-based approach for targeting AD.

Keywords: Alzheimer's disease, cyclin-dependent kinase-5, Sp1, tau, therapy, tolfenamic acid.

J. Neurochem. (2015) **133**, 266–272.

Over 100 years have passed since the identification of Alzheimer's disease (AD) and no disease modifying drug has been found for this disorder. Current therapies try to recover the deteriorating mental functions by targeting symptoms, but fail to alter the debilitating course of the disease that ultimately leads to total memory loss and death. None of the few available medications targets the characteristic pathological aggregates in AD, the extracellular senile amyloid plaques and the intracellular neurofibrillary tau tangles.

The microtubule-associated protein tau was first isolated and recognized for its role in microtubule assembly in 1975 (Weingarten *et al.* 1975). In AD and other tauopathies, tau assembles forming pathological deposits. AD is the most common tauopathy where hyperphosphorylated tau aggregates as paired helical filaments (PHFs) and tangles (Grundke-Iqbal *et al.* 1986; Lee *et al.* 1991, 2001; Goedert 1997; Brunden *et al.* 2009). The normal function of tau is to stabilize microtubules, and the exact cause of its aggregation remains unknown. Mutations in the tau gene have been

coupled with frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17) where tau aggregates and not plaques are the characteristic deposits (Hong *et al.* 1998; Hutton *et al.* 1998; Gao *et al.* 2005). Tau mutations are responsible for 5% of frontotemporal dementia cases (Goedert and Spillantini 2011). Tau hyperphosphorylation reduces its binding to microtubules and plays a role in its

Received July 29, 2014; revised manuscript received September 12, 2014; accepted September 29, 2014.

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Abbreviations used: AD, Alzheimer's disease; APP, amyloid precursor protein; A β , amyloid β ; BACE1, beta-site APP cleaving enzyme 1; CDK5, cyclin-dependent kinase-5; GSK3 β , glycogen synthase kinase-3 beta; MAPs, microtubule-associated proteins; PHFs, paired helical filaments; P-tau, phosphorylated tau; SP1, Sp1 protein; Sp1, Specificity protein 1.

aggregation (Drechsel *et al.* 1992; Iqbal *et al.* 1994; Alonso *et al.* 1997; Goedert 1997; Brunden *et al.* 2009). Hyperphosphorylated tau lacks its normal function of binding to microtubules and forms neurofibrillary aggregates (Beyreuther and Masters 1996). Moreover, hyperphosphorylated tau suppresses microtubules assembly and can sequester normal tau and high molecular weight microtubule binding proteins, restraining their normal functions (Drechsel *et al.* 1992; Alonso *et al.* 1997; Iqbal *et al.* 2009, 2010; Medina 2011). This suggests that phosphorylation regulates the functions of tau. The main enzymes responsible for tau phosphorylation are glycogen synthase kinase-3 beta (GSK3 β) and cyclin-dependent kinase-5 (CDK5).

Specificity protein 1 (Sp1) is a transcription factor involved in AD pathology. Sp1 gene expression and protein levels are elevated within the frontal cortex of AD patients and animal models with AD-like pathology (Basha *et al.* 2005; Zawia and Basha 2005; Santpere *et al.* 2006; Brock *et al.* 2008; Citron *et al.* 2008). Sp1 binds to GC rich promoter regions within the amyloid precursor protein (APP), beta-site APP cleaving enzyme 1 (BACE1), and tau genes and promotes their transcription (Salbaum *et al.* 1988; Pollwein *et al.* 1992; Hoffman and Chernak 1995; Heicklen-Klein and Ginzburg 2000; Christensen *et al.* 2004; Docagne *et al.* 2004; Gao *et al.* 2005; Citron *et al.* 2008). Sp1 regulates the expression of tau and mutations on the Sp1-binding regions on the tau promoter decrease tau expression (Heicklen-Klein and Ginzburg 2000; Gao *et al.* 2005). Sp1 protein (SP1) is co-localized with hyperphosphorylated tau in AD tangles (Santpere *et al.* 2006). Sp1 also regulates the transcription of CDK5 activators p39 and p35 with Sp1 binding motifs found on CDK5, p39, and p35 promoter regions (Ohshima *et al.* 1995, 1996; Ross *et al.* 2002; Valin *et al.* 2009). CDK5 is responsible for the phosphorylation of tau on sites that are unusually hyperphosphorylated in AD (Paudel *et al.* 1993; Ohshima *et al.* 1995).

Tolfenamic acid, a drug used in Europe for migraine headaches, promotes SP1 degradation, and hence lowers the expression of genes regulated by Sp1 including APP and BACE1 and reduces their cleavage product amyloid beta ($A\beta$) (Abdelrahim *et al.* 2006; Adwan *et al.* 2011, 2014). Tolfenamic acid also improves cognition in mice (Subaiea *et al.* 2013), and is currently scheduled for a biomarker study in AD patients. Data obtained by our collaborators demonstrated that chronic administration of tolfenamic acid was not toxic and had no adverse effects on animals' weight, hematocrit, stomach, or intestinal lining integrity compared to control (Sankpal *et al.* 2013).

As lowering of hyperphosphorylated tau correlates with cognitive improvement (Iqbal *et al.* 2009; O'Leary *et al.* 2010; Medina 2011), this study was designed to test the ability of tolfenamic acid to down-regulate the expression of tau and CDK5 via its unique capability to promote the degradation of SP1 (Fig. 1). This would provide more

evidence for tolfenamic acid as a broad spectrum drug able to interrupt multiple pathways in the neurodegenerative process and offer more promise in its use in the upcoming clinical studies.

Materials and methods

Chemicals and reagents

All materials used were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

Animals

APP YAC transgenic mice, line R1.40, were used in this study (The Jackson Laboratory, Bar Harbor, ME, USA). These animals were particularly used to demonstrate in the same mouse model, the ability of tolfenamic acid to impact pathways associated with both amyloid and tau pathology. The ability of tolfenamic acid to decrease the levels of SP1, APP and $A\beta$ and reduce BACE1 mRNA and activity in these same animals has already been published by us, along with behavioral tests demonstrating cognitive improvement following tolfenamic acid treatment in these mice (Subaiea *et al.*

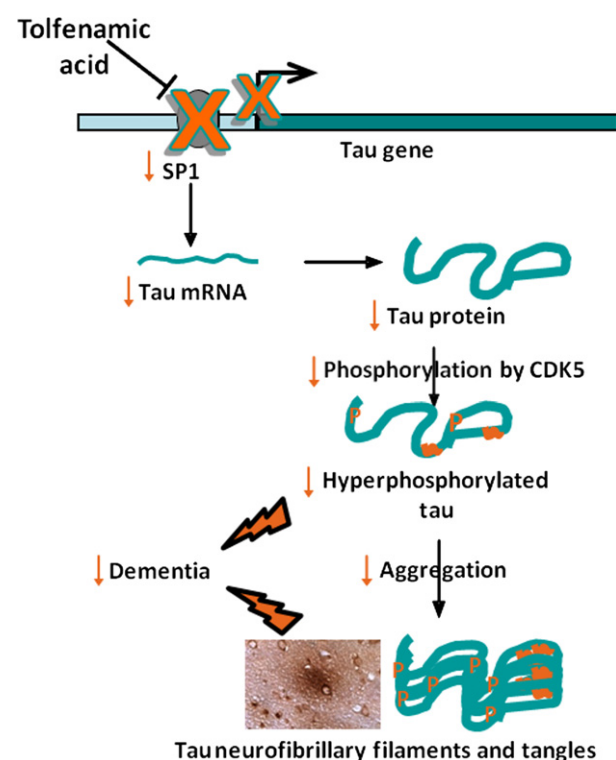


Fig. 1 Proposed transcription-based mechanism of tau and cyclin-dependent kinase-5 (CDK5) down-regulation by tolfenamic acid. Tolfenamic acid induces the degradation of the transcription factor Specificity protein 1 (Sp1) (Abdelrahim *et al.* 2006; Adwan *et al.* 2011; Subaiea *et al.* 2013), which reduces the transcription of its target genes such as tau and CDK5 activators, resulting in a decrease in the total levels of tau as well as the pathogenic phosphorylated tau species.

2013; Adwan *et al.* 2014). Animals were housed in designated rooms within the animal facility at the University of Rhode Island. Female hemizygous mice bred in-house were assigned into three groups of similar age variations between 14 and 21 months of age, $n = 6$ in each group. Animals were administered 0, 5, or 50 mg/kg tolfenamic acid in corn oil everyday by oral gavage for 34 days. On day 35, mice were killed and brain tissues were collected and stored at -80°C until further use. The detailed information on the breeding and exposure scenario have been already published along with the results from the biochemical and behavioral experiments conducted on these animals (Subaiea *et al.* 2013). All experiments were performed in accordance with the standard guidelines and the protocol approved by the Institutional Animal Care and Use Committee of the University of Rhode Island.

RNA isolation, cDNA synthesis, and real-time PCR

RNA was isolated from cerebral cortex tissue following the TRIzol® reagent method (Invitrogen, Carlsbad, CA, USA), checked for integrity by NanoDrop (Thermo Scientific, Wilmington, DE, USA), and reverse transcribed to cDNA using iScript™ Select cDNA Synthesis Kit following manufacturer's instructions (Bio-Rad, Hercules, CA, USA). About 1000 ng of RNA were diluted to 19.5 μL with nuclease free water, then 3 μL Oligo (dT) mix, 6 μL 5x iScript Select reaction mix, and 1.5 μL of iScript reverse transcriptase were added. Samples were incubated at 42°C for 90 min then at 85°C for 5 min to terminate the reaction. All incubations were conducted using MJ Research MiniCycler™ (Bio-Rad). Primer pairs for mouse tau, CDK5, β -actin, and GAPDH were obtained from Invitrogen as follows: tau sense: 5'-GTGGCCAGG TGGAAGTAAA-3' and antisense: 5'-TGGAAGACACATTGC TGAGG-3'; CDK5 sense: 5'-GGCTAAAACCGGGAAACTC-3', and antisense: 5'-CCATTGCAGCTGTCGAAATA-3'; β -actin sense: 5'-TGTTACCAACTGGGACGACA-3', and antisense: 5'-TCTCAGCTGTGGTGGTGAAG-3'; GAPDH sense: 5'-AGCTGAA CGGGAAGCTCACT-3', and antisense: 5'-AGGTCCACCACTGA CACGTTG-3'. Each real-time PCR reaction mix contained 2 μL of cDNA, 1 μL of each primer, 8.5 μL nuclease free water, and 12.5 μL SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Real time PCR was conducted using the 7500 Real Time PCR System (Applied Biosystems) following the standard protocol: 50°C for 2 min followed by 95°C for 10 min, then 40 cycles of 95°C for 15 s, and 60°C for 1 min. Results were analyzed using the 7500 system software with relative quantification method and β -actin or GAPDH as endogenous control.

Protein extraction and western blot analyses

Cerebral cortex tissue was homogenized with radio-immunoprecipitation assay 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 (Thermo Fisher Scientific, Waltham, MA, USA), and 0.1% protease inhibitor cocktail). The homogenates were centrifuged at 10,600 g for 10 min at 4°C and supernatants were collected. Protein concentration was determined using the Micro BCA Protein Assay Kit (Thermo Scientific Pierce, Rockford, IL, USA). Protein extracts were stored at -80°C until further use. For western blot analyses, approximately 40 μg of protein samples was separated onto 4–15% precast polyacrylamide gels (Bio-Rad) at 150 V for 1–2 h and then transferred to polyvinylidene fluoride

membranes (GE-Healthcare, Piscataway, NJ, USA). Membranes were blocked and incubated with the appropriate dilution of the specific primary antibody for 1–2 h. The antibodies used were as follows: 1 : 1000 dilution of T9450 for total tau levels (Sigma-Aldrich); 1 : 1000 of CDK5 #2506 (Cell Signaling, Beverly, MA, USA); 1 : 1000 of phosphorylated tau (P-tau) at Thr 181 #5383 (Cell Signaling); 1 : 1000 of P-tau at Ser 235 ab30664 (Abcam, Cambridge, MA, USA); 1 : 5000 of β -actin A2013 (Sigma-Aldrich); or 1 : 2000 of GAPDH T9450 (Sigma-Aldrich), then the membranes were washed with tris-buffered saline containing 0.05% Tween 20 and incubated with the appropriate infrared dye-labeled secondary antibody (Li-Cor, Lincoln, NE, USA) for 1 h at room temperature (25°C) in the dark. Infrared signal of western blot bands was detected and quantified using Odyssey® Infrared Imaging System (Li-Cor). Western blot protein levels for tau, CDK5, and P-tau were normalized against the levels of the house keeping proteins β -actin or GAPDH.

Statistical analysis

Data were represented as the mean \pm SEM. Statistical analysis was performed using GraphPad InStat software (GraphPad software, San Diego, CA, USA) and statistical significance was determined by one-way ANOVA and Tukey–Kramer multiple comparisons post-test. Results with a p -value of < 0.05 were considered statistically significant, and were marked with asterisks accordingly.

Results

Targeting neurofibrillary tau pathology of AD by influencing the transcription factor Sp1 is a new therapeutic approach that can be extended to other tauopathies. Studies from our laboratory have already provided evidence that tolfenamic acid crosses the blood brain barrier and is able to lower SP1 and subsequently reduce APP and BACE1 transcription and A β levels within mice brains as well as improve cognitive functions (Adwan *et al.* 2011, 2014; Subaiea *et al.* 2011, 2013). The safety profile of tolfenamic acid has already been established. This drug has been approved and used in Europe for the management of migraine headaches and rheumatoid arthritis for decades. In our experiments, we did not observe any toxic effects on animals administered tolfenamic acid. In this study tolfenamic acid was given daily to mice for 34 days to study the effects on tau gene expression and protein levels. The data reported below also show the effects of tolfenamic acid treatment on various intermediates in tau pathology including CDK5 and P-tau at Ser 235 and Thr 181.

Tolfenamic acid lowers tau gene expression and total tau levels *in vivo*

By inducing SP1 degradation and reducing its levels in these animals (Subaiea *et al.* 2013), we hypothesized that tolfenamic acid would also reduce the gene expression of its transcriptional targets like tau (Abdelrahim *et al.* 2006; Adwan *et al.* 2011). Following the administration of tolfenamic acid to mice daily for 34 days, tau gene expression was lowered by 48% with both the 5 and 50 mg/kg doses as

demonstrated by real time PCR (Fig. 2). Statistical significance was determined by one-way ANOVA [$F(2, 14) = 10.287$, $p = 0.0018$], followed by Tukey–Kramer multiple comparisons post-test $p < 0.001$ for the control (C) versus 5 mg/kg group, $p < 0.05$ for the C versus the 50 mg/kg group. Furthermore, tolfenamic acid decreased total tau protein levels by 46% with both doses as measured by western blot analysis (Fig. 3). One-way ANOVA $F(2, 11) = 6.446$, $p = 0.014$. Tukey–Kramer post-test $p < 0.05$ for the C versus the 5 mg/kg group and for the C versus the 50 mg/kg group.

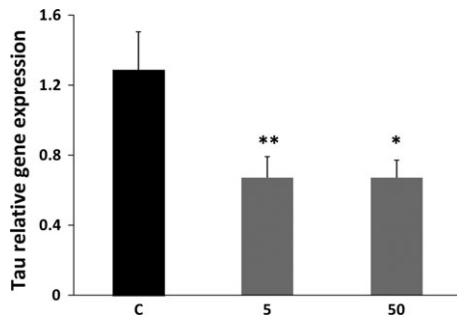


Fig. 2 Tau relative gene expression in cerebral cortex tissues from mice treated with tolfenamic acid daily for 34 days. Mice were administered 0, 5 or 50 mg/kg tolfenamic acid everyday for 34 days. Tau mRNA levels were measured in the cerebral cortex by real time PCR, with β -actin used as an endogenous control as mentioned in the methods section. Values shown are for the mean \pm SEM, $n = 6$ in each group, $p = 0.018$ as determined by one-way ANOVA with Tukey–Kramer post-test * $p < 0.05$; ** $p < 0.01$.

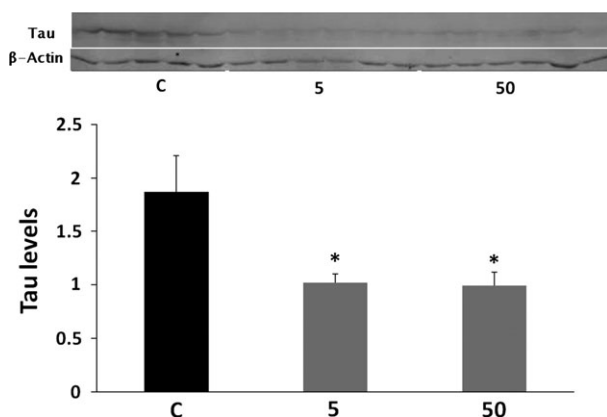


Fig. 3 Tau levels following tolfenamic acid administration. Total tau protein levels were analyzed in the cerebral cortex following daily administration of tolfenamic acid to transgenic mice for 34 days by western blot analysis. Values shown are for the mean \pm SEM, $n = 5$. Tau levels were normalized to the levels of the house keeping protein β -actin. One-way ANOVA $p = 0.014$, with Tukey–Kramer post-test * $p < 0.05$. Insert shows representative control (C), 5 or 50 mg/kg treatment tau or β -actin western blot bands.

Tolfenamic acid decreases the gene and protein expression of CDK5 in mice

As Sp1 also regulates CDK5 activators (Valin *et al.* 2009), we tested the effects of tolfenamic acid on CDK5. We found that daily administration of tolfenamic acid to mice for a month lowered the gene expression of CDK5 in the cerebral cortex by about 50% (Fig. 4). One-way ANOVA $F(2, 13) = 59.647$, $p = 2.8 \times 10^{-7}$. Tukey–Kramer post-test $p < 0.05$ for the C versus the 5 mg/kg group and for the C versus the 50 mg/kg group. There was a lowering trend in CDK5 levels (Fig. 5) that was not significant when analyzed with one-way ANOVA [$F(2, 8) = 4.086$, $p = 0.059$]. However, when comparing the 50 mg/kg dose group to the control group by Tukey–Kramer test, the 40% lowering in CDK5 from control was statistically significant ($p < 0.05$).

Tolfenamic acid reduces the expression of phosphorylated tau

As phosphorylation of tau affects its function and its ability to bind to microtubules (Alonso *et al.* 1997, 2008; Sengupta

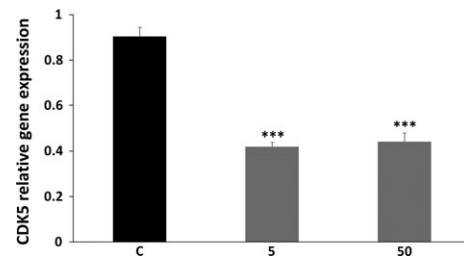


Fig. 4 Cyclin-dependent kinase-5 (CDK5) gene expression after tolfenamic acid treatment. CDK5 mRNA levels in mice cortices were measured with real time PCR with GAPDH as an endogenous control as mentioned in the methods section. Values shown are for the mean \pm SEM, $n = 5$. One-way ANOVA *** $p < 0.0001$ as determined by Tukey–Kramer post-test.

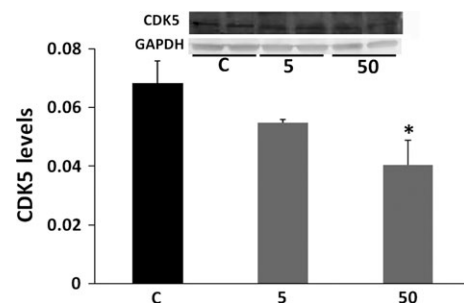


Fig. 5 Cyclin-dependent kinase-5 (CDK5) levels following tolfenamic acid treatment. CDK5 levels in cerebral cortices of mice administered tolfenamic acid or control for 34 days were obtained by western blot analysis. CDK5 levels were normalized to GAPDH levels. Values shown are for the mean \pm SEM, $n = 4$. One-way ANOVA $p = 0.059$. * $p < 0.05$ according to Tukey–Kramer post-test. Insert shows representative control (C), 5 or 50 mg/kg treatment CDK5 or GAPDH western blot bands.

et al. 1998), it was important to test how phosphorylated tau is affected by the treatment. P-tau levels were analyzed by western blotting using specific antibodies. P-tau at Ser 235 and P-tau at Thr 181 were lowered by both doses of tolfenamic acid (Fig. 6 and 7). Tau phosphorylated at Ser 235 was lowered by about 15% as indicated by one-way ANOVA [$F(2, 11) = 6.105, p = 0.0165$], Tukey–Kramer post-test $p < 0.05$ for the C versus the 5 mg/kg group and for the C versus the 50 mg/kg group. P-tau at Thr 181 was lowered by about 30%, one-way ANOVA $F(2, 10) = 7.272, p = 0.0112$, Tukey–Kramer post-test $p < 0.05$ for the C versus the 5 mg/kg group and for the C versus the 50 mg/kg group.

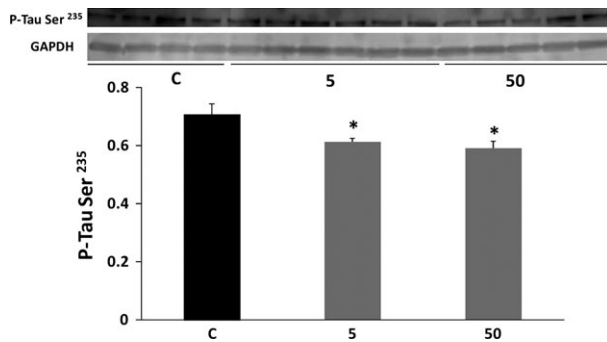


Fig. 6 Levels of tau phosphorylated at Ser 235 after tolfenamic acid treatment. P-tau levels were measured by western blot analysis and normalized to GAPDH as mentioned in the methods section. Values shown are for the mean \pm SEM, $n = 5$. One-way ANOVA $p = 0.0165$. * $p < 0.05$ according to Tukey–Kramer post-test. Insert shows representative C, 5 or 50 mg/kg treatment P-tau at Ser 235 or GAPDH western blot bands.

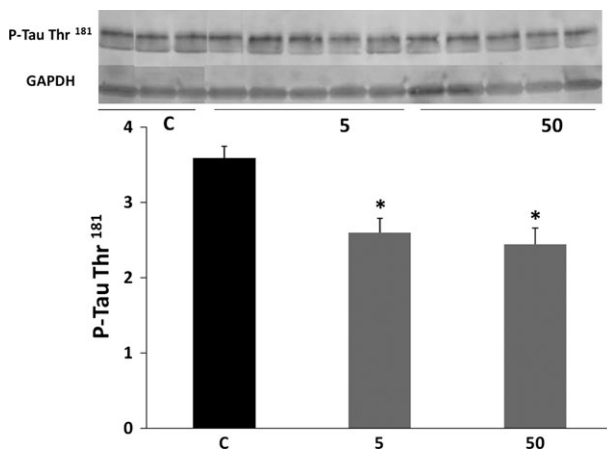


Fig. 7 Levels of tau phosphorylated at Thr 181 following tolfenamic acid exposure. P-tau levels were measured by western blot and normalized to GAPDH levels. Values shown are for the mean \pm SEM, $n = 5$. One-way ANOVA $p = 0.0112$. * $p < 0.05$ according to Tukey–Kramer post-test. Insert shows representative C, 5 or 50 mg/kg treatment P-tau at Thr 181 or GAPDH western blot bands.

Discussion

Tolfenamic acid, a drug already available in the European market for the management of migraine headaches, represents a novel class of drugs that could be repurposed for AD due to its unique ability to promote the degradation of SP1 (Abdelrahim *et al.* 2006; Adwan *et al.* 2011), a transcription factor that has been linked to AD tau and A β pathology (Docagne *et al.* 2004; Santpere *et al.* 2006; Brock *et al.* 2008; Citron *et al.* 2008). Previous studies from our laboratory demonstrate that by lowering SP1, tolfenamic acid was able to decrease the transcription of APP as well as A β levels in mice following 2 weeks of daily administration (Adwan *et al.* 2011). Our studies show that tolfenamic acid is readily available in the brain after dosing (Adwan *et al.* 2011; Subaiea *et al.* 2011). Behavioral and biochemical analyses have also revealed that tolfenamic acid lowers Sp1, APP, BACE1 mRNA and activity in addition to A β and improves cognition in the APP transgenic mouse model used in this study (Subaiea *et al.* 2013; Adwan *et al.* 2014).

Drug discovery for AD has focused on targeting intermediates mentioned in the amyloid hypothesis of AD including APP and A β , and so far no successful disease-modifying candidate has been found for this devastating disorder. Much less attention was paid to tau which is abnormally hyperphosphorylated and forms aggregates in AD. More recent studies have found a better correlation between tau and memory impairment in AD (Medina 2011). In a transgenic mouse model that expresses plaques and tangles, lowering both soluble tau and A β caused cognitive improvement, whereas lowering only soluble A β did not improve cognition (Oddo *et al.* 2006). Tangles are later manifestations of tau pathology and soluble phosphorylated tau is the species responsible for neurodegenerative damage (Iqbal *et al.* 2009; Medina 2011).

As cognitive impairment is better correlated with the presence of tau and as Sp1 regulates tau expression (Heicklen-Klein and Ginzburg 2000; Iqbal *et al.* 2009; Medina 2011), we sought to study the effects of tolfenamic acid on the tau pathology in the same animals where we observed its cognitive benefits (Subaiea *et al.* 2013). Data presented within this manuscript demonstrate that tolfenamic acid lowers tau and CDK5 levels by inhibiting their transcription. However, the exact mechanism of action by which tolfenamic acid enhances SP1 degradation still remains to be established. Interestingly, we do not see much difference between the two doses used, suggesting that to get a dose–response relationship we need to go lower beyond the 5 and 50 mg/kg doses used. Such low doses would resemble those approved for migraine headaches management in Europe.

Tau and its abnormal phosphorylation are becoming targets for AD therapeutics. Tau knockdown by siRNA *in vitro* does not alter cell viability or the availability of microtubules (Morris *et al.* 2011). Probably because other

microtubule-associated proteins like MAP1b carry out similar functions to tau (Morris *et al.* 2011). The ability of tolfenamic acid to lower total tau levels is of great importance (Fig. 3). It was found that lowering soluble hyperphosphorylated tau rather than the insoluble tangles correlates with cognitive improvement (Iqbal *et al.* 2009; O'Leary *et al.* 2010; Medina 2011). In fact, in a neurodegenerative mouse model, tau inhibition recovered memory function even though the buildup of tangles continued suggesting that tangles by themselves are not responsible for cognitive dysfunction (Santacruz *et al.* 2005).

It is important to note that tolfenamic acid has been used for years, and that its interference with Sp1 should not be alarming since it was found that Sp1 is vital during early embryonic development only but not necessary for the following later stages of cell growth and differentiation (Marin *et al.* 1997). CDK5 is also important during nervous system development but not crucial later in life and thus is considered a promising target for AD where aberrant hyperphosphorylation and aggregation of tau is a major pathological finding (Lau *et al.* 2002; Piedrahita *et al.* 2010; Lopez-Tobon *et al.* 2011).

Administration of tolfenamic acid reduced the levels of tau phosphorylated at two sites, Ser 235 and Thr 181 (Fig. 6 and 7). Both sites are phosphorylated by CDK5 and other kinases (Baumann *et al.* 1993; Liu *et al.* 2002). Tau phosphorylation occurs on multiple sites and is regulated by different kinases (Liu *et al.* 2006). Ser 235 was found to be one of three sites whose phosphorylation inhibits tau binding to microtubules (Sengupta *et al.* 1998). Moreover, it is one of the sites that are especially phosphorylated in paired helical filament tau (Morishima-Kawashima *et al.* 1995; Hoffmann *et al.* 1997).

Decreasing the levels of the tangle forming tau protein by reducing its transcription is a novel approach for targeting AD and other tauopathies. Data from this study demonstrate that this can be achieved by promoting the degradation of the transcription factor Sp1. Tolfenamic acid is able to lower tau, CDK5, phosphorylated tau at Ser 235 and Thr 181. Hence, tolfenamic acid represents a promising candidate that targets both the amyloid and tau neurofibrillary pathways of AD and improves cognition through a unique transcription driven mechanism.

Acknowledgments and conflict of interest disclosure

This research was supported by the Intramural Research Program of the National Institutes of Health (NIH), National Institute of Environmental Health Sciences (NIEHS) and by grants NIH-ES015867 and AG042695 awarded to NHZ. The RI-INBRE Research Core Facility was supported by an Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health under grant number 8 P20 GM103430-12. Provisional patent application US Serial No.

61/739930 (N.H.Z.) was filed related to the work in this manuscript. The authors declare no other conflict of interest.

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