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Pharmacodynamic Mechanisms of Tolfenamic Acid Induced SP1 Degradation Relative to Alzheimer's Disease Pathology

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PHARMACODYNAMIC MECHANISMS OF TOLFENAMIC ACID INDUCED SP1 DEGRADATION RELATIVE TO ALZHEIMER'S DISEASE PATHOLOGY

BY

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ABSTRACT

Alzheimer's disease (AD) continues to disrupt the lives of millions of patients and caregivers around the world. The few drugs currently used for AD have modest effects on the symptoms and do not prevent the progression of the disease into total memory loss and death. With the increase in the number of AD cases and the high social and economical costs of the disease, there is a great need to find disease-modifying therapeutics that target the core pathology of the disease as well as improve the symptoms and the patients' everyday quality of life. Two types of pathological aggregates are found in AD. The senile plaques are composed of amyloid beta (Aβ), which is cleaved off the amyloid precursor protein (APP) by beta-site APP cleaving enzyme (BACE) and γ-secretase. The other deposits are the neurofibrillary tangles (NFTs), which are mainly composed of hyperphosphorylated tau. These aggregates and factors involved in the production or clearance of Aβ, as well as the phosphorylation of tau are being investigated for potential AD treatments but so far no successful drug candidate has been found. The transcription factor specificity protein 1 (Sp1) has been linked to pathological intermediates in AD. Sp1 regulates the transcription of APP, BACE1, tau and its cyclin dependent kinase-5 (CDK5) activators p39 and p35. Previous experiments from our lab have shown that AD like pathology develops later *in vitro* and *in vivo* following early lead (Pb) exposure including elevated levels of SP1, APP, Aβ, tau and CDK5 as well as cognitive decline in mice. Studies from our lab demonstrated that decreasing Sp1 protein (SP1) levels following oral administration of tolfenamic acid to mice was able to reduce APP and Aβ levels and improve cognition. In this dissertation, we first provided an introduction

to AD with a review on the role of epigenetics in the disease and the various means by which transcriptional pathways can provide therapeutic alternatives for AD. We then examined the ability of tolfenamic acid to affect the expression of AD targets that are regulated by Sp1 including tau, phosphorylated tau, CDK5 and BACE1 in mice by using Western blot, real time PCR and enzyme activity assays. In addition, we studied the ability of tolfenamic acid to prevent the increase in SP1, APP and Aβ in differentiated neuroblastoma cells that was triggered by prior exposure to Pb. After treatment of cells with Pb, tolfenamic acid or both, we used real time PCR, ELISA and Western blot analyses to examine the effects of both agents on AD related intermediates compared to control. In addition to providing a summary of the current knowledge on epigenetic therapeutic targets for AD, the major findings of this dissertation provide proof that tolfenamic acid was able to decrease the transcription and translation of proteins involved in AD like tau, BACE1 and CDK5 as well as the phosphorylation of tau in mice. Moreover, in differentiated neuroblastoma cells, tolfenamic acid decreased the expression of SP1, APP gene and Aβ which was previously upregulated by Pb. Hence, tolfenamic acid represents a novel oral drug candidate that can be beneficial for AD by affecting both the amyloid and tangle pathology of the disease through a unique transcription driven mechanism.

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PREFACE

This dissertation was prepared following the manuscript format. It was divided into four manuscripts that relate to the effects of tolfenamic acid on Alzheimer's disease (AD) associated genes and proteins. The first manuscript is a review article on epigenetic treatments for AD as an emerging field of study, it was prepared following the guidelines of Pharmacology and Therapeutics journal. The second manuscript examines the effects of tolfenamic acid administration on the tau pathway of AD in mice, it was prepared according to the Journal of Neuroscience guidelines. The third manuscript examines the consequences of tolfenamic acid exposure in APP transgenic mice on the enzyme β-secretase, it was written in accordance with the Neuropharmacology journal. The last manuscript summarizes our observations after the sequential exposure of neuroblastoma cells to Pb and tolfenamic acid and was prepared following Toxicology and Applied Pharmacology guidelines.

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Epigenetics: A Novel Therapeutic Approach for the Treatment of Alzheimer's Disease

Lina I. Adwan and Nasser H. Zawia

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Epigenetics: A novel therapeutic approach for the treatment of Alzheimer's disease

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Abstract

Alzheimer's disease (AD) is the most common type of dementia in the elderly. It is characterized by the deposition of two forms of aggregates within the brain, the amyloid β plaques and tau neurofibrillary tangles. Currently, no disease-modifying agent is approved for the treatment of AD. Approved pharmacotherapies target the peripheral symptoms but they do not prevent or slow down the progression of the disease. Although several disease-modifying immunotherapeutic agents are in clinical development, many have failed due to lack of efficacy or serious adverse events. Epigenetic changes including DNA methylation and histone modifications are involved in learning and memory and have been recently highlighted for holding promise as potential targets for AD therapeutics. Dynamic and latent epigenetic alterations are incorporated in AD pathological pathways and present valuable reversible targets for AD and other neurological disorders. The approval of epigenetic drugs for cancer treatment has opened the door for the development of epigenetic drugs for other disorders including neurodegenerative diseases. In particular, methyl donors and histone deacetylase inhibitors are being investigated for possible therapeutic effects to rescue memory and cognitive decline found in such disorders. This review explores the area of epigenetics for potential AD interventions and presents the most recent findings in this field.

Keywords:
Alzheimer's disease
DNA methylation
Epigenetics
Histone modification
Memory
Therapy

Abbreviations: Aβ, amyloid β; AD, Alzheimer's disease; APP, amyloid β precursor protein; BACE, β-site APP cleaving enzyme; CBP, CREB binding protein; CREB, cAMP response element-binding protein; CSF, cerebrospinal fluid; DNMT, DNA methyltransferase; FAD, familial AD; HAT, histone acetyltransferase; HDAC, histone deacetylase; LOAD, late onset AD; MeCP2, methyl CpG binding protein 2; NFTs, neurofibrillary tangles; PS, presenilin; SAM, *S*-adenosyl methionine; SIRT, sirtuin; Sp1, specificity protein 1.

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1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder, with over 35 million cases worldwide (Selkoe, 2012). The four acetylcholinesterase inhibitors, donepezil rivastigmine, galantamine and tacrine, along with the NMDA receptor antagonist memantine are the only FDA-approved drugs for AD; however, they merely target the symptoms and do not prevent the progressive loss of memory, cognitive and executive functions in AD patients. With the increase in life expectancy and the absence of disease-modifying agents, the number of people with AD is expected to triple within the upcoming 40 years (Barnes & Yaffe, 2011; Huang & Mucke, 2012; Tricco et al., 2012). The annual costs for AD and other dementias in the US in 2013 are estimated to be over $200 billion and are expected to reach $1.2 trillion in 2050 (Alzheimer's Association, 2013). With such a heavy socioeconomic burden, there is an urgent need to find novel and improved treatments for AD. Throughout the last century, the advances acquired in health related fields were able to increase the lifespan of AD patients, yet this needs to be matched with discoveries that improve the quality of life of people with this debilitating disorder. New targets should be identified and investigated for possible AD therapeutics that tackle its core pathophysiology as well as prevent the decline in memory and cognitive functions associated with the disease.

AD is characterized by progressive loss of memory and other cognitive and executive functions, with two types of pathological deposits found in the brain, the extracellular amyloid β (Aβ) plaques and the intracellular tau neurofibrillary tangles (NFTs). Senile plaques are mainly composed of Aβ which is cleaved off the larger amyloid β

precursor protein (APP) by β-site APP cleaving enzyme (BACE), also known as β-secretase, and γ-secretase (Citron et al., 1995; Shoji et al., 1992). According to the amyloid hypothesis, Aβ and its aggregates are responsible for the neurodegeneration and dementia in AD through mechanisms that involve disturbances in calcium homeostasis which make cells more vulnerable to toxicants that can cause further damage and NFTs (Hardy & Higgins, 1992; Mattson et al., 1992; Selkoe, 1993). The hypothesis was supported by the fact that mutations on APP are connected to hereditary types of AD (Hardy & Higgins, 1992). Early onset familial AD (FAD) could also be due to mutations on genes encoding the presenilin (PS) membrane proteins PS1 and PS2 (Czech et al., 2000; Tanzi et al., 1996). PS mutations increase the production of the more aggregative 42 amino acid-long Aβ ($A\beta_{42}$) from APP and elevated $A\beta_{42}$ levels were observed in the blood and brains of FAD patients with PS abnormalities (Czech et al., 2000). In addition, neurons lacking the PS1 gene fail to produce Aβ peptides (De Strooper et al., 1998; Naruse et al., 1998). PS1 was found to be related to the enzyme γ-secretase (De Strooper et al., 1998; Shimojo et al., 2007). These findings suggest that Aβ and its aggregates are involved in the pathology of AD as proposed in the amyloid hypothesis.

NFTs are composed of tau protein which belongs to a family of microtubule-associated proteins that normally promote microtubule assembly (Weingarten et al., 1975). When hyperphosphorylated, tau loses its normal function and becomes prone to form pathological aggregates causing disorders known as tauopathies of which AD is the most common (Alonso et al., 1997; Lee et al., 2001). Both Aβ and tau have been associated with neurodegeneration and memory decline and have been extensively

targeted for AD interventions such as immunotherapeutics, enzyme modulators, and aggregation inhibitors (Hardy & Higgins, 1992; Hardy & Selkoe, 2002; Hutton et al., 1998; Iqbal et al., 2009; Lee et al., 2001; Selkoe, 1993).

Epigenetics deals with acquired and heritable modifications on DNA that regulate the expression and functions of genes without affecting the DNA nucleotide sequence. These include DNA methylation and hydroxymethylation, histone modifications and non-coding RNA regulation. Histone modifications consist of acetylation, methylation, crotonylation, ubiquitination, sumoylation, phosphorylation, hydroxylation and proline isomerization (Davie & Spencer, 1999; Houston et al., 2013; Kouzarides, 2007; Peterson & Laniel, 2004). All these pathways act as mediators between the environment and the genome, these epigenetic changes are activated by various conditions such as stress or exposure to environmental toxicants and in turn they result in a variety of responses including gene transcription or silencing. Epigenetic changes are dynamic and unlike genetic mutations, they can be reversed for therapeutic purposes by targeting enzymes or other factors that control or maintain them (Caraci et al., 2012; Feinberg, 2008; Henikoff & Matzke, 1997; Liu et al., 2008; Mill, 2011). As changes within the genetic makeup itself are limited and the environment cannot freely amend the DNA sequence, epigenetics is the mechanism through which the environment can affect gene expression and function which can be employed as a medical intervention for diseases where epigenetics play a pathological role (Jaenisch & Bird, 2003; Mill, 2011). Furthermore, some age related changes are also mediated through epigenetics (Feinberg, 2008).

The majority of AD cases are sporadic or late onset AD (LOAD). Only about 5% of cases are familial or early onset AD which is associated with rare mutations on the APP, PS1 and PS2 genes (Goate et al., 1991; Sherrington et al., 1995; Tanzi, 2012). The sporadic nature of AD suggests that epigenetics plays an important role in the pathology of the disease; a hypothesis that is supported by recent findings from our laboratory and others (Lahiri et al., 2009; Lahiri et al., 2008; Mastroeni et al., 2011; Mill, 2011; Wang et al., 2008a; Wu et al., 2008b; Zawia & Basha, 2005; Zawia et al., 2009). This review explores epigenetic mechanisms as possible targets for AD therapeutics and highlights the current status of epigenetics in AD pathology and drug discovery.

1.1. Epigenetics of the brain and memory formation

Epigenetic dynamics within cells play a major role in their differentiation and in determining their functional type as hepatocytes in the liver, neurons in the brain, skin cells, or other cells, as well as becoming cancerous or not (Chadwick, 2012; Feinberg, 2008). Epigenetics is involved in various brain related disorders and physiologic responses that genetics alone does not completely explain including AD, depression, schizophrenia, glioma, addiction, Rett syndrome, alcohol dependence, autism, epilepsy, multiple sclerosis and stress (Heim & Binder, 2012; Inkster et al., 2013; Jaenisch & Bird, 2003; Kreth et al., 2012; Maric & Svrakic, 2012; Maze & Nestler, 2011; Mifsud et al., 2011; Nguyen et al., 2010; Orr et al., 2012; Qureshi & Mehler, 2010; Shahbazian & Zoghbi, 2002; Taqi et al., 2011; Zawia et al., 2009). As neurons do not divide and cannot be replaced after degeneration, epigenetic changes resulting

in neuronal dysfunction need to be targeted and modified to prevent neurodegeneration (Bird, 2007; Selvi et al., 2010).

Recent studies have pointed out the importance of epigenetics in brain development and functions including learning and memory (Feng et al., 2007; Miller & Sweatt, 2007; Molfese, 2011; Sultan & Day, 2011). In particular, DNA methylation and histone acetylation both play an important role in memory formation (Levenson et al., 2006; Miller et al., 2008). Other histone modifications involved in memory are methylation and phosphorylation (Chwang et al., 2006; Gupta et al., 2010; Molfese, 2011).

DNA methylation is catalyzed by DNA methyltransferases (DNMTs) in the presence of the methyl donor *S*-adenosyl methionine (SAM) (Yen et al., 1992). The DNMT family of enzymes includes DNMT1, DNMT2, DNMT3a, and DNMT3b (Okano et al., 1999). It is found that DNMT3a and DNMT3b are responsible for *de novo* methylation and establish DNA methylation patterns while DNMT1 has preference for hemi-methylated DNA (Chen et al., 2003; Hsieh, 1999; Okano et al., 1999). DNA methylation occurs on the 5' position of cytosine in CpG rich regions (Bird, 1986). This epigenetic mechanism regulates gene transcription and plays a particular role in memory functions (Day & Sweatt, 2010; Korzus, 2010; Liu et al., 2009). Memory and learning abilities decline with age which correlates with an overall reduction in DNA methylation (Liu et al., 2009). Furthermore, methylation on certain locations of the APP promoter in the human cortex is reduced with age (Tohgi et al., 1999).

DNMTs are considered crucial for memory functions (Miller & Sweatt, 2007). DNMTs regulate methylation within the promoter of reelin, an extracellular

glycoprotein that is involved in memory formation in the adult brain (Levenson et al., 2006; Weeber et al., 2002). Protein levels of DNMT1 and DNMT3a are reduced in the cortex of aged monkeys compared to early time points (Bihaqi et al., 2011). Moreover, conditional knockout mice lacking the expression of *Dnmt1* and *Dnmt3a* genes in forebrain neurons perform worse on the Morris water maze hippocampus related memory task than wild-type littermates or knockout mice lacking the expression of only one of the genes (Feng et al., 2010). The gene expression of *Dnmt3a2* decreases with age in mouse cortex and hippocampus (Oliveira et al., 2012). This age-related decline in *Dnmt3a2* gene expression is linked to memory decline that can be recovered by restoring DNMT3a2 levels (Oliveira et al., 2012).

Hydroxylation of 5-methylcytosine to 5-hydroxymethylcytosine by ten-eleven translocation (TET) enzymes is an important regulatory pathway involved in brain development, aging and disease (Szulwach et al., 2011). Levels of 5-hydroxymethylcytosine are significantly higher in neurons than in cells of other tissues (Globisch et al., 2010; Szulwach et al., 2011). DNA hydroxymethylation, levels of 5-hydroxymethylcytosine and 5-methylcytidine increase with age, and alterations in DNMT3a have also been reported with aging in mouse hippocampus (Chouliaras et al., 2011; Chouliaras et al., 2012a; Chouliaras et al., 2012b). Such epigenetic changes could be prevented by 50% caloric restriction diet throughout the mice lifetime after weaning (Chouliaras et al., 2011; Chouliaras et al., 2012a; Chouliaras et al., 2012b).

Moreover, mutations on methyl CpG binding protein 2 (MeCP2) may contribute to the development of Rett syndrome; a life-long neurodevelopmental disorder with marked learning disabilities (Amir et al., 1999). Other neurological abnormalities such as

autism and infantile encephalopathy have been associated with disturbances in MeCP2 (Chahrour et al., 2008; Moretti & Zoghbi, 2006). MeCP2 binds to methylated cytosine in CpG dinucleotides and inhibits or promotes gene expression by recruiting transcription repressors or activators like cAMP response element-binding protein 1 (CREB1) (Chahrour et al., 2008; Jones et al., 1998; Nan et al., 1998). This also involves MeCP2 binding to histone deacetylase complex (Jones et al., 1998; Nan et al., 1998). MeCP2 levels are found to decrease with age in primates (Bihaqi et al., 2011).

Histone acetylation is also involved in the regulation of learning and memory (Levenson et al., 2004; Martin & Sun, 2004). The most widely studied histone modification is regulated by two groups of enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs). The HDACs family of enzymes has been studied extensively for implications in cancer. Aberrant overexpression of various HDACs has been reported in different cancer types including gastric, pancreatic, breast, lung and colon cancer (Barneda-Zahonero & Parra, 2012; Johnstone, 2002; New et al., 2012). Many HDAC inhibitors are in clinical trials for cancer therapy, vorinostat and romidepsin have been approved by the FDA for cutaneous T-cell lymphoma (Kim et al., 2012; Mann et al., 2007; Nebbioso et al., 2012). There are 18 HDACs identified that belong to four classes I, II, III and IV according to their sequence homology (Xu et al., 2007). Class I HDAC2, class IIb HDAC6 and class III sirtuins (SIRTs) 1 and 2 are linked to AD pathology (de Oliveira et al., 2012; Karagiannis & Ververis, 2012). Acetylation of histone H3 in the hippocampus accompanies long-term memory formation in rats as determined in hippocampal

tissues collected 1 hour after fear conditioning experiments (Levenson et al., 2004). Increasing histone acetylation by the administration of the HDAC inhibitor sodium butyrate to rats prior to contextual fear conditioning improves memory formation (Levenson et al., 2004). Class I HDAC inhibitors sodium butyrate, sodium valproate and vorinostat enhance cognition in APP/PS1 double transgenic AD mouse model as evaluated by contextual fear conditioning tests (Kilgore et al., 2010). Overexpression of HDAC2 and not HDAC1, both Class I HDACs, in mice impairs memory and chronic administration of the HDAC inhibitors vorinostat or sodium butyrate enhances cognition (Guan et al., 2009). HDAC2 knockout mice display improved memory in fear conditioning experiments over wild-type mice (Guan et al., 2009). Consequently, downregulation or inhibition of HDACs 2 and 6 constitute important therapeutic targets for memory related disorders.

1.2. Epigenetic changes in AD

1.2.1. Histone modifications in AD

Increased levels of HDAC2 have been associated with cognitive impairment in CK-p25 AD mouse model which seems to be mediated through glucocorticoid receptor induced HDAC2 transcription (Graff et al., 2012). Postmortem studies reported that HDAC2 and not HDAC1 or HDAC3 is increased within the hippocampus of AD patients (Graff et al., 2012). Class II HDAC6 levels are elevated in AD cortex and hippocampus by 52% and 91% respectively (Ding et al., 2008). Tau co-localizes with HDAC6 in AD hippocampus and *in vitro,* and downregulation of HDAC6 decreases tau phosphorylation at Thr231 (Ding et al., 2008). Hyperphosphorylation of tau inhibits its normal functions and promotes its aggregation (Alonso et al., 1997). In

particular tau phosphorylation at Thr231 restrains its normal function of binding to microtubules (Sengupta et al., 1998). Class III HDACs or sirtuins is a family of enzymes that includes seven members named SIRT1-7 (Gray & Ekstrom, 2001). In addition to histones, SIRTs are responsible for deacetylation of other molecules like some proteins involved in AD pathology. For example SIRT1 accounts for tau deacetylation which is considered neuroprotective while tau acetylation contributes to tau dysfunction and aggregation (Cohen et al., 2011; Min et al., 2010; Stunkel & Campbell, 2011). SIRT1 is lower in the AD cortex which correlates with presence of tau pathology and memory impairment (Julien et al., 2009). There is evidence that SIRT1 also stimulates α-secretase which cleaves APP within the Aβ sequence and protects against Aβ accumulation (Donmez et al., 2010; Raghavan & Shah, 2012; Wang et al., 2010). SIRT1 is the most studied sirtuin, other sirtuins are also expressed in the brain and SIRT2 has been presented as a drug target for neurodegenerative diseases such as Parkinson's and Huntington's diseases (de Oliveira et al., 2012).

1.2.2. DNA methylation in AD

DNA methylation and factors such as DNMT1 are significantly reduced in neurons of entorhinal cortex layer II in AD patients (Mastroeni et al., 2010). Reductions in methylation are particularly localized in tangles containing neurons (Mastroeni et al., 2010). Other studies have demonstrated that there is abnormal methylation in AD patients (Bakulski et al., 2012; Wang et al., 2008a). When studying DNA methylation within the cerebral cortex of AD and control subjects, two out of the fifty loci examined were differentially methylated in AD which represent an acceleration of aging-linked alterations (Siegmund et al., 2007). In an AD discordant pair of

monozygotic twins, extensive plaques and NFTs were present and less methylation was found in the cortex of the AD twin compared to the non-AD twin (Mastroeni et al., 2009). However, some studies found no differences in the methylation patterns of AD-related genes (Barrachina & Ferrer, 2009). The difficulties in obtaining postmortem AD brain tissues for such studies and the variability among the available tissues as well as the different end points of methylation analyzed within these studies account for their various findings.

The promoter region within the APP gene is GC rich suggesting that it can be modulated through methylation (Pollwein et al., 1992). APP promoter displays differential methylation within the human brain (Rogaev et al., 1994). Hypomethylation of the APP promoter was reported to correlate with APP overexpression in AD (West et al., 1995). DNA methylation controls BACE and PS1 expression and consequently Aβ levels (Fuso et al., 2005). PS1 expression and methylation is altered in LOAD (Wang et al., 2008a). However, the changes on PS1 gene methylation in AD brains were not significant (Wang et al., 2008a). Another study did not detect significant changes in PS1, APP and tau genes methylation in the cortex and hippocampus of AD patients compared to controls (Barrachina & Ferrer, 2009). Lower paternal age was significantly associated with the increase in LOAD risk which might involve DNA methylation (Farrer et al., 1991). The challenges in acquiring and handling human brain tissues make it difficult to have a large number of matched controls and AD samples, however the available studies along with the sporadic and non-mendelian inheritance nature of the disease suggest that epigenetics is indeed involved in AD. Further research is needed to examine the epigenetic

changes affecting AD biomarkers including APP, tau, BACE, PS1 and PS2 among others, as well as global gene methylation patterns which would help with the early diagnosis of the disease.

1.2.3. Non-coding RNA in AD

Non-coding RNA can influence gene expression via epigenetic mechanisms affecting DNA methylation, histone modifications and chromatin remodeling (Costa, 2008). Various microRNAs are differentially expressed in AD and alter the expression of AD pathological intermediates (Cogswell et al., 2008; Nunez-Iglesias et al., 2010; Provost, 2010). An example is microRNA-101 which negatively regulates APP levels and is reduced within the brain cortex of AD patients (Hebert et al., 2008; Vilardo et al., 2010). Another example is microRNA-107 which is lowered early in AD and regulates BACE1 expression (Wang et al., 2008b). *BACE1*-AS is a long non-coding RNA antisense transcript of BACE1 that improves BACE1 stability and expression and is upregulated in the hippocampus and cortex of AD patients (Faghihi et al., 2008). Additional changes on non-coding RNAs are reported in AD and have been reviewed recently (Schonrock & Gotz, 2012). However, due to the current limitations and the absence of methods that can target or modify non-coding RNAs for therapeutic purposes, only few are mentioned within this review.

2. Epigenetic therapeutic approaches for AD

2.1. HDAC inhibitors

HDAC inhibitors show promise for cognitive improvement and are being considered for drug development for AD (Abel & Zukin, 2008; Fischer et al., 2007; Guan et al., 2009). Epigenetic changes play a role in cognitive decline and reversing such changes

by inhibiting HDAC2 improves memory and cognitive functions (Graff et al., 2012). Treatment of hippocampal neurons with Aβ promotes HDAC2 transcription suggesting that the traditional target of Aβ lowering in AD should be complemented with the reversal of epigenetic changes that were caused by increased Aβ levels (Graff et al., 2012). This might explain why Aβ lowering is not always successful in improving memory and cognitive deficits when subsequent epigenetic changes are not reversed as well (Graff et al., 2012). Crebinostat, an HDAC inhibitor, improves memory in mice (Fass et al., 2013). Administration of any of the three Class I HDAC inhibitors sodium valproate, sodium butyrate and vorinostat, which is an HDAC inhibitor approved by the FDA for cancer, improve memory in the APPswe/PS1dE9 AD mouse model (Kilgore et al., 2010). Hence, HDAC inhibitors could be promising therapeutic agents for AD and other disorders associated with dementia and cognitive impairments.

Valproic acid, which is used as an anticonvulsant in epileptic patients and as a mood stabilizer in bipolar disorder patients (Phiel et al., 2001), is a known HDAC inhibitor and has therefore been proposed for use in cancer and AD (Gottlicher et al., 2001; Kramer et al., 2003; Nalivaeva et al., 2009). In addition, valproate seems to have multi-target effects that can be useful for AD including inhibition of the enzyme responsible for tau phosphorylation glycogen synthase kinase 3 beta (GSK3β) (Loy & Tariot, 2002). Valproic acid lowers Aβ in the PDAPP transgenic mouse model of AD (Su et al., 2004). However, in a 2-year clinical trial, valproate did not improve cognitive function or slow memory decline in moderate AD patients and was

associated with adverse effects such as somnolence, tremor, weakness and dyspnea (Fleisher et al., 2011; Tariot et al., 2011).

Another HDAC inhibitor, sodium phenylbutyrate was found to improve memory and lower tau phosphorylation by GSK3β in APPswe transgenic AD mice (Ricobaraza et al., 2009). EVP-0334 is an HDAC inhibitor developed for AD by EnVivo Pharmaceuticals that successfully completed phase I clinical trials and was deemed safe for further testing, however, detailed information on the trial have not been made available yet (Arrowsmith et al., 2012; Caraci et al., 2012; Mack, 2010). A class II HDAC inhibitor referred to as W2 lowers Aβ, tau phosphorylated at Thr181 and improves cognition in hAPP transgenic mice (Sung et al., 2013). The authors also found that W2 and I2, a class I and II HDAC inhibitor, both downregulate genes involved in Aβ production and promote genes responsible for Aβ degradation *in vitro* (Sung et al., 2013).

2.2. Sirtuins

Class III HDACs or SIRTs are epigenetic targets for cancer and AD (Albani et al., 2010; Huber & Superti-Furga, 2011; Outeiro et al., 2008). The natural product found in red grapes skin and wine resveratrol is a SIRT1 activator that improves cognition in mice (Kim et al., 2007). However, resveratrol cognitive benefits involve other mechanisms besides SIRT1 activation and its epigenetic functions (Huber & Superti-Furga, 2011; Kim et al., 2007). A phase II study is currently recruiting mild to moderate AD patients to study the effects of resveratrol on AD biomarkers including cerebrospinal fluid (CSF) tau and Aβ levels as well as memory and daily performance using tests like Mini-Mental State Examination (MMSE) and Alzheimer's Disease

Assessment Scale-Cognitive (ADAS-Cog) (ClinicalTrials.gov., identifier: NCT01504854). Two SIRT activators developed by GSK were in phase I clinical trials, SRT2104 and SRT2379, recently the results from one of the trials were published and showed that SRT2104 was well tolerated by human subjects and suitable for further clinical trials (Hoffmann et al., 2013; Townsend, 2011). Interestingly, the nonselective SIRT inhibitor nicotinamide lowers phosphorylated tau and improves cognition in mice demonstrating that SIRT modulation involves complex mechanisms (Green et al., 2008; Stunkel & Campbell, 2011). Nicotinamide is in phase II clinical trial for AD (ClinicalTrials.gov., identifier: NCT00580931). Furthermore, administration of the SIRT2 inhibitor AK1 directly into the hippocampus protects against neurodegeneration in tau transgenic mice without altering tau tangles (Spires-Jones et al., 2012).

2.3. HATs

Less attention has been given to HAT enzymes as epigenetic targets for AD. Three HATs are involved in memory formation CREB binding protein (CBP), p300 and p300/CBP associated factor (PCAF) which might represent more specific targets than HDACs (Korzus et al., 2004; Selvi et al., 2010). CBP plays an important role in memory as CBP deficient mice display impaired long-term memory formation (Oike et al., 1999; Wood et al., 2005). In humans, mutations on the CBP gene result in Rubinstein-Taybi syndrome which is characterized by mental retardation (Petrij et al., 1995; Rubinstein & Taybi, 1963). Inducing the expression of CBP within the brains of 3xTg-AD triple transgenic AD mouse model recovers the impaired memory functions in these mice (Caccamo et al., 2010). On the other hand, inhibition of the HAT p300

by using the commercially available p300 inhibitor C646 reduces the levels of acetylated tau and phosphorylated tau at Ser202 *in vitro* (Min et al., 2010). The natural plant product curcumin possesses p300/CBP HAT inhibitor activity and is in phase II clinical trial to study its cognitive effects and Aβ lowering potential in AD patients (Balasubramanyam et al., 2004; ClinicalTrials.gov., identifier: NCT01383161; Marcu et al., 2006). Previous trials with a smaller number of AD subjects reported no significant changes between curcumin- and placebo- treated groups (Hamaguchi et al., 2010; Ringman et al., 2012). While in transgenic animal models, curcumin decreased oxidative damage and Aβ pathology by affecting anti-inflammatory pathways (Begum et al., 2008; Hamaguchi et al., 2010; Lim et al., 2001; Yang et al., 2005).

2.4. DNA methylation

There are multiple ways for targeting DNA methylation for therapeutic purposes (Klose & Bird, 2006). DNA methylation affects the expression of the AD-related intermediates APP, PS1 and Aβ (Fuso et al., 2005). It has been hypothesized that hypomethylation of the promoter regions of such genes like PS1 leads to the overexpression of their products including Aβ (Mulder et al., 2005). Overexpression of DNMT3a2 within the hippocampus of old mice increases overall methylation and improves memory (Oliveira et al., 2012).

The levels of the methyl donor SAM are lower in the CSF and within the brains of AD patients (Bottiglieri et al., 1990; Bottiglieri et al., 1994; Morrison et al., 1996). However, in another study, there was no difference in SAM-CSF levels in AD patients vs. healthy subjects (Mulder et al., 2005). Nevertheless, treatment with SAM reduces BACE1, PS1 and Aβ production *in vitro* in human neuroblastoma cells (Fuso et al.,

2005; Scarpa et al., 2003). Moreover, administration of SAM adjunct to antidepressants in depressed patients enhances their cognitive symptoms and ability to remember as determined by cognitive and physical symptoms questionnaire (CPFQ) (Levkovitz et al., 2012).

Betaine, the methyl donor used conventionally for homocystinuria treatment (Key, 2000), was tested in 8 AD patients for 24 weeks and failed to demonstrate cognitive improvement (Craig, 2004; Knopman & Patterson, 2001). However, the small number of patients and the lack of a placebo-treated control group suggest that further trials are needed to properly evaluate betaine's efficacy in AD (Knopman & Patterson, 2001), especially that elevated homocysteine has been associated with dementia and AD and betaine lowers homocysteine (Seshadri et al., 2002). In a more recent study in mice, betaine was able to improve memory that was compromised by prior lipopolysaccharide administration (Miwa et al., 2011).

2.5. Non-coding RNA

Several non-coding RNAs are involved in AD pathology and could present specific diagnostic and therapeutic targets for the disease (Costa, 2008; Provost, 2010). These include *BACE1*-AS, microRNA-34c, microRNA-101, and microRNA-107 (Cogswell et al., 2008; Faghihi et al., 2008; Vilardo et al., 2010; Wang et al., 2008b; Zovoilis et al., 2011). However, concerns about ways to alter such targets, off target effects, and delivery methods still need to be adequately addressed before having epigenetic treatments capable of affecting non-coding RNAs. Targeting non-coding RNA regions on APP by the antibiotic erythromycin, the antidepressant paroxetine and N-acetyl

cysteine has been found to reduce Aβ in TgCRND8 transgenic mice (Tucker et al., 2005; Tucker et al., 2006).

2.6. Beyond epigenetics: Epigenetics and transcription

Epigenetics is an important mediator that influences DNA transcription and translation. The aim of AD therapy is to enhance the transcription of genes involved in memory formation and reduce the transcription of pathogenic intermediates in the disease process like tau, APP, and BACE1. Hence, transcription factors constitute valid targets for developing novel treatments for AD. One of the important transcription factors for learning and memory is CREB (Silva et al., 1998). CREB is an essential mediator of memory improvement following HDAC inhibition as CREB has histone acetylation activity through recruitment of the histone acetyltransferase CBP (Vecsey et al., 2007). HDAC inhibitors, such as phenylbutyrate or crebinostat promote the transcription of genes involved in memory functions as seen with crebinostat which upregulates the CREB target gene early growth response 1 (egr1), which is involved in memory formation (Fass et al., 2013; Ricobaraza et al., 2009). A clinical trial studying the effects of the antiplatelet drug cilostazol on cognition in AD patients co-administered with donepezil is currently in progress (ClinicalTrials.gov., identifier: NCT01409564). The rationale behind choosing cilostazol is to promote the phosphorylation of CREB which regulates its activity and consequential expression of genes that are controlled by CREB (Bito et al., 1996; ClinicalTrials.gov., identifier: NCT01409564; Silva et al., 1998). Cilostazol protects against Aβ triggered cognitive impairment in mice and improves memory following cerebral hypoperfusion damage in rats (Hiramatsu et al., 2010; Watanabe et al., 2006).

An important transcription factor involved in AD is specificity protein 1 (Sp1). It binds to GC-rich regions within the promoters of APP, tau and BACE1 and upregulates their expression (Docagne et al., 2004; Hoffman & Chernak, 1995; Pollwein et al., 1992). Sp1 is able to bind to CpG sites in genes promoters that have such specific binding motifs and activate their transcription whether they are methylated or non-methylated (Holler et al., 1988). Furthermore, Sp1 can trigger epigenetic modifications as it regulates the expression of DNMT1 (Kishikawa et al., 2002). Tolfenamic acid promotes Sp1 protein degradation and lowers APP, tau, and BACE1 expression as well as Aβ levels and improves cognition in mice (Abdelrahim et al., 2006; Adwan et al., 2011; Adwan et al., unpublished observation; Subaiea et al., in press). Tolfenamic acid is scheduled to be tested in AD patients in the near future.

3. Discussion and conclusions

Epigenetic changes that occur early in life can impact our health decades later. Various studies suggest that pathologic changes in AD can be reversed prior to the development of symptoms through epigenetic modifications (Fig. 1). Developmental exposure to lead (Pb) upregulates genes involved in AD late in life through mechanisms that involve DNA methylation and histone acetylation (Bihaqi et al., 2011; Bihaqi & Zawia, 2012; Wu et al., 2008a). Persistent bidirectional changes in DNA methylation in response to earlier Pb exposure are reported with hypermethylation resulting in a latent reduction in gene expression (Alashwal et al., 2012; Dosunmu et al., 2012). Moreover, cognitive impairment accompanies overexpression of Sp1, BACE1, APP and Aβ late in life following early exposure to Pb and consequential epigenetic alterations (Bihaqi et al., in press). Such

environmentally-induced changes on AD-related intermediates could be reversed via epigenetic mechanisms. Alternatively, active epigenetic changes are involved in memory formation and could be targeted for AD therapy. Modulation of epigenetic intermediates could be a means for upregulation of genes that promote learning and memory, or reversing epigenetic changes that are responsible for the overexpression of genes involved in AD pathology. As neurons have a very limited ability to regenerate, reversing pathological changes through targeting epigenetic intermediates seems to be a promising therapeutic approach.

Interestingly, epigenetic targets in AD are also implicated in the pathophysiology of schizophrenia and depression (Covington et al., 2009; Gavin & Sharma, 2010). Depression is a common comorbidity in demented patients (Alzheimer's Association, 2012; Holtzer et al., 2005). Psychotic symptoms, especially at later stages of the disease, are also frequent in AD (Alzheimer's Association, 2012; Ropacki & Jeste, 2005). Besides AD, HDAC inhibitors have been explored for other disorders including schizophrenia and depression. For example, the HDAC inhibitor sodium butyrate shows antidepressant activity in mice (Schroeder et al., 2007). Sodium butyrate also protects against phencyclidine induced psychotic-like behavior in mice (Koseki et al., 2012). It would be interesting to study the effects of HDAC inhibitors as epigenetic modifiers on cognitive as well as depressed and psychotic symptoms in AD patients.

Epigenetic alterations reported in AD are summarized in Table 1. A major challenge for AD management is early diagnosis. Currently, no standard criteria are available for early or accurate detection of AD through reference values of biomarkers from patients CSF and blood samples or imaging results. Epigenetic changes in AD could

offer a diagnostic tool for the disease especially that some changes occur long before the molecular pathology of AD develops. If such changes are identified and detected early, reversing them via epigenetic therapeutic approaches would prevent the triggering of alterations in gene expression and transcriptional cascade associated with the neuropathology of AD. Also establishing criteria for epigenetic changes in AD can help administer disease-modifying drugs, once they become available, early in the disease process. The use of epigenetics will likely be even more crucial as the field moves towards early and pre-symptomatic case detection and earlier attempts at intervention (Sperling et al., 2011).

The side effects of epigenetic targeting should also be studied. Attention should be made for the consequences of epigenetic modifications that are involved in multiple pathways and which might serve various functions within different cells and organs. Identification of more specific targets and agents could be a way for minimizing toxicity. It is important to realize that drugs with epigenetic effects are already present on the market. Some drugs used for years like the antihypertensive agent hydralazine and the antiepileptic drug valproate are found to interfere with epigenetic pathways which explains their previously unknown mechanisms of action or some of their adverse effects and suggests that their use could be repurposed for other disorders where epigenetic alterations are desired (Csoka & Szyf, 2009).

Our knowledge about epigenetics is still limited, some mechanisms have been studied more thoroughly like histone acetylation and DNA methylation, yet much remains to be revealed, especially when it comes to AD, memory and cognitive functions. Epigenetics is more upstream in AD pathology than the more common or conventional

targets such as BACE, γ-secretase, Aβ and tau and thus could be beneficial especially in early stages of the disease to prevent further transcription and accumulation of pathological intermediates. Screening for such modifications and diagnosis of AD at an early stage remain a challenge. Nevertheless, promoting epigenetic mechanisms that trigger memory formation and inhibit pathological events could be a novel and effective therapeutic approach for preventing or at least delaying the development of dementia. Epigenetics offer potential for AD where epigenetic changes are integrated in the disease pathology. While no disease-modifying candidate is available, more research is needed for the refinement of epigenetic targets and identification of specific agents that can improve cognition and prevent or slow AD. Although knowledge is still being gathered about this field of study, there is evidence that epigenetics could provide multi-target therapeutic approaches for AD.

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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Fig. 1. Epigenetic targets and therapeutic approaches for AD. DNA methylation and histone modification mediators involved in AD and potential therapeutic interventions under development reported in literature. Barrels = histones; (M) = methyl group; (Ac) = acetyl group. Abbreviations in the figure are as follows: DNMT, DNA methyltransferase; HAT, histone acetyltransferase; HDAC, histone deacetylase; SIRT, sirtuin.

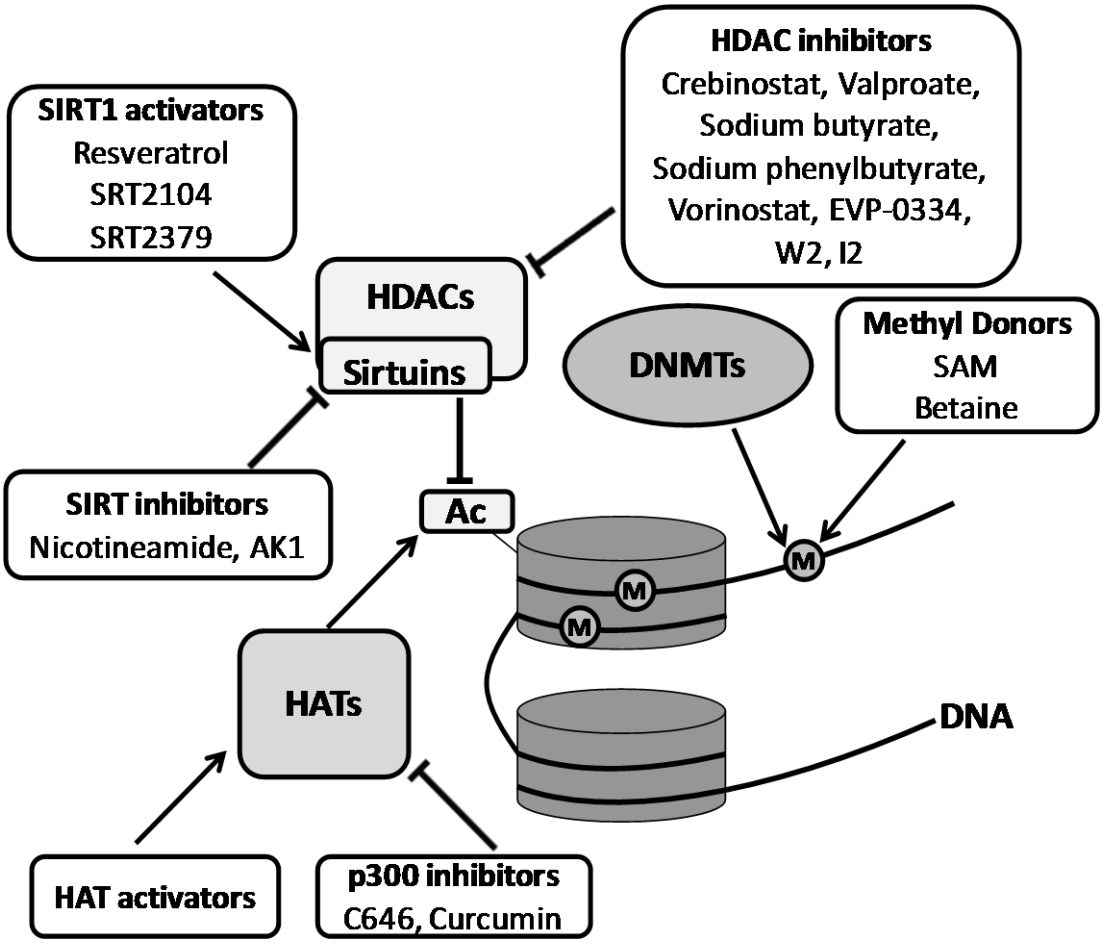
HDAC inhibitors
Crebinostat, Valproate,
Sodium butyrate,
Sodium phenylbutyrate,
Vorinostat, EVP-0334,
W2, I2
SIRT1 activators
Resveratrol
SRT2104
SRT2379
HDACs
Sirtuins
DNMTs
Methyl Donors
SAM
Betaine
SIRT inhibitors
Nicotineamide, AK1
Ac
M
M
M
HATs
DNA
HAT activators
p300 inhibitors
C646, Curcumin

Table 1 Some epigenetic changes in AD reported in literature.

Epigenetic Mark	Change	Reference
HDAC2	Increase	Graff et al., 2012
HDAC6	Increase	Ding et al., 2008
SIRT1	Decrease	Julien et al., 2009
DNMT1	Decrease	Mastroeni et al., 2010
microRNA-101	Decrease	Hebert et al., 2008
microRNA-107	Decrease early in AD	Wang et al., 2008b
BACE1-AS	Upregulated	Faghihi et al., 2008
Methylation on APP gene	Hypomethylation	West et al., 1995
Methylation on APP, PS1, tau	No change	Barrachina & Ferrer, 2009

MANUSCRIPT II

Tolfenamic Acid Reduces Total, Phosphorylated Tau and CDK5 Levels via a Transcriptional Pathway: Implications for Dementia and Tauopathies

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Abbreviated title: Tolfenamic acid suppresses tau and CDK5 transcription

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Abstract

Tau and its aggregates are linked to the pathology of Alzheimer's disease (AD) and other tauopathies. Currently, they are being targeted to find the much needed treatments for such disorders. Tau belongs to a family of microtubule-associated proteins (MAPs) that promote microtubule assembly. When hyperphosphorylated, tau loses its normal function of binding to microtubules and becomes prone to form aggregates. Increased levels of hyperphosphorylated tau in the brain correlate with dementia. Specificity protein 1 (Sp1) is a transcription factor that is elevated in AD and is responsible for the transcription of AD-related genes including the amyloid precursor protein (APP), tau, and its cyclin dependent kinase-5 (CDK5) activators. Tolfenamic acid promotes the degradation of Sp1; previous studies from our lab demonstrated its ability to downregulate transcriptional targets of Sp1 like APP and thereby reduce amyloid beta (Aβ) levels, the main component of AD plaques. In this study, we administered two different doses of tolfenamic acid daily to APP knockin mice for one month, and used real time PCR and Western blot analyses to examine the changes in tau and CDK5 gene and protein expression within the cerebral cortex. Our results demonstrate that tolfenamic acid lowers tau mRNA and protein, as well as the levels of its phosphorylated form. Moreover, tolfenamic acid decreases the levels of the kinase involved in tau phosphorylation, CDK5. Thus, this repurposed drug inhibits the transcription of multiple intermediates in AD pathology through a common mechanism and may offer a therapeutic solution subsequent to its impending human biomarker study.

Introduction

The microtubule associated protein tau (MAPT) was first isolated and recognized for its role in microtubule assembly in 1975 (Weingarten et al., 1975). In Alzheimer's disease (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; Goedert, 1997; Lee et al., 2001; Brunden et al., 2009). The normal function of tau is to stabilize microtubules, and the exact cause of its aggregation is unknown. It has been found that tau hyperphosphorylation reduces its binding to microtubules and is suspected to play a role in its 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; Iqbal et al., 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 (GSK3β) and cyclin-dependent kinase 5 (CDK5) among others.

Specificity protein1 (sp1) is a transcription factor that is 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) and tau genes and promotes their transcription (Salbaum et al., 1988; Pollwein et al., 1992; Hoffman and Chernak, 1995; Heicklen-Klein and Ginzburg, 2000; 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 the promoter regions of CDK5, p39 and p35 (Ohshima *et al.*, 1995; Ohshima *et al.*, 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 available on the European market for migraine headaches, promotes SP1 degradation and lowers the expression of genes regulated by Sp1 including APP (Abdelrahim et al., 2006; Adwan et al., 2011). It is currently scheduled for a human biomarker study involving AD patients. This research study is designed to test the ability of tolfenamic acid to downregulate the expression of tau and CDK5 as players in the tangle pathology of AD by its unique capability to promote the degradation of SP1 (Fig. 1). This would provide more evidence for tolfenamic acid use in clinical neurodegenerative studies.

Materials and Methods

Animals.

Female hemizygous APP YAC transgenic mice line R1.40 (14-20 months old) were used in this study (The Jackson Laboratory). Animals were housed in designated

rooms within the animal facility at the University of Rhode Island. Mice were assigned into 3 groups of similar age variations, n= 6 in each group. Animals were administered 0, 5 or 50 mg/kg tolfenamic acid (Sigma-Aldrich) in corn oil everyday by oral gavage for 34 days. On day 35, mice were sacrificed and brain tissues were collected and stored at -80°C until further use. 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), checked for integrity by NanoDrop (Thermo Scientific), and reverse transcribed to cDNA using iScript™ Select cDNA Synthesis Kit following manufacturer's instructions (Bio-Rad). 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 minutes then at 85°C for 5 minutes 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'- GTG GCC AGG TGG AAG TAA AA -3' and antisense: 5'- TGG AAG ACA CAT TGC TGA GG -3'; CDK5 sense: 5'- GGC TAA AAA CCG GGA AAC TC -3', and antisense: 5'- CCA TTG CAG CTG TCG AAA TA -3'; β-actin sense: 5'- TGT TAC CAA CTG GGA CGA CA -3', and antisense: 5'- TCT CAG CTG TGG TGG TGA AG -3'; GAPDH sense: 5'- GCCTTCTCTCCATGGTGGTAAA AGC TGA ACG GGA AGC TCA CT -3', and antisense: 5'- AGG TCC ACC ACT GAC

ACG TTG-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). Real time PCR was conducted using the 7500 Real Time PCR System (Applied Biosystems) following the standard protocol: 50°C for 2 minutes followed by 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. 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 RIPA lysis buffer (10 mM Tris-HCl ([pH 7.4], 150 mM NaCl, 1% Triton X-100, 0.1 SDS, 1 mM EDTA (Fisher Scientific), and 0.1% protease inhibitor cocktail (Sigma-Aldrich)). The homogenates were centrifuged at 10,600 x g for 10 minutes at 4°C and supernatants were collected. Protein concentration was determined using the Micro BCA Protein Assay Kit (Pierce). Protein extracts were stored at -80°C until further use. For Western blot analyses, approximately 40 µg of protein samples were separated onto 4–15% precast polyacrylamide gels (Bio-Rad) at 150 V for 1-2 hours and then transferred to PVDF membranes (GE-Healthcare). Membranes were blocked and incubated with the appropriate dilution of the specific primary antibody for 1-2 hours. The tested antibodies were used as follows: 1:1000 dilution of T9450 for total tau levels (Sigma-Aldrich); 1:1000 of CDK5 #2506 (Cell Signaling); 1:1000 of P-tau Thr^{181} #5383 (Cell Signaling); 1:1000 of P-tau Ser^{235} ab30664 (Abcam); 1:5000 dilution of β-actin A2013 (Sigma-Aldrich); or 1:2000 of GAPDH T9450 (Sigma-Aldrich), then the membranes were washed with TBST and incubated with the appropriate infrared dye-labeled

secondary antibody (Li-Cor) for 1 hour at room temperature in the dark. Infrared signal of Western blot bands was detected and quantified using an Odyssey® Infrared Imaging System (Li-Cor). Western blot protein levels were normalized against the levels of the house keeping proteins β-actin or GAPDH.

Statistical analysis

Data were represented as the mean ± the standard error of the mean (SEM). Statistical analysis was performed using GraphPad Instat software (GraphPad software) and statistical significance was determined by one-way analysis of variance (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 the 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 lab have already provided evidence that tolfenamic acid crosses the blood brain barrier and is able to lower SP1 and subsequently reduce APP transcription and Aβ levels in mice brain (Adwan et al., 2011; Subaiea et al., 2011). 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 years. 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 of promoting SP1 degradation by the drug on tau gene expression and protein levels. The data reported below also show the effects

of reducing SP1 on various intermediates in the tau pathology including CDK5 and phosphorylated tau (P-tau) on Ser 235 and Thr 181.

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

By inducing SP1 degradation we hypothesized that tolfenamic acid would also reduce the gene expression of Sp1 transcription 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 determined by real time PCR (Fig. 2). One-way ANOVA p=0.0018, Tukey-Kramer multiple comparisons post-test p<0.001 for the control (C) vs 5 mg/kg group comparison, p<0.05 for C vs 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 p=0.014, Tukey-Kramer post-test p<0.05 for C vs 5 mg/kg and for C vs 50 mg/kg.

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

As Sp1 also regulates CDK5 activators (Valin et al., 2009), we next 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 $p=2.8\times10^{-7}$, Tukey-Kramer post-test p<0.05 for C vs 5 mg/kg and for C vs 50 mg/kg. There was a lowering trend in CDK5 levels (Fig. 5) that was not significant when analyzed with one-way ANOVA p=0.059. However when comparing the 50 mg/kg dose group to the control 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; Sengupta et al., 1998; Alonso et al., 2008), it was important to test how phosphorylated tau is affected by the treatment. Levels of phosphorylated forms were analyzed by Western blotting using specific antibodies. P-tau Ser^{235} and P-tau Thr^{181} were lowered by both doses of tolfenamic acid (Fig. 6, 7). Tau phosphorylated on Ser 235 was lowered by about 15%, one-way ANOVA p=0.0165, Tukey-Kramer post-test p<0.05 for C vs 5 mg/kg and for C vs 50 mg/kg. P-tau Thr^{181} was lowered by about 30%, one-way ANOVA p=0.0112, Tukey-Kramer post-test p<0.05 for C vs 5 mg/kg and for C vs 50 mg/kg.

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 Aβ pathology (Docagne et al., 2004; Santpere et al., 2006; Brock et al., 2008; Citron et al., 2008). Previous studies from our lab demonstrate that by lowering SP1, tolfenamic acid is able to decrease the transcription of APP as well as Aβ levels in mice following 2 weeks of daily administration (Adwan et al., 2011). Our studies also show that tolfenamic acid is readily available in the brain after dosing (Adwan et al., 2011; Subaiea et al., 2011).

Behavioral and immunohistochemical studies that took place at our lab have revealed that tolfenamic acid lowers the plaque burden and improves cognition in the APP transgenic mouse model used in this study (G. M. Subaiea, and N. H. Zawia, unpublished observations). These transgenic mice were chosen as a model of the amyloid pathology of AD, as they express Aβ plaques that are not found in wild type mice. Since cognitive impairment is better correlated with tau and Sp1 regulates the expression of tau (Heicklen-Klein and Ginzburg, 2000; Iqbal et al., 2009; Medina, 2011), we wanted to study the effects of tolfenamic acid on the tau pathology in the same animals where we observed its cognitive benefits. Data presented within this manuscript demonstrate that tolfenamic acid lowers tau and CDK5 levels by inhibiting their transcription. The exact mechanism of action by which tolfenamic acid enhances SP1 degradation still remains to be established.

During the past twenty years, drug discovery has focused on targeting intermediates mentioned in the amyloid hypothesis of AD including APP and Aβ, 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 Aβ caused cognitive improvement, while lowering only soluble Aβ 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).

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 (MAPs) 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, 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 3 sites whose phosphorylation inhibits tau binding to microtubules

(Sengupta et al., 1998). Moreover, it is one of the sites that are especially phosphorylated in PHF tau (Morishima-Kawashima et al., 1995; Hoffmann et al., 1997).

Interestingly we do not see much difference between the two doses used, suggesting that in order 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.

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 through a unique transcription driven mechanism.

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Figure II-1. The proposed transcriptional based mechanism of tau and CDK5 downregulation by tolfenamic acid. Tolfenamic acid induces the degradation of the transcription factor Sp1, which reduces the transcription of tau and the activator for CDK5, resulting in a decrease in the total levels of tau as well as the pathogenic phosphorylated tau species and ultimately reducing the associated dementia.

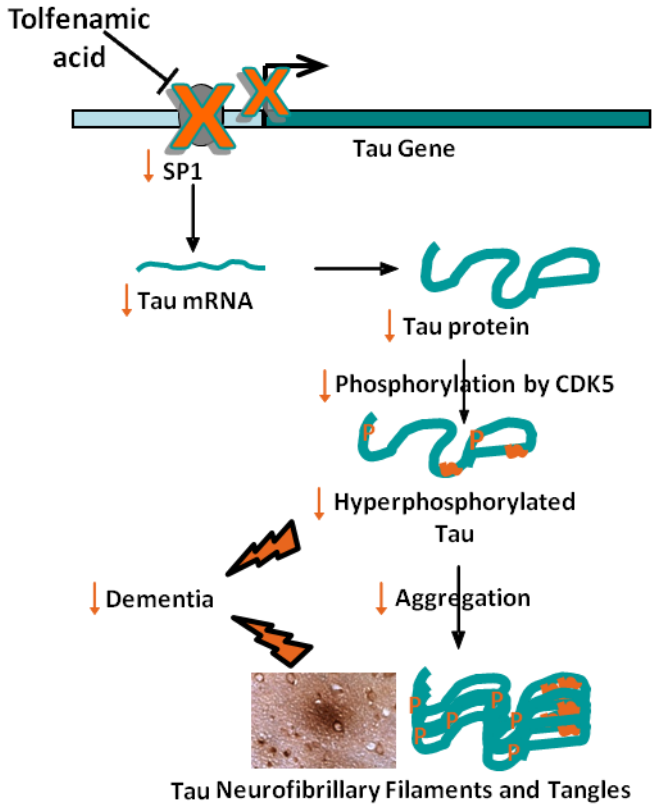
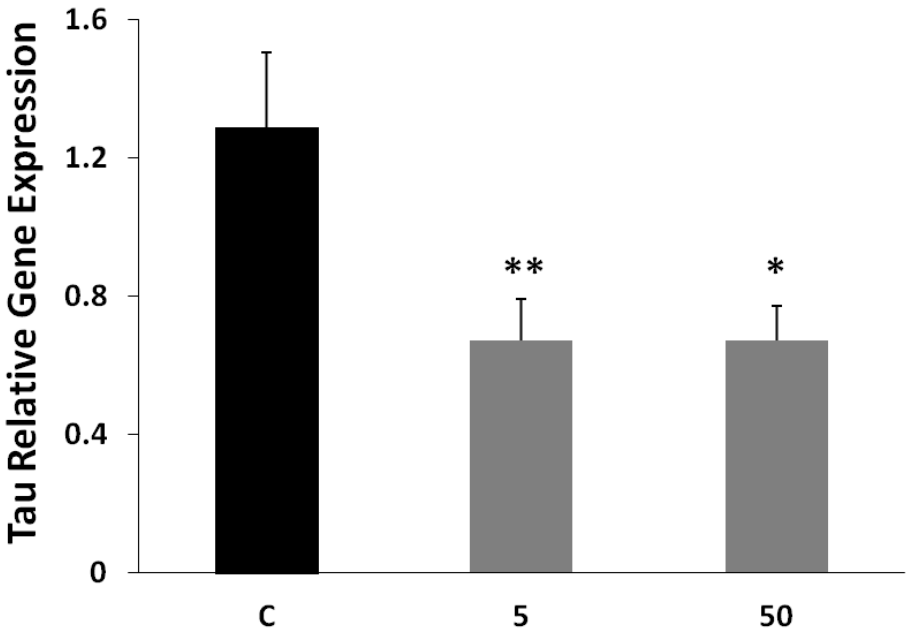

Tolfenamic acid
Tau Gene
SP1
Tau mRNA
Tau protein
Phosphorylation by CDK5
Hyperphosphorylated Tau
Dementia
Aggregation
Tau Neurofibrillary Filaments and Tangles

Figure II-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 as endogenous control as illustrated in the methods section. Values shown are the mean ± 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.


Tau Relative Gene Expression
1.6
1.2
0.8
0.4
0
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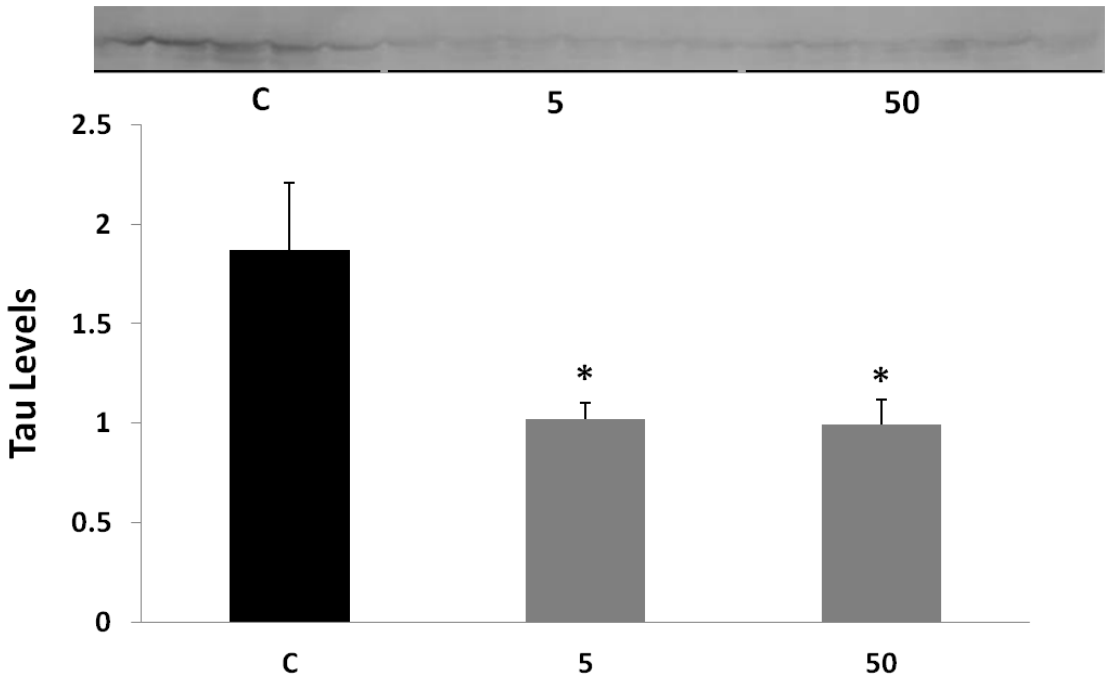
C
5
50

Figure II-3. Tau levels with tolfenamic acid administration. Total tau levels were analyzed in the cerebral cortex following daily administration of tolfenamic acid to mice for 34 days by Western blot analysis. Value shown are the mean ± 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 Western blot bands.


C
5
50
2.5
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Tau Levels
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5
50

Figure II-4. CDK5 gene expression after tolfenamic acid treatment. CDK5 mRNA levels in mice cortex were measured with real time PCR with GAPDH as endogenous control as illustrated in the methods section. Values shown are the mean ± SEM, n=5. One-way ANOVA $p<0.0001$, ***$p<0.001$ as determined by Tukey-Kramer post-test.

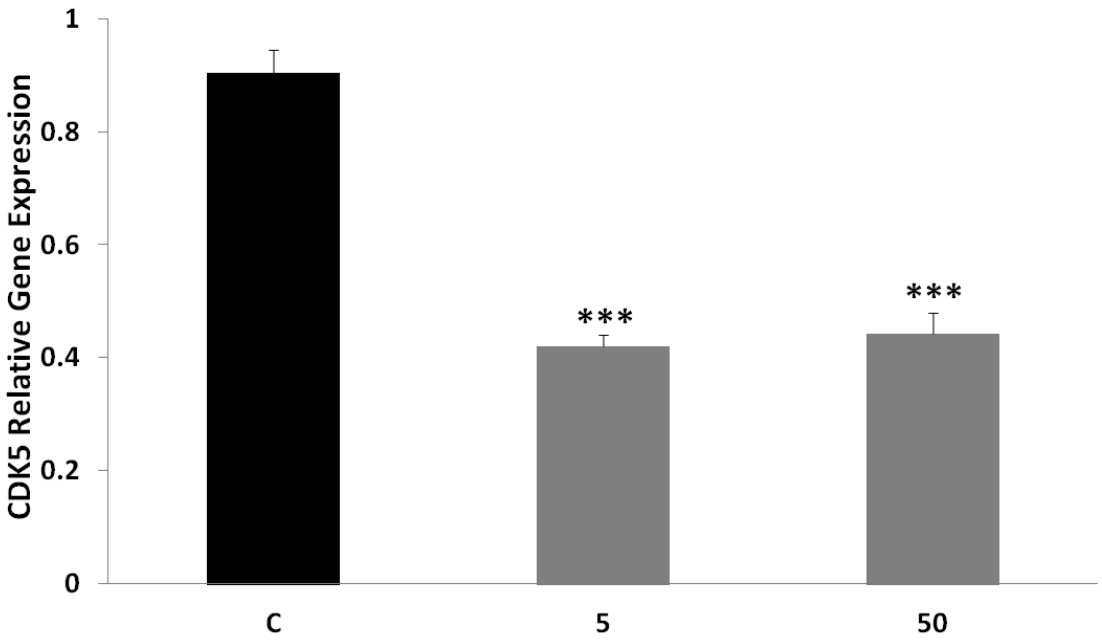

CDK5 Relative Gene Expression
1
0.8
0.6
0.4
0.2
0
***
***
C
5
50

Figure II-5. CDK5 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 the mean ± 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 Western blot bands.

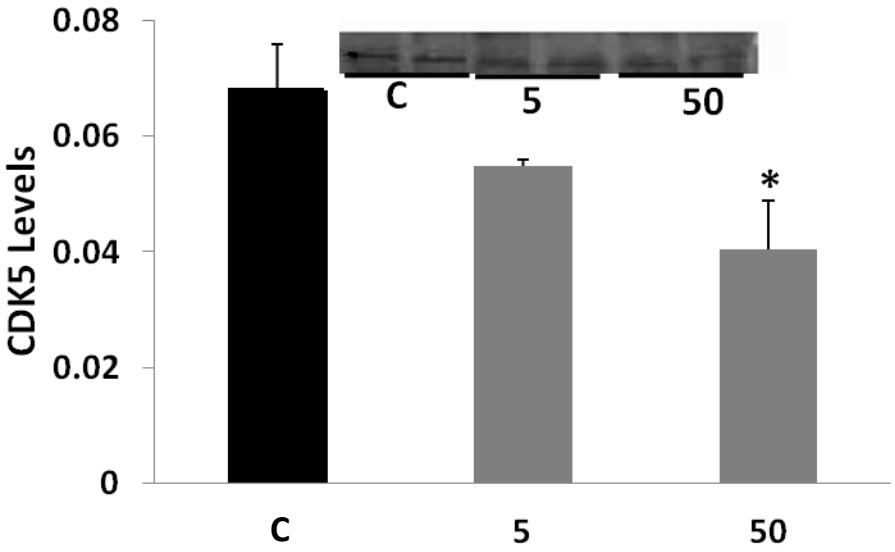

0.08
0.06
0.04
0.02
0
CDK5 Levels
C
5
50
*
C
5
50

Figure II-6. Levels of tau phosphorylated on Ser 235 after tolfenamic acid treatment. P-tau levels were measure by Western blot analysis and normalized to GAPDH as illustrated in the methods section. Values shown are the mean ± 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 Western blot bands.

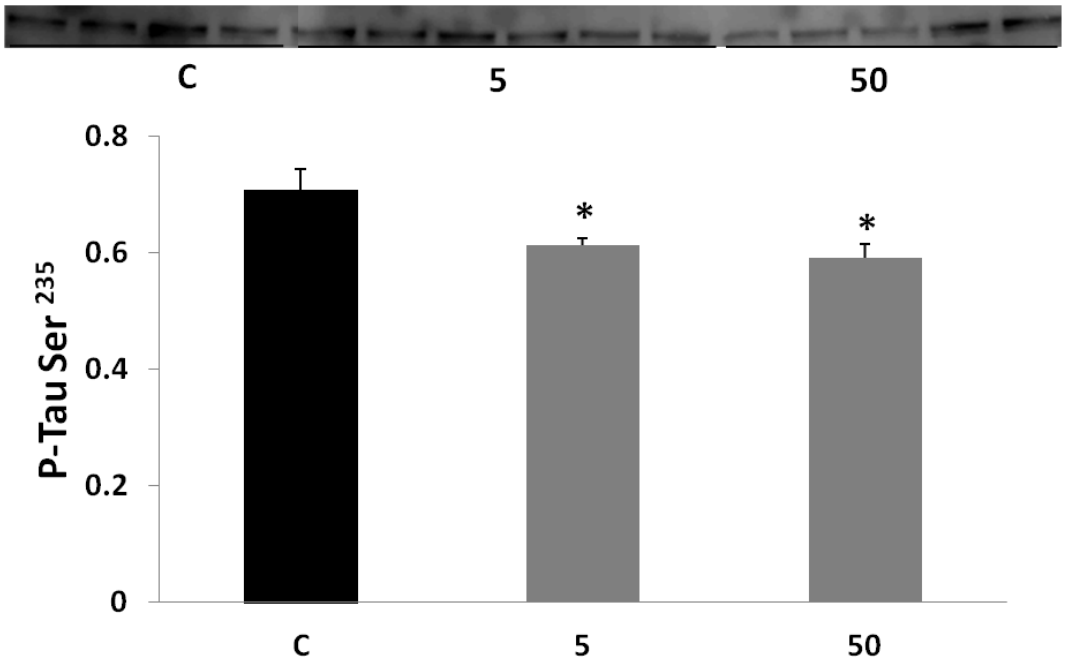
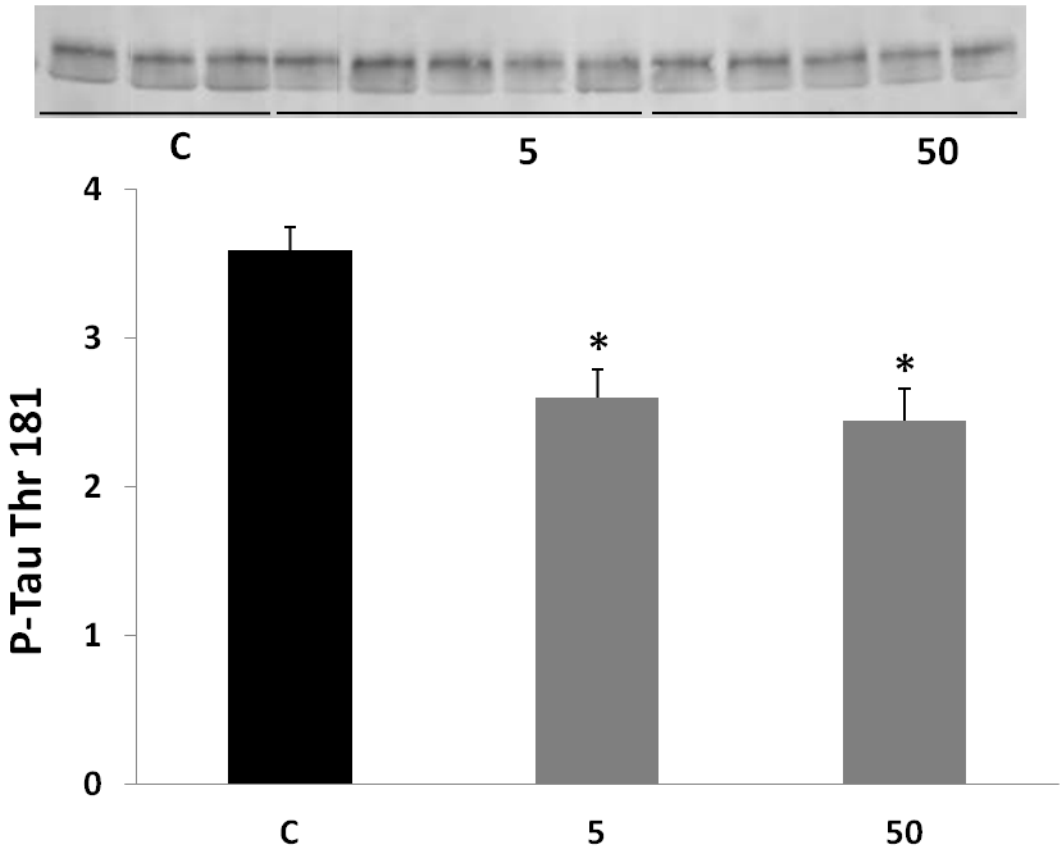

C
5
50
0.8
0.6
0.4
0.2
0
P-Tau Ser 235
*
*
C
5
50

Figure II-7. Levels of tau phosphorylated on Thr 181 following tolfenamic acid exposure. P-tau levels were measured by Western blot and normalized to GAPDH levels. Value shown are the mean ± 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 Western blot bands.


C
5
50
4
3
2
1
0
P-Tau Thr 181
*
*
C
5
50

MANUSCRIPT III

Tolfenamic Acid, a Multi-target Drug Candidate for Alzheimer's Disease, Downregulates BACE1 via a Transcriptional Mechanism

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(Prepared for Neuropharmacology Journal)

Tolfenamic Acid, a Multi-target Drug Candidate for Alzheimer's Disease, Downregulates BACE1 via a Transcriptional Mechanism

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Keywords

Alzheimer's disease, Amyloid Beta, APP, BACE1, Sp1, Tolfenamic acid.

Abbreviations

Aβ, amyloid β; AD, Alzheimer's disease; ANOVA, analysis of variance; APP, amyloid β precursor protein; BACE, beta-site APP cleaving enzyme; SEM, standard error of the mean; Sp1, specificity protein 1; SP1, Sp1 protein; YAC, yeast artificial chromosome.

Abstract

Since the introduction of the amyloid hypothesis of Alzheimer's disease (AD) two decades ago, the plaque forming protein amyloid beta (Aβ) has been extensively targeted for AD therapy. However, so far no Aβ-lowering or any other mechanism-based disease-modifying drug for AD is available. The effects of the drugs approved for AD are only symptomatic and cannot slow or stop the disease progression. Studies from our lab demonstrated that tolfenamic acid was able to lower the levels of the amyloid β precursor protein (APP) and its aggregative cleavage product Aβ by inducing the degradation of the transcription factor specificity protein 1 (Sp1). Similarly, tolfenamic acid also decreased the levels of tau, the main component of the neurofibrillary tangles in AD, and related deposits in other tauopathies. In this study, we examined whether tolfenamic acid alters the expression of the beta site APP cleaving enzyme 1 (BACE1) which is responsible for Aβ production and like APP and tau is under the transcriptional regulation of Sp1. Mice were administered two different doses of tolfenamic acid for one month, at the end of the study, BACE1 gene and protein levels as well as its activity were analyzed in the cerebral cortex. We found that tolfenamic acid was able to downregulate the expression of BACE1 and reduce its activity. Therefore, tolfenamic acid, a drug that has been used for years as anti-migraine, represents a novel class of AD therapeutics that targets the amyloid and tangle pathology of AD through multiple pathways due to its unique Sp1 lowering ability.

1. Introduction

A century has passed since the disease was first described by Alois Alzheimer and about 35 million patients around the world suffer today from Alzheimer's disease (AD) without any potential cure (Anstey et al., 2013; Selkoe, 2012). Furthermore, as no means for prevention of AD is available, the number of cases and the enormous economic cost of this devastating disease will continue to grow at an alarming rate. Knowledge on the pathophysiology of the disease continues to be gathered and reveal more possible drug targets and disease biomarkers. Two types of deposits are found in the AD brain, the amyloid plaques and the tau neurofibrillary tangles (Terry et al., 1964; Tomlinson, 1982). A lot of attention has been directed to the plaques and their main constituent amyloid beta (Aβ) as well as intermediates in Aβ production or degradation, especially after the development of the amyloid cascade hypothesis which views Aβ as a major trigger in the pathology of AD (Hardy and Selkoe, 2002; Hardy and Higgins, 1992).

Aβ is generated by the sequential enzymatic processing of the amyloid β precursor protein (APP) by β-secretase and γ-secretase (Shoji et al., 1992). The produced Aβ is normally secreted, but also can accumulate and form insoluble aggregates (Shoji et al., 1992; Urbanc et al., 1999). The levels and activity of β-secretase are elevated in AD brains compared to control (Holsinger et al., 2002; Li et al., 2004). β-APP cleaving enzyme 1 (BACE1) is the main form of β-secretase that cleaves APP to generate Aβ (Cai et al., 2001). In an alternative pathway for processing APP, it can be cleaved by the enzyme α-secretase within the Aβ fragment resulting in non-amyloidogenic products (Selkoe, 1994). Aβ is found as 36-43 amino-acid-long peptides of which

$A\beta_{40}$ is the most abundant and $A\beta_{42}$ is the most aggregative and is proposed to trigger plaque formation in AD (Iwatsubo et al., 1994; Nakano et al., 1999; Naslund et al., 2000).

Up to now, five drugs that belong to two classes have been approved for AD, the cholinesterase inhibitors and the NMDA receptor antagonist memantine. These interventions aim at improving memory functions to some extent but do not stop the dementia and the ultimate loss of daily functioning caused by AD. Many other candidates were in preclinical and clinical trials but failed to demonstrate safety or efficacy. Several AD targets under investigation are within the amyloid pathway of AD including APP, β-secretase, γ-secretase and Aβ itself. Yet, no successful candidate that can change the course of AD has been found.

Specificity protein 1 (Sp1) is a transcription factor that has been associated with the pathology of AD (Basha et al., 2005; Santpere et al., 2006; Zawia and Basha, 2005). Sp1 acts as a co-activator of APP transcription and regulates the expression of BACE1 (Christensen et al., 2004; Docagne et al., 2004). Sp1 regulates gene transcription by binding to GC rich promoter regions in genes like APP and BACE1 whose binding to Sp1 increases their transcription (Christensen et al., 2004; Docagne et al., 2004; Hoffman and Chernak, 1995; Pollwein et al., 1992). Overexpression of Sp1 increases BACE1 promoter activity, while the decline in Sp1 reduces BACE1 gene transcription (Christensen et al., 2004). Immunohistochemical studies from our laboratory demonstrated that (Sp1 protein) SP1, APP, and Aβ co-localize in brain neurons, and that cortical and hippocampal areas with higher SP1 levels express more Aβ (Brock et al., 2008). Moreover, depletion of SP1 by siRNA silencing of the Sp1 gene reduces the

responsiveness of the human APP promoter by approximately 70% (Basha et al., 2005). Therefore, changes in Sp1 expression can influence APP and BACE1 transcription and consequently alter the levels of their downstream product Aβ. Sp1 represents a potential AD target, where its abnormal and elevated expression has been associated with the disease decline (Brock et al., 2008; Christensen et al., 2004; Citron et al., 2008; Hoffman and Chernak, 1995; Santpere et al., 2006; Zawia and Basha, 2005).

Tolfenamic acid is a non-steroidal anti-inflammatory drug approved for migraine headaches in Europe. Tolfenamic acid induces the degradation of SP1 (Abdelrahim et al., 2006). Previous studies from our lab demonstrated that tolfenamic acid reduces the levels of SP1, APP, and Aβ (Adwan et al., 2011). Since the transcription factor Sp1 is vital for the regulation of several genes involved in AD including APP and BACE1, this research study was conducted to assess the effect of tolfenamic acid administration to APP yeast artificial chromosome (YAC) transgenic mice on BACE1, as a major enzyme in the production of Aβ, that is under Sp1 regulation. The hypothesis behind the use of tolfenamic acid for targeting Aβ in AD is illustrated in Fig. 1.

2. Materials and Methods

2.1. Animals

Female hemizygous APP YAC transgenic mice line R1.40 were used in this study. The B6.129-Tg(APPSw)40Btla/Mmjax strain was obtained from the Jackson Laboratory, Bar Harbor, ME. Animals were bred in-house and the age of mice used in

this study was between 14-20 months. This AD animal model contains the entire human APP gene including the regulatory fragments and expresses elevated levels of Aβ especially the longer more aggregative forms $A\beta_{42}$ and $A\beta_{43}$ (Lamb et al., 1999; Lamb et al., 1997; Lehman et al., 2003). Animals were housed in designated rooms within the animal facility at the University of Rhode Island under standard conditions with food and water freely available. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques, if available. Mice were assigned into 3 groups of similar age variations, n= 6 in each group. Animals were administered 0, 5 or 50 mg/kg tolfenamic acid (Sigma-Aldrich, St. Louis, MO) in corn oil every day by oral gavage for 34 days. On day 35, mice were sacrificed and brain tissues were collected and stored at -80°C until further use. Studies of animal weights before and after tolfenamic acid administration came from previous animal exposures, Hartley guinea pigs were administered control or 50 mg/kg tolfenamic acid 3 times a week for 4 weeks (n=3), and wild type C57BL/6 mice were treated with 0, 1, 5, 10, 25 or 50 mg/kg/day tolfenamic acid for 15 days, the full exposure scenario and other effects on APP and Aβ have already been published (Adwan et al., 2011). 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.

2.2. RNA isolation, cDNA synthesis and real time PCR

RNA was isolated from cerebral cortex tissue following the TRIzol® Reagent method (Invitrogen, Carlsbad, CA), checked for integrity by NanoDrop (Thermo Scientific, Wilmington, DE), and reverse transcribed to cDNA using iScript™ Select cDNA

Synthesis Kit following manufacturer's instructions (Bio-Rad, Hercules, CA). 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 minutes then at 85°C for 5 minutes to terminate the reaction. All incubations were conducted using MJ Research MiniCycler™ (Bio-Rad, Hercules, CA). Primer pairs for mouse BACE1 or β-actin were obtained from Invitrogen (Carlsbad, CA) as follows: BACE1 sense: 5'-ATGTGCACGATGAGTTCAGG-3' and antisense: 5'- GCA GAG TGG CAA CAT GAA GA -3'; β-actin sense: 5'- TGT TAC CAA CTG GGA CGA CA -3', and antisense: 5'- TCT CAG CTG TGG TGG TGA AG -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). Real time PCR was conducted using the 7500 Real Time PCR System (Applied Biosystems, Foster City, CA) following the standard protocol: 50°C for 2 minutes followed by 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Results were analyzed using the 7500 system software with relative quantification method and β-actin as endogenous control.

2.3. Protein extraction and Western blot analyses

Cerebral cortex tissue was homogenized with RIPA lysis buffer (10 mM Tris-HCl ([pH 7.4], 150 mM NaCl, 1% Triton X-100, 0.1 SDS, 1 mM EDTA (Fisher Scientific, Rockford, IL), and 0.1% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO)). The homogenates were centrifuged at 10,600 x g for 10 minutes at 4°C and supernatants were collected. Protein concentration was determined using the Micro

BCA Protein Assay Kit (Thermo Scientific Pierce, Rockford, IL). Protein extracts were stored at -80°C until further use. For Western blot analyses, approximately 40 µg of protein samples were separated onto 4–15% precast polyacrylamide gels (Bio-Rad, Hercules, CA) at 150 V for 1-2 hours and then transferred to PVDF membranes (GE-Healthcare, Piscataway, NJ). Membranes were blocked and incubated with the appropriate dilution of the specific primary antibody for 1-2 hours, either 1:500 dilution of MAB5308 for BACE1 levels (Millipore, Billerica, MA) or 1:5000 dilution of β-actin A2013 (Sigma-Aldrich, St. Louis, MO) were used. Then the membranes were washed with TBST four times and incubated with the appropriate infrared dye-labeled secondary antibody (Li-Cor, Lincoln, NE) for 1 hour at room temperature in the dark. Infrared signal of Western blot bands was detected and quantified using an Odyssey® Infrared Imaging System (Li-Cor, Lincoln, NE). Western blot bands were normalized against the levels of the house keeping protein β-actin.

2.4. BACE1 activity assay

BACE1 activity within the cerebral cortices of control and treated mice was measured using SensiZyme BACE1 activity assay kit CS1060 (Sigma-Aldrich, St. Louis, MO) following the manufacturer's instructions. Briefly 100 µL of blank, standards, or samples containing 450 µg protein were loaded into wells pre-coated with anti-BACE1 antibody. Samples were incubated for 2 hours at 4°C, after that, wells were washed 4 times then 50 µL of BACE1 substrate A was added to each well and incubated overnight at room temperature in a humidified chamber. On the next day 50 µL of the colorimetric substrate B reagent mixture was added to the wells and incubated at room temperature for 3 hours. At the end of the incubation period,

absorbance was measured at 405 nm using Spectra Max UV/Vis Spectrometer (GMI, Ramsey, MN) and BACE1 activity was calculated in ng/mL using the standard curve.

2.5. Statistical analysis

Data was represented as the mean ± the standard error of the mean (SEM). Statistical analysis was performed using GraphPad Instat software (GraphPad software, San Diego, CA) and statistical significance was determined by one-way analysis of variance (ANOVA) and Tukey-Kramer multiple comparisons post-test or using a two-tailed Student's *t*-test. Results marked with asterisks were significantly different from the control group ($p<0.05$).

3. Results

3.1. Tolfenamic acid is safe and well-tolerated by exposed animals

Tolfenamic acid has been used for years in humans for migraine headaches and rheumatoid arthritis. In our experiments, we did not observe any toxic effects of tolfenamic acid on animals. Tolfenamic acid was well tolerated and no changes in weight occurred in wild type mice administered 0, 1, 5, 10, 25 or 50 mg/kg/day doses for 15 days and Hartley guinea pigs administered 50 mg/kg 3 times a week for 4 weeks (Fig. 2A and B). Data obtained by our collaborators at M. D. Anderson Cancer Center also found 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 (M. R. Basha, unpublished observations).

3.2. Tolfenamic acid lowers BACE1 gene and protein expression in vivo

Following the administration of tolfenamic acid to APP YAC transgenic mice daily for 34 days, BACE1 gene expression within the cerebral cortex was lowered by 30% with both the 5 and 50 mg/kg doses as determined by real time PCR (Fig. 3). One-way ANOVA p=0.0116, Tukey-Kramer multiple comparisons post-test p<0.05 for the control vs 5 mg/kg group and for control vs 50 mg/kg group. Similar results were found in wild type animals administered 0 or 5 mg/kg/day tolfenamic acid for 15 days. On Day 4 there was a lowering trend that did not reach statistical significance, Student's test p=0.122 (Fig. 4A), while after 15 days of daily dosing BACE1 gene expression was lowered by 33%, Student's test p=0.0312 (Fig. 4B). Furthermore, tolfenamic acid daily administration for about a month decreased BACE1 protein expression levels by 6% and 16% in the transgenic APP YAC mice with the 5 and 50 mg/kg doses respectively as measured by Western blot analysis (Fig. 5). Student's t-test p<0.05.

3.3. Tolfenamic acid reduces BACE1 activity

We then checked how the activity of the enzyme BACE1 in the cerebral cortex was affected following the exposure of APP YAC transgenic mice to tolfenamic acid for 34 days. BACE1 enzyme activity was reduced by 45% with the 5 and 50 mg/kg/day doses as determined by BACE1 activity assay (Fig. 6). One-way ANOVA p=0.0197, Tukey-Kramer multiple comparisons post-test p<0.05 for the control vs 5 mg/kg and for C vs 50 mg/kg group.

4. Discussion

Research studies including those conducted in our lab demonstrate that the transcription factor Sp1 is involved in AD pathology (Brock et al., 2008; Christensen et al., 2004; Citron et al., 2008; Docagne et al., 2004; Santpere et al., 2006). Sp1 regulates the expression of APP, BACE1 and tau (Christensen et al., 2004; Docagne et al., 2004; Heicklen-Klein and Ginzburg, 2000; Hoffman and Chernak, 1995). SP1 co-localizes with APP and Aβ in brain neurons as well as with tau in tangles (Brock et al., 2008; Santpere et al., 2006). Due to its unique role in the transcription of AD related genes, targeting Sp1 is a novel and promising approach for discovering disease-modifying drugs for AD. In cancer, the reduction of SP1 by tolfenamic acid is beneficial due to the subsequent drop in the transcription of certain genes that are involved in tumor growth and formation such as the vascular endothelial growth factor and survivin (Abdelrahim et al., 2006; Basha et al., 2011; Eslin et al., 2011; Konduri et al., 2009).

So far, no drug has been found to slow or stop the progression of AD, all available medications alleviate symptoms of the disease to a certain limit, but do not affect any of its pathological features or prevent its progression. Aβ and other factors involved in its processing are being targeted for AD. Vaccines against Aβ are under development although several have failed in clinical trials due to life threatening adverse effects such as meningoencephalitis (Delrieu et al., 2012; Schnabel, 2011). The structural properties of the BACE active site limit the ability for development of inhibitors for this enzyme (Tamagno et al., 2012). Whereas γ-secretase inhibitors have failed due to

toxicity associated with the inhibition of Notch signaling (Mattson, 2004; Ross and Imbimbo, 2010).

Our previous work demonstrated that tolfenamic acid was able to downregulate proteins implicated in AD pathology including APP and Aβ (Adwan et al., 2011). In this study, we confirmed that tolfenamic acid also lowers BACE1, another protein that is regulated by Sp1 and takes part in the amyloidogenic pathway of AD (Christensen et al., 2004). Following tolfenamic acid daily administration for about 1 month, the expression and activity of BACE1 were reduced in APP YAC transgenic mice. In these animals, tolfenamic acid also lowered SP1, APP and Aβ as well as improved cognition as determined by behavioral tests using the Morris water maze and the Y-maze (G. M. Subaiea and N. H. Zawia, unpublished observations). By lowering SP1, tolfenamic acid was able to decrease tau gene and protein expression in the same animals as well (L. I. Adwan and N. H. Zawia, unpublished observations).

The safety of tolfenamic acid has already been established and the drug has been used for migraine headaches in Europe for years (Hakkarainen et al., 1982; Hakkarainen et al., 1979; Myllyla et al., 1998; Tokola et al., 1984). In our studies, no signs of toxicity were observed throughout the exposure periods, the weights of wild type mice and Hartley guinea pigs administered tolfenamic acid in our preliminary studies were presented in Fig. 2, with no irregular changes in weights throughout the duration of dosing. This exposure resulted in the reduction of the levels of brain APP and Aβ (Adwan et al., 2011).

Tolfenamic acid is a multi-target drug candidate for AD that affects both the amyloid and neurofibrillary tau pathology of AD. By decreasing Sp1, tolfenamic acid was able to lower BACE1 expression and activity. The safety of tolfenamic acid use in humans has already been established as it has been approved and used for years in Europe for migraine headaches. Hence it represents a promising agent that can be repurposed for AD and was recently scheduled to be tested in AD patients.

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Figure III-1. Downregulation of BACE1 and APP by tolfenamic acid. Tolfenamic acid stimulates the degradation of the transcription factor Sp1, which reduces the transcription of APP and BACE1, consequently reducing the expression of BACE1 and APP as well as the aggregative product Aβ and the associated AD pathology.

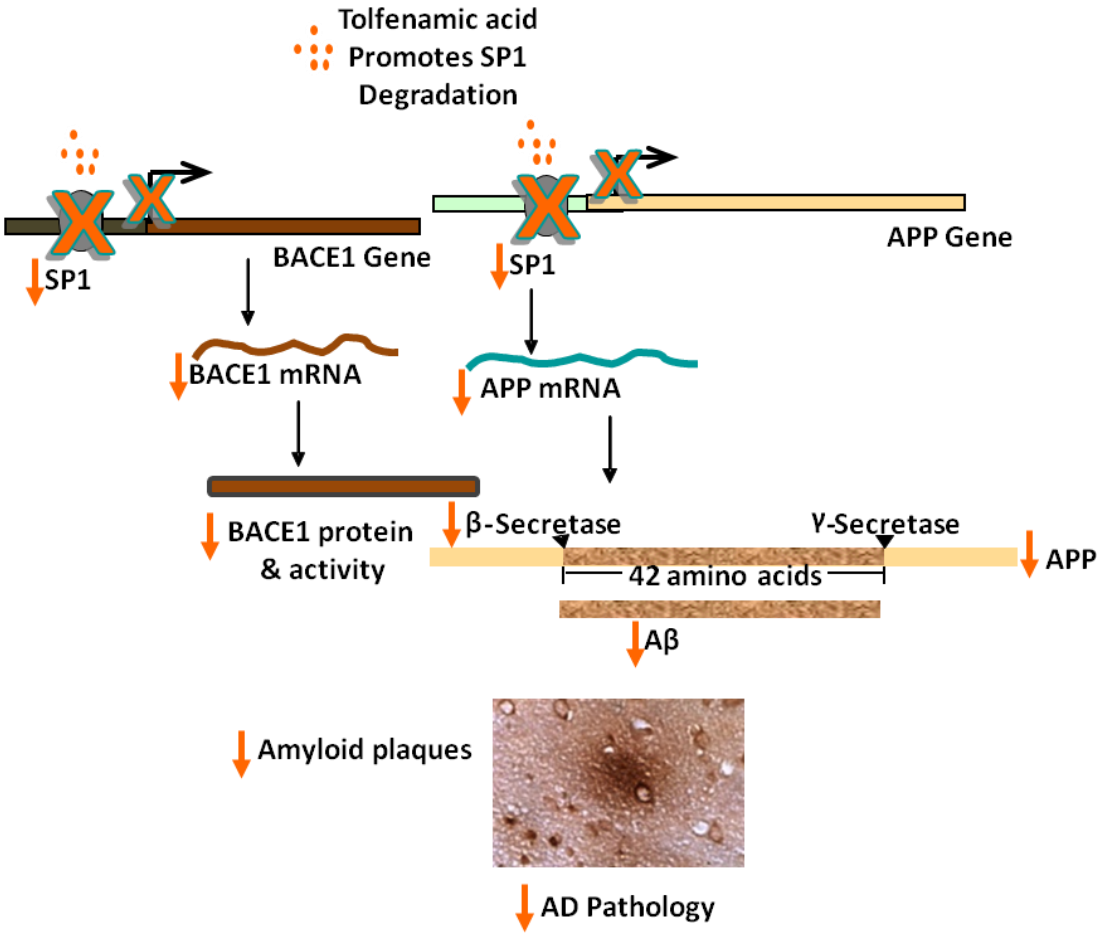
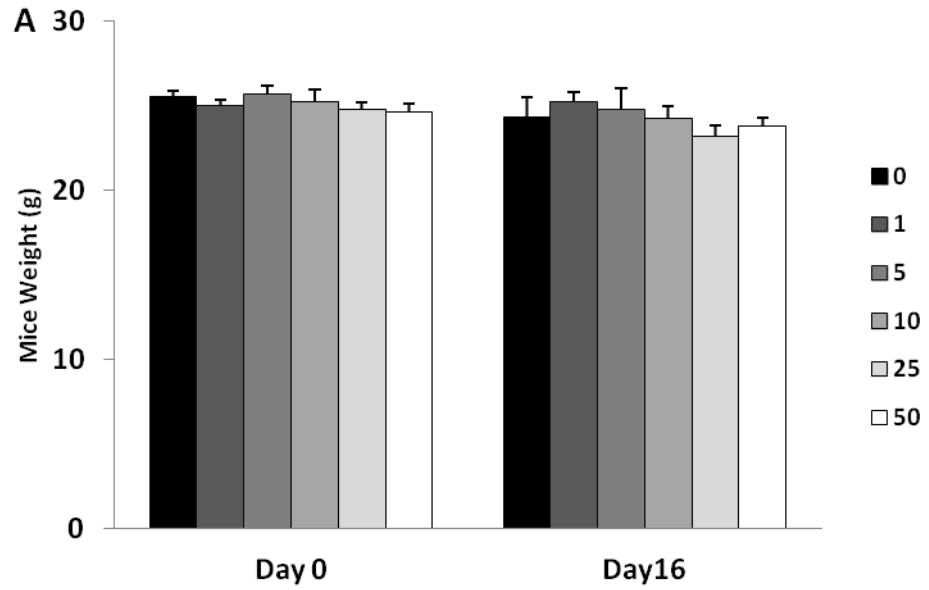

Tolfenamic acid
Promotes SP1
Degradation
SP1
BACE1 Gene
SP1
APP Gene
BACE1 mRNA
APP mRNA
BACE1 protein
& activity
β-Secretase
γ-Secretase
42 amino acids
APP
Aβ
Amyloid plaques
AD Pathology

Figure III-2. Weights of animals following treatment with tolfenamic acid. (A) Averaged weights of wild type mice before and after daily treatment with 0, 1, 5, 10, 25, or 50 mg/kg/day tolfenamic acid for 15 days, n=5. **(B)** Weekly measurements of the weights of Hartley guinea pigs administered 0 or 50 mg/kg tolfenamic acid 3 times a week for 4 weeks, n=3. Values shown are the mean ± SEM.


A
30
20
10
0
Mice Weight (g)
Day 0
Day16
0
1
5
10
25
50

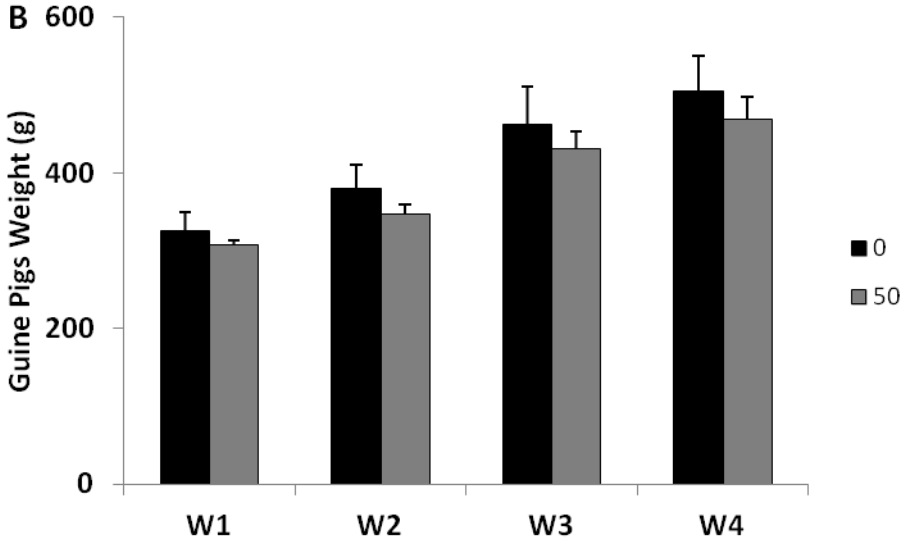

B
600
400
200
0
Guine Pigs Weight (g)
W1
W2
W3
W4
0
50

Figure III-3. BACE1 relative gene expression within the cerebral cortices of R1.40 transgenic mice following tolfenamic acid exposure. Hemizygous transgenic APP YAC mice were administered 0, 5 or 50 mg/kg/day tolfenamic acid for 34 days. BACE1 mRNA levels were measured in the cerebral cortex by real time PCR with β-actin as endogenous control as illustrated in the methods section. Values shown are the mean ± SEM, n=5 in each group, p=0.0116 as determined by one-way ANOVA with Tukey-Kramer post-test *p<0.05.

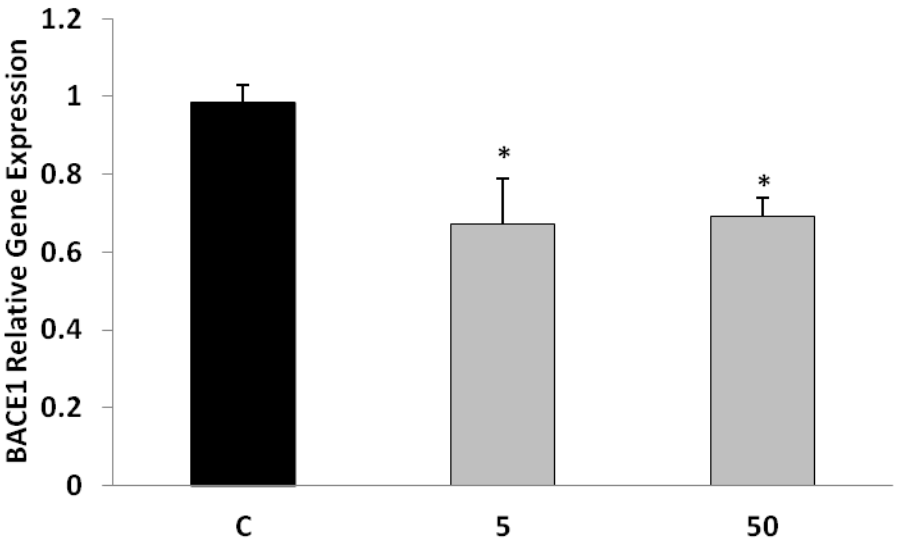

BACE1 Relative Gene Expression
1.2
1
0.8
0.6
0.4
0.2
0
*
*
C
5
50

Figure III-4. BACE1 relative gene expression in cerebral cortex tissues from mice treated with tolfenamic acid daily. Wild type C57BL/6 mice were given 0 or 5 mg/kg/day tolfenamic acid for 15 days. BACE1 mRNA levels were measured on **(A)** day 4 and **(B)** day 16 after dosing within the cerebral cortex by real time PCR with β-actin as endogenous control. For full details on the exposure scenario refer to Adwan et al., (2011). Values shown are the mean ± SEM, n=4, *p<0.05 as determined by Student's t-test.

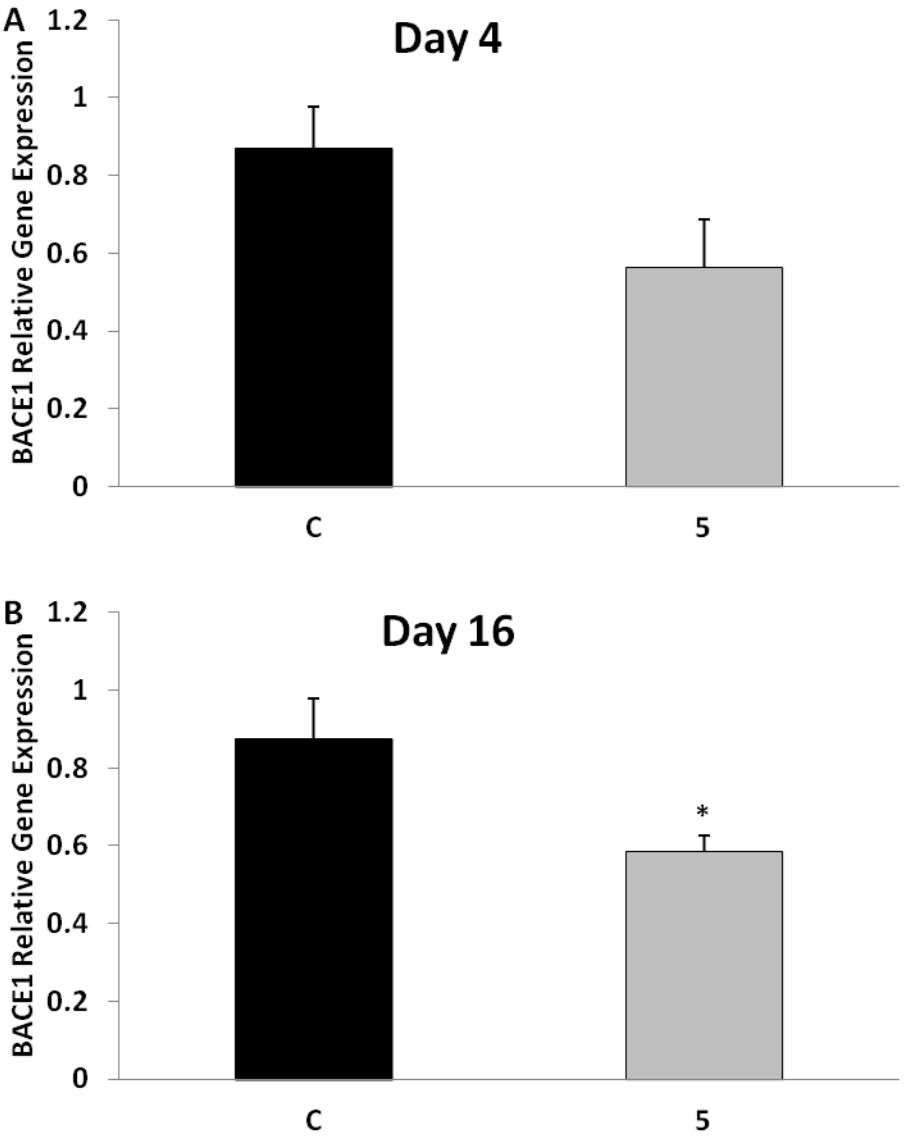

A
Day 4
BACE1 Relative Gene Expression
1.2
1
0.8
0.6
0.4
0.2
0
C
5
B
Day 16
BACE1 Relative Gene Expression
1.2
1
0.8
0.6
0.4
0.2
0
*
C
5

Figure III-5. BACE1 levels in R1.40 APP transgenic mice cortex after tolfenamic acid administration. BACE1 levels were analyzed in the cerebral cortex following daily administration of 0, 5, or 50 mg/kg tolfenamic acid to mice for 34 days by Western blot analysis. Value shown are the mean ± SEM, n=4. BACE1 levels were normalized to the levels of the house keeping protein β-actin. *p<0.05 according to Student's t-test.

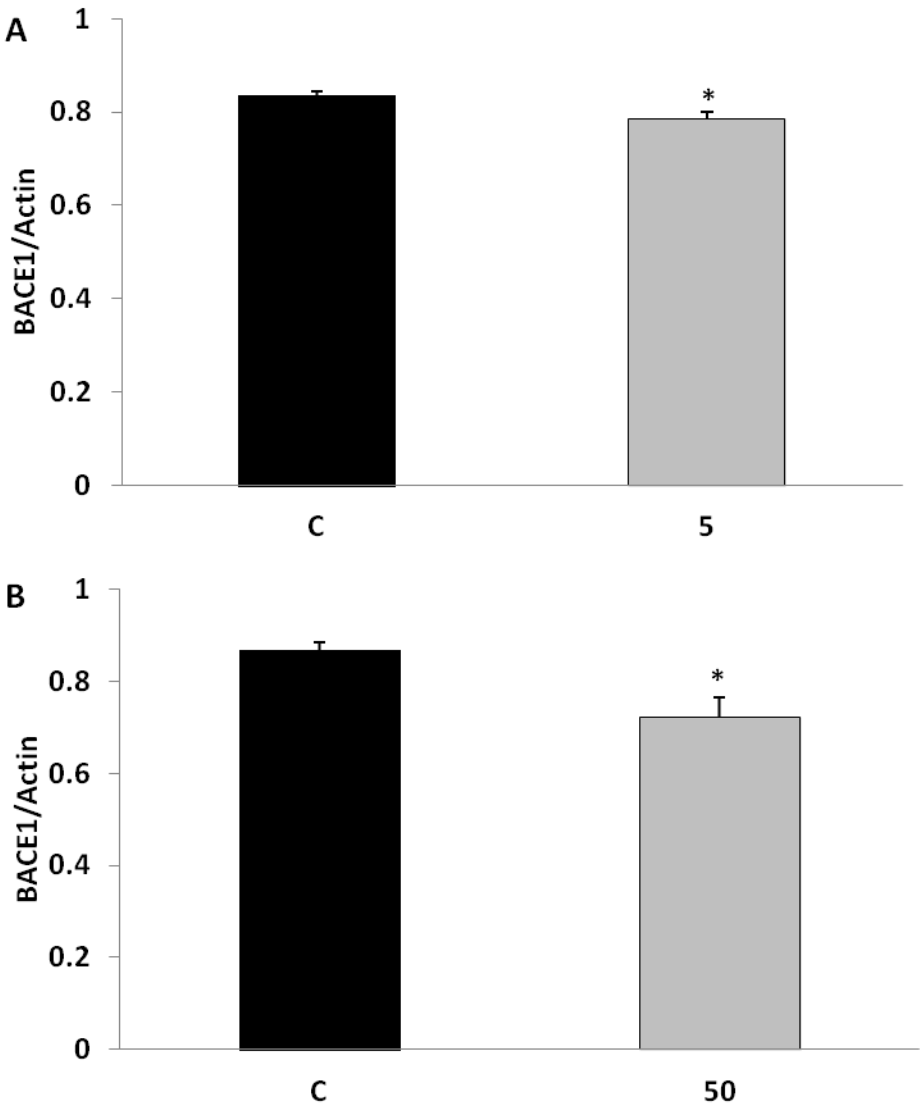
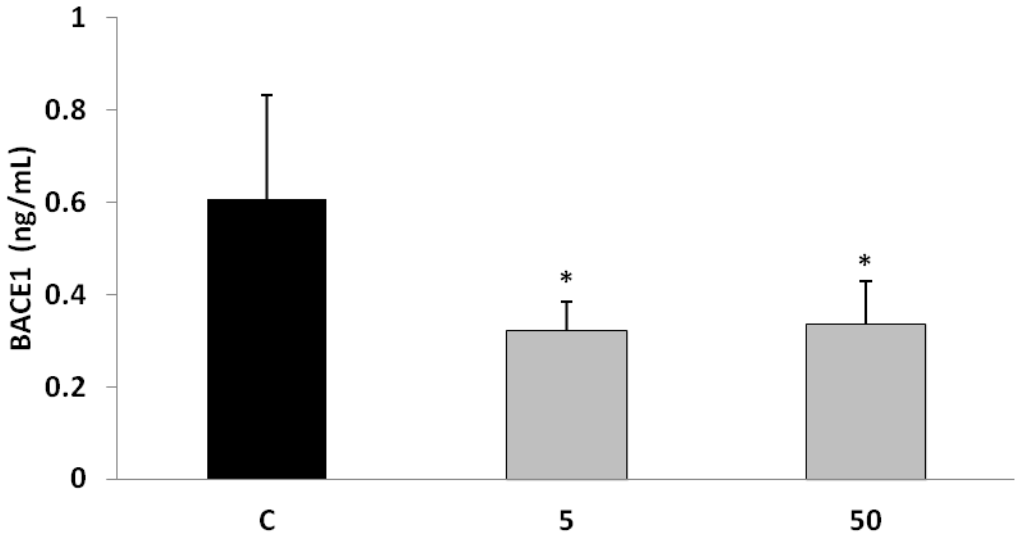

A
1
0.8
0.6
0.4
0.2
0
BACE1/Actin
*
C
5
B
1
0.8
0.6
0.4
0.2
0
BACE1/Actin
*
C
50

Figure III-6. BACE1 enzyme activity in the cerebral cortex of APP YAC transgenic mice following tolfenamic acid exposure. BACE1 activity was measured using SensiZyme BACE1 activity assay kit from Sigma-Aldrich as illustrated in the methods section. Values shown are the mean ± SEM, n=5. One-way ANOVA p=0.0197. *p<0.05 as determined by Tukey-Kramer post-test.


1
0.8
0.6
0.4
0.2
0
BACE1 (ng/mL)
*
*
C
5
50

MANUSCRIPT IV

Tolfenamic Acid Protects against Lead Toxicity *in vitro*: Effects on the Levels of Basal and Pb-Induced Alzheimer's Disease Related Genes and Proteins

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(Prepared for Toxicology and Applied Pharmacology Journal)

Tolfenamic Acid Protects against Lead Toxicity *in vitro*: Effects on the Levels of Basal and Pb-Induced Alzheimer's Disease Related Genes and Proteins

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Abstract

Tolfenamic acid reduces the levels of the transcription factor specificity protein 1 (Sp1) and as a result decreases the transcription of genes regulated by Sp1 like the amyloid precursor protein (APP), beta-site APP cleaving enzyme 1 (BACE1) and tau. These genes are implicated in the pathology of Alzheimer's disease (AD). They are major mediators in the formation of the characteristic plaques and tangles. Exposure to the environmental toxicant lead (Pb) is associated with AD pathology. Early life Pb exposure results in the upregulation of AD-related genes later in life. The gene and protein expression of APP and its aggregative product amyloid beta (Aβ) were increased following earlier Pb exposure. These results were replicated in neuroblastoma cells, rodents and primates. The effects of Pb were mediated by the upregulation of Sp1. This study was designed to examine the effects of tolfenamic acid on basal and Pb-induced levels of intermediates within the amyloid cascade hypothesis. Differentiated SH-SY5Y neuroblastoma cells were exposed to control, tolfenamic acid, or sequentially to Pb followed by control or tolfenamic acid. Our results show that while Pb upregulated SP1, APP and Aβ, tolfenamic acid was able to lower their expression. These results along with previous data from *in vivo* experiments provide evidence that tolfenamic acid represents a drug candidate, which can reduce the pathology of AD and may mitigate the damage of environmental risk factors associated with this disease which is mainly sporadic in nature.

Keywords

Alzheimer's disease, Amyloid β, Lead, SH-SY5Y cells, Sp1, Tolfenamic acid.

Abbreviations

Aβ, amyloid β; AD, Alzheimer's disease; ANOVA, analysis of variance; APP, amyloid precursor protein; BACE, β-site APP cleaving enzyme; FBS, fetal bovine serum; SEM, standard error of the mean; Sp1, specificity protein 1; SP1, Sp1 protein.

Introduction

Tolfenamic acid induces the degradation of the transcription factor specificity protein 1 (Sp1) (Abdelrahim et al., 2006). In mice, lowering Sp1 protein (SP1) resulted in the reduction of the amyloid precursor protein (APP) and its cleavage product amyloid β (Aβ), which are involved in Alzheimer's disease (AD) pathology (Adwan et al., 2011). This reduction is attributed to the transcriptional regulation of APP by Sp1 (Hoffman and Chernak, 1995; Docagne et al., 2004). Sp1 also regulates the transcription of the beta site APP cleaving enzyme 1 (BACE1) that processes APP and generates Aβ (Christensen et al., 2004). Overexpression of SP1 increases BACE1 promoter activity, while the decline in SP1 reduces BACE1 gene transcription (Christensen et al., 2004).

AD is characterized by the deposition of β-amyloid plaques and neurofibrillary tau tangles within the brain. Senile plaques are aggregates of Aβ peptides that are about 40 amino acids long (Glenner and Wong, 1984; Masters et al., 1985). Aβ is normally secreted, but also can accumulate resulting in the formation of insoluble aggregates which depends on the rates of Aβ synthesis and elimination (Shoji et al., 1992). The majority of AD cases are sporadic and the exact causes of the disease are unknown. According to the amyloid cascade hypothesis of AD, Aβ and its plaque aggregates formed by the amyloidogenic breakdown of APP trigger events that cause the neurodegeneration and dementia in AD, and therefore have been targeted for potential therapeutics (Hardy and Higgins, 1992; Hardy and Selkoe, 2002). However, so far no disease-modifying drug for AD is available.

Sp1 is a co-activator of APP transcription and siRNA silencing of the Sp1 gene reduces the responsiveness of the human APP promoter by 70% (Docagne et al., 2004; Basha et al., 2005). Immunohistochemical studies from our lab demonstrated that SP1, APP, and Aβ co-localize in rodent and primate brain neurons, and that cortical and hippocampal areas with higher SP1 levels express more Aβ (Brock et al., 2008). Therefore, any process that affects Sp1 could also influence APP transcription and alter the expression of its downstream pathogenic product Aβ. This makes Sp1 a plausible target for AD therapeutics.

Exposure to the environmental toxicant lead (Pb) is considered a risk factor with detrimental effects on various organs especially the brain (Zawia and Basha, 2005; White et al., 2007; Zawia et al., 2009). Experiments conducted at our lab demonstrated that Pb exposure early in life results in AD like pathology *in vitro* and *in vivo*, in rodents and primates. Pb administration caused the upregulation of Sp1, APP, Aβ as well as other intermediates implicated in AD later in life (Basha et al., 2005; Wu et al., 2008; Zawia et al., 2009; Bihaqi et al., 2011; Huang et al., 2011; Bihaqi and Zawia, 2012). Our most recent studies revealed that these molecular changes were accompanied by cognitive deterioration in mice administered Pb compared to controls (Bihaqi et al., in press).

In this study, we utilized an *in vitro* model of Pb exposure established in our lab to test the ability of tolfenamic acid to rescue proteins upregulated following early Pb exposure, which induces molecular consequences that resemble pathological events observed in late onset AD (Huang et al., 2011; Bihaqi and Zawia, 2012). Following cell viability studies, differentiated SH-SY5Y cells were exposed to Pb, tolfenamic

acid or both agents in chronological order and the changes on SP1, APP and Aβ were examined in comparison to control.

Materials and methods

Cell culture. Human neuroblastoma SH-SY5Y cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM)/F12 (Life technologies, Grand Island, NY) with 10% fetal bovine serum (FBS) and 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine at 5% CO_2 and 37°C. Cells were subcultured at 10^5 cells/mL in flasks containing 10 mL each and were allowed to attach over night then differentiated in 10 μM all-trans retinoic acid (Sigma-Aldrich, St. Louis, MO) in DMEM/F12 containing 1% FBS and 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine for 1 week following previously published methods (Jamsa et al., 2004; Huang et al., 2011). Neurite outgrowth was examined at 48, 72 h and 6 days (Jamsa et al., 2004) and the medium was changed every 48 h. Following differentiation, cells were exposed to control, tolfenamic acid, Pb or both.

Exposure to Pb and tolfenamic acid. For treatments, stock solutions of 10 mM Pb acetate in sterile distilled water and 100 mM tolfenamic acid in DMSO were prepared. The stock solutions were diluted in DMEM/F12 media containing 1% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine for the different exposures. The concentration of DMSO in the cell culture media was maintained at 0.05% for control and all other treatments. Differentiated SH-SY5Y cells were exposed to 0 or 25 μM tolfenamic acid for 96 h with the media changed every 48 h.

Cells were also exposed to 50 μM Pb for 48 h after which the media was removed and replaced with media containing 0, 25 or 50 μM tolfenamic acid for another 48 h. Cells exposed to media containing 0.05% DMSO, 1% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine with no Pb or tolfenamic acid were used as controls.

Cell viability assay. SH-SY5Y cells were loaded at 10^4 cells per 100 μL in each well onto 96-well plates and were allowed to attach overnight then were differentiated using 100 μM all-trans retinoic acid. Differentiated cells were exposed to 0, 1, 2.5, 5, 10, 50, or 100 μM tolfenamic acid for 12, 24, or 72 h with six replicates per group. Cells were incubated at 37˚C with 5% CO_2 and 90% humidity. Cell viability was determined using the Vybrant® MTT cell proliferation assay kit following the manufacturer's instructions (Life technologies, Grand Island, NY). Absorbance at 570 nm was measured using Spectra Max UV/Vis Spectrometer (GMI, Ramsey, MN) and cell viability was determined in treatment groups as a percentage from control.

RNA isolation, cDNA synthesis and real time PCR. RNA was isolated from cells following the TRIzol® Reagent method (Invitrogen, Carlsbad, CA), checked for integrity by NanoDrop (Thermo Scientific, Wilmington, DE), and reverse transcribed to cDNA using iScript™ Select cDNA Synthesis Kit following the manufacturer's instructions (Bio-Rad, Hercules, CA). 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 minutes followed by 85°C for 5 minutes to terminate the reaction. All incubations were conducted using MJ Research MiniCycler™ (Bio-Rad, Hercules,

CA). Primer pairs for human APP or GAPDH were obtained from Invitrogen (Carlsbad, CA) as follows: APP sense: 5′- GCC AAA GAG ACA TGC AGT GA -3' and antisense: 5′- CCA GAC ATC CGA GTC ATC CT -3′; GAPDH sense: 5′- AGC TGA ACG GGA AGC TCA CT -3′, and antisense: AGG TCC ACC ACT GAC ACG TTG -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). Real time PCR was conducted using the 7500 Real Time PCR System (Applied Biosystems, Foster City, CA) following the standard protocol: 50°C for 2 minutes followed by 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Results were analyzed using the 7500 system software with relative quantification method and GAPDH as endogenous control.

Protein extraction and Western blot analysis. Cytoplasmic and nuclear extractions were carried out using NE-PER nuclear and cytoplasmic extraction reagents according to the manufacturer's instructions (Thermo Scientific Pierce, Rockford, IL). Protein concentration was determined with the Micro BCA protein assay kit (Pierce, Rockford, IL). For SP1 Western blotting, samples containing 20 µg nuclear protein were separated onto 4–15% precast polyacrylamide gels (Bio-Rad Hercules, CA) at 150 V for 1-2 h and then transferred to PVDF membranes (GE-Healthcare, Piscataway, NJ). Membranes were blocked and incubated with the appropriate primary antibody for 1-2 h, either 1:500 dilution of ABE135 for SP1 levels (Millipore, Billerica, MA) or 1:2000 of GAPDH T9450 (Sigma-Aldrich, St. Louis, MO), then the membranes were washed with TBST and incubated with the appropriate infrared dye-labeled secondary antibody (Li-Cor, Lincoln, NE) for 1 h at room temperature in the

dark. Infrared signal of Western blot bands was detected and quantified using Odyssey® Infrared Imaging System (Li-Cor, Lincoln, NE). Western blot results were normalized against the levels of the house keeping protein GAPDH.

ELISA $A\beta_{40}$ assay. Levels of $A\beta_{40}$ in cell culture media were measured using human $A\beta_{40}$ kit JP27713 (IBL, Gunma, Japan). The kit is solid-phase sandwich ELISA with highly specific antibody that is 100% reactive with human $A\beta_{40}$ with a sensitivity of 5.00 pg/mL. The kit measures $A\beta_{40}$ cleaved N terminal side by any cause. The assay was conducted following manufacturer's instructions with minor modifications. One hundred µg protein as determined by Micro BCA protein assay kit (Thermo Scientific Pierce, Rockford, IL) in 100 µL EIA buffer and assay standards were added in triplicates to 96-well plates pre-coated with anti-human Aβ mouse IgG MoAb. The plates were incubated overnight at 4°C, and washed 7 times using the 40X diluted wash buffer supplied with the kit (0.05% Tween 20 in phosphate buffer), and 100 µL labeled antibody was added and incubated for 1 h at 4°C, the wells were washed again 9 times, and then 100 µL of tetramethylbenzidine was added as a coloring agent, and incubated in the dark for 30 minutes at room temperature. Finally 100 µL of 1N H_2SO_4 was added to stop the reaction, and absorbance was measured at 450 nm using Spectra Max UV/Vis Spectrometer (GMI, Ramsey, MN). The concentration of Aβ in unknown samples was calculated as pg/mg total protein using the standard curve obtained.

Statistical analysis. Data was represented as the mean ± the standard error of the mean (SEM). Statistical analysis was performed using GraphPad Instat software (GraphPad software, San Diego, CA) and statistical significance was determined by one-way

analysis of variance (ANOVA) and Tukey-Kramer multiple comparisons post-test. Results with *p*-values <0.05 were considered significantly different from the group in comparison and were marked accordingly.

Results

Tolfenamic acid cell viability studies in differentiated neuroblastoma cells

The viability of SH-SY5Y cells differentiated using all-trans retinoic acid was examined following treatments with 0-100 μM tolfenamic acid. The results show that tolfenamic acid did not cause any cytotoxicity until the highest dose of 100 μM after 24 h and 72 h of exposure ($p<0.001$) (Fig. 1). This suggests that the effects of tolfenamic acid on cell viability are time and dose-dependent. Overall one-way ANOVA reported a *p*-value less than 0.0001; one-way ANOVA $p=0.8164$ for groups in the 12 h exposure; $p<0.0001$ for the 24 h treatment groups; and $p<0.0001$ for groups in the 72 h exposure. Based on these results, we chose the doses of 25 and 50 μM of tolfenamic acid for the following exposure experiments.

Tolfenamic acid lowers SP1

Exposure of differentiated SH-SY5Y cells to 50 μM Pb for 48 h followed by control for 48 h induced the expression of SP1 by 47% which did not reach statistical significance according to Tukey-Kramer post-test when compared to control exposed cells. However, when Pb treatment for 48 h was succeeded by exposure to 25 μM tolfenamic acid for 48 h, SP1 levels were decreased by 75% compared to control which was deemed statistically significant according to Tukey-Kramer post-test ($p<0.05$), and by 83% when compared to SP1 levels in cells exposed to Pb for 48 h

followed by control for 48 h (p<0.01). Overall one-way ANOVA between all groups reported a p-value equal to 0.003 (Fig. 2).

Effects of tolfenamic exposure on APP gene expression

Treatment of cells with tolfenamic acid for 96 h reduced the gene expression of APP compared to control by 18% that was not statistically significant. Whereas the exposure of cells to Pb for 48 h and control for 48 h increased APP gene expression by 23% which did not reach statistical significance when compared to control. Tolfenamic acid treatment after Pb lowered the Pb-induced APP gene expression in differentiated neuroblastoma cells by 60% from control (p<0.05) and by 67% from cells exposed to Pb for 48 h followed by control for 48 h (p<0.01). Overall one-way ANOVA reported a p-value of 0.001 (Fig. 3).

Tolfenamic acid lowers the levels of $A\beta_{40}$ induced by Pb

Aβ levels were increased by 42% in differentiated SH-SY5Y cells after treatment with Pb for 48 h followed by control for additional 48 h (Fig. 4). This increase was significant when compared to cells treated with control for 96 h with the media changed every 48 h according to Tukey-Kramer multiple comparison test (p<0.01). When treatment of SH-SY-5Y cells by Pb for 48 h was followed by treatment with 25 µM tolfenamic acid for 48 h, there was a trend of reduction in Aβ levels in the media by 10% compared to treatment with Pb for 48 h and control for 48 h. Aβ levels were decreased by 56% with the 50 µM tolfenamic concentration for 48 h following prior Pb exposure for 48 h which was significant compared to Aβ levels in the media of cells exposed to Pb for 48 h and control for 48 h (p<0.001); and Aβ levels were reduced by 37% compared to cells exposed to control for 96 h (p<0.05). However,

treatment of cells with 25 μM tolfenamic acid for 96 h did not change Aβ levels within the media. The overall one-way ANOVA *p*-value between groups was $p<0.0001$.

Discussion

The transcription factor Sp1 has been linked to the pathology of AD (Zawia and Basha, 2005; Santpere et al., 2006; Citron et al., 2008). Sp1 promotes the transcription of APP, BACE1 and tau, which are considered to be key pathological intermediates in AD. Data from our lab demonstrated that the non-steroidal anti-inflammatory drug tolfenamic acid lowers SP1, APP, Aβ, BACE1, and tau in mice (Adwan and Zawia, Unpublished observations; Adwan et al., 2011). The toxic effects of Pb on health have been described in the literature, experiments from our lab showed that Pb induced the expression of AD related genes and proteins including Sp1, APP, Aβ, and tau (Basha et al., 2005; Wu et al., 2008; Huang et al., 2011; Bihaqi and Zawia, 2012). Hence, tolfenamic acid and Pb represent two agents that have opposing effects when it comes to AD related processes.

Tolfenamic acid has been used for rheumatoid arthritis and migraine headaches in Europe for years and its safety for use in humans was established. In neuroblastoma cells, low doses of tolfenamic acid did not affect cell viability (Fig. 1). A decrease in cell viability with tolfenamic acid was observed at the higher concentration of 100 μM and at the longer periods of exposure of 24 h and 72 h. The outcomes of tolfenamic acid on cell viability were dose and time dependent.

To study the effects of tolfenamic acid on AD related genes and proteins in neuroblastoma cells, we chose the 25 and 50 μM concentrations which did not affect

cell viability based on our results. The 50 μM dose chosen for Pb exposure came from our previous cell viability and exposure studies with the same cell line (Huang et al., 2011; Bihaqi and Zawia, 2012). Our results show that the exposure of differentiated SH-SY5Y neuroblastoma cells to tolfenamic acid for 48 h after 48 h of Pb exposure decreased SP1 levels significantly compared to cells exposed to control for 96 h or Pb followed by control for 48 h each (Fig. 2). Furthermore, tolfenamic acid significantly reduced APP gene and Aβ expression that was induced by Pb exposure but not the basal levels of APP and Aβ (Figs. 3 and 4).

The 25 μM dose of tolfenamic acid was able to decrease SP1 levels and APP gene expression induced by prior Pb exposure. However, Aβ levels were only decreased significantly by the 50 μM tolfenamic acid exposure after Pb. As tolfenamic acid affects transcription, time is an important factor for observing its effects and in this study, although the 25 μM tolfenamic acid was very effective in lowering SP1 and APP gene expression following Pb administration, this drastic change was not translated into Aβ lowering probably due to insufficient time. For example, our previous studies showed that even though APP gene transcription was lowered with tolfenamic acid daily administration in mice for three days, APP protein levels were not lowered at that time (Adwan et al., 2011). Whereas the levels of both the APP gene and protein were decreased after two weeks of tolfenamic acid daily administration to mice (Adwan et al., 2011).

About 90% of AD cases are sporadic and are referred to as late onset AD with age being the major risk factor (Alzheimer's Association, 2012). Our lab has demonstrated that early Pb exposure replicates pathological events observed late in life in AD within

various *in vitro* and *in vivo* models (Basha et al., 2005; Wu et al., 2008; Bihaqi et al., 2011; Huang et al., 2011; Bihaqi and Zawia, 2012). In this manuscript, we use prior Pb exposure as a model that produces an upregulation of AD related intermediates including APP and Aβ by inducing the transcription factor Sp1, in a matter that resembles the environmentally inflicted late onset AD. After Pb exposure, we exposed the cells to tolfenamic acid, in order to test its ability to reverse the events caused by Pb. Our results show that tolfenamic acid was able to rescue the cells from the pathological increase in SP1, APP and Aβ. Hence tolfenamic acid represents a promising agent for AD which has been selected for testing in AD patients and may be useful in environmentally-induced AD pathogenesis.

Acknowledgements

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Figure IV-1. Cell viability of differentiated SH-SY5Y cells following tolfenamic acid exposure. Neuroblastoma cells were differentiated and exposed to 0, 1, 2.5, 5, 10, 25, 50 or 100 μM tolfenamic acid for 12 h, 24 h, or 72 h and cell viability was studied using MTT as illustrated in the methods section. Values shown are the mean ± SEM, n=6 in each group, overall one-way ANOVA $p<0.0001$, one-way ANOVA $p=0.8164$ for groups in the 12 h exposure; $p<0.0001$ for groups in the 24 h; and $p<0.0001$ for groups in the 72 h exposure. ***$p<0.001$ compared to the corresponding control group from the same time duration of exposure as determined by Tukey-Kramer post-test.

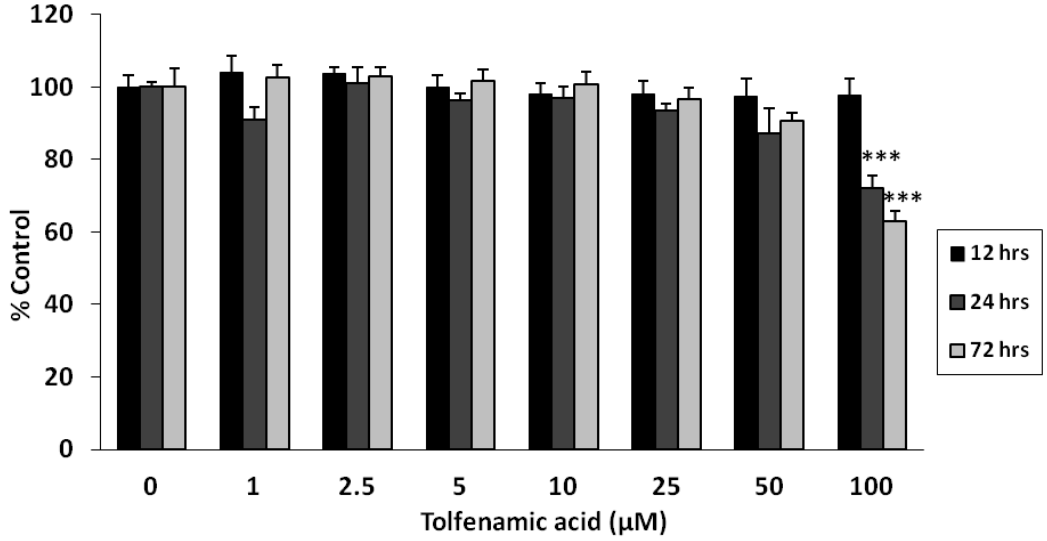

120
100
80
60
40
20
0
% Control
***
***
12 hrs
24 hrs
72 hrs
0
1
2.5
5
10
25
50
100
Tolfenamic acid (μM)

Figure IV-2. SP1 levels in differentiated SH-SY5Y cells after treatment with control or Pb followed by control or by tolfenamic acid. SH-SY5Y cells were differentiated using 100 μM all-trans retinoic acid and exposed to control (C) for 96 h with the media changed every 48 h; 50 μM Pb for 48 h followed by control for another 48 h; or 50 μM Pb for 48 h followed by 25 μM tolfenamic acid (TA) for 48 h. Values shown are the mean ± SEM. Three independent experiments were performed in triplicates. SP1 levels were normalized to the levels of the house keeping protein GAPDH. One-way ANOVA p=0.003, with Tukey-Kramer post-test *p<0.05 compared to C, ††p<0.01 compared to 48 h Pb followed by 48 C exposure group. Insert shows representative SP1 and GAPDH Western blot bands from 96 h control (C); 48 h Pb treatment followed by 48 h control; or 48 h Pb exposure followed by 48 h tolfenamic acid (TA) treatment.

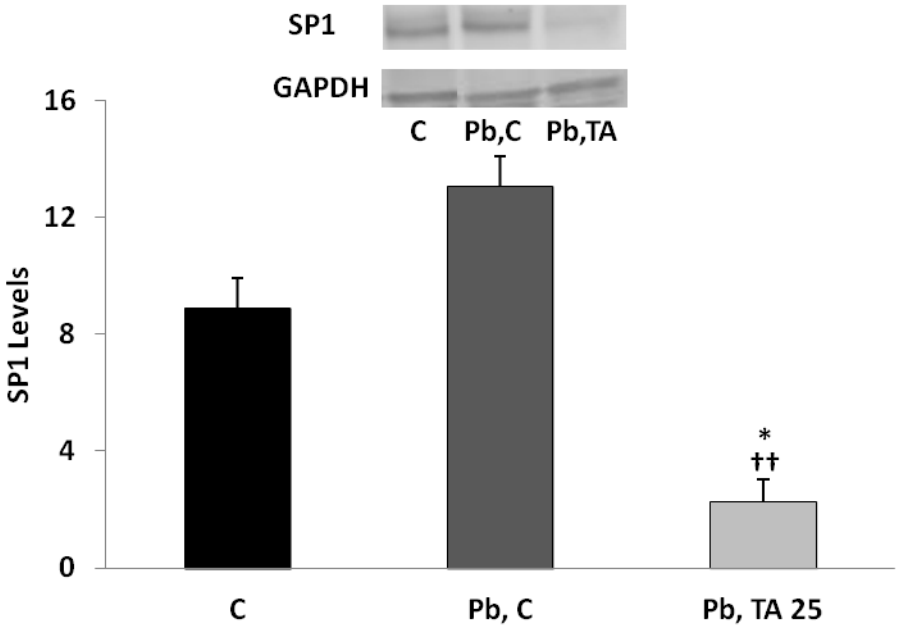

SP1
GAPDH
C
Pb,C
Pb,TA
16
12
8
4
0
SP1 Levels
*
††
C
Pb, C
Pb, TA 25

Figure IV-3. APP gene expression in differentiated SH-SY5Y cells exposed to tolfenamic acid, Pb or both. Differentiated neuroblastoma cells were exposed to control (C) or 25 μM tolfenamic acid (TA) for 96 h with the media changed every 48 h, cells were also exposed to 50 μM Pb for 48 h followed by C, 25 or 50 μM tolfenamic acid for 48 h. APP gene expression was measured by real time PCR with GAPDH as endogenous control as illustrated in the methods section. One-way ANOVA p=0.001. Tukey-Kramer post-test *p<0.05 compared to C, ††p<0.01 compared to 48 h Pb followed by 48 C exposure group.

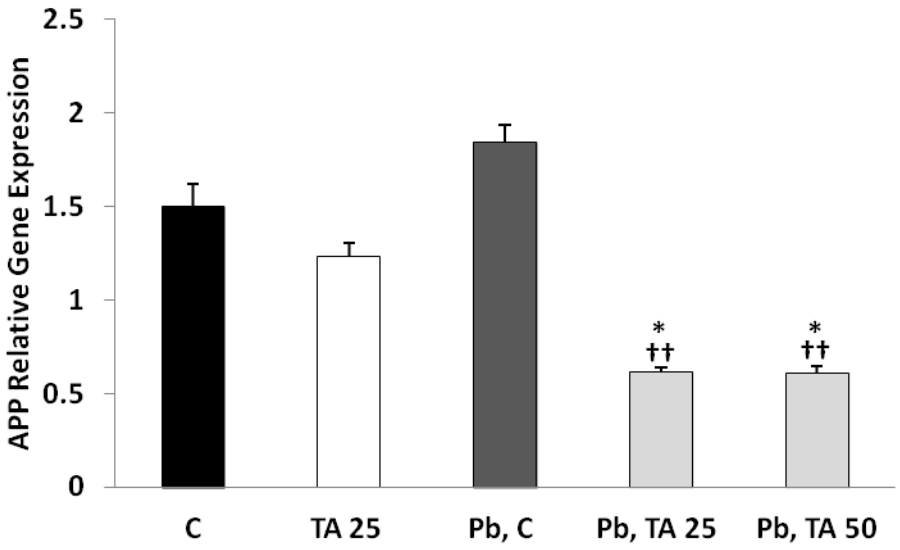
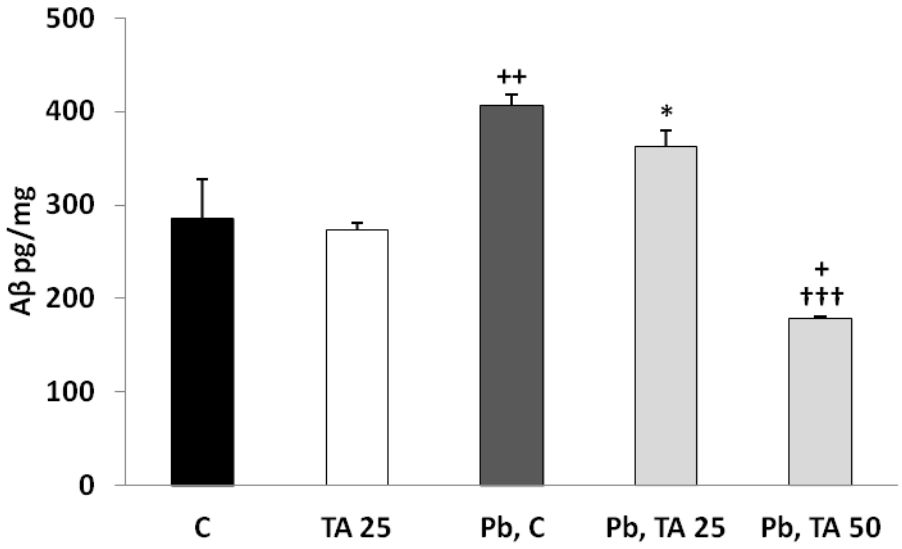

APP Relative Gene Expression
2.5
2
1.5
1
0.5
0
*
††
*
††
C
TA 25
Pb, C
Pb, TA 25
Pb, TA 50

Figure IV-4. Aβ levels in differentiated SH-SY5Y cells exposed to tolfenamic acid, Pb or both. Differentiated neuroblastoma cells were exposed to control (C) or 25 µM tolfenamic acid (TA) for 96 h with the media changed every 48 h; or 50 µM Pb for 48 h followed by either C, 25, or 50 µM tolfenamic acid for 48 h. Aβ levels within the media were measured using ELISA as explained in the methods section. Overall one-way ANOVA $p<0.0001$ between all groups. Tukey-Kramer post-test *$p<0.05$ compared to 25 µM TA group; +$p<0.05$, ++$p<0.01$ compared to 96 h C group and 25 µM TA group; †††$p<0.001$ compared to 48 h Pb followed by 48 C group and compared to 48 h Pb followed by 48 h 25 µM TA group


500
400
300
200
100
0
Aβ pg/mg
++
*
+
†††
C
TA 25
Pb, C
Pb, TA 25
Pb, TA 50