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# BRAIN PENETRATION OF TOLFENAMIC ACID AND ITS ABILITY TO ENHANCE THE COGNITIVE FUNCTIONS AND TO LOWER AMYLOID PATHOLOGY IN ANIMAL MODELS OF ALZHEIMER'S DISEASE

BY

GEHAD MOHAMMED SUBAIEA

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE

REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

PHARMACEUTICAL SCIENCES

UNIVERSITY OF RHODE ISLAND

2012

DOCTOR OF PHILOSOPHY DISSERTATION

OF

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2012

# ABSTRACT

Alzheimer's disease (AD) is a neurodegenerative disease that is responsible for up to 80% of all dementia cases. Hallmarks of AD include the presence of beta-amyloid (Aβ) deposits and twisted strands of abnormal hyperphosphorylated tau forming neurofibrillary tangles (NFTs), which ultimately lead to damage and death of nerve cells in the brain. Symptoms of the disease include impairments in cognitive and functional abilities that gradually worsen as the disease progress. Finally, in advanced AD, patients become unable to perform basic daily activities, become more susceptible to infections, and ultimately die. Only five drugs are FDA-approved for use in AD, which only provide temporary symptomatic relief without capabilities of modifying the progression of the disease. Tolfenamic acid is a non-steroidal anti-inflammatory drug that has the potential for slowing the progression of AD by inducing the degradation of the transcription factor specificity protein 1 (Sp1). Sp1 regulates the expression of several AD-related genes including amyloid precursor protein (APP), tau and beta-site APP-cleaving enzyme 1 (BACE1).

To investigate the ability of tolfenamic acid to cross the blood brain barrier (BBB), multiple approaches were utilized. *In silico* computational models to predict logBB and logPS for CNS penetration indicated that tolfenamic acid could transfer passively through the BBB. In addition, immobilized artificial membrane (IAM) chromatography using a phosphatidylcholine column was utilized for the *in vitro* determination of the brain penetration capacity factor $[(K_{IAM}/MW^4)x10^{10}]$ of tolfenamic acid. The results showed that the $[(K_{IAM}/MW^4)x10^{10}]$ for tolfenamic acid

was 2.73 indicating its ability to cross the BBB and penetrate into the brain. Upon intravenous (IV) administration of tolfenamic acid to guinea pigs and mice, LC-MS analysis revealed the presence of tolfenamic acid in the extracted brain tissue. This *in vivo* approach was also used to estimate the brain-to-plasma ratio (*Kp*) for tolfenamic acid, which was 0.11. Thus, using *in silico*, *in vitro* and *in vivo* methods, we confirmed the ability of tolfenamic acid to cross the BBB. The study offers a guide of using multiple approaches to predict the ability of different compounds to penetrate the brain.

Next, we investigated the ability of tolfenamic acid to attenuate the cognitive deficits in a transgenic mouse model of AD, namely R1.40. First, we demonstrated that hemizygous R1.40 mouse model exhibits spatial working and long-term memory deficits when tested in the Morris water maze and Y-maze. We found that short-term administration of tolfenamic acid for 34 days was able to reverse the observed cognitive deficits in hemizygous R1.40 mice. These mnemonic improvements were parallel to reductions in SP1 protein, APP expression and protein, and soluble and insoluble $A\beta_{40-42}$ levels. These findings suggest that the reductions in the biochemical markers of AD were also accompanied by functional improvements in the treated mice.

We further examined the ability of tolfenamic acid to improve spatial learning and memory as well as to reduce Aβ plaque pathology in old homozygous R1.40. Homozygous R1.40 mice exhibit cognitive deficits that are accompanied by fibrillar Aβ deposition in different regions of the brain by the age of 14-15 months. Our

immunohistochemical results indicated that short-term tolfenamic acid treatment for 34 days was able to produce a drastic reduction in Aβ plaque pathology. In addition, spatial working memory, assessed by the spontaneous alternations in the Y-maze, was improved by tolfenamic acid treatment. Further, we investigated if tolfenamic acid would improve the age-related learning and memory impairments in senescent C57BL/6 mice and we found that treatment with tolfenamic acid for 34 days resulted in improvements in the spatial reference memory functions as well. The major findings from these studies suggest that tolfenamic acid crosses the BBB into the brain, is able to decrease AD-related proteins including Aβ plaques, and is able to produce mnemonic functional improvements in AD transgenic mouse model and senescent wild type mice as well. Thus, tolfenamic acid could be proposed as a disease-modifying therapy for AD via its unique mechanism.

## ACKNOWLEDGEMENTS

In the name of Allah, the most gracious, the most merciful. Thanks to Allah, glory be to Him, for his help and guidance throughout my life.

I wish to thank and express my respectful gratitude to my major advisor Dr. Nasser H. Zawia for providing me the opportunity to conduct my research in his lab in the College of Pharmacy at the University of Rhode Island. His mentorship, supervision and encouragement guided me through my research and I thank him for his comments and suggestions on my dissertation.

I wish to thank my core committee members, Dr. Keykavous Parang, Dr. Karen Stevens and Dr. Walter Besio, and my dissertation readers, Dr. Constantine Anagnostopoulos and Dr. Angela Slitt for taking the time and effort to read my dissertation and provide me with valuable suggestions.

I am grateful to all my colleagues in the Zawia lab and in the Department of Biomedical and Pharmaceutical Sciences in the College of Pharmacy for their kind assistance.

I would like to express my deepest appreciation to my parents and all my family members for their kind support and encouragement throughout the years I spent in higher education. In particular, I thank my wife, to whom this dissertation is dedicated to, for her support, endurance and caring for me and my children; Alaa, Rana and Eyad.

## PREFACE

This dissertation addresses the brain penetration of tolfenamic acid using multiple approaches as well as it address its effects in altering various AD-related pathology and improving the mnemonic deficits in a transgenic mouse model of AD and senescent wild type mice. It discusses the molecular biology, behavioral and immunohistochemical experiments used to investigate the effects of tolfenamic acid as a proposed disease-modifying drug candidate for AD.

The dissertation is structured in manuscript format and it comprises three manuscripts. The first manuscript "The Ability of Tolfenamic Acid to Penetrate the Brain: A Model for Testing the Brain Disposition of Candidate Alzheimer's Drugs Using Multiple Platforms" was published in 2011 in Current Alzheimer Research. The second manuscript "Reversal of Cognitive Deficits by Tolfenamic Acid is Accompanied by Reductions in Intermediates Associated with Amyloid pathology in a Transgenic Mouse Model of Alzheimer's Disease" is formatted in accordance for submission to Neurobiology of Aging. The third manuscript "Reduction of β-Amyloid Deposition and Attenuation of Memory Deficits by Tolfenamic Acid" is formatted in accordance for submission to Alzheimer's & Dementia.

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## MANUSCRIPT 1

# The Ability of Tolfenamic Acid to Penetrate the Brain: A Model for Testing the Brain Disposition of Candidate Alzheimer's Drugs Using Multiple Platforms

**By**

**Gehad M. Subaiea, Bothaina H. Alansi, David A. Serra, Maged Alwan, and Nasser H. Zawia**

**(Published in Current Alzheimer Research, 2011, 8, 860-867)**

## Abstract

Evidence from our laboratory suggests that tolfenamic acid has a potential for slowing the progression of Alzheimer's disease (AD) through lowering cortical levels of the β-amyloid precursor protein (APP) and its pathogenic amyloid beta (Aβ) intermediates [1]. In this study, we examined the ability of tolfenamic acid to cross the blood brain barrier (BBB) by predicting its logBB and logPS values, the indexes of BBB permeability, using computational models. We also determined, via *in vitro* methods, the brain penetration capacity factor $[(K_{IAM}/MW^4)x10^{10}]$ using phosphatidylcholine column chromatography. The obtained logBB, logPS and $(K_{IAM}/MW^4)x10^{10}$ values predicted that tolfenamic acid can passively transfer into the central nervous system (CNS). These results were validated *in vivo* using LC-MS analysis after administration of tolfenamic acid intravenously to guinea pigs and mice. The present study provides the first evidence of the ability of tolfenamic acid to cross the BBB and offers a comparative analysis of approaches used to predict the ability of compounds to penetrate into the brain.

**Keywords:** Alzheimer's disease, blood brain barrier, HPLC, mass spectrometry, tolfenamic acid.

## 1. Introduction

Alzheimer's disease (AD), a progressive neurodegenerative disease marked by dementia evolving till death, is characterized by plaques. These plaques are composed mainly of amyloid beta (Aβ) peptides that result from the cleavage of β-amyloid precursor protein (APP) by the enzyme β-site APP cleaving enzyme 1 (BACE1) followed by γ-secretase [2]. Specificity protein 1 (Sp1) is a transcription factor that is involved in co-activation of APP transcription and regulation of BACE1 expression [3, 4] and is critical for the activation of the human APP promoter [5]. Tolfenamic acid is a non-steroidal anti-inflammatory drug (NSAID) used in Europe for the treatment of migraine, and has been found to degrade the transcription factors Sp1, Sp3, and Sp4 proteins and thus inhibiting the growth of pancreatic tumors by diminishing the expression of vascular endothelial growth factor (VEGF) [6].

Data from our lab indicate that tolfenamic acid lowers cortical Sp1 protein (SP1) levels and consequently APP expression and its plaque-forming Aβ products [1]. Compounds designed to exhibit their mode of action in the central nervous system (CNS) need to be assessed for their ability to cross the blood brain barrier (BBB) and thus we conducted several studies to examine the ability of tolfenamic acid to penetrate into the brain.

There are various *in silico, in vitro* and *in vivo* models that are used in drug discovery for the assessment of the ability of potential drug candidates to transfer into the CNS [7]; however, few studies have compared the potential of these methods using a single compound tested across platforms. In this study, we studied the brain transfer potential

of tolfenamic acid using three approaches. Comparison of these approaches offers a guide for any study that seeks to examine brain penetration of compounds and also provides data relevant for future screening strategies of candidate drugs tested for AD therapeutics.

First, we utilized an *in silico* computational model using prediction software developed by Advanced Chemistry Development (ACD) to predict and analyze the indexes of BBB permeability logBB and logPS of tolfenamic acid. Reference adrenergic blockers of known CNS classification such as propranolol, a compound known for its ability to cross the BBB [8], timolol and atenolol, both of which poorly cross the BBB [9, 10], were used. Second, The Immobilized Artificial Membrane (IAM) chromatography utilizing phosphatidylcholine column method [11] was used as a valid non cell-based *in vitro* predictor of BBB permeation. The IAM surface of the column resembles the lipid phase of the brain capillary endothelium and is chemically and thermally stable for chromatographic conditions [7]. Finally, the *in silico* and *in vitro* BBB permeation predictions of tolfenamic acid were validated using *in vivo* analysis where mice and guinea pigs, the two animal models we conduct our studies on, were administered tolfenamic acid intravenously followed by analysis of mass spectra data and calculating the brain-to-plasma concentration ratio, $K_p$, of tolfenamic acid after a single time point [7].

## 2. Materials and Methods

### *2.1.* Materials and Standards

Tolfenamic acid, propranolol, timolol, diclofenac sodium, potassium chloride (KCl), monobasic potassium phosphate ($KH_2PO_4$) and sodium chloride (NaCl) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium hydroxide 97% pellets and formic acid were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Hydrochloric acid (HCl) 37%, acetonitrile, sodium phosphate dibasic dodecahydrate ($Na_2HPO_4.12H_2O$), and water were HPLC grade from Acros Organics (Morris Plains, NJ, USA). Other chemicals and reagents were of analytical grade.

#### *2.1.1. In silico Computational Approach*

LogBB and LogPS were calculated for tolfenamic acid, propranolol, timolol and atenolol from values obtained through using two prediction software developed by ACD (Toronto, Ontario, Canada). Values used to calculate logBB were obtained from ACD/ChemSketch 12.0 software [12] which included octanol-water partition coefficient (LogP). Other brain transfer descriptors including molecular weight, hydrogen bond donor/acceptor number ($H_d$ and $H_a$), polar surface area (PSA), free rotatable bond (FRB) and acid dissociation constant (pKa) were obtained from literature [13]. LogPS values were obtained utilizing ACD/ADME Suite software [14].

#### *2.1.2. In vitro Experiment*

##### ***2.1.2.1. HPLC Conditions***

Hitachi analytical HPLC system consisting of an L-7100 low- pressure gradient pump, a 4-channel degasser, an L-7200 sequential auto-sampler, and a high sensitivity diode array detector (190-800 nm) was used. This system is controlled by a D-7000 HPLC system manager software package. The HPLC apparatus was fitted with an analytically sized (10 cm x 4.6 mm) IAM.PC.DD2 column from Regis technologies (Morton Grove, IL, USA). The Dulbecco's phosphate-buffered saline (DPBS) consisted of 2.7 mM KCL, 1.5 mM $KH_2PO_4$, 137 mM NaCl, and 8.1 mM $Na_2HPO_4$. The mobile phase consisted of acetonitrile and DPBS (20:80 v/v, pH of 5.5). Samples were eluted using the mobile phase with a flow rate of 0.5 ml/min at 37°C, and UV absorption at 210 nm.

#### ***2.1.2.2. Selection of Reference Compounds***

Propranolol and timolol were used as reference compounds for the *in vitro* experiment. Propranolol is classified in the literature as a high brain penetration (CNS+) compound [8] while timolol is classified as a low brain penetration (CNS-) compound [9]. Both compounds were reported to show the expected CNS penetration trend when screened by using IAM phosphatidylcholine column chromatography [11].

#### ***2.1.2.3. Determination of the IAM partition coefficients***

Samples were prepared by dissolution in absolute ethanol with concentrations of 20 µg/µl, and the IAM partition coefficients were calculated using equations from literature [11]. $K_{IAM}$, the IAM capacity factor, was calculated from the following equation:

$$k_{IAM} = \left( \frac{t_r - t_0}{t_0} \right)$$

Where $t_r$ is the retention time of the drug and $t_0$ is the holdup time of the column. The permeability of a drug through the BBB by passive diffusion ($P_m$) is expressed following correction by the molecular weight of the drug, $K_{IAM}$ /$MW^n$ [11]. It was reported that the most successful differentiation was obtained when the power function of $K_{IAM}$ /$MW^n$ was set at n = 4 at pH 5.5 [11].

### ***2.1.3. In Vivo Validation Using Mass Spectrometry***

#### ***2.1.3.1. Animals and Dosing***

C57BL/6 mice weighing between 25–30 g and Hartley guinea pigs weighing between 900-1000 g were obtained from Charles River Laboratories (Wilmington, MA, USA). Animal experiments were carried out according to the Institutional Animal Care and Use Committee (IACUC) guidelines for the care and use of laboratory animals. Guinea pigs were grouped and anaesthetized with 20% isoflurane/PEG (v/v). One group was then injected with 200µl of 100 mg/kg tolfenamic acid (n=3), while the other group was administered 200µl vehicle (n=2) intravenously through the saphenous vein by the university Attending Veterinarian (AV). The animals were euthanized within 5-10 minutes, decapitated and their brains were collected and stored at -80$^o$C. For LC/MS studies, mice were anaesthetized with 20% isoflurane/PEG (v/v) then injected either with 20µl of 100 mg/kg tolfenamic acid (n=3) or with vehicle (n=1) into the lateral tail vein by the AV. After 5 minutes, mice were anaesthetized and euthanized by exsanguination through cardiac puncture. Blood was collected into

7.5 units/ml heparin solution-treated tubes. Blood samples were then centrifuged at 1700 x g for 10 minutes and plasma was collected and stored at -80$^{o}$C. Brain tissue was extracted and stored in the -80$^{o}$C freezer.

#### ***2.1.3.2. Extraction Procedure and Sample Preparation***

A previously published extraction process was followed [15] and 20µl of the extract was injected into the mass spectrometry (MS) system. An alternate extraction method was also applied for brain homogenate, plasma and calibration standard samples analyzed by LC/MS. Each sample was treated with 4:1 0.25 M HCl to sample volume followed by addition of 50µl of 10 µg/ml diclofenac sodium, the internal standard (IS). Samples were vortexed for 1 minute followed by the addition of 2 ml ethyl acetate. After mixing for another minute, samples were centrifuged at 2800 x g for 10 minutes. The organic layer was collected and evaporated to dryness under nitrogen stream. The residue was reconstituted in 250µl acetonitrile and filtered, then 20µl of each sample was injected in the LC/MS system for separation. Using a stock solution of 500 µg/ml tolfenamic acid in acetonitrile, brain samples were spiked with 50µl of 10 µg/ml diclofenac sodium and with tolfenamic acid to produce the final concentrations of 0.05, 0.1, 0.25, 0.5, 1, 5, 10, 25 and 50 µg/ml tolfenamic acid. Plasma samples were spiked with 50µl of 10 µg/ml diclofenac sodium and with tolfenamic acid to produce the final concentrations of 1, 5, 10, 25, 50, 100 and 150 µg/ml tolfenamic acid.

#### ***2.1.3.3. Apparatus and Chromatographic Conditions***

**MS**: Applied Biosystems Mariner® system was used which consisted of an orthogonal time-of-flight (TOF) mass spectrometer equipped with standard electrospray ionization (ESI), PE SCIECX's Turbo Ion Spray™ (TIS) and Atmospheric Pressure Chemical Ionization (APCI) source. The instrument was outfitted with an integrated syringe pump with dual syringe rack for direct infusion into the mass spectrometer. Extracted samples and standards were injected directly at a flow rate of 20 µl/min and the negative ion mode was used for ion peak detection.

**LC-MS Method**: LC unit used was an Agilent 1100 series HPLC (Agilent Technologies, Santa Clara, CA, USA) and separation was performed using Luna pentafluorophenyl propyl, PFP(2), 50 x 2.0 mm column with a 3 µm particle size (Phenomenex, Torrance, CA, USA). The Mobile phase used consisted of a mixture of water with 0.1% formic acid (v/v) (A) and acetonitrile with 0.1% formic acid (v/v) (B). Gradient elution was employed as follow: 0-15 min, 100% eluent A; 15-30 min, 100% to 0% eluent A; 30-35 min, 100% eluent B; 35-45 min 100% to 0% eluent B; 45-55 min 100% eluent A under a flow rate of 0.2 ml/min. Mass spectroscopy equipment used was QSTAR® Elite Hybrid LC/MS equipped with a high performance quadruple time of flight (QqTOF) mass spectrometer, a turbo ion spray, and Analyst® QS 2.0 software. For each sample, 20µl was injected and MS detection was conducted using the negative ion mode.

## 3. Results

### 3.1. *In Silico* Computational Brain Transfer Prediction of Tolfenamic Acid

LogBB values for tolfenamic acid and some adrenergic blockers including propranolol, timolol and atenolol were calculated (Table 1) using Clark's equation [16]. Compounds that have logBB value of > 0.3 distribute freely to the brain however, compounds of logBB value of < -1 distribute poorly to the brain [17]. The logBB for tolfenamic acid was calculated to be 0.75 predicting its ability to distribute well into the brain. Propranolol had a calculated logBB of 0.1 that was less than the cutoff value to cross freely into the brain (0.3). However, it was much higher than -1, the reported value of which less than that a compound is predicted to be of poor CNS penetration. The calculated logBB for timolol and atenolol, both of which have poor CNS penetration as documented in the literature [9, 10], were -0.9 and -1.0, respectively, indicating their low ability to distribute into the brain.

Another index of BBB permeability, LogPS, was obtained for tolfenamic acid and the three antiadrenergic drugs (Table 1) using ACD/ADME Suite software [14]. The program predicts compounds with logPS values less than -5 to be unable to transfer into the brain. The calculated LogPS values for tolfenamic acid and propranolol were -2.1 and -2 respectively and thus both are predicted to transfer into the brain. The values of logPS for timolol and atenolol were -3 and -3.9 respectively which were close to the cutoff value of -5.0 for drugs that are predicted not to transfer into the brain.

Tolfenamic acid values of other brain transfer descriptors including octanol-water partition coefficient (logP), molecular weight, free rotatable bonds (FRB), hydrogen-bond donor/acceptor number ($H_d$ and $H_a$) and polar surface area (PSA) were consistent with the optimum values for drugs that distribute into the brain (Table 1). LogP is an indicator of lipophilicity; the higher the number the higher the lipophilicity. Predicted LogP for tolfenamic acid was 5.86 indicating it can passively cross the BBB. The molecular weight of tolfenamic acid is 261.7 Da which is less than 450 Da, a requirement for brain penetration [18]. Tolfenamic acid's predicted FRB was 3 and compounds with FRB >7 are considered of low BBB permeability [16]. Compounds that permeate into the CNS usually have 0-4 hydrogen-bond donors and 1-5 hydrogen-bond acceptors [19] and the drug has two hydrogen-bond donors and three hydrogen-bond acceptors. The PSA value for tolfenamic acid was predicted to be 49.3 $Å^2$ and for brain penetration, the PSA should be <90 $Å^2$ [18]. Thus, the predicted values of logBB, logPS and other brain transfer descriptors strongly suggest that tolfenamic acid would passively distribute into the brain to a great extent.

### 3.2. *In vitro* Brain Transfer Prediction of Tolfenamic Acid

Using the IAM phosphotidylcholine column chromatography method described by Yoon *et al,* (2006) [11], the $(K_{IAM}/MW^4)$ x$10^{10}$ values at pH 5.5 for tolfenamic acid and the reference compounds, propranolol and timolol, were obtained (Table 2). Compounds with IAM capacity factors $(K_{IAM}/MW^4)x10^{10} > 1.01$ have high CNS penetration while compounds with $(K_{IAM}/MW^4)x10^{10} < 0.64$ values have low CNS penetration [11]. Although we had higher IAM capacity factor values than reported by

Yoon *et al*. for propranolol and timolol, the order of the values was consistent. The classification of CNS penetration of tolfenamic acid, propranolol and timolol based on their IAM capacity factor values was determined (Fig. 1). With a $(K_{IAM}/MW^4)x10^{10}$ value of 2.73, our *in vitro* prediction model indicated the ability of tolfenamic acid to transfer into the CNS.

### 3.3. Presence of Tolfenamic Acid in the Brains of Guinea Pigs after IV Administration

Mass spectra data of tolfenamic acid presence in the brain after intravenous administration in guinea pigs were the first evidence of the ability of tolfenamic acid to have brain disposition criteria (Fig. 2). These data suggest that tolfenamic acid's presence in the brain following IV administration validates our computation and *in vitro* prediction results; however, we still need to obtain the evidence of this disposition of tolfenamic acid in the brain relative to the plasma concentration and such relativity is expressed as the brain-to-plasma ratio of tolfenamic acid.

### 3.4. Determination of Brain-to-Plasma Concentration Ratio of Tolfenamic Acid

Using the LC-MS method, the brain-to-plasma concentration ratio of a single time-point, *Kp*, was determined after intravenous administration of 20µl of 100 mg/kg tolfenamic acid to mice via their lateral vein. Tolfenamic acid peak was identified in the brain (Fig. 3), confirming the earlier results of tolfenamic acid detection after IV administration into guinea pigs, as well as in the plasma (Fig. 4). The areas under the tolfenamic acid peaks in the brain and plasma were plotted versus the standard calibration curves of brain and plasma and were quantified using the quantification

wizard Analyst® QS 2.0 software. The concentration of tolfenamic acid in the brain was 1.21 µg/g whereas the measured concentration of tolfenamic acid in the blood was 10.85 µg/ml and thus the *Kp* value calculated after 5 minutes following IV administration was 0.11.

## 4. Discussion

The delivery of therapeutic agents to the CNS is of great importance in CNS drug discovery. The BBB prevents many drugs from accessing the CNS, and hence poses a significant challenge for drug delivery into the CNS [20]. A survey of the Comprehensive Medicinal Chemistry (CMC) database showed that only 5% of over 7,000 drugs listed were CNS active [21]. Presently, several methods are available to assess the potential of candidate drugs to penetrate the BBB including *in silico, in vitro and in vivo* approaches. We applied these approaches comparatively in assessment of the ability of tolfenamic acid to cross the BBB as a drug that we have demonstrated experimentally that it affects processes associated with amyloidogenesis in the brain [1].

Computational approach, compared to *in vitro* and *in vivo* methods, is cost effective and requires less time for analysis in brain penetration studies, however, the performance of such a model depends on the data input, descriptor used and modeling approaches [7]. The calculated LogBB value for tolfenamic acid using ACD/ChemSketch 12.0 software [12] showed its ability to cross the BBB and this prediction was consistent with its physicochemical properties (Table 1). With some controversy about the validity of using logBB as indicator of brain penetration [22],

ACD/ADME Suite software [14] was used to predict brain penetration by analyzing logPS value which also indicated the ability of tolfenamic acid to cross into the CNS. The predicted logBB of propranolol (0.1) was much less than the actual experimental value (1.57) which can be explained by the presence of active transport mechanisms that deliver propranolol into the brain [8]. A limitation of computational models is that they can only predict brain penetration as a function of passive permeability and do not consider other factors such as efflux and influx transporters and plasma protein binding which can affect brain transfer of drugs. With limited number of compounds to design logBB and logPS models and the fact that experimental logBB and logPS were generated from different protocols, the computational prediction can sometimes be contradictory and uncertain [7]. Thus, we advanced into comparing our computational results with an *in vitro* model.

*In vitro* models to determine brain penetration are developed in a way to mimic *in vivo* brain penetration, hence, there are various *in vitro* cell-based and non-cell-based models [7]. IAM chromatography technique has been experimented to predict BBB permeability [11, 23] with less complexity compared to other cell based models that require cell culture cycles. Using this method, our results showed that tolfenamic acid had a $k_{IAM}$ that reflected its ability to permeate through the BBB. The obtained $k_{IAM}$ value for tolfenamic acid was intermediate between that of propranolol and timolol. While it was not close to that of propranolol, it was much higher than that for timolol, thus we concluded that tolfenamic acid was CNS+ and that its ability to penetrate the brain was intermediate in nature. Although IAM chromatography is a rapid and simple

method which provides a promising tool for assessing BBB permeability, it has few limitations which include the fact that lipid molecular dynamics including lateral diffusion are not mimicked by IAM and that BBB permeation prediction becomes unreliable by IAM if the drug brain uptake is influenced by transporters or plasma protein binding [7, 11, 23]. However, there are numerous cellular systems that can be used to evaluate the influence of BBB efflux transporters including P-glycoprotein (PGP) and multi-drug resistant proteins (MRP) on CNS distribution of drugs. For examples, human colon adenocarcinoma (Caco-2) and MDR1-trasfectted Madin-Darby Canine Kidney (MDCK) cell lines have been good tools for evaluating the BBB efflux transporters effect on different compounds [7]. Nevertheless, some limitations of using such models are valid. The fact that the *in vivo* BBB contains other efflux transporters which could exert a major effect in the overall BBB penetration and the fact that MDCK cell line also expresses the organic cation transporter (OCT-2) could lead to complications when interpreting generated data [7, 24]. Thus, the *in vitro* findings, inevitably, needed to be validated *in vivo*.

Several invasive and noninvasive *in vivo* methods are designed for brain uptake experiments, which represent the most reliable validation of other models [7, 25]. Tolfenamic acid was present in the brain after intravenous administration in guinea pigs as detected by MS (Fig. 2). However, as already mentioned, we still need to obtain the evidence of this disposition of tolfenamic acid in the brain relative to the plasma concentration and such relativity is expressed as the brain-to-plasma ratio of tolfenamic acid.

The plasma-to-brain concentration ratio, the most widely used term for evaluating brain penetration [26], was assessed for tolfenamic acid after 5 minutes and was calculated to be 0.11 indicating its disposition into the brain in low levels that might be due to the early time point chosen. With no pharmacokinetic (PK) data available regarding the disposition of tolfenamic acid through the mouse body, multiple time-point assessment and determination of area under the curve (AUC) concentration in brain and plasma is needed for more accurate experimental calculations of the drug PK. Also, $K_p$ is heavily affected by the binding of the drug in brain and plasma [26] and it is known that tolfenamic acid has a very high plasma binding level up to 99.7% in human [27] and this could explain well the low $K_{p\ total.}$ Thus, to avoid the effect of the non-specific binding, determination of the free unbound drug levels in the brain and plasma to generate the unbound brain-to-plasma concentration ratio, $Kp,uu,$ could provide a better analysis of the brain penetration of tolfenamic acid. In addition, $Kp,uu$ could describe the effect of efflux and influx transporters at the BBB on the CNS distribution of tolfenamic acid, if any.

To conclude, comparative analysis of multiple approaches has predicted and validated the ability of tolfenamic acid to penetrate into the CNS and exert its effects that are manifested in lowering amyloidogenesis and treatment of migraine.

**Acknowledgements**

This work was supported by the grants ES13022, AG027246, 1R56ES015867-01A1, and 1R01ES015867-01A2 from the National Institutes of Health awarded to N.H.Z. The research was made possible by the use of the RI-INBRE Research Core Facility, supported jointly by NCRR/NIH Grant # P20 RR016457 and the network institutions. The authors thank Dr. Aftab Ahmed, Dr. Roberta S. King, Dr. Vaidyanathan V.G., Lina Adwan and Gail Golomb-Mello for their help in this research.

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**Table I-1. LogBB and logPS values obtained using *in silico* computational prediction software with related parameters for tolfenamic acid, propranolol, timolol and atenolol.**

| Structure | MW[a] | PSA[b] | $H_d$[c] | $H_a$[d] | FRB[e] | pKa[f] | LogP[g] | LogBB[h] | LogPS[i] |
|---|---|---|---|---|---|---|---|---|---|
| Tolfenamic Acid | 261.7 | 49.3 | 2 | 3 | 3 | 4.2 | 5.86 | 0.75 | -2.1 |
| Propranolol | 259.3 | 41.5 | 2 | 3 | 6 | 9.5 | 3.1 | 0.1 | -2.0 |
| Timolol | 316.4 | 108 | 2 | 6 | 7 | 9.8 | 1.56 | -0.9 | -3.0 |
| Atenolol | 266.3 | 84.6 | 3 | 4 | 8 | 9.5 | 0.20 | -1.0 | -3.9 |

$$\text{LogBB} = (0.205 \times LogP) + 0.18 - (0.0094 \times PSA) - (0.055 \times FRB)^{j}$$

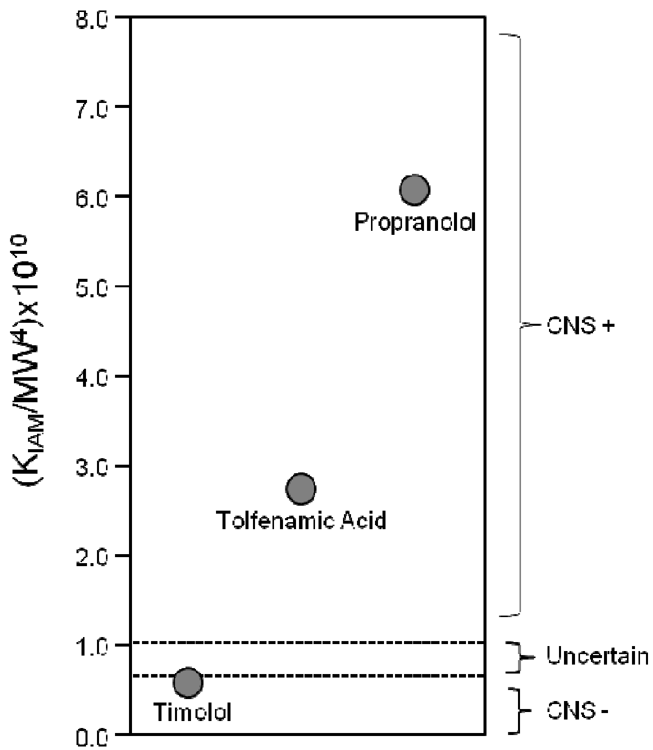
[a] Molecular weight in Dalton; [b] Polar Surface Area; [c] The number of hydrogen-bond donors; [d] The number of hydrogen-bond acceptors; [e] Free rotatable bond; [g] Octanol-water partition coefficient; [h] Equilibrium distribution between blood and brain; [i] Passive permeability-surface area product; [j] Equation for calculating logBB which was extracted by analyzing logBB values of a database of 179 data points measured in rats which showed that logBB determination is strongly dependent on logP, PSA and FRB [16].

**Table I-2. Human CNS penetration evaluation of propranolol, timolol and tolfenamic acid from literature and calculated from the Immobilized Artificial Membrane (IAM) capacity factor [($K_{IAM}/MW^4$)x$10^{10}$] using IAM Chromatography [11].**

| Compound | CNS Penetration[a] | $(K_{IAM}/MW^4)x10^{10}$ | CNS Penetration[b] |
|---|---|---|---|
| Propranolol | CNS+ | 6.16 | CNS+ |
| Timolol | CNS- | 0.73 | CNS- |
| Tolfenamic Acid | - | 2.73 | CNS+ |

[a] CNS penetration obtained from literature [8, 9]; [b] CNS penetration based on $(K_{IAM}/MW^4)x10^{10}$ values at pH 5.5; CNS+ $(K_{IAM}/MW^4)x10^{10} > 1.01$, CNS- $(K_{IAM}/MW^4)x10^{10} < 0.64$ [11].

**Figure I-1. Classification of CNS penetration of tolfenamic acid, propranolol and timolol using IAM chromatography.** The IAM capacity factor [$(K_{IAM}/MW^4)x10^{10}$] at pH 5.5 was determined. High BBB penetration (CNS+); $(K_{IAM}/MW^4)x10^{10} > 1.01$, low BBB penetration (CNS-); $(K_{IAM}/MW^4)x10^{10} < 0.64$, Uncertain BBB penetration; $(K_{IAM}/MW^4)x10^{10} = 0.65\text{-}1$.



8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
$(K_{IAM}/MW^4)x10^{10}$
Propranolol
Tolfenamic Acid
Timolol
CNS +
Uncertain
CNS -

**Figure I-2. Mass spectra analysis of tolfenamic acid in the brain of guinea pigs following intravenous administration.** Tolfenamic acid was administered through the saphenous vein (200µl of a 100 mg/kg dose) and its levels were analyzed. Ovals denote the desired tolfenamic acid peak with m/z of 259.8. **(a)** Tolfenamic acid standard in ethanol (1 mg/ml); **(b)** Brain extract from vehicle dosed animals; **(c)** Brain extract from tolfenamic acid dosed animals; **(d)** Extracted tolfenamic acid peak from mass spectrum(c).

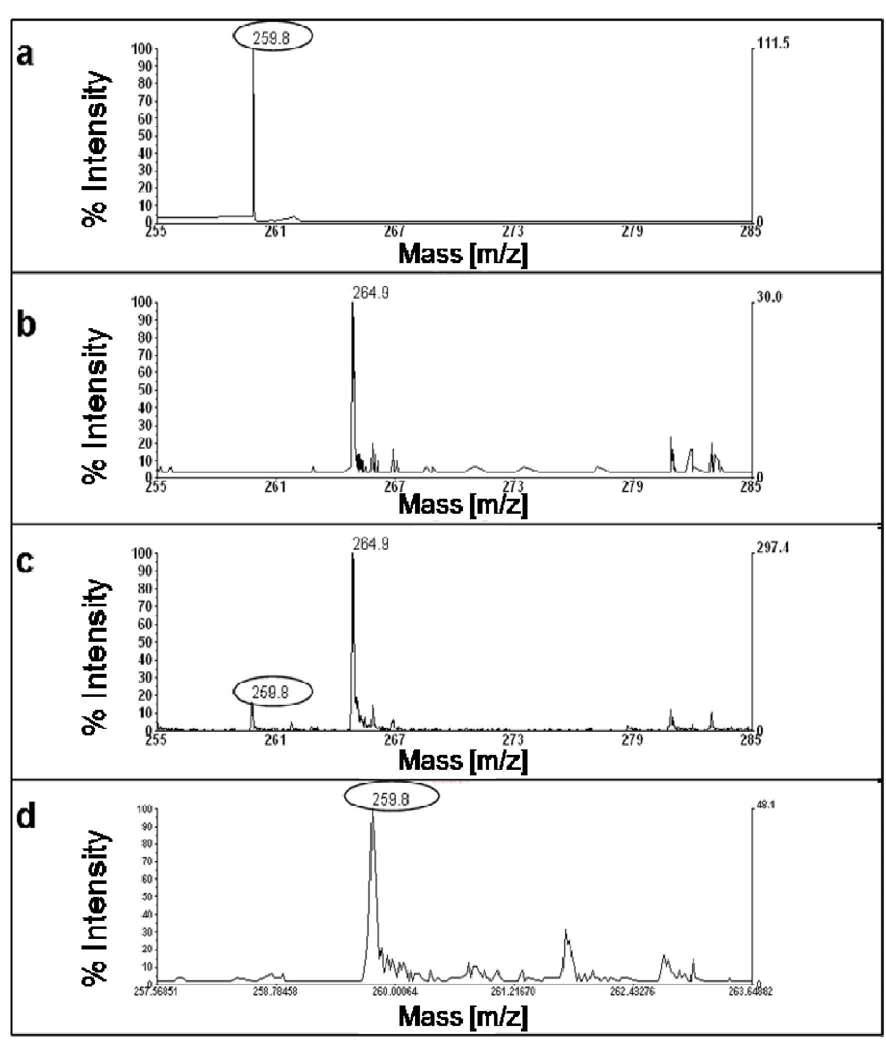

a
259.8
% Intensity
Mass [m/z]
b
264.9
% Intensity
Mass [m/z]
c
264.9
259.8
% Intensity
Mass [m/z]
d
259.8
% Intensity
Mass [m/z]

**Figure I-3. Extracted ion count chromatogram of tolfenamic acid in the brain of mice following intravenous administration.** Tolfenamic acid was administered through the lateral tail vein (20µl of a 100 mg/kg dose) and its levels in the brain were analyzed using the extraction and LC-MS methods described in the methods section. The area under tolfenamic acid peak was used for brain-to-plasma concentration ratio calculation. TA = Tolfenamic acid; IS = Internal standard (diclofenac sodium).

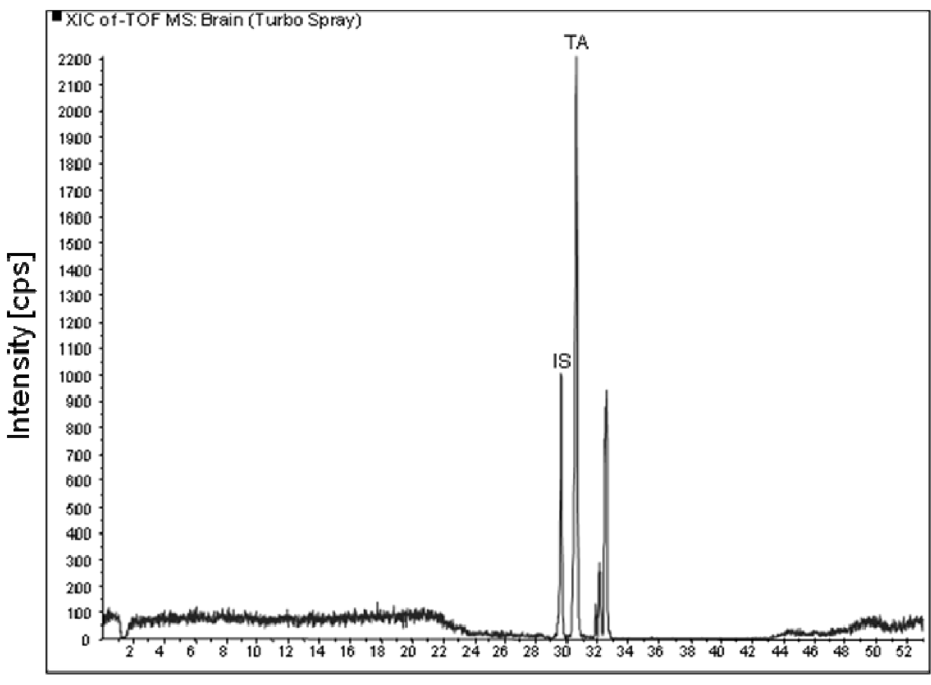
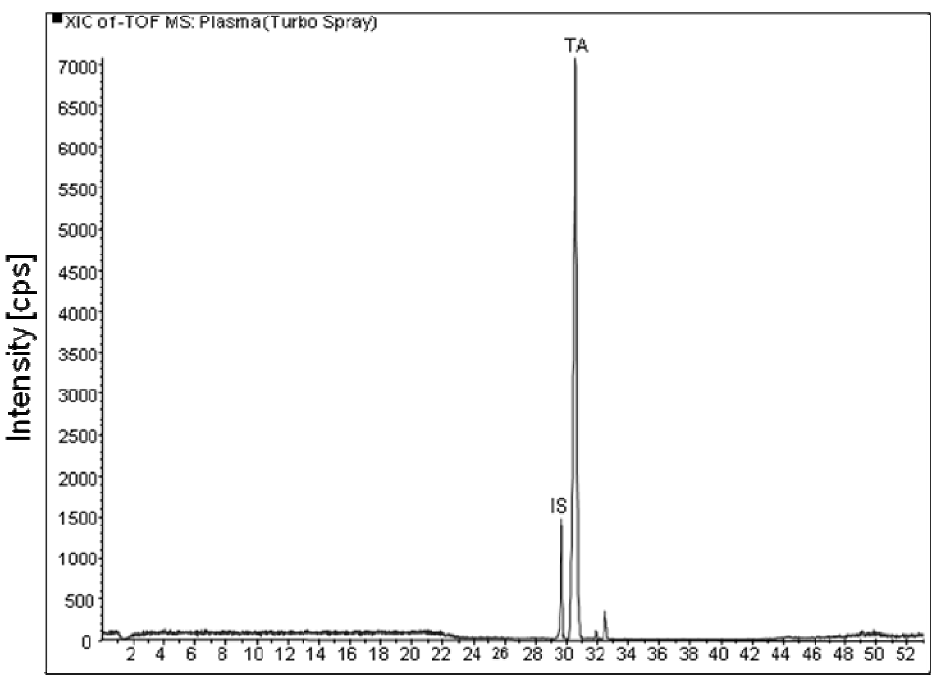

XIC of -TOF MS: Brain (Turbo Spray)
TA
IS
Intensity [cps]
0
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
1500
1600
1700
1800
1900
2000
2100
2200
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
42
44
46
48
50
52
Time [min]

**Figure I-4. Extracted ion count chromatogram of tolfenamic acid in the plasma of mice following intravenous administration.** Tolfenamic acid was administered through the lateral tail vein (20µl of a 100 mg/kg dose) and its levels in the plasma were analyzed using the extraction and LC-MS methods described in the methods section. The area under tolfenamic acid peak was used for brain-to-plasma concentration ratio calculation. TA = Tolfenamic acid; IS = Internal standard (diclofenac sodium).


XIC of -TOF MS: Plasma(Turbo Spray)
TA
IS
Intensity [cps]
Time [min]
7000
6500
6000
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52

## MANUSCRIPT 2

# Reversal of Cognitive Deficits by Tolfenamic Acid is Accompanied by Reductions in Intermediates Associated with Amyloid Pathology in a Transgenic Mouse Model of Alzheimer's Disease

**By**

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**(Intended publication in Neurobiology of Aging)**

**Abstract**

Data from our lab had shown that tolfenamic acid lowers the levels of the amyloid precursor protein (APP) and amyloid beta (Aβ) peptide when administered to C57BL/6 mice by lowering the levels of their transcriptional regulator specificity protein 1 (Sp1). In this study, we examined the ability of tolfenamic acid to improve the cognitive deficits in a transgenic mouse model of Alzheimer's disease (AD), R1.40, as well as to reduce the expression of genes and the levels of proteins associated with AD, including SP1, APP and Aβ. Following characterization of the ensuing cognitive deficits in aged hemizygous R1.40 transgenic mice, we administered variable doses of tolfenamic acid or vehicle for thirty four days. Behavioral assessment of spatial learning and memory functions began on Day 15 using the Morris water maze. Working memory was tested using the Y-maze. Results showed that tolfenamic acid improved both long-term spatial memory deficits as well as working memory in these mice. These improvements were accompanied by reductions in the levels of the transcriptional activator SP1, followed by decreases in both the mRNA and protein levels of APP, and subsequent Aβ levels. These findings provide evidence of the ability of tolfenamic acid to disrupt the development and the progression of the pathological processes associated with AD, manifested by the reduction of amyloidogenic proteins, and amelioration of cognitive dysfunctions.

***Keywords:*** Alzheimer's disease; tolfenamic acid; AD transgenic mouse model; learning and memory; Morris water maze; Y-maze.

## 1. Introduction

Alzheimer's disease (AD) represents the most prevalent neurodegenerative diseases in the elderly. During the course of the disease, memory, cognitive performance, and other daily activities are all impaired as a result of extensive neuronal loss (Berg, et al., 1993; Braak and Braak, 1997; Nelson, et al., 2012). AD is characterized by the presence of neuropathological deposits consisting of extracellular senile plaques of β-amyloid core and intraneuronal neurofibrillary tangles (NFTs) in different brain regions, especially in the cerebral cortex, hippocampus, subcortical nuclei and amygdala (Selkoe, 2001; Reddy, et al., 2010; Ballard, et al., 2011; Harrington, 2012).

The amyloid precursor protein (APP) is processed by beta-site APP-cleaving enzyme 1 (BACE1) and γ-secretase proteases to generate various β-amyloid (Aβ) peptide isoforms that can accumulate resulting in the formation of insoluble aggregates of amyloid plaques (Shoji, et al., 1992; Urbanc, et al., 1999; Querfurth and LaFerla, 2010; Zhang, et al., 2012). $A\beta_{1-40}$ and $A\beta_{1-42}$ are the major generated isoforms with $A\beta_{1-42}$ found to be more aggregative triggering amyloid plaque formation (Finder and Glockshuber, 2007). Accumulation of Aβ into amyloid plaques initiates a pathological cascade resulting in synaptic dysfunction and neuronal death that contribute to the neurodegeneration observed in AD according to the amyloid hypothesis (Hardy and Higgins, 1992; Selkoe, 2001).

Over the last two decades, more evidence has been garnered as to the association between chronic NSAIDs intake and up to 80% reduction in risk of AD incidence, slowing of disease progression, and reduction of microglial activation (Andersen, et

al., 1995; McGeer, et al., 1996; Stewart, et al., 1997; in t' Veld, et al., 2001; Lindsay, et al., 2002; Zandi, et al., 2002; Etminan, et al., 2003; Vlad, et al., 2008; Cote, et al., 2012). However, several prospective clinical trials failed to demonstrate the efficacy of NSAIDs in altering AD risk (in 't Veld, et al., 1998; Hayden, et al., 2007; Lyketsos, et al., 2007; Arvanitakis, et al., 2008). Nonetheless, the findings of the ability of a subset of NSAIDs to alter AD pathology and improve cognitive performance in murine mouse models of AD by mechanisms other than their classic cyclooxygenase (COX) inhibition have led to a renewed interest in these compounds as potential disease-modifying agents in AD (Weggen, et al., 2001; Zhou, et al., 2003; Lleo, et al., 2004).

No cure has been discovered for AD yet and FDA-approved drugs for use in AD include four cholinesterase inhibitors and one N-methyl-D-aspartate receptor antagonist. However, these medications are not disease-modifying therapies and they do not stop the progression of the disease (Ozudogru and Lippa, 2012). Current research focuses on interventions that target Aβ production and aggregation, and the production of hyperphosphorylated tau (Gotz, et al., 2012; Ozudogru and Lippa, 2012); however, no therapeutic strategy has explored more upstream interventions at the transcriptional level. Specifitcity protein 1 (Sp1) co-activates the transcription of APP, BACE1 and tau genes (Heicklen-Klein and Ginzburg, 2000; Christensen, et al., 2004; Docagne, et al., 2004). Consequently, changes in its levels can alter the downstream pathways related to amyloidogenesis (Basha, et al., 2005; Adwan, et al., 2011) and tau (unpublished data) pathology representing an ideal target for a disease-

modifying intervention. Tolfenamic acid, a non-steroidal anti-inflammatory drug (NSAID), induces the degradation of SP1 protein (Abdelrahim, et al., 2006) and data from our lab have shown that the treatment of wild type C57BL/6 mice with tolfenamic acid lowers the levels of SP1 and the expression of AD-related Sp1 target genes such as APP (Adwan, et al., 2011). Unpublished data from our lab also indicate that it can do the same to both BACE1 and tau.

Transgenic mouse models of AD represent useful tools for elaborating mechanisms involved in the development and progression of AD. In addition, they allow for testing of new therapies *in vivo* to provide more accurate data to justify testing in human clinical trials (Hock and Lamb, 2001; Duff and Suleman, 2004). Thus, we decided to examine the ability of tolfenamic acid to lower AD proteins, including SP1, APP, and Aβ, and to examine whether such reductions are commensurate with an improvement in cognitive functions in a mouse model of AD. Since the endogenous APP promoter is largely driven by Sp1 (Docagne, et al., 2004), we chose to study the effects of tolfenamic acid, which degrades SP1, in R1.40 mouse model which utilizes the human APP promoter.

Thus, the purpose of this experiment was to investigate the presence of memory deficits in the hemizygous R1.40 mice, a genomic-base transgenic mouse model that harbor the Swedish mutation APPK670N/M671L (Lamb, et al., 1993), and to examine the ability of tolfenamic acid to attenuate such deficits and to decrease AD-related pathology. Therefore, following characterization of the learning and memory impairment in female hemizygous R1.40 transgenic mice, 5 and 50 mg/kg/day

tolfenamic acid was administered to female R1.40 mice aging between 14-21 months via oral gavage for 34 days and the learning and memory functions were assessed in the Morris water maze and the Y-maze. On Day 35, mice were euthanized and AD associated proteins including SP1, APP and soluble and insoluble $A\beta_{1-40}$ and $A\beta_{1-42}$ were assessed in the frontal cortex, which displays extensive AD pathology in this animal model (Kulnane and Lamb, 2001; Lehman, et al., 2003).

## 2. Methods

### *2.1. Animal model*

The transgenic mouse model R1.40 was utilized for this study. These transgenic mice, B6.129-Tg(APPSw)40btla/J, were obtained from the Jackson laboratory (Bar Harbor, ME, USA) and colonies of hemizygous and homozygous strains were established in-house. R1.40 is a genomic-based transgenic mouse model that was developed by Bruce T. Lamb, and it utilizes a yeast artificial chromosome (YAC) that contains the full 400 kb human APP gene and flanking sequence of approximately 250 kb to harbor the Swedish mutation APPK670N/M671L including the human transcriptional regulatory elements needed for proper spatial and temporal expression (Reaume, et al., 1996; Lamb, et al., 1997; Hock and Lamb, 2001). The developed hemizygous R1.40 line shows a great increase in APP and Aβ production as early as 6 months of age with Aβ plaque deposition occurring at 24-26 months of age compared to the wild type mice and it was concluded that the mnemonic deficits in R1.40 were similar to those observed in AD (Lamb, et al., 1999; Hock, et al., 2009).

*2.2. Genotyping of R1.40 transgenic mice*

In order to establish transgenic mice colonies of hemizygous and homozygous R1.40, mice were bred and genotyped in-house at the University of Rhode Island. To ensure the accuracy of genotyping results, two genotyping techniques were performed: standard PCR followed by gel electrophoresis on 1.5% agarose gel, and the TaqMan® allelic discrimination assay (Applied Biosystems, Foster City, CA, USA). The breeding and genotyping methods were approved by University of Rhode Island Institutional Animal Care and Use Committee (IACUC).

*2.2.1. DNA isolation*

DNA was isolated from mouse tissue as described in the literature (Truett, et al., 2000). In brief, tissue was obtained through tail snipping (2 mm) and was placed in Eppendorf tubes followed by adding 75µl of a mix of 25 mM sodium hydroxide (NaOH) and 0.2 mM ethylenediaminetetraacetic acid (EDTA). The tubes were placed in a thermocycler (Bio-Rad, Hercules, CA, USA) at 98°C for 1 hr followed by cooling at 15 ºC. After that, 75µl of 40 mM Tris hydrochloride (HCl) of pH 5.5 was added and mixed. Tubes were centrifuged at 1700 × g for three minutes and supernatants were collected. DNA concentration and quality was checked using the NanoDrop 2000 Micro-Volume UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

*2.2.2. Standard PCR and agarose gel electrophoresis*

Standard PCR genotyping method for R1.40 mice was performed as provided by the Jackson laboratory (Bar Harbor, ME, USA). The primer pairs for the APP transgene used were: IMR6938 (5'-CTT CAC TCG TTC TCA TTC TCT TCC A-3') and IMR6939 (5'-GCG TTT TTA TCC GCA TTT CGT TTT T-3'). Each reaction consisted of a mixture of 10µl TaqMan® genotyping master mix (Applied Biosystems, Foster City, CA, USA), 2µl of each primer and 2µl DNA from test samples. PCR amplification step was performed using a thermocycler (Bio-Rad, Hercules, CA, USA) as follows: 94°C for 3 min and then 35 cycles of 94°C for 30 sec, 65°C for 30 sec and 72°C for 30 sec, and then at 72°C for 2 min. Bands were then separated by gel electrophoresis on 1.5% agarose gels containing ethidium bromide and bands were visualized using Typhoon™ 9410 Variable Mode Imager (GE Healthcare, Piscataway, NJ, USA).

*2.2.3. TaqMan® allelic discrimination assay*

To distinguish between hemizyougs and homozygous R1.40 YAC APP and WT, TaqMan® allelic discrimination assay was performed. SNP genotyping assay, catalogue: M_23312848_20, containing the context sequence ([VIC/FAM]): TAC TGC TCA CTC TTT TAA CAA ACA T[A/G]G GAA ACT AAT GAA ATG GTA TCA CAA was obtained from ABI (Applied Biosystems, Carlsbad, CA, USA) and the assay was performed according to ABI protocol. Briefly, each reaction consisted of 6.25µl 2× TaqMan® genotyping master mix (Applied Biosystems, Foster City, CA, USA), 0.625µl 20× SNP genotyping assay mix, 3.125µl RNase free water and 2.5µl

genomic DNA from test samples. The pre-read run and PCR amplification were conducted in a 7500 Real-Time PCR System following ABI standard protocol, which was 95°C for 10 min followed by 40 cycles of 92°C for 15 sec and 60°C for 1 min. Following the post-read run, results were analyzed using the sequence detection software (SDS, version 1.3) from ABI and the allelic discrimination scatter plots were automatically generated.

*2.3. Assessment of cognitive deficits in hemizygous R1.40 transgenic mice*

In order to characterize memory and cognitive deficits in the hemizygous R1.40 transgenic mouse model, behavioral testing in mazes that are dependent on the integrity of the hippocampus and brain cortex was conducted using the Morris water maze and spontaneous alternations in the Y-maze. Female hemizygous APP transgenic (n=19) and female control wild type (n=18) groups of ages ranging between 9-20 months were used.

*2.3.1. Morris water maze*

We have tested the mice in the hidden version of the Morris water maze. In this task, the mice had to locate the hidden platform by learning multiple spatial relationships between the platform and the distal extra-maze cues (Vorhees and Williams, 2006; Gulinello, et al., 2009; Laczo, et al., 2009). The apparatus consisted of a white 48" diameter pool that is 30" in height and was filled with water to a depth of 14". The water was kept opaque by the addition of white non-toxic liquid washable paint. The pool was surrounded by distinct fixed visual cues that the animals used to navigate

to reach the escape platform. A clear Plexiglas platform square of 10 cm was kept submerged 0.5 cm below the surface of the water. The temperature of the water was kept 25 ±2°C during all procedures in the water maze. On Day 15 of tolfenamic acid administration, mice received a habituation trial in which they were allowed to swim freely for 60 sec. On the following day and for a total of 8 days, mice received training sessions of 3 trials daily. The starting position for each trial was randomly assigned between the four possible positions while the platform position was fixed in each trial. Each animal was allowed to swim until they found the immersed hidden platform or for a maximum duration of 60 sec. If the mouse failed to locate the platform, it would be gently guided to sit on the platform for a maximum duration of 30 sec. Mice were also left to sit on the platform for a maximum of 10 sec upon successful trial. Upon conducting the 8 acquisition sessions, probe trials for up to 60 sec on Day 1 and Day 11 following the last day of training were performed to assess the long term memory retention by studying the preference of the mice for the correct quadrant that previously contained the hidden platform. The swim paths and latencies to locate the platform were videotaped and tracked with a computerized video tracking system (ObjectScan, Clever Sys. Inc., Reston, VA, USA) and the resultant data were analyzed.

*2.3.2. Spontaneous alternations in the Y-maze*

The spontaneous alternation ratio, defined as the percentage of the number of arm entries, different from the previous two entries, divided by the total arm entries minus two (King, et al., 1999; Hock, et al., 2009) was measured. Testing was

conducted in a white Y-maze with arms that were 12" (long) by 3" (wide) with 8" height walls. Mice were placed into one of the Y-maze arms and were left to explore the maze freely for 5 minutes. After each trial, the maze was cleaned with 70% ethanol. Y-maze trials were videotaped and tracked with a computerized video tracking system (ObjectScan, Clever Sys. Inc., Reston, VA, USA) and the data were analyzed.

*2.4. Animal exposure to tolfenamic acid and behavioral testing*

Tolfenamic acid was obtained from Sigma Aldrich (St. Louis, MO, USA) and female hemizygous APP YAC transgenic mice aging between 14-21 months were used to examine the ability of tolfenamic acid to reduce the amyloidogenic load and to improve the learning and memory deficits of the mice. The animals were divided into three groups of similar age variation and were administered tolfenamic acid daily via oral gavage for 34 days; one group was administered 5 mg/kg/day tolfenamic acid in corn oil (n=6); the second group was administered 50 mg/kg/day tolfenamic acid in corn oil (n=7) and the third group was administered corn oil, the vehicle, (n=6). After 14 days of daily dosing, mice were tested in the Morris water maze as described in section 2.3.1. On Day 33, mice were tested for spontaneous alternations in Y-maze as described in section 2.3.2. Finally, on Day 35, mice were euthanized and brain tissue was extracted, dissected and stored at -80°C. Figure 1 provides a timeline for the experimental events involved in this study of drug administration and behavioral assessment.

*2.5. Protein extraction and Western blot analysis*

Brain cortices were homogenized with RIPA lysis buffer containing 10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, and 0.1% protease inhibitor cocktail. The homogenates were centrifuged at 10,000 × g for 10 minutes at 4°C and supernatants were collected. Protein levels were determined using the BCA kit (Pierce Biotechnology Inc., Rockford, IL, USA). Forty μg of total protein was separated on 8% SDS polyacrylamide gel and then transferred onto polyvinylidiene diflouride (PVDF) membranes (GE Healthcare, Piscataway, NJ, USA). Membranes were blocked using 5% non-fat milk in TBST (Tris buffered saline + 0.5% Tween-20, pH 7.4) for 1 hr. After that, membranes were incubated overnight at 4°C with the specific antibody diluted in TBST for 1 hr (1:2000 dilution of 6E10 for APP (Covance Research Products Inc., Dedham, MA, USA) and 1:500 dilution of 1C6 for SP1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing membranes 4 times with TBST, membranes were incubated for one hour with the appropriate anti-mouse IRDye® 680 (Li-Cor Bioscience, Lincoln, NE, USA) (1:5000) at room temperature. After washing the membranes twice with TBST, the images were developed using the Li-Cor Odyssey infrared imaging system (Li-Cor Bioscience, Lincoln, NE, USA). The membranes were also re-probed for β-actin (Sigma Aldrich, St. Louis, MO, USA) at a dilution of 1:2500 in TBST to obtain the APP/β-Actin ratio. The intensities of the obtained Western blot bands were determined using Odyssey V1.2 software (Li-Cor Bioscience, Lincoln, NE, USA).

*2.6. RNA isolation, synthesis of cDNA and quantitative Real-Time PCR*

RNA was isolated from cortical tissue following the TRIzol® Reagent method (Invitrogen, Carlsbad, CA, USA), checked for integrity using NanoDrop 2000 Micro-Volume UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and gel electrophoresis. RNA was reverse transcribed to cDNA using the iScript™ Select cDNA Synthesis Kit following manufacturer's instructions (Bio-Rad, Hercules, CA, USA). About 1000ng of RNA was diluted to 19.5µl with nuclease free water, then 3µl Oligo (dT) mix, 6µl 5x iScript Select reaction mix, and 1.5µl of iScript reverse transcriptase was added. Samples were incubated at 42°C for 90 min then at 85°C for 5 min to terminate the reaction. All incubations were conducted using MJ Research MiniCycler™ PTC-150 (Bio-Rad, Hercules, CA, USA). Primer pairs for Sp1, APP and β-actin were obtained from Invitrogen (Carlsbad, CA, USA) as follows: Sp1 sense: 5'-CAA GCC CAA ACA ATC ACC TT-3', and antisense, 5'-CAA TGG GTG TGA GAG TGG TG-3'; APP sense: 5'-TGC AGC AGA ACG GAT ATG AG-3', and antisense: 5'-ACA CCG ATG GGT AGT GAA GC-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 2µl of cDNA, 1µl of each primer, 8.5µl nuclease free water and 12.5µl SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Real-Time PCR was conducted using 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) following the standard protocol: 50°C for 2 min followed by 95°C for 10 min, then 40 cycles of

95°C for 15 sec and 60°C for 1 min. Results were analyzed using SDS software and expression data were reported relative to β-actin mRNA using the $2^{-\Delta\Delta Ct}$ method.

*2.7. ELISA $A\beta_{1–40}$ and $A\beta_{1–42}$ assay*

Amyloid beta (Aβ) levels both soluble and insoluble fractions were determined by enzyme-linked immunosorbent assay (ELISA) using human $A\beta_{1–40}$ and $A\beta_{1–42}$ assay IBL kits JP27713 and JP27711 (Immuno-Biological Laboratories, Gunma, Japan). These kits are solid-phase sandwich ELISA with two types of highly specific antibodies that are 100% reactive with mouse $A\beta_{1-40}$ with a sensitivity of 5.00 pg/mL (JP27713), and 70% reactive with mouse $A\beta_{1-42}$ with 4.03 pg/mL sensitivity (JP27711). The assay conditions were followed according to a method described in the literature (Morishima-Kawashima, et al., 2000) with slight modifications. Brain tissue was homogenized in Tris-saline (TS) [50 mM Tris-HCl buffer, pH 7.4, 150 mM NaCl, 1 μg/ml TLCK (*N*-α-*p*-tosyl-L-lysine chloromethyl ketone), 1μg/ml antipain, 0.5 mM DIFP (diisopropyl fluorophosphates), 0.5 mM PMSF, 0.1% protease inhibitor mixture] and centrifuged at 100,000 × g for 20 min at 4°C. The supernatant was collected as the soluble fraction of Aβ. The pellet was resuspended in 4 vol of TS and centrifuged at 70,000 × g for 20 min at 4°C. The resultant pellet was dissolved in 500µl of 6 M guanidine-HCl (in 50mM Tris buffer, pH 7.6), incubated at room temperature for 30 min, and centrifuged at 70,000 × g for 20 min at 4°C. The resultant supernatant was collected as the insoluble fraction of Aβ and diluted by enzyme immunoassay (EIA) buffer (supplied with the kit) to 12× to reduce sample guanidine-HCl concentration. Protein levels were determined using the BCA kit (Pierce

Biotechnology Inc., Rockford, IL, USA). Aliquots (100µg of protein in 100µl of EIA buffer) and assay standards were added to 96-well plates [pre-coated with anti-human $A\beta_{1-40}$ and $A\beta_{1-42}$] and were incubated overnight at 4°C. The wells were washed seven times with EIA buffer. Then, 100µl of labeled antibody was added to each well containing sample or standard and incubated at 4°C for 1 hr. The wells were washed nine times with EIA buffer followed by the addition of 100µl of tetramethylbenzidine (TMB) buffer, and incubated in the dark for 30 min at room temperature. The reaction was stopped by adding 100µl of 1N $H_2SO_4$, and the colorimetric absorption was measured at 450 nm using Spectra Max UV/Vis Spectrometer (GMI Inc., Ramsey, MN, USA). The levels of Aβ in the test samples were calculated relative to the standard curve generated on each plate.

*2.8. Statistical analysis*

Biochemical data are expressed as the mean ± the standard error of the mean (SEM). The significance of difference in behavioral performance in the Morris water maze daily training sessions between the hemizygous R1.40 and the wild type groups was determined using repeated measure analysis of variance (ANOVA) while probe trails difference between both groups was determined by two-tailed student t-test. The significance of difference between different treatment groups was determined by repeated measure ANOVA and Tukey-Kramer multiple comparison post-hoc test. All statistical analyses were conducted using GraphPad InStat 3 software (GraphPad Software, La Jolla, CA, USA). Probability (*p*) value of $<0.05$ was considered statistically significant.

# 3. Results

### *3.1. Genotyping of R1.40 mice*

The primer pairs IMR6938 and IMR6938 amplified the 84 bp PCR product from the mutant human APP whereas no PCR product was expected from the non-transgenic mice (Fig. 2A). Thus, the detected bands at 84 bp in agarose gels represent either a hemizygous or homozygous mutant APP alleles. Allelic discrimination scatter plots were generated showing three cluster groups classifying the unknown samples as hemizygous APP mice having both allele A and allele G, and as homozygous APP mice having allele G or wild type mice having only Allele A (Fig. 2B). Thus, by allelic discrimination assay we were able to identify hemizygous and homozygous transgenic APP mice, and the wild type mice as well.

### *3.2. Hemizygous R1.40 exhibits cognitive impairments when tested in Morris water maze and in the Y-maze*

Spatial navigation in the Morris water maze is one type of the tests used to assess long-term memory functions, which is usually impaired in AD. In the daily sessions that consisted of three trials per day for a total of eight days, repeated measure ANOVA indicated no significant difference in the performance in this task, assessed by measuring escape latency, between the transgenic group and the control wild type mice ($F(7,245) = 0.924$, $p \geq 0.05$) (Fig.3A). However, probe trials that assessed the percent time in the correct quadrant that contained the hidden platform showed that as the retention delay increased from Day 1 following the last day of the daily training sessions to Day 11, the transgenic mice exhibited memory impairment as

they failed to remember the location of the hidden platform. As shown in Fig. 3B, probe trial in Day 1 following the last day of daily training sessions showed no significant difference between both groups ($p > 0.1$). However, after a longer delay on Day 11 following the last day of daily trainings sessions, the hemizygous R1.40 transgenic mice significantly spent less time (M = 23% ; SEM = 1.99%) compared to the control wild type mice (M = 29.8% ; SEM= 2.58%), $p = 0.045$ (Fig. 3B).

Working memory function was assessed by measuring the spontaneous alternations in the Y-maze defined as the percentage of the number of arm entries, different from the previous two entries, divided by the total arm entries minus two. This task is based on the innate nature of the mice to visit arms that have not been recently visited (King, et al., 1999; Hock, et al., 2009). Results revealed that hemizygous transgenic R1.40 mice exhibited impaired working memory function as they had a significantly lower spontaneous alternation ratio (M = 0.413 ; SEM = 0.021) than the control wild type mice (M = 0.496 ; SEM = 0.019), $p = 0.0047$ (Fig. 4).

*3.3. Treatment with tolfenamic acid reverses the cognitive deficits in hemizygous R1.40 mouse model*

We have determined the presence of impaired behavioral performance in the hemizygous R1.40 mice manifested by a decline in the long-term memory and the working memory functions as identified by the retention of the spatial location of the platform in the Morris water maze and the spontaneous alternations in the Y-maze, respectively.

Thus, we examined the ability of a tolfenamic acid treatment of 5 and 50 mg/kg/day to attenuate such learning and memory deficits in groups of female hemizygous R1.40 transgenic mice aging between 14-21 months. In the Morris water maze task, escape latency among the treatment and the vehicle groups showed no significant difference in daily learning acquisition (Fig. 5A). However, ANOVA analysis indicated an improvement in long-term memory retention in Day 11 probe trial ($F(2,15) = 4.145$, $p = 0.0369$) (Fig. 5B). The Tukey-Kramer HSD post-hoc test showed that mice group treated with 50 mg/kg/day tolfenamic acid had a significant improvement (HSD = 3.12, $p < 0.05$) while the treatment with 5 mg/kg/day failed to reach significance (HSD = 0.71 $p \geq 0.5$) (Fig. 5B).

Results from the ANOVA analysis of the spontaneous alternation ratio in the Y-maze showed a significant improvement in working memory ($F(2,15) = 4.479$, $p = 0.0298$) (Fig. 6). The Tukey-Kramer HSD post-hoc test revealed a significant improvement with tolfenamic acid treatment of 50 mg/kg/day (HSD = 4.139, $p < 0.05$), however, treatment with 5 mg/kg/day did not reach significance (HSD = 1.30, $p \geq 0.05$) (Fig. 6)

*3.4. Tolfenamic acid treatment lowers SP1 protein without altering Sp1 gene expression*

The protein expressions of cortical SP1 normalized to β-actin was analyzed by Western blot following treatment with 5 and 50 mg/kg/day for 34 days or with vehicle. Results analyzed with ANOVA revealed a significant difference in SP1 protein levels ($F(2.11) = 8.747$, $p = 0.0053$) (Fig. 7A). The Tukey-Kramer HSD post-hoc test indicated a significant decrease of SP1 after treatment with 5 mg/kg/day tolfenamic

acid (HSD = 4.145, $p < 0.05$, % change = - 43%) and after treatment with 50 mg/kg/day tolfenamic acid (HSD = 5.712, $p < 0.01$, % change = - 52%) (Fig. 7A).

Gene expression of Sp1 was evaluated for the different treatment groups as well. Analysis of Sp1 mRNA levels showed that treatment with tolfenamic acid did not alter the gene expression of Sp1 ($F(2,16) = 0.082$, $p = 0.922$) indicating that the decrease in SP1 protein levels is not due to a change in its *de novo* gene expression (Fig. 7B).

*3.5. Reduction of APP protein and mRNA following treatment with tolfenamic acid*

The protein expressions of cortical APP normalized to β-actin was analyzed by Western blot following treatment with 5 and 50 mg/kg/day for 34 days or with vehicle. Results analyzed with ANOVA revealed that APP levels were lowered significantly ($F(2,9) = 8.377$, $p = 0.009$) (Fig. 8A). The Tukey-Kramer HSD post-hoc test indicated a significant decrease in APP after treatment with 5 mg/kg/day tolfenamic acid (HSD = 4.877, $p < 0.05$, % change = - 30.3%) and after treatment with 50 mg/kg/day tolfenamic acid (HSD = 5.139, $p < 0.05$, % change = - 32.5%) (Fig. 8A).

In addition to analysis of APP protein levels, APP gene expression was evaluated for the different treatment groups by Real-Time PCR. ANOVA analysis of APP mRNA levels revealed a significant decrease in APP gene expression ($F(2,15) = 5.238$, $p = 0.019$) (Fig. 8B). The Tukey-Kramer HSD post-hoc test indicated a significant decrease of APP mRNA after treatment with 5 mg/kg/day tolfenamic acid (HSD = 3.885, $p < 0.05$, % change = - 50.3%) and after treatment with 50 mg/kg/day tolfenamic acid (HSD = 4.039, $p < 0.05$, % change = - 52.3%) (Fig. 8B).

*3.6. Tolfenamic acid lowers both soluble and insoluble $A\beta_{1-40}$ and $A\beta_{1-42}$ levels*

We measured the levels of cortical $A\beta_{1-40}$ and $A\beta_{1-42}$ levels in the Tris-saline-soluble and in the insoluble fraction using IBL ELISA kits as described in literature (Morishima-Kawashima, et al., 2000). ANOVA analysis showed that there was a significant reduction in soluble $A\beta_{1-40}$ fraction ($F(2,14) = 3.89$, $p = 0.04$) (Fig. 9A) and the insoluble $A\beta_{1-40}$ fraction ($F(2,14) = 4.95$, $p = 0.02$) (Fig. 9B). The Tukey-Kramer HSD post-hoc test indicated a significant decrease in soluble $A\beta_{1-40}$ after treatment with 50 mg/kg/day (HSD = 3.91, $p < 0.05$, % change = - 30.1%), however, the lowering of soluble $A\beta_{1-40}$ after treatment with 5 mg/kg/day did not reach significance (HSD = 2.55, $p \geq 0.05$, % change = - 21.3%) (Fig. 9A). Multiple group comparison showed a significant decrease in the insoluble $A\beta_{1-40}$ fraction after treatment with 5 mg/kg/day (HSD = 3.73, $p < 0.05$, % change = - 35.2%) and after treatment with 50 mg/kg/day tolfenamic acid (HSD = 3.97, $p < 0.05$, % change = - 37.4%) (Fig. 9B).

Also, ANOVA analysis showed a significant decrease in soluble $A\beta_{1-42}$ fraction ($F(2,14) = 6.174$, $p = 0.012$) (Fig. 9C) and the insoluble $A\beta_{1-42}$ fraction ($F(2,14) = 6.343$, $p = 0.011$) (Fig. 9D). The Tukey-Kramer HSD post-hoc test indicated a significant decrease in soluble $A\beta_{1-42}$ after tolfenamic acid treatment with 5 mg/kg/day (HSD = 4.18, $p < 0.05$, % change = - 22.3%) and after treatment with 50 mg/kg/day (HSD = 4.518, $p < 0.05$, % change = - 24.9%) (Fig. 9C). Multiple group comparison showed a significant decrease in the insoluble $A\beta_{1-42}$ fraction after treatment with 5 mg/kg/day (HSD = 4.72, $p < 0.05$, % change = - 31.8%) and after treatment with 50 mg/kg/day (HSD = 4.79, $p < 0.05$, % change = - 37.7%) (Fig. 9D).

We also observed that 5 and 50 mg/kg/day tolfenamic acid treatment reduced total $A\beta_{1-42}/A\beta_{1-40}$ ratio -16.3% and -19.9%, respectively. However, this reduction in $A\beta_{1-42}$ $/A\beta_{1-40}$ did not reach statistical significance ($F(2.14) = 1.788$ , $p = 0.203$).

## 4. Discussion

Several mechanisms have been proposed to explain how NSAIDs could exert their effects on AD independent of their COX inhibitory pathways. Certain NSAIDs including indomethacin, fulfinamic acid, ibuprofen and fenoprofen are agonists for the nuclear transcriptional regulator peroxisome proliferator-activated receptor-γ (PPARγ) (Lehmann, et al., 1997; Heneka, et al., 2011) that has been reported to be reduced in AD brains (Sastre, et al., 2006). PPARγ activation results in a reduction in the levels of Aβ-induced proinflammatory response of microglia and monocytes, and promotes Aβ clearance by astrocytes and microglia (Matsuo, et al., 1996; Combs, et al., 2000; Daynes and Jones, 2002; Yue and Mazzone, 2009; Mandrekar-Colucci, et al., 2012). In addition, a PPARγ responsive element (PPRE) has been identified in the BACE1 gene promoter that is repressed by PPARγ binding resulting in a decrease in BACE1 expression (Sastre, et al., 2003; Sastre, et al., 2006). Weggen et al., reported that a subset of NSAIDs specifically lowers $A\beta_{1-42}$ that was accompanied by a parallel increase in $A\beta_{1-38}$ suggesting a minor γ-secretase modulatory effect without alteration of the Notch pathway (Weggen, et al., 2001). The specific $A\beta_{1-42}$ inhibition by that subset of NSAIDs was further studied by Zhou et al., and concluded that the involvement of Rho-Rock pathway in regulating APP processing, and inhibition of

Rho activity by specific NSAIDs could preferentially reduce A$\beta_{1-42}$ (Zhou, et al., 2003).

Tolfenamic acid, of all other NSAIDs, has the unique mechanism of inducing the proteasome dependent degradation of SP transcription factors including SP1, SP3 and SP4 and thereby, decreasing the expression of vascular endothelial growth factors (VEGF) (Abdelrahim, et al., 2006), which is involved in tumor progression and metastases. Because of its SP degradation effects, tolfenamic acid has been studied and research has confirmed its antitumor effects (Abdelrahim, et al., 2006; Konduri, et al., 2009; Basha, et al., 2011; Colon, et al., 2011).

Sp1 involvement in AD has been researched and it was found that its expression was elevated in the frontal cortex of human AD brains as well as in the brains of transgenic mouse models of AD (Santpere, et al., 2006; Citron, et al., 2008). The elevation of Sp1 was accompanied by an increase in COX-2, another Sp1 target gene, suggesting interplay between inflammatory processes and Sp1 driven gene expression (Citron, et al., 2008). Sp1 binds to 5'-GGGGCGGGGC-rich promoter genes such as APP and BACE1 genes, which are involved in AD pathology, and Sp1 overexpression leads to induction of their protein expression (Cai, et al., 2001; Christensen, et al., 2004; Citron, et al., 2008). We have reported that silencing of Sp1 gene by siRNA resulted in 70% decrease in the responsiveness of the human APP promoter (Basha, et al., 2005). Also, we have shown the co-localization of APP, Sp1 and Aβ in various regions of the brain (Brock, et al., 2008). Thus, we had proposed the hypothesis that tolfenamic acid could interrupt the *de novo* synthesis of APP and consequently, alter the downstream

levels of Aβ. This hypothesis was confirmed when oral treatment with tolfenamic acid resulted in degradation of SP1 that was accompanied by a reduction in cortical and hippocampal APP gene expression and protein and Aβ peptide levels (Adwan, et al., 2011). This was further supported by measurements which demonstrated the presence of tolfenamic acid in the brain following IV administration (Subaiea, et al., 2011).

In the present study, we demonstrate that hemizygous R1.40 transgenic mice exhibit cognitive deficits upon assessment of their working memory and long-term memory retention even before the reported age at which hemizygous R1.40 start to show extensive β-amyloid deposition (Lamb et al., 1999). Spatial learning was assessed in the hidden version of the Morris water maze and we found that the transgenic mice were able to learn escaping to the hidden platform in the daily training sessions (Fig. 3A). However, they exhibited memory retention impairments in the probe trials as they failed to memorize the spatial location of the hidden platform with increased delay (Fig. 3B, Day 11). In addition, working memory assessed in the Y-maze was impaired in these mice with numerous alternation errors and a lowered alternation ratio compared to the WT control group (Fig. 4). It has been proposed that recent spatial memory is hippocampal-dependent, while remote spatial memory is dependent on the integrity of the medial prefrontal cortex (King, et al., 1999; Frankland and Bontempi, 2005; Teixeira, et al., 2006). In turn, working memory assessed in the Y-maze is hippocampal and cortical-dependent (Lalonde, 2002). That, together with the findings that the R1.40 mouse model exhibits extensive amyloid deposition in the

cortical region compared to that observed in the hippocampal formation (Hock, et al., 2009), may explain the observed results.

We report here that short-term tolfenamic acid administration was able to improve the mnemonic deficits observed in hemizygous R1.40. We found that 50 mg/kg/day tolfenamic acid was able to reverse the long-term memory retention deficits seen in delayed probe trial of the Morris water maze experiment (Fig. 5B). In addition, the 50 mg/kg/day treatment group showed significant improvement in the Y-maze with less error in spontaneous alternations (Fig. 6). However, the short-term treatment with 5 mg/kg/day showed only modest effects in reversing the memory deficits in the mice. Analysis of amyloid pathology markers indicated that both treatment regimens, 5 and 50 mg/kg/day, were able to significantly lower the protein level of SP1 without altering it gene expression (Fig. 7A and 7B), similar to our previous findings in the C57BL/6 treated with tolfenamic acid (Adwan, et al., 2011). In addition, both tolfenamic acid doses significantly reduced the protein levels of APP and its gene expression (Fig. 8A and 8B). Furthermore, levels of the soluble and insoluble $A\beta_{1-40}$ and $A\beta_{1-42}$ were reduced by tolfenamic acid with a trend toward lowering the insoluble fractions of Aβ more than that of the soluble fraction. It is possible that tolfenamic acid may aid in clearance of insoluble Aβ by a PPARγ activation, however, it is not clear yet if tolfenamic acid activates PPARγ as certain other NSAIDs do.

While tolfenamic acid was effective in lowering the amyloidogenic proteins with both doses, we observed dose-dependent improvement in the cognitive deficits. Whether

longer period of treatment with low dose tolfenamic acid could result in significant improvements in cognitive function is yet to be studied.

In conclusion, tolfenamic acid is unique among NSAIDs in its ability to inhibit Sp1 and thus interrupt APP *de novo* synthesis and its Aβ products. In this study, we demonstrated that short-term administration of tolfenamic acid for 34 days reversed the cognitive deficits in hemizygous R1.40 transgenic mice. Consistent with our previous published findings in C57BL/6 mice, SP1, the amyloidogenic levels of APP and soluble and insoluble $A\beta_{1-40}$ and $A\beta_{1-42}$ were markedly lowered by tolfenamic acid in AD transgenic mice. These data provide further evidence that tolfenamic acid could be a promising therapeutic approach as a repurposed AD drug acting through an alternative mechanism.

**Acknowledgements**

This work was supported by the grants ES13022, AG027246, 1R56ES015867-01A1, and 1R01ES015867-01A2 from the National Institutes of Health awarded to N.H.Z. The research was made possible by the use of the RI-INBRE Research Core Facility, supported jointly by NCRR/NIH Grant # P20 RR016457 and the network institutions. Special thanks from the authors to Dr. Karen E. Stevens from University of Colorado Denver, College of Medicine, Department of Psychiatry for her assistance in the behavioral experiments conducted in this study.

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**Figure II-1. A timeline of tolfenamic acid administration and behavioral assessment.** Mice were administered tolfenamic acid daily for 34 days. Behavioral testing in the Morris water maze began on the 15$^{th}$ day of dosing with daily training sessions that lasted until the 23$^{rd}$ day of the study. On the 24$^{th}$ day of dosing, we conducted the first session of probe trials (Day 1) and then we performed a second session of probe trials on the 34th of dosing (Day 11), to assess for long-term memory retention. Trials for the spontaneous alternations in the Y-maze were conducted on the 33$^{rd}$ day of dosing as well. Animals were euthanized on the 35$^{th}$ day of the study and brain tissue was dissected, collected and stored in the -80°C freezer.

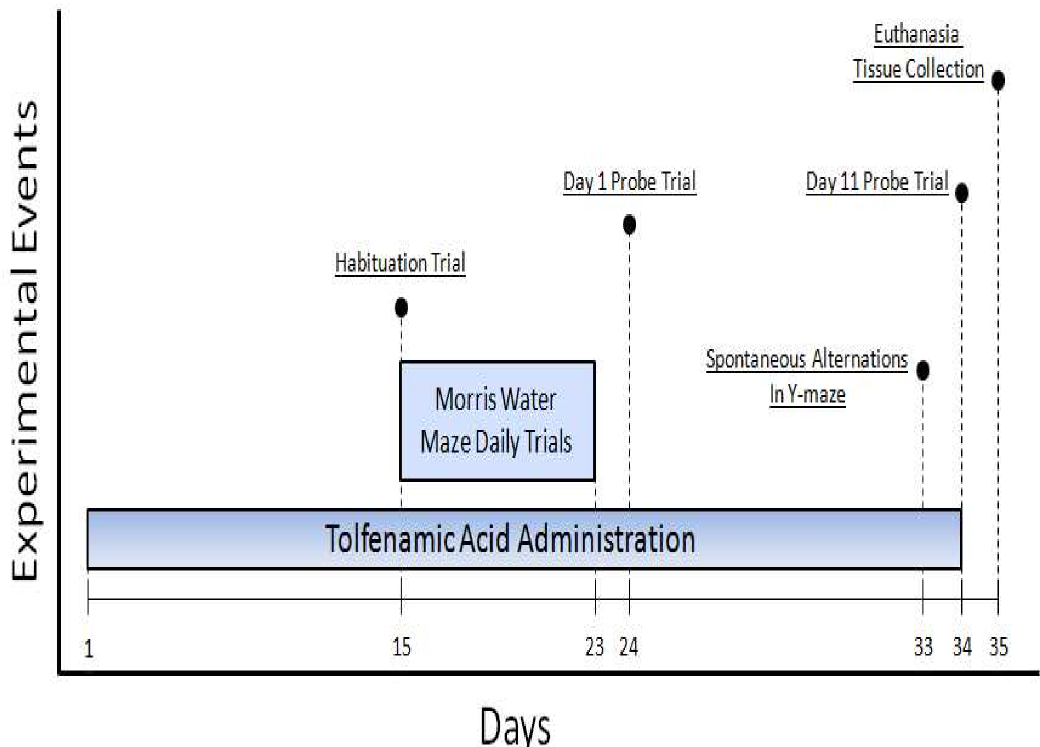
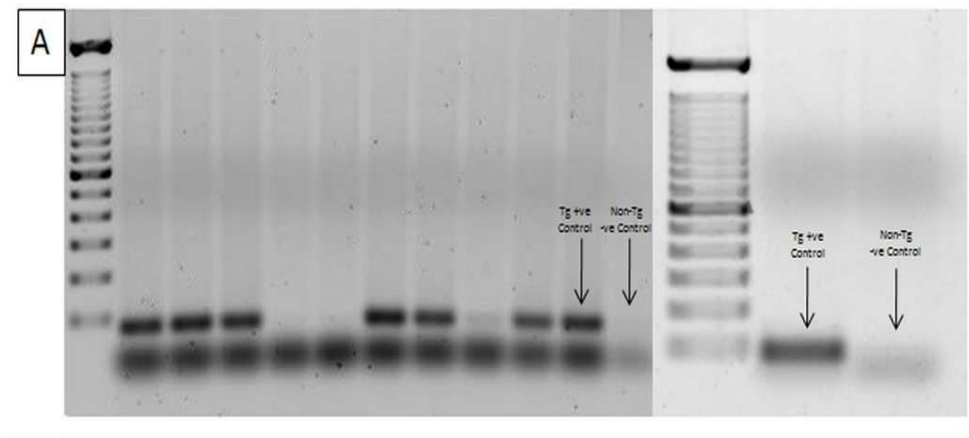

Euthanasia
Tissue Collection
Day 1 Probe Trial
Day 11 Probe Trial
Habituation Trial
Spontaneous Alternations
In Y-maze
Morris Water
Maze Daily Trials
Tolfenamic Acid Administration
1
15
23
24
33
34
35
Experimental Events
Days

**Figure II-2. Genotyping of in-house bred APP YAC transgenic mice, line R1.40**. Genetic identification and allelic discrimination was performed using standard protocols (see methods section). **A)** Genetic identification of YAC APP transgenic (Tg) and non-transgenic (non-Tg) mice using standard PCR protocol; **B)** A scatter plot of allelic discrimination analysis.


A
Tg +ve Control
Non-Tg -ve Control
Tg +ve Control
Non-Tg -ve Control

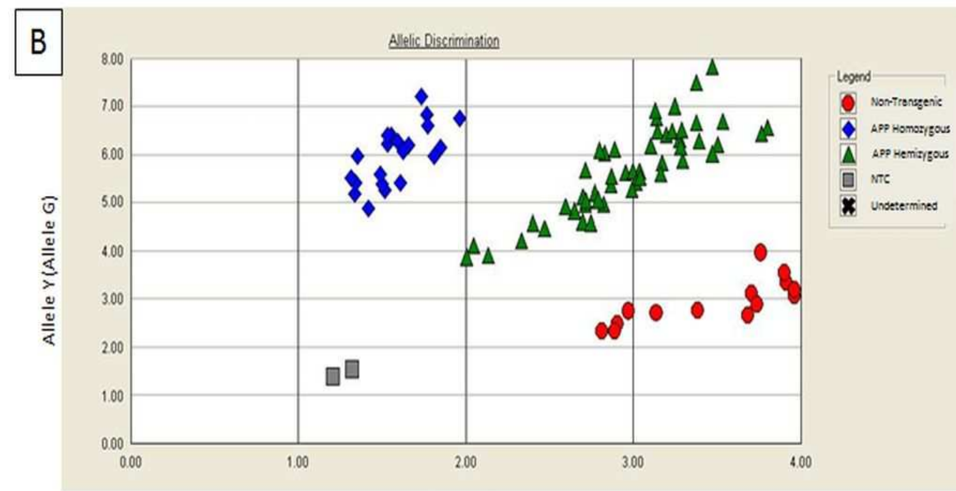

B
Allelic Discrimination
Legend
Non-Transgenic
APP Homozygous
APP Hemizygous
NTC
Undetermined
Allele Y (Allele G)
Allele X (Allele A)

**Figure II-3. Memory impairments in hemizygous APP YAC transgenic mice, line R1.40, assessed by the swimming task in the Morris water maze. A)** Acquisition patterns during daily training. **B)** Probe trials for long-term memory retention on Days 1 and 11 following acquisition-training trials. “*” indicates that the values are significantly different from control, as determined by a Student’s *t*-test ($p<0.05$).

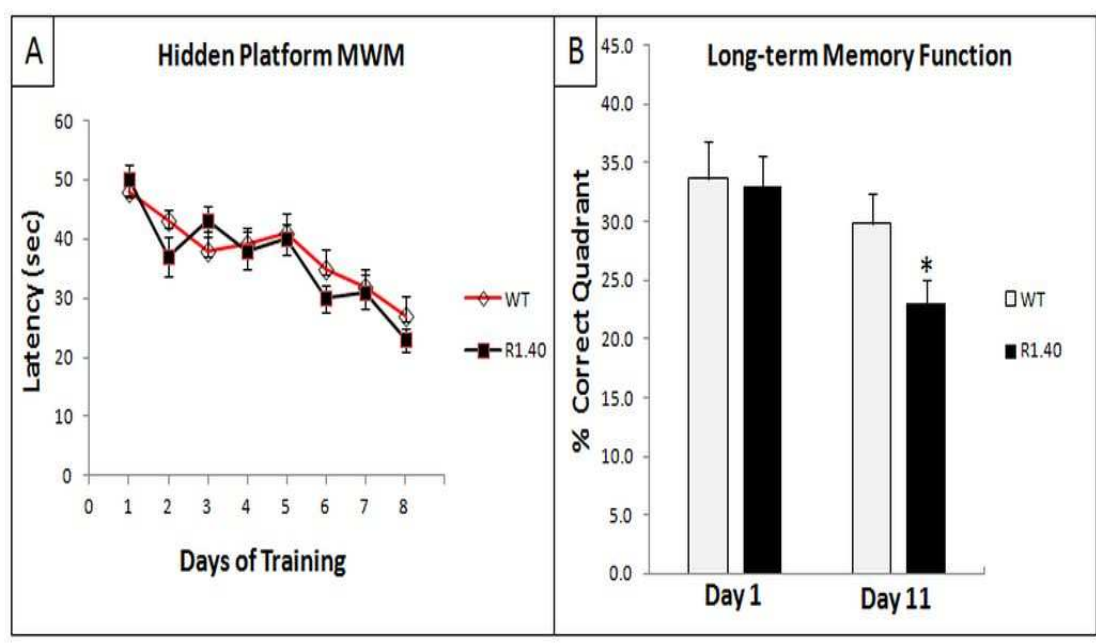

A
Hidden Platform MWM
Latency (sec)
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8
Days of Training
WT
R1.40
B
Long-term Memory Function
% Correct Quadrant
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
*
Day 1
Day 11
WT
R1.40

**Figure II-4. Working memory impairments in hemizygous APP YAC transgenic mice, line R1.40.** Mice were examined for working memory function assessed by measuring the spontaneous alternation ratio in the Y-maze. "**" indicates that the values are significantly different from control, as determined by a Student's $t$-test ($p<0.01$)

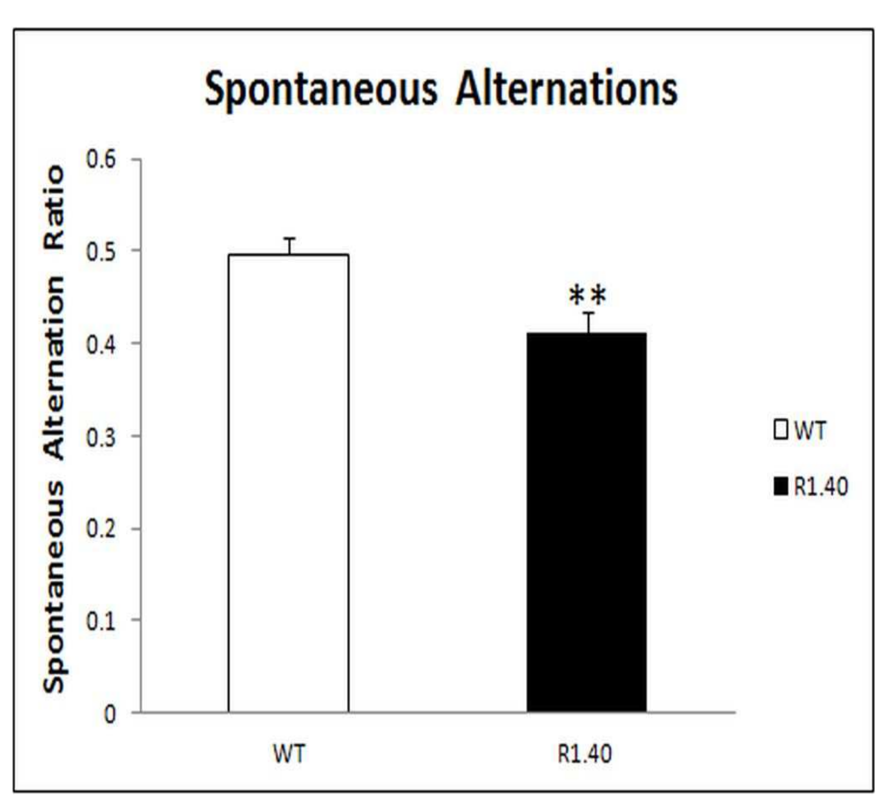

Spontaneous Alternations
Spontaneous Alternation Ratio
0.6
0.5
0.4
0.3
0.2
0.1
0
**
WT
R1.40
WT
R1.40

**Figure II-5. Effects of the administration of tolfenamic acid to hemizygous APP YAC transgenic mice, line R1.40.** Tolfenamic acid was administered daily by oral gavage for 34 days. See the methods section for details. **A)** Acquisition patterns during daily training trials of 5 mg/kg/day, 50 mg/kg/day and vehicle groups **B)** Probe trials assessing the long-term memory retention on Days 1 and 11 following acquisition-training trials. "*" indicates that values are significantly different from control, as determined by ANOVA analysis with Tukey-Kramer post-hoc test to compare all pairs of columns ($p<0.05$), obtained using GraphPad InStat 3 software. Vehicle n=6; 5 mg/kg/day n=6; 50 mg/kg/day n=7.

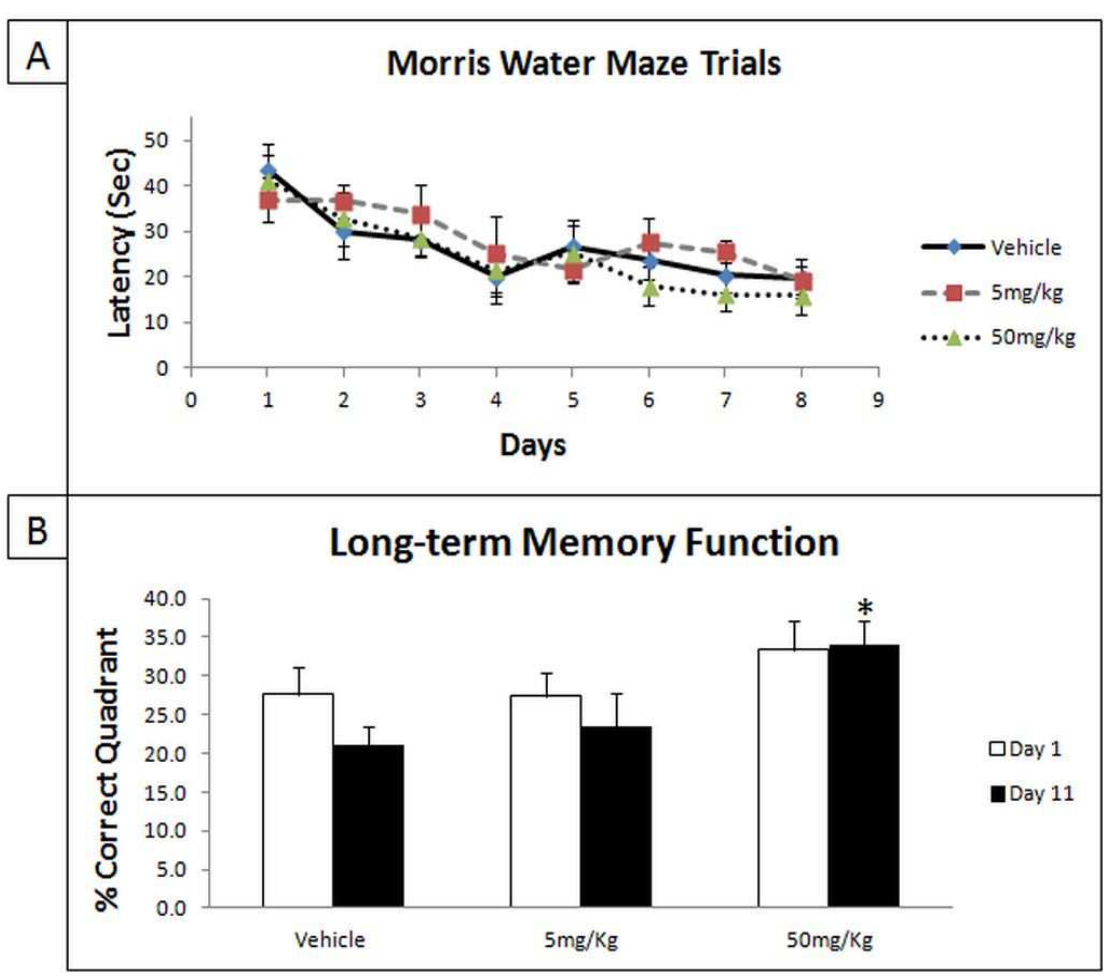

A
Morris Water Maze Trials
Latency (Sec)
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
9
Days
Vehicle
5mg/kg
50mg/kg
B
Long-term Memory Function
% Correct Quadrant
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
*
Vehicle
5mg/Kg
50mg/Kg
Day 1
Day 11

**Figure II-6. Working memory improvement in hemizygous APP YAC transgenic mice, line R1.40, after administration of tolfenamic acid assessed by the spontaneous alternations in Y-maze.** Tolfenamic acid was administered daily by oral gavage for 34 days. See the methods section for details. "*" indicates that values are significantly different from control vehicle, as determined by ANOVA analysis with Tukey-Kramer post-hoc test to compare all pairs of columns ($p<0.05$), obtained using GraphPad InStat 3 software. Vehicle n=6; 5 mg/kg/day n=6; 50 mg/kg/day n=7.

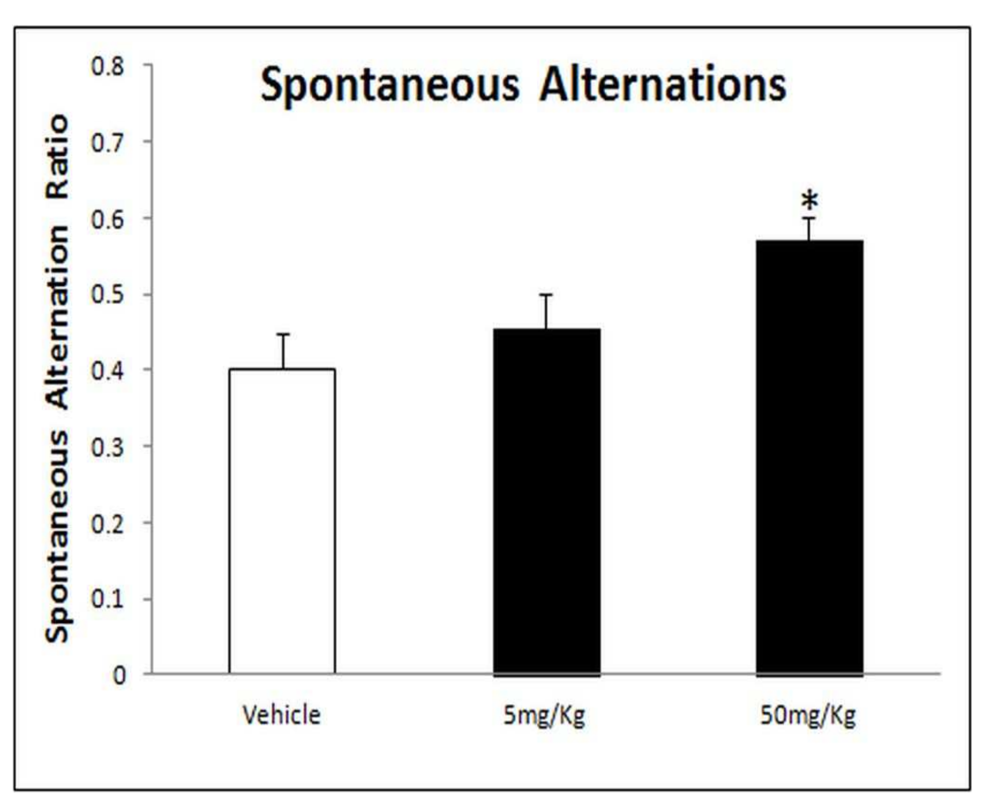

Spontaneous Alternations
Spontaneous Alternation Ratio
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
Vehicle
5mg/Kg
50mg/Kg

**Figure II-7. Reduction of SP1 protein levels in the brain after treatment with tolfenamic acid.** Tolfenamic acid was administered daily by oral gavage for 34 days. See the methods section for details. **A)** Western blot analysis of cortical SP1 levels as a ratio to the house keeping protein β-actin after treatment with 5 and 50 mg/kg/day of tolfenamic acid for 34 days. **B)** Cortical Sp1 mRNA levels determined by Real-Time PCR. "*" indicates that values are significantly different from control, as determined by ANOVA analysis with Tukey-Kramer post-hoc test to compare all pairs of columns (*$p<0.05$, **$p<0.01$), obtained using GraphPad InStat 3 software.

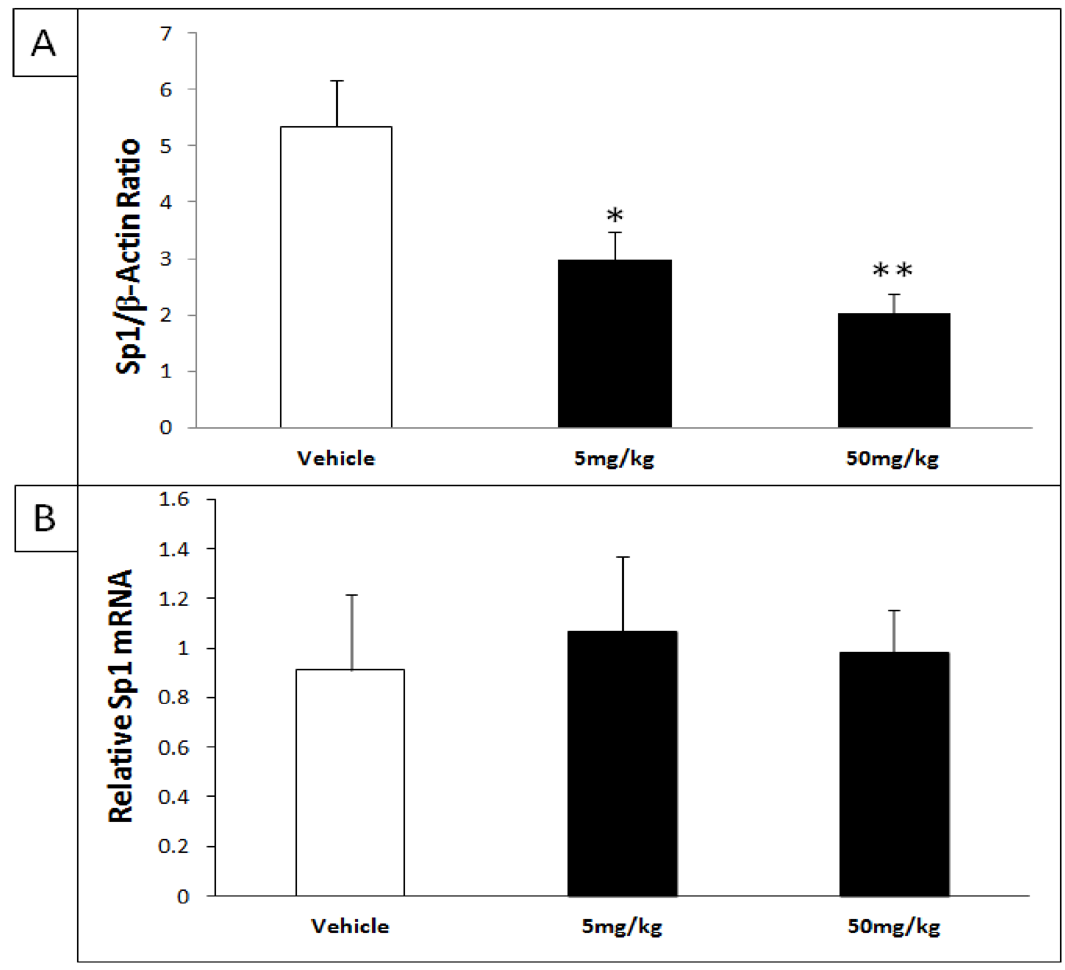

A
7
6
5
4
3
2
1
0
Sp1/β-Actin Ratio
*
**
Vehicle
5mg/kg
50mg/kg
B
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Relative Sp1 mRNA
Vehicle
5mg/kg
50mg/kg

**Figure II-8. Reduction of amyloid precursor protein (APP) levels in the brain after treatment with tolfenamic acid.** Tolfenamic acid was administered daily by oral gavage for 34 days. See the methods section for details. **A)** Western blot analysis of cortical APP levels as a ratio to the house keeping protein β-actin after treatment with 5 and 50 mg/kg/day of tolfenamic acid for 34 days. B) Cortical APP mRNA determined by Real-Time PCR. "*" indicates that values are significantly different from control, as determined by ANOVA analysis with Tukey-Kramer post-hoc test to compare all pairs of columns ($p$<0.05), obtained using GraphPad InStat 3 software.

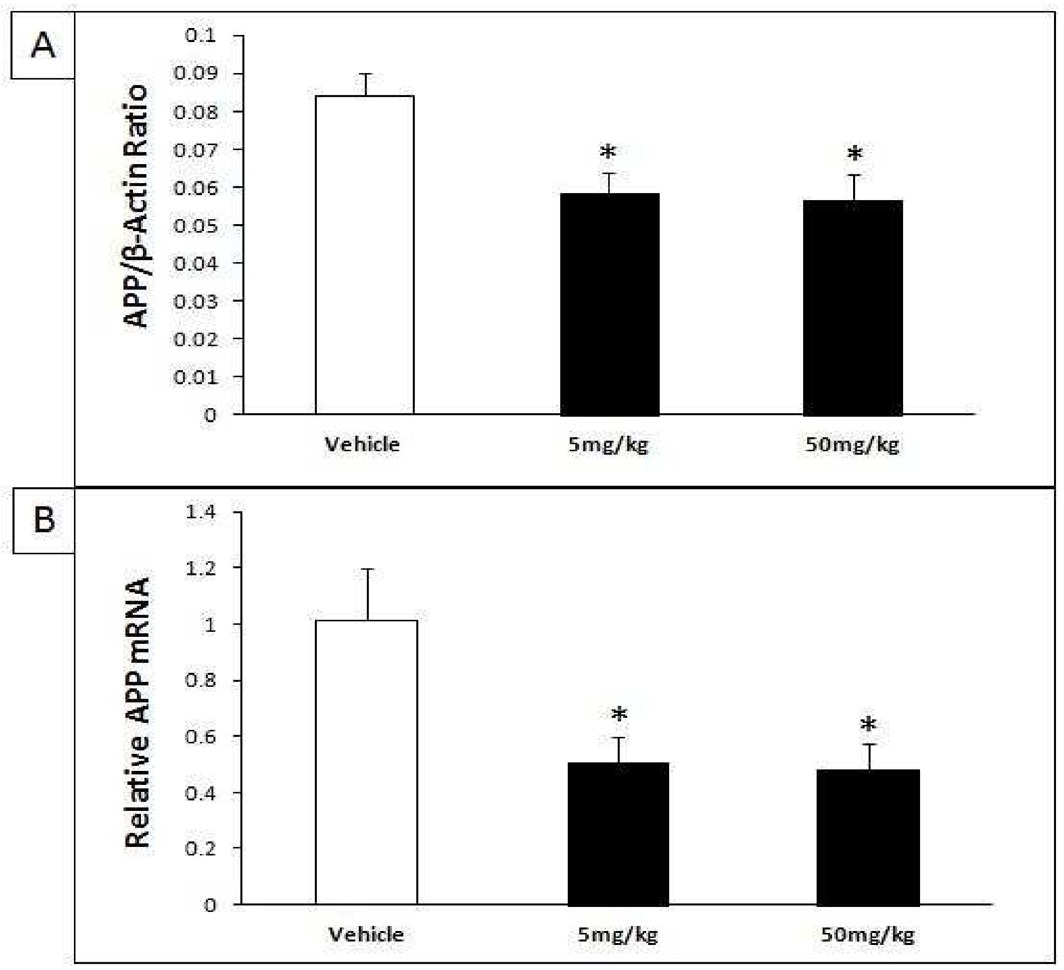
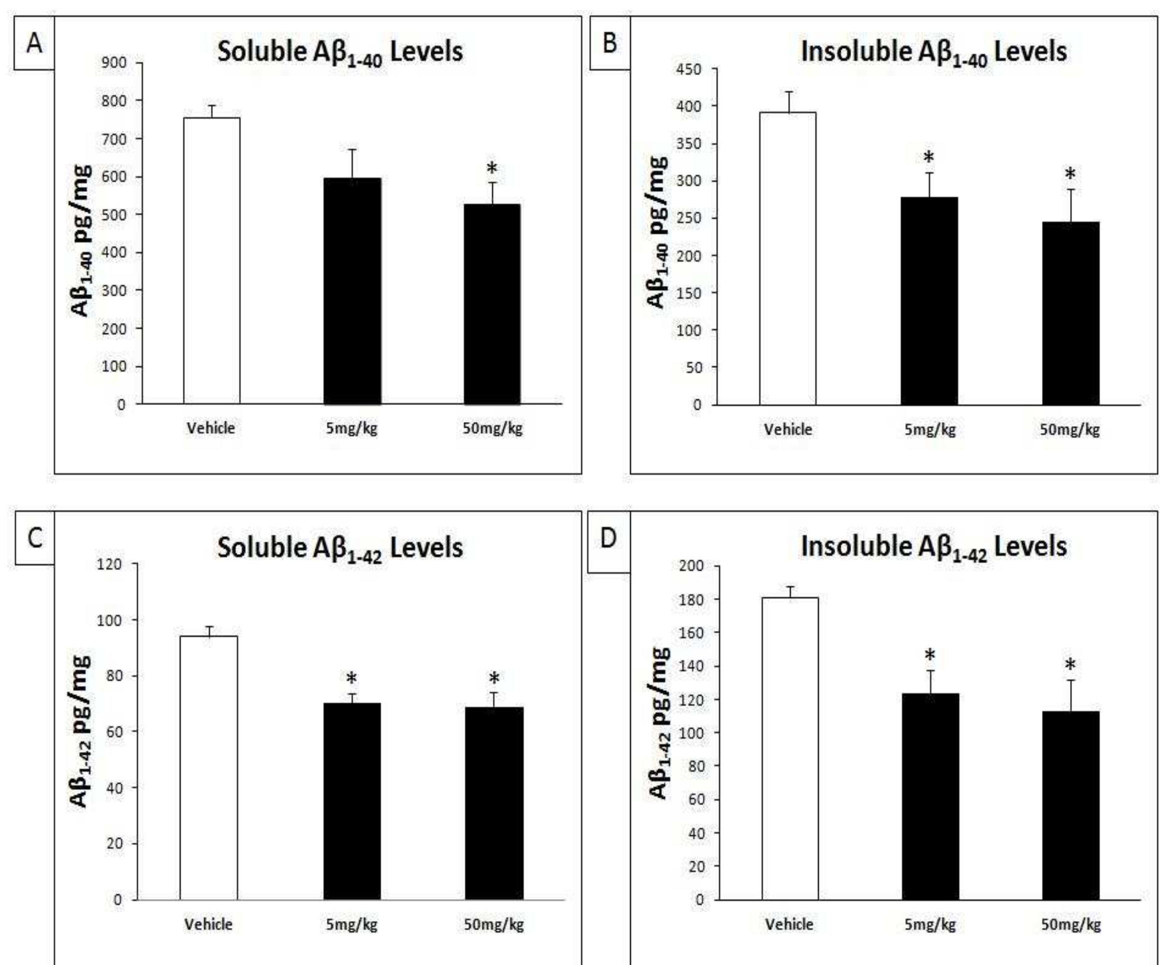

A
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
APP/β-Actin Ratio
*
*
Vehicle
5mg/kg
50mg/kg
B
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Relative APP mRNA
*
*
Vehicle
5mg/kg
50mg/kg

**Figure II-9. Reduction of soluble and insoluble A$\beta_{1-40}$ and A$\beta_{1-42}$ levels in the brain after treatment with tolfenamic acid.** Tolfenamic acid was administered daily by oral gavage for 34 days. See the methods section for details. Aβ levels were quantified by ELISA in the cortices of mice treated with 5 and 50 mg/kg/day of tolfenamic acid for 34 days. **(A)** Soluble A$\beta_{1-40}$, **(B)** Insoluble A$\beta_{1-40}$, **(C)** Soluble A$\beta_{1-42}$ and **(D)** Insoluble A$\beta_{1-42}$ levels. "*" indicates that values are significantly different from control, as determined ANOVA analysis with Tukey-Kramer post-hoc test to compare all pairs of columns ($p<0.05$), obtained using GraphPad InStat 3 software.


A
Soluble Aβ1-40 Levels
Aβ1-40 pg/mg
900
800
700
600
500
400
300
200
100
0
*
Vehicle
5mg/kg
50mg/kg
B
Insoluble Aβ1-40 Levels
Aβ1-40 pg/mg
450
400
350
300
250
200
150
100
50
0
*
*
Vehicle
5mg/kg
50mg/kg
C
Soluble Aβ1-42 Levels
Aβ1-42 pg/mg
120
100
80
60
40
20
0
*
*
Vehicle
5mg/kg
50mg/kg
D
Insoluble Aβ1-42 Levels
Aβ1-42 pg/mg
200
180
160
140
120
100
80
60
40
20
0
*
*
Vehicle
5mg/kg
50mg/kg

## MANUSCRIPT 3

# Reduction of β-Amyloid Deposition and Attenuation of Memory Deficits by Tolfenamic Acid

**By**

**Gehad M. Subaiea, Lina I. Adwan, Aseef H. Ahmed and Nasser H. Zawia**

**(Intended publication in Alzheimer's & Dementia)**

**ABSTRACT**

**BACKGROUND:** We have reported that tolfenamic acid treatment decreases amyloidogenic proteins in wild type and transgenic mice via lowering of the transcription factor specificity 1 protein (Sp1).

**OBJECTIVES:** In this study, we examine the ability of tolfenamic acid to reduce the amyloid plaque burden in R1.40 mouse model, as well as to ameliorate spatial learning and memory deficits in R1.40 and senescent wild type mice.

**METHODS:** Behavioral and immunohistochemical analyses were performed following the oral administration of multiple doses of tolfenamic acid to homozygous R1.40 and senescent wild type mice for 34 days.

**RESULTS:** Quantitative immunohistochemical analysis indicated that tolfenamic acid treatment resulted in a profound decrease in cerebral amyloid beta (Aβ) plaque burden. The reduction in the amyloid pathology was accompanied by improvements in spatial working memory assessed by spontaneous alternation ratio in the Y-maze. In addition, senescent wild type mice treated with tolfenamic acid exhibited improved spatial reference memory performance in the delayed probe trials of the Morris water maze compared to the non-treated group.

**CONCLUSION:** These results provide further evidence that tolfenamic acid could be utilized as a repurposed drug to modify Alzheimer's disease (AD) pathogenesis.

***Keywords:*** Alzheimer's disease; tolfenamic acid; AD transgenic mouse model; learning and memory; Morris water maze; Y-maze; immunohistochemistry; amyloid plaque burden.

## 1. Introduction

Alzheimer's disease (AD) patients suffer from profound general memory loss and dementia until death and the disease is known as the most prevalent neurodegenerative disorder [1]. Even though Alois Alzheimer has described the disease for more than a century, no cure has been discovered yet [2, 3]. Different brain regions especially in the cerebral cortex, hippocampus, subcortical nuclei and amygdala suffer extracellular senile plaques and intraneuronal neurofibrillary tangles (NFTs) formation that characterize the neuropathological deposits identified in AD [2, 4]. Neuritic plaques induce the proliferation of astrocytes and microglia with altered morphology leading to inflammatory responses that contribute to the brain degeneration observed in the disease [5, 6].

Senile plaques are mainly composed of aggregated amyloid beta (Aβ) peptides, which are 38-43 amino acids long, that are generated by the cleavage of the amyloid precursor protein (APP) by the beta-site APP-cleaving enzyme 1 (BACE1) and γ-secretase [7, 8]. The formation of the insoluble Aβ depositions depends on the rate of Aβ production and the rate of its elimination. Thus, the amyloid hypothesis of AD was developed, suggesting that amyloid plaque aggregates formed by the amyloidogenic breakdown of APP cause the neurodegeneration and dementia seen in AD [9].

Current FDA approved medications for AD are not disease-modifying therapies and do not stop the progression of AD [10]. Tolfenamic acid, a non-steroidal anti-inflammatory drug (NSAID), induces the degradation of the transcription factor specificity protein 1 (Sp1) [11]. Sp1 regulates the expression of several AD-related

genes, and its expression is elevated in the frontal cortex of human AD brains as well as in the brains of transgenic mouse models of AD [12, 13]. We have previously provided proof-of-concept that tolfenamic acid interrupts the *de novo* synthesis of APP and consequently, alters the downstream levels of Aβ [14]. Treatment of wild type C57BL/6 mice with tolfenamic acid lowered the levels of Sp1 protein (SP1) and the expression of AD-related Sp1 target genes including APP [14], tau and BACE1 (unpublished observations), and was supported by measurements which demonstrated the presence of tolfenamic acid in the brain following IV administration [15]. In addition, data from our lab showed that tolfenamic acid reversed the cognitive deficits in the hemizygous R1.40 transgenic mouse model of AD and lowered the levels of SP1, APP and soluble and insoluble $A\beta_{1-40}$ and $A\beta_{1-42}$.

The purpose of the current study was to investigate the ability of tolfenamic acid to lower Aβ plaque load in old homozygous R1.40 mice that exhibit fibrillar plaque depositions between the ages of 14 and 15 months [16, 17]. Furthermore, we investigated if tolfenamic acid would ameliorate the cognitive impairments even long after plaque deposition has begun in homozygous R1.40 mice. In addition, we also administered tolfenamic acid to senescent wild type mice to determine whether the treatment could enhance their mnemonic functions as they express age-related spatial working and reference memory decline in the absence of plaque deposition.

## 2. Methods

### *2.1. Animal model*

Wild type mice and a genomic-based transgenic mouse model, R1.40, were utilized for this study. The transgenic mice, developed by Bruce T. Lamb, were obtained from the Jackson laboratory (Bar Harbor, ME, USA) and colonies of homozygous strains were established in-house. The R1.40 mouse model utilizes a yeast artificial chromosome (YAC) that includes the full 400 kb human APP gene and the flanking sequence of approximately 250 kb to harbor the Swedish mutation APPK670N/M671L and human transcriptional regulatory elements, allowing for proper spatial and temporal expression [18-20]. The mnemonic deficits in homozygous R1.40 are similar to those observed in AD and the model shows a great increase in APP and Aβ production with Aβ deposition occurring at 14-15 months of age compared to the wild type [21, 22].

Mice colonies were established in-house at the University of Rhode Island (URI) animal quarter facility. Mice were bred and genotyped using two genotyping techniques including standard PCR followed by gel electrophoresis, and was confirmed using the TaqMan® allelic discrimination assay (Applied Biosystems, Foster City, CA, USA). Mice of different genotypes were housed in standard mouse cages in rooms with temperature maintained at 22±2°C with humidity levels of 55±5%. The rooms were set for 12:12 hr light-dark cycle (light on 6:00 AM, light off at 6:00 PM) and the animals were provided with food and water ad libitum. A URI veterinarian continuously supervised the animals during the entire period of the study

and assisted in drug administration. All the protocols including breeding and genotyping techniques were approved by the URI Institutional Animal Care and Use Committee (IACUC).

*2.2. Animal exposure to tolfenamic acid and behavioral testing*

Tolfenamic acid was obtained from Sigma Aldrich (St. Louis, MO, USA) and homozygous APP YAC transgenic mice of mixed gender aging between 19-24 months were used to examine the ability of the drug to reduce the amyloidogenic plaque load and to improve the learning and memory deficits observed in these mice. The animals were divided into three groups of similar age and gender variation and were administered tolfenamic acid daily via oral gavage for 34 days; one group was administered 5 mg/kg/day tolfenamic acid in corn oil (n=7); the second group was administered 50 mg/kg/day tolfenamic acid in corn oil (n=6) and the third group was administered corn oil, the vehicle, (n=7). In addition, tolfenamic acid was administered to old wild type mice of mixed gender aging between 18-24 months in order to study its effects on learning and memory. The animals were divided into three groups of similar age and gender variation and were administered tolfenamic acid daily via oral gavage for 34 days; one group was administered 5 mg/kg/day tolfenamic acid in corn oil (n=11); the second group was administered 50 mg/kg/day tolfenamic acid in corn oil (n=10) and the third group was administered corn oil, the vehicle, (n=13).

*2.3. Assessment of cognitive functions following tolfenamic acid treatment in homozygous R1.40 transgenic mice and wild type mice*

Memory and cognitive functions in homozygous R1.40 transgenic and wild type mice were characterized through behavioral testing in mazes that examine the integrity of the hippocampus and the brain cortex including the Morris water maze and spontaneous alternations in the Y-maze. After 14 days of daily dosing, testing began in the Morris water maze and on Day 33 mice were tested for spontaneous alternations in Y-maze. Finally, on Day 35, mice were euthanized and brains were extracted.

*2.3.1. Morris water maze*

Mice were tested in the hidden version of the Morris water maze where they had to locate a hidden platform by learning multiple spatial relationships between the platform and the distal extra-maze cues [23-25]. The apparatus consisted of a white 48” diameter pool that is 30” in height and was filled with water to a depth of 14”. Non-toxic washable liquid paint was added to the water to keep it opaque. Distinct fixed visual cues surrounded the pool that the animals used for navigation while trying to reach the escape platform. A clear Plexiglas platform square of 10 cm was kept submerged 0.5 cm below the surface of the water. The temperature of the water was always kept around 25 ±2°C during all procedures in the water maze.

Habituation trial in which mice were allowed to swim freely for 60 sec to acclimate to the procedure was conducted on Day 15 of drug administration. On the following day and for a total of 8 days, mice received training sessions of three trials daily. For

each trial, the starting position was randomly assigned between the four possible positions while the platform position was kept the same for the entire training sessions.

Each animal was allowed to swim until it found the immersed hidden platform or for a maximum duration of 60 sec. A mouse that failed to locate the platform in the first 60 sec would be gently guided to sit on the platform for a maximum duration of 30 sec. Upon a successful trial, the mouse would be left to sit on the platform for a maximum of 10 sec. Following conducting the eight acquisition sessions, probe trials for up to 60 sec on Day 1 and Day 11 following the last day of training were performed to assess the long-term memory retention. This was achieved by studying the preference of the mice to swim in the correct quadrant that previously contained the hidden platform. All trials including the swim paths and latencies to locate the platform were videotaped and tracked with a computerized video tracking system (ObjectScan, Clever Sys. Inc., Reston, VA, USA) and the resultant data were analyzed.

*2.3.2. Spontaneous alternations in the Y-maze*

The spontaneous alternation ratio, defined as the percentage of the number of arm entries, different from the previous two entries, divided by the total arm entries minus two was measured [22, 26]. Testing trials were conducted in a white Y-maze with arms that were 12” (long) by 3” (wide) with 8” height walls. Mice were placed into one of the Y-maze arms and were left to explore the maze freely for 5 minutes. After each trial, the maze was cleaned with 70% ethanol. Y-maze trials were

videotaped and tracked with a computerized video tracking system (ObjectScan, Clever Sys. Inc., Reston, VA, USA) and the data were analyzed.

### *2.4. Tissue preparation*

Following 34 days of daily administration of tolfenamic acid and on Day 35, homozygous R1.40 mice were deeply anesthetized with an intraperitoneal injection of 0.1 ml/10g of xylazine-ketamine mixture (100 mg/ml-10 mg/ml) and were perfused transcardially with 100 ml of perfusion wash that consisted of 0.8% sodium chloride, 0.8% sucrose, 0.4% dextrose, 0.034% anhydrous sodium cacodylate and 0.023% calcium chloride. After that, mice were perfused with 100 ml of perfusion fix that consisted of 4% paraformaldehyde, 4% sucrose and 1.07% anhydrous sodium cacodylate and the brains were removed. The extracted brains were post-fixed in the perfusion fix solution overnight and then they were cryopreserved in 30% sucrose solution. Fixed brains were subject to coronal sectioning (40 µm) and collection using MultiBrain® Technology (NeuroScience Associates, Knoxville, TN, USA) and sections were stored in preservative fix at -20°C. MultiBrain® Technology allows brains from different groups to be embedded together in the solid matrix and to be processed as a single unit. That enabled treatment of different brain sections under the same conditions providing more built-in quality control leading to obtaining uniform staining. For the wild type group, on Day 35, the mice were anesthetized with carbon dioxide, euthanized and their brains were extracted and stored at -80°C.

*2.5. Immunohistochemistry*

To identify and quantify amyloid plaques in brain sections from different groups, free-floating sections were taken from the preservative solution and were washed with distilled water. That was followed by washing with 1X phosphate buffer saline (PBS) three times, three min each. After that, sections were immersed in a solution of 3% hydrogen peroxide ($H_2O_2$) for 30 min to quench the endogenous peroxidase activity. Sections were rinsed three times with PBS for three min each and then incubated in 70% formic acid solution for 30 min at room temperature. The sections were washed with PBS three times, three min each and were incubated in 3% bovine serum albumin (BSA) and 0.1% Triton X-100 for 30 min. After rinsing with PBS, sections were Incubated with 1□ antibody against Aβ (6E10, Sig-39320, Covance, Dedham, MA, USA) of 1:200 dilution overnight at 4°C. That was followed by incubation with the appropriate species-specific biotinylated secondary antibody (Vector Labs, Burlingame, CA, USA) of 1:500 dilution for 30 min and then the sections were incubated with horseradish peroxidase HRP-conjugated streptavidin (Vector Labs, Burlingame, CA, USA) for 30 min. After that, sections were washed with PBS and the immunoreactivity was detected and visualized with the substrate 3-3, diaminobenzidine (DAB) (Vector Labs, Burlingame, CA, USA). Brain sections were counterstained with hematoxylin. The sections were mounted on microscope slides according to procedure provided by NeuroScience Associates and coverslips were mounted with permanent mounting medium (Vector Labs, Burlingame, CA, USA).

### 2.6. *Quantification of Aβ plaques*

Evaluation of the extracellular Aβ plaque load in the cerebral cortex was performed using a Nikon Eclipse E600 microscope (Nikon, Melville, NY, USA) attached with a Diagnostic Instruments digital camera and using SPOT Diagnostic Instruments software (Diagnostic Instruments, Sterling Heights, MI, USA). Serial images of 10X magnification were captured on four sections per animal that were 80 µm apart from each other. Using ImageJ software from NIH (Bethesda, MD, USA), the images were binarized to 16-bit grey scale and were segmented to distinguish the aggregates from background. The minimum and maximum size was set to exclude objects in the image that were clearly not of interest and data were collected including size, number and area of the plaques. Amyloid burden was expressed as the percent area stained positive for Aβ to the total area analyzed.

### 2.7. *Statistical analysis*

The significance of difference between different treatment groups was determined by repeated measure analysis of variance (ANOVA) and Tukey-Kramer multiple comparison post-hoc test. Data are expressed as the mean ± the standard error of the mean (SEM). All statistical analyses were conducted using GraphPad InStat 3 software (GraphPad Software, La Jolla, CA, USA)and probability (*p*) value of $<0.05$ was considered acceptable for statistical significance.

## Results

*3.1. Treatment with tolfenamic acid attenuates the cognitive deficits in homozygous R1.40 mouse model*

We examined the effects of tolfenamic acid treatment of 5 and 50 mg/kg/day on learning and memory in groups of old homozygous R1.40 transgenic mice aging between 19-24 months. There was a significant effect of training as ANOVA analysis showed that the difference in escape latency between the first and the last day of training sessions was statistically significant ($F(7,152) = 7.293$, $p < 0.0001$). The results showed that in the Morris water maze, the difference in the escape latency during the training phase between different experimental groups was not statistically significant. However, the tolfenamic acid treated groups showed a trend of better performance than the control vehicle group through days 4-8 of the training sessions (Fig. 1A). Analysis of probe trials Day 1 and Day 11 indicated that while both tolfenamic acid treatment groups scored higher percentages in the correct quadrant than the control group in both probe trials, the difference was not statistically significant ($p \geq 0.05$) (Fig. 1B).

Nevertheless, results from repeated measures ANOVA analysis of the spontaneous alternation ratio in the Y-maze showed a significantly improved working memory function ($F(2,14) = 4.495$, $p = 0.0311$) (Fig. 2). The Tukey-Kramer HSD post-hoc test indicated a significant effect of 50 mg/kg/day tolfenamic acid treatment in improving spatial working memory (HSD = 3.702, $p < 0.05$), while the treatment with 5 mg/kg/day did not reach significance (HSD = 0.91, $p \geq 0.05$) (Fig. 2).

*3.2. Effects of tolfenamic acid treatment on spatial learning and memory in old wild type mice*

Several studies have reported age-related spatial memory impairments and thus we wanted to examine whether tolfenamic acid treatment could alter learning and memory in old C57BL/6 mice. Again, there was a significant effect of training as ANOVA analysis showed that the difference in escape latency between the first to the last daily training sessions was statistically significant ($F(7,264) = 8.858$, $p < 0.0001$) (Fig. 3A). Our results showed that in the Morris water maze task, the escape latency during the training phase of the different experimental groups was not statistically significant. However, repeated measures ANOVA analysis of probe trial Day 11 revealed a significant improvement in the spatial reference memory function ($F(2,29) = 4.811$, $p = 0.0291$) (Fig. 3B). The Tukey-Kramer HSD post-hoc test showed that mice group treated with 5 mg/kg/day tolfenamic acid dose had a significant improvement (HSD = 3.828, $p < 0.05$) while difference in percent time mice spent in the correct quadrant after treatment with 50 mg/kg/day versus the control group did not reach statistical significance (HSD = 2.632, $p \geq 0.5$) (Fig. 3B).

In the Y-maze, ANOVA analysis of spontaneous alternation ratio did not indicate a significant difference between different treatment groups ($F(2,30) = 0.451$, $p = 0.641$) (Fig. 4).

*3.3. Tolfenamic acid treatment significantly reduces Aβ amyloid deposition in aged homozygous R1.40 mice*

As described in the literature, the homozygous R1.40 mouse model develops extracellular Aβ plaques that start to be evident between the ages of 14 and 15 months [16, 17]. Tolfenamic acid treatment with both doses greatly reduced amyloid plaque pathology in multiple cerebral cortical regions such as the primary somatosensory cortex, parietal association cortex, auditory cortex and insular cortex, compared to non-treated mice (Fig. 5A). Quantitative immunohistochemistry analysis revealed that tolfenamic acid treatment for 34 days significantly lowered Aβ plaque deposition $F(2,14) = 6.685$, $p = 0.009$) (Fig. 5B). The Tukey-Kramer HSD post-hoc test indicated that 5 and 50 mg/kg/day tolfenamic acid doses lowered amyloid deposition significantly (HSD = 3.779, $p < 0.05$ and HSD = 5.012 $p < 0.01$, respectively) (Fig. 5B). Consistent with previously reported data describing the pathology and the behavioral deficits in homozygous R1.40 [22], there was no correlation between the extent of Aβ deposition and the time spent in the correct quadrant during the probe trials conducted in Day 1 and Day 11 following the training sessions (Day 1 retention, $r(17) = 0.049$, $p > 0.1$; Day 11 retention, $r(17) = -0.113$, $p > 0.1$). However, there was a significant correlation between the plaque burden and the spontaneous alternation ratio ($r(17) = -0.482$, $p = 0.042$).

## 3. Discussion

Certain NSAIDs have been shown to alter AD pathology and attenuate learning and memory deficits in murine mouse models of AD by mechanisms other than their

classic cyclooxygenase (COX) inhibition pathway and have led researchers to consider such NSAIDs as potential disease-modifying agents for AD [27-29]. Tolfenamic acid, of all other NSAIDs, has the unique mechanism of inducing the proteasome-dependent degradation of SP transcription factors including SP1, SP3 and SP4 and thereby, decreasing the expression of the vascular endothelial growth factors (VEGF), which is involved in tumor progression and metastases [11]. Elevated levels of Sp1 have been found in the frontal cortex of the brains of AD patients as well as in the brains of transgenic mouse models of AD [12, 13]. Interplay between inflammatory processes and Sp1-driven gene expression has been suggested, as the elevation of Sp1 was accompanied by an increase in COX-2 expression, an Sp1 target gene [12].

Sp1 induces the expression of genes involved in AD pathology which include APP, BACE1 and tau as their promoter regions are rich with 5'-GGGGCGGGGC- sequence that Sp1 binds to [12, 30, 31]. Thus, we hypothesized and we showed that tolfenamic acid could interrupt the *de novo* synthesis of APP and alter the downstream levels of Aβ in C57BL/6 mice [14] and hemizygous R1.40 transgenics leading to improvements in learning and memory profiles.

As a genomic-based transgenic mouse model of AD, R1.40 line represents a valuable tool for assessing the effects of potential AD therapeutics in reducing amyloidogenic pathology and in improving learning and memory profiles. At 14-15 months of age, homozygous R1.40 mice exhibit depositions of extracellular Aβ plaques accompanied by cognitive deficits [16, 17, 22]. The mice demonstrate the ability to learn the

location of the hidden platform during daily training session trials in the Morris water maze; however, they exhibit a decline in long-term memory retention compared to non-transgenic mice as assessed by probe trials. In addition, when these mice were assessed for the spontaneous alternation ratio in the Y-maze, impairments in the working memory functions were observed [22].

The present study provides the first evidence of the ability of a relatively short-term tolfenamic acid treatment to reduce Aβ plaque burden drastically in homozygous R1.40 compared to longer treatments for 6-9 months in other transgenic models with another NSAID, namely ibuprofen [32, 33]. It was also observed that tolfenamic acid treatment results in a greater lowering of Aβ peptide levels in wild type C57BL/6 and hemizygous R1.40 mice compared to the reduction of APP ([14]; unpublished data), indicating that additional mechanisms responsible for plaque degradation and clearance of Aβ are involved.

We also examined the ability of tolfenamic acid treatment to attenuate the spatial memory deficits observed in old homozygous R1.40 mice aging between 19-24 months, which have been suffering from Aβ plaque deposition reported to start as early as 14 months of age [21]. We found that both doses of 5 and 50 mg/kg/day resulted in a trend of shorter latency during daily Morris water maze training trials and higher percentage in the correct quadrant during both probe trials compared to the vehicle treated group. However, those differences from the vehicle treated group did not reach statistical significance.

We exposed animals to tolfenamic acid at senescence whilst the combined effect of age and the mutant transgene persisted. Thus, lowering the amyloidogenic pathology may not have been sufficient enough to retrieve enough spatial reference memory capabilities to perform significantly well in the Morris water maze compared to the non-treated group. At the onset of tolfenamic acid exposure, the extensive plaque pathology in different cortical regions has been in effect for a long period of time. It is well known that Aβ plaques are toxic, affect synaptic plasticity, and stimulate the production of reactive oxygen species (ROS) leading to elevations in the oxidative stress and neuronal cell death. It is possible that the damage by Aβ deposition on memory networks was extensive and irreversible by the time treatment started, even though tolfenamic acid treatment reduced the plaque burden significantly.

Alternatively, there may be a lesser connection between spatial memory functions and the plaque burden. The previous explanation is supported by the fact that tolfenamic acid significantly improved mice performance in the spontaneous alternations in the Y-maze. The spontaneous alternations task is mainly dependent on the hippocampus and the prefrontal cortex [34], and as described in the literature, R1.40 mice only show scattered Aβ plaque deposition in hippocampal formation [16, 22] which is in consistency with our observations (data not shown).

In aged wild type C57BL/6 mice, short-term tolfenamic acid administration was able to improve certain spatial mnemonic deficits. Data analysis of Morris water maze probe trial in Day 11 shows that tolfenamic acid significantly improved spatial reference memory while there was no effect in improving the spatial working memory

assessed by spontaneous alternations in the Y-maze. Wild type mice do not develop amyloid plaque pathology, however, tolfenamic acid reduces APP and Aβ levels in C57BL/6 mice [14]. It is not clear if the improved spatial reference memory was due to the reduction of Aβ levels or due to tolfenamic acid's effects on other pathways mediated by COX inhibition or by non-COX effects.

We have found that Aβ levels in hippocampus were lowered by tolfenamic acid [14], however, spontaneous alternation ratio was not altered by tolfenamic acid treatment. It is possible that non-amyloidogenic processes that are not altered by tolfenamic acid treatment are contributing strongly to the age-related spatial working memory impairments in C57BL/6 mice. For example, those deficits were explained as due to alterations in the synaptic plasticity within the hippocampal formation [35] that were attributed to changes in the functional properties of NMDA receptors and changes in calcium homeostasis [36-38]. Fordyce and Wehner suggested that reduction in the activity membrane-bound protein kinase C (PKC) in the hippocampus was associated with the age-related decline in spatial learning and memory [39]. In addition, the increase in reactive oxygen species (ROS) and brain oxidative stress is another mechanism involved in age-related behavioral deficits [40, 41]. Furthermore, it has been reported that hippocampal glucose levels depletion contributes to memory dysfunction, and that systemic glucose administration reverses the age-dependent decrease in the spontaneous alternation ratio [42, 43].

Consistent with our prior findings in hemizygous R1.40 mice, the present study indicates that tolfenamic acid is effective in lowering the amyloidogenic proteins and

Aβ plaques with both doses, with dose-dependent improvements in cognitive deficits. We still have to study the effects of extended period of tolfenamic acid exposure with low doses as it may result in significant improvements in cognitive functions. It is essential to mention that compared to other FDA approved AD drugs and NSAIDs, tolfenamic acid significantly decreased plaque pathology and produced significant behavioral improvements in a relatively short duration of treatment. For example, 0.58 mg/kg/day donepezil, an FDA-approved anticholinesterase drug for AD, improved performance in the Morris water maze after two months of treatment in APP23 transgenic mice [44]. Another FDA-approved drug for use in moderate to severe AD, memantine, improved cognition in 3x TG-AD mice after three months of treatment [45]. In addition, ibuprofen was able to produce improvement in cognitive functions in triple transgenic mice (APPswe, PS1M146V and tauP301L) following its administration for six months [46].

In conclusion, we report that tolfenamic acid treatment drastically reduces Aβ plaque pathology that is accompanied by improvement in spatial learning and memory in a murine mouse model of AD. This occurs within a period that is much shorter than that observed with other NSAIDs. Our data suggest that tolfenamic acid treatment can also combat certain age-related mnemonic deficits in wild type mice. The data provide further evidence of the ability of tolfenamic acid, through Sp1 reduction, to disrupt the development and the progression of the pathological processes of AD. Thus, tolfenamic acid, as a repurposed AD drug, could be a promising disease-modifying therapeutic agent acting through an alternative mechanism.

**Acknowledgements**

This work was supported by the grants ES13022, AG027246, 1R56ES015867-01A1, and 1R01ES015867-01A2 from the National Institutes of Health awarded to N.H.Z. The research was made possible by the use of the RI-INBRE Research Core Facility, supported jointly by NCRR/NIH Grant # P20 RR016457 and the network institutions. Special thanks from the authors to Dr. Karen E. Stevens from University of Colorado Denver, College of Medicine, Department of Psychiatry for her assistance in the behavioral experiments conducted in this study.

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**Figure III-1. Effects of tolfenamic acid on spatial reference memory in homozygous R1.40 mice.** Tolfenamic acid was administered by oral gavage daily for 34 days and behavioral testing was conducted in the Morris water maze. See the methods section for details. **A)** Acquisition patterns during daily training trials for the 5 mg/kg/day, 50 mg/kg/day and vehicle groups; **B)** Probe trials assessing the long-term memory retention on Days 1 and 11 following acquisition training trials. Data were analyzed by ANOVA with Tukey-Kramer post-hoc test to compare all pairs of columns, obtained using GraphPad InStat 3 software. Vehicle n=7; 5 mg/kg/day n=7; 50 mg/kg/day n=6.

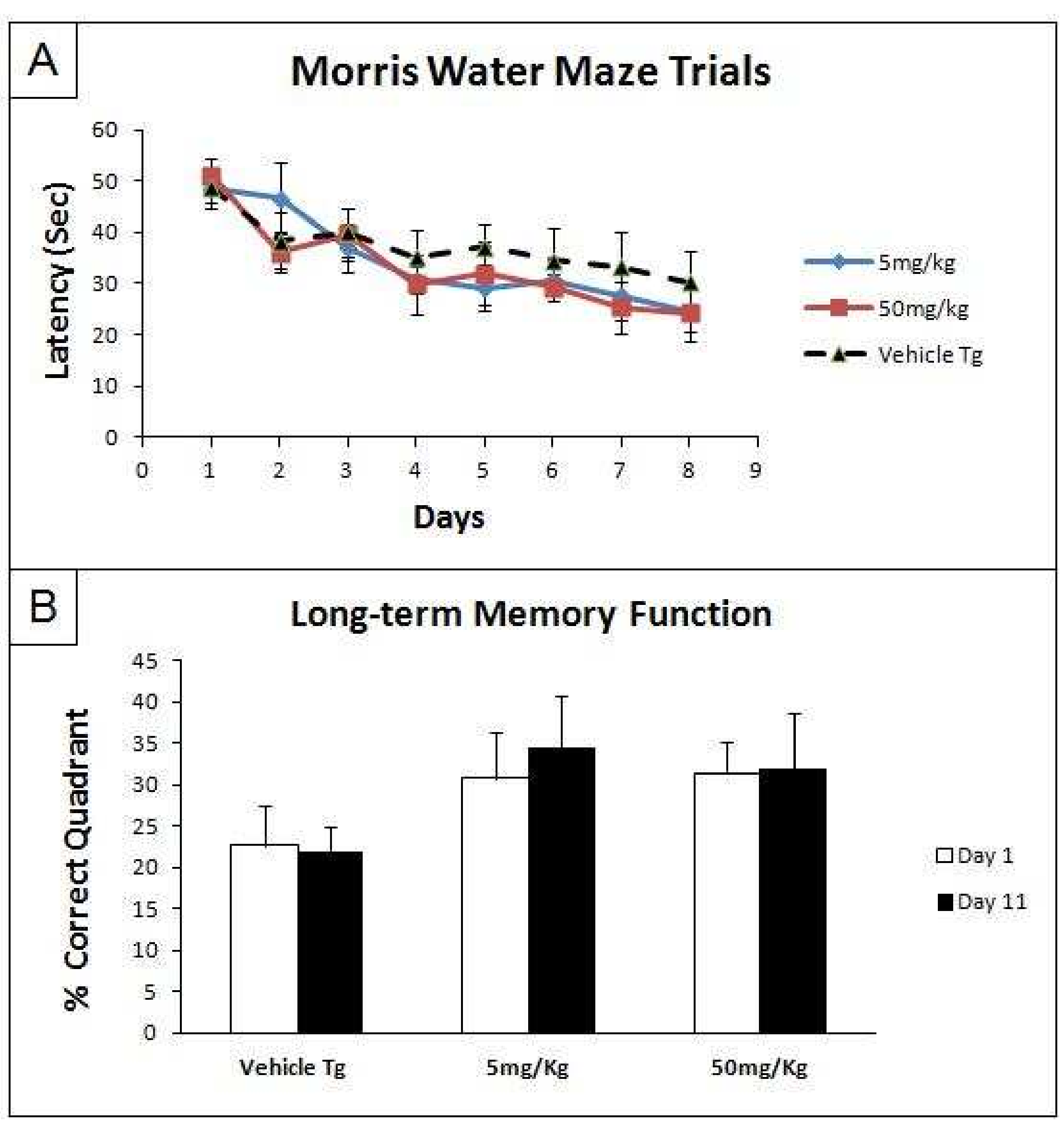

A
Morris Water Maze Trials
Latency (Sec)
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
9
Days
5mg/kg
50mg/kg
Vehicle Tg
B
Long-term Memory Function
% Correct Quadrant
45
40
35
30
25
20
15
10
5
0
Vehicle Tg
5mg/Kg
50mg/Kg
Day 1
Day 11

**Figure III-2. Working memory improvement in homozygous R1.40 transgenic mice after administration of tolfenamic acid.** Tolfenamic acid was administered by oral gavage daily for 34 days. Working memory was assessed by spontaneous alternations in the Y-maze. See the methods section for details. “*” indicates that values are significantly different from control vehicle, as determined by ANOVA analysis with Tukey-Kramer post-hoc test to compare all pairs of columns ($p<0.05$), obtained using GraphPad InStat 3 software. Vehicle n=7; 5 mg/kg/day n=7; 50 mg/kg/day n=6.

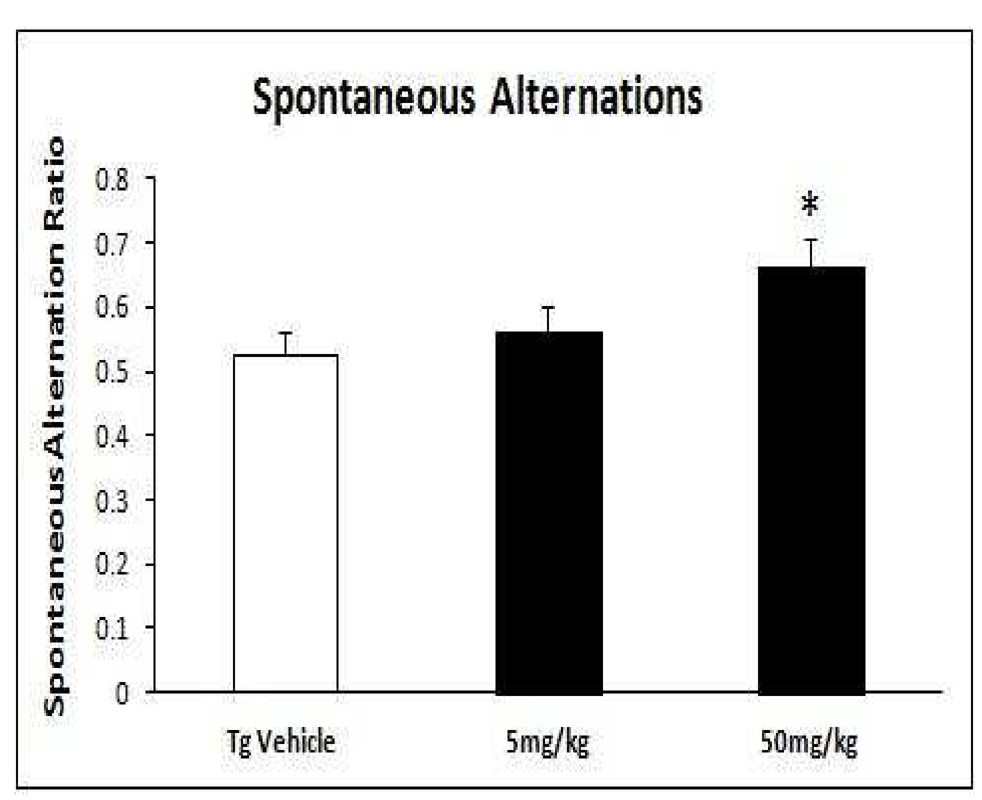

Spontaneous Alternations
Spontaneous Alternation Ratio
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
Tg Vehicle
5mg/kg
50mg/kg

**Figure III-3. Administration of tolfenamic acid to old wild type mice alters spatial reference memory.** Tolfenamic acid was administered by oral gavage daily for 34 days. Spatial reference memory was tested in the Morris water maze. See the methods section for details. **A)** Acquisition patterns during daily training trials for the 5 mg/kg/day, 50 mg/kg/day and vehicle groups. **B)** Probe trials assessing the long-term memory retention on Days 1 and 11 following acquisition training trials. "*" indicates that values are significantly different from control, as determined by ANOVA analysis with Tukey-Kramer post-hoc test to compare all pairs of columns ($p<0.05$), obtained using GraphPad InStat 3 software. Vehicle n=13; 5 mg/kg/day n=11; 50 mg/kg/day n=10.

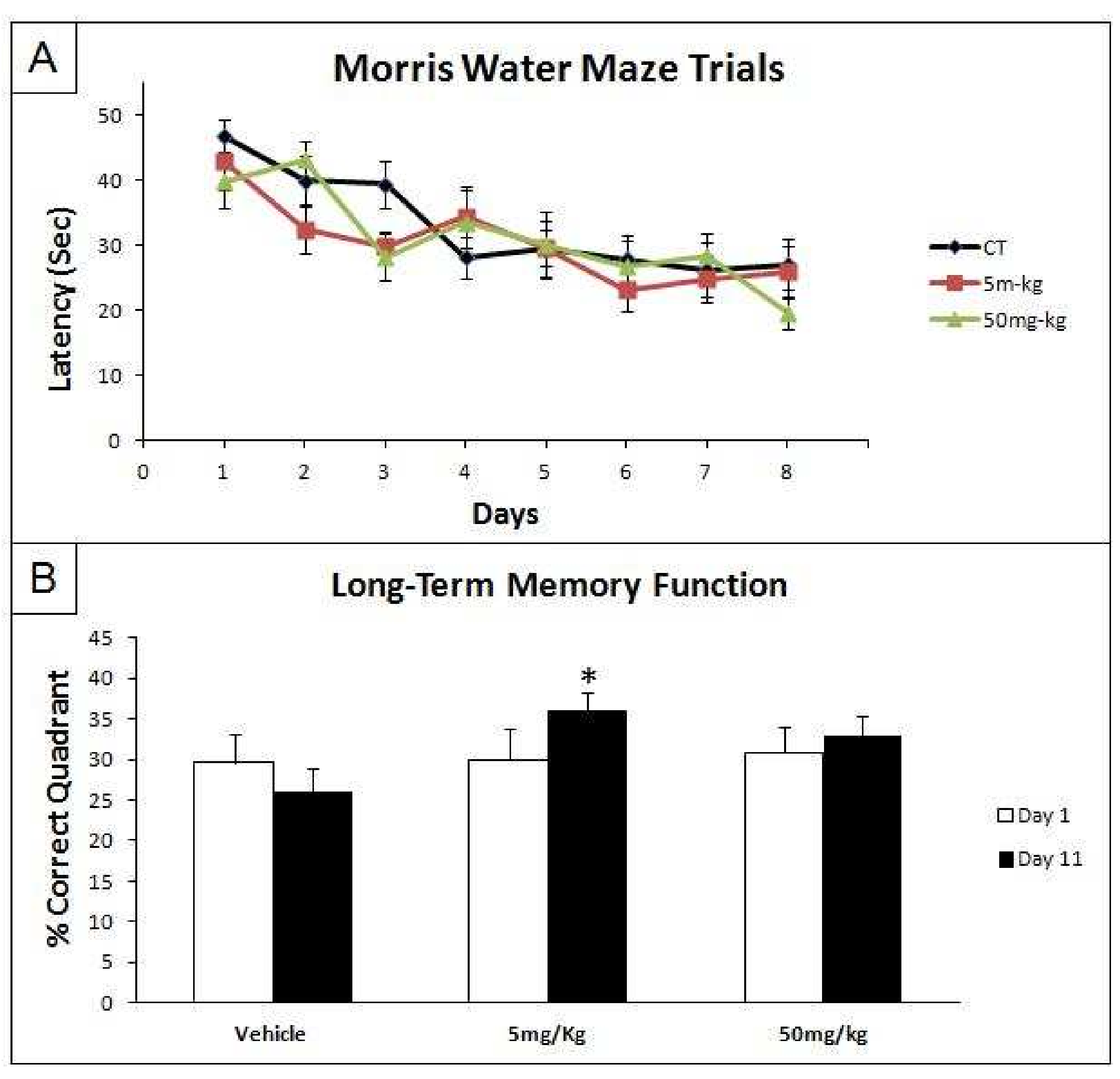

A
Morris Water Maze Trials
Latency (Sec)
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
Days
CT
5m-kg
50mg-kg
B
Long-Term Memory Function
% Correct Quadrant
45
40
35
30
25
20
15
10
5
0
*
Vehicle
5mg/Kg
50mg/kg
Day 1
Day 11

**Figure III-4. Performance of old wild type mice in the Y-maze following administration of tolfenamic acid.** Tolfenamic acid was administered by oral gavage daily for 34 days. See the methods section for details. Vehicle n=13; 5 mg/kg/day n=11; 50 mg/kg/day n=10.

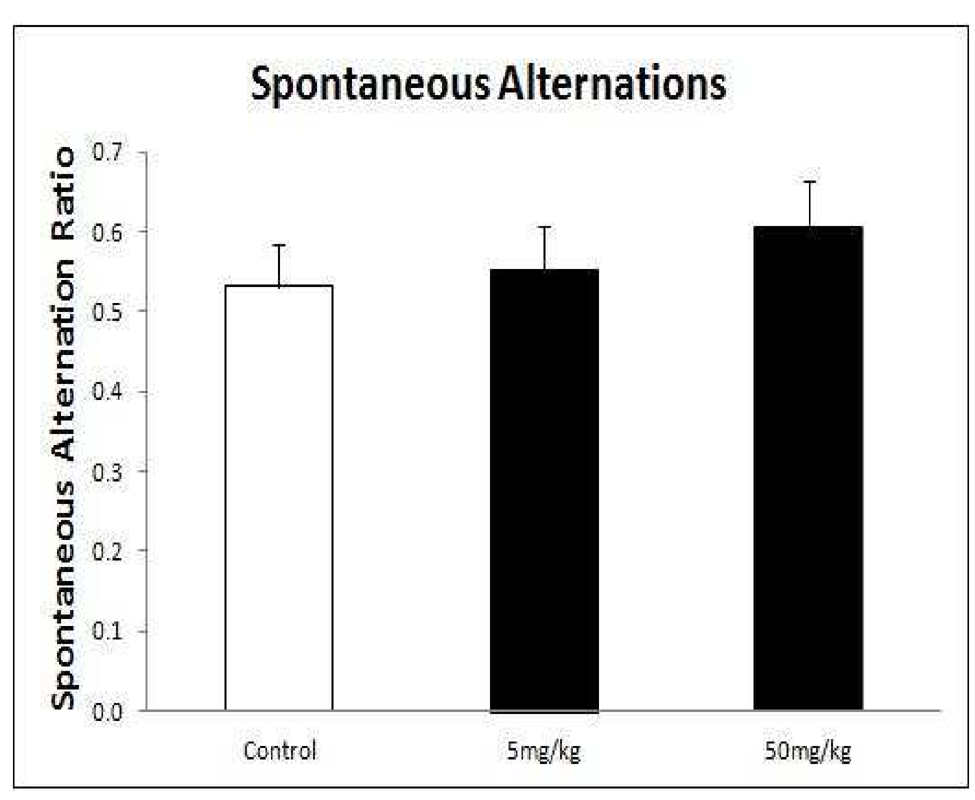
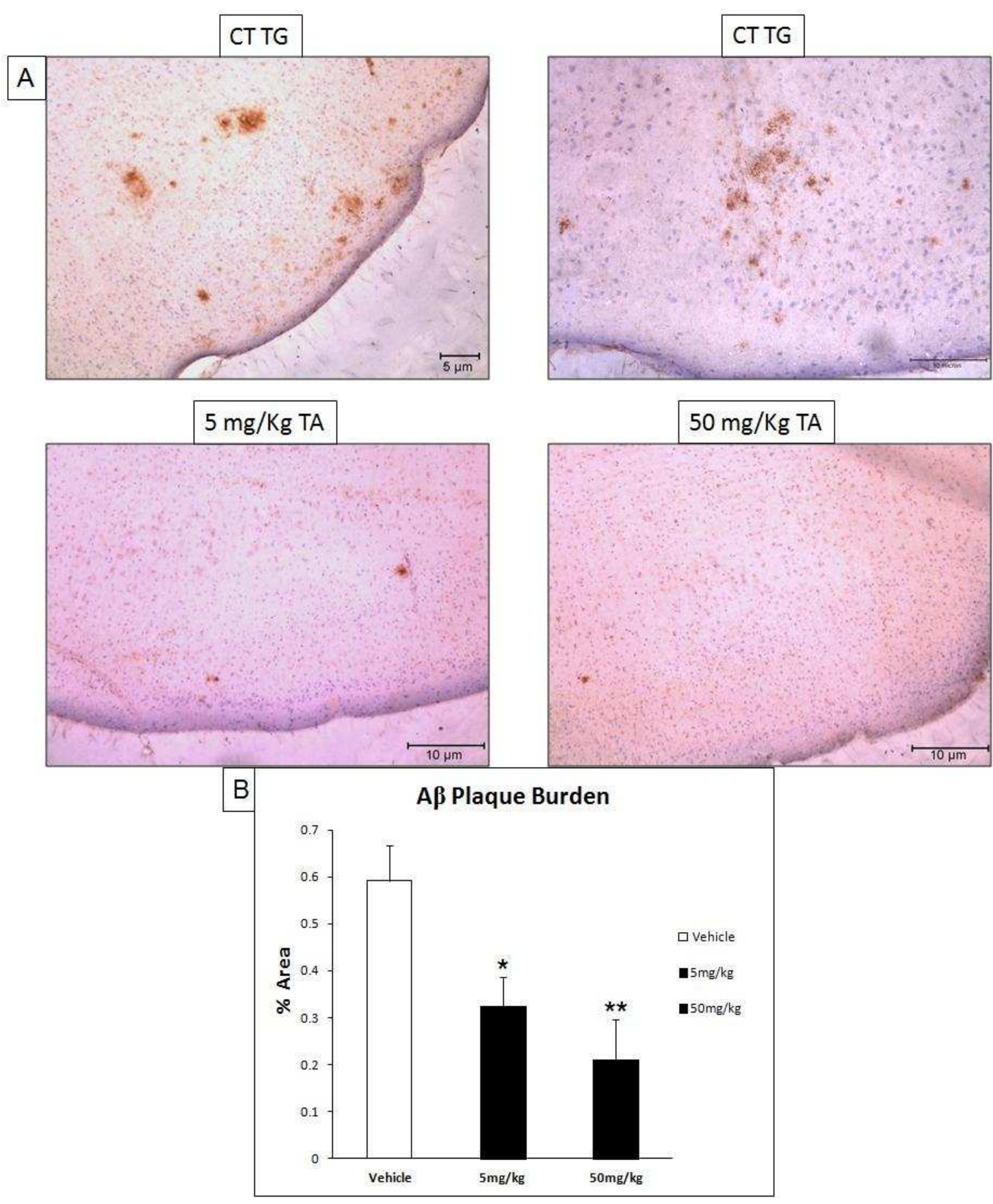

Spontaneous Alternations
Spontaneous Alternation Ratio
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Control
5mg/kg
50mg/kg

**Figure III-5. Treatment with tolfenamic acid reduces cortical Aβ plaque burden in homozygous R1.40 mice.** Tolfenamic acid was administered by oral gavage daily for 34 days. See the methods section for details. **A)** Represents images of Aβ plaque morphology and density in the cerebral cortex in different treatment groups after immunohistochemical staining against Aβ using 6E10 antibody. CT TG = control transgenic **B)** Quantification of total Aβ plaque area. "*" indicates that values are significantly different from control, as determined by ANOVA analysis with Tukey-Kramer post-hoc test to compare all pairs of columns (*$p<0.05$, **$p<0.01$), obtained using GraphPad InStat 3 software. Vehicle n=5; 5 mg/kg/day n=7; 50 mg/kg/day n=5.


CT TG
A
5 μm
CT TG
5 mg/Kg TA
10 μm
50 mg/Kg TA
10 μm
B
Aβ Plaque Burden
% Area
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Vehicle
5mg/kg
50mg/kg
*
**
Vehicle
5mg/kg
50mg/kg