2008

Tolfenamic Acid Interrupts the De Novo Synthesis of Plaque-Forming Amyloid Beta Peptides: Implications for Alzheimers Disease

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TOLFENAMIC ACID INTERRUPTS THE DE NOVO SYNTHESIS OF PLAQUE-FORMING AMYLOID BETA PEPTIDES: IMPLICATIONS FOR ALZHEIMER'S DISEASE

BY

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE

REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

PHARMACOLOGY AND TOXICOLOGY

UNIVERSITY OF RHODE ISLAND

2008

MASTER OF SCIENCE THESIS

OF

LINA I. ADWAN

APPROVED:

Thesis Committee:

Major Professor _____

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DEAN OF THE GRADUATE SCHOOL

UNIVERSITY OF RHODE ISLAND

2008

ABSTRACT

Alzheimer's disease (AD) distresses the lives of millions of people around the world. Its costs were estimated to be at least 100 billion dollars each year (Fillit, 2000). And so far, no cure has been found to stop or slow the progression of the disease. Senile plaques and neurofibrillary tangles are considered to be central in the pathogenesis of AD. The plaques are mainly composed of aggregations of amyloid β (Aβ) peptides, which are generated upon the sequential cleavage of the larger amyloid precursor protein (APP) first by the enzyme β-secretase, and then by the enzyme γ-secretase. These plaques are one of the main targets being explored for newer therapeutic interventions in AD. The transcription factor specificity protein 1 (Sp1) is associated with the pathology of AD, as it was found to be involved in the transcription of APP and beta site APP cleaving enzyme 1 (BACE1) (Christensen et al., 2004, Basha et al., 2005, Santpere et al., 2006). Tolfenamic acid is an NSAID that was reported to lower Sp1 levels (Abdelrahim et al., 2006). Accordingly, this study was designed to examine the capability of tolfenamic acid to decrease the levels of the transcription factor Sp1, and therefore, other AD involved proteins and peptides like APP and Aβ within animals' brains. Consequently, the drug was administered to C57BL/6 mice and Hartley guinea pigs. Different doses of the drug were tried to find the dosage range where efficacy is achieved. Animals also were sacrificed at different time points, in order to study the drug effects over the time course of the study. Data showed that tolfenamic acid was able to lower SP1, APP and Aβ in the brains of animal models in a time dependent manner, and within certain doses. These

studies demonstrate that tolfenamic acid is a potential candidate for further development as a treatment for AD.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my advisor, Dr. Nasser Zawia, who accepted me into his laboratory, and gave me this opportunity along with all the support, knowledge, and guidance I needed to work on this project. I want to extend my appreciation to my colleagues at Dr. Zawia's lab, and our collaborators, who have greatly contributed to this study at many occasions. I also want to thank the Fulbright Foreign Student Scholarship program for generously funding my study and stay in the United States throughout the past two years and a half. And finally, I am extremely grateful to my family and my friends, for their precious help and support.

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1.0 Introduction

1.1 Background and significance

Alzheimer's disease (AD) is a progressive neurodegenerative disorder marked by dementia evolving till death. During the course of the disease, memory, cognitive performance, and other daily activities are all impaired. In 2000, the number of AD cases in the US was about 4.5 million (Hebert et al., 2003), this number has reached 5.2 million in 2008 (Alzheimer's Association, 2008), it was estimated it would grow up to 13.2 million in 2050 (Hebert et al., 2003). In addition, Brookmeyer et al. (2007) predicted that that the number of AD cases around the world grow from 26.6 million in 2006 to 106.8 million in 2050. Annual AD-associated costs were estimated to be at least 100 billion dollars solely in the US (Fillit, 2000). So far, no cure was found for AD, and all available medications are prescribed for symptomatic or adjuvant therapy.

The characteristic neuropathological deposits identified in AD are the extracellular Amyloid beta (Aβ) plaques and intracellular tau tangles (Terry et al., 1964; Thal et al., 2004; Goedert and Spillantini, 2006). Alois Alzheimer described those neuritic plaques and neurofibrillary tangles more than a hundred years ago (Goedert and Spillantini, 2006). Since then, numerous studies have explored these plaques and tangles (Terry et al., 1964; Evin and Weidemann, 2002; Thal et al., 2004; Goedert and Spillantini, 2006). Under the electron microscope, these

deposits display abnormal filaments (Terry et al., 1964). A recent study confirmed these findings, as extensive Aβ depositions were found in the neocortex and caudate nuclei of AD patients, and these deposits were more abundant in AD than in healthy controls; dementia with Lewy bodies; frontotemporal dementia; and mild cognitive impairment (Rowe et al., 2007).

The main component of AD plaques is the Aβ peptide. It is 39-43 amino acids long, and is generated by cleavage of the amyloid precursor protein (APP) (Haass et al., 1991; Urbanc et al., 1999). This amyloidogenic pathway involves cleavage of APP by the enzyme β-secretase, followed by γ-secretase. The produced Aβ is normally secreted, but also can accumulate resulting in formation of insoluble aggregates (Shoji et al., 1992; Urbanc et al., 1999). Two forms of Aβ can be generated, $A\beta_{40}$ and $A\beta_{42}$, the difference between the two is not only in the number of amino acids in their composition, but also $A\beta_{42}$ was found to be more aggregative, and it was proposed that $A\beta_{42}$ triggers plaque formation in AD (Näslund et al., 2000). Furthermore, Shoji et al. (1992) suggest that formation of the insoluble Aβ depositions depends on the rate of Aβ production and the rate of its elimination. Accordingly, 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 (Selkoe, 1993; Hardy and Selkoe, 2002). On the other hand, APP also can be processed by a non-amyloidogenic pathway, where it is cleaved within the Aβ

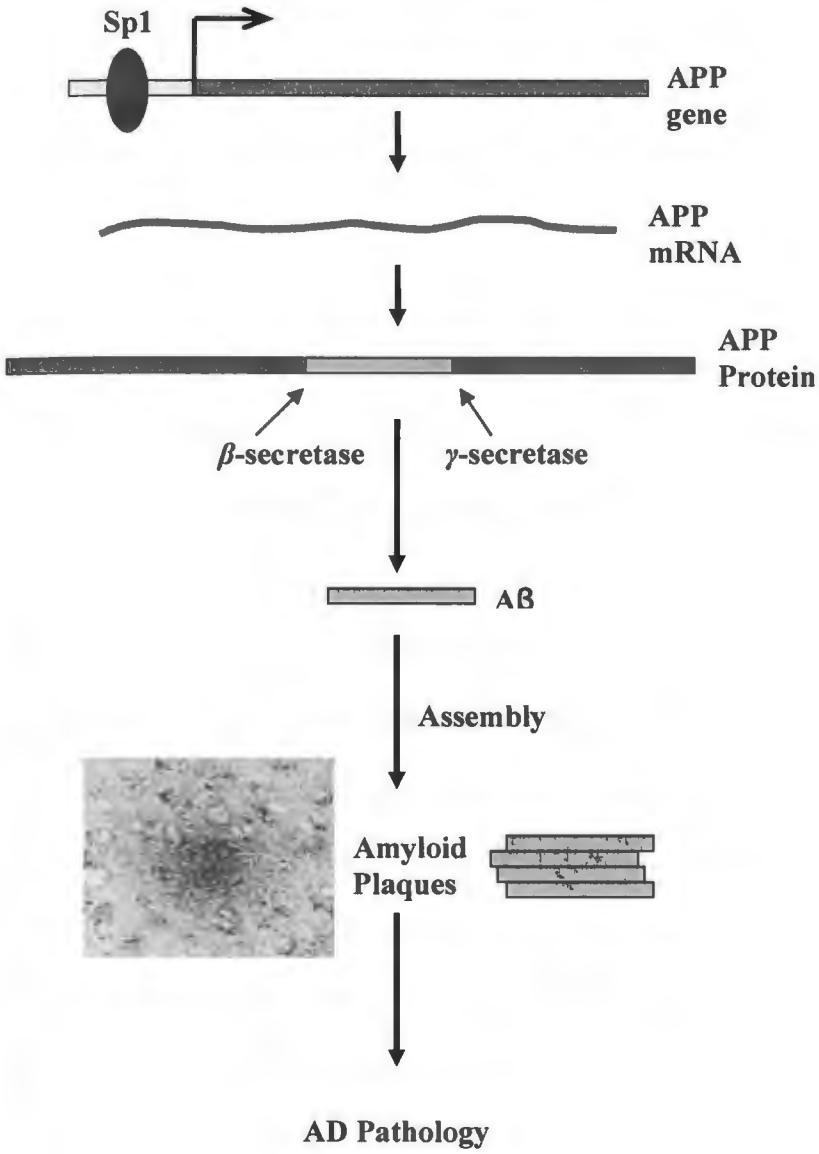
region, resulting in secreted products that do not form deposits (Shoji et al., 1992). This occurs following APP cleavage by the enzyme α-secretase.

The specificity protein 1 (Sp1) is a transcription factor involved in the pathology of AD (Zawia and Basha, 2005; Basha et al., 2005; Santpere et al., 2006). Sp1 acts as a co-activator in the process of the transcription of APP (Docagne et al., 2004). Previous studies have shown that the APP promoter includes several Sp1 binding sites (Lukiw et al., 1994; Hoffman and Chernak, 1995; Querfurth et al., 1999; Zawia and Basha, 2005). Furthermore, Sp1 greatly regulates the expression of beta site APP cleaving enzyme 1 (BACE1) (Christensen et al., 2004). BACE1 is the main form of β-secretase that cleaves APP to generate Aβ (Cai et al., 2001). Christensen et al. (2004) found that overexpression of SP1 increases BACE1 promoter activity, while the decline in Sp1 protein reduces transcription of the BACE1 gene. Likewise, silencing the Sp1 gene dramatically reduces APP promoter activity (Basha et al., 2005). In addition, in vivo immunohistochemical results have shown that Sp1, APP, and Aβ co-localize in brain neurons (Brock et al., 2008). In addition, Sp1 regulates the expression of phosphorylated tau (Heicklen-Klein and Ginzburg, 2000). The buildup of hyper-phosphorylated tau results in the formation of the pathogenic tangles found in AD and some other neurodegenerative disorders like Pick's disease (Santpere et al., 2006).

Early-onset familial AD (FAD) compromises 5-10% of all AD cases. The majority of FAD is related to mutations on genes encoding the presenilin (PS)

membrane proteins, PS-1, PS-2 (Czech et al., 2000). Presenilin mutations increase production of $A\beta_{42}$ from APP, and increased $A\beta_{42}$ levels were observed in the blood and brains of FAD patients with presenilin abnormalities (Czech et al., 2000). In addition, neurons lacking the PS-1 gene fail to produce Aβ peptides (De Strooper et al., 1998; Naruse et al., 1998). Consequently, PS-1 was found to be related to the activity of the enzyme γ-secretase (De Strooper et al., 1998; Shimojo et al., 2007). Aβ and its aggregates also were found to cause synaptic dysfunction and loss (Mattson, 1997; Takahashi, 2002; Fein et al., 2008). Collectively, these findings confirm the involvement of Aβ in the pathology of AD. The role of Sp1, APP and Aβ in plaque formation and AD is illustrated in Fig. 1.

Figure 1. A proposed transcriptional pathway for brain amyloid plaque formation. Sp1 binds to the promoter region of the APP gene, resulting in enhancement of APP transcription and tarnslation. APP is then cleaved first by the enzyme β-secretase, and then by γ-secretase to produce Aβ, which can aggregate forming the amyloid plaques that contribute to the pathology of AD.


Sp1
APP
gene
APP
mRNA
APP
Protein
β-secretase
γ-secretase
Aß
Assembly
Amyloid
Plaques
AD Pathology

To date, no drug has been found to stop the progression of AD. All available medications are prescribed as symptomatic or adjuvant therapy. FDA approved drugs for AD include acetylcholinesterase inhibitors and the *N*-methyl-D-aspatarte (NMDA) receptor antagonist memantine. The rational for using cholinesterase inhibitors in AD is based on the cholinergic hypothesis, in which the decline in cholinergic function with age was proposed to contribute to the cognitive dysfunction in AD (Bartus et al., 1982). Accordingly, cholinesterase inhibitors like donepezil, rivastigmine, and galantamine are currently prescribed for mild to moderate AD, and have produced improvement in disease symptoms (Francis et al., 1999). The other drug approved for treating AD is memantine, an NMDA receptor antagonist that blocks the excitotoxicity effects of glutamine, and is used in moderate to severe cases of AD (Reisberg et al., 2003). Improved AD symptoms were observed with memantine administration (Möbius and Stöffler, 2003; Reisberg et al., 2003). Additionally, antipsychotics and antidepressants are sometimes prescribed to manage corresponding ailments that can arise in AD patients (Lesser and Hughes, 2006). Non-prescription medications also are used for their anti-oxidant properties like the vitamins C and E, however, their use in AD is controversial (Boothby and Doering, 2005).

Considering the large and increasing number of AD cases and the devastating course of the disease, there is a great need for developing drugs that target critical pathologic mechanisms in AD. Continuing research revealing additional insights into the pathogenesis and molecular mechanisms behind AD has created more

hope for disease modifying therapies, rather than merely symptomatic treatment. New mechanisms are under investigation for the design of new drugs for AD. Many approaches targeting the pathological plaques of AD are being examined, particularly those exploring different factors in the production, secretion, and degradation of Aβ peptides (Roberson and Mucke, 2006).

Epidemiological studies have demonstrated that chronic exposure to non-steroidal anti-inflammatory drugs (NSAIDs) has a positive effect on AD. NSAIDs exert their anti-inflammatory effects by blocking the cyclooxygenase (COX) mediated production of prostaglandins. Various mechanisms have been suggested for NSAIDs' role in AD. According to a meta-analysis of different studies on NSAIDs in AD, it was found that NSAIDs exposure reduced the risk for AD development (Szekely et al., 2004). However, these effects were not consistent among all NSAIDs, and in all studies. It was proposed that NSAIDs decrease BACE1 levels as they activate the peroxisome proliferator-activated receptor-γ (PPARγ) and that would be how they exert their protective effect in AD (Sastre et al., 2006). Other studies suggested that NSAIDs act by affecting γ-secretase activity, resulting in the decrease of $A\beta_{42}$ production, apart from their COX inhibition activity (Weggen et al., 2001; Eriksen et al., 2003).

Tolfenamic acid is an NSAID currently used to treat migraine symptoms in humans in Europe, and for pain management in veterinary medicine in the US. Recently, tolfenamic acid also was found to decrease the levels of transcription

factors Sp1, Sp3, and Sp4 in pancreatic tumors, and thus inhibit their growth (Abdelrahim et al., 2006). Since Sp1 is associated with the pathology of AD (Basha et al., 2005; Santpere et al., 2006), particularly through APP transcription coactivation (Docagne et al., 2004), and regulating BACE1 expression (Christensen et al., 2004), this study was designed to examine the potential of tolfenamic acid as a new therapeutic intervention for AD.

1.2 Objectives

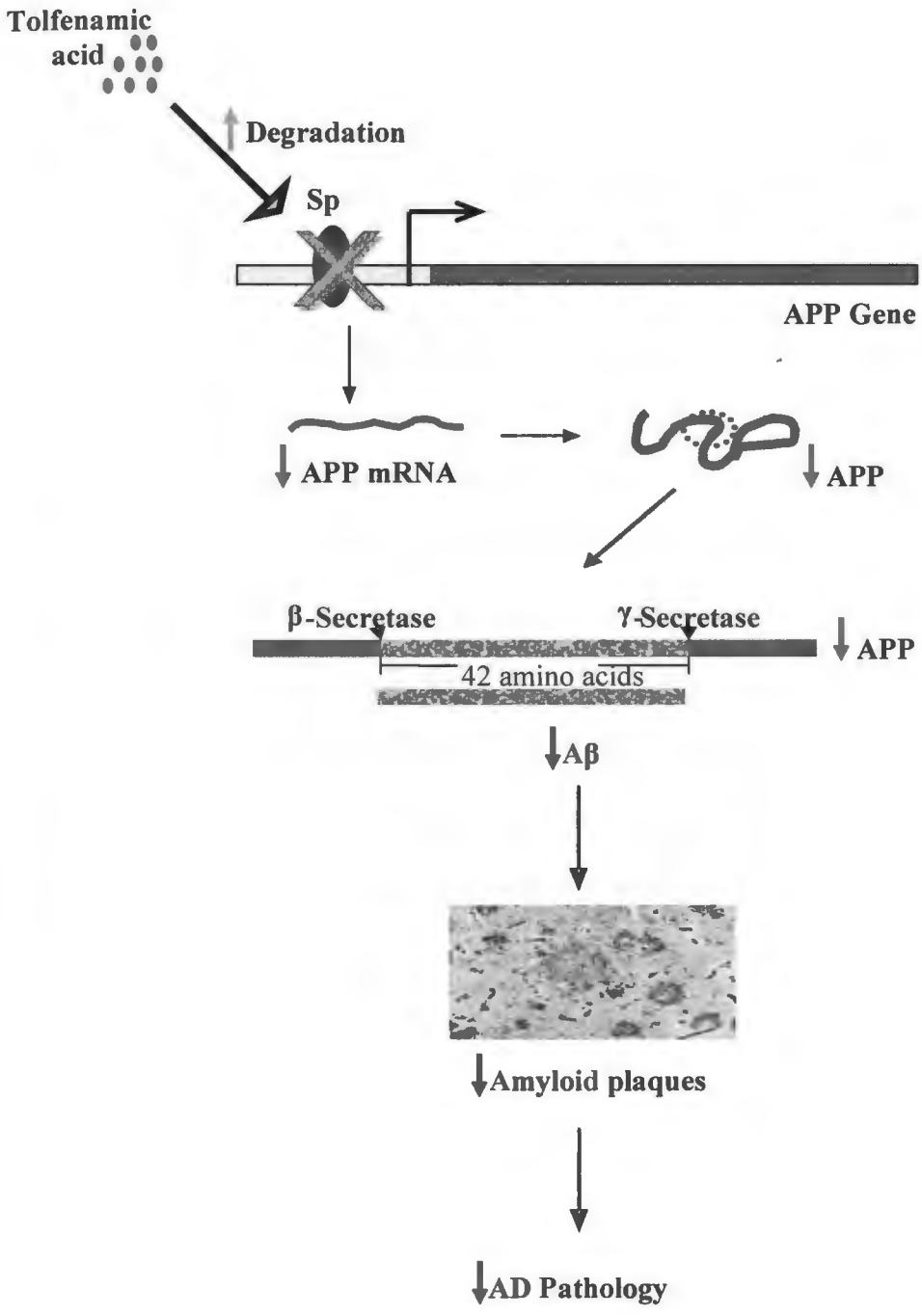
Previous studies from our lab have shown that the transcription factor Sp1 is associated with the pathology of AD. Increased APP mRNA expression later in life following prior lead exposure early in life was coupled with increased Sp1 activity (Basha et al., 2005, Wu et al. 2008). Furthermore, recently published in vivo immunohistochemical results have confirmed the formerly established link between Sp1 and APP, and have proposed that Aβ is more likely to be found in cerebral cortex and hippocampal areas with higher Sp1 levels (Brock et al., 2008). This study was designed to examine Sp1 as a target involved in a possible therapeutic intervention of Alzheimer's disease. Tolfenamic acid is an NSAID found to lower Sp1 levels, and subsequently inhibit the growth and angiogenesis of pancreatic tumors (Abdelrahim et al, 2006). Accordingly, this research work was conducted to investigate the ability of tolfenamic acid to lower AD associated proteins like APP and Aβ through its action on Sp1, as illustrated in Fig. 2.

Specific aims

1. Examine the ability of tolfenamic acid to reduce APP and Aβ protein levels in the brains of wild type animal models exposed to the drug.

2. Test the ability of tolfenamic acid to decrease levels of the transcription factor Sp1 within the brains of animal models dosed with tolfenamic acid.

Figure 2. Proposed down-regulation of the APP gene by tolfenamic acid. Tolfenamic acid promotes the degradation of the transcription factor Sp1. Sp1 decline results in a decrease in APP transcription, and thus lowers APP and Aβ levels. This would be expected to diminish amyloid plaque pathology.


Tolfenamic acid
Degradation
Sp
APP Gene
APP mRNA
APP
β-Secretase
γ-Secretase
APP
42 amino acids
Aβ
Amyloid plaques
AD Pathology

1.3 Experimental design

C57BL/6 mice were exposed to different doses of tolfenamic acid or vehicle in order to examine whether the drug can affect the following proteins: APP, SP1, and Aβ in comparison to vehicle. At first, a pilot study was conducted using a few doses of tolfenamic acid in a small number of animals. The study was repeated with a wide but safe selection of doses of tolfenamic acid, in order to examine the dose range where the drug was affecting AD related proteins. Moreover, animals were sacrificed at different time points during the study, in order to examine the changes in those proteins over time. In addition, tolfenamic acid also was tested in Hartley guinea pigs, as they have been suggested to represent a better animal model for studying pathological changes related to AD, since their Aβ peptides are identical to the ones found in humans (Beck et al., 2003). Following each study, the brains of these animals were tested for changes in the levels of their APP, SP1, and Aβ peptides by the proper analytical techniques.

2.0 Materials and methods

Materials and instruments

Most chemicals and reagents were purchased form Thermo Fisher Scientific Inc. (Rockford, IL) unless otherwise noted. Tolfenamic acid was purchased from LKT Laboratories Inc. (St. Paul, MN). Micro BCA Protein Assay Kit and Super signal Ultra from Pierce (Rockford, IL). Nitrocellulose transfer membranes from Bio-Rad Laboratories (Hercules, CA). PVDF transfer membranes from GE Osmonics Labstore (Minnetonka, Mn). ECL Plus Western Blotting Detection Reagents, ImageQuant™ 5.2 software, and the Typhoon™ Variable Mode Imager from GE Healthcare Bio-Sciences Corporation. (Piscataway, NJ). KODAK Image Station 2000MM from Eastman Kodak (Wood Dale, IL). Primary antibodies against APP, actin, and GAPDH were from Sigma Chemical Co. (St. Louis, MO). Additionally, APP primary antibody was purchased from Millipore Corporation. (Billerica, MA). SP1 antibody was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Human Aβ (1-40) and (1-42) ELISA assay kits were from Immuno-Biological Laboratories (Gunma, Japan).

Animal Exposure

Mice (C57BL/6 strain) were either bred in-house or obtained from Charles River Laboratories (Shrewsbury, MA) or Jackson Laboratory (Bar Harbor, ME). Guinea

pigs (Hartley strain) were purchased from Charles River Laboratories (Shrewsbury, MA). Animals were housed in rooms designated for each species within the animal facility at URI.

Mice of the C57BL/6 strain were used as they have already been employed for AD research. Guinea pig Aβ is identical to that of humans (Beck et al., 2003; Anderson et al.,2005; Du et al., 2007), and like humans, guinea pigs were found to be one of the animal models that express neuritic plaques in vivo, while plaques can not be found in smaller animals like mice and rats (Beck et al., 2003). However, no plaques were seen within the brains of 10 guinea pigs with ages up to 10 years old (Rossner, personal communication, December 22, 2007). The fact that these animals express structures similar to neutritic plaques found in human brains was first cited in a German source that dates back to 1910, nevertheless, this book did not provide any images of those plaques (Previous source; Beck et al., 1997). Since then, numerous articles have mentioned that plaques are naturally present within the guinea pig brain, while a thorough literature review revealed no further information or images supporting such statements.

Animals were exposed to tolfenamic acid prepared in corn oil through gavage (oral dose) for 4-5 weeks. Animals were divided into groups of 2-6 animals in each, including a control group receiving corn oil; and experimental groups receiving either 1, 5, 10, 25, 50, or 150 mg/kg body weight doses of tolfenamic acid in corn oil. Cremophor was used as the solvent for the tolfenamic acid

formulation administered to guinea pigs, since the 50 mg/kg dose could not be achieved in a small volume of corn oil, as the animals were 10 times the weight of mice. The standard guidelines and the protocol approved by IACUC were followed for animal handling. Animals were euthanized by CO_2 asphyxiation in a specially designed canister for that purpose. They were left in the canister to be rendered unconscious and dead. The dead animals also were decapitated to eliminate any uncertainty. This method is consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Association. The brains of these animals were isolated, and the various regions; cerebral cortex, hippocampus, cerebellum and brain stem; were separated on dry ice and stored at -80°C. The effect of tolfenamic acid on APP and Aβ peptides was examined within the cerebral cortices of brain samples.

Accordingly, and due to study requirements, four separate and independent animal experiments were conducted. First, a small number of 2 months old male C57BL/6 mice were dosed every other day (i.e. 3 times a week), with either corn oil, 10, 50, or 150 mg/kg tolfenamic acid in corn oil for 5 weeks. In the following study, 1 month old male Hartley guinea pigs were dosed every other day with 50 mg/kg tolfenamic acid solution in cremophor or vehicle for 4 weeks. The third experiment conducted included dosing of female C57BL/6 mice with either 5, 10, or 25, mg/kg tolfenamic acid in corn oil, or vehicle every other day for four weeks. And finally tolfenamic acid was administered daily to male C57BL/6 mice in one of the following doses 0 (vehicle) 1, 5, 10, 25, or 50 mg/kg. All tolfenamic

acid formulations for the first three studies were kindly provided by Dr. Maen Abdelrahim (Anderson Cancer Center, Orlando Cancer Research Institute, Orlando, FL). Guinea pig brains were kindly donated by Dr. Thomas Mather (University of Rhode Island, Kingston, RI), to conduct Western blot analyses and ELISA assays necessary for early optimization of experimental conditions. Female C57BL/6 mice were kindly given by Dr. Angela Slitt (University of Rhode Island, Kingston, RI).

Protein extraction

Approximately 0.1 gm of cerebral cortex tissue was homogenized with 500 µl of the RIPA lysis buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 0.1 SDS, 1 mM EDTA). The homogenate was then centrifuged at 10,000 rpm for 10 minutes at 4°C, and the supernatant was collected. Protein concentration was determined using the Micro BCA Protein Assay Kit. Extracts were stored at -80°C until used.

Western Blot Analysis

Western blot analysis was performed to observe the changes in the levels of SP1, APP, and the housekeeping proteins actin and GAPDH among control and treatment tissues. The method mentioned in previous studies was followed (Atwood et al., 1998; Nunomura et al., 1999; Atkins et al., 2003). In brief,

samples were loaded onto 8% gels (5.35 H_2O, 2.5 ml of 1.5 M Tris-HCl, [pH 8.8], 100 µl of 10% SDS, 2 ml of 40% Acrylamide/Bis, 50 µl of 10% Amonium Persulfate, and 5 µl TEMED). The samples were then run for about 1-2 hours at 20-30 mA, transferred to nitrocellulose or PVDF membranes, fixed, and blocked with 5% non-fat milk overnight. After that, the membranes were incubated with the specific primary antibody for 1-2 hours at room temperature, then with the secondary antibody for 1 hour at room temperature, and finally with ECL plus reagent for 1 minute. The florescence of Western blot bands was detected using the Typhoon™ Variable Mode Imager. In some experiments, Super signal Ultra was used to detect chemilumescence where the membranes were incubated with the substrate for 3-5 minutes and the signal was detected using the KODAK Image Station 2000MM. Quantitative assay of the bands was performed using ImageQuant™ 5.2 software.

ELISA Aβ (1–40) and Aβ (1–42) Assay

The levels of Aβ were measured using human Aβ (1–40) and (1–42) assay kits. These kits are solid-phase sandwich ELISA with two kinds of highly specific antibodies, which are 100% cross reactive with mouse Aβ (1-40), and 70% cross reactive with mouse Aβ (1-42). The assay was conducted as mentioned within the manufacturer's instructions (Immuno-Biological Laboratories, Gunma, Japan) and Morishima-Kawashima et al. (2000) with minor modifications. 100 µg of protein samples in 100 µl of EIA buffer and assay standards were added to a 96-well

plates pre-coated with anti-human Aβ mouse IgG MoAb. The plates were incubated overnight at 4°C. Plates were washed using the 40X diluted wash buffer supplied with the kit (0.05% Tween20 in phosphate buffer) for 7 times, afterwards, 100 µL of labeled antibody were added to the samples and standards, and they were incubated for one hour at 4°C. The plates were washed again 9 times, and then 100 µL of tetramethylbenzidine were added as a coloring agent. The plates were incubated in the dark for 30 minutes at room temperature. Finally 100 µL of 1N H_2SO_4 were added to stop the reaction, and absorbance was measured at 450 nm using a UV/Vis Spectrometer. The concentration of Aβ in unknown samples was obtained as pg per mg protein after plotting the absorbance of standards against the standard concentrations.

Statistical Analysis

Data were represented as the mean ± the standard error of the mean (SEM). Statistical analysis was performed using two-tailed Student's *t*-test, and results marked with an asterisk were significantly different from the control group ($p<0.05$).

3.0 Results

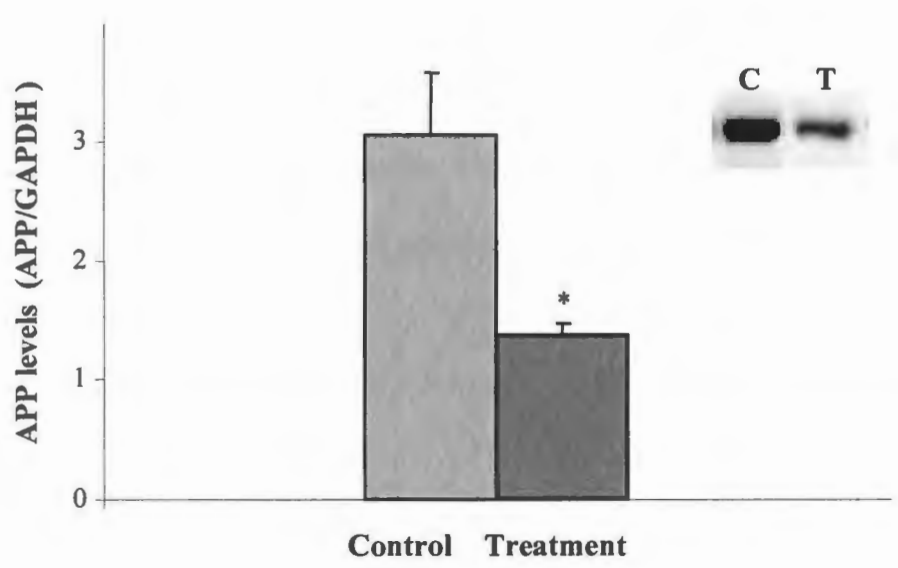
The transcription factor Sp1 is an essential factor within the pathology of AD (Christensen et al., 2004, Basha et al., 2005, Santpere et al., 2006). Tolfenamic acid was found to stimulate the degradation of Sp1 in pancreatic tumor cells (Abdelrahim et al., 2006). Accordingly, the effects of tolfenamic acid administration on proteins related to AD pathology were examined in animal models. Four studies were conducted to observe changes in APP and Aβ levels, proteins that are affiliated with the neurodegeneration in AD according to the amyloid hypothesis. Within these studies, different doses of tolfenamic acid were tested and compared to the vehicle controls. The dose range effects were examined in samples obtained from the animals' cerebral cortexes.

3.1 A pilot study in male C57BL/6 mice

Treatment with 10 and 50 mg/kg of tolfenamic acid every other day for five weeks significantly reduced APP protein levels in the cerebral cortex of male mice when compared to controls (student's *t* test $p < 0.05$) (Fig. 3A). SP1 protein levels were lower in 10 mg/kg treated animals as demonstrated by Western blot analysis (Fig.3B). Aβ (1-40) protein levels also were significantly reduced within the cerebral cortex of the same treatment groups in comparison to controls (student's *t* test $p < 0.05$) (Fig. 4).

Figure 3. APP and SP1 levels in mice treated with tolfenamic acid. Animals were two months of age at the beginning of the study. They were given 0, 10, or 50 mg/kg tolfenamic acid (n=3 in each dose group). Doses were administered three times a week for 5 weeks by oral gavage. (A) APP levels in cerebral cortex samples from control and tolfenamic acid treated animals. APP protein levels were expressed as a ratio of the housekeeping protein GAPDH as measured by Western blot analysis, described in the methods section. Values are shown as the mean ± SEM. Results for treatment animals (n=6) were pooled together as their values compared to each other were not statistically significant. The "*" indicates that values are significantly different from control, as determined by a student's *t*-test ($p<0.05$). (B) SP1 Western blot results of samples from mouse brain extracts from controls and animals treated every other day with 10 mg/kg tolfenamic acid for 5 weeks.

A


APP levels (APP/GAPDH)
3
2
1
0
*
Control
Treatment
C
T

B

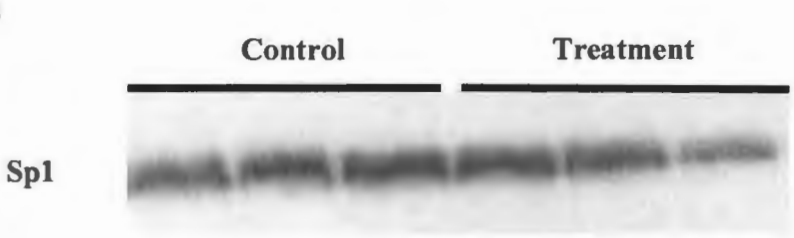
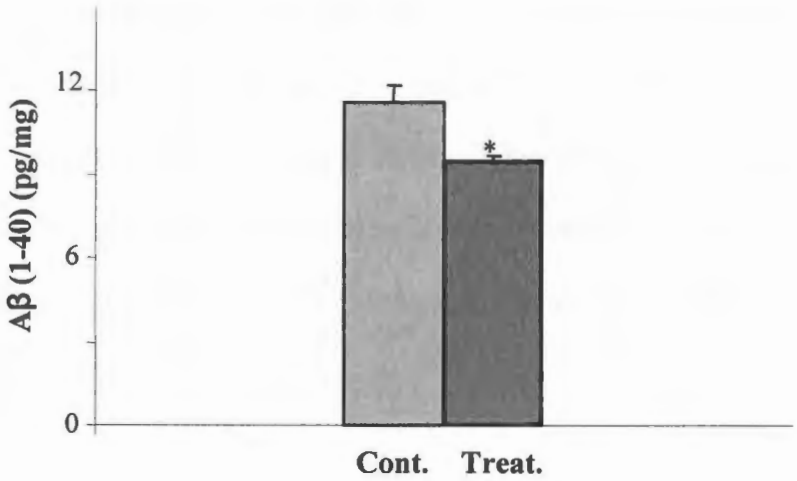

Control
Treatment
Sp1

Figure 4. Aβ (1-40) levels in male mice treated with tolfenamic acid. Animals were two months of age at the beginning of the study. They were given vehicle, 10, or 50 mg/kg tolfenamic acid (n=3 in each dose group). Doses were administered three times a week for 5 weeks by oral gavage. Aβ levels were measured in pg/mg protein using an ELISA assay as described in the methods section. Values shown are the mean ± SEM. Results for treatment animals (n=6) were pooled together as their values compared to each other were not statistically significant. The "*" indicates that values are significantly different from control, as determined by a student's *t*-test ($p<0.05$).


Aβ (1-40) (pg/mg)
12
6
0
*
Cont.
Treat.

3.2 A pilot study in male Hartley guinea pigs

In guinea pigs 50 mg/kg tolfenamic acid in cremophor decreased APP cerebral cortex levels by thirty percent in comparison to controls as determined by Western blot analysis, following 4 weeks of every other day dosing (n=3). This change was significant (p=0.01) according to student's t-test (Fig. 5). Aβ (1-42) displayed a lowering trend, but it was not statistically significant according to student's t-test (p= 0.23) (Fig. 6).

3.3 A study in female mice

After tolfenamic acid was administered to female mice, three times a week, for four weeks, cerebral cortex APP levels were significantly lowered with 10 and 25 mg/kg doses by 52.18% and 40.58% respectively (student's t-test $p<0.05$). APP also was lower within the cerebral cortex samples obtained from animals dosed with 5 mg/kg, however, this change was not statistically significant as determined by student's t-test (p=0.11) (Fig. 7).

On days 4 and 8, the APP/GAPDH ratio was not changed. However, on day 16 there was a clear downward trend (23%). By day 29, 10 mg/kg tolfenamic acid decreased APP levels by 52.18% as a percentage change from control, which was statistically significant, student's t test p =0.01 (Fig. 8).

Figure 5. Changes in APP levels in guinea pigs treated with tolfenamic acid. Animals' age was one month at the beginning of the study. They were given either vehicle or 50 mg/kg tolfenamic acid orally three times a week for 4 weeks. APP protein levels in cerebral cortex samples were expressed as a ratio of the housekeeping protein GAPDH as measured by Western blot analysis described in the methods section. Values shown are the mean ± SEM (n=3). The "*" indicates that the change is statistically significant, as determined by student's *t*-test (p=0.01).

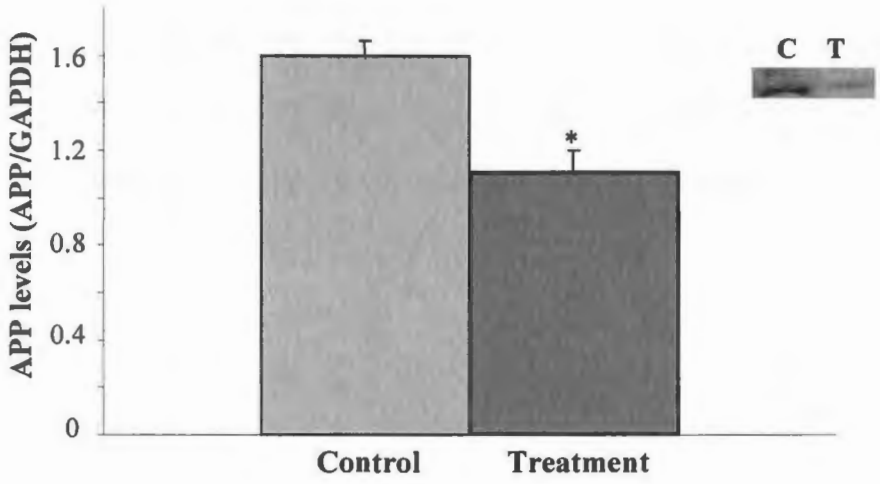

APP levels (APP/GAPDH)
1.6
1.2
0.8
0.4
0
*
Control
Treatment
C T

Figure 6. Aβ (1-42) levels in guinea pigs exposed to tolfenamic acid. Animals were two months old at the beginning of the study. They were dosed with either vehicle or 50 mg/kg tolfenamic acid orally three times a week for 4 weeks. Aβ (1-42) levels in cerebral cortex protein samples were measured as pg/mg protein by an ELISA assay as described in the methods section. Values shown are the mean ± SEM (n=4).

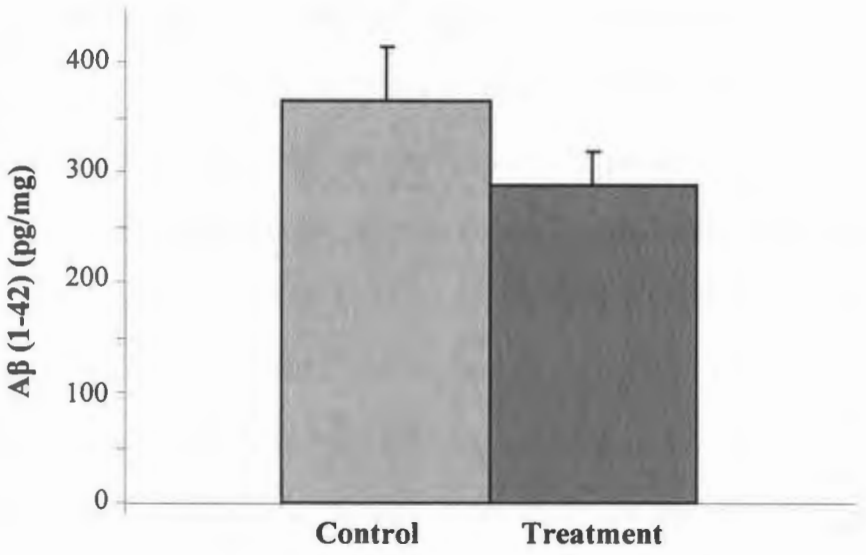

Aβ (1-42) (pg/mg)
400
300
200
100
0
Control
Treatment

Figure 7. APP levels in the cerebral cortex of female mice treated with various doses of tolfenamic acid. Animals' age was two-three months at the beginning of the study. Vehicle, 5, 10, or 25 mg/kg tolfenamic acid were administered three times a week by oral gavage for four weeks. APP protein levels in cerebral cortex samples were expressed as the ratio of the housekeeping protein GAPDH, as measured by Western blot analysis described in the methods section. Values shown are the mean ± SEM. The number of animals in each group was: control (n=2); 5 mg/kg (n=3); 10 mg/kg (n=5); and 25 mg/kg (n=2). The "*" indicates that values are significantly different from control, as determined by a student's *t*-test ($p<0.05$).

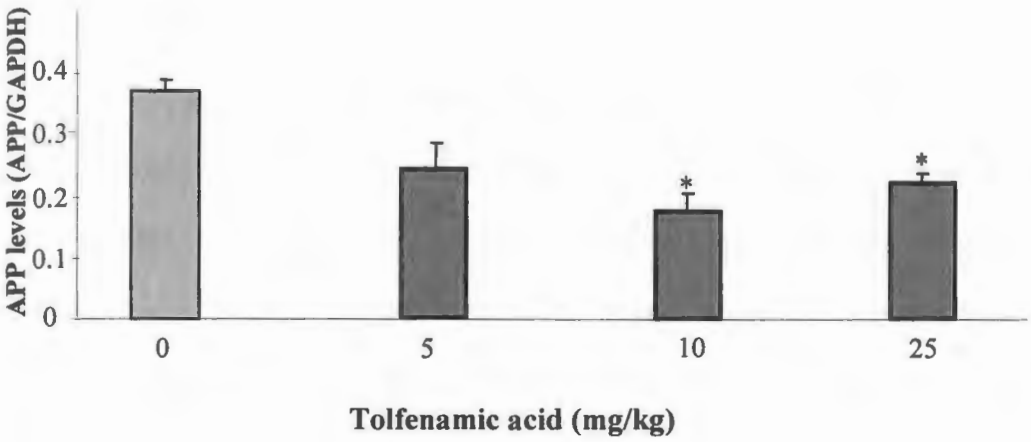
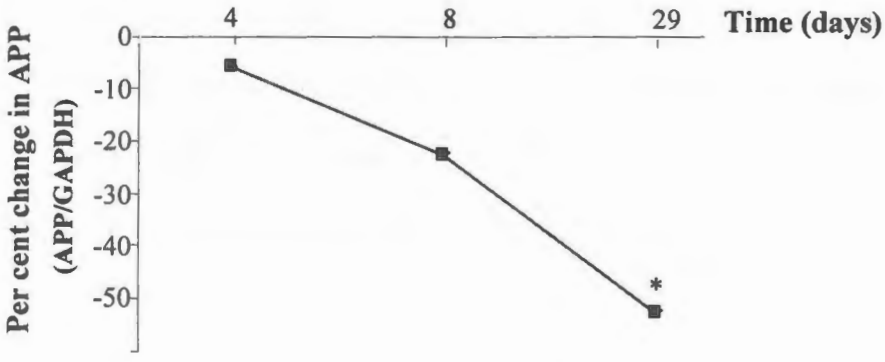

APP levels (APP/GAPDH)
0.4
0.3
0.2
0.1
0
*
*
0
5
10
25
Tolfenamic acid (mg/kg)

Figure 8. Temporal changes in APP levels in cerebral cortex of female mice treated with tolfenamic acid. Animals' age was two months at the beginning of the study. They were given either vehicle or 10 mg/kg tolfenamic acid three times a week by oral gavage. Control and treatment animals were sacrificed on day 4, 8, and 29 after the beginning of the study. Results are expressed as the percentage of APP/GAPDH change from each group's control as obtained by Western blot analysis described in the methods section. The number of animals in each group: day 4 control (n=2), treatment (n=4); day 8 control (n=2), treatment (n=4); day 29 control (n=2), treatment (n=5). The "*" indicates that values are significantly different from control, as determined by a student's *t*-test ($p<0.05$).


Per cent change in APP
(APP/GAPDH)
0
-10
-20
-30
-40
-50
4
8
29
Time (days)
*

3.4 A time and dose range finding study

To determine the range of effective doses, tolfenamic acid was administered daily at the following concentrations: 1, 5, 10, 25, and 50 mg/kg. It was observed that cerebral cortex APP levels were significantly lowered (student's *t*-test p=0.04) by 18.52% with the 5 mg/kg dose. The remaining four doses resulted in no change (Fig. 9).

Moreover, changes in APP with the 5 mg/kg treatment were examined at different time points. On days 4 and 8, there were non-significant fluctuations in APP levels. However, there was as a nearly significant 20% lowering trend (p=0.13) of APP levels 8 days following daily tolfenamic acid administration. On day 16, tolfenamic acid significantly decreased APP levels by 18.52%, student's *t* test p=0.04 (Fig. 10).

Changes in SP1 levels also varied in response to different doses of tolfenamic acid (Fig. 11). SP1 protein was significantly less than control in the cerebral cortex of animals treated daily with 5 and 10 mg/kg tolfenamic acid, with the percentages of change from control being 57.65%, and 30.13% respectively (Student's *t*-test p=0.02, p=0.05).

Changes in levels of Aβ (1-40) at day 16 following daily doses of 1, 5, 25, 50 mg/kg tolfenamic acid compared to controls are shown in Fig. 12. Treatment

animals' Aβ (1-40) was lower than controls by the following percentages; 24.32% with the 1 mg/kg dose (p=0.01); 14.27% with 5 mg/kg (p=0.06); 25.29% with 25 mg/kg (p=0.02); and 21.61% with 50 mg/kg (p=0.05). Aβ (1-42) changes can be examined from Fig. 13, Aβ (1-42) levels were significantly lower than controls by the following percentages; 32.81% with the 1 mg/kg dose (p=0.05) 68.2% with 5 mg/kg (p=0.02); 32.96% with 25 mg/kg (p=0.02); and 29.45% with 50 mg/kg (p=0.0004).

The overall changes in SP1, APP, and Aβ following daily dosing with different doses of tolfenamic acid for 15 days are summarized in Table 1. Accordingly, higher doses of tolfenamic acid and a dose as low as 1 mg/kg administered daily did not alter SP1 (Fig. 11), but still had a lowering effect on Aβ (Fig. 12, Fig. 13).

The changes occurring in Aβ (1-40) during the time course of the 5 mg/kg daily treatment are demonstrated in Fig. 14. Only on day 16 post treatment, Aβ (1-40) levels dropped by 14.06% (p=0.06). The changes in Aβ (1-42) were examined following daily 5 mg/kg administration of tolfenamic acid for 15 days (Fig. 15). Exclusively on day 16 a statistically significant change could be observed with -68.2% percentage change in Aβ (1-42) (p=0.02).

Figure 9. APP levels in the cerebral cortex of mice treated with various doses of tolfenamic acid. Animals' age was 3 months at the beginning of the study. Animals were dosed with 0, 1, 5, 10, 25, or 50 mg/kg tolfenamic acid by oral gavage daily for 15 days. APP levels detected by Western blot are shown as a ratio of the levels of the housekeeping protein actin. Values shown are the mean ± SEM. The number of animals in each group: control n=2; 1 mg/kg n=3; 5 mg/kg n=3; 10 mg/kg n=4; 25 mg/kg n=4; 50 mg/kg n=3. The "*" indicates that values are significantly different from controls, as determined by a student's *t*-test ($p<0.05$).

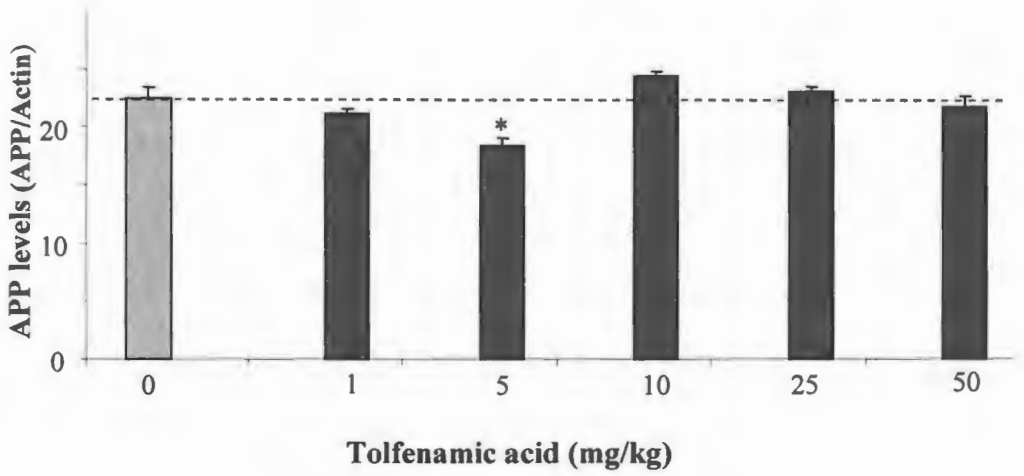

APP levels (APP/Actin)
0
10
20
*
0
1
5
10
25
50
Tolfenamic acid (mg/kg)

Figure 10. Temporal changes in APP levels in the cerebral cortex of mice treated with tolfenamic acid. Animals' age was 3 months at the beginning of the study. Vehicle or 5 mg/kg tolfenamic acid were administered daily, and animals were sacrificed at days 4, 8, and 16 following commencement of the treatment. Results are expressed as the percentage of APP/actin change from each group's controls as obtained by the Western blot analysis described in the methods section. The number of animals in each group, day 4 control n=4, treatment n=5; day 8 control n=3, treatment n=3; day 16 control n=2, treatment n=3. The "*" indicates that values are significantly different from control, as determined by a student's *t*-test ($p<0.05$).

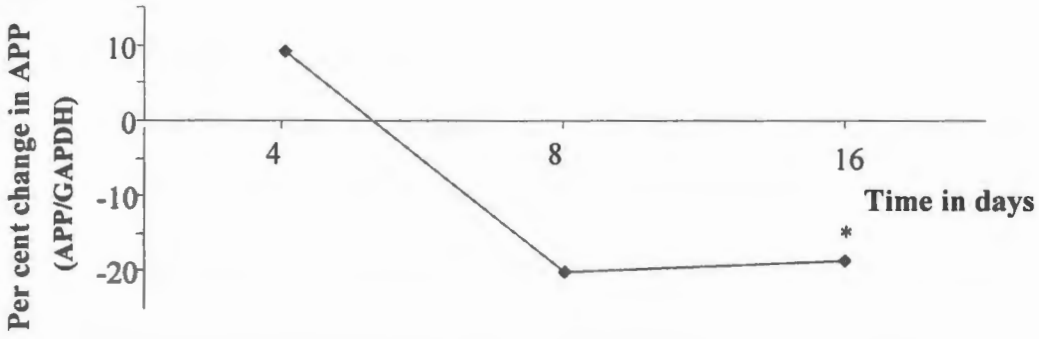

Per cent change in APP
(APP/GAPDH)
10
0
-10
-20
4
8
16
Time in days
*

Figure 11. SP1 levels in the cerebral cortex of mice treated with different doses of tolfenamic acid. Animals' age was 3 months at the beginning of the study. They were treated with 0, 1, 5, 10, 25, or 50 mg/kg tolfenamic acid daily by oral gavage for 15 days. SP1 protein levels were expressed as a ratio of the housekeeping protein actin measured by Western blot analysis, described in the methods section. Values shown are the mean ± SEM. The number of animals in each group: control n=2; 1 mg/kg n=4; 5 mg/kg n=3; 10 mg/kg n=4; 25 mg/kg n=5; 50 mg/kg n=5. The "*" indicates that values are significantly different from control, as determined by a student's *t*-test ($p<0.05$).

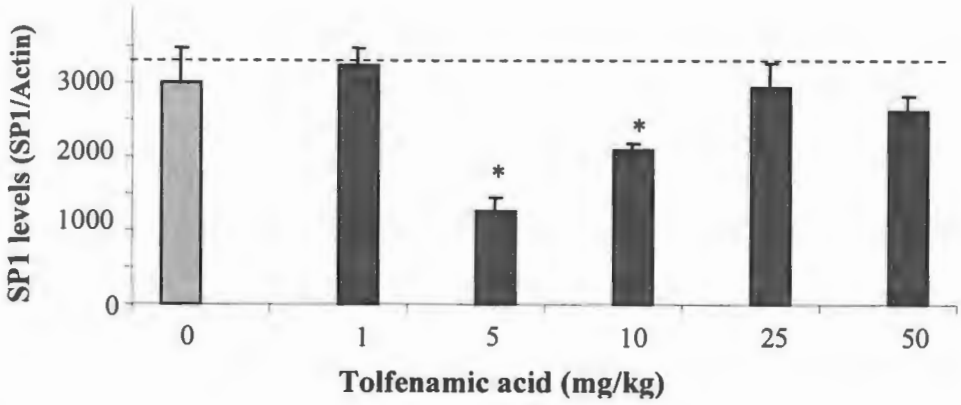

SP1 levels (SP1/Actin)
3000
2000
1000
0
*
*
0
1
5
10
25
50
Tolfenamic acid (mg/kg)

Figure 12. Aβ (1-40) levels in the cerebral cortex of mice treated with various doses of tolfenamic acid. Animals were 3 months at the beginning of the study. They were given one of the following doses of tolfenamic acid 0, 1, 5, 25, 50 mg/kg. The drug was administered daily by oral gavage for 15 days. Aβ (1-40) levels were measured by an ELISA assay according to the procedures illustrated in the methods section. Results shown are the mean ± SEM. The number of animals in each group, control n=3; 1mg/kg n=4; 5 mg/kg n=4; 25 mg/kg n=5; and 50 mg/kg n=4. The "*" indicates that values are significantly different from control, as determined by a student's *t*-test ($p<0.05$).

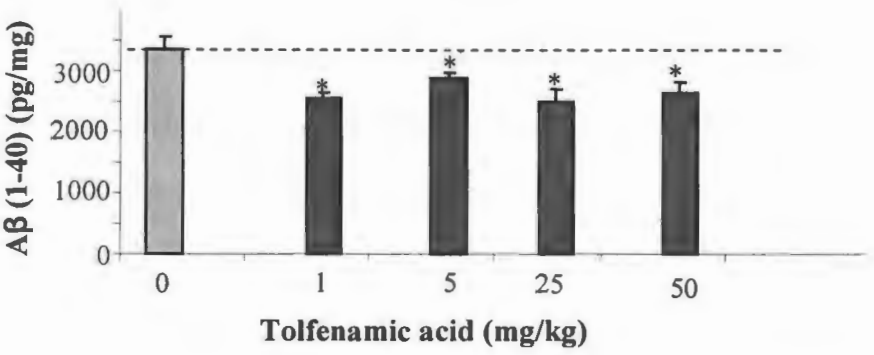

Aβ (1-40) (pg/mg)
3000
2000
1000
0
0
1
5
25
50
Tolfenamic acid (mg/kg)

Figure 13. Aβ (1-42) levels in the cerebral cortex of mice treated with various doses of tolfenamic acid. Animals were 3 months at the beginning of the study. They were given one of the following doses of tolfenamic acid 0, 1, 5, 25, 50 mg/kg. The drug was administered daily by oral gavage for 15 days. Aβ (1-42) levels were measured by an ELISA assay according to the procedures illustrated in the methods section. Values shown are the mean ± SEM. The number of animals in each group: control n=3; 1mg/kg n=4; 5 mg/kg n=3; 25 mg/kg n=4; and 50 mg/kg n=5. The "*" indicates that values are significantly different from control, as determined by a student's *t*-test ($p<0.05$).

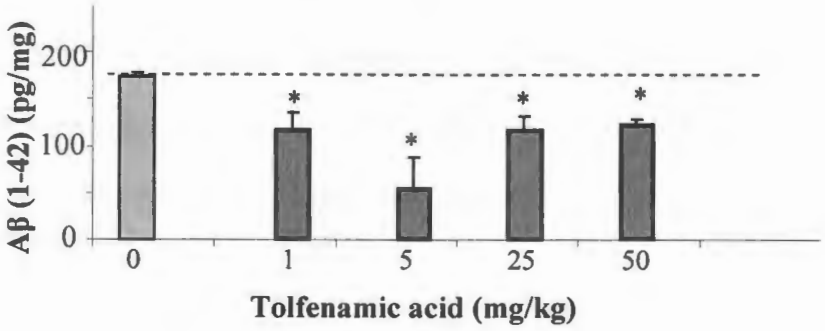

Aβ (1-42) (pg/mg)
200
100
0
*
*
*
*
0
1
5
25
50
Tolfenamic acid (mg/kg)

Table 1. A Summary of the effect of daily dosing with various doses of tolfenamic acid on SP1, APP and Aβ. Animals' age was 3 months at the beginning of the study. Animals were given one of the following doses of tolfenamic acid 0, 1, 5, 25, or 50 mg/kg, daily for 15 days. Protein changes are expressed as percentage change from controls.

Dose (mg/kg)	SP1	APP	Aβ (1-40)	Aβ (1-42)
1	No change	No change	**-24.32%**	**-32.81%**
5	**-57.65%**	**-18.52%**	**-14.27%**	**-68.19%**
25	No change	No change	**-25.29%**	**-32.96%**
50	No change	No change	**-21.61%**	**-29.54%**

Figure 14. Temporal changes in Aβ (1-40) levels in the cerebral cortex of mice treated with tolfenamic acid. Animals' were 3 months of age at the beginning of the study. Vehicle or 5 mg/kg tolfenamic acid were administered daily, and animals were sacrificed at days 4, 8, and 16 after the beginning of the study. Aβ (1-40) levels were measured by an ELISA assay as illustrated in the methods section. Results expressed are the mean ± SEM. The number of animals in each group, day 4 control n=4, treatment n=5; day 8 control n=4, treatment n=5; day 16 control n=3, treatment n=4. (p=0.06) according to student's t-test, at day 16.

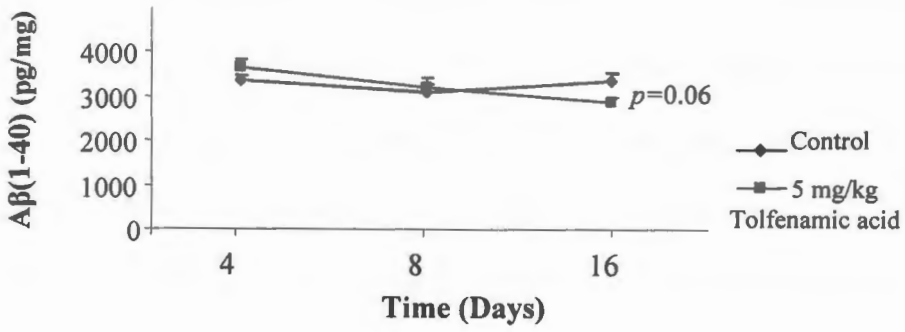
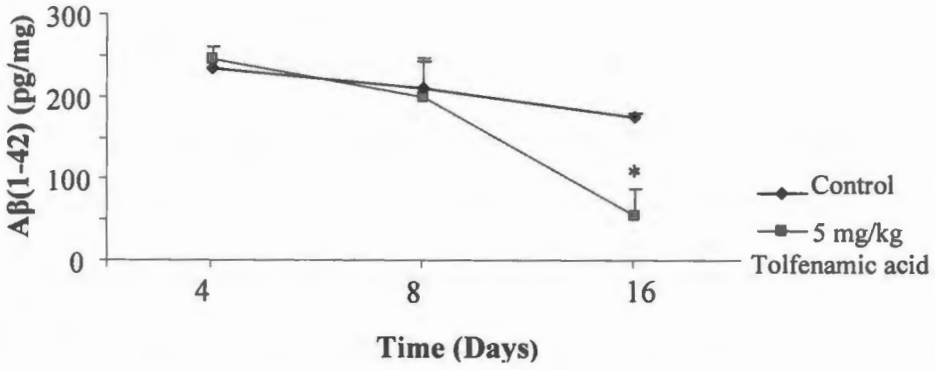

Aβ(1-40) (pg/mg)
4000
3000
2000
1000
0
4
8
16
Time (Days)
p=0.06
Control
5 mg/kg
Tolfenamic acid

Figure 15. Temporal changes in Aβ (1-42) levels in the cerebral cortex of mice treated with tolfenamic acid. Animals' were 3 months at the beginning of the study. Vehicle or 5 mg/kg tolfenamic acid were administered daily, and animals were sacrificed at days 4, 8, and 16 after the beginning of the study. Aβ (1-42) levels were measured using an ELISA assay as illustrated in the methods section. Values shown are the mean ± SEM. The number of animals in each group, day 4 control n=4, treatment n=5; day 8 control n=4, treatment n=5; day 16 control n=3, treatment n=3. The "*" indicates that values are significantly different from control, as determined by a student's *t*-test ($p<0.05$).


Aβ(1-42) (pg/mg)
300
200
100
0
4
8
16
*
Control
5 mg/kg
Tolfenamic acid
Time (Days)

4.0 Discussion

Alzheimer's disease is a neurodegenerative disorder causing progressive memory depreciation until death. While the exact cause remains unknown, no disease modifying drug is available, and all existing medications only target the symptoms. The presence of Aβ plaques and tau neurofibrillary tangles within the cerebral cortex and hippocampal regions of the brain are the two major known characteristics of the disease. Aβ is the product of the sequential cleavage of APP by the enzymes β-secretase and γ-secretase. Since the amyloid hypothesis has closely linked these proteins with the pathology of AD (Hardy and Selkoe, 2002), Aβ and other elements involved in its production have been targets for possible therapeutic interventions.

The transcription factor Sp1 has been linked to the pathology of AD. It is involved in regulating the APP gene as well as BACE1 (Christensen et al., 2004; Docagne et al., 2004; Basha et al., 2005; Santpere et al., 2006). Therefore, any process that impacts Sp1 is likely to have an effect on APP levels as well as its downstream products. Tolfenamic acid was shown to inhibit pancreatic tumors growth by promoting the degradation of the transcription factors Sp1, Sp3, and Sp4, and suppressing VEGF mRNA and protein expression (Abdelrahim et al., 2006). Thus, tolfenamic acid is likely to lower APP levels in the brain.

In our studies, tolfenamic acid demonstrated a time dependent effect on APP and Aβ levels within certain doses; these are major pathological mediators in AD according to the amyloid hypothesis. These effects were accompanied by a correlated drop in the levels of the transcription factor SP1 at certain doses. Those results were first observed in a pilot trial in male mice where APP levels were lowered by about 50% (Fig. 3A), and Aβ levels by 18% (Fig. 4). SP1 protein bands also were of lower intensity in the treated group than the control (Fig. 3B).

APP levels also were lower in male guinea pigs following tolfenamic acid treatment (Fig. 5); however, the reduction in APP was less than that seen in mice. Furthermore, Aβ (1-42) levels were mitigated but not significantly (Fig. 6). This is more likely due to the small sample number used in this study. The findings in guinea pigs demonstrate that the effects of tolfenamic acid on APP are not species-specific.

In female mice, the same reduction levels were achieved after 28 days of alternate day dosing (Fig. 7). This indicated that the effects of tolfenamic acid are not gender-specific and appear to follow the same dose and time course requirements. The relationship between AD and gender has been disputed as some studies concluded that AD is indifferent to gender, while others argued that there was a higher risk in women for developing AD (Barnes et. al., 2005). Our studies show that tolfenamic acid had a similar effect on APP in male and female C57BL/6 mice.

While alternate day dosing was selected in pilot trials to identify the time and dose ranges where tolfenamic acid effects can be observed, a more accurate determination can be achieved by daily dosing. Daily administration of tolfenamic acid resulted in a reduction in all three endpoints namely APP, SP1, and Aβ at certain doses within 16 days (Table 1). The daily dosing time course was consistent with previous alternate day dosing as the time was cut in half in daily dosing.

The delay in the observed reduction of APP and Aβ levels in animals strongly suggests that the lowering of these AD-related proteins is achieved through interruption of processes associated with gene expression and regulation (Fig 8, 10, 14, 15). In pancreatic tumors, Sp1 inhibition was masked following treatment with proteasome inhibitor, proposing that tolfenamic acid degradation of Sp1 is mediated by a proteasome (Abdelrahim et al., 2006). Such degradation would deplete a cell from SP1 reserves, and over time, less APP expression would be expected due to a decreased pool of SP1. Likewise, the fact that Aβ levels take weeks to exhibit a decline, suggests that the lowering of this peptide is linked to the proteolytic elimination of SP1 and lowered transcriptional activation of APP.

Although the temporal condition of lowering these intermediates was relative to the dosing schedule, the effective dose range was very different. While higher doses of 10, 25 mg had an effect on alternate day dosing, the effects of daily dosing were only seen at 5 mg for APP and 5-10 mg for SP1. It is not clear why

the dose range was narrowed following daily dosing, but this maybe related to the half-life of the drug. In daily dosing a residual amount of the drug appears to persist that saturates the system and renders higher doses ineffective, while in alternate day dosing, the drug maybe washed out of the system before the next dose is administered. In any event, the lower sensitivity is greatly advantageous from a therapeutic perspective.

The expression of the APP gene is regulated by Sp1 (Basha et al., 2005). In the daily dosing study, it appeared that a 50% reduction in SP1 levels had to be achieved before any lowering of APP could be detected. This suggests that the pools of SP1 have to be reduced in half through tolfenamic acid induced degradation at a rate that overwhelms SP1 synthesis. Tolfenamic acid thus acts to disrupt the balance between the de novo synthesis of APP and its degradation.

While a narrowing of the dose range was observed relative to SP1 and APP, Aβ levels on the other hand were lowered at daily doses tested from 1-50 mg/kg (Fig. 12, 13). While both Aβ (1-40) and (1-42) were lowered, there was a greater lowering of Aβ (1-42) at the 5 mg dose. This is the dose where parallel reductions were seen in both SP1 and APP. This leads to the conclusion that the effects on Aβ at the dose range of 1-10 mg is related to SP1 and APP, while the lowering of Aβ at higher dose may be associated with other mechanisms.

The lowering of Aβ (1-42) is highly relevant because the 42 amino acid-long Aβ is considered more aggressive than the other forms, and resulted in more rapid aggregations in solutions with Aβ of varying sequences (Snyder et al., 1994; Näslund et al., 2000). In solution, Aβ (1-40) aggregates were found to inhibit the aggregation of Aβ (1-42) (Snyder et al., 1994). The greater Aβ (1-42) decline compared to Aβ (1-40) following the 5 mg/kg dose of tolfenamic acid suggests that in vivo Aβ production might be shifted more towards the Aβ (1-40) fragment.

It is not clear why at higher doses the drug did not affect Sp1 and APP while Aβ was still lowered. A possible explanation could be that at higher doses the drug was aggregating, and thus was trapped in tissue before it could get into the brain in a concentration sufficient to cause Sp1 and APP decline, in that case, cerebral cortex Aβ lowering could be explained by the tolfenamic acid triggered peripheral decline in Aβ acting as an Aβ sink from central compartments. This can be tested by studying the effects of the drug on Sp1, APP, and Aβ in peripheral tissue.

The pharmacokinetics of the drug could also play a role in the narrowing of the dosage range where effects are observed on Sp1, APP and Aβ. The elimination half-life of the drug from plasma was about 2.5 hours with 60 % bioavailability and 99.7% protein binding (Pentikäinen et al., 1981; Sidhu et al., 2006). However, the terminal clearance half-life of tolfenamic acid metabolites was as long as 19 hours (Pentikäinen et al., 1984). Unpublished studies from our lab have confirmed that tolfenamic acid is available in the brain following oral or intramuscular

administration, still it is not yet proven whether the effects observed on Sp1, APP, and Aβ are due to the parent compound or one of its metabolites. Nevertheless, one would expect the metabolites to be more polar and larger in size, and thus with lesser brain penetration potential than the parent drug; however, it is possible that some derivative of tofenamic acid is responsible for these effects.

Furthermore, considering the fact that the rate and the degree of tolfenamic acid absorption and elimination are not dose dependent (Pentikäinen et al., 1981), higher doses of tolfenamic acid administered daily could have resulted in the depletion of the target proteasome that degrades Sp1, and thus at such doses of tolfenamic acid, the target could have been used up, this would affect the response at the pharmacodynamic level. On the other hand, every other day administration might have allowed more time during which the body is drug free and the targets can be regenerated to be available for the next dosage. Nevertheless, doses as low as 5 mg/kg would still be more favorable, since they would be associated with fewer NSAID's related gastrointestinal side effects, and they are close to the doses approved for migraine headaches in humans, and pain control in veterinary medicine.

5.0 Conclusion

These studies were primarily conducted to examine whether or not tolfenamic acid is capable of altering Aβ and APP protein levels, as important pathological intermediates in neurodegeneration and AD. Additionally, the studies aimed at finding the effective dose and time range at which such outcomes occur. Tolfenamic acid was able to lower the levels of cerebral SP1, APP, and Aβ with certain doses in wild type male mice. Temporal studies showed that the decline in APP and Aβ was time dependent. These effects seemed to be mediated through SP1 degradation at a lower dose of the drug, i.e. 5 mg/kg administered daily. At higher doses Aβ lowering does not appear to be associated with the decrease in SP1 decrease suggesting that there is an alternate mechanism underlying the Aβ change. In addition, tolfenamic acid lowered APP in the cerebral cortex of female mice, and Hartley guinea pigs. Accordingly, tolfenamic acid could be beneficial for AD by targeting essential pathological mediators within the disease process. Further behavioral tests in AD animal models are necessary to determine the relationship between the molecular changes and cognitive improvement. Moreover, the connection between Aβ lowering and plaque burden needs to be examined. More research is needed in order to reveal the exact mechanism by which tolfenamic acid affects AD related proteins and the consequential effect on behavior and plaque pathology.

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