Tolfenamic Acid Interrupts the *De Novo* Synthesis of the β-Amyloid Precursor Protein and Lowers Amyloid Beta Via a Transcriptional Pathway

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Abstract: Amyloid beta (Aβ) peptides are related to the pathogenesis of Alzheimer's disease (AD). The search for therapeutic strategies that lower these peptides has mainly focused on the proteolytic processing of the β-amyloid precursor protein (APP), and other post-transcriptional pathways. The transcription factor specificity protein 1 (Sp1) is vital for the regulation of several genes involved in AD including APP and the beta site APP cleaving enzyme 1 (BACE1). We have previously reported that tolfenamic acid promotes the degradation of Sp1 protein (SP1) in pancreatic human cancer cells and mice tumors. This study examines the ability of tolfenamic acid to reduce SP1 levels, and thereby decrease APP transcription and Aβ levels in rodent brains. Tolfenamic acid was administered by oral gavage to C57BL/6 mice at variable dosages and for different time periods. Results have shown that tolfenamic acid was able to downregulate brain protein levels of SP1, APP, and Aβ. These findings demonstrate that interference with upstream transcriptional pathways can lower pathogenic intermediates associated with AD, and thus tolfenamic acid represents a novel approach for the development of a therapeutic intervention for AD.

Keywords: Alzheimer's disease, amyloid beta, APP, Sp1, tolfenamic acid, transcription.

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder marked by dementia deteriorating until death. The extracellular neuritic plaques and intracellular neurofibrillary tangles are the characteristic neuropathological deposits of AD described by Alois Alzheimer in 1906 [1, 2]. Plaques are aggregates of the 36-43 amino acid peptide amyloid beta (Aβ), which is generated following the sequential cleavage of the β-amyloid precursor protein (APP) by the enzymes β-secretase and γ-secretase [3-5]. Produced Aβ is normally secreted but can also accumulate forming insoluble aggregates [4, 6]. $A\beta_{1-40}$ and $A\beta_{1-42}$ are the two major forms of Aβ, with the 42-amino acid peptide being more aggregative and proposed to provoke plaque formation in AD [7]. Aβ and its aggregates cause synaptic dysfunction and loss [8-10] and according to the amyloid hypothesis, amyloid plaques trigger the neurodegeneration and dementia associated with AD [11, 12].

To date, no drug has been found to stop or slow the progression of AD and the few available medications are used for symptomatic or adjunctive therapy. With no cure available, innovative mechanisms are under investigation for the design of new therapeutic agents. Ongoing research revealing additional insights on AD pathogenesis and molecular mechanisms has created further prospects for disease modifying therapies. Amyloid plaques along with factors involved in the production, secretion and degradation of Aβ peptides are common targets [13]. Our novel approach targets the selective transcription factor specificity protein 1 (Sp1) to inhibit particular downstream pathways related to AD pathology. Transcription is the process by which DNA is copied into mRNA and is a fundamental step that triggers certain cellular responses leading to protein formation [14]. Transcription factors play a significant role in regulating transcription and could constitute important therapeutic targets for modulating endogenous protein expression [14, 15]. Sp1 is a zinc finger motif transcription factor that is involved in AD as it co-activates APP transcription [16]. The APP promoter is rich in its GC content, providing several Sp1 binding sites [17-21]. It was found that silencing the Sp1 gene dramatically decreases the APP promoter activity [22], while enhancing Sp1 activity increases APP mRNA expression [22, 23]. Furthermore, Sp1 regulates the expression of beta site APP cleaving enzyme 1 (BACE1) [24], the main β-secretase that cleaves APP to generate Aβ [25]. Sp1 protein (SP1) overexpression increases BACE1 promoter activity, while SP1 decline reduces BACE1 gene transcription [24]. In addition, Sp1 regulates the expression of tau [26]. 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 [27]. SP1 levels are elevated in the frontal cortex of AD patients as well as in transgenic animal models that develop AD-like pathology [28]. These findings along with immunohistochemical co-localization of SP1, APP, and Aβ within neurons throughout the brain [29] suggest that Sp1 could be targeted to interrupt the *de novo* synthesis of APP and consequently alter Aβ levels in AD.

Sp1 represents a potential AD target, where its abnormal and elevated expression has been associated with the disease deterioration [18, 20, 24, 27-29]. Sp1 regulates gene tran-

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1567-2050/11 $58.00+.00

scription by binding to GC rich promoter regions in genes like APP, whose binding to Sp1 increases the production of APP mRNA [16, 18, 30]. While concerns about a widespread effect on gene expression when a transcription factor is targeted are legitimate, most genes would not be altered by interference with Sp1 because it is involved in the activation of genes possessing GC box elements [31], since less than 10% of genes have such GGGCGG sequences in their promoter regions [32], impacts of altering SP1 levels would be limited. Moreover, it was found that Sp1 is vital during early embryonic development only but not necessary for the consequent later stages of cell growth and differentiation [33]. Genes targeted by Sp1 are mostly housekeeping genes and rarely exhibit regulatory or transcriptional functions [32]. Thus, lowering SP1 as a target in AD patients should not affect the normal functioning of cells.

Following the screening of fourteen compounds for their SP lowering potential, as transcription factors that control proliferation and are upregulated in various cancer cell lines [34], it was found that tolfenamic acid inhibits the growth of pancreatic tumors by promoting the degradation of the transcription factors SP1, SP3, and SP4 [34]. Tolfenamic acid is used in Europe and other countries to treat migraine headaches. Recent studies have confirmed tolfenamic acid antitumor effects targeting key genes associated with tumor progression and metastasis through inhibition of Sp transcription factors [35, 36]. Since Sp1 is involved in AD pathology, this study has been designed to investigate the ability of tolfenamic acid to lower AD associated proteins like SP1, APP and Aβ through its unique property of promoting SP1 degradation and therefore altering APP transcription and Aβ production as illustrated in Fig. (**1**).

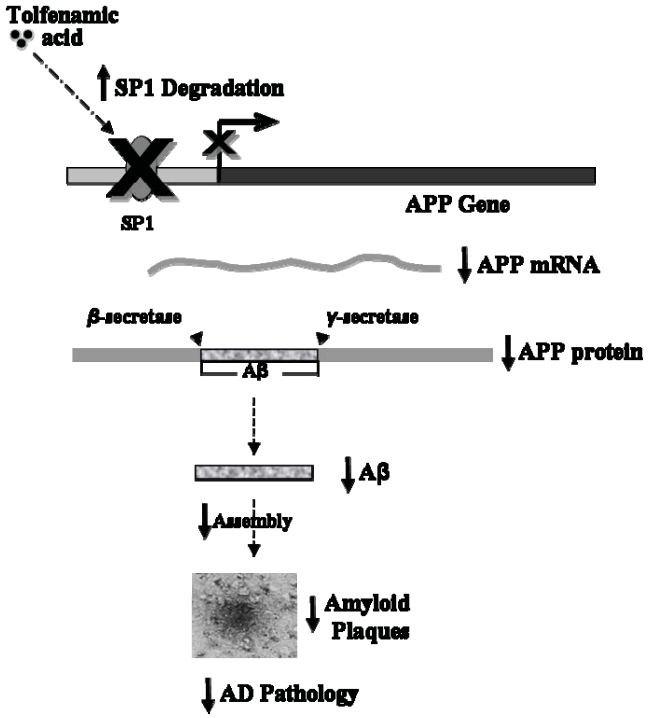

Fig. (1). Proposed pathway for tolfenamic acid downregulation of the APP gene. Tolfenamic acid promotes the degradation of the transcription factor Sp1. This results in a decrease in APP transcription, and lowers APP and Aβ protein levels, ultimately reducing the amyloid plaque burden and pathology of AD.

MATERIALS AND METHODS

Reagents

Chemicals and reagents were purchased from Thermo Fisher Scientific Inc. unless otherwise noted.

Animal Exposure

Adult C57BL/6 mice were obtained from Jackson Laboratory or bred in-house, and were kept in a designated room within the animal facility at the University of Rhode Island. In an initial study, 0 and 50 mg/kg tolfenamic acid was administered by oral gavage on alternate days to 2 months old mice for 5 weeks after which brain tissues were collected, and changes in APP, SP1 and Aβ levels were assessed within cortices. To examine the effects of tolfenamic acid on a wider dosage range, another study was conducted where tolfenamic acid in corn oil was administered to 3 months old mice in one of the following doses 0 (vehicle), 1, 5, 10, 25, or 50 mg/kg/day for 15 days. On day 16, brain tissues were collected and changes in Sp1, APP, and Aβ within the cerebral cortices and hippocampi were examined. In addition, animals were also euthanized 24 hours after 3 and 7 days of dosing to establish a temporal pattern for effects. All experiments were performed in accordance with the standard guidelines and the protocol approved by the Institutional Animal Care and Use Committee of the University of Rhode Island.

Protein Extraction

Tissue was homogenized with RIPA lysis buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 0.1 SDS, 1 mM EDTA, and 0.1% protease inhibitor cocktail). The homogenates were centrifuged at 10,600 x g for 10 minutes at 4°C and supernatants were collected. Protein concentration was determined using the Micro BCA Protein Assay Kit from Pierce. Protein extracts were stored at -80°C.

Western Blot Analysis

SP1, APP, and the housekeeping proteins β-actin and GAPDH levels were examined by Western blot analysis. The method mentioned in previous studies was followed [37-39]. Protein (20-40 μg) was electrophoresed onto 8% SDS-PAGE gels at 20-30 mA for 1-2 hours, transferred to PVDF membranes (GE) and blocked with 5% non-fat milk or 3% bovine serum albumin (Sigma) overnight. Membranes were incubated with the appropriate dilution of the specific primary antibody, i.e. 1:5000 dilution of APP MAB348 (Millipore), 1:500 SP1 sc-420 (Santa Cruz Biotechnology), 1:5000 dilution of β-actin A2013 (Sigma), or 1:5000 GAPDH G9545 (Sigma), then the membranes were washed with TBST and incubated with either 1:20000 goat anti-rabbit secondary antibody 31460 (Thermo Fisher Scientific) or 1:1000 dilution of goat anti-mouse secondary antibody 1858413 (Pierce). Finally, membranes were developed using the ECL plus reagent and Typhoon™ Variable Mode Imager, and quantitative analysis of the bands was performed by ImageQuant™ 5.2 software (GE).

ELISA $A\beta_{1-40}$ and $A\beta_{1-42}$ Assay

Levels of Aβ were measured using human $A\beta_{1-40}$ and $A\beta_{1-42}$ assay kits JP27713 and JP27711 (IBL). These kits are solid-phase sandwich ELISA with two types of highly specific antibodies which are 100% reactive with mouse $A\beta_{1-40}$ with a sensitivity of 5.00 pg/mL (JP27713), and 70.9% reactive with mouse $A\beta_{1-42}$ with 4.03 pg/mL sensitivity (JP27711). Each kit measures the respective $A\beta_{1-40}$ or $A\beta_{1-42}$ variants cleaved N terminal side by any cause. The assay was conducted following manufacturer's instructions [40] with minor modifications. One hundred μg protein in 100 μL EIA buffer and assay standards were added in duplicates to 96-well plates pre-coated with anti-human Aβ mouse IgG MoAb. The plates were incubated overnight at 4°C, and washed 7 times using the 40X diluted wash buffer supplied with the kit (0.05% Tween 20 in phosphate buffer), and 100 μL labeled antibody was added and incubated for 1 hour at 4°C, the wells were washed again 9 times, and then 100 μL of tetramethylbenzidine was added as a coloring agent, and incubated in the dark for 30 minutes at room temperature. Finally 100 μL of 1N H_2SO_4 was added to stop the reaction, and absorbance was measured at 450 nm using Spectra Max UV/Vis Spectrometer (GMI). The concentration of Aβ in unknown samples was obtained as pg/mg total protein after plotting the absorbance of standards against the standard concentrations.

Total RNA Isolation, Synthesis of cDNA, and Real Time PCR

RNA was isolated from cortex tissue following the TRIzol® Reagent method (Invitrogen). RNA was reverse transcribed to cDNA using iScript™ Select cDNA Synthesis Kit (Bio-Rad). About 500 ng of RNA was added to 8 μL of nuclease free water and 2 μL Oligo (dT) mix and mixtures were incubated at 65˚C for 5 minutes, followed by 1 minute incubation on ice. Next, 4 μL of 5x iScript Select reaction mix and 1 μL of iScript reverse transcriptase were added. Samples were incubated at 42ºC for 90 minutes then at 85ºC for 5 minutes to terminate the reaction. All incubations were conducted using MJ Research MiniCycler™ (Bio-Rad). Primer pairs used for mouse APP, Sp1 and β-actin were obtained from Invitrogen as follows: APP sense: 5'-GCT GGC TGA ACC CCA GAT-3' and antisense: 5'-CCC ACT TCC CAT TCT GGA CAT-3'; Sp1 sense: 5'-CAA GCC CAA ACA ATC ACC TT-3', and antisense, 5'-CAA TGG GTG TGA GAG TGG TG-3'; β-actin sense: 5'-TGT TAC CAA CTG GGA CGA CA-3', and antisense: 5'-TCT CAG CTG TGG TGG TGA AG-3'. Each real time PCR reaction mix contained 2 μL of cDNA, 1 μL of each primer, 8.5 μL water and 12.5 μL SYBR® Green PCR Master Mix (Applied Biosystems). Real time PCR was conducted using 7500 Real Time PCR System (Applied Biosystems) following the standard protocol: 50°C for 2 minutes followed by 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds, and 60°C for 1 min. Results were analyzed using the 7500 system software with relative quantification method and β-actin as endogenous control.

Determination of the Presence of Tolfenamic Acid in the Brain Following Oral Administration

C57BL/6 mice were administered 200 μL of 100 mg/kg tolfenamic acid in corn oil by oral gavage (n=5). After 1.5 hours, the estimated Tmax for tolfenamic acid [41], the mice were euthanized, decapitated and their brains were separated and stored at -80°C until the time of use. A previously published extraction method was followed [42]. First, 1 g of brain tissue was homogenized with 5 mL aqueous HCl solution (0.25 M). The homogenate was placed on a shaker and agitated for 16 hours at room temperature. After hydrolysis, 500 μL of 0.3 M sodium phosphate dibasic dodecahydrate solution was added and the pH was neutralized to 7.1±0.2 with 2N sodium hydroxide. The samples were extracted three times with 4 mL of ethyl acetate. Every time, the samples were vortexed for 1 minute and centrifuged at 3000 x g for 8 minutes. The organic extracts were transferred into polypropylene tubes and evaporated to dryness under nitrogen stream. The residues were reconstituted with 400 μL of ethanol solution and filtered through 0.2 μm filter of which 20 μL was injected into the mass spectrometry system. Applied Biosystems Mariner® Mass Spectrometer system was used which consists of an orthogonal time-of-flight mass spectrometer equipped with standard electrospray ionization, PE SCIECX's Turbo Ion Spray™ and Atmospheric Pressure Chemical Ionization source. The instrument was outfitted with an integrated syringe pump with a dual syringe rack for direct infusion into the mass spectrometer. Extracted samples and standards were injected directly with a flow rate of 20 μL/min and the negative ion mode was used for ion peak detection.

Statistical Analysis

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

RESULTS

Lowering SP1 levels in the brain is a novel mechanism that could be beneficial for AD through consequent lowering of APP and Aβ, Fig. (**1**). In an initial study, adult C57BL/6 mice were orally gavaged with 0 or 50 mg/kg of tolfenamic acid every other day for five weeks, at the end of the study cortical APP levels were significantly reduced in treatment groups compared to controls, Fig. (**2A**); $p<0.05$. $A\beta_{1-40}$ protein levels also were significantly lowered within the cerebral cortices of treatment groups in comparison to controls, Fig. (**2B**); $p<0.05$, these changes were accompanied by a decrease in SP1, Fig. (**2C**). To determine a more specific range of effective doses administered daily to a larger number of animals, adult C57BL/6 mice were administered tolfenamic acid everyday for 15 days at the following concentrations: 0, 1, 5, 10, 25, and 50 mg/kg. The data presented below demonstrate the effects of tolfenamic acid on Sp1, APP and Aβ following daily administration to wild type mice.

Tolfenamic Acid Downregulates APP by Lowering SP1

Daily administration of 5 mg/kg tolfenamic acid for 15 days resulted in a 58% decline in cortical SP1 levels, Fig. (**3A**); $p<0.05$, at the same dose, relative mRNA expression was significantly reduced by 54% on day 4 following dosing

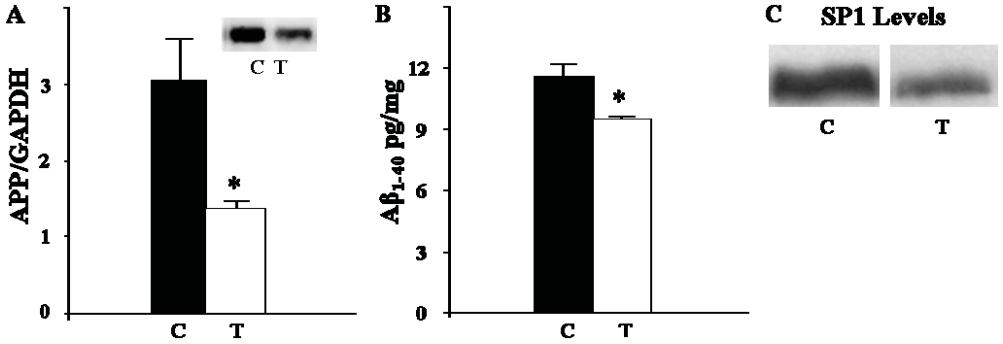

Fig. (2). Changes in cortical APP, $A\beta_{1-40}$ and SP1 protein levels in adult mice treated with tolfenamic acid. C57BL/6 mice were dosed with 50 mg/kg tolfenamic acid or vehicle on alternate days for 5 weeks. **(A)** APP levels as a ratio of the house keeping protein GAPDH as obtained by Western blot. Insert shows representative APP Western blot bands from control (C) and treatment (T). **(B)** $A\beta_{1-40}$ levels measured by ELISA. **(C)** Western blot bands representing the change in SP1 levels following drug administration. Values shown are the mean ± SEM (n=6). "*" indicates that values are significantly different from control, as determined by a Student's *t*-test ($p<0.05$).

and 55% on day 16, Fig. (**3B**) $p<0.05$. APP levels within the cerebral cortex were lowered by 19%, Fig. (**3C**) $p<0.05$, after 15 days of the 5 mg/kg/day treatment, while non-significant fluctuations in APP occurred on days 4 and 8 following daily treatment with the same dose, with a 20% lowering trend on day 8 that did not reach statistical significance (data not shown). Within the hippocampus, another area associated with memory and affected by AD, tolfenamic acid decreased SP1 and APP levels with the 10-50 mg/kg/day doses by 30-40%, Fig. (**4**) $p<0.05$.

Tolfenamic Acid Reduces Aβ Protein Levels

Following daily treatment with tolfenamic acid for 15 days, $A\beta_{1-40}$ levels were significantly reduced compared to controls by the following percentages: 24% with 1 mg/kg, 25% with 25 mg/kg and 22% with 50 mg/kg, Fig. (**5A**) $p<0.05$. Results show that tolfenamic acid generated a much greater decline in the levels of the more aggregative Aβ form, $A\beta_{1-42}$, whose levels were significantly lower than controls by the following percentages: 33% with 1 mg/kg, 68% with 5 mg/kg, 33% with 25 mg/kg, and 29% with 50 mg/kg Fig. (**5B**); $p<0.05$.

Changes in cortical $A\beta_{1-40}$ and $A\beta_{1-42}$ levels as well as the ratio of the later to the former were examined at 3 time points following treatment with 5 mg/kg/day tolfenamic acid, Fig. (**6**). On day 16 post-treatment, $A\beta_{1-40}$ levels dropped by 14%, Fig. (**6A**); $p=0.06$ and $A\beta_{1-42}$ levels were significantly less than controls by 68%, Fig. (**6B**) $p<0.05$, while the ratio of $A\beta_{1-42}/A\beta_{1-40}$ was lowered by 65%, Fig. (**6C**) $p<0.05$.

Tolfenamic Acid is Present in the Brain Following Oral Administration

Mass spectra data provide evidence that tolfenamic acid was able to cross the blood brain barrier and was available in the brain after oral dosing, Fig. (**7**). The peak of tolfenamic acid in brain extract identified at m/z 259.7, Fig. (**7B**) indicated the ability of the drug to transfer into the CNS following oral administration.

DISCUSSION

The transcription factor Sp1 is associated with the pathology of AD, as it regulates APP and BACE1 gene expression [16-20, 24, 27]. Depletion of SP1 by siRNA silencing of

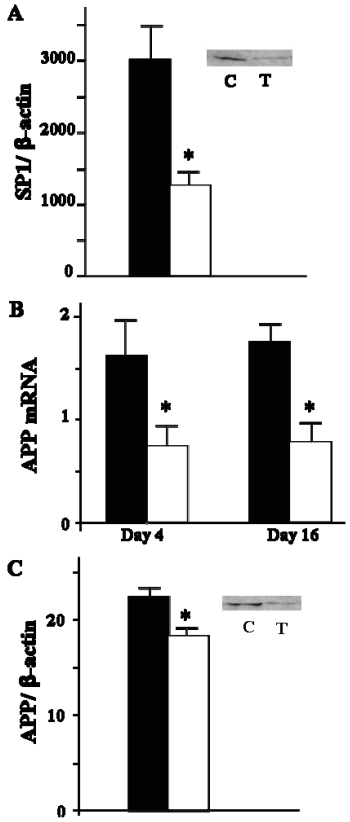

Fig. (3). Cortical SP1 and APP mRNA and protein levels in mice treated with 5 mg/kg/day tolfenamic acid. C57BL/6 mice were given 5 mg/kg/day tolfenamic acid or vehicle for 15 days. Animals were euthanized on days 4 and 16 after the beginning of the study. **(A)** Cortical SP1 levels on day 16 measured by Western blot as a ratio to the housekeeping protein β-actin. Insert shows representative control (C) and treatment (T) SP1 Western blot bands. **(B)** Temporal changes in cortical APP mRNA expression on days 4 and 16 as obtained by real time PCR. **(C)** Cortical APP levels on day 16 after dosing measured by Western blot as a ratio to the house keeping protein β-actin. Insert shows representative control and treatment APP Western blot bands. Black bars denote control, open bars denote 5 mg/kg treatment. An average of 3 animals per dosing group was used. Values shown are the mean ± SEM. "*" indicates that values are significantly different from control, as determined by a Student's *t*-test ($p<0.05$).

the Sp1 gene reduces the responsiveness of the human APP promoter by approximately 70% [22]. Immunohistochemical studies from our lab have shown that SP1, APP, and Aβ co-localize in brain neurons, and that cortical and hippocampal areas with higher SP1 levels express more Aβ [29]. Consequently, any process that affects Sp1 could also influence APP transcription and as a result alter the expression of its downstream product Aβ as depicted in Fig. (**1**).

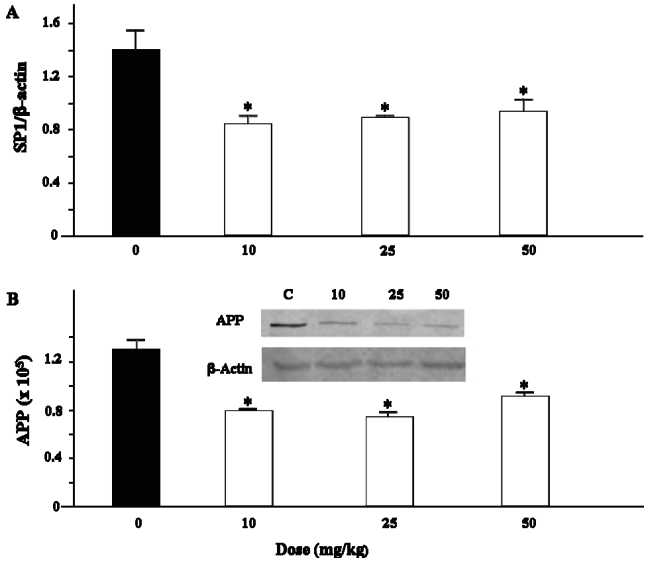

Fig. (4). Hippocampal SP1 and APP levels in mice treated daily with different doses of tolfenamic acid. C57BL/6 mice were administered 0, 10, 25, or 50 mg/kg/day tolfenamic acid by oral gavage for 15 days. **(A)** SP1 levels expressed as a ratio of the housekeeping protein β-actin measured by Western blot analysis. **(B)** APP levels as measured by Western blot analysis. Insert shows representative APP and β-actin bands. Values shown are the mean ± SEM. The average number of animals per dosing group was 4. "*" indicates that values are significantly different from control, as determined by a Student's *t*-test ($p<0.05$).

Tolfenamic acid inhibits pancreatic tumor growth by promoting the degradation of the transcription factors SP1, SP3 and SP4, therefore suppressing vascular endothelial growth factor (VEGF) mRNA and protein expression [34]. These findings and others by our collaborators led to the acquisition of FDA approval for tolfenamic acid to be tested as an investigational new drug for cancer treatment, and the drug was deemed safe for human use by the FDA. Tolfenamic acid is currently available in the European market for the treatment of migraine headaches as 200 mg tablets. This is a drug with a large therapeutic index that has been used in humans for decades and is relatively safe.

By inducing the degradation of SP1 in the brain, tolfenamic acid can inhibit the *de novo* synthesis of APP, thus it represents a novel class of mechanism-based AD drugs that could be administered orally. In our studies, tolfenamic acid was given daily to wild type mice by oral gavage in doses ranging from 1 to 50 mg/kg. The drug was well tolerated and no signs of toxicity or discomfort were observed. Tolfenamic acid affected the amyloidogenic pathway by knocking down SP1 in rodents, and hence lowering the levels of APP mRNA, APP as well as Aß peptides, which are involved in cellular processes that may contribute to AD pathology [7-12]. Since APP gene regulation is highly conserved [43, 44], we believe that tolfenamic acid would also do the same in humans, compromising a new mechanism-based disease modifying drug for AD whose effects occur following oral administration, unlike most other Aβ lowering approaches under investigation where delivery is a major challenge.

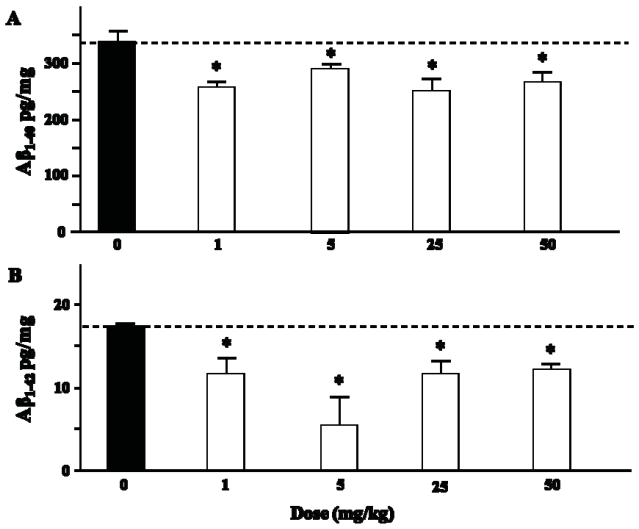

Fig. (5). Aβ levels in the cerebral cortex of mice treated with various doses of tolfenamic acid. Adult animals were given tolfenamic acid daily in one of the following doses 0, 1, 5, 25, 50 mg/kg by oral gavage for 15 days. **(A)** $A\beta_{1\text{-}40}$ and **(B)** $A\beta_{1\text{-}42}$ levels as measured by ELISA. An average of 4 animals per group was used. Values shown are the mean ± SEM. "*" indicates that values are significantly different from control, as determined by a Student's t-test ($p<0.05$).

In Panc-1 cells, SP1 and VEGF lowering by tolfenamic acid was blocked by the proteasome inhibitor lactacystin, suggesting that tolfenamic acid promotes SP1 proteasome-mediated degradation, thereby inhibiting VEGF transcription [34]. In the brain, such degradation would deplete cellular SP1 reserves, and over time, less APP expression is expected due to the decreased SP1 pool. The delay observed in the reduction of APP (data not shown) and Aβ Fig. (**6**) strongly suggests that lowering of these AD-related proteins is achieved through interruption of processes related to gene expression and regulation. The fact that APP and Aβ levels took time to exhibit a decline strengthens the association between lowering of these peptides and the proteolytic clearance of SP1 along with the consequent decline in the transcriptional activation of the APP gene. Lowering of relative APP mRNA expression occurring as early as 3 days after of treatment supports this hypothesis, Fig. (**3B**).

In our studies, tolfenamic acid induced SP1 degradation causing a drop in the levels of this transcription factor, Figs. (**2C**, **3A** and **4A**). Tolfenamic acid demonstrated a rapid decline in APP mRNA and a time dependent effect on APP and Aβ; which are the major pathological mediators in AD according to the amyloid cascade hypothesis, Figs. (**3** and **6**).

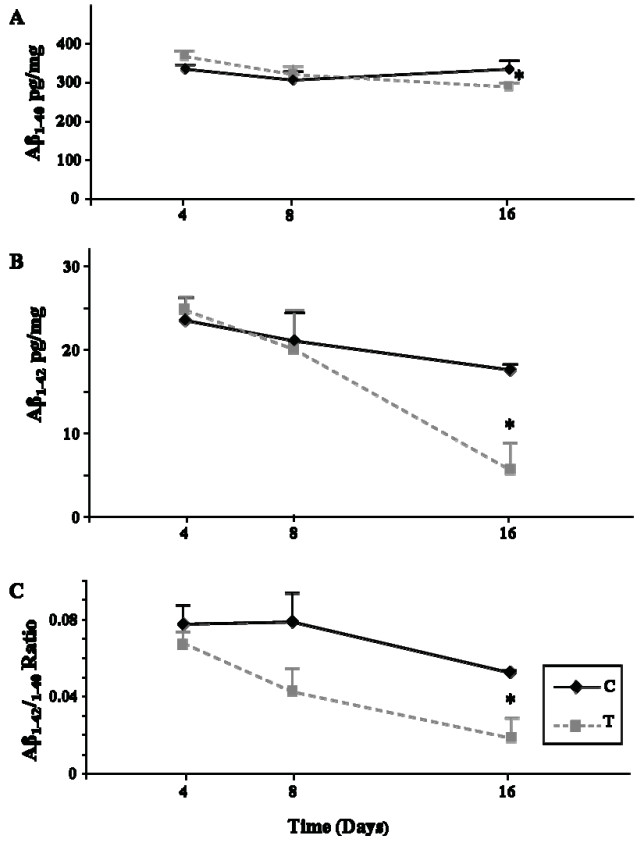

Fig. (6). Temporal changes in Aβ levels in the cerebral cortex of C57BL/6 mice treated with tolfenamic acid. Twenty-four animals were administered vehicle or 5 mg/kg tolfenamic acid daily, animals were euthanized on days 4, 8, and 16 after the beginning of the study. **(A)** $A\beta_{1-40}$ levels. **(B)** $A\beta_{1-42}$ levels. **(C)** The ratio of $A\beta_{1-42}/A\beta_{1-40}$ following treatment with tolfenamic acid for 15 days. Aβ levels were measured by ELISA. The average number of animals in each group was 4. Values shown are the mean ± SEM. "*" indicates that values are significantly different from control, as determined by a Student's *t*-test ($p<0.05$).

Daily administration resulted in reduction in all three endpoints; SP1, APP, and Aβ after treatment with 5, 25, or 50 mg/kg/day for 15 days, Figs. (**3**, **4** and **5**). However, cortical Sp1 mRNA levels did not change with tolfenamic acid treatment (data not shown), hence tolfenamic acid changes occurred on the protein level of Sp1 and not on the gene expression level, similar to the pattern observed in pancreatic tumors where tolfenamic acid promoted SP degradation [34].

Tolfenamic acid reduced both $A\beta_{1-40}$ and $A\beta_{1-42}$, however, a much greater lowering occurred in $A\beta_{1-42}$. The larger $A\beta_{1-42}$ decline compared to $A\beta_{1-40}$ suggests that *in vivo* Aβ production is shifted more towards the $A\beta_{1-40}$ fragment, Fig. (**5**). The greater lowering of $A\beta_{1-42}$ is highly critical because the 42-amino acid long Aβ is more aggregative than the other forms, and is the fastest to develop aggregations in solutions containing various Aβ peptides [7, 45]. Moreover, $A\beta_{1-40}$ aggregates inhibit $A\beta_{1-42}$ aggregation in solutions [45]. Tolfenamic acid is available in the brain following oral administration, Fig. (**7**). However, it is still possible that cortical Aβ lowering is enhanced by peripheral Aβ decline acting as a sink for Aβ from central compartments [46], such as with the lower dose of 1 mg/kg, where changes in APP and SP1 were not observed in the cortex, while cortical Aβ levels were reduced.

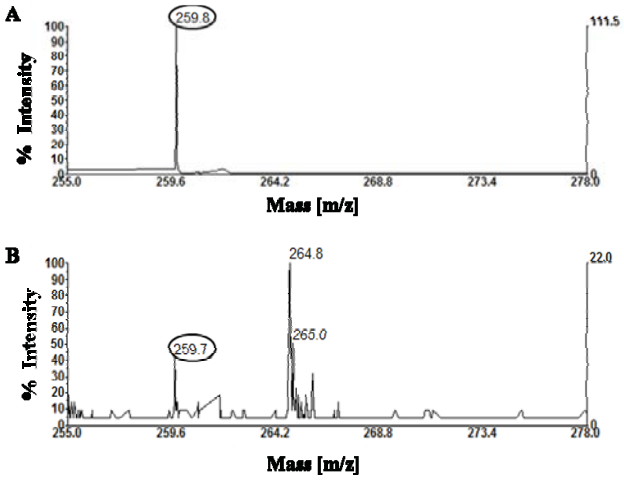

Fig. (7). Mass spectra analysis of tolfenamic acid in the brain of mice following oral administration of tolfenamic acid. Mice were orally dosed with 200 µL of 100 mg/kg dose. Tolfenamic acid levels were analyzed using mass spectrometry as described in the methods section. Ovals denote the desired tolfenamic acid peak with m/z of 259.8. **(A)** Tolfenamic acid standard in ethanol (1 mg/mL). **(B)** Brain extract from tolfenamic acid dosed animals.

CONCLUSION

This research study was designed to examine the ability of tolfenamic acid to alter Aβ and APP levels, as key pathological intermediates in AD through a mechanism that involves SP1 lowering. The study aimed at finding the effective doses and time ranges at which such outcomes occur. Tolfenamic acid was able to lower cortical SP1, APP, and Aβ levels within certain doses in wild type mice. Temporal studies showed that the decline in APP and Aβ protein levels was time dependent, occurring at the end of the study, while APP mRNA lowering occurred earlier. The effects appeared to be mediated by inducing SP1 degradation, at a rate that overwhelms SP1 production and disrupts the balance between APP *de novo* synthesis and degradation. Accordingly, oral administration of tolfenamic acid could be beneficial for AD by targeting essential pathological mediators within the disease process.

ACKNOWLEDGEMENTS

This work was supported by National Institutes of Health grants ES13022, AG027246, 1R56ES015867-01A1, and 1R01ES015867-01A2 awarded to N.H.Z. The research was made possible by using the RI-INBRE Research Core Facility, supported jointly by NCRR/NIH Grant # P20 RR016457 and the network institutions. The authors would like to thank Gail Mello, Vijay More, Dr. Remi Dosunmu, and Dr. David Serra for their help with animal handling and dosing.

ABBREVIATIONS

Aβ = Amyloid beta

AD = Alzheimer's disease

APP = β-Amyloid precursor protein

BACE1 = Beta site APP cleaving enzyme 1

SEM = Standard error of the mean

Sp1 = Specificity protein 1

SP1 = Sp1 protein

VEGF = Vascular endothelial growth factor

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Received: September 13, 2010 Revised: January 06, 2011 Accepted: January 10, 2011