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Combination therapy in a transgenic model of Alzheimer's disease

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Abstract

The pathological accumulation of the β -amyloid protein ($A\beta$) has been closely associated with synaptic loss and neurotoxicity contributing to cognitive dysfunction in Alzheimer's disease (AD). Oligomers of $A\beta_{42}$ appear to be the most neurotoxic form. Two of the most promising attempts to reduce $A\beta$ accumulation have been with scyllo-inositol, an inositol stereoisomer, that stabilizes $A\beta_{42}$ peptide and prevents it from progressing to oligomers and fibrils and *R*-flurbiprofen, a purified enantiomer of the classical racemic non-steroidal anti-inflammatory drugs (NSAID), flurbiprofen, that retains the ability to specifically lower $A\beta_{42}$. In the present study we evaluated the effects of scyllo-inositol and the combination treatment of scyllo-inositol + *R*-flurbiprofen on amyloid pathology and hippocampal-dependent memory function in 5XFAD mice, a model of $A\beta$ pathology characterized by an enormous production of $A\beta_{42}$. Our expectations were that the combination treatment of scylloinositol + *R*-flurbiprofen would have an additive effect in preventing $A\beta$ accumulation and that cognition would be improved. Mice treated with scyllo-inositol exhibit 41 and 35% reduction in the deposition of the amyloid plaques stained by antibody against $A\beta_{42}$ and $A\beta_{40}$ respectively. Scyllo-inositol was not more effective when combined with *R*-flurbiprofen for the measures tested. Scyllo-inositol treated mice performed significantly better at the radial arm water maze (RAWM) task than untreated and scyllo-inositol + *R*-flurbiprofen treated mice.

Keywords

Alzheimer's disease; Scyllo-inositol; *R*-flurbiprofen; Magnetic resonance spectroscopy

Introduction

Alzheimer's disease (AD) is an incurable age-related neurodegenerative disease, characterized by progressive memory loss and irreversible cognitive decline. $A\beta$

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neurotoxicity is established to be a critical event in AD pathogenesis and correlated with dendritic spine loss, which causes synaptic failure resulting in cognitive dysfunction (Selkoe, 2008). Although multiple other complex systems are likely involved in the pathogenesis of AD, there is substantial evidence that A β neurotoxicity, especially non-fibrillar oligomers derived from A β 42, is a fundamental event (Selkoe, 2004; Walsh and Selkoe, 2004). Many studies in both AD patients and animal models have shown that the brain levels of soluble oligomeric form of A β strongly correlate with senile plaques, one of the pathological hallmarks of AD (Koffie et al., 2009; Shankar et al., 2008). Therefore, many therapeutic strategies aiming to reduce A β accumulation and/or to accelerate its clearance are currently being developed in transgenic mouse models and cell culture models of AD.

In recent years, studies focused on cyclohexanols (inositols) have provided evidence that the scyllo-inositol interacts with A β 42 peptide promoting its conformational change and stabilizing it in small, non-fibrillar complexes that are significantly less toxic to neuronal cells (McLaurin et al., 2000). In animal models of AD, scyllo-inositol was shown to reduce plaque burden, improve performance on memory tasks and restore hippocampal synaptic plasticity (McLaurin et al., 2006; Townsend et al., 2006). Scyllo-inositol is an orally bioavailable molecule that easily crosses the blood brain barrier and can be detected in the brain by magnetic resonance spectroscopy (MRS). Using this technique we could detect increased levels of scyllo-inositol in the hippocampus and frontal cortex in mice orally treated with scyllo-inositol in water (16.5 mg/L) (Choi et al., 2010a). Scyllo-inositol has recently been in human clinical trials for the treatment of patients with mild to moderate AD (Salloway et al., 2011). The effect of scyllo-inositol treatment on the concentration of A β 42 in the cerebrospinal fluid (CSF) and on ventricular volume was evaluated in mild to moderate AD patients treated with 250 mg dose of scyllo-inositol twice daily for 78 weeks. Treatment with 250 mg dose of scyllo-inositol treatment significantly reduced CSF A β 42 concentration compared to placebo group at 78 weeks. Moreover, scyllo-inositol at 250 mg dose showed a significant increase in ventricular volume measured by MRI (Salloway et al., 2011).

The protective effects of NSAIDs against AD pathologies are observed in various experimental models and NSAIDs have been one of the therapeutic approaches for AD treatment by either delaying onset or preventing progression of AD (Eriksen et al., 2003; in t' Veld et al., 2001; Jantzen et al., 2002; Weggen et al., 2001).

This approach has been supported in transgenic mouse models of AD with studies using subsets of NSAIDs such as ibuprofen and *R*-flurbiprofen to reduce amyloidogenic A β 42 production (Eriksen et al., 2003; Kukar et al., 2007).

We have previously reported the beneficial effects of ibuprofen in lowering the A β levels in the triple transgenic mouse model of AD (3xTg-AD) (McKee et al., 2008) and in double transgenic Alzheimer mice (APPxPS1) (Choi et al., 2010b). Prophylactic treatment of 3xTg-AD mice with ibuprofen at 6 months of age showed a significant decrease in intraneuronal oligomeric A β and hyperphosphorylated tau immunoreactivity in the hippocampus, and prevented cognitive deficiency compared to untreated 3xTg-AD mice. In double transgenic mouse model of AD (APPxPS1) treatment with ibuprofen also provided significant protection against neuronal markers *N*-acetyl aspartate (NAA) and glutamate detected by MRS. In that study, we examined the effects of chronic NSAID, ibuprofen treatment on amyloid plaque deposition, A β peptide levels, and neurochemical profiles using MRS at two different ages in APPxPS1 mice. Ibuprofen significantly lowered the plaque burden in the 16- to 18-month-old mice as measured by the percent of cortical area. At 17 months of age, there was a decrease in the neuronal markers NAA and glutamate and an increase in the

astrocytic markers glutamine and myo-inositol in APPxPS1 mice compared to age matched WT mice. Ibuprofen provided significant protection against NAA and glutamate loss, however it did not significantly affect myo-inositol or glutamine levels (Choi et al., 2010b).

Although the mechanism of the A β 42 lowering NSAIDs has not been definitively established, the most convincing mechanism of the A β 42 lowering activity of selected NSAIDs suggests a direct allosteric modulation of γ -secretase activity. This action of these NSAIDs was independent of their COX activity (Weggen et al., 2001).

R-Flurbiprofen is an enantiomer of flurbiprofen, a class of NSAIDs. *R*-Flurbiprofen practically lacks COX-activity and in humans undergoes very limited chiral inversion to the *S*-enantiomer that maintains its activity against COX (Geisslinger et al., 1994; Wechter et al., 1994). This enables the use of high levels of *R*-flurbiprofen in the absence of COX mediated side effects. It has been reported that chronic administration of *R*-flurbiprofen attenuates the spatial learning deficits if given prior to plaque deposition in Tg2576 mice (Kukar et al., 2007).

However, the clinical results from phase III trial of tarenflurbil (formerly *R*-flurbiprofen) failed to slow cognitive and functional decline over 18 months in patients with mild Alzheimer's disease (Green et al., 2009).

AD remains an incurable disease. Despite promising results in preclinical studies over the past several years, clinical trials have failed to show efficacy in AD patients. The combination of pharmacological therapies has been successfully developed for the treatment of several diseases, including cancer and diabetes. We designed this study to test the hypothesis that the combination therapy of scyllo-inositol with *R*-flurbiprofen would have an additive effect and further ameliorate and/or reverse amyloid pathology and cognitive decline in 5XFAD mice.

Material and methods

Mice

5XFAD transgenic mice, developed by Oakley (Oakley et al., 2006), coexpress human APP and PS1 by combining multiple FAD mutations [APP K670N/M671L (Swedish) + I716V (Florida) + V717I (London) and PS1 M146L + L286V] with neuronal expression driven by the Thy-1 promoter were purchased from Jackson Laboratory. WT mice from 129/C57BL6 background strain were used as control animals. Only female mice were used in the current study (10 mice per group). All animal experiments were carried in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the local animal care committee.

Diet and drug protocol

Mice were housed on a 12 h light:12 h dark schedule. All mice were given access to food and water ad libitum. Scyllo-inositol (AG Scientific, 3.3 mg/kg/day) was added into animal drinking water. Water has been changed twice weekly. *R*-Flurbiprofen was formulated into animal diets (Research Diets Inc., NJ, USA, 10 mg/kg/day). Food that contains *R*-flurbiprofen has been changed at least once a week. We monitored mice for general well being, food and water consumption and no side effects related to either treatment have been observed.

Tissue collection

At 8 months of age, the cognitive ability of the mice (regular (untreated) and scyllo-inositol + *R*-flurbiprofen or scyllo-inositol -treated mice) was tested in the radial water maze spatial memory test. Upon completion of the cognitive test, mice were euthanized and brains removed for histological analysis. The left hemisphere was post-fixed with 4% paraformaldehyde solution for 24 h and cryoprotected in a graded series of 10% and 20% glycerol/2% DMSO solution for histological analysis. The right hemisphere was dissected at the frontal level and saved for the Enzyme-linked immunosorbent assay (ELISA) assay.

Histology/immunohistochemistry

Brains were serially cut at 50 μ m on a freezing microtome and were immunostained for A β 1–40 and A β 1–42 (Invitrogen, Grand Island, NY #44348A, #44–344) to define A β deposits. Immunohistochemical procedures were performed as previously described (Kowall et al., 2000). In brief, free-floating sections were incubated overnight in primary antibody followed by PBS (Phosphate buffered saline) washes and incubation in peroxidase-conjugated secondary antibody followed by development using 3,3'-diaminobenzidine tetrahydrochloride (DAB) as a chromagen.

Quantitative analysis of A β deposits

For the estimation of A β plaque intensities, three serial sections per mouse brain were analyzed blindly using custom software written in Matlab. The most rostral section analyzed was at the anterior commissure level ($\sim$ 0.1 mm anterior to bregma), and each successive section was at 0.34 mm increments caudal to the first. The cortex area used for A β plaque analysis was delineated from bregma -0.1 to -0.34 mm. Images were acquired by using a Nikon Optiphot microscope. In each section, the area of interest in the cortex was manually outlined at 2 $\times$ magnification to determine the area occupied by the plaque and stained plaque area was automatically detected to give a percent area.

A custom written MatLab program was used to measure the intensity of staining for A β staining in the cortex. Background intensity for each image was measured on an area away from all A β staining. Background was subtracted from A β stained area.

Radial arm water maze

At 8 months of age, 5XFAD treated mice and control mice were trained to find an invisible submerged platform of 14 cm in diameter, in a 6-arm-maze created inside a 4 feet diameter pool, using a variety of visual extra maze cues located surrounding it. Testing took place until a group reached the pre-set criteria of committing less than 2 errors in the last acquisition trial. Each testing day consisted of four acquisition trials (T1–4) and one memory retention trial (T5) 30 min after the last acquisition trial. For any given day of testing, the submerged escape platform was positioned near the end of a randomly designated goal arm for that day and the starting position for T1–4 was chosen in a semi-random sequence from the remaining 5 arms. The memory retention trial T5 started from the same arm as the last acquisition trial (T4) of the day. Mice were allowed to swim for up to 1 min to find the submerged platform and then they were allowed to remain on the platform for 30 s. Alternatively, if a mouse did not find the platform in the 60 s trial, it was guided to the submerged platform where it stayed for 30 s. Errors were charged upon entering all 4 paws in an incorrect arm, upon entering the goal arm but failing to find the submerged platform or upon failing to select an arm after 20 s. Every time an error occurred, the mouse was gently pulled back to the start arm for that trial. The number of errors during all trials was recorded and used to calculate the index of daily learning (the difference of errors between the first, T1, and the last acquisition trial, T4) and the index of memory (the

difference in errors between the last acquisition trial, T4, and the memory retention trial, T5). Testing lasted until a group reached the pre-set criteria of committing less than 2 errors in the last acquisition trial of the day (T4). The errors for the T1 and T4 of testing of each mouse were averaged and used for statistical analysis.

ELISA assay

Dissected frontal brain tissue was homogenized with 5× volumes (w/v) of Tris-buffered saline (TBS) and centrifuged at 16,000 *g* for 1 h at 4°C. After removing supernatants, the resulting pellet was resuspended with 8× cold 5 M guanidine HCl buffer to analyze insoluble A β . To determine A β levels in extractions from 8-month-old 5XFAD mice, human A β 40 and A β 42 ELISA kits (Invitrogen, Grand Island, NY) were used according to the manufacturer's specifications. Briefly, homogenized samples were added into the wells of a 96-well plate, samples were then mixed with a cleavage-specific antibody to either A β 40 or A β 42. After an overnight incubation at 4 °C, plates were washed and incubated with the secondary antibody for 30 min at 25 °C. Washed wells were developed by the addition of a substrate. The substrate reaction was then stopped and color intensity was measured at 450 nm.

High resolution magic angle spinning spectroscopy (HRMAS)

In vitro MRS was collected as previously published (Choi et al., 2010a,b). We collected high resolution magic angle spinning (HRMAS) spectra on Bruker 14 T (Billerica, MA). We obtained tissue punches of freshly frozen cortex from mice. The punches were 1 mm in diameter and were taken from M1 and M2 motor cortices at about the center and at approximately 0.2 mm from bregma. The MRS punch was just posterior to the cortex tissue punches used for the ELISA measurements. The dissected tissue sample was placed into a glass cylinder positioned in a 3 mm zirconium oxide MAS rotor (volume 50 μ L). HRMAS measurements were performed using a sample spinning rate of 3.6 kHz selected to push the spinning side bands outside the frequency region of the metabolites. The experiments were performed at 4 °C to minimize tissue degradation.

Data were acquired using a rotor synchronized, T₂-filtered Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence [90 – (τ – 180 – τ – Acq)_n] with two different effective TEs (100 ms/10 ms). The longer TE serves to remove the lipid/macromolecular resonances and the short TE retains them. The interpulse delay, τ , is synchronized to the rotor frequency, and is 272 μ s. The *n* value for the relatively short T₂ filter was 36 and for the long TE it was 360. The short τ value removes all the T₂*-like effects on the line shapes. The long T₂ filter yields approximately 95% of the total spectral intensity of all metabolites of interest compared to the short TE. Other acquisition parameters were a 90° pulse of 5–10 μ s, a spectral width of 8 kHz, 16 K complex points, 256 averages and a TR of 5 s. Samples were placed in the rotor with a small amount of D₂O (Sigma-Aldrich, Milwaukee, WI) for locking and shimming.

Data were analyzed using the Chenomx (Edmonton, Alberta, Canada) package fitting the entire metabolite spectrum for each neurochemical. HRMAS data were reported as molar ratios to creatine since our prior studies of the absolute concentrations in multiple different AD transgenic mouse models showed no change in total creatine between WT and any of the AD models (Choi et al., 2010a,b; Dedeoglu et al., 2004). In a paper recently published by Mlynarik et al. (2012) examining the 5XFAD mice using a water normalization method, no significant change in the creatine concentrations was noted in the 5XFAD mice compared to WT. Statistical analysis of the MRS data was performed using a one-way ANOVA with the Tukey HSD post-hoc tests for group comparisons for each metabolite. Classification of the data was performed using WEKA (Mark Hall, Eibe Frank, Geoffrey Holmes, Bernhard

Pfahring, Peter Reutemann, Ian H. Witten (2009); The WEKA Data Mining Software: An Update; SIGKDD Explorations, Volume 11, Issue 1).

Results

Effect of scyllo-inositol alone and in combination with R-flurbiprofen treatments on spatial learning memory

Treatment of 5XFAD mice with scyllo-inositol or scyllo-inositol + *R*-flurbiprofen combination started at 7 months of age and continued for one month. No side effects were observed to either treatment. Body weights of 5XFAD treated mice, untreated 5XFAD and WT mice were comparable at all times. At 8 months of age, spatial learning and memory were tested in a RAWM. In this test, mice are trained in 4 consecutive trials per day (T1-T4) for 60 s each to escape onto a submerged platform placed at the end of one of the 6 arms using extramaze cues. An error is counted every time a mouse enters a wrong arm or enters the right arm but fails to find the platform. The number of errors/trial is recorded and used as a measure of learning. A group of mice is considered to have learned the task when they reach the milestone of committing 3 or less errors/trial. Fig. 1 shows the number of errors committed by each group of mice in T4 during the consecutive testing days. Scyllo-inositol treated mice reached the milestone of committing 3 or less errors/trial in day 12 of training however, untreated 5XFAD mice and 5XFAD mice treated with the combination therapy of scyllo-inositol + *R*-flurbiprofen were unable to reach the learning stage. Control untreated WT mice reached 3 or less errors/trial by day 6. In order to facilitate inter-group comparisons we binned the data from days 1–3 (baseline values), days 4–6, 7–9, and 10–13. The comparisons showed (Table 1) that there were significant differences in days 10–12 between the WT, regular diet (untreated mice) and scyllo-inositol + *R*-flurbiprofen treated group but not with the scyllo-inositol treated group. After day 6 we tested the short-term memory in an additional trial (T5) performed 30 min after T4. The results in T5 during days 10–12 were pooled and plotted together with the results in T1 and T4. In this graph, learning is shown by the difference between the number of errors in T1 and T4 while the memory is shown by the difference between T4 and T5. For WT and scyllo-inositol treated 5XFAD mice, the number of errors in T4 is significantly lower than in T1 and the number of errors in T5 are not different to that in T4 (Table 2). These results indicate that mice learned the task of escaping from the water onto the platform and that they have no impaired short-term memory. On the other hand, the number of errors in T1 and T4 committed by untreated 5XFAD and 5XFAD treated with scyllo-inositol + *R*-flurbiprofen are not significantly different from each other indicating again that these two groups of mice did not learn the task of escaping from the water onto the platform. There were significant differences between the scyllo-inositol treated and regular diet mice in T5.

These results suggest that scyllo-inositol treatment alone might have the potential to improve behavioral deficits induced by AD pathology.

Effect of scyllo-inositol alone and in combination with R-flurbiprofen treatment on A β plaque load in 5XFAD mice

At 8 months of age, the immunohistochemical staining of the brains with specific A β 42 antibody showed advanced A β pathology in 5XFAD mice, including intraneuronal A β and extracellular plaques, throughout the cortex, hippocampus, and the amygdala (Fig. 2A). The densitometric analysis of the immunostained brains indicates that scyllo-inositol treatment significantly decreased A β plaque burden. We observed a 41% and 35% decrease in A β 42 and A β 40 plaque burden respectively in 5XFAD mice treated with scyllo-inositol compared to untreated group (Fig. 2B, * $p < 0.05$). Treatment with both scyllo-inositol alone or with scyllo-inositol + *R*-flurbiprofen resulted in significant decreases in A β plaque burden. The

decrease in A β 40 plaque burden was significant ($F_{29,2} = 8.46$, $p < 0.01$) with regular diet being different from scylloinositol ($|q| = 5.75$, $p < 0.01$) and scyllo-inositol + *R*-flurbiprofen ($|q| = 3.65$, $p < 0.05$). A similar pattern was found for A β 42 plaque burden ($F_{29,2} = 10.45$, $p < 0.001$) with regular diet being different from scylloinositol ($|q| = 6.46$, $p < 0.001$) and scyllo-inositol + *R*-flurbiprofen ($|q| = 3.04$, $p < 0.1$). The scyllo-inositol and scyllo-inositol + *R*-flurbiprofen groups were not significantly different from one another.

Effect of scyllo-inositol alone and in combination with *R*-flurbiprofen on A β levels in 5XFAD mice

To evaluate the effect of scyllo-inositol and scyllo-inositol + *R*-flurbiprofen treatments on A β levels, we assayed frontal cortex brain extracts by ELISA. There was remarkable high levels of A β 40 and A β 42 in the 5XFAD mice at 8 months of age and the levels of both A β 40 and A β 42 were decreased in the scyllo-inositol treated group compared to untreated-5XFAD mice after the 1 month treatment, however the decreases did not reach significance. The levels of A β 40 and A β 42 were not statistically different in scyllo-inositol + *R*-flurbiprofen treated group compared to scyllo-inositol treated group. We detected increased soluble A β 40 and A β 42 levels in SCY + RF treatment and increased soluble A β 40 level in SCY-treated group, however the increases were not significant (Figs. 2C, D).

Neurochemical alterations in 5XFAD cortex

The 5XFAD mice show numerous neurochemical alterations compared to WT mice using tissue punches from the cortex. There were significant changes between the WT and 5XFAD mice in numerous neurochemicals at eight months of age including decreases in GABA, glutamate and NAA, and increases in glutamine and myo-inositol as shown in the averaged HRMAS spectra from WT and regular diet 5XFAD mice (Fig. 3). Treatment with scyllo-inositol or scylloinositol + *R*-flurbiprofen both yielded an increase in scyllo-inositol concentrations in the brain (Fig. 4). Both treatments showed some protection against the changes observed in the regular diet mice compared to the WT (Fig. 5). In general, the changes brought the values closer to the WT for the dual treatment of scyllo-inositol + *R*-flurbiprofen except for the changes in myo-inositol for which the scyllo-inositol alone treatment was better (i.e. brought the values closer to the WT values).

We previously published on using metabolite ratios in APPxPS1 mice to get a better discrimination for potential AD treatments (Choi et al., 2010a). Since the 5XFAD mice show increased glutamine (gln) and myo-inositol (myo) and decreased GABA, glutamate (glt) and *N*-acetyl aspartate (NAA), we used a ratio composed of those metabolites (i.e. $(\text{myo} \times \text{gln}) / (\text{GABA} \times \text{glt} \times \text{NAA})$). This ratio showed excellent discrimination between the groups (Fig. 5), however the ANOVA post-hoc comparisons between groups using a Tukey HSD showed that this ratio was only better than NAA alone for the regular diet scyllo-inositol-fed group (the p values for separation of the treated groups from the regular diet animals using a post-hoc Tukey HSD for the complex ratio were WT vs. regular diet $p < 0.0001$, regular diet vs scyllo-inositol + *R*-flurbiprofen $p = 0.02$ and regular diet vs. scyllo-inositol $p = 0.03$ compared to the values for NAA alone of WT vs. regular diet $p < 0.0001$, regular diet vs. scyllo-inositol + *R*-flurbiprofen $p = 0.028$ and regular diet vs. scyllo-inositol $p = 0.27$). The ratio of gln/NAA was almost as good as the more complicated ratio. Measuring effect sizes using Cohen's d showed that the ordering of the effect sizes for comparing AD vs. WT (Cohen's d) was: NAA ($d = 3.184$) > GABA (-1.985) = Glt (-1.984) > Gln (0.946) > myo-inositol (0.622).

We found a reasonable correlation between the NAA and the cortical plaque burden as shown in Fig. 6. This correlation was not driven by the outliers as determined by a jack-

knife analysis. Further, there was a similar magnitude of correlation between the plaque burden (A β 40 and A β 42) and the ELISA measurements (Fig. 6).

We also addressed the question of whether the decreased plaque burden and/or A β 40 and A β 42 levels were related to the brain concentrations of scyllo-inositol (Fig. 7). There is a strong correlation between the brain scyllo-inositol levels and the plaque burden that was highly significant for A β 40 ($p < 0.001$) and A β 42 ($p < 0.01$). The correlations with the ELISA data were weaker and in the case of A β 42 was not significant (Fig. 7).

In order to better assess the ability of the MRS data to classify the data with respect to the therapeutic attributes we examined a number of different classifier schemes. Although the numbers of mice we studied were too small to make firm conclusions about which variables and classifier schemes would be optimal in a large population study, the data support some rather strong conclusions. First we examined the MRS data using the four metabolites: GABA, glutamate, glutamine and NAA to examine the neuroprotection. We chose not to use myo-inositol since it is unknown and how it may be influenced by dietary intake of scyllo-inositol. Further, given the prior results of McLaurin's lab it is possible that myo-inositol could be protective (McLaurin et al., 2000, 2006). The first classifier we used was linear discriminant analysis (LDA). Using LDA showed that 97% of the variance could be explained by the first discriminant function which was the only significant function via Wilk's lambda (Fig. 8). The distance of the centroids of the AD groups from WT in a 3 function space was regular diet (4.52) > scyllo-inositol (3.39) > scyllo-inositol + *R*-flurbiprofen (2.77). The classification was quite efficient for the regular diet and WT animals. If one includes the scyllo-inositol and scyllo-inositol + *R*-flurbiprofen treatment groups together the overall classification efficiency, using hold-out analysis, was 79.4% for LDA. Other classifiers, such as an unsupervised, multi-layer perceptron (with a linear activation function) or support vector machines with simple logistic regression yielded confusion matrices that showed similar classification efficiencies to the LDA. Areas under the receiver operator characteristic (ROC) curves ranged between 0.78–0.9 for the latter two classifiers for the regular and WT animals.

We also examined the effects of combining the MRS and plaque data. Since we noted a reasonable correlation between the NAA and plaque burden (Fig. 6) we chose to use only three variables: NAA which had the largest effect size, glutamine (since the ratio of gln/NAA performed better than most other metrics for post-hoc statistical testing between group differences) and A β plaque burden. For this analysis, since the WT animals show no plaques it is obvious that perfect separation for this group could come from this parameter alone, thus we did not include the WT in the classification. Using the combination of the two MRS variables with the plaques produced a much better separation than using either the MRS data alone, or plaques and the ELISA data (Table 3).

Discussion

Imaging studies have shown that A β plaques start to accumulate prior to onset of clinical symptoms and drugs currently being developed mostly target A β for prevention of AD. In view of the potential benefit of scyllo-inositol inhibiting the aggregation of A β into oligomers and A β neurotoxicity, we proposed combining scyllo-inositol with a complementary therapy, which targets A β through a distinct mechanism to further increase the beneficial effects of scyllo-inositol. To test this concept, we combined scyllo-inositol with *R*-flurbiprofen, a compound that has been previously shown to reduce amyloid deposition in Tg2576 mouse model of AD (Eriksen et al., 2003; Kukar et al., 2007) by decreasing the production of A β 42.

Eight months old 5XFAD mice present cognitive dysfunction shown by the inability of untreated 5XFAD mice to learn the task of finding the submerged platform in a radial arm water maze test. We also found that oral treatment of 5XFAD mice with scyllo-inositol for 1 month (from 7 to 8 months of age) results in improved cognition as shown in 8 months old treated 5XFAD mice in learning the task of finding the submerge platform. On the other hand, the behavior of 5XFAD mice treated with the combination of scyllo-inositol + *R*-flurbiprofen did not differ from that of untreated transgenic mice. Improved cognitive function in scyllo-inositol treated mice was accompanied by a significant reduction of A β plaque burden and decreased levels of insoluble A β 40 and A β 42.

Using NMRS, we previously detected a 2- to 3-fold increase in brain scyllo-inositol levels following dietary supplementation of scylloinositol in APPxPS1 and 3xTg-AD mice (Choi et al., 2010a). Consistent with these findings, we now found a significant increase in scylloinositol levels in the brains of 5XFAD mice following scyllo-inositol administration (alone or in combination with *R*-flurbiprofen) by means of NMR analysis (Fig. 4). These data show a similar increase in scyllo-inositol (ca. a factor of 2) to that we noted earlier in the frontal cortex of triple transgene mice and slightly smaller than that we noted in the frontal cortex of APPxPS1 mice (Choi et al., 2010a). Thus, it is clear that scyllo-inositol can be increased in the brains of 5XFAD mice. Similar to a previous study of the 5XFAD mice by Mlynarik et al. (2012) we noted a decrease in NAA and GABA. Decreases were also noted in glutamate along with increases in myo-inositol and glutamine. Protection of these changes by the two treatments was noted, with the largest effect size for NAA. Considering all the MRS data (shown in Figs. 3–6) it is clear that there was somewhat a better protection noted for the scylloinositol + *R*-flurbiprofen treatment compared to the scyllo-inositol alone. This is counter to what was observed for the behavioral and plaque data where the scyllo-inositol alone performed better. Interestingly, the scyllo-inositol alone treatment normalized the myo-inositol levels in the AD mice to WT levels (Fig. 3). Given that there is some evidence that myo-inositol can be protective against A β pathology in vitro and in AD mice (McLaurin et al., 2000, 2006), the increase in myoinositol noted in both AD mice and in human AD patients may actually be beneficial. This issue requires further investigation. No matter how the data is analyzed, however, the added value of the flurbiprofen is rather modest.

We also found that the scyllo-inositol concentration in the brain correlated well with the plaque density, especially with the A β 40 plaques. This was a feature of all the correlations, that the highest R values were obtained for the A β 40 plaques. This may relate to the noise created by evaluation of the diffuse borders of the A β 42 plaques. Using different classifiers we were able to correctly classify a high fraction of the mice into different groups (AD, AD treated, WT). The combination of the pathology and MRS data was quite successful, lending support to classification schemes using multimodal data in human studies. The addition of the classification schemes becomes important when trying to find additional metrics using in vivo imaging with which AD can be diagnosed and additionally finding ways to combine multi-modal data (e.g. imaging and behavior or cognitive tests, imaging and pathology) to assess potential therapies. Using classifiers may aid, in intuitive fashions, to predict which patients may go on to progress to AD from an MCI population (Sabuncu et al., 2012).

Similar to our findings, studies lead by Dr. J. McLaurin (Fenili et al., 2007; McLaurin et al., 2006) showed that oral scyllo-inositol treatment increased the scyllo-inositol concentration within the brain, inhibited A β levels and plaque formation, and improve cognition in TgCRND8 mice. In another study Townsend et al. (2006) demonstrated that scyllo-inositol prevented the inhibition of long term potentiation (LTP) caused by soluble A β oligomers in mouse hippocampal slices and cognitive deficiency in vivo (Townsend et al., 2006). Increased scyllo-inositol levels were also detected in the brain of patients with AD using

MRS (Griffith et al., 2007). In earlier studies, a 2-week therapeutic treatment with *R*-flurbiprofen at 20 month-old Tg2576 AD mice resulted in a significant decrease in A β plaque burden and levels of insoluble A β 42. Reduction in A β 42 levels and plaque burden did not improve spatial learning in these mice but chronic administration of *R*-flurbiprofen was able to attenuate spatial learning deficits when given prior to plaque deposition in Tg2576 mice (Kukar et al., 2007).

Nitric oxide-releasing derivatives of flurbiprofen (HCT 1026 and NCX2216) have also been shown to reduce amyloid pathology following a long-term administration to A β -depositing mice (Gasparini et al., 2005; Jantzen et al., 2002).

Given these A β -decreasing effects of *R*-flurbiprofen, we decided to test whether combining *R*-flurbiprofen and scyllo-inositol would potentiate their effect on A β levels.

Administration of *R*-flurbiprofen and scyllo-inositol resulted in a significant decrease in A β plaque burden in the brains of 8 months old 5XFAD mice however trends towards lower levels of insoluble A β 40 and A β 42 were not statistically significant. Surprisingly, the soluble A β 40 and A β 42 levels were increased in this group however the increases did not reach significance.

However, treatment with scyllo-inositol along with *R*-flurbiprofen did not show additive effect to decrease A β level and plaque burden in contrast to scyllo-inositol treatment alone in 5XFAD mice model.

Long-term treatment with ibuprofen (for 3 months) did not decrease A β plaque load in 5XFAD mice at the age of 6 months (Hillmann et al., 2012) and similar to our findings, increased levels of TBS-soluble A β 42 were detected in ibuprofen-treated 5XFAD mice brain. This phenomenon can be explained by the presence of mutations in the APP and PSEN1 genes in 5XFAD mice model leading to insensitivity to the A β 42 lowering effects of ibuprofen.

Notably, the results from other studies supported the conclusion that the A β 42 lowering effects of certain NSAIDs and related small molecules γ -secretase modulators (GSMs) are altered by mutations in the PSEN1 (Presenilin 1) gene in cell culture and in an AD mouse model (Czirr et al., 2007; Page et al., 2008; Weggen et al., 2003).

Most preclinical studies in AD have relied on the transgenic mouse models of AD carrying different mutations. Using these transgenic models, these studies show that certain NSAIDs such as ibuprofen and *R*-flurbiprofen exert their potential neuroprotective effect at reducing A β by modulation of γ -secretase activity.

One caveat in our results is that we did not test the effect of *R*-flurbiprofen alone in 5XFAD mice model. We designed this study on improving the efficacy of scyllo-inositol treatment in combination with a modulator of A β production, *R*-flurbiprofen, to test if these two therapies might have synergistic or additive therapeutic effects to reduce A β . Further studies are needed to determine whether the outcome of scyllo-inositol and *R*-flurbiprofen combination treatment could be improved when these drugs are administered for a longer period of time starting the treatment before the A β pathology develops.

Conclusions

These results show that scyllo-inositol treatment lead to significant reduction in the A β plaque burden in the brains of 5XFAD mice, protected the decrease in NAA, GABA and glutamate and improved memory thus providing a rationale for the potential benefit of the

use of scyllo-inositol in treatment of AD. However, treatment with scyllo-inositol combined with *R*-flurbiprofen was overall not more effective to decrease A β levels and plaque burden or protect against neurochemical changes when compared to scyllo-inositol treatment alone in 5XFAD mice.

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Fig. 1.

Radial arm water maze learning (Trial 4; T4) and memory retention (Trial 5; T5). Mice were tested in a 12-day, 6 arm version of the RAWM. A. Graph shows mean number of errors made over the 12-day trial period following 1 month of scyllo-inositol (SCY) and scyllo-inositol + *R*-flurbiprofen (SCY + RF) treatment. WT mice learn the task of finding a submerged platform by day 6. SCY treated 5XFAD mice learned it by day 9 but SCY + RF and untreated mice did not learn it. B. Performance in Trials 1, 4 and 5 were average for the last 3 days of testing (days 10, 11 and 12). WT and SCY treated 5XFAD mice acquire the RAWM task (number of errors in T4 are different than in T1) and have short-term memory (number of errors in T5 are different than in T1 and not different than in T4). Regular and SCY + RF 5XFAD mice do not learn (number of errors in T4 and T5 are not different than in T1). * Significance compared to untreated # significance compared to number of errors in T1. $p < 0.01$, ($n = 10$).

Fig. 2.

A. Representative pictures showing Aβ42 immunostained brain sections of 5XFAD mice from regular (untreated) and treated with scyllo-inositol (SCY), and with scyllo-inositol and

R-flurbiprofen (SCY + RF) (Magnification $\times 20$, $\times 100$). B. Effects of scyllo-inositol and scyllo-inositol + *R*-flurbiprofen treatments on A β plaque burden. Brain sections of representative groups were stained for A β plaque using A β 40 and A β 42 antibodies. Plaque burden (expressed as a fraction of cortical areas) at 8 months was measured as a function of scyllo-inositol and scyllo-inositol + *R*-flurbiprofen combination treatment. Scyllo-inositol significantly lowered the plaque burden after 1 month of treatment in the 8 month-old mice compared to the regular diet as measured by the percent of cortical area. C. Analysis of the effect of scyllo-inositol and scyllo-inositol + *R*-flurbiprofen treatment on levels A β 40 and A β 42 by ELISA using soluble (TBS-soluble) and D. insoluble (Guanidine-HCL soluble) protein extracts from pre-frontal cortex. Scyllo-inositol and scyllo-inositol + *R*-flurbiprofen combination treatment were decreased and the levels of insoluble A β 40 and A β 42 however did not reach significant decrease. The increased soluble A β 40 and A β 42 levels were detected in the treatment groups however the increases were not significant, (* $p < 0.05$), (n = 10).

Fig. 3.

Averaged HRMAS spectra from cortex in WT and regular diet AD animals. The spectra have been averaged 6 WT and 9 AD animals. The spectra are normalized to creatine which our previous studies in AD mice showed and doesn't change (Choi et al., 2010a,b). There are large decreases in GABA, glt and NAA and smaller increases in myo-inositol and gln.

Fig. 4. Average HRMAS spectra from WT (n = 6), regular diet AD (n = 9), scyllo-inositol treated AD (n = 9) and scyllo-inositol + *R*-flurbiprofen treated AD (n = 10). There is an increase in the scyllo-inositol peak as shown on the bar graph on the right.

Fig. 5.

Individual neurochemical values in the four groups of mice showing the decreases in GABA, glutamate and NAA and the increases in glutamine, myo-inositol and the ratio of (gln*myo)/(GABA*glut*NAA). The p values shown are the p-values determined from the omnibus one-way ANOVA for the four groups. Other metabolites not shown (lactate, alanine, succinate, cholines, taurine) were not significantly different between the groups.

Fig. 6.

Correlations between cortical plaque density and NAA and ELISA measurements. Shown are the correlations between the NAA and ELISA Aβ40 and Aβ42 and cortical plaque densities separated into Aβ40 and Aβ42 plaques. The correlations for NAA were significant for both the Aβ40 and Aβ42 using jack-knife analysis (R values = 0.543 for Aβ40 ($p < 0.01$) and 0.45 for Aβ42 ($p < 0.05$)) and were not driven by the points on the far left. The correlations were also significant between the plaque density and ELISA measurements for both Aβ40 ($R = 0.54$; $p < 0.01$) and Aβ42 ($R = 0.43$; $p < 0.05$). Units for the ELISA are pg Aβ/mg tissue.

Fig. 7.

Correlations between plaque levels and Aβ levels measured using ELISA with the scyllo-inositol levels measured in the brain using HRMAS. The correlations were all significant at $p < 0.05$ for all the values except for the ELISA Aβ42.

Fig. 8.

Linear discriminant analysis showing the separation between the four groups of animals using GABA, glt, gln and NAA as variables. There is excellent separation between the WT and regular diet AD animals. The two treated groups cluster in-between the WT and regular diet animals as shown in the classification matrix. The only significant function was the first which accounted for 97% of the variance.

Table 1

Contrast table for water maze learning.

	Pre	Days 4–6	Days 7–9	Days 10–12
WT vs. REG	NS (0.157)	NS (0.132)	0.013	0.003
WT vs. SCY +RF	NS (0.879)	NS (0.087)	<.00001	0.009
WT vs. SCY	NS (0.136)	0.037	0.008	NS (0.083)
SCY vs. REG	0.007	NS (0.402)	NS (0.671)	NS (0.169)
SCY +RF vs. REG	NS (0.22)	NS (0.872)	NS (0.191)	NS (0.69)
SCY vs. SCY +RF	NS (0.108)	NS (0.461)	NS (0.465)	NS (0.324)

Data were analyzed using a within-subjects controlled trial approach. To minimize total contrast numbers data were binned in three day intervals as pre (days 1–3) etc. Significant contrasts are shown in BOLD.

Table 2

Contrast table for water maze memory.

	Pre	T4	T5
WT vs. REG	NS (0.155)	0.005	0.004
WT vs. SCY+RF	NS (0.081)	0.039	0.015
WT vs. SCY	0.042	NS (0.163)	NS (0.215)
SCY vs. REG	NS (0.845)	NS (0.056)	0.013
SCY +RF vs. REG	NS (0.926)	NS (0.47)	NS (0.597)
SCY vs. SCY +RF	NS (0.914)	NS (0.314)	NS (0.092)

Data were analyzed using a within-subjects controlled trial approach (Spreadsheets for Analysis of Controlled Trials, with Adjustment for a Subject Characteristic, Will G Hopkins, Sportsmedicine 10, 46-50, 2006; A Decision Tree for Controlled Trials Alan M Batterham, Will G Hopkins, Sportsmedicine 9, 33-39, 2005). Significant contrasts are shown in BOLD.

Table 3

Classifiers for MRS and plaque data.

Classified as:	REG	SCY	SCY+RF	WT	% Correct ^a
<i>MRS only</i> ^b					
Reg	7	1	1	0	0.778
SCY	2	6	1	0	0.778
SCY +RF	2	4	4	2	0.8
WT	0	1	0	5	0.833
LDA overall					79.4%
Reg	4	3	2	0	0.444
SCY	0	5	3	1	0.889
SCY +RF	3	1	3	3	0.4
WT	0	0	1	5	0.833
SVM+Logistic					61.8%
Reg	6	2	1	0	0.667
SCY	3	2	4	0	0.667
SCY +RF	2	1	4	3	0.5
WT	0	0	1	5	0.833
Perception					64.7%
<i>Plaques+ELISA</i> ^c					
Reg	7	0	3		0.7
SCY	0	7	3		1
SCY +RF	4	1	5		0.6
SVM+Logistic					76.7%
<i>MRS +Plaques</i> ^d					
Reg	9	0	0		1
SCY	1	6	2		0.889
SCY +RF	0	3	7		1
SVM+Logistic					96.4%

^aSCY and SCY+RF are allowed to mix categories for a correct assignment.^bMRS classifications based upon GABA, glt, gln and NAA.^cClassified using both A β 42 and A β 40 plaque density and ELISA values.^dClassified using NAA, gln and total A β plaque density. Incorporation of the plaque data means dropping the WT from the analysis since that group has no plaques.