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Dynamics of ß-Amyloid Reductions in Brain, Cerebrospinal Fluid and Plasma of ß-Amyloid Precursor Protein Transgenic Mice Treated with a γ-Secretase Inhibitor

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Running Title: Aß in Transgenic Mice Treated with a γ-Secretase Inhibitor

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Non-Standard Abbreviations:

Aß beta amyloid peptide

AD Alzheimer's disease

APP amyloid precursor protein

CHAPS 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propane sulfonate

CNS central nervous system

CSF cerebrospinal fluid

CTF C-terminal fragment of APP

ELISA enzyme-linked immunosorbent assay

FA formic acid

ISF interstitial fluid

kDa kilodalton

LC/MS/MS liquid chromatography tandem mass spectrometry

PBS phosphate buffered saline

PS presenilin

TCR T cell receptor

TBS Tris buffered saline

TBST Tris buffered saline with Tween-20

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ABSTRACT

γ-Secretase inhibitors are one promising approach to the development of a therapeutic for Alzheimer's disease (AD). γ-Secretase inhibitors reduce brain ß-amyloid peptide (Aß), which is believed to be a major contributor in the etiology of AD. Transgenic mice overexpressing the human ß-amyloid precursor protein (APP) are valuable models to examine the dynamics of Aß changes with γ-secretase inhibitors in plaque-free and plaque-bearing animals. BMS-299897, a γ-secretase inhibitor, showed dose and time dependant reductions of Aß in brain, cerebrospinal fluid (CSF) and plasma in young transgenic mice, with a significant correlation between brain and CSF Aß levels. Because CSF and brain interstitial fluid (ISF) are distinct compartments in composition and location, this correlation could not be assumed. In contrast, aged transgenic mice with large accumulations of Aß in plaques showed reductions in CSF Aß in the absence of measurable changes in plaque Aß in the brain following up to two weeks of treatment. Hence, CSF Aß levels were a valuable measure of γ-secretase activity in the central nervous system (CNS) in either the presence or absence of plaques. Trangsenic mice were also used to examine potential side effects due to Notch inhibition. BMS-299897 was 15 fold more effective at preventing the cleavage of APP than of Notch in vitro. No changes in the maturation of CD8+ thymocytes or of intestinal goblet cells were observed in mice treated with BMS-299897, showing that it is possible for γ-secretase inhibitors to reduce brain Aß without causing Notch mediated toxicity.

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Diagnosis of AD is based on key pathological features at autopsy in the presence of dementia: loss of neuronal mass, intracellular tangles, and extracellular plaques. Plaques are composed predominantly of Aß. Aß has both N and C-terminal heterogeneity, with the C-terminus usually ending at residues 40 or 42. Although Aß40 is 80-90% of the total, Aß42 is most associated with disease and is predominant in plaques. Aß is produced by two cleavage events in APP (Wolfe, 2001). ß-secretase, now identified as ß-site APP cleaving enzyme, makes the initial N-terminal cleavage, releasing a soluble N-terminal form of APP. The remaining, membrane-bound C-terminal fragment (CTF) of APP is cleaved by γ-secretase to release soluble Aß along with the APP intracellular domain. Alternatively, APP can also be cleaved by α-secretase, which creates a shorter CTF and a non-amyloidogenic peptide, p3, after γ-secretase cleavage. γ-Secretase is a large protein complex with presenilin (PS)-1 or PS-2, nicastrin, Aph-1 and Pen-2 as minimal critical components (Selkoe and Kopan, 2003). γ-Secretase inhibitors are being actively pursued in the pharmaceutical industry for the treatment of AD (Wolfe, 2001; Josien, 2002).

When using γ-secretase inhibitors in vivo, it is important to understand the dynamics of Aß movement between and within brain, CSF and plasma. Extracellular brain Aß can exist in 3 basic forms: fibrillar deposits or plaques, oligomeric Aß, and monomeric Aß. Oligomeric and monomeric Aß are soluble and exist in the ISF within the brain, although only a small fraction of this Aß is freely diffusible (Cirrito et al., 2003). The ISF predominantly flows into the lymphatic system after moving through periarterial sheaths and into the cervical lymph nodes (Weller, 1998). Only an estimated 10% of CSF originates from ISF. CSF is created by the choroid plexus, which can also produce, transport, and possibly degrade Aß (Chodobski and Szmydynger-Chodobska, 2001; Ghersi-Egea and Strazielle, 2001). Intracellular Aß

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(predominantly Aß42) also exists and accumulates within neurons, further complicating the analysis of brain Aß (Wilson et al., 2003). Unlike most peptides, Aß is actively transported from brain to plasma via lipoprotein related protein (Shibata et al., 2000) and from plasma to brain via RAGE (Deane et al., 2003). There is also evidence for a dynamic equilibrium between CSF and plasma Aß in transgenic mice, which is altered when plaques begin to form (DeMattos et al., 2002). Plasma Aß is known to be rapidly cleared by the liver and kidneys in an ApoE-dependant mechanism (Hone et al., 2003). If Aβ is to be used as a biomarker during clinical development of γ-secretase inhibitors, the changes in Aβ in each of these compartments should be understood.

The γ-secretase complex cleaves not only APP, but also Notch 1-4, ErbB4, CD44, cadherin and others (Selkoe and Kopan, 2003). Multiple substrates create greater possibilities for toxic side effects of γ-secretase inhibitors. In fact, major alterations in the differentiation of intestinal stem cells and lymphocytes has been reported for two γ-secretase inhibitors (Searfoss et al., 2003; Wong et al., 2004), suggesting toxicity due to Notch inhibition. Whether or not a therapeutic window can be achieved for γ-secretase inhibitors is a major issue that needs to be addressed.

Two structurally-related γ-secretase inhibitors have been shown to reduce Aß in brain, CSF and plasma of transgenic mice (Dovey et al., 2001; Cirrito et al., 2003; Lanz et al., 2003; Lanz et al., 2004; Wong et al., 2004) . In this paper, BMS-299897, from a novel chemical series (Smith et al., 2000), was used to address several questions related to the potential clinical development of a γ-secretase inhibitor. What are the changes in Aß in various compartments following administration of a γ-secretase inhibitor? Does the presence of large accumulations of Aß in plaques alter the pharmacodynamics of Aß decreases in plasma and CSF and is the plaque Aß

reduced? In addition, is there any Notch-related toxicity after two weeks of dosing at efficacious doses? These questions were addressed using Tg2576 mice.

METHODS

Handling of Mice- The Tg2576 mice were developed by Karen Hsaio (Hsiao et al., 1996) and licensed from the Mayo Foundation for Medical Education and Research (Rochester, MN). Male Tg2576 transgenic mice were bred to normal C57BL6/SJL females at the Bristol-Myers Squibb facility in Wallingford, CT. Mice were housed with a 6 AM-6 PM light/dark cycle and allowed free access to food and water. Both male and female mice were used in these studies, and, although no differences in Aß were observed between them, only one sex was used in a single study. Young Tg2576 mice were used between 3-6 months of age, while aged animals were used at 14-17 months. BMS-299897 was synthesized by the Medicinal Chemistry groups of SIBIA Neurosciences, Inc. (now Merck Research Laboratories, San Diego, CA) and Bristol-Myers Squibb. Animals were dosed by oral gavage in a volume of 6 ml/kg in polyethylene glycol, average molecular weight of 400, or a vehicle consisting of 10% propylene glycol, 7.5% ethanol, and 82.5% Solutol HS-15® (polyethylene glycol/hydroxystearate; BASF Corp., Mount Olive, NJ) by weight. Mice were handled strictly according to Bristol-Myers Squibb Animal Care and Use Committee guidelines.

CSF was collected under anesthesia using a glass pulled micropipette after exposure of the cisterna magna, taking care not to contaminate the CSF with blood. Two to ten µl was routinely collected. The CSF was immediately diluted 1:10 in 1% 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propane sulfonate (CHAPS) in phosphate buffered saline (PBS) with protease inhibitors (Roche Diagnostics, Mannheim, Germany) prior to freezing in liquid nitrogen and storage at –80^{o}C. Blood was collected by cardiac puncture and placed into ethylene-diaminetetraacetic acid microtainer tubes for the preparation of plasma. The cerebellum and

hindbrain were collected for the determination of compound concentration, and the remaining brain was separated into quarters prior to freezing in liquid nitrogen.

Aß40 ELISA- Human Aß40 was measured in a standard sandwich ELISA using an Aß40 terminus specific monoclonal antibody to capture the Aß40 (TSD9S3.2, Bristol-Myers Squibb) and a monoclonal antibody recognizing human Aß1-12 (26D6, SIBIA Neurosciences, Inc.) for detection of brain and CSF Aß. An antibody to Aß 17-24 (4G8, Signet Laboratories, Dedham, MA) was used for detection of plasma Aß. Young brain tissue was prepared for assay by homogenization in 1% CHAPS + protease inhibitors. Centrifugation followed at 100,000 x g at 4^{o}C for 1 hour and the supernatant was diluted 10 fold more. If aged brains were used, they were homogenized first in CHAPS as described above. The pellets were then extracted with 70% formic acid (FA), then neutralized with 1M Tris (pH 11). The pH of each sample was neutralized with 1 M sodium hydroxide if necessary. For the FA extracts 3-6 dilutions between 2 x 10^3 and 10^5 ml/g were examined to make sure the signal for each sample was on the linear part of the standard curve. Plasma was diluted 1:15 in 0.1% CHAPS in PBS, and mouse CSF was further diluted 1:15 in 1% CHAPS + protease inhibitors prior to loading on the plates.

A standard sandwich ELISA was performed using TSD9S3.2 to coat the plates. Washes between steps were done in PBS + 0.1% Tween-20. The plates were blocked with 0.1% bovine serum albumin in PBS for brain and CSF, and 1% bovine serum albumin in PBS for plasma. Samples were loaded in triplicate, and an Aß 1-40 standard curve and positive control were run on each plate. Plates were incubated overnight at 4^{o}C (brain and CSF) or for 2 hours at room temperature (plasma). Brain and CSF plates were washed prior to addition of horse radish peroxidase

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conjugated 26D6 for 1 hour, while the plasma samples were incubated with 4G8-biotin at for 1 hour prior to the addition of horse radish peroxidase conjugated neutravidin (Pierce Chemical Co. Rockford, IL) for 45 minutes. 3,3',5,5'-tetramethylbenzidine peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg MD) was used to develop the signal, which was stopped with 1 M phosphoric acid. Plates were read at 450 nm.

Brain Aß42 ELISA- The protocol follows that for Aß40, with the following changes. Capture of Aß42 occurred with a Aß42 terminus-specific antibody, 1163 (polyclonal, affinity purified on Protein A, then on an Aß35-42 column, Bristol- Myers Squibb). Brain CHAPS extracts were diluted to 1% CHAPS + 2.5% non-fat dry milk in PBS with protease inhibitors. The Aß40 and Aß42 specific ELISAs were shown not to detect each other by immunodepletion and spiking experiments (data not shown).

Immunoprecipitation/Western Blot assays

For the APP and CTF assays, brain extracts as prepared for ELISA were immunoprecipitated overnight with a polyclonal antibody specific for the last 12 amino acids of APP (85461, Bristol-Myers Squibb) at a 1:63 dilution and 40µl Protein A Sepharose-4 Fast Flow® slurry (Amersham Pharmacia Biotech, Piscataway, NJ). The Protein A beads were preblocked with 1% bovine serum albumin for 1 hour prior to use. The complexes were washed twice with wash dilution buffer [50mM Tris, 0.15M sodium chloride, 0.05% Tween-20 at pH 8.0], followed by one wash with Tris buffered saline (TBS) and a final wash with 50mM Tris, pH 6.8. The complexes were

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resuspended in 2X tricine sodium dodecyl sulfate sample buffer, denatured by heating at 95°C for 5 minutes and electrophoresed on 16% tricine polyacrylamide gels (NOVEX, San Diego, CA). The proteins were transferred onto Hybond ECL™ nitrocellulose membranes (Amersham Pharmacia Biotech) and blocked overnight with 8% non-fat dry milk in TBS with 0.1% Tween-20 (TBST). The blots were incubated with G369, a polyclonal antibody to the last 50 amino acids of APP at 1:3000 (generously provided by Dr. Sam Gandy, Farber Institute of Neuroscience, Thomas Jefferson University, Philadelphia, PA). This was followed by a secondary horse radish peroxidase conjugated anti-rabbit antibody (1:4000 dilution, Amersham Pharmacia Biotech). Blots were developed by chemiluminescence (SuperSignal Ultra™, Pierce, Rockford, IL) and visualized by exposure to x-ray film.

To detect Aß by Western blot analysis, Aß was first immunoprecipitated from brain homogenates with a polyclonal antibody specific for the first 16 amino acids of Aß (36935, Bristol-Myers Squibb) at a 1:200 dilution. Samples were run on bicine urea gels (Klafki et al., 1996) and blotted onto Immobilon-P transfer membranes (Millipore, Bedford, MA). After the blots were boiled for 5 minutes in PBS, they were blocked with 8% non-fat dry milk in TBST overnight. Blots were incubated with horse radish peroxidase-conjugated 26D6 at 1:1000 in TBST + 1% bovine serum albumin for 1-2 hours, then detected with chemiluminescence.

Analysis of APP/Notch Cleavage in Cells:

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HEK293 human embryonic kidney cells were transfected using Lipofectamine Plus™ (Life Technologies, Rockville, MD) with either mouse ΔE-Notch cDNA with a C-terminal myc tag or a construct containing human placental alkaline phosphatase linked to the C-terminal 164 amino acids of APP with the Swedish mutation. Separate transfections were done to eliminate any effect of competition of the substrates. For analysis of ΔE-Notch cleavage, cells were radioactively labeled with [^{35}S]-methionine using Easy Tag™ (New England Nuclear, Beverly, MA). Cells were rinsed and incubated with methionine-free labeling medium + 5% dialyzed fetal bovine serum to reduce intracellular methionine levels. Cells were then incubated with the BMS-299897 + 100 μCuries/ml of Easy Tag™ for 5 hours. Cells were rinsed off the plate and washed twice with PBS prior to lysis by RIPA buffer (150 mM NaCl, 1% nonidet-P40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris, pH8.0 with protease inhibitors). After a 15 minute incubation, cell debris was removed by centrifugation. The cell extracts were pre-cleared with Protein G agarose beads (Pierce, Rockford, IL), then incubated with the 9E10, monoclonal anti-myc-tag antibody (Zymed Laboratories, S. San Francisco, CA) and Protein G agarose at 4°C overnight. The beads were washed three times with 0.25X RIPA buffer and were extracted with Tris-glycine reducing sample buffer. The samples were run on 6% polyacrylamide gels. The gels were fixed, dried and analyzed by phosphoimage analysis (Amersham Pharmacia Biotech, Piscataway, NJ). For APP cleavage analysis, cells were also incubated with the compound for 5 hours and Aß was measured by ELISA as above.

Thymocyte Isolation and FACS Analysis- The thymus was collected from each mouse and teased apart with fine forceps in Hanks balanced salt solution + 10% FCS. The cells were passed through a 100 micron mesh screen, washed, and counted in trypan blue. Cell viability was

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greater than 90%. 2 x 10^6 live cells were blocked with 1% bovine serum albumin and rat γ-globulin (11 μg/ml Pharmingen) for 45 minutes at 4^0C. After two washes in Hanks balanced salt solution, the cells were resuspended in Hanks balanced salt solution + 5% FCS, 0.1% sodium azide with 2 μl each of an anti-αβ T cell receptor antibody conjugated to fluorescein isothiocyanate and an anti-CD8 T cell antibody conjugated to Cy-Chrome® (Pharmingen). After a 1 hour incubation at 4^0C , the samples were washed and resuspended in 200 μl of 2% paraformaldehyde in PBS. The samples were analyzed on a Becton Dickenson FACSVantage SE (San Jose, CA). Live cell gating was accomplished with forward and side scatter analysis, after analyzing approximately 100,000 cells per sample.

Analysis of BMS-299897 Concentrations:

The transgenic mouse plasma and brain samples were analyzed by a liquid chromatography-tandem mass spectrometry (LC-MS-MS) method. The LC-MS-MS system consisted of two Shimadzu LC-10AD pumps, a Perkin-Elmer series 200 autosampler, a Keystone BDS C18 2 × 20 mm (3 μ) column at 60°C, and a Micromass Quattro LC mass spectrometer. The mobile phase consisted of (A) 10 mM ammonium acetate buffer / methanol (75/25, pH 5.5) and (B) acetonitrile delivered at 0.3 ml/min using a gradient program. The initial elution condition was 15% B, which was increased to 85% B in 0.5 min and maintained for 0.5 min. It was then returned to 15% B in 0.1 min and maintained for 1.2 min. The MS-MS analysis was performed using the electrospray interface under negative ion mode with the source and desolvation temperatures at 150°C and 300°C, respectively. The capillary voltage was 3.5 kV, cone voltage 35 V, and collision energy 20 eV. The mass-to-charge ratio of 510 (parent ion) and 302

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(daughter ion) were used for multiple reaction mode monitoring of BMS-299897. The lower limit of detection and upper limit of detection for BMS-299897 were 3.9 ng/mL and 8000 ng/mL, respectively. Plasma samples were deproteinized and extracted with two portions of acetonitrile. Brain samples (0.1 g) were homogenized in 0.4 mL of acetonitrile.

Statistical Analyses:

Routine statistical analyses were done using analysis of variance and Dunnett's post-hoc test with Instat version 3.0 (GraphPad Software, San Diego, CA). Results are reported as the mean ± the standard error of the mean.

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RESULTS

Treatment of Young Transgenic Mice with a γ-Secretase Inhibitor

BMS-299897 (Figure 1) (Smith et al., 2000) is a γ-secretase inhibitor (Wagner S, Polson C, Munoz B, Srinivasan K, Franco D, Hendrick J, Robertson B, Vinitsky A, Izzarelli D, Wang R, Roome J, Roberts SB, Smith D, Barten D, Kounnas M, and Felsenstein KM, Manuscript in preparation). BMS-299897 preferentially inhibits cleavage of the APP CTF cleavage over that of Notch-1 in vitro (Figure 2). In HEK293 cells, BMS-299897 had a 15 fold lower IC50 for APP cleavage than Notch-1 cleavage (APP IC50 of 7.1 nM, and a Notch IC50 of 105.9 nM). This compound has also been described as a γ-secretase inhibitor in a detergent solubilized in vitro assay (Tian et al., 2002). In Tg2576 mice, BMS-299897 was orally bioavailable, showing dose dependent inhibition of brain and plasma Aß40 (Figure 3). The ED50s for brain (18 mg/kg) and plasma (15 mg/kg) were similar. Total brain and plasma levels of the compound increased linearly with dose, but the levels of compound in the plasma were much higher than in the brain. This was consistent with high plasma protein binding of this compound (99.6% by equilibrium dialysis of [^{14}C]-labeled compound).

The relative rates of Aß changes in brain, plasma and CSF were examined after dosing with BMS-299897. Brain Aß40, brain Aß42, and CSF Aß40 declined and recovered at similar rates (Figure 4A). Plasma Aß40 decreased faster than the other Aß pools, as was investigated in more detail in a separate experiment (Figure 4B). A delay in BMS-299897 penetration into the brain was unlikely because compound concentrations in brain and plasma tracked in a constant ratio, suggesting a rapid equilibrium (<15 min) across the blood brain barrier. The more rapid decline

of Aß40 in the plasma of these mice was most likely due to a difference in the elimination half life of Aß40 in brain and plasma. Kinetic models (KineticaTM v.1.0) were employed to calculate a half life of 15 minutes in plasma and 38 minutes in the brains of these transgenic mice.

Immunoprecipitation followed by Western blots using antibodies to the C-terminal end of APP showed no changes in APP levels, but increases in the α- and ß- cleaved CTF of APP were observed after dosing with BMS-299897 (Figure 5A). These 5 CTF bands represent unphosphorylated and phosphorylated versions of each fragment, along with a CTF beginning at the 11 residue, an alternative product of ß-secretase cleavage (Buxbaum et al., 1998; Cai et al., 2001). All CTF were affected, as expected for a γ-secretase inhibitor. The in vivo turnover rate of CTF was high, as previously suggested (Savage et al., 1998). The CTF increased and decreased with a similar timecourse to the changes in Aß (Figure 5B). In addition, all 3 major isoforms of Aß in Tg2576 mice (Aß40, Aß42 and Aß38) were shown using immunoprecipitation and Western blot analysis to decline after dosing with BMS-299897, confirming the results observed by ELISA.

Brain and CSF Aß levels of individual mice treated with BMS-299897 were compared to determine the predictive ability of CSF Aß as a biomarker for what was occurring in the brain parenchyma (Figure 6). The correlation between CSF and brain Aß40 was 0.909 ($r^2 = 0.825$, $p < 0.001$). For young mice without Aß accumulated into plaques, brain and CSF Aß correlated well.

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Treatment of Young and Aged, Plaque-Bearing Transgenic Mice with a γ-Secretase Inhibitor

It was of interest to determine if the effects of BMS-299897 on Aß would be consistent as the Tg2576 mice began to age and deposit plaques, creating a new, dense pool of Aß in the brain. BMS-299897 was administered to Tg2576 mice from 4 – 14 months of age (Figure 7). Plasma Aß was reduced at all ages. Brain Aß40 was sequentially extracted with CHAPS detergent, then with FA to solubilize the deposited Aß. In 4 - 6 month animals this second FA extraction resulted in little to no signal. At 8, 10, and 14 months, the FA extractable brain Aß was 910, 5680 and 86,700 ng/g, respectively, and this pool of Aß was not reduced by a single dose of BMS-299897. While CHAPS extractable brain Aß did not increase in 8 and 10 month old animals, it was reduced by BMS-299897, although only significantly so at 8 months. The CHAPS extractable Aß increased significantly in the 14 month old mice having extensive plaque formation, and BMS-299897 was no longer able to reduce this pool. There were no significant differences in the concentration of the compound in animals between 4 and 14 months of age (see figure legend).

Tg2576 mice were then dosed over a 2 week period with BMS-299897 to determine whether a longer dosing regimen would enable reductions of brain Aß in aged mice. Aged mice and a set of young mice dosed for 2 weeks were compared with a matched group given only a single dose. Aß40 was measured in brain, plasma and CSF. The results from this experiment are shown in Figure 8. In young mice, plasma, CSF and brain Aß were all reduced after 1 dose or following

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14 days of BMS-299897 treatment. In aged mice, CHAPS extractable Aß was elevated by 2.8 fold compared to young animals, but was still only a small percentage (0.5%) of the FA extractable pool. BMS-299897 did not influence either the CHAPS or the FA extractable brain Aß pools in transgenic mice containing plaques within this two week time frame. Even so, the compound did demonstrate efficacy in both the peripheral (plasma) and CNS (CSF) compartments.

The efficacy of the compound after 14 days was not as great as that observed after an acute dose in both young and old animals. Concentrations of BMS-299897 were lower following repeated dosing (Table 1) in these mice which also had significantly elevated liver weights (data not shown). These results suggest autoinduction of drug metabolizing enzymes has occurred, a phenomenon whereby a compound triggers the upregulation of its own metabolizing enzymes upon continual exposure. The resulting increase in compound clearance causes decreased systemic exposure. The aged animals (17 month males in this study) also had approximately four fold lower exposure than young animals at the same dose (Table 1), even though there was no difference in exposure in the 14 month females used in the previous study. The reduced exposure is consistent with the diminished effectiveness of the same dose in the aged animals. This reduced exposure in aged males (average weight 39 g) was observed repeatedly, but was not observed in aged females (average weight 26 g) or young males (average weight 31 g). It is unclear why such exposure differences were observed. There were no compound formulation differences between studies, not is it likely that the presence of plaques could account for the changes in BMS-299897 levels in aged males. It is speculated that weight, or other sex and age

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specific alterations in absorption, metabolism or elimination of this compound mice could also contribute to this observation.

Examination of Potential Notch Related Effects of a γ-Secretase Inhibitor in Young Transgenic Mice

The potential effects of Notch inhibition were also investigated, focusing on T cell and intestinal cell differentiation. Unless a therapeutic window can be generated in vivo, γ-secretase inhibitors will not be useful for the treatment of AD. For T cell differentiation, the number of CD8+ T cells developing in the thymus was examined for a possible reduction. Thymocyte development goes through a proscribed series of steps. CD4-/CD8- double negative thymocytes with no T cell Receptor (TCR) expression choose between the αβ and γδ TCR, then become CD4+/CD8+ double positive thymocytes with low αβ TCR expression, and finally differentiate to CD4 or CD8 single positive cells with high αβ TCR expression (Allman et al., 2002; Germain, 2002) . Thymocytes were isolated from young Tg2576 mice that were treated with BMS-299897 for 14 days. Although the inhibition of Aß was diminished at the end of the 14 day dosing regimen, it was still significant. Brain Aß was reduced 19 % ($p < 0.05$) at 15 mg/kg and 56% ($p< 0.01$) at 100 mg/kg in this study. The total number of thymocytes isolated was the same for vehicle and for BMS-299897 treated mice at both 15 and 100 mg/kg ($5.4 \pm 0.4 \times 10^7$, $6.3 \pm 1.0 \times 10^7$, and $5.5 \pm 0.7 \times 10^7$ cells, respectively). The number of CD8+, αß T Cell receptor high (TCR Hi) expressing thymocytes was used as an indicator of mature CD8 thymocytes. Using FACS, there were no observable differences in the total number of CD8+ thymocytes (64.9 ± 3.7, 71.9 ± 1.6, and 65.5 ± 2.0 % of total cells), total αß TCR Hi thymocytes (21.9 ± 1.6, 18.5 ± 1.3, and $25.2 \pm$

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1.6 % of total cells) or in the differentiated CD8+, αß TCR High thymocytes (Figure 9). Complete blood counts and basic serum chemistries done on a separate group of mice treated for 2 weeks with BMS-299897 also showed no differences between vehicle and treated animals. In addition, no gastrointestinal lesions or differentiation abnormalities were observed histologically (data not shown). There were no obvious Notch-based toxic effects as has previously been reported for other γ-secretase inhibitors (Searfoss et al., 2003; Wong et al., 2004).

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DISCUSSION

Tg2576 mice were used to study changes in Aß levels in different biological compartments with a γ-secretase inhibitor. The mice have high levels of human Aß in brain, plasma and CSF, and low levels in peripheral tissues where there is some limited transgene expression (Kawarabayashi et al., 2001). We demonstrated that BMS-299897 reduced Aß in brain, CSF and plasma of young mice with a similar timecourse, except for a slightly faster rate of decline in plasma Aβ within the first hour. The value of brain and plasma Aβ half-life we estimated (38 and 15 minutes) agrees well with previous estimates of brain Aß turnover of 1 - 2.5 hours (Savage et al., 1998) and of plasma Aß turnover of 15 min (Savage et al., 1998; Hone et al., 2003). These results differ from those observed by Lanz et al. using LY-411575 (Lanz et al., 2004), with a significant delay in maximal reductions of brain Aβ (9 hours) relative to that observed in CSF and plasma (3 hours). The transgenic mice used in the two studies are both Tg2576, so disparate results are likely due to differences in the extraction techniques for brain Aβ, or to pharmacokinetic differences between the compounds. Their extraction method uses 5M guanidine HCl and is believed to extract both extracellular and intracellular Aß. We used CHAPS detergent for extraction, which does not extract most plaque Aß (not present in young mice), and also may not extract intracellular Aß. It is unknown how much intracellular Aß contributes to the total pool of Aß in the brain, but its turnover is likely to be slower than secreted Aß. If significant, differences in the extraction of intracellular Aß could explain their longer apparent half-life, and the increased efficacy observed with repeated dosing. The previous estimate of brain Aß half-life was determined using a diethylamine brain extract, adding yet another extraction method to the existing data (Savage et al., 1998). Differences in the pharmacokinetics and distribution of BMS-299897 and LY-411575 within the brain itself could

also account for differences in the two studies. Total brain levels of compound in both studies were measured, but differences in compound distribution between different anatomical areas of the brain (ie myelin rich or poor areas), or even within different cellular membranes (possibly through differences in lipid composition) could also contribute to different results. It is possible that it takes longer for LY-411575 to equilibrate into the compartment where brain active γ-secretase resides. These will be important questions to pursue in further studies, which should involve side by side comparisons of the two compounds.

Our studies also showed the expected increases in APP CTF with a timecourse mirroring the decreases in brain Aβ. Increases in CTF with γ-secretase inhibitors have been reported using N-[N-(3,5-difluorophenylacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (Dovey et al., 2001; Lanz et al., 2003). We also observed equivalent findings using BMS-299897 in wild-type APP-yeast artificial chromosome transgenic mice and in guinea pigs [Anderson JJ, Holtz G, Baskin PP, Turner M, Rowe B, Wang B, Kounnas MZ, Barten DM, Felsenstein KM, McDonald I, Srinivasan K, Munoz B, and Wagner S Reductions in ß-amyloid concentrations in vivo by the γ-secretase inhibitors BMS-289948 and BMS-299897 (manuscript under review)].

In this report, studies were also performed with BMS-299897 in aged Tg2576 mice containing plaques. Once plaque formation was sufficient to increase CHAPS extractable Aß, BMS-299897 was no longer able to reduce either that pool or FA extractable Aß. The CHAPS extract in young animals was likely all soluble Aß, but in aged animals it likely contained easily exchanged Aß from the plaques and/or high levels of Aß oligomers. FA extractable brain Aß from aged mice likely represented plaque Aß, but may have included intracellular Aß as well. LY-411575

also did not reduce brain Aß in aged Tg2576 mice with a single dose (Lanz et al., 2003). After 14 days of once daily dosing, BMS-299897 was also unable to reduce plaque Aß in aged Tg2576 mice, but it is possible that this dosing regimen was not sufficient to influence plaque Aß. It is possible that continuous exposure to compound over 24 hours or for longer than 14 days would be required to see reductions in plaque Aß. Apparent autoinduction and lower exposure of BMS-299897 in aged animals worked against increasing exposure with this compound. Attempts to give continuous exposure using BMS-299897 in chow were also limited by significant apparent autoinduction (data not shown). Reductions in plaque formation in APP transgenic mice have been reported following various treatments, all of which required 1-6 months of treatment (Schenk et al., 1999; Lim et al., 2000; Cherny et al., 2001; Haugabook et al., 2001; Refolo et al., 2001; Carro et al., 2002; Jantzen et al., 2002; Permanne et al., 2002; Deane et al., 2003; Matsuoka et al., 2003; Yan et al., 2003). Two weeks of treatment with a nitric oxide-releasing non-steroidal anti-inflammatory drug was not sufficient to reduce plaque load, although 5 months of treatment was effective (Jantzen et al., 2002). In light of these reports, it is not surprising that we did not observe changes in FA extractable Aß in these mice treated for 2 weeks, and BMS-299897 is probably not the optimal compound with which to perform these studies in transgenic mice due to its pharmacokinetic profile.

In spite of this tremendous pool of unchanging plaque Aß in the brains of aged Tg2576 mice, CSF and plasma Aß in aged Tg2576 mice were reduced by BMS-299897 by one or fourteen days of treatment. These data are in agreement with those generated by others in transgenic mice (Lanz et al., 2003). Differences in ISF and CSF Aß have also been reported using microdialysis (Cirrito et al., 2003). In addition, disparities in brain and CSF Aß are also observed in AD

patients, where soluble brain Aß is 50 times higher than CSF Aß (Kuo et al., 1996). The dynamics of Aß in the brain and CSF compartments were independant in the presence of plaques, even though there was a strong correlation between brain and CSF Aß in young mice. CSF Aß was a valid indicator of CNS activity in any age group, and could potentially be a valuable biomarker in AD patients.

Notch is part of a major signaling pathway during development. Notch was discovered in experiments with Drosophila, where homozygous loss causes major developmental deformities, and loss of 1 copy causes a notched wing phenotype. Ligand binding induces two proteolytic cleavages in the Notch receptor. The second protease activity occurs within the membrane, and requires PS. This second cleavage releases the Notch intracellular domain, which causes transcription of many genes. Eliminating functional PS in Drosophila, C. elegans or mice has severe effects during development, similar to Notch-1 loss of function mutants. γ-secretase inhibitors have also been shown to induce notched wings in Drosophila (Micchelli et al., 2003) and to alter embryonic development in zebrafish (Geling et al., 2002).

In an adult mammal, Notch signaling plays a significant role in the differentiation of cells in the intestinal epithelium (Brittan and Wright, 2002) and during the multistep process of lymphopoeisis. Inhibition of Notch processing could prevent the development of γ-secretase inhibitors for the treatment of AD, so this is a critical point to be examined in efficacy studies with these compounds. Notch is involved in Lymphopoeisis at multiple steps including the T cell/B cell lineage decision, the γδ verses αβ TCR decision, and the CD4 verses CD8 T cell commitments (Allman et al., 2002; Germain, 2002). The data supporting a role for Notch in

CD8 T cell development include experiments with transgenic mice overexpressing a truncated active form of Notch in the thymus showing an increased number of CD8 single positive thymocytes. γ-secretase inhibitors at concentrations that eliminate activity interfere with thymocyte development in fetal thymic organ cultures at the T/B cell and αß/γδ TCR interfaces, along with the CD4/CD8 T cell decision point (Allman et al., 2002). Dose response studies suggest that the CD4/CD8 T cell decision point might be most sensitive to a γ-secretase inhibitor. Toxic, Notch-related effects of some γ-secretase inhibitors have already been described in rodents. Recently, LY-411575 was shown to profoundly affect the differentiation of T cells in the thymus, and B cells in the spleen of transgenic mice treated for 5 – 15 days (Wong et al., 2004). For intestinal stem cell differentiation, LY-411575 (Wong et al., 2004) and Compound X (Searfoss et al., 2003) have both been shown to increase differentiation to goblet cells. The role of Notch-2 and Notch-3, both of which are also expressed in the thymus and intestine, are currently unknown and may also influence differentiation. Some γ-secretase inhibitors are known to inhibit all known forms of Notch (Mizutani et al., 2001).

In this report, mice treated for 14 days with a γ-secretase inhibitor did not show any significant changes in CD8 T cell development in the thymus. No changes in villus structure or goblet cell numbers were observed in our studies. The possibility exists that the doses and/or the dosing regimen were not sufficient to alter Notch processing in the periphery, especially considering that BMS-299897 was 15 fold more efficient at inhibiting APP than Notch-1 cleavage in vitro and Aß levels were not inhibited by more than 80% in these animals. It is widely accepted that only partial inhibition of Aß production is the clinical goal for secretase inhibitors (Wolfe, 2001;

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Selkoe and Kopan, 2003). These data encourage the possibility of using γ-secretase inhibitors to reduce APP processing in humans without generating an unacceptable side effect profile.

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Figure Legends

Figure 1. BMS-299897

Figure 2. BMS-299897 inhibits cleavage of APP CTF more effectively than cleavage of Notch-1. Transfected HEK293 cells were treated with BMS-299897 for 5 hours prior to the analysis of secreted Aß or the Notch intracellular domain cleavage product.

Figure 3. Dose response relationship of Aß reduction and drug concentration in young Tg2576 mice. Brain and plasma samples were collected 3 hours after a single oral dose. Each group contained 8-20 mice and were compared to vehicle dosed animals.

Figure 4. Reduction of Aß levels in young Tg2576 mice after a single oral dose of 100 mg/kg BMS-299897 in Tg2576 mice. Male mice (n = 8-9) were used in (A), and female mice (n = 6-8) were used in (B). In (A) control brain Aß40 levels were 262 ± 7 ng/g, brain Aß42 levels 13.9 ± 0.6 ng/g, CSF Aß40 levels 199 ± 6 ng/ml, and plasma Aß40 levels 14.8 ± 1.3 ng/ml. In B, control brain and plasma Aß40 levels were 277 ± 7 ng/g and 18.5 ± 0.9 ng/ml, respectively.

Figure 5. Immunoprecipitation/Western blot analysis of APP, CTF and Aß in the brains of BMS-299897 treated mice. Each lane shows a representative mouse from a group of 7 mice for each treatment. Mice were dosed orally with 100 mg/kg BMS-299897. The western blots

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were probed with an antibody to the C-terminal end of APP, G369 (A), or to residues 1-12 of Aß, 26D6 (B).

Figure 6. Brain and CSF Aß in young Tg2576 mice. Data were collected from a number of experiments in which Tg2576 mice were treated with BMS-299897 or vehicle, n = 120.

Figure 7. Treatment of Tg2576 mice of increasing age with BMS-299897. Female mice were administered a single oral dose of 100 mg/kg BMS-299897 in polyethylene glycol-400, n = 3. The concentrations of BMS-299897 in plasma were: 55 ± 18, 14 ± 2, 18 ± 7, 47 ± 24, and 23 ± 7 μM at 4, 6, 8, 10 and 14 months respectively. The brain concentrations were 5.3 ± 1.8, 1.4 ± 0.2, 1.9 ± 0.4, 7.4 ± 3.5, and 2.5 ± 0.3 μM at the same timepoints. * $p < 0.05$.

Figure 8. Reductions in Aß40 following acute or 14 days of oral dosing inTg2576 mice. Male mice were dosed once daily (QD) with 100 mg/kg BMS-299897. Mice in this study (n = 10-11) were either 5 (A) or 17 (B) months old. ND is not determined. * $p < 0.05$, ** $p < 0.01$.

Figure 9. Levels of mature CD8+ thymocytes in young Tg2576 mice treated with 15 or 100 mg/kg BMS-299897 for 14 days. Each point represents one mouse and the line indicates the mean.

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Table 1. Brain and plasma concentrations of BMS-299897 in Tg2576 mice treated for 1 or 14 days. Samples were collected three hours after the final 100 mg/kg dose. Each group consisted of 10-11 mice 5 or 17 months of age and were the same mice as shown in Figure 8.

	Plasma BMS-299897 (nM)		**Brain BMS-299897 (nM)** [a]	
Group	**Day 1**	**Day 14**	**Day 1**	**Day 14**
Young	26,860 ± 8,220	11,210 ± 4,450	3,330 ± 890	1,580 ± 510
Aged	7,060 ± 2,680	5,560 ± 1,470	890 ± 300	410 ± 70

[a] *Concentration of BMS-299897 was actually measured in pmol/g, but the assumption was made that 1g tissue = 1 ml to allow a better comparison with plasma data.*

Figure 1

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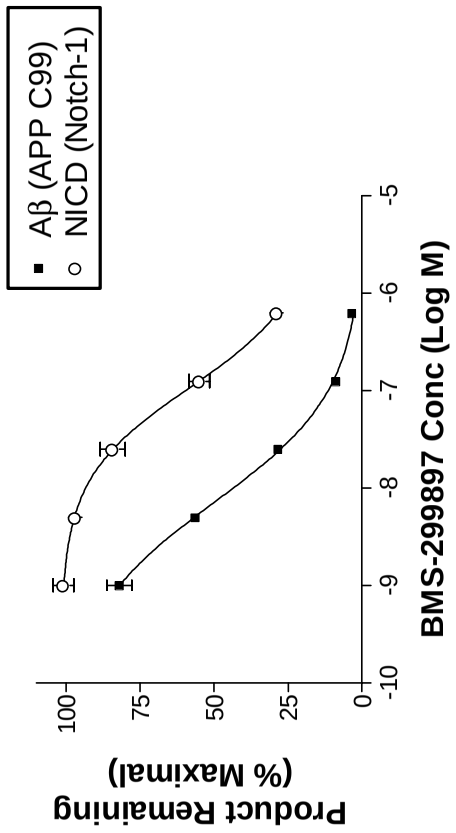

Figure 2

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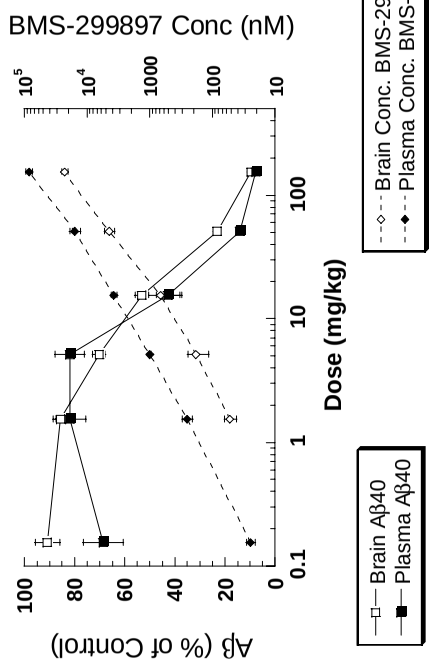

Figure 3

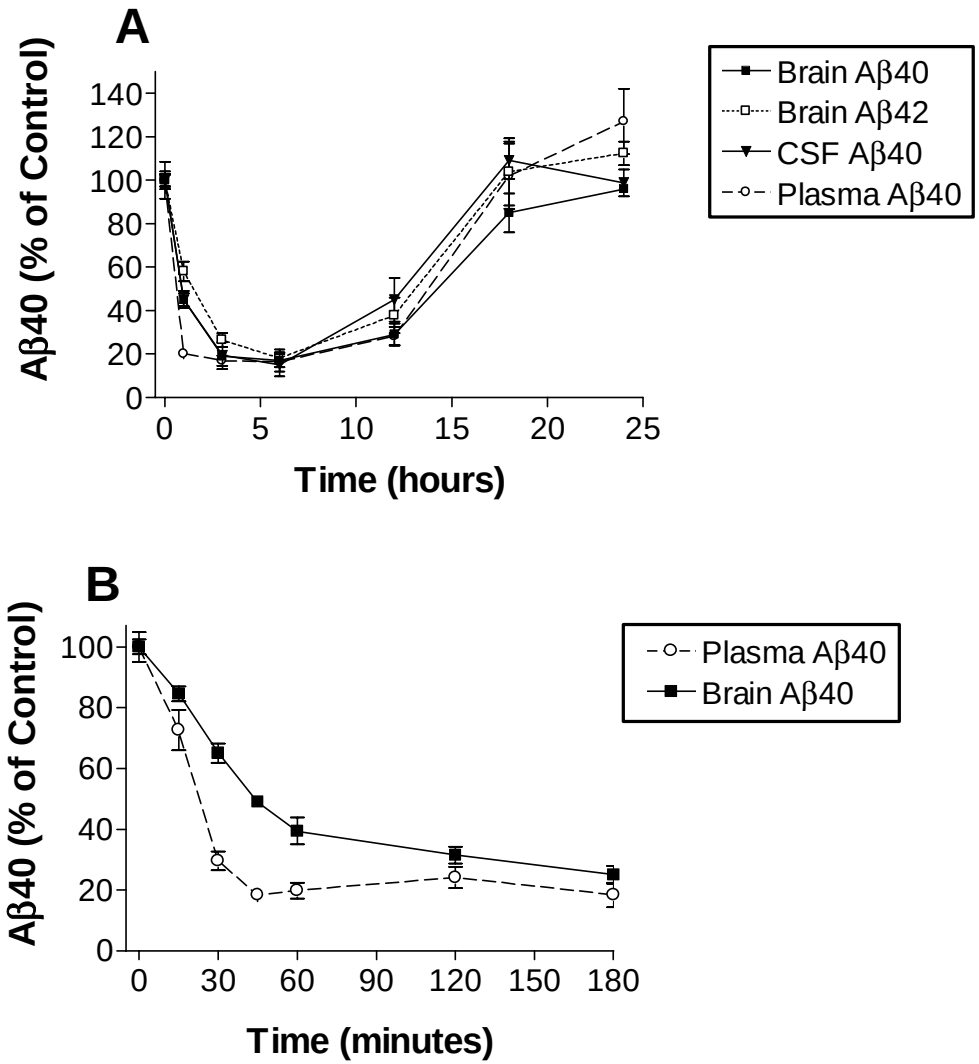

Figure 4

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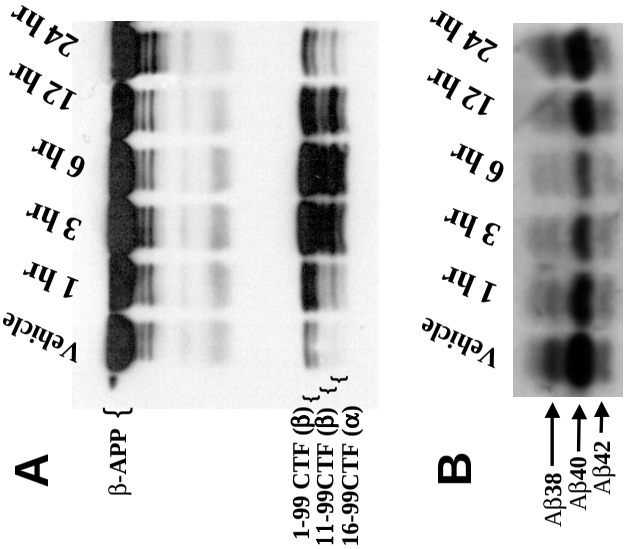

Figure 5

Figure 6

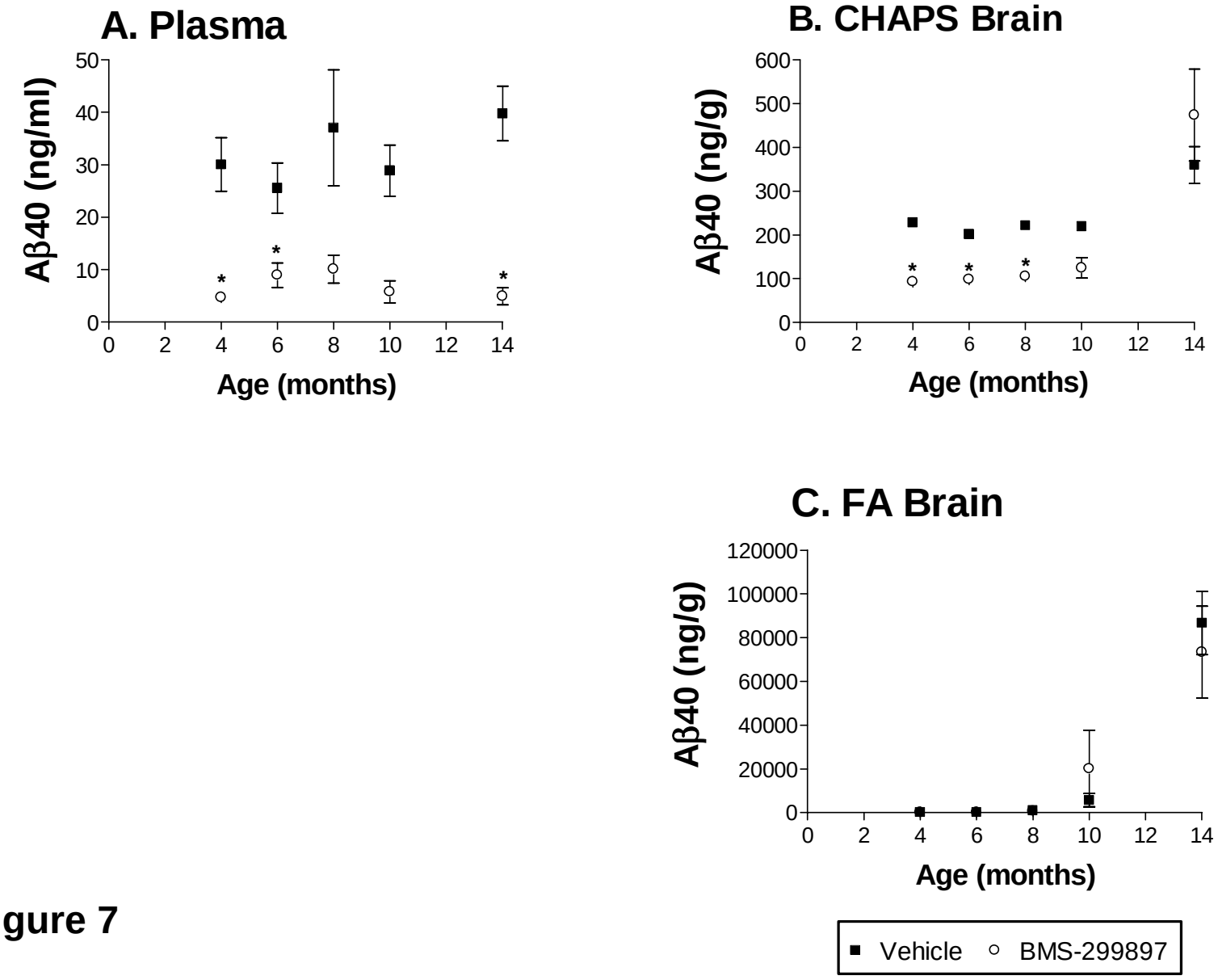

Figure 7

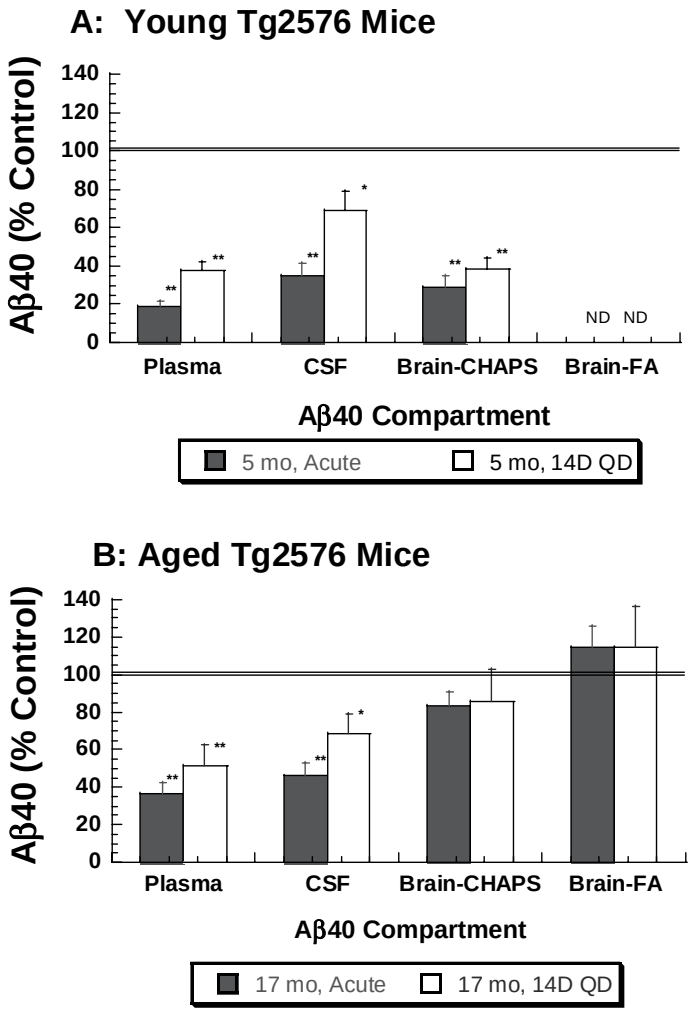

Figure 8

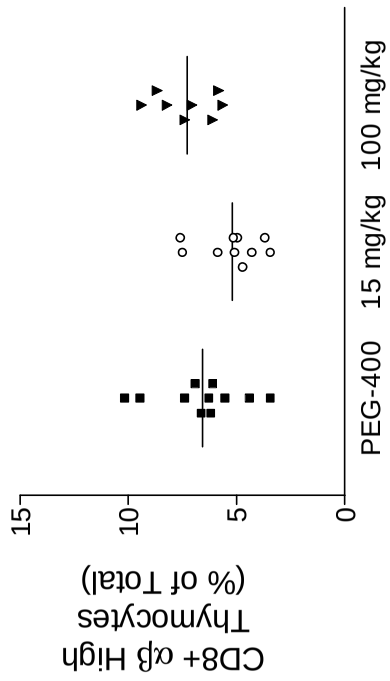

Figure 9