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Studies of Aβ Pharmacodynamics in the Brain, CSF and Plasma in Young (Plaque-free) Tg2576 Mice Using the γ-secretase Inhibitor, LY-411575.

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Running Title: Aβ dynamics in Tg2576 mice after γ-secretase inhibition.

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Abbreviations: Aβ, amyloid-β; BACE, β-amyloid precursor protein cleavage enzyme; BID, twice-daily dosing; CSF, cerebrospinal fluid; DAPT, *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester; ELISA, enzyme-linked immunosorbent assay; PO, oral; SRM, selective reaction monitoring.

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ABSTRACT

Our previous study (Lanz et al., 2003) suggests the utility of CSF and plasma Aβ as biomarkers of β- or γ-secretase inhibition. The present study characterized further Aβ pharmacodynamics in these tissues from Tg2576 mice and examined their correlation with brain Aβ after acute treatment with a potent γ-secretase inhibitor, LY-411575 (N^2-[(2S)-2-(3,5-difluorophenyl)-2-hydroxyethanoyl]-N^1-[(7S)-5-methyl-6-oxo-6,7-dihydro-5H-dibenzo[b,d]azepin-7-yl]-L-alaninamide). A single dose of LY-411575 dose-dependently (0.1 to 10 mg/kg, PO) reduced Aβ(1-40) and Aβ(1-42) in the CSF and the brain. In contrast, plasma Aβ levels were increased by 0.1 mg/kg of LY-411575 and were followed by a dose-dependent reduction at higher doses. The time courses of Aβ reduction and recovery were distinct for the three tissues: maximal declines in Aβ levels were evident by 3 h in the CSF and plasma but not until 9 h in the brain. A recovery in Aβ levels was underway in the CSF by 9 h and nearly completed by 24 h in all tissues. The differential time courses in the three compartments do not appear to be due to pharmacokinetic factors. Five days of twice daily treatment with LY-411575 not only sustained the Aβ reductions in all tissues, but significantly augmented the efficacy in the brain and plasma. The increased efficacy occurred in the absence of compound accumulation and was consistent with the recovery rates in each compartment. Overall, Aβ in the CSF and not plasma appears to be a better biomarker of brain Aβ reduction; however, the time course of Aβ changes needs to be established in clinical studies.

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The hallmark of Alzheimer's disease (AD) is the presence of amyloid-β containing senile plaques in the brain. Aβ peptides are generated by sequential proteolytic clevage of amyloid precursor protein, APP, by β- and γ-secretase activity. Of the Aβ isoforms, Aβ(1-40) is the most abundant and Aβ(1-42) is the most fibrillogenic and both have been studied intensively. Genetic mutations that lead to early onset AD are associated with greater levels of Aβ, specifically the Aβ(1-42) isoform. Hence, reducing cleavage of the APP holoprotein into amyloidgenic fragments holds much therapeutic promise. To this end, one of the dominant strategies currently being pursued is the development of a drug that inhibits the activity of β- or γ-secretase and thereby reduces the production of Aβ peptides in the brain. Indeed, animal studies have validated this approach. Thus the γ-secretase inhibitor, *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT), has been shown to dose-dependently reduce Aβ levels in the brains of two different strains of APP-transgenic mice (PDAPP and Tg2576) after only a single dose (Dovey et al., 2001; Lanz et al., 2003). Additionally, chronic administration of LY-411575 to PDAPP mice prior to the onset of plaque deposition also significantly prevents amyloid plaque accumulation in the brain (May et al., 2001).

The development of a clinical candidate aimed at reducing Aβ levels in the brain would be facilitated greatly by the availability of a biomarker that is easily accessible to assess the pharmacological efficacy of the treatment. Secreted Aβ(1-40) and Aβ(1-42) can be readily detected in the cerebrospinal fluid (CSF) and plasma of AD patients and normal controls (Ida et al., 1992; Seubert et al., 1992). Furthermore, acute treatment of Tg2576 mice with DAPT dose-dependently reduces Aβ levels in the CSF and plasma, independent of the plaque burden (Lanz et al., 2003). These data suggest that Aβ levels in the CSF and/or

plasma are a promising biomarker to establish proof of pharmacology of β- or γ-secretase inhibitors. The aim of the present studies was to characterize further the pharmacodynamics of Aβ(1-40) and Aβ(1-42) in order to evaluate their utility as biomarkers for clinical and preclinical studies.

One of the limitations of DAPT is its poor potency, which required administration of high doses to observe efficacy *in vivo* (Dovey et al., 2001). Hence, for the present studies we used a more potent γ-secretase inhibitor, LY-411575, that has an IC_{50} of 119 pM for reduction of Aβ levels in APP-transfected cell lines (Lewis et al., 2003). Young, non-plaque-bearing Tg2576 mice were selected for the studies in order to be able to assess Aβ reductions in not only the CSF and plasma but also the brain from the same animals. The studies had three specific goals: One, to determine whether the reduction in Aβ levels in the CSF or plasma correlates best with brain Aβ reduction using a dose response assessment. Secondly, to establish the time course of Aβ reduction in the brain, CSF and plasma to understand whether turnover rates of Aβ are similar in the three tissues. The final goal was to determine whether Aβ reductions are sustained in the three compartments after a subchronic treatment paradigm. The present results suggest that CSF Aβ levels may be monitored to assess γ-secretase inhibition in the brain. However, differences in turnover rates of Aβ between the brain and CSF exist. A better understanding of the turnover rates in AD patients is therefore necessary to be able to utilize these peptides in the CSF as biomarkers of pharmacological inhibition of γ-secretase activity or BACE (β-amyloid precursor protein cleavage enzyme).

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MATERIALS AND METHODS

Animals and *in vivo* drug administration

Three- to five-month-old, female, transgenic mice over-expressing the human *APP* gene with the Swedish double mutation (K670N/M671L) under the transcriptional control of the hamster prion protein promoter (Tg2576 line; Hsiao et al., 1996) were used for the studies (N=12 per group for each experiment). The use of female mice allowed direct comparisons with results of our previous study (Lanz et al., 2003), which also utilized only female mice. The preference for female mice for these experiments is based solely on practical issues concerning the maintenance of the transgenic colony. Male Tg2576 mice are too aggressive to permit group housing whereas the female Tg2576 mice can be group housed, which allows conservation of space and other resources. All animal treatment protocols were approved by Pharmacia's Institutional Animal Care and Use Committee and were compliant with the Animal Welfare Act Regulations.

For all studies, mice were dosed orally (PO) with vehicle (5% EtOH, 10% polysorbate, 5% soybean oil, 80% water) or LY-411575 suspended in the vehicle at a final volume of 5 or 10 mL/kg. For study one, mice received LY-411575 (0.1 mg/kg, 0.3 mg/kg, 1 mg/kg, or 10 mg/kg at 10 mL/kg) or the vehicle and reduction in Aβ levels in the plasma, CSF and brain tissue were assessed at 3 hours post-dose. The doses were selected to produce a broad dose-response curve and the 3 h time point was selected on the basis of previous studies with DAPT (Lanz et al, 2003). In the second study, the time course of Aβ reduction and recovery following a single dose of LY-411575 (1 mg/kg, PO, at 10 mL/kg) was determined by comparing Aβ levels at 9 and 24 hours post-dosing, with the inclusion of time-appropriate vehicle controls. The dose of 1 mg/kg was selected for the time-course experiment since it was the minimum effective dose that produced consistent reductions in

all tissues at 3 hours. In the third experiment, mice were treated twice daily (12 h apart) with vehicle or LY-411575 (0.3 mg/kg, 10 mg/kg) for 5 days in order to determine whether Aβ reduction can be sustained after subchronic administration of the γ-secretase inhibitor. The dose volume was reduced to 5 mL/kg for this experiment to minimize potentially harmful effect of the vehicle by itself as a result of twice daily dosing. The two extreme doses from the dose-response study were selected for this purpose to be able to assess the potential for either augmentation or attenuation in efficacy after repeated dosing. Tissues were collected 3 hours after the final dose to enable comparison with effects seen after a single dose of LY-411575 at 0.3 or 10 mg/kg.

Tissue isolation and processing

At the specified time after vehicle or drug administration, mice were anaesthetized with a mixture of ketamine/xylazine (200/5 mg/kg, SC; Butler Company, Columbus, OH). Muscle tissue was dissected away from the cisterna magna, the skull around the cisterna magna was cleaned to remove traces of blood. Cerebrospinal fluid (CSF) was collected (approximately 15-20 μL per animal) from the cisterna magna via a fine tipped pipette. Samples were frozen immediately on dry ice. Blood samples were obtained by cardiac puncture, collected in EDTA-coated tubes, and centrifuged (2400 rpm; 20 min) within 1 hr of collection to separate the plasma. The entire hippocampus (bilateral) and overlying cerebral cortex from one hemisphere were removed immediately after excising the brains from the skulls and frozen in separate tubes on dry ice. The brain samples were homogenized in 5M guanidine buffer (5M guanidine HCl in 50mM Tris-Cl, pH 8.0; Sigma-Aldrich, St. Louis, MO) at 1:10 dilution. Homogenates were agitated at room temperature for 3-4 hours, then stored at –20^{o}C. The day before running the ELISA, the brain homogenates (1:10 in 5M guanidine) were diluted further 1:10 for a final guanidine concentration of 0.5M. The

homogenates were then spun down at 14,000 rpm for 20 min at 4°C and supernatants were used for Aβ determination by ELISA.

Aβ ELISA

Aβ(1-40) and Aβ(1-42) were assayed using ELISA as detailed in Lanz et al. (2003). Briefly, CSF and plasma samples were thawed and diluted in blocking buffer (PBS, 0.05% Tween 20, 1% bovine serum albumin); CSF was diluted 1:20 for Aβ(1-42) and 1:40 for Aβ(1-40), plasma was diluted 1:2.5 for Aβ(1-42) and 1:5 for Aβ(1-40). Brain extracts were assayed as already diluted in 0.5M guanidine.

Each sample was assayed in duplicate wells and the average signal was used to determine Aβ concentrations for the sample. Half-volume, 96-well plates were coated overnight at 4°C with capture antibody 6E10 (Signet Laboratories, Dedham, MA) diluted 1:250 in 0.1 M $NaHCO_3$, pH 8.2. Standard curves were prepared in blocking buffer for plasma and CSF Aβ assays and in guanidine buffer for brain homogenates from stock solutions of Aβ(1-40) and Aβ(1-42). Standards and samples were incubated for 3 hrs at room temperature. Detection of Aβ(1-40) and Aβ(1-42) was carried out using biotinylated rabbit polyclonal antibodies (purchased from Dr. Mehta, N.Y. State Institute of Basic Research, Staten Island, NY) as described previously (Mehta et al., 1998). After signal amplification with HRP-conjugated neutravidin, protein levels were determined using a TMB peroxidase substrate kit (Kirkegaard & Perry Labs, Gaithersburg, MD); color development was stopped after 1 hr with 1M H_3PO4. Plates were read at 450 nm on a microplate reader and 4-par logistics were used to determine unknown values for the samples using the standard curves.

Measurement of drug levels in brain tissue and plasma

Brain tissue was homogenized 1:2 in methanol/water (75:25) using a sonic probe. Brain or plasma samples (30 μL) were deproteinized with acetonitrile (200 μL) containing an internal standard (BMS-494). Supernatant were mixed with 1 μL propylene glycol and evaporated to dryness followed by reconstitution in 20 μL methanol plus 50 μL water. Samples were chromatographed on a Phenomenex Luna C18(2) reversed-phase column (150 mm x 2.0 mm ID; 5 μm) incorporating a mobile phase consisting of acetonitrile/5 mM aqueous ammonium formate and a step gradient system (50:50 to 90:10, v:v) at a flow rate of 0.3 mL/min. ESI-LC/MS/MS was performed on a Sciex API4000 equipped with ESI source operated in negative ion mode; neutral loss of 351 amu observed in product mass spectrum. Quantitation was achieved by SRM using peak area ratios and quadratic regression parameters calculated using concentration weighting.

Statistical Analysis and Data Presentation

For each study, one-way analysis of variance was used to detect a significant treatment effect on Aβ(1-40) and Aβ(1-42) in each tissue compartment. To facilitate comparisons between studies, all values were normalized to the corresponding vehicle controls. Data are presented as mean % of vehicle control ± standard error of the mean (SEM). Following a significant main effect by ANOVA, individual group differences were analyzed using Dunnett's multiple comparison test; $p<0.05$ was set as a statistically significant level.

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RESULTS

Dose response study of LY-411575: 3h time point

LY-411575 dose-dependently reduced Aβ(1-40) and Aβ(1-42) in the brain, CSF, and plasma of Tg2576 mice (Fig. 1*A, B*). A main effect of treatment was evident for cortical Aβ(1-40) ($F_{4,8}$=25.2, p<0.001), cortical Aβ(1-42) ($F_{4,8}$=7.99, p<0.001) as well as hippocampal Aβ(1-40) ($F_{4,8}$=77.6, p<0.001) and hippocampal Aβ(1-42) ($F_{4,8}$=26.7, p<0.001). Compared to the vehicle controls, both 1 mg/kg and 10 mg/kg doses significantly reduced Aβ(1-40) in the cortex (p<0.01 for each dose group), but cortical Aβ(1-42) was significantly reduced only by the 10 mg/kg dose (p<0.01). Hippocampal Aβ(1-40) was significantly reduced from vehicle base-line by both 1 and 10 mg/kg LY-411575 (p<0.01). Similarly Aβ(1-42) was reduced by the 1 and 10 mg/kg doses of LY-411575 (p<0.01).

As seen in the brain, a significant main effect of treatment could be observed in the CSF for both Aβ(1-40) ($F_{4,8}$=25.2, p<0.001) and Aβ(1-42) ($F_{4,8}$=23.2, p<0.001). Group-wise comparisons showed that significant reductions in both amyloid peptides were produced by LY-411575 administered at 1 or 10 mg/kg doses (p<0.001). Plasma Aβ(1-40) and Aβ(1-42) also showed a significant treatment effect ($F_{4,8}$=76.04, p<0.001 and $F_{4,8}$=93.08, p<0.001, respectively). However, amyloid peptide levels were raised by the 0.1 mg/kg dose (significance was reached only for Aβ(1-42), p<0.01), which was followed by dose-dependent reductions in both Aβ(1-40) and Aβ(1-42) levels at 0.3, 1, and 10 mg/kg (p<0.01 for each dose).

Time course of changes in Aβ levels after treatment with LY-411575 at 1 mg/kg

In the cortex and the hippocampus, a significant treatment effect was evident for both Aβ(1-40) ($F_{3,9}$=21.8, p<0.001, $F_{3,9}$=22.1, p<0.001, respectively) and Aβ(1-42) ($F_{3,9}$=4.05,

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$p<0.05$, $F_{3,9}=8.07$, $p<0.001$, respectively) as a result of treatment with 1 mg/kg LY-411575 (Fig. 2; 3 hour data from the dose response study is reproduced to facilitate direct comparisons of time points). Compared to the 3 h time point, there was greater efficacy in both brain regions at 9 hours, which was followed by a partial or complete recovery in Aβ levels at 24 h after treatment. In the cortex, Aβ(1-40) was approximately 50% of vehicle controls ($p<0.01$) at 9 hours, but only 10% lower than the vehicle control by 24 hours. Cortical Aβ(1-42) level was significantly reduced only at the 9 hour time point ($p<0.01$ versus vehicle control); at 3 and 24 hours it was statistically similar to that in the vehicle-treated mice. In LY-411575 treated animals, cortical Aβ(1-40) levels at 9 h were significantly different from those at 3 h ($p<0.05$) and 24 h ($p<0.01$). In the hippocampus as well, Aβ(1-40) levels showed greater reductions at 9 h than at the 3h time point ($p<0.01$) and showed a significant recovery at 24 h ($p<0.01$ vs 9 h time point). By 24 hours, although hippocampal Aβ(1-40) remained lower than the vehicle control, Aβ(1-42) was not significantly different from vehicle.

Like the brain, a significant treatment effect was evident in the CSF for both amyloid peptides ($F_{3,9}=20.5$, $p<0.001$ for Aβ(1-40) and $F_{3,9}=10.2$, $p<0.001$ for Aβ(1-42)). Unlike the brain, CSF showed smaller reductions in Aβ levels at 9 h than those at 3 h ($p<0.05$ for Aβ(1-40) and $p<0.05$ for Aβ(1-42)) and showed a full recovery at 24 h after LY-411575 treatment ($p<0.01$ versus the 3 h time point). Thus about 20-25% reductions in Aβ(1-40) and Aβ(1-42) levels were evident in the CSF at 9 h ($p<0.01$ versus the vehicle controls) compared to approximately 40% reductions evident at 3 h after LY-411575.

A significant treatment effect was evident for plasma Aβ(1-40) ($F_{3,9}=76.7$, $p<0.001$) and Aβ(1-42) ($F_{3,9}=37.0$, $p<0.001$) reductions and the magnitude of the effect appeared to be

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greater in the plasma than the other tissues examined. The time course of Aβ reductions in the plasma was somewhat distinct from that in the brain as well as the CSF. Thus the efficacy of LY-411575 at reducing Aβ levels was similar at 3 and 9 h followed by a partial recovery in Aβ(1-40) and an almost complete recovery in Aβ(1-42) level at 24 h ($p<0.05$ for Aβ(1-40), $p<0.01$ versus 3 or 9 h time points for both amyloid peptides).

5-day repeat-dosing with LY-411575

As shown in Figure 3, BID dosing at 0.3 or 10 mg/kg LY-411575 produced greater Aβ reductions at 3 hours after the final (10^{th}) dose than those seen after a single dose; 3 hour acute data from Figure 1 is shown alongside the repeat-dosing data for direct comparison. After the subchronic treatment, the greatest increase in LY-411575 efficacy was evident in the brain followed by the plasma; however, CSF Aβ reductions were comparable between the acute and subchronic treatment groups. The 0.3 mg/kg dose, which was a threshold dose acutely for brain effects, showed robust efficacy in both brain regions after 10 doses administered over 5 days. In the cortex and hippocampus, a significant treatment effect on Aβ(1-40) ($F_{4,8}=109.2$, $p<0.001$, $F_{4,8}=10.3$, $p<0.001$, respectively) and Aβ(1-42) ($F_{4,8}=22.5$, $p<0.001$, $F_{4,8}=39.7$, $p<0.001$, respectively) levels were evident. Compared to the vehicle controls, 50% to 60% reductions in Aβ levels were observed after the 0.3 mg/kg, BID dose ($p<0.001$). At the 10 mg/kg, BID dose LY-411575 reduced Aβ(1-40) to background levels and Aβ(1-42) by 80% of control levels ($p<0.01$). For each dose level, the reductions in both amyloid peptides were significantly greater in magnitude than those seen after the corresponding single dose administration ($p<0.01$ for all comparisons except Aβ(1-42) in the hippocampus, which did not achieve statistical significance).

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Although a significant treatment effect could be seen in the CSF ($F_{4,8}$=32.5, $p<0.001$ for Aβ(1-40) and $F_{4,8}$=33.2, $p<0.001$ for Aβ(1-42), the augmentation in efficacy after repeated treatment with LY-411575 was marginal in this tissue. Thus both Aβ peptides were reduced by 25% after 0.3 mg/kg, BID, LY-411575 ($p<0.05$) and by greater than 90% after the 10 mg/kg, BID dose ($p<0.01$). However, neither subchronic treatment dose was significantly different from the corresponding single acute dose

Significant treatment effects on plasma Aβ(1-40) ($F_{4,8}$=42.8, $p<0.001$) and Aβ(1-42) ($F_{4,8}$=35.8, $p<0.001$) levels were also observed. As with the other tissues, effects of the subchronic treatment were dose-dependent with the 10 mg/kg dose reducing both Aβ peptide levels to below 20% of vehicle controls whereas 0.3 mg/kg BID reduced them to 30-40% of controls ($p<0.01$). The augmentation of the efficacy after subchronic treatment compared to the acute dosing was evident only for the 0.3 mg/kg dose for both Aβ peptides ($p<0.05$).

Concentrations of LY-411575 in the brain and plasma

After oral dosing with 1 mg/kg of LY-411575, plasma Cmax was 25.1 ± 3.5 nM, Tmax was 30 min and $T_{1/2}$ was calculated to be 1.83 hours. The only dose for which both plasma and brain levels of LY-411575 could be consistently measured was 10 mg/kg. Table 1 shows the concentration of the compound in the plasma and brain at 3 h (from the dose response study) and 9 hours (from the time course study) after a single dose of LY-411575 at 10 mg/kg. Additionally, data on LY-411575 levels after 10 mg/kg, BID, from the repeated dose study are included. After a single dose, brain compound levels were higher at 3 hours than at 9 h, though efficacy was greater at 9 hours in the brain. Repeated dosing, 12 h apart for 5 days, did not cause the compound to accumulate either in the plasma or the brain; rather the brain and plasma levels of LY-411575 at the 3 hour time point after 5 days of dosing appear to be lower than those at 3 hours after a single acute dose.

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DISCUSSION

The present study extends the results of Lanz et al. (2003) and provides further support to the contention that assessment of Aβ levels in the CSF and plasma offers a promising biomarker strategy for clinical studies of γ- and likely β-secretase (BACE) inhibitors. To our knowledge, this is the first published report characterizing Aβ pharmacodynamics simultaneously in the brain, CSF and plasma in a mouse model of amyloid pathology associated with Alzheimer's disease.

One of the main requirements for a pharmacology-based biomarker is to demonstrate that it changes dose-dependently with the treatment. Indeed, brain and CSF Aβ peptides were dose-dependently reduced by both acute and subchronic treatment of young, plaque-free Tg2576 mice with LY-411575. On the other hand, its effects in the plasma followed a more complex dose relationship with the lowest dose (0.1 mg/kg) increasing Aβ peptide levels, and dose-dependent reductions at higher doses. The mechanism underlying the increase in plasma Aβ levels is unclear, but a similar phenomenon (increases in secreted Aβ(1-40) and Aβ(1-42) levels) was observed by us (Li et al., 2003) in studies of fibroblast cultures derived from mice with partial deficiency of the γ-secretase component, nicastrin (i.e., nicastrin$^{+/-}$ mice) when compared to nicastrin$^{+/+}$ fibroblasts. Taken together, these data indicate that partial inhibition of γ-secretase activity by pharmacological or genetic means may result in higher Aβ secretion in some cell types. It is interesting that neither the CSF nor the brain tissues showed an increase in Aβ levels even though several studies have demonstrated an active transport of Aβ from the blood to the central compartments (Zlokovic et al., 1993; Maness et al., 1994; Martel et al., 1996; Poduslo et al., 1999, Deane et al., 2003). The implications of increases in plasma Aβ levels at low doses of γ-secretase inhibitors

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remain unclear and require further studies aimed at assessing long-term effects on end points such as brain amyloid plaque load. The plasma also appeared to be more sensitive to LY-411575-induced reduction in Aβ levels than the CSF and brain. Thus at 0.3 mg/kg, plasma Aβ levels were about 50% of controls whereas brain and CSF Aβ peptide levels were minimally affected. Measurements of LY-411575 levels demonstrate that the differential sensitivity of the plasma from that of the brain or the CSF to LY-411575 cannot be attributed to differences in tissue concentrations of the compound and likely involves pharmacodynamic factors. Thus overall the dose-response study demonstrates that CSF Aβ levels appear to correlate better with brain Aβ reductions following treatment with LY-411575 and supports the idea that Aβ reductions in the CSF may be used as a marker of brain Aβ reduction following γ- secretase, and likely BACE, inhibition.

The apparently differential sensitivity of plasma versus central compartments to LY-411575 raises an interesting opportunity to characterize further the dynamics of Aβ between plasma and central compartments. Thus the so-called "peripheral sink" hypothesis proposed by Demattos et al. (2001) and supported by Matsuoka et al. (2003) suggests that the binding of the plasma Aβ pool by a high affinity antibody or Aβ binding proteins facilitates Aβ efflux from the brain. Taken together with the recent evidence of RAGE-mediated Aβ transport from the plasma to the brain (Deane et al., 2003), it would be interesting to determine whether chronic treatment with a γ-secretase inhibitor that significantly reduces plasma Aβ (without affecting brain Aβ) could lower brain amyloid plaque deposition as a result of reduced influx of amyloid peptides from the periphery. The dose- and time-response data shown here provide the critical information needed for the design of such a study.

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Comparison of the time courses of Aβ changes after LY-411575 treatment, demonstrated that the time course of Aβ reduction and recovery in the CSF was distinct from those in the brain. Within the time periods examined, maximal reductions in CSF Aβ were evident at 3 hours with a recovery underway by 9 hours and a full restoration of Aβ levels by 24 h. In contrast, brain Aβ levels continued to go down from 3 to 9 hours after a single dose treatment with LY-411575 and showed partial recovery by 24 h. It is noteworthy that the pharmacodynamic effects of LY-411575 in the brain are shifted temporally rightwards in relation to the tissue levels of the compound. Thus although the compound levels in the brain were higher at the 3 hour time point, the efficacy was greater at 9 h in this tissue. One possible explanation for the distinction between the CSF and brain Aβ turnover rates is that brain Aβ levels, as measured here, reflect total Aβ consisting of both the secreted, extracellular pool and an intracellular pool, the turnover rates of which are likely to be different. The half-life of extracellular Aβ (analyzed by microdialysis of interstitial fluid) has been estimated at 2 h (Cirrito et al., 2003), while that of combined pools (as extracted by guanidine) has been estimated as between 1 and 2.5h (Savage MJ, 1998). The CSF Aβ pool likely represents, or is at least in equilibrium with, the brain extracellular pool, which appears to have a faster turnover rate than the intracellular pool. This is consistent with the observation that under non-equilibrium dosing conditions occurring at 3 h after a single dose, 10 mg/kg LY-411575 produced significantly greater decrease in Aβ levels in the CSF than in the brain. On the other hand, after repeated dosing, which may approach equilibrium conditions, total brain Aβ reduction is similar to that seen in the CSF (<80% of control). The differential turnover rates of CSF and brain Aβ peptides also appear to be responsible for the profound augmentation in the efficacy of LY-411575 in the brain, but only marginal

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improvement in the CSF, after 0.3 mg/kg BID dosing for 5 days. The daily doses were administered 12 hours apart, a time point at which the CSF Aβ levels have likely recovered to baseline values (on the basis of extrapolation from the effect sizes at 3, 9 and 24 h time points). Thus the second dose of LY-411575 may produce the same magnitude of effect (change from baseline) in the CSF as that seen after the first dose, resulting in no net augmentation in efficacy after 5-days of BID dosing in this tissue. On the other hand, the time course data shown here indicate that at 12 h brain Aβ levels are likely to be significantly lower than the baseline such that the administration of a second dose of LY-411575 at 12 h will reduce Aβ to a level lower than that seen after a single dose. Thus the cumulative effect of 5 days of BID dosing would be expected to result in greater efficacy in the brain than in the CSF, as was demonstrated in the present study. It should be noted that the subchronic, BID dosing paradigm did not lead to accumulation of LY-411575 in the brain. As such, it is the pharmacodynamic factors (namely turnover rates), and not pharmacokinetic factors, that appear to be solely responsible for the differential effects of subchronic treatment in the CSF versus the brain. Although not to the same extent as the brain, the efficacy of LY-411575 was augmented in the plasma after 5 days of dosing than that seen after a single dose. As with the brain, the slower recovery of Aβ in the plasma (sustained reduction at 9 hours post treatment) is the most likely explanation for this observation.

Although our data support the use of CSF Aβ as a biomarker to monitor the efficacy of γ-secretase inhibitors (and likely BACE inhibitors as well), they raise critical issues that need to be considered or resolved for validation of CSF Aβ as a biomarker for brain Aβ changes. One complicating issue relates to the pools of Aβ measured in the CSF and plasma. Given the association of Aβ with various proteins in these fluids, it is unclear whether the

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ELISA used here reflects total Aβ in the CSF and plasma (Matsubara et al., 1999; Kuo et al., 2000) and more importantly whether γ-secretase inhibition may differentially affect the dynamics of Aβ in the various pools. Future studies using differential extraction techniques will help clarify this issue. Another important factor to consider for the translation of the present data to clinical application is that the Tg2576 transgenic mouse produces significantly higher levels of amyloid peptides than those seen in the corresponding human tissues (Kawarabayashi et al., 2001) or the PDAPP mouse (Masliah et al., 1996). Whether the absolute levels of Aβ peptides could affect the turnover rates of amyloid peptides in the CSF is unclear. However, a comparison of our previous study (Lanz et al., 2003) of the γ-secretase inhibitor, DAPT, in the Tg2576 mice to that of Dovey et al (2001) in the PDAPP mice indicates that the acute potency and efficacy of DAPT are similar between the two mouse models. Nonetheless, given the complexity of mechanisms proposed to regulate Aβ production and clearance, it will be important to determine the turnover rates of amyloid peptides in clinical CSF samples. Additionally, although the presence of plaques does not affect the acute efficacy (CSF Aβ reduction) of a given dose of a γ-secretase inhibitor (Lanz et al., 2003), one needs to determine whether the presence of plaques could affect the rates of recovery in Aβ levels in the CSF following treatment with a γ-secretase inhibitor. This is especially important in view of studies by DeMattos et al (2002) and Cirrito et al (2003) that indicate that that the presence of plaques affects the dynamics of Aβ as measured by peripheral antibody-induced accumulation of plasma Aβ or turnover rate of interstitial fluid Aβ.

In summary, the data provide a rationale for clinical studies of CSF Aβ as a pharmacology-based biomarker for γ-secretase, and likely BACE, inhibitors. However,

differential turnover rates in CSF versus plasma Aβ shown here as well other factors discussed above indicate that further clinical validation of this biomarker is required. Once validated, CSF Aβ has the potential to be used as a biomarker for clinical dose-setting and proof of concept studies; thereby aiding development of novel therapeutic agents for Alzheimer's disease.

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FIGURES AND TABLES

Figure 1. Effects of acute LY-411575 administration (PO) on Aβ(1-40) (*A*) and Aβ(1-42) (*B*) in various compartments of Tg2576 mice at 4 different doses. Bars represent mean Aβ concentrations shown as % of vehicle Aβ concentration + SEM for cortex (black), hippocampus (white), CSF (grey), and plasma (hatched). The average Aβ(1-40) concentrations (nM) for each tissue in vehicle-treated controls were as follows: cortex, 25.0 ± 1.5; hippocampus, 32.5 ± 1.1; CSF, 13.0 ± 1.0; plasma, 1.9 ± 0.08. The average Aβ(1-42) concentrations (nM) for each tissue in vehicle-treated controls were as follows: cortex, 4.0 ± 0.2; hippocampus, 5.0 ± 0.3; CSF, 1.7 ± 0.5; plasma, 0.2 ± 0.02. *p<.05, **p<.01 versus corresponding vehicle controls.

Figure 2. Effects of acute LY-411575 administration (PO) on Aβ(1-40) (*A*) and Aβ(1-42) (*B*) in various compartments of Tg2576 mice at 3 different time points. Bars represent mean Aβ concentrations shown as % of vehicle control Aβ concentration + SEM at 3 hours post-dose (black), 9 hours post-dose (white), or 24 hours post-dose (grey). *p<.05, **p<.01 versus the corresponding vehicle-treated controls, #p<0.05, ##p<0.01 versus the corresponding 3 h time point.

Figure 3. Effects of acute versus subchronic LY-411575 administration (PO) on Aβ(1-40) (*A*) and Aβ(1-42) (*B*) in various compartments of Tg2576 mice at 2 different doses. Bars represent mean % of vehicle Aβ concentration + SEM after a single dose at 0.3 mg/kg (black), 5 days of BID dosing at 0.3 mg/kg (white), a single dose at 10 mg/kg (grey), or 5 days of BID dosing at 10 mg/kg (hatched). The average Aβ(1-40) concentrations (nM) for each tissue in subchronic vehicle-treated controls were as follows: cortex, 23.4 ± 1.1; hippocampus, 29.7 ± 0.7; CSF, 8.1 ± 0.7; plasma, 1.2 ± 0.08. The average Aβ(1-42) concentrations (nM) for each tissue in subchronic vehicle-treated controls were as follows: cortex, 9.7 ± 0.6; hippocampus, 9.8 ± 0.6; CSF, 0.9 ± 0.3; plasma, 0.4 ± 0.05. *p<.05, **p<.01 versus the corresponding vehicle treated controls; # p<0.05, ## p<0.01 versus the corresponding acute dose.

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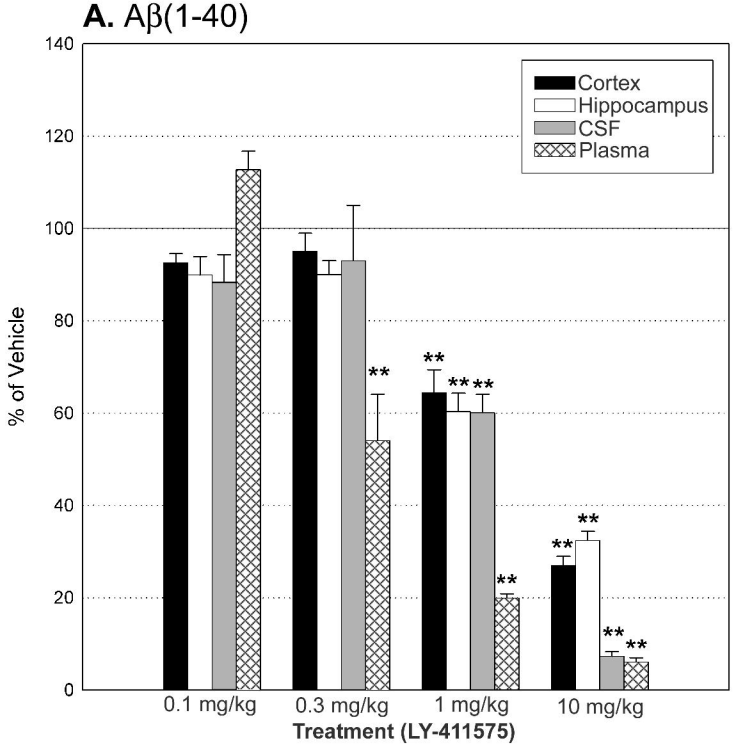
Table 1. Concentration of LY-411575 in brain and plasma 3 hours after a single dose at 10 mg/kg, 9 hours after a single dose at 10 mg/kg, or 3 hours after 5 days of BID dosing at 10 mg/kg. Values are given as mean ± SEM.

Figure 1.


A. Aβ(1-40)
Cortex
Hippocampus
CSF
Plasma
% of Vehicle
0
20
40
60
80
100
120
140
**
0.1 mg/kg
0.3 mg/kg
1 mg/kg
10 mg/kg
Treatment (LY-411575)

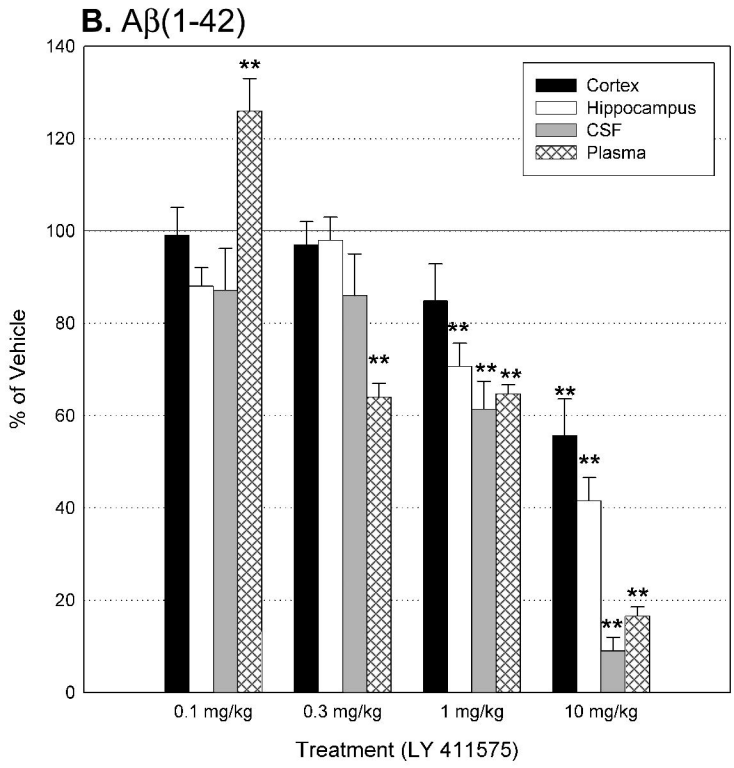
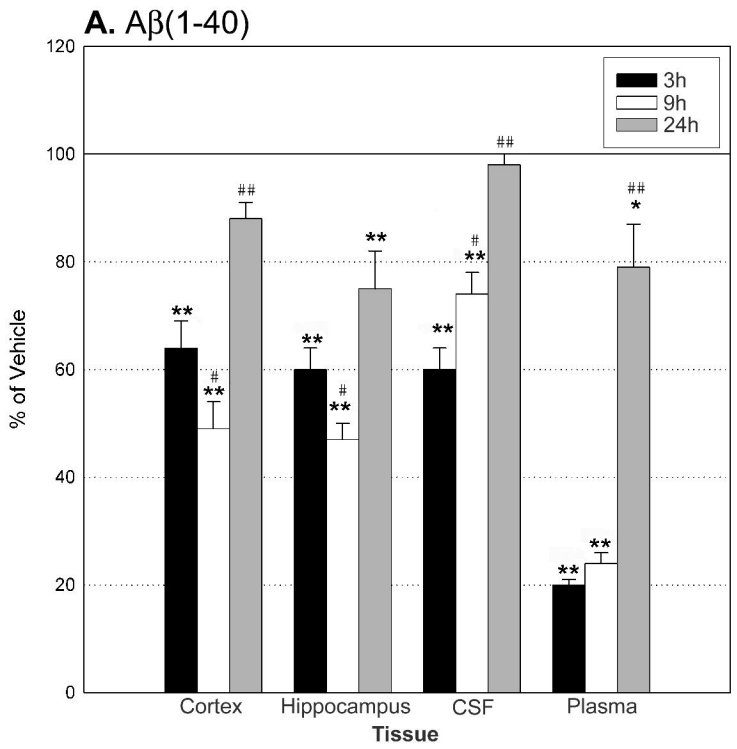

B. Aβ(1-42)
Cortex
Hippocampus
CSF
Plasma
% of Vehicle
0
20
40
60
80
100
120
140
**
0.1 mg/kg
0.3 mg/kg
1 mg/kg
10 mg/kg
Treatment (LY 411575)

Figure 2.


A. Aβ(1-40)
3h
9h
24h
% of Vehicle
120
100
80
60
40
20
0
Cortex
Hippocampus
CSF
Plasma
Tissue

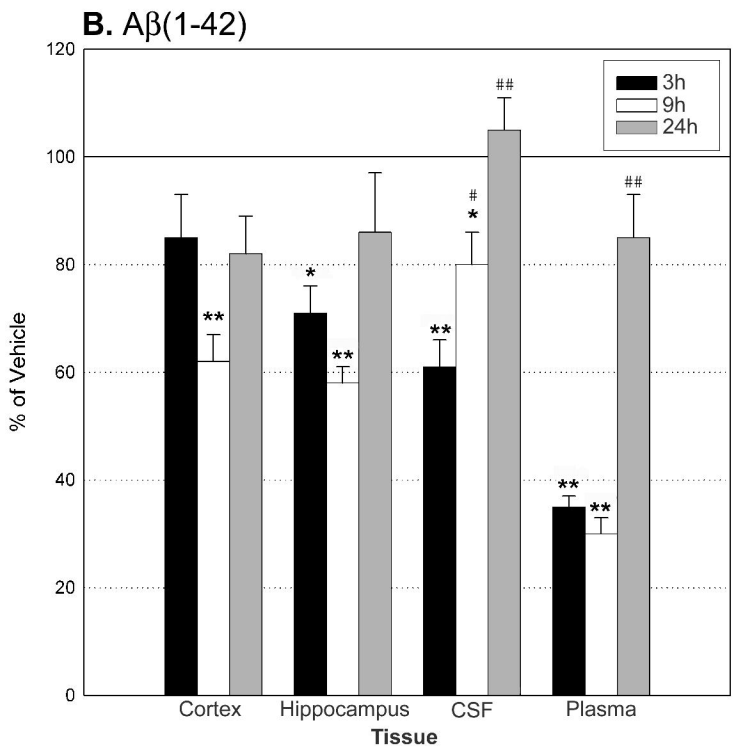

B. Aβ(1-42)
3h
9h
24h
% of Vehicle
120
100
80
60
40
20
0
Cortex
Hippocampus
CSF
Plasma
Tissue

Figure 3.

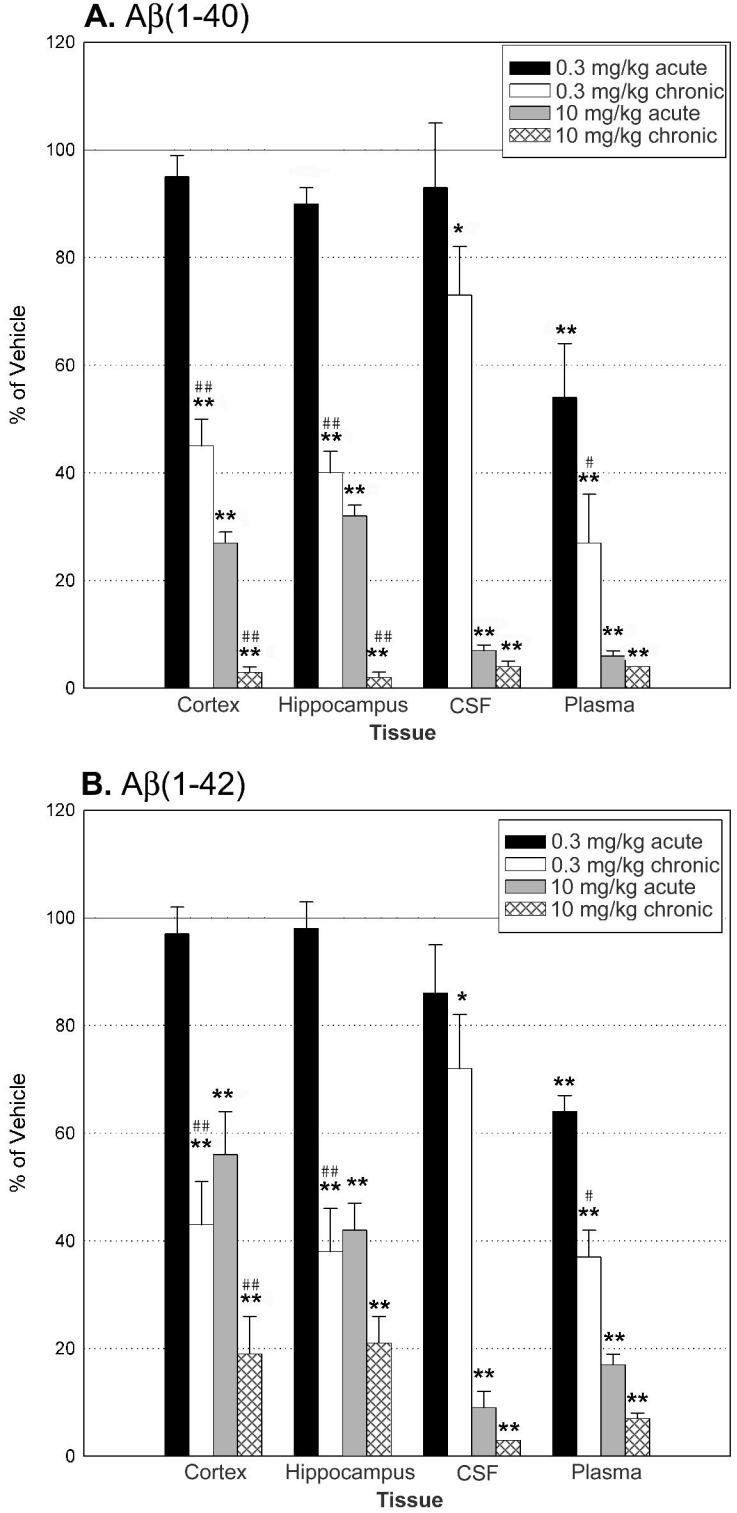

A. Aβ(1-40)
0.3 mg/kg acute
0.3 mg/kg chronic
10 mg/kg acute
10 mg/kg chronic
% of Vehicle
120
100
80
60
40
20
0
Cortex
Hippocampus
CSF
Plasma
Tissue
B. Aβ(1-42)
0.3 mg/kg acute
0.3 mg/kg chronic
10 mg/kg acute
10 mg/kg chronic
% of Vehicle
120
100
80
60
40
20
0
Cortex
Hippocampus
CSF
Plasma
Tissue

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Table 1.

Dosing schedule	Brain concentration (nM)	Plasma concentration (nM)
3 hours – single dose	31.7 ± 2.7	47.2 ± 5.9
9 hours – single dose	16.5 ± 1.3	9.9 ± 1.36
5 days – chronic dosing	17.8 ± 1.3	17.5 ± 3.9