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Concentration-Dependent Modulation of Amyloid-β in Vivo and in Vitro Using the γ-Secretase Inhibitor, LY-450139

Thomas A. Lanz, Michael J. Karmilowicz, Kathleen M. Wood, Nikolay Pozdnyakov, Ping Du, Mary A. Piotrowski, Tracy M. Brown, Charles E. Nolan, Karl E. G. Richter, James E. Finley, Qing Fei, Charles F. Ebbinghaus, Yuhpyng L. Chen, Douglas K. Spracklin, Barbara Tate, Kieran F. Geoghegan, Lit-Fui Lau, David D. Auperin, and Joel B. Schachter

CNS Discovery, Pfizer, Inc., Groton, Connecticut

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ABSTRACT

LY-450139 is a γ-secretase inhibitor shown to have efficacy in multiple cellular and animal models. Paradoxically, robust elevations of plasma amyloid-β (Aβ) have been reported in dogs and humans after administration of subefficacious doses. The present study sought to further evaluate Aβ responses to LY-450139 in the guinea pig, a nontransgenic model that has an Aβ sequence identical to that of human. Male guinea pigs were treated with LY-450139 (0.2–60 mg/kg), and brain, cerebrospinal fluid, and plasma Aβ levels were characterized at 1, 3, 6, 9, and 14 h postdose. Low doses significantly elevated plasma Aβ levels at early time points, with return to baseline within hours. Higher doses inhibited Aβ levels in all compartments at early time points, but elevated plasma Aβ levels at later time points. To determine whether this phenomenon occurs under steady-state drug exposure, guinea pigs were implanted with subcutaneous minipumps delivering LY-450139 (0.3–30 mg/kg/day) for 5 days. Plasma Aβ was significantly inhibited at 10–30 mg/kg/day, but significantly elevated at 1 mg/kg/day. To further understand the mechanism of Aβ elevation by LY-450139, H4 cells overexpressing the Swedish mutant of amyloid-precursor protein and a mouse embryonic stem cell-derived neuronal cell line were studied. In both cellular models, elevated levels of secreted Aβ were observed at subefficacious concentrations, whereas dose-responsive inhibition was observed at higher concentrations. These results suggest that LY-450139 modulates the γ-secretase complex, eliciting Aβ lowering at high concentrations but Aβ elevation at low concentrations.

The pathological accumulation of amyloid-β peptide into dense core plaques in the brains of Alzheimer's disease patients is the ultimate target of multiple disease-modifying drug discovery efforts. One strategy that has entered the clinic is the use of a γ-secretase inhibitor to reduce central Aβ production. Preclinically, multiple γ-secretase inhibitors have demonstrated central and peripheral Aβ-lowering activity in transgenic mouse lines overexpressing human mutant amyloid precursor protein (Dovey et al., 2001; Cirrito et al., 2003; Lanz et al., 2003, 2004; Wong et al., 2004; Barten et al., 2005), as well as nontransgenic species (Anderson et al., 2005; Best et al., 2006; El Mouedden et al., 2006). Whereas acute treatment of old, plaque-bearing mice should have little immediate impact on plaque load (insoluble Aβ), these inhibitors have been shown to inhibit Aβ in CSF (Lanz et al., 2003; Barten et al., 2005) and interstitial fluid (Cirrito et al., 2003) similarly in both plaque-free and plaque-bearing mice. In addition, plasma Aβ has been shown to be reduced similarly by γ-secretase inhibition in both young and old Tg2576 mice (Lanz et al., 2003; Barten et al., 2005). These findings indicate that despite the presence or absence of insoluble Aβ plaques, these compounds had similar potency in reducing soluble, secreted Aβ in young and old transgenic mice.

The ability of plasma and CSF Aβ to track pharmacological changes in brain Aβ provides a useful method for tracking the efficacy of γ-secretase inhibitors in the clinic. Because each compartment may have varying degrees of drug exposure and different clearance rates for both drug and Aβ, it is important to understand the dynamics of Aβ in each compartment. Dose-response and time course studies with γ-secretase inhibitors in transgenic mice have revealed dif-

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ABBREVIATIONS: LY-450139, (*S*)-2-hydroxy-3-methyl-*N*-[(*S*)-1-((*S*)-3-methyl-2-oxo-2,3,4,5,-tetrahydro-1*H*-benzo[*d*]azepin-1-carbamoyl)-ethyl]-butyramide; Aβ, amyloid-β; CSF, cerebrospinal fluid; APP, amyloid-precursor protein; H4(Sw), H4 cells overexpressing the Swedish mutation of APP; IP/MS, immunoprecipitation/mass spectroscopy; ELISA, enzyme-linked immunosorbent assay; DMSO, dimethyl sulfoxide; QC, quality control.

ferences in sensitivity, time of maximal efficacy, and rate of Aβ recovery in each of the different compartments. At lower doses or earlier time points, Aβ levels in plasma seemed to be more dynamic than those in CSF or brain (Lanz et al., 2004; Barten et al., 2005). In addition, a low dose of LY-411575 in Tg2576 mice has been shown to elevate plasma Aβ1–40 and Aβ1–42 while having no effect upon CSF or brain Aβ (Lanz et al., 2004). A structurally related γ-secretase inhibitor, LY-450139, has since been shown to elevate plasma Aβ levels in beagles and in human volunteers either at low doses that had no effect upon CSF Aβ or at higher doses that initially lowered plasma Aβ but later elevated plasma Aβ above baseline (Hyslop et al., 2004; Siemers et al., 2005).

In the present series of experiments, we sought to further characterize the multicompartment efficacy of LY-450139 in guinea pigs, whose Aβ sequence is identical to that of human (Johnstone et al., 1991). To obtain both dose-response and time course information in brain, CSF, and plasma, a six-point dose response was repeated in different cohorts of animals that were evaluated at 1, 3, 6, 9, and 14 h postadministration. At early time points subefficacious doses raised plasma Aβ, whereas higher doses lowered Aβ levels in brain, CSF, and plasma, as expected. At later time points, Aβ levels in animals receiving lower doses had returned to baseline, whereas animals receiving the higher doses exhibited elevated levels of plasma Aβ. To test the hypothesis that this potentiation phenomenon is driven by drug concentration, guinea pigs were implanted with minipumps delivering vehicle or LY-450139 for 5 days to establish steady-state drug levels in plasma. On the final day, brain, CSF, and plasma were collected and analyzed. At high drug concentrations, inhibition of plasma and brain Aβ was observed. A low concentration (1 mg/kg/day) significantly elevated plasma Aβ.

To rule out the possibility that Aβ elevation at low concentrations might be caused by intercompartmental dynamics, two cell lines were dosed with LY-450139, and Aβ was analyzed in media. Both APP overexpressing H4(Swe) cells and embryonic stem cell-derived mixed neuronal cultures showed a similar pattern: dose-responsive Aβ inhibition until a certain low concentration range in which Aβ levels were elevated. IP/MS of H4(Swe) media confirmed ELISA data and showed multiple Aβ fragments responding to concentration-dependent elevation and inhibition.

Materials and Methods

In Vivo Experiments

Animals and Drug Administration. Male Hartley guinea pigs (Charles River) were used at ~225 to 250 g (n = 6 per group). All animal treatment protocols were approved by Pfizer's Institutional Animal Care and Use Committee and were compliant with Animal Welfare Act Regulations. For acute dosing, animals were administered vehicle (0.5% methylcellulose) or 0.2, 0.6, 2, 6, 20, or 60 mg/kg LY-450139 (see Fig. 1 for structure) by oral gavage. Doses and time points were selected on the basis of pilot experiments (data not shown). For steady-state drug exposure, 7-day minipumps (10 μl/h) were implanted s.c. in male guinea pigs (n = 6 per group). Minipumps were loaded with vehicle (20% DMSO, 20% EtOH, and 60% PEG400) or LY-450139 at concentrations corresponding to a delivery rate of 0.3, 1, 3, 10, or 30 mg/kg/day, and animals were euthanized after 5 days.

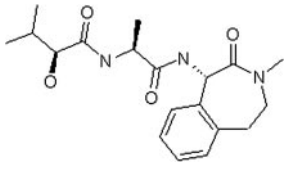

Fig. 1. Structure of LY-450139.

Tissue Isolation and Processing. At specified intervals after dosing, guinea pigs were euthanized with CO_2. Once breathing had ceased, a blood sample was obtained via cardiac puncture and stored on ice in EDTA-coated tubes for separation of plasma. The cisterna magna was then exposed by dissection, and a sample of CSF was drawn through a 25-gauge butterfly needle (BD Biosciences, Franklin Lakes, NJ) and frozen on dry ice. Brain hemispheres were harvested into conical tubes and frozen in liquid nitrogen. All tissue was stored at −80°C. Brain tissue was homogenized in 9 volumes of 0.2% diethylamine-50 mM sodium chloride, incubated 3 h on ice, and then centrifuged at 135,000g (1 h, 4°C). Supernatants were applied to 60-mg HLB 96-well plates (Waters, Milford, MA) and concentrated as described previously (Lanz and Schachter, 2006).

Aβ Assay. Half-volume 96-well plates were coated overnight at 4°C with 6E10 (Signet Laboratories, Dedham, MA) diluted (4 μg/ml) in 0.1 M sodium bicarbonate, pH 8.2, and then blocked for 3 h at room temperature with 1% bovine serum albumin in phosphate-buffered saline with 0.05% Tween 20 (Sigma-Aldrich, St. Louis, MO). Standard curves were prepared from stock solutions of species-specific Aβ peptides (Bachem Biosciences, King of Prussia, PA) in blocking buffer. Aβ1–40 peptide was used to prepare the standard curve for Aβ1–X in addition to Aβ1–40. Standards and samples were incubated with capture antibody overnight at 4°C. For detection of Aβ1–X, biotinylated 4G8 (0.2 μg/ml) was incubated for 2 h at room temperature. For detection of Aβ1–40 and Aβ1–42, rabbit polyclonal antibodies directed against each of the fragments (Mehta et al., 1998) were used in place of 4G8 at dilutions of 1:1000 to 1:2000. The signal was amplified with europium-conjugated streptavidin followed by incubation with Delfia enhancement solution (both steps at room temperature for 1 h). Plates were read on a Wallac Victor plate reader (europium-Delfia reagents and equipment from PerkinElmer Life and Analytical Sciences, Boston, MA). Standards were fit to a fourth-order polynomial curve, and sample values were extrapolated with GraphPad Prism 4.0. Each time point was treated as a separate experiment: each was assayed and analyzed separately.

Measurement of Drug Levels in CSF and Plasma

Calibration Standards and QC Samples. Stock solutions of LY-450139 (1 mg/ml) and internal standard (1 mg/ml) were prepared individually in methanol. Standard working solutions of LY-450139 (100 μg/ml) and internal standard (10 μg/ml) were prepared by serial dilution of their stock solutions with methanol-water. Calibration standard samples were prepared by serial dilution with blank guinea pig plasma or ultrafiltered rat plasma to yield concentrations of 1, 2, 5, 10, 20, 50, 100, 500, and 1000 ng/ml. Likewise, QC samples at concentrations of 3.2, 400, and 800 ng/ml were also prepared in each matrix.

Plasma Sample Preparation. Aliquots (100 μl) of plasma sample, calibration standards, and QC samples were pipetted into individual wells of a 96-well plate. All samples were spiked with 10 μl of internal standard (10 μg/ml) followed by 400 μl of acetonitrile. The plate was vortexed, followed by centrifugation at 3000 rpm for 10 min; 300 μl of acetonitrile was drawn into a clean 96-well plate and evaporated to dryness under a stream of nitrogen. Samples were reconstituted in 100 μl of methanol-water and vortexed for approximately 2 min, and 20 μl was injected onto the liquid chromatograph-tandem mass spectrometer.

CSF Sample Preparation. Aliquots (50 μl) of CSF sample, ultrafiltrate calibration standards, and ultrafiltrate QC samples were pipetted into a 96-well plate containing glass inserts. All samples were spiked with 5 μl of internal standard (10 μg/ml) and vortexed,

and 20 μl was injected onto the liquid chromatograph-tandem mass spectrometer.

Liquid Chromatography-Tandem Mass Spectrometry Conditions. An LC-10AD chromatograph (Shimadzu) equipped with a degasser and a CTC Analytics HTS PAL autosampler (Leap Technologies, Carrboro, NC) was used to fractionate 20-μl aliquots of processed samples on a Phenomenex Synergi Polar-RP column (2.0 mm × 50 mm, 4 μm). The mobile phase was a gradient mixture of solvents A (10 mM ammonium acetate, pH 7.0) and B (acetonitrile). Quantitation was achieved by tandem mass spectrometry detection in positive ion modes for analyte and internal standard using a Micromass Quattro Ultima mass spectrometer. Detection of the ions was performed in the multiple reaction-monitoring mode, monitoring the transition of *m/z* 362.4 → 191.1 for LY-450139 and *m/z* 407.4 → 153.8 for the internal standard. The analytical data were processed by MassLynx software (version 4.0). Calibration curves were acquired by plotting the peak area ratio of analyte to internal standard against the nominal ratio of concentrations of calibration standards. The results were fitted to linear regression analysis using $1/\chi^2$ as the weighting factor.

Whole-Cell Assay

Drug Treatment. H4 cells overexpressing the Swedish mutation of human APP were plated in 96-well plates at 30,000 cells/well grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and penicillin-streptomycin (Invitrogen, Carlsbad, CA). For ELISA analysis, cells were treated in triplicate with vehicle (0.5% DMSO in medium) or LY-450139 at concentrations ranging from 200 pM to 2 μM. For IP/MS analysis, vehicle or a single concentration was administered to every well in a 96-well plate. Cells were treated with drug for 20 h, at which time medium was harvested and frozen. For IP/MS analysis, all wells for a given treatment group were pooled before freezing.

Aβ Assay. Cell medium was assayed at a 1:2 dilution in blocking buffer for Aβ1–X using the ELISA configuration described for in vivo samples.

IP/MS of Conditioned Media. Aβ was immunoprecipitated from conditioned media of vehicle or LY-450139-treated H4(Swe) cells with monoclonal antibodies 6E10 and 4G8. Matrix-assisted time-of-flight mass spectroscopy analysis was performed as described elsewhere (Wood et al., 2005).

Mouse Embryonic Stem Cell-Derived Neurons

Differentiation. Undifferentiated mouse embryonic stem cells were incubated for 5 days in the presence of transferrin, leukemia inhibitory factor, and noggin. Once embryoid bodies formed, cells were dissociated and replated in the presence of basic fibroblast growth factor, sonic hedgehog signal, fibroblast growth factor-8, noggin, laminin, and G418. Medium was replaced after 6 days, and neuronal precursors were allowed to expand for 4 days. The resulting culture contained both cells of neuronal and non-neuronal phenotype.

Drug Treatment. Mouse neuronal precursor cells were adhered to 24-well poly-D-lysine-coated plates at 1.2×10^5 cells/well in a NeuroIII medium (1 μM cAMP, 200 μM ascorbic acid, 100 ng/ml brain-derived neurotrophic factor, and 5 mg/l laminin added to a mixture of 80% Neurobasal medium with B27, L-glutamine, and 20% Dulbecco's modified Eagle's medium F-12/N_2) and incubated at 2% O_2. At day 8 medium was replaced with 0.5 ml of fresh NeuroIII with 10% fetal bovine serum, and differentiated neurons were treated for 48 h with vehicle (1% DMSO) or LY-450139 at concentrations ranging from 30 pM to 3 μM. Conditioned medium was collected, and viability of neurons was assessed using the 3-(4,5-dimethylthyazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric microassay.

Aβ Assay. AβX–40 levels were quantified by an electrochemiluminescence assay (Origen M-8 analyzer; BioVeris, Gaithersburg, MD). Conditioned medium (100 μl/well) was incubated with biotinylated 4G8 diluted 1:2000 into 100 μl of phosphate-buffered saline with 1% bovine serum albumin, streptavidin M-280 Dynabeads (0.1 mg/ml; BioVeris), and 0.5 μg/ml ruthenylated 8D2 (in-house monoclonal antibody raised against amino acids 33–40 of Aβ).

Statistical Analysis and Data Presentation

At each time point, one-way analysis of variance was used to detect a significant treatment effect on Aβ (GraphPad Prism software). After a significant main effect by ANOVA, individual differences versus vehicle were analyzed with Dunnett's multiple comparison test using $p < 0.05$ as a statistically significant level. To facilitate comparisons between all studies, data are presented as percentages of the corresponding vehicle control ± S.E.M. Absolute values for the vehicle group are included in the figure legends.

Results

Dose- and Time-Dependent Modulation of γ-Secretase in Guinea Pigs by LY-450139. Male guinea pigs were treated orally with vehicle or LY-450139 at doses ranging from 0.2 to 60 mg/kg, and complete dose responses were repeated at multiple time points. As early as 1 h after dosing (Fig. 2), significant inhibition of brain Aβ1–X production was observed at 20 to 60 mg/kg (32–34% reduction, $p < 0.05$). These doses also significantly lowered plasma Aβ1–X, Aβ1–40 (84–97% reduction, $p < 0.01$), and Aβ1–42 (63–74% reduction, $p < 0.01$). Doses of 2 to 6 mg/kg significantly lowered plasma Aβ1–X and 1–40 (63–90% reduction, $p < 0.01$) but had no effect in CSF or brain at 1 h. The lowest dose, 0.2 mg/kg, significantly elevated plasma Aβ1–X, 1–40, and 1–42 to between 159 and 214% of vehicle ($p < 0.01$). This dose also elevated CSF Aβ1–42 to 261% of vehicle ($p < 0.05$). The next higher dose, 0.6 mg/kg, had no effect upon Aβ1–X or Aβ1–40 in any compartment but significantly elevated plasma Aβ1–42 (161% of vehicle, $p < 0.01$).

At 3 h after treatment (Fig. 3), low-dose elevation of Aβ was still observed in plasma, although it only reached statistical significance for Aβ1–40 (183–212% of vehicle, $p < 0.05$). Aβ1–X and 1–40 were significantly reduced in all compartments at 20–60 mg/kg. As with the 1-h time point, less reduction of Aβ1–42 was observed relative to Aβ1-X and 1–40. At 2 to 6 mg/kg, plasma Aβ1–X and 1–40 still exhibited significant reduction, although the magnitude of effect was less at 3 h versus 1 h, suggesting a very short time of maximal effect at these doses.

By 6 h (Fig. 4), the 2 to 6 mg/kg doses no longer reduced plasma Aβ, but rather raised Aβ levels over baseline. Aβ1–X and Aβ1–40 reached 171 and 220% of vehicle, respectively ($p < 0.01$) at the 6 mg/kg dose. Plasma Aβ1–42 was also significantly elevated at the 6 mg/kg dose (128% of vehicle, $p < 0.01$). Only the maximum dose, 60 mg/kg, still elicited significant reduction at 6 h, and this efficacy was restricted to plasma.

No further reduction of brain or plasma Aβ was observed at 9 h (Fig. 5). The 2 mg/kg dose, which lowered plasma Aβ at 1–3 h and elevated plasma Aβ at 6 h, had no effect on any compartment by 9 h. The 6 mg/kg dose, which had earlier lowered Aβ in all three compartments, caused an elevation of plasma Aβ1–X and 1–40 at 9 h. By 14 h, Aβ levels in all compartments had returned to baseline (data not shown).

Thus, the lowest doses assessed in this series of experiments produced only elevation of Aβ, predominantly in plasma. Middle doses showed inhibition of plasma Aβ followed by elevation of plasma Aβ, without any significant

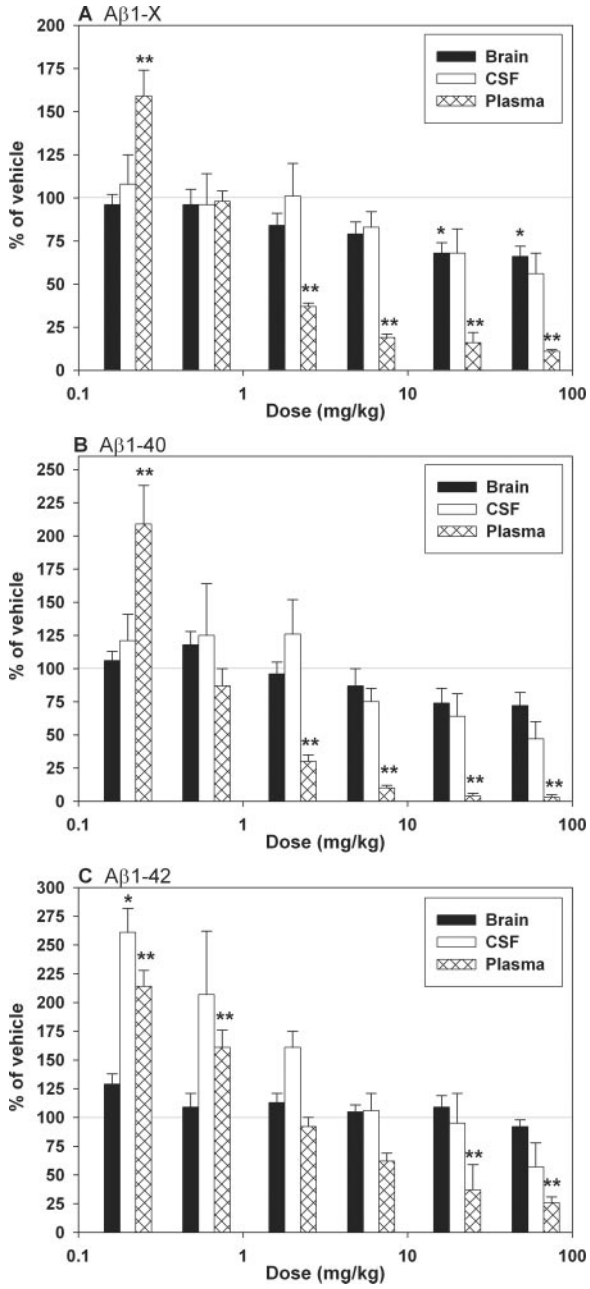

Fig. 2. One-hour dose response of LY-450139 in male guinea pigs. Mean ± S.E.M. percentages of vehicle Aβ levels after drug treatment are shown for brain (■), CSF (□), and plasma (▨). A, Aβ1–X; vehicle Aβ1–X concentration was 9588 ± 873 pg/g in brain, 9043 ± 1274 pg/ml in CSF, and 488 ± 41 pg/ml in plasma. B, Aβ1–40; vehicle Aβ1–40 concentration was 4171 ± 499 pg/g in brain, 4734 ± 836 pg/ml in CSF, and 253 ± 15 pg/ml in plasma. C, Aβ1–42; vehicle Aβ1–42 concentration was 816 ± 77 pg/g in brain, 375 ± 49 pg/ml in CSF, and 38 ± 3 pg/ml in plasma. *, $p < 0.05$; **, $p < 0.01$ versus vehicle.

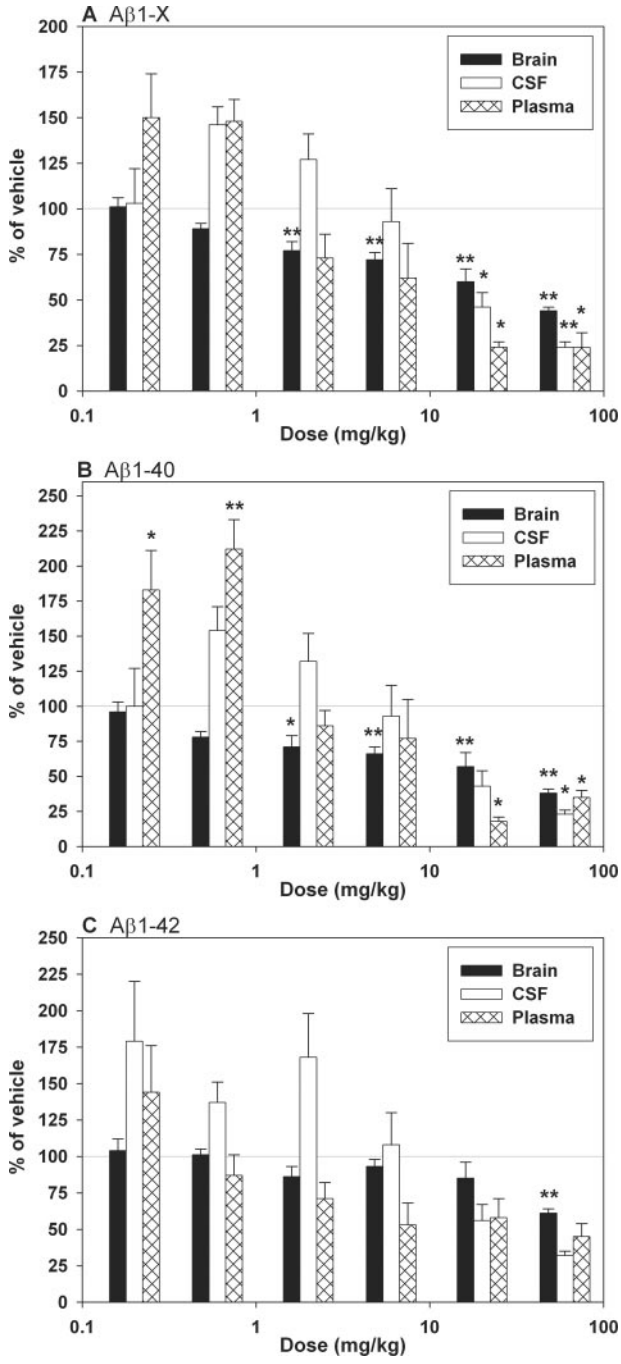

Fig. 3. Three-hour dose response of LY-450139 in male guinea pigs. Mean ± S.E.M. percentages of vehicle Aβ levels after drug treatment are shown for brain (■), CSF (□), and plasma (▨). A, Aβ1–X; vehicle Aβ1–X concentration was 5371 ± 98 pg/g in brain, 12,260 ± 1794 pg/ml in CSF, and 588 ± 73 pg/ml in plasma. B, Aβ1–40; vehicle Aβ1–40 concentration was 1926 ± 54 pg/g in brain, 6949 ± 1277 pg/ml in CSF, and 328 ± 40 pg/ml in plasma. C, Aβ1–42; vehicle Aβ1–42 concentration was 554 ± 18 pg/g in brain, 742 ± 141 pg/ml in CSF, and 49 ± 6 pg/ml in plasma. *, $p < 0.05$; **, $p < 0.01$ versus vehicle.

effect in CSF or brain. The highest two doses showed efficacy in plasma by 1 h, with CSF and brain showing Aβ significant reduction by 3 h. The lower of these two doses eventually caused an elevation of plasma Aβ at 9 h, by which time levels in CSF had returned to baseline.

Dose-Dependent Modulation of Aβ in Guinea Pigs with Steady-State Ly-450139 Infusion. To evaluate whether the Aβ elevations observed in plasma could occur under steady-state conditions, guinea pigs were treated with vehicle or LY-450139 for 5 days through a s.c. minipump. At infusion rates of 10 and 30 mg/kg/day, Aβ1–X (Fig. 6*A*) was significantly lowered in brain (16–22% reduction, $p < 0.05$) and plasma (66–73% reduction, $p < 0.01$). Plasma Aβ1–X was significantly elevated (141% of vehicle) at 1 mg/kg/day ($p < 0.01$). Plasma Aβ1–40 (Fig. 6*B*) was similarly lowered at 10 to 30 mg/kg/day (81% reduction, $p < 0.01$) and was elevated at 1 mg/kg/day (141% of vehicle, $p < 0.01$). No treat-

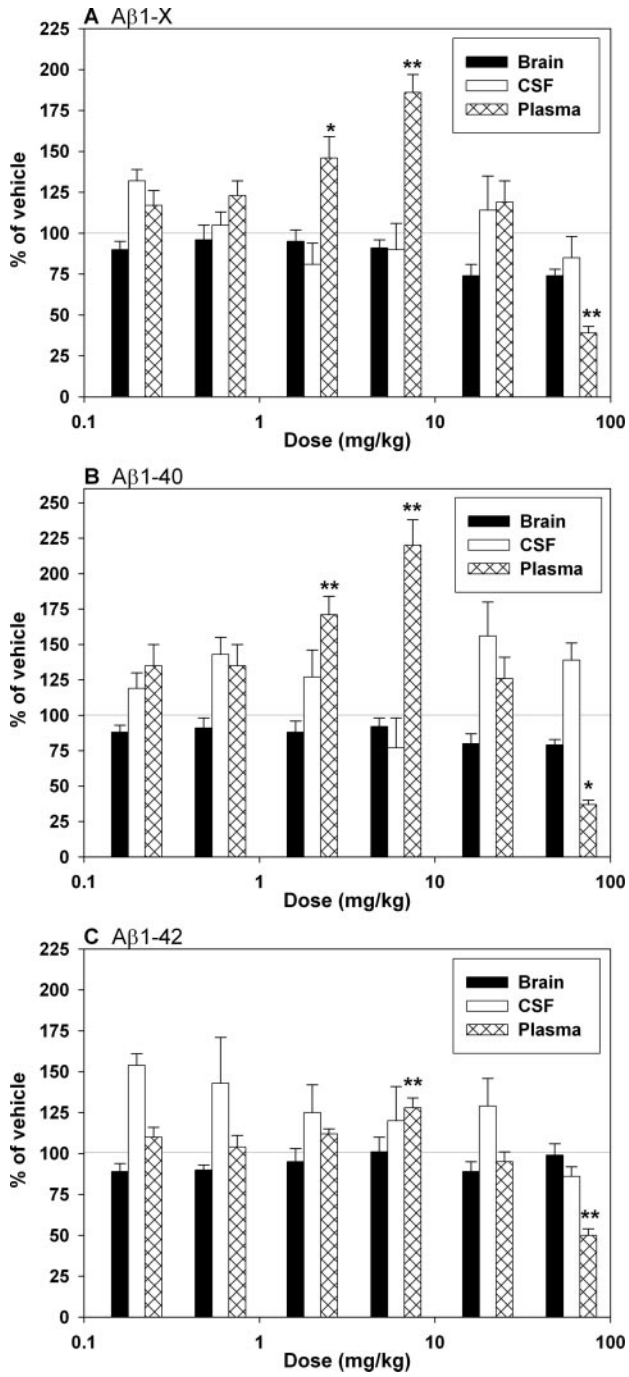

Fig. 4. Six-hour dose response of LY-450139 in male guinea pigs. Mean ± S.E.M. % of vehicle Aβ levels after drug treatment are shown for brain (■), CSF (□), and plasma (▨). A, Aβ1–X; vehicle Aβ1–X concentration was 7905 ± 924 pg/g in brain, 16,208 ± 1033 pg/ml in CSF, and 1253 ± 92 pg/ml in plasma. B, Aβ1–40; vehicle Aβ1–40 concentration was 3475 ± 374 pg/g in brain, 5837 ± 634 pg/ml in CSF, and 617 ± 53 pg/ml in plasma. C, Aβ1–42; vehicle Aβ1–42 concentration was 655 ± 52 pg/g in brain, 753 ± 75 pg/ml in CSF, and 131 ± 10 pg/ml in plasma. *, $p < 0.05$; **, $p < 0.01$ versus vehicle.

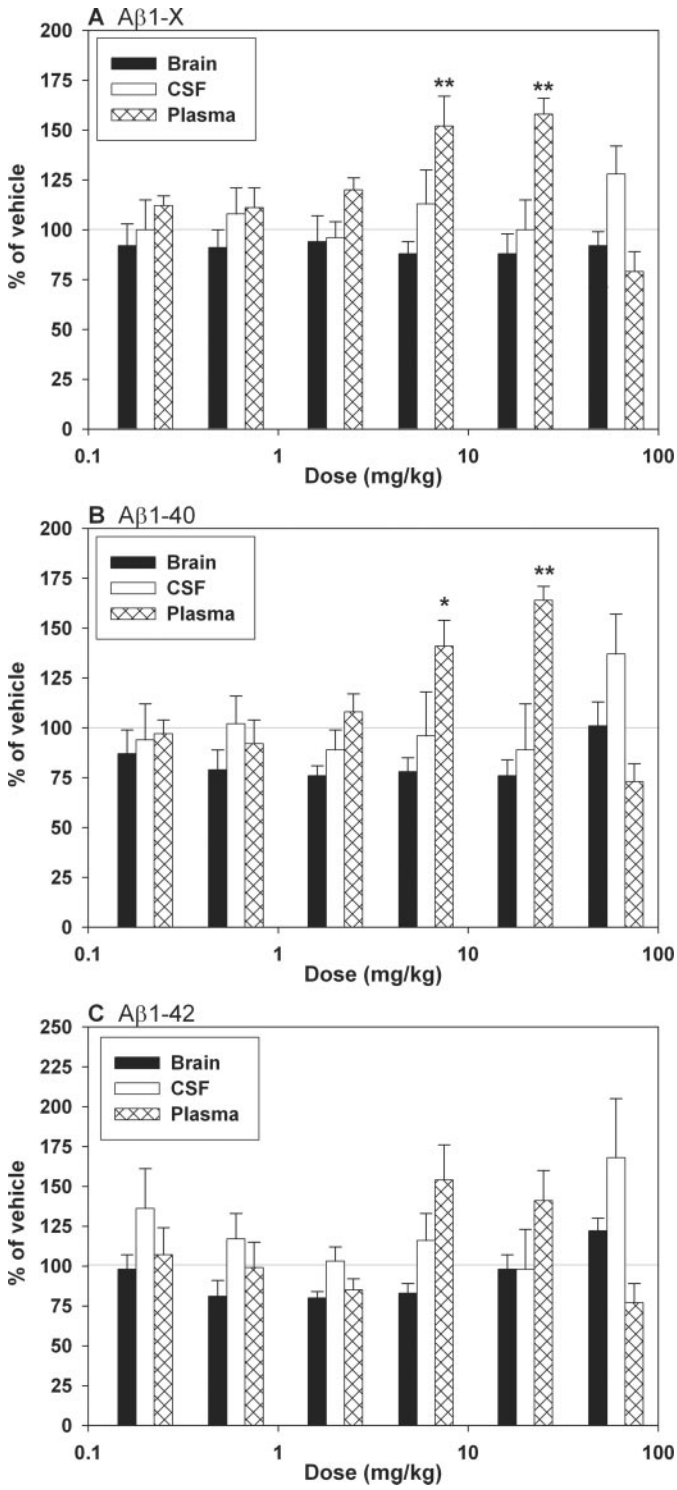

Fig. 5. Nine-hour dose response of LY-450139 in male guinea pigs. Mean ± S.E.M. percentages of vehicle Aβ levels after drug treatment are shown for brain (■), CSF (□), and plasma (▨). A, Aβ1–X; vehicle Aβ1–X concentration was 7130 ± 679 pg/g in brain, 16,730 ± 2445 pg/ml in CSF, and 742 ± 77 pg/ml in plasma. B, Aβ1–40; vehicle Aβ1–40 concentration was 3432 ± 416 pg/g in brain, 9471 ± 1741 pg/ml in CSF, and 441 ± 59 pg/ml in plasma. C, Aβ1–42; vehicle Aβ1–42 concentration was 381 ± 21 pg/g in brain, 759 ± 143 pg/ml in CSF, and 32 ± 4 pg/ml in plasma. *, $p < 0.05$; **, $p < 0.01$ versus vehicle.

ment effect was detected in brain Aβ1–40. Although 10 to 30 mg/kg/day significantly reduced Aβ1–42 by 32 to 35% ($p < 0.05$), no potentiation was observed at any of the doses tested (Fig. 6*C*). The only significant change in brain Aβ1–42 was an 18% increase at 30 mg/kg/day ($p < 0.05$). Whereas CSF Aβ1–42 showed a trend toward elevation at the high doses, treatment effects did not reach significance in any Aβ fragment measured in this compartment.

Drug levels in CSF and plasma are listed in Table 1.

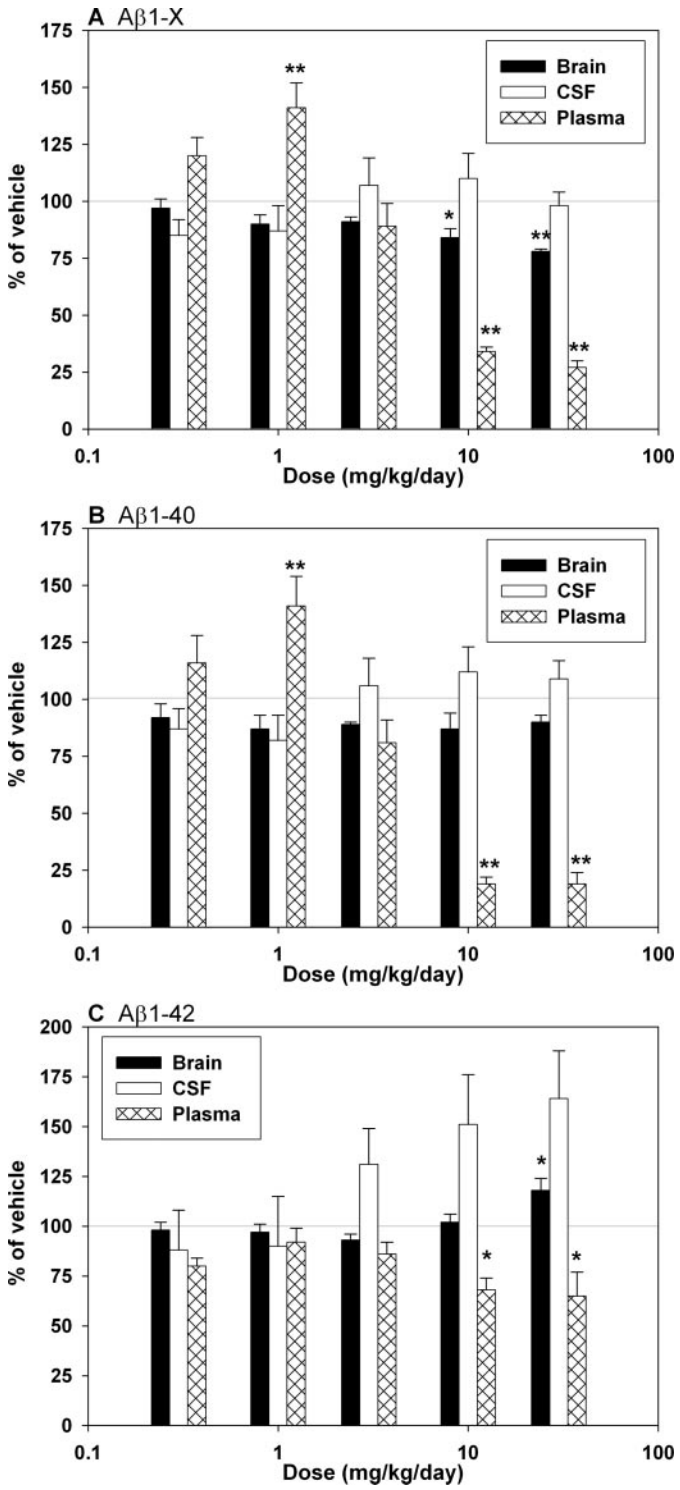

Fig. 6. Five-day minipump delivery of LY-450139 in male guinea pigs. Mean ± S.E.M. percentages of vehicle Aβ levels after drug treatment are shown for brain (■), CSF (□), and plasma (▨). A, Aβ1–X; vehicle Aβ1–X concentration was 8261 ± 378 pg/g in brain, 17,638 ± 1714 pg/ml in CSF, and 704 ± 57 pg/ml in plasma. B, Aβ1–40; vehicle Aβ1–40 concentration was 3160 ± 236 pg/g in brain, 10,748 ± 1217 pg/ml in CSF, and 413 ± 28 pg/ml in plasma. C, Aβ1–42; vehicle Aβ1–42 concentration was 507 ± 18 pg/g in brain, 642 ± 123 pg/ml in CSF, and 75 ± 10 pg/ml in plasma. *, $p < 0.05$; **, $p < 0.01$ versus vehicle.

Detectable levels of LY-450139 were measured in plasma at every dose. The 1 mg/kg/day delivery rate resulted in a plasma drug concentration of 40 nM, which caused significant elevation of plasma Aβ. No significant alteration of Aβ levels in any compartment was observed at 116 nM plasma LY-450139, whereas significant reductions of Aβ1–X, 1–40, and 1–42 were detected at plasma drug concentrations of 368 nM and above.

TABLE 1

LY-450139 levels in CSF and plasma of guinea pigs after 5 days of dosing via s.c. minipump

CSF exposure for 0.3 and 1 mg/kg/day was below the limit of quantitation (LOQ) (3 nM). Values are means ± S.E.M.

Dose	Plasma	CSF
	nM	
0.3 mg/kg/day	14 ± 1	<LOQ
1 mg/kg/day	40 ± 5	<LOQ
3 mg/kg/day	116 ± 45	14 ± 2
10 mg/kg/day	368 ± 51	40 ± 6
30 mg/kg/day	894 ± 165	98 ± 25

Concentration-Dependent Aβ Modulation in Cell Culture Models. After the observation of Aβ elevation in plasma at low measurable concentrations of LY-450139, the compound was evaluated in H4 cells overexpressing the "Swedish" mutation of human APP. Cells were treated with LY-450139 at concentrations ranging from 0.6 nM to 2 μM. After exposure to the drug for 20 h, Aβ1–X levels were measured in media (Fig. 7). Across two trials, the IC_{50} was approximately 60 nM, with near-complete inhibition at higher concentrations. At 6 and 19 nM, Aβ1–X was 30 to 40% elevated. At lower concentrations, Aβ1-X levels were similar to baseline.

Once a concentration range that reproducibly elevated Aβ levels was identified, an inhibitory concentration was compared with a potentiating concentration at multiple time points up to 24 h (Fig. 8). With vehicle treatment, cell media continued to accumulate Aβ1–X over a 24-h period. When a high concentration (95 nM) of LY-450139 was present, the rate of Aβ production remained low at every time point. When cells were incubated with 9.5 nM LY-450139, however, the rate of Aβ production was consistently higher than that of vehicle, resulting in elevated Aβ levels at every time point.

To further probe the identity of Aβ species affected by

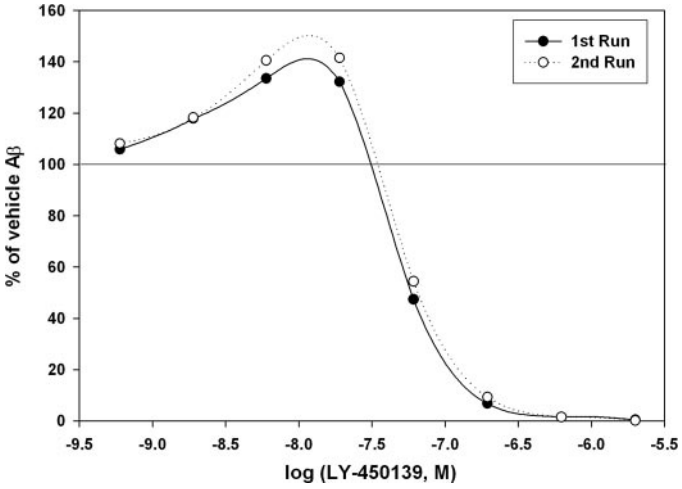

Fig. 7. H4(Swe) cells were treated in triplicate with escalating doses of LY-450139. Medium was collected at 20 h and Aβ1–X levels were measured. This procedure was repeated on a second set of cells; ● versus ○ represents the two different samples. Mean vehicle Aβ1–X concentration was 10.5 ng/ml.

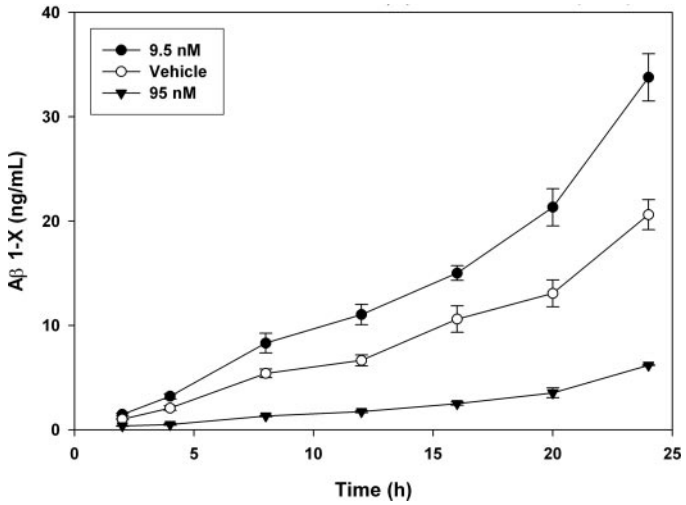

Fig. 8. Time course of Aβ production in H4(Swe) cells treated with LY-450139 at a high, inhibitory dose (95 nM, ▼) or a potentiating dose (9.5 nM, ●). Time 0 denotes a medium change with addition of drug. The rate of Aβ production in the absence of drug is represented by ○.

LY-450139 in this cell culture system, media were pooled from vehicle-treated samples and separately from drug-treated samples for IP/MS analysis (Fig. 9). Fixed concentrations of ^{15}N-substituted synthetic Aβ1–40, 1–42, and 1–43 peptides were included in each set of samples to enable accurate quantitation of these peptides in the sample. At 6 nM LY-450139, Aβ1–40 and Aβ1–42 were elevated 30 to 50%; these changes could be visualized in the spectra by a change in the relative size of these peaks relative to the ^{15}N standards. Aβ11–40 also showed an increase at 6 nM LY-450139. At 60 nM, Aβ1–40 was inhibited by 50%, and Aβ1–42 was inhibited 25%. Aβ11–40, however, showed further elevation beyond the level detected at 6 nM LY-450139. By 600 nM, all Aβ peptides were almost completely reduced; the only major peaks in the spectra were the ^{15}N standards.

LY-450139 was then dosed in a nonmutant cell culture system, in which APP and Aβ are generated at physiological rates. Mixed neuronal cultures derived from mouse embryonic stem cells were treated with LY-450139 for 48 h (Fig. 10). As in H4(Swe) cells, high drug concentrations depleted the media of AβX-40. At 30 and 100 nM LY-450139, Aβ levels were elevated by 60% or more in all three repeats. At drug concentrations of ≤10 nM, Aβ levels resembled those of vehicle-treated cells. None of the drug treatments used in these experiments caused a significant change in cell viability as assessed by 3-(4,5-dimethylthyazol-2-yl)-2,5-diphenyltetrazolium bromide activity.

Discussion

The present data show that the γ-secretase inhibitor, LY-450139, differentially modulates guinea pig plasma Aβ in a concentration-dependent manner. At high doses, the compound reduces brain, CSF, and plasma Aβ in a similar fashion within the first 3 h after dosing. At these early time

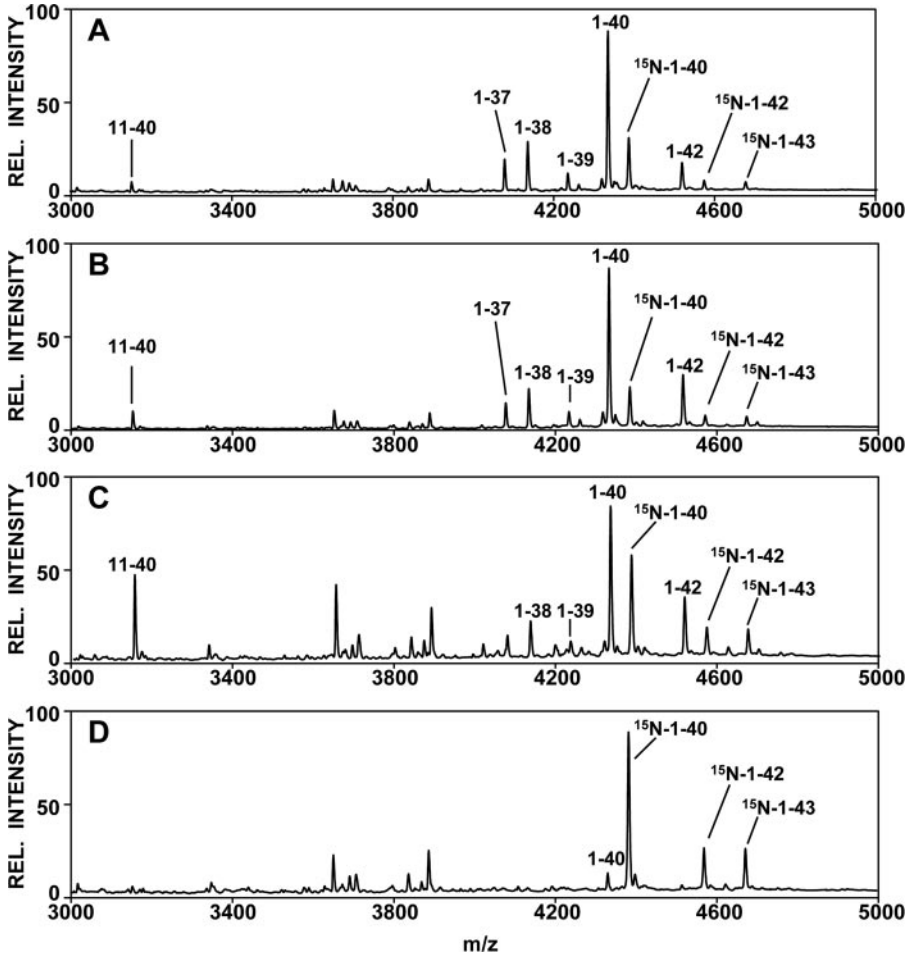

Fig. 9. IP/MS of Aβ from H4(Swe) conditioned media after treatment with vehicle (A) or LY-450139 at concentrations of 6 (B), 60 (C), or 600 nM (D). Fixed concentrations of ^{15}N standards for Aβ1–40, 1–42, and 1–43 were run along with the media as quantitative references.

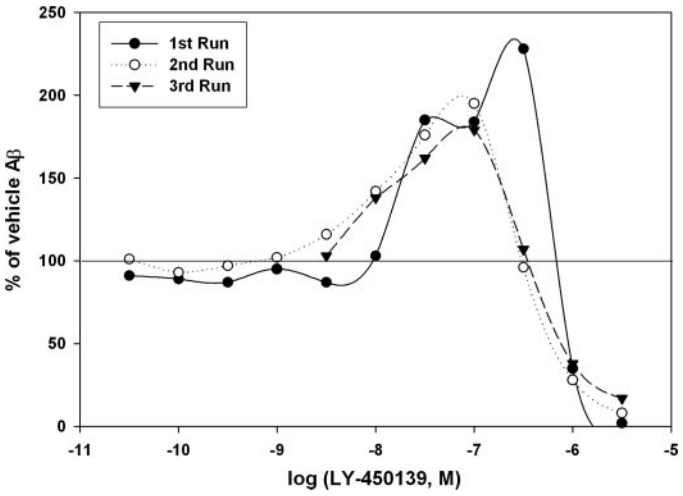

Fig. 10. Neuronal cultures derived from mouse embryonic stem cells were treated in triplicate with LY-450139. Medium was collected at 48 h and AβX-40 levels were measured. The experiment was performed 3 times; each run is denoted by a different symbol. All three runs showed potentiation within a common concentration range. The mean vehicle AβX-40 concentration across the three runs was 1110 ± 26 pg/ml.

points, low doses of the inhibitor significantly elevate plasma Aβ. Higher doses that reduced plasma Aβ within the first 3 h were shown to significantly elevate plasma Aβ at later time points, presumably as drug exposure waned. After 5 days of steady-state delivery, LY-450139 reduced plasma Aβ at high doses and elevated plasma Aβ at low doses, similar to the pattern seen at early time points after acute dosing. Furthermore, elevation of Aβ at low drug concentrations was observed in two different in vitro model systems. Maintaining steady-state drug levels of LY-450139 both in vitro and in vivo produced sustained inhibition or potentiation of γ-secretase activity depending on drug concentration.

The acute dosing data in guinea pigs reproduced a similar pattern of reduction versus elevation of plasma Aβ1–X shown previously in beagles (Hyslop et al., 2004) and in healthy human patients (Siemers et al., 2005). In addition, significant elevation of plasma Aβ by a low dose of LY-411575 has been previously demonstrated in Tg2576 mice (Lanz et al., 2004). If potentiation occurred only after a period of inhibition, it is reasonable to hypothesize that substrate may accumulate while drug is present; once the enzyme is no longer inhibited, a transient overproduction of Aβ could occur as the substrate stock is consumed. Our results, however, suggest that an initial inhibition of γ-secretase activity is not required for LY-450139 to produce elevated Aβ levels. When LY-450139 was maintained at a low concentration (40 nM) in plasma for several days, Aβ levels remained in an elevated state. It is reasonable to assume that after an acute dose, plasma exposure quickly reaches a potentiating concentration, whereas high doses may take several hours before normal clearance mechanisms reduce circulating drug levels to such a concentration. Second, in two different whole-cell models, sustained nanomolar concentrations of LY-450139 also increased levels of multiple Aβ peptides. The potentiation occurred in the earliest time point studied (4 h) and was maintained throughout the whole incubation period with LY-450139. Differences in the IC_{50} and potentiating concentrations between the two cell systems were observed, possibly due to substrate availability, cell density, and media formulation. However, the detection of this concentration-dependent phenomenon in two very different cell systems using both immunoassay and IP/MS suggests that in vitro potentiation is not merely a model-specific artifact.

Despite the demonstration of reproducible Aβ potentiation in vitro, it is interesting to note that in vivo potentiation in the present work was predominantly restricted to plasma. In CSF, some elevations in Aβ were seen, although few changes were statistically significant because of the high variability in this compartment. Additional variability in this and other compartments in extrapolated vehicle Aβ levels is also observed due to analytical issues: because each time point was run as a separate experiment, each set of samples was extrapolated against a separate standard curve. Absolute Aβ levels therefore exhibit higher variance between assays than within an assay.

Brain Aβ was inhibited by multiple doses across time, but significant potentiation of multiple Aβ peptides was not observed. Because of the gross homogenization of brain tissue, the mixing of intracellular and secreted Aβ pools may account for some differences between this compartment and those of CSF and plasma, which contain only secreted Aβ. In cell culture, potentiation and inhibition was measured in terms of Aβ secreted into the media over several hours. Technical considerations prohibited robust detection of intracellular Aβ at these potentiating concentrations. In vivo, the dynamics of Aβ clearance and transport in and out of the brain are also quite complex, including active transport by receptor for advanced glycation end products, P-glycoprotein, and other proteins (Deane et al., 2003; Cirrito et al., 2005). In a healthy animal, therefore, homeostatic mechanisms may prevent significant accumulation of Aβ in the central nervous system.

The accumulation of secreted Aβ under low, steady-state concentrations of LY-450139 could be due to direct or indirect effects of the compound. Whereas inhibition of an Aβ-degrading enzyme could lead to an increased pool of Aβ, cells have multiple mechanisms for degrading this peptide. Nonspecific effects upon multiple proteases would not be expected at the low drug concentrations that produce potentiation. Aβ peptides have been shown to modulate various intracellular pathways, including synthesis of cholesterol, which can affect Aβ production or transport (Grimm et al., 2005). It is therefore feasible that a subtle change in Aβ levels could feed back into a pathway that influences γ-cleavage or Aβ secretion. If such a mechanism were in place, however, one might expect that Aβ levels would eventually return to baseline, rather than show continued elevation. In contrast, a mechanism in which both potentiation and inhibition are a result of direct action on γ-secretase activity, although paradoxical, could be responsible for the sustained effects observed in vivo and in vitro.

In a multisubunit enzyme complex such as γ-secretase, it is conceivable that more than one binding site is available, and this results in subtle effects on enzymatic activity. The apparent activation of γ-secretase that results in potentiation could be caused if binding of a first molecule of drug prevents access of the substrate to an unproductive binding mode and so increases the rate of proteolysis. Binding of a second molecule of drug would then result in inhibition. On presenilin alone, at least three sites have been identified whose binding by various substrates affects γ-secretase cleavage of

APP: γ-secretase substrates bind first to an exosite spanning the N- and C-terminal fragments before entering the active site (Kornilova et al., 2005) and a nucleotide binding site has been shown to selectively modulate APP proteolysis (Fraering et al., 2005).

There is ample literature demonstrating that various genetic modifications of presenilin-1 elevate the cleavage of certain C-terminal Aβ fragments (for review, see German and Eisch, 2004). Gene dosage experiments with other components of the enzyme complex have also been shown to either inhibit or potentiate Aβ production. Neurons and fibroblasts from homozygous nicastrin knockout mice produce no detectable Aβ, whereas the heterozygous knockouts show increased Aβ1–40 and Aβ1–42 production versus wild-type cells (Li et al., 2003). Altered gene dosage of Aph-1b has been shown to affect cleavage of APP, but not that of other γ-secretase substrates such as ErbB4, neuregulin-2, or p75 in rat cortex (Coolen et al., 2006). Whereas potentiation of Aβ has been shown for more than one peptidomimetic γ-secretase inhibitors, further work is necessary to determine whether structurally unrelated inhibitors and those with different binding sites modulate Aβ production in a concentration-dependent manner.

Pharmacological manipulations of γ-secretase have been previously shown to modulate specific γ-cleavage sites. Select nonsteroidal anti-inflammatory drugs have been shown to increase Aβ1–38 while reducing Aβ1–42 in vitro (Weggen et al., 2001; Beher et al., 2005). In another in vitro model, Qi et al. (2003) demonstrated elevation of Aβ1–42 levels at concentrations that reduced Aβ1–40 with the γ-secretase inhibitor, difluoroketone. The well-characterized γ-secretase inhibitor, *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-(*S*)-phenylglycine *t*-butyl ester, has elicited modulation in vitro and in vivo: doses that reduced Aβ1–40 and Aβ1–42 simultaneously elevated levels of longer Aβ fragments such as Aβ1–43 and Aβ1–46 (Qi-Takahara et al., 2005; Yagashita et al., 2006). In the present studies, two forms of modulation were observed. Low concentrations of LY-450139 caused Aβ elevations in vivo and in vitro. In addition, greater potency in reducing Aβ1–40 versus Aβ1–42 was observed at multiple doses and in multiple compartments in vivo. The preferential inhibition of Aβ1–40 has also been demonstrated previously using the γ-secretase inhibitor LY-411575 in Tg2576 mice (Lanz et al., 2004). Furthermore, in the present studies, IP/MS revealed that Aβ11–40 was elevated at a concentration of LY-450139 at which both Aβ1–40 and Aβ1–42 were inhibited. Only at high concentrations (>200 nM) were all detectable Aβ peptides significantly reduced. Whether these forms of modulation occur with γ-secretase inhibitors from more diverse chemical series remains to be determined.

In the development of a γ-secretase inhibitor as a therapeutic agent, it is important to understand its pharmacodynamics in measurable compartments. There is ample evidence that γ-secretase is capable of being modulated to up-regulate select products while down-regulating others. In addition, we and others have observed both elevation and reduction of the same Aβ fragment using a γ-secretase inhibitor, with the direction of effect being dependent upon drug concentration. In vitro evidence suggests that although high drug concentrations effectively reduce the rate of γ-secretase activity, a certain range of low concentrations actually increases the rate of γ-secretase cleavage. This unusual pharmacology is further complicated by the fact that Aβ elevation was not clearly detected in the brain in vivo, despite demonstration of the phenomenon in a mixed culture containing cells with a neuronal phenotype. Whether this discrepancy is due to methodological issues or basic physiology remains to be answered. Even if potentiation is restricted to plasma, characterizing its appearance and disappearance will have an impact on a biomarker strategy dependent upon Aβ endpoints. The fact that elevations in plasma Aβ have already been detected in patients treated with a γ-secretase inhibitor (Siemers et al., 2005) underscores the importance of more fully understanding the dynamics of γ-secretase inhibition or modulation, if indeed such a distinction can be made.

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Address correspondence to: Dr. Thomas A. Lanz, Pfizer, Inc., Eastern Point Rd., MS# 8220-4183, Groton, CT 06340. E-mail: thomas.a.lanz@pfizer.com