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Studies to Investigate the in Vivo Therapeutic Window of the γ-Secretase Inhibitor N^2-[(2*S*)-2-(3,5-Difluorophenyl)-2-hydroxyethanoyl]-N^1-[(7*S*)-5-methyl-6-oxo-6,7-dihydro-5*H*-dibenzo[*b*,*d*]azepin-7-yl]-L-alaninamide (LY411,575) in the CRND8 Mouse

Lynn A. Hyde, Nansie A. McHugh,[1] Joseph Chen, Qi Zhang, Denise Manfra, Amin A. Nomeir, Hubert Josien, Thomas Bara, John W. Clader, Lili Zhang, Eric M. Parker, and Guy A. Higgins[2]

Departments of Neurobiology (L.A.H., N.A.M., J.C., Q.Z., L.Z., E.M.P., G.A.H.), Inflammation and Infectious Diseases (D.M.), Drug Metabolism and Pharmacokinetics (A.A.N.), and Chemical Research (H.J., T.B., J.W.C.), Schering Plough Research Institute, Kenilworth, New Jersey

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ABSTRACT

Accumulation of amyloid β-peptide (Aβ) is considered a key step in the etiology of Alzheimer's disease. Aβ is produced by sequential cleavage of the amyloid precursor protein by β- and γ-secretase enzymes. Consequently, inhibition of γ-secretase provides a promising therapeutic approach to treat Alzheimer's disease. Preclinically, several γ-secretase inhibitors have been shown to reduce plasma and brain Aβ, although they also produce mechanism-based side effects, including thymus atrophy and intestinal goblet cell hyperplasia. The present studies sought to establish an efficient screen for determining the therapeutic window of γ-secretase inhibitors and to test various means of maximizing this window. Six-day oral administration of the γ-secretase inhibitor N^2-[(2*S*)-2-(3,5-difluorophenyl)-2-hydroxyethanoyl]-N^1-[(7*S*)-5-methyl-6-oxo-6,7-dihydro-5*H*-dibenzo[*b*,*d*]azepin-7-yl]-L-alaninamide (LY411,575) reduced cortical $A\beta_{40}$ in young (preplaque) transgenic CRND8 mice ($ED_{50} \approx$ 0.6 mg/kg) and produced significant thymus atrophy and intestinal goblet cell hyperplasia at higher doses (>3 mg/kg). The therapeutic window was similar after oral and subcutaneous administration and in young and aged CRND8 mice. Both the thymus and intestinal side effects were reversible after a 2-week washout period. Three-week treatment with 1 mg/kg LY411,575 reduced cortical $A\beta_{40}$ by 69% without inducing intestinal effects, although a previously unreported change in coat color was observed. These studies demonstrate that the 3- to 5-fold therapeutic window for LY411,575 can be exploited to obtain reduction in Aβ levels without induction of intestinal side effects, that intermittent treatment could be used to mitigate side effects, and that a 6-day dosing paradigm can be used to rapidly determine the therapeutic window of novel γ-secretase inhibitors.

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[1] Current affiliation: Huntingdon Life Sciences, East Millstone, New Jersey.
[2] Current affiliation: NPS Pharmaceuticals, Toronto, Ontario, Canada.

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The amyloid hypothesis of Alzheimer's disease (AD) posits that amyloid β-peptide (Aβ) production, accumulation, or both trigger neuronal dysfunction and neurodegeneration, which ultimately results in the clinical expression of this disease (Hardy and Selkoe, 2002). The sequential cleavage of the amyloid precursor protein (APP) by β- and γ-secretase enzymes produces Aβ. Consequently, preventing Aβ formation by inhibiting either enzyme represents an attractive approach for AD treatment. Several low-molecular-weight γ-secretase inhibitors have been described, e.g., *N*-[*N*-(3,5-difluorophenacetyl)-*l*-alanyl]-*S*-phenylglycine *t*-butyl ester

ABBREVIATIONS: AD, Alzheimer's disease; Aβ, amyloid β-peptide; APP, amyloid precursor protein; LY411,575, N^2-[(2*S*)-2-(3,5-difluorophenyl)-2-hydroxyethanoyl]-N^1-[(7*S*)-5-methyl-6-oxo-6,7-dihydro-5*H*-dibenzo[*b*,*d*]azepin-7-yl]-L-alaninamide; DAPT, *N*-[*N*-(3,5-difluorophenacetyl)-*l*-alanyl]-*S*-phenylglycine *t*-butyl ester; BMS-299897, 2-[(1*R*)-1-[[(4-chlorophenyl)-sulfonyl](2,5-difluorophenyl)amino]ethyl]-5-fluoro-benzenepropanoic acid; Compound E, (2*S*)-2-{[(3,5-diflurophenyl)acetyl]amino}-*N*-[(3*S*)-1-methyl-2-oxo-5-phenyl-2,3-dihydro-1*H*-1,4-benzodiazepin-3-yl]propanamide; MRK-560, *N*-[*cis*-4-[(4-chlorophenyl)sulfonyl]-4-(2,5-difluorophenyl)cyclohexyl]-1,1,1-trifluoromethanesulfonamide; LY450,139, 2(*S*)-hydroxy-3-methyl-*N*-[1(*S*)-methyl-2-oxo-2-[(2,3,4,5-tetrahydro-3-methyl-2-oxo-1*H*-3-benzazepine-1(*S*)-yl)amino]ethyl]butanamide; PAS, periodic acid-Schiff; BLD, below the level of detection; LY-D, N^2-[(2*R*)-2-(3,5-difluorophenyl)-2-hydroxyethanoyl]-N^1-[(7*R*)-5-methyl-6-oxo-6,7-dihydro-5*H*-dibenzo[*b*,*d*]azepin-7-yl-L-alaninamide.

(DAPT), N^2-[(2*S*)-2-(3,5-difluorophenyl)-2-hydroxyethanoyl]-N^1-[(7*S*)-5-methyl-6-oxo-6,7-dihydro-5*H*-dibenzo[*b*,*d*]azepin-7-yl]-L-alaninamide (LY411,575), 2-[(1*R*)-1-[[(4-chlorophenyl)-sulfonyl](2,5-difluorophenyl)amino]ethyl]-5-fluoro-benzenepropanoic acid (BMS-299897), (2*S*)-2-{[(3,5-diflurophenyl)acetyl]amino}-*N*-[(3*S*)-1-methyl-2-oxo-5-phenyl-2,3-dihydro-1*H*-1,4-benzodiazepin-3-yl]propanamide (Compound E), and *N*-[*cis*-4-[(4-chlorophenyl)sulfonyl]-4-(2,5-difluorophenyl)cyclohexyl]-1,1,1-trifluoromethanesulfonamide (MRK-560); each lowers Aβ production in cell-based systems, transgenic mice overexpressing human APP containing familial AD mutation(s) or nontransgenic animals (Dovey et al., 2001; May et al., 2001; Lanz et al., 2003, 2004; Milano et al., 2004; Anderson et al., 2005; Barten et al., 2005; Best et al., 2005, 2006; Grimwood et al., 2005; El Mouedden et al., 2006). Furthermore, a recent clinical study reported that the γ-secretase inhibitor 2(*S*)-hydroxy-3-methyl-*N*-[1(*S*)-methyl-2-oxo-2-[(2,3,4,5-tetrahydro-3-methyl-2-oxo-1*H*-3-benzazepine-1(*S*)-yl)amino]ethyl]butanamide (LY450,139) lowered plasma Aβ in Alzheimer's disease patients (Siemers et al., 2006).

γ-Secretase is a complex composed of at least four proteins: presenilin-1 or -2, nicastrin, PEN-2 and APH-1 (Kimberly and Wolfe, 2003). γ-Secretase has proved to be a promiscuous enzyme with a large and continually increasing number of known substrates. In addition to the C-terminal fragment of APP, other known substrates for γ-secretase include Notch (De Strooper et al., 1999), the Notch ligands Delta1 and Jagged2 (Ikeuchi and Sisodia, 2003), ErbB4 (Lee et al., 2002), CD44 (Lammich et al., 2002), and E-cadherin (Marambaud et al., 2002). Notch, a critical factor in the regulation of cell growth and lineage, seems to be vital for T- and B-lymphocyte differentiation, plays a role in intestinal epithelial stem cell fate, and is critical for normal embryonic development (Shen et al., 1997; Wong et al., 1997; Jensen et al., 2000; Hadland et al., 2001).

Using the APP transgenic CRND8 mouse model (Chishti et al., 2001), we previously reported that 15-day oral administration of the γ-secretase inhibitor LY411,575 lowered plasma and brain Aβ and also decreased thymus cellularity (1 and 10 mg/kg) and induced extensive goblet cell hyperplasia within the small and large intestine (10 mg/kg) (Wong et al., 2004). Others have reported similar observations following chronic γ-secretase inhibition (Searfoss et al., 2003; Milano et al., 2004). These side effects are almost certainly mechanism-based because they were not observed following treatment with a less active diastereoisomer of LY411,575 (Wong et al., 2004) and can be attributed to inhibition of Notch cleavage (Jensen et al., 2000; Fre et al., 2005; van Es et al., 2005).

The early preclinical identification of target related side effects of novel AD-related therapeutics is a valuable application for APP transgenics, such as the CRND8 mouse (e.g., Higgins and Jacobsen, 2003). In the current study, we developed a methodology to evaluate both efficacy and side effects in the same animal, thereby establishing a therapeutic window, i.e., the ratio between doses required to lower Aβ and doses that produce side effects. Using this methodology, the therapeutic window of the γ-secretase inhibitor LY411,575 was more specifically defined and various means of maximizing the window were explored. Since there are suggestions that the therapeutic window of γ-secretase inhibitors may be compound-dependent (Milano et al., 2004; Barten et al., 2005), our method can also be used as an efficient screening paradigm to compare structurally diverse members of this drug class.

Materials and Methods

Animals and Housing

Transgenic CRND8 male and female mice (equally represented in each group) were bred and housed at the Schering Plough Research Institute (Kenilworth, NJ) as described previously (Hyde et al., 2005). Unless otherwise noted, studies were conducted in transgenic CRND8 mice that were 5 to 8 weeks old because at this age cortical Aβ is primarily in a soluble form (Hyde et al., 2005), and thus it is more amenable to reduction following relatively short-term γ-secretase inhibition (Lanz et al., 2003; Barten et al., 2005). Mice from the aged cohort (16–26 months old) were either retired breeders or experimentally naive mice. Before dosing began and for the duration of the study, mice were singly housed with a plastic igloo and nesting material. Mice were sacrificed 2 to 4 h after their final dosing. All in vivo procedures adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of the Schering Plough Research Institute, an Association for Assessment and Accreditation of Laboratory Animal Care-accredited institution.

Drugs

In all studies, we used LY411,575 (May et al., 2001) as a tool γ-secretase inhibitor compound due to its excellent potency and bioavailability (Lanz et al., 2004) and because of the availability of its less active diastereoisomer, LY-D, to control for potential nonspecific effects of LY411,575 unrelated to γ-secretase inhibition (Wong et al., 2004). For oral dosing, LY411,575 and LY-D were formulated as 10 mg/ml solutions and diluted 1:10 with 0.4% methycellulose as described previously (Wong et al., 2004). In the case of subcutaneous dosing, the 10 mg/ml stock solution was diluted 1:10 with 20% hydroxyl-propyl-β-cyclodextrin. If necessary, serial dilutions were made from the 1 mg/ml solution using the appropriate 1:10 vehicle. The dosing volume was 10 ml/kg. After oral administration of 10 mg/kg LY411,575, inhibition of plasma Aβ is still significant 24, but not 48, h after dosing (Wong et al., 2004), so in an effort to maintain continuous γ-secretase inhibition, we dosed LY411,575 and LY-D once per day in all studies.

β-Amyloid Quantification

Blood and brains were collected and $A\beta_{40}$ levels in plasma and cortex (following guanidine extraction) were quantified as described previously (Zhang et al., 2001; Wong et al., 2004). We focused on $A\beta_{40}$ as the primary marker of efficacy because we have previously shown that in young mice LY411,575 affected $A\beta_{40}$ and $A\beta_{42}$ to a similar extent (Wong et al., 2004).

Determination of Thymocyte Number

The thymus was weighed. The cell number was determined as described previously (Wong et al., 2004).

Histology and Immunohistochemistry

Histology. The ileum (2 cm in length harvested just proximal to the ileocecal junction) was processed, sectioned (3 μm), and stained as described previously (Wong et al., 2004). Intestinal goblet cell hyperplasia, characterized by elevated mucin expression, was quantified as percentage of villi area covered by periodic acid-Schiff (PAS) stain (average of three slices per mouse). Digital imaging and quantification of the stained sections were performed with a Nikon E400 microscope (Nikon, Tokyo, Japan) equipped with a digital camera and Image-Pro Plus (Media Cybernetics, Inc., Silver Spring, MD) image analysis software for Windows.

Immunohistochemistry. Brains from a separate group of 3- and 18-month-old mice were perfusion-fixed with 10% formalin, dehydrated, and embedded in paraffin. Five-micrometer sections were rehydrated, permeabilized with 2 N HCl, quenched with 0.3% H_2O_2, and blocked with 1% donkey serum. The slices were incubated overnight with D-17 primary antibody to the Aβ peptide (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Eighteen to 24 h later, a biotinylated secondary antibody (donkey anti-goat; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) was used with a diaminobenzidine detection system (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) to visualize plaques.

Data Analyses

Analyses of variance with group as the between-subject factor followed by Dunnett's post hoc *t* tests were used to analyze most data. In a few instances, Student's *t* tests were used to compare vehicle-treated mice to LY-D-treated mice and to age-matched LY411,575-treated mice. On occasion, Fisher's post hoc *t* tests were used to compare the group of interest to both vehicle-treated mice and to either dose-matched washout mice or mice treated with LY-D. Data from statistical outliers (defined as more than 2 S.D.s from the mean) were not included in the analyses.

To calculate a dose causing 50% inhibition or reduction (ED_{50}), data were expressed as a percentage of vehicle and fitted with a nonlinear regression equation (GraphPad Prism software, version 4.0; GraphPad Software Inc., San Diego, CA).

On occasion, mice would become "sickly" (e.g., hypothermic, hypoactive, poorly groomed, and hunched posture) or die during these studies. This was primarily seen in mice treated with the high dose (10 mg/kg) of LY411,575. If a mouse died before the tissue collection day, no tissues were used. If an animal died on the tissue collection day (in between the last dose and tissue collection), only cortex was isolated. If an animal was sickly on the collection day, it was sometimes not possible to obtain enough blood for Aβ quantification, but all other tissues were used.

TABLE 1

Percentage of inhibition of plasma and cortical $A\beta_{40}$ and body weight change from baseline (mean ± S.E.M.) after 2 to 6 days of oral dosing with 10 mg/kg LY411,575 in CRND8 mice

Group	$A\beta_{40}$ Inhibition		Body Weight Change
	Plasma	Cortex	
	%		*g*
Vehicle			+1.1 ± 0.3
Day 2	100†	83†	−0.4 ± 0.2
Day 3	100†	85†	−0.8 ± 0.3*
Day 4	100†	87†	−1.5 ± 0.3*
Day 5	100†	91†	−1.6 ± 0.6*
Day 6	100†	92†	−2.7 ± 0.6*

* $p < 0.01$ vs. vehicle, Dunnett's post hoc *t* test.
† $p < 0.0001$ vs. vehicle, Dunnett's post hoc *t* test on raw data, not percentage of inhibition data.

Results

Time Course of Mechanism-Based Side Effects Produced by LY411,575

Inhibition of thymocyte maturation and intestinal goblet cell hyperplasia have been identified previously as mechanism-based side effects of γ-secretase inhibition (Wong et al., 2004). To determine the minimum number of days needed to observe these side effects following administration of a high dose of LY411,575, CRND8 mice were orally dosed with 10 mg/kg LY411,575 for 2, 3, 4, 5, or 6 days or with vehicle for 6 days.

Efficacy. Levels of plasma and cortical $A\beta_{40}$ were significantly reduced following 2 to 6 days of treatment with LY411,575 at 10 mg/kg [Table 1; main effect of group: $F(5,38) = 319.48$ and $F(5,40) = 216.35$, $p < 0.0001$, for plasma and cortex, respectively].

Body Weight. Body weight change from baseline (last day of dosing compared with day 1) was proportional to treatment duration [Table 1; $F(5,41) = 9.71$, $p < 0.0001$]. Although vehicle-treated mice gained weight after 6 days, animals treated with LY411,575 progressively lost weight over the 6-day dosing period (Table 1).

Thymus. Dosing with 10 mg/kg LY411,575 produced a time-dependent reduction in the weight and cellularity of the thymus [Fig. 1, A and B; $F(5,24) = 13.90$ and 13.69, $p < 0.0001$, for weight and cellularity, respectively]. The reduction was statistically significant after treatment with LY411,575 for 4, 5, or 6 days with virtually complete atrophy of the thymus on day 6. Because within each group there was a positive correlation between thymus weight and cell number (Pearson product $r = 0.84–1.0$), only thymus weight was analyzed in subsequent studies.

Ileum. Substantial goblet cell hyperplasia, as visualized by increased PAS staining, can be observed after 6 days of administration of 10 mg/kg LY411,575 (Fig. 2, compare E with D). In this study, the percentage of villi area covered by PAS stain (a quantitative index of intestinal goblet cell hyperplasia) increased as the number of days of dosing with 10 mg/kg LY411,575 increased [Fig. 1C; $F(5,40) = 68.92$, $p < 0.0001$]. This effect was statistically significant by day 3 of treatment and was maximal by day 5.

Based on these findings suggesting maximal efficacy and

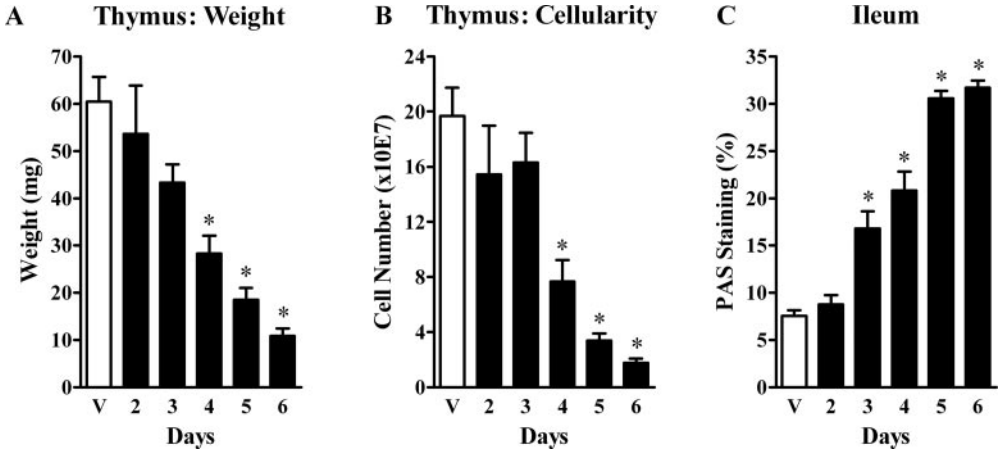

Fig. 1. Time course of mechanism-based side effect onset caused by oral administration of 10 mg/kg LY411,575 in CRND8 mice. A time-dependent reduction in thymus weight (A; $n = 5$/group) and cell number (B; $n = 5$), and a time-dependent increase in percentage of villi area covered by PAS stain in the ileum (C; $n = 7–9$) were observed. * $p < 0.002$ versus vehicle (V; white bars), Dunnett's post hoc *t* test.

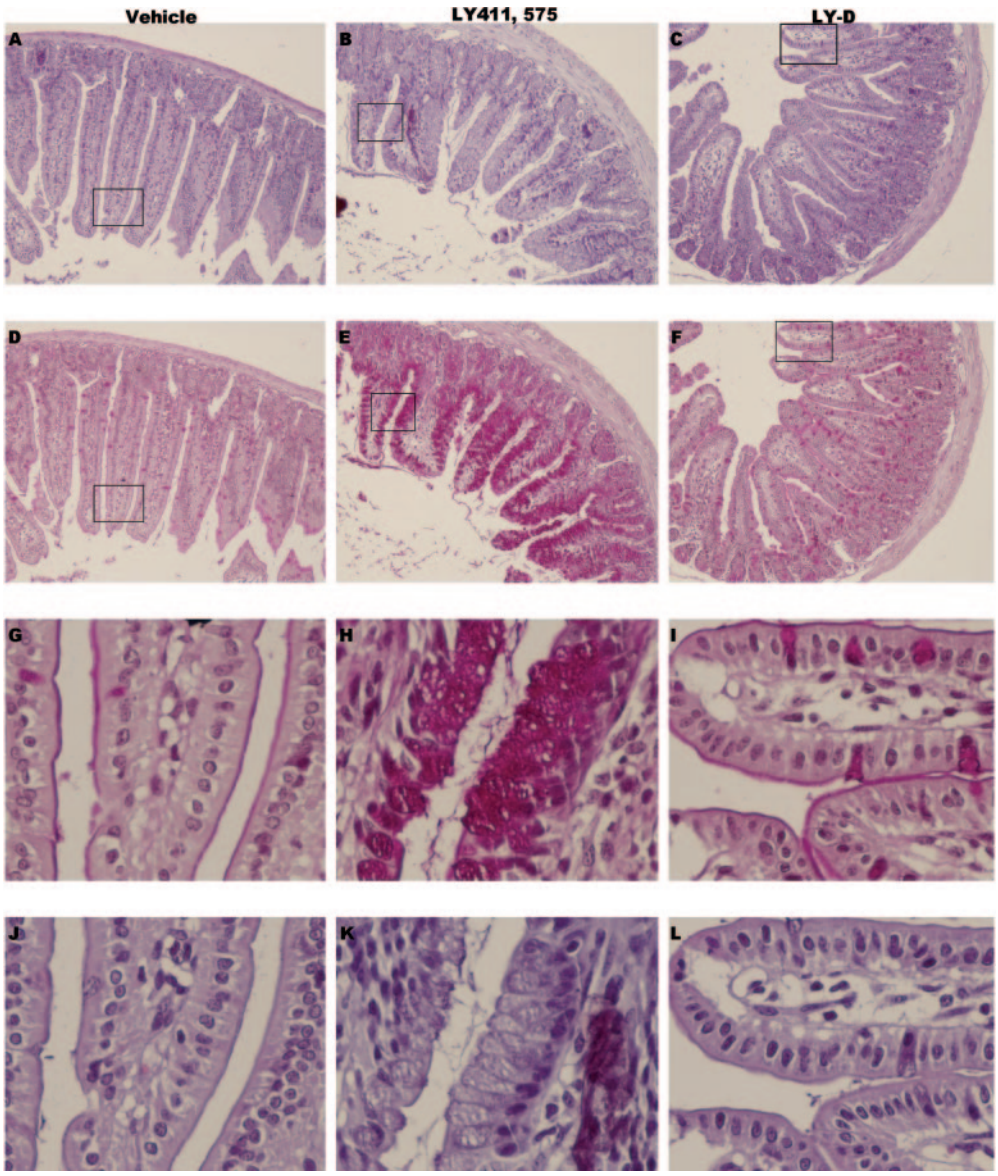

Fig. 2. Representative ileum sections from CRND8 mice treated with vehicle (left column), LY411,575 (10 mg/kg p.o.; middle column), or LY-D (10 mg/kg p.o.; right column). The top row (A–C) shows ileum stained with H&E to visualize cells of the intestinal villi. Higher magnification of boxed areas of H&E-stained slices are shown in J to L. Goblet cells are the elongated cells with displaced nuclei and are much more prevalent in the ileum from an LY411,575-treated mouse (K) compared with the ileum from a vehicle-treated (J) and a LY-D-treated (L) mouse. Adjacent ileum slices were stained with PAS to enable quantification of goblet cell hyperplasia (D–F). Higher magnification of boxed areas of PAS-stained slices are shown in G to I. Note the strong staining of hyperplastic goblet cells in the LY411,575-treated mouse [bright magenta; E (29% of villi covered by PAS stain) and H], compared with the weak PAS staining in vehicle-treated [D (2%) and G] and LY-D-treated [F (4%) and I] mice.

mechanism-based side effects after 6 days of treatment with 10 mg/kg LY411,575, we chose to use a 6-day treatment protocol for further studies.

The Therapeutic Window of LY411,575 Was Similar following Oral and Subcutaneous Administration

Intestinal goblet cell hyperplasia might be reduced, and thus the therapeutic window increased, if LY411,575 was given via a nonoral route of administration. To test this hypothesis, CRND8 mice were dosed either orally or subcutaneously for 6 days with vehicle or 0.1, 0.3, 1, 3, or 10 mg/kg LY411,575 or orally for 6 days with 10 mg/kg LY-D (to confirm that side effects were indeed mechanism-based; Wong et al., 2004). Among the vehicle-treated mice, there was no difference between the routes of administration on any measure, so the vehicle data were combined.

Efficacy. There was a dose-dependent inhibition of plasma and cortical $A\beta_{40}$ following both oral and subcutaneous administration of LY411,575 [Fig. 3, A and B; oral: main effect of dose: $F(5,41) = 47.99$ and 25.01, $p < 0.0001$, for plasma and cortex, respectively; subcutaneous: $F(5,41) = 95.51$ and 138.95, $p < 0.0001$]. The ED_{50} values for inhibition of plasma and cortical $A\beta_{40}$ were 0.3 and 0.6 mg/kg (oral) and 0.1 and 0.4 mg/kg (subcutaneous). LY411,575 was slightly less potent at inhibiting plasma $A\beta_{40}$ when it was dosed orally compared with when it was dosed subcutaneously [$F(4,70) = 11.84$, $p < 0.0001$]. In contrast, the potency of LY411,575 in inhibiting cortical $A\beta_{40}$ levels was similar for both routes ($F < 0.6$, N.S.). LY-D (10 mg/kg) did not have any effect on plasma or cortical $A\beta_{40}$ after oral administration (Fig. 3, A and B).

Body Weight. Among the mice dosed orally, 10 mg/kg LY411,575 treatment had a statistically significant effect on day 6 body weight change from baseline [Table 2; $F(5,41) = 6.17$, $p < 0.0003$; Dunnett's post hoc t test, $p < 0.0006$]. Body weight change was not significantly affected by LY411,575 dosed subcutaneously or by LY-D (Table 2).

Thymus. Both oral and subcutaneous administration of LY411,575 dose-dependently reduced thymus weight [Fig. 3C; $F(5,24) = 10.44$ and 11.79, $p < 0.0001$, respectively]. The oral ED_{50} for thymus weight reduction was 2.9 mg/kg, whereas the subcutaneous ED_{50} was 1.8 mg/kg. Thus, the

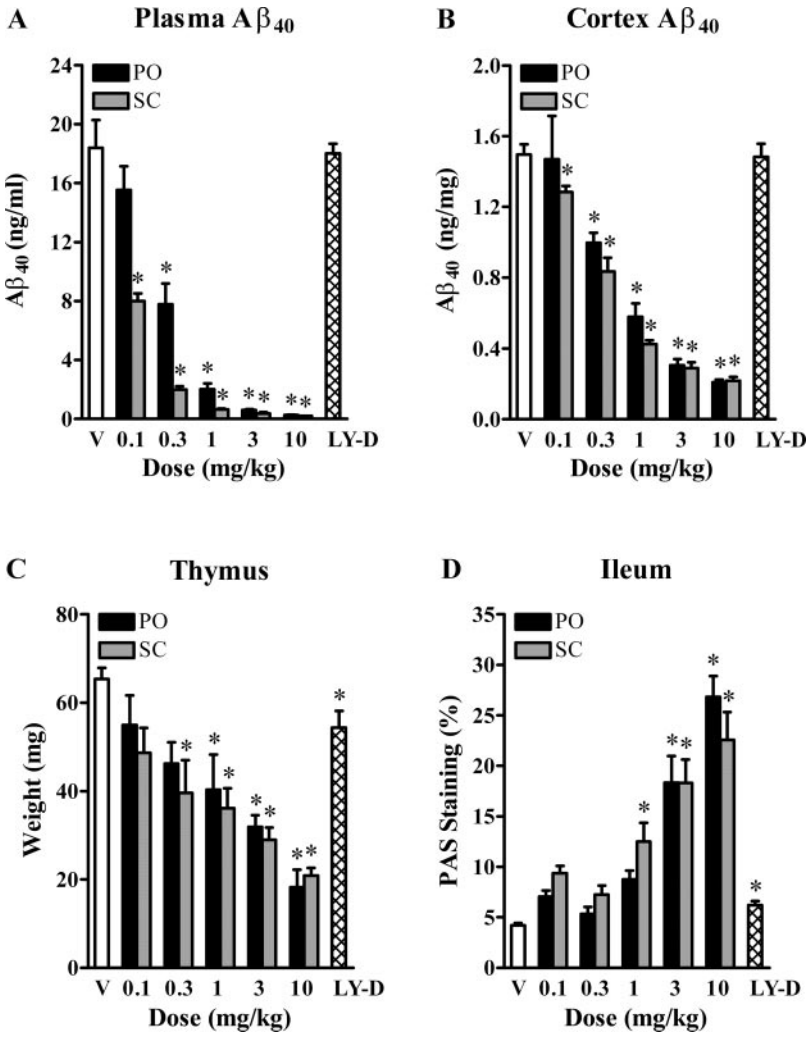

Fig. 3. Effects of LY411,575 (0.1–10 mg/kg) dosed orally (black bars) or subcutaneously (gray bars) and LY-D (10 mg/kg) dosed orally (cross-hatched bars) on efficacy and side effect measures assessed in CRND8 mice. Dose-dependent inhibition of $A\beta_{40}$ levels in plasma (A; $n = 7–8$/group) and cortex (B; $n = 7–8$) and reduction in thymus weight (C; $n = 5$) and increase in percentage of villi area covered by PAS stain in the ileum (D; $n = 5–8$) were observed with both routes of administration of LY411,575. There were no significant effects of 10 mg/kg LY-D on $A\beta_{40}$ levels, and the effects on thymus weight and ileum PAS staining were minimal. *, $p < 0.05$ versus vehicle (V; white bars), within route Dunnett's post hoc t test for LY411,575 or t test for LY-D.

TABLE 2

Body weight change from baseline and plasma drug levels (mean ± S.E.M.) following 6 days of oral or subcutaneous administration of various doses of LY411,575 or oral administration of 10 mg/kg LY-D in CRND8 mice

Group	Body Weight Change	Plasma Levels (n Included)[a]
	g	*ng/ml*
Vehicle	+1.6 ± 0.6	
Oral		
0.1	+1.7 ± 0.3	BLD
0.3	+1.6 ± 0.3	4.08 ± 4.08 ($n = 1$)
1	+1.2 ± 0.4	7.82 ± 2.60 ($n = 3$)
3	+1.3 ± 0.3	16.37 ± 4.11 ($n = 6$)
10	−0.7 ± 0.3*	26.53 ± 11.66 ($n = 7$)
LY-D	+1.6 ± 0.5	38.73 ± 12.24 ($n = 6$)
Subcutaneous		
0.1	+1.5 ± 0.4	BLD
0.3	+1.7 ± 0.3	3.10 ± 0.28 ($n = 2$)
1	+1.4 ± 0.2	9.05 ± 2.08 ($n = 5$)
3	+1.3 ± 0.3	26.90 ± 12.27 ($n = 5$)
10	+0.7 ± 0.4	19.06 ± 6.90 ($n = 8$)

* $p < 0.0006$ vs. vehicle, Dunnett's post hoc t test.
[a] Only mice with drug levels >2.5 ng/ml are included; BLD, below the level of detection (<2.5 ng/ml) for all mice in the group.

therapeutic window of LY411,575, defined as ED_{50} for inhibition of cortical $A\beta_{40}/ED_{50}$ for thymus weight reduction, was similar for the oral (4.8) and subcutaneous (4.5) routes.

Although administration of 10 mg/kg LY-D significantly reduced thymus weight [Fig. 3C; $t(8) = 2.41$, $p < 0.05$], the magnitude of reduction (17%) was considerably less than that observed following oral (72%) or subcutaneous (68%) administration of 10 mg/kg LY411,575.

Ileum. Figure 2, A to L, shows lower and higher magnification views of representative ileum sections from mice treated orally with vehicle, 10 mg/kg LY411,575, or 10 mg/kg LY-D. Substantial goblet cell hyperplasia, as visualized by increased PAS staining (Fig. 2, D–I), can be observed after administration of 10 mg/kg LY411,575, whereas the extent of PAS staining in mice treated with LY-D was similar to that observed in vehicle-treated mice.

Both oral and subcutaneous administration of LY411,575 resulted in a dose-dependent increase in intestinal goblet cell hyperplasia as indicated by an increase in percentage of villi area covered by PAS stain [Fig. 3D; $F(5,27) = 22.51$ and 12.69, $p < 0.0001$, respectively]. The minimal effective dose (first statistically significant dose) was 3 mg/kg for oral dosing and 1 mg/kg for subcutaneous dosing. The therapeutic window of LY411,575, defined as ED_{50} for inhibition of cortical $A\beta_{40}$/minimal effective dose for PAS staining, was similar for the oral and subcutaneous routes (5 and 2.5, respectively).

The percentage of area covered by PAS stain in mice

TABLE 3
Percentage of inhibition of plasma $A\beta_{40}$, body weight change from baseline, thymus weight, and plasma drug levels (mean ± S.E.M.) following 6 days of oral dosing with 10 mg/kg LY411,575 in young and aged CRND8 mice

Group	Plasma $A\beta_{40}$ Inhibition	Body Weight Change	Thymus Weight	Plasma Levels
	%	g	mg	ng/ml
Young (5–6 weeks)				
Vehicle		+1.2 ± 0.4	47.36 ± 5.48	
10	100†	−0.6 ± 0.1*	16.62 ± 1.47*	16.39 ± 5.18
Aged (16–25 months)				
Vehicle		−1.7 ± 2.2	6.35 ± 1.66	
10	100†	−3.8 ± 1.0	4.43 ± 0.82	27.18 ± 5.60

* $p < 0.003$ vs. age-matched vehicle, t test.
† $p < 0.0001$ vs. age-matched vehicle, t test on raw data, not percentage of inhibition.

treated with 10 mg/kg LY-D was slightly more (6%) than that covered by vehicle-treated mice (4%) [Fig. 3D; $t(8) = 4.75$, $p < 0.002$], but it was considerably less than that observed following oral or subcutaneous administration of 10 mg/kg LY411,575 (27 and 23%, respectively).

Plasma Concentrations. Three to 4 h after the final dose on day 6, plasma levels of LY411,575 and LY-D were determined (Table 2). There was a dose-related increase in plasma concentrations for both the oral and subcutaneous routes, and plasma concentrations at a given dose of LY411,575 were similar after oral and subcutaneous dosing. The plasma levels of LY-D (10 mg/kg orally) were similar to the equivalent oral dose of LY411,575.

The Extent of Intestinal Goblet Cell Hyperplasia Induced by LY411,575 Was Similar in Young and Aged Mice

Notch signaling in muscle has been shown to be reduced in aged mice (Conboy et al., 2003). To determine whether the intestinal side effects of LY411,575, which are thought to be due to inhibition of Notch signaling (Jensen et al., 2000; Fre et al., 2005; van Es et al., 2005), would differ in aged mice, young (5- to 6-week-old) or aged (16- to 26-month-old) CRND8 mice were orally dosed with 10 mg/kg LY411,575 or vehicle for 6 days.

Efficacy. Young and aged vehicle-treated mice did not differ in baseline levels of plasma $A\beta_{40}$ (18.67 ± 1.89 and 13.70 ± 0.56 ng/ml, respectively). There was 100% inhibition of plasma $A\beta_{40}$ production following treatment with 10 mg/kg LY411,575 for both age groups [Table 3; $t(9) = 11.28$ and $t(7) = 61.28$, $p < 0.0001$). Figure 4 shows an example of a relatively plaque-free brain from a 12-week-old CRND8 mouse (Fig. 4A) and the extensive plaque burden that is characteristic of an 18-month-old CRND8 mouse (Fig. 4B). As expected, young vehicle-treated mice had much less cortical $A\beta_{40}$ than aged vehicle-treated mice (Fig. 4C). In young mice, 6-day treatment with 10 mg/kg LY411,575 significantly reduced cortical $A\beta_{40}$ by 66% [$t(8) = 22.91$, $p < 0.0001$], whereas LY411,575 had no effect on cortical $A\beta_{40}$ in the aged mice (Fig. 4C).

Body Weight. Young vehicle-treated mice gained weight over the 6 days of dosing, whereas young mice treated with 10 mg/kg LY411,575 lost weight [Table 3; $t(9) = 4.25$, $p < 0.003$]. Both groups of aged mice lost weight by day 6 and did not significantly differ from each other (Table 3).

Thymus. Consistent with previous findings, treatment of young mice with 10 mg/kg LY411,575 significantly reduced the weight of the thymus [Table 3; $t(9) = 5.90$, $p < 0.0003$]. LY411,575 did not affect the thymus weight in aged mice, but this may reflect a floor effect given the small thymus size in aged vehicle-treated mice (Table 3).

Ileum. In young and aged mice, the percentage of villi area covered by PAS stain significantly increased following treatment with 10 mg/kg LY411,575 [Fig. 4D; $t(9) = 14.79$ and $t(8) = 9.38$, respectively, $p < 0.0001$]. The percentage of increase over vehicle was 387% for young and 329% for aged LY411,575-treated mice, which was not a significant difference.

Plasma Concentrations. Plasma concentrations 3 to 4 h after the final 10 mg/kg dose of LY411,575 were comparable across both age groups (Table 3).

The Thymus and Intestinal Side Effects Induced by LY411,575 Were Reversible

To assess the reversibility of the thymus and intestinal side effects induced by chronic dosing with higher doses of LY411,575, CRND8 mice were orally dosed with either 1) 3

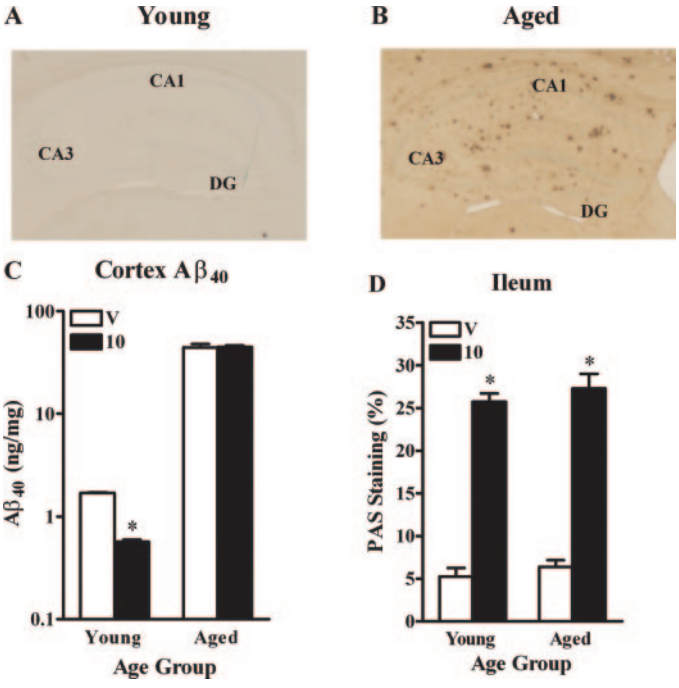

Fig. 4. Representative sections showing the dentate gyrus (DG), CA1, and CA3 regions of the hippocampus from a 12-week-old (A) and an 18-month-old (B) CRND8 mouse. Effects of oral dosing of 10 mg/kg LY411,575 (black bars) or vehicle (white bars) on cortical $A\beta_{40}$ levels (C; $n = 4–6$/group) and percentage of villi area covered by PAS stain in the ileum (D; $n = 4–6$) in young (6-week-old) and aged (16- to 26-month-old) CRND8 mice. *, $p < 0.05$ versus vehicle (V), within age t test.

mg/kg LY411,575 for 6 days followed by vehicle for an additional 14 days (washout 3 mg/kg), 2) 10 mg/kg LY411,575 for 6 days followed by vehicle for an additional 14 days (washout 10 mg/kg), 3) vehicle for 14 days followed by 6 days of 3 mg/kg LY411,575 (immediate 3 mg/kg), 4) vehicle for 14 days followed by 6 days of 10 mg/kg LY411,575 (immediate 10 mg/kg), or 5) vehicle for 20 days (vehicle). All mice were sacrificed on day 20.

Efficacy. On day 20, the groups differed in plasma and cortical $A\beta_{40}$ levels [main effect of group: $F(4,27) = 98.66$ and 15.32, $p < 0.0001$; data not shown and Fig. 5A]. Plasma $A\beta_{40}$ levels were reduced to below the limit of quantification in the immediate 3 and 10 mg/kg LY411,575 groups, but they returned to levels near the vehicle-treated animals after the 14-day washout period (immediate group differed from both the vehicle and dose-matched washout group, $p < 0.0001$; Fisher's post hoc t test; data not shown). Likewise, cortical $A\beta_{40}$ levels were reduced by 85 and 89% following 6 days of treatment with 3 or 10 mg/kg LY411,575, respectively, and they returned to vehicle-treated levels following the washout period (Fig. 5A). As confirmation that the washout mice did indeed receive sufficient amounts of LY411,575 during the first 6 days of dosing, a separate group of mice that were dosed along with the mice in this study were sacrificed after 6 days of dosing with 10 mg/kg LY411,575; these mice showed 100 and 66% inhibition of plasma and cortical $A\beta_{40}$, respectively.

Body Weight. Body weight change from baseline remained relatively stable (± 1 g) throughout the study for mice in the vehicle group (Fig. 5B) and immediate and washout 3 mg/kg groups (data not shown). Alternatively, mice in both 10 mg/kg groups lost weight while LY411,575 was being administered for 6 days compared with when vehicle was administered for the same 6 days [Fig. 5B; $t(12) = 2.81$, $p < 0.02$ and $t(15) = 3.40$, $p < 0.004$, for immediate and washout groups, respectively]. The body weight change from baseline of washout 10 mg/kg mice slowly returned to vehicle-treated levels after cessation of drug treatment.

Thymus. Thymus weight at the end of the study was different across the groups [Fig. 5C; $F(4,19) = 3.15$, $p < 0.04$]. Thymus weight in mice treated for 6 days with 10 mg/kg LY411,575 (immediate 10 mg/kg) was reduced by 61% compared with vehicle-treated mice, but thymus weight for this group was not significantly different from that for the 10 mg/kg washout animals. Thymus weight for the immediate 3 mg/kg group was not significantly different from that for vehicle-treated mice or the washout group.

Ileum. The groups differed in percentage of area covered by PAS stain at the end of the study [Fig. 5D; $F(4,27) = 53.51$, $p < 0.0001$]. After 6 days of treatment with 3 or 10 mg/kg LY411,575 (immediate groups), PAS staining was significantly increased, but it returned to vehicle-treated levels for both dose groups following the 14-day washout period. Figure 5, E to G, shows representative ileum sections from mice treated with vehicle, 10 mg/kg LY411,575 for 6 days, and 10 mg/kg LY411,575 for 6 days followed by 14 days of washout, indicating that PAS staining was greatly increased in the immediate group but had returned to vehicle levels following a 2-week washout period.

Coat Color. All of the mice in the washout 3 mg/kg LY411,575 group and 50% of the mice in the washout 10 mg/kg group showed some evidence of lightening of fur, similar to what was observed in the partial inhibition study described in the next section (Fig. 5, H–I). The changes were first noted in 3 mg/kg washout mice approximately 3 days into the washout period after 6 days of dosing with LY411,575 (i.e., 9 days from first dose). Both black and agouti mice were affected. The areas affected were patchy and ranged from the snout, forehead, back of head, lower back, and shoulders. The extent of lightening varied from slight to quite noticeable by the end of the study. None of the vehicle-treated or immediate LY411,575-treated mice showed any coat color changes.

Partial Inhibition of Cortical $A\beta_{40}$ with LY411,575 Did Not Produce Intestinal Side Effects Even with Longer Term Treatment

To determine whether a dose of LY411,575 that partially inhibited Aβ production would produce side effects after 3 weeks of treatment, CRND8 mice were orally dosed with either 1) 1 mg/kg LY411,575 for 20 days, 2) vehicle for 14 days followed by 6 days of 1 mg/kg LY411,575, or 3) vehicle for 20 days. All mice were sacrificed on day 20.

Efficacy. Treatment with 1 mg/kg LY411,575 for 6 or 20 days reduced plasma $A\beta_{40}$ levels by more than 94% [Table 4; main effect of group: $F(2,19) = 149.61$, $p < 0.0001$] and reduced cortical $A\beta_{40}$ by 44 and 69%, respectively [Table 4; $F(2,18) = 6.17$, $p < 0.01$].

Body Weight. There were no differences in body weight change from baseline among the groups across the 20-day study (Table 4).

Thymus. Twenty-day, but not 6-day, treatment with 1 mg/kg LY411,575 significantly reduced thymus weight [Fig. 6A; $F(2,12) = 3.95$, $p < 0.05$].

Ileum. Ileum PAS staining was marginally affected by LY411,575 treatment [Fig. 6B; $F(2,18) = 3.43$, $p < 0.06$], but neither 6 nor 20 days of treatment significantly increased PAS staining.

Coat Color. Similar to what was observed in washout mice from the previous study, all of the mice dosed with 1 mg/kg LY411,575 for 20 days showed some evidence of lightening or fading of fur color (Fig. 5, H–I). The changes were first noted after approximately 12 days of dosing. None of the vehicle-treated mice and only one mouse dosed with 1 mg/kg for 6 days showed evidence of coat color changes

Plasma Exposure. There was no significant difference between the 6- and 20-day groups in plasma levels of LY411,575 at 2 to 3 h postdose on the last day of dosing (Table 4).

Discussion

Pharmacological inhibition of the γ-secretase enzyme represents a promising therapeutic approach to reduce Aβ production in AD patients. Both acute and chronic treatment with various γ-secretase inhibitors robustly decreases plasma and brain Aβ in transgenic and nontransgenic animals (Dovey et al., 2001; May et al., 2001; Lanz et al., 2003, 2004; Milano et al., 2004; Wong et al., 2004; Anderson et al., 2005; Barten et al., 2005; Best et al., 2005, 2006; Grimwood et al., 2005; El Mouedden et al., 2006). However, there remains the distinct possibility that above a certain exposure

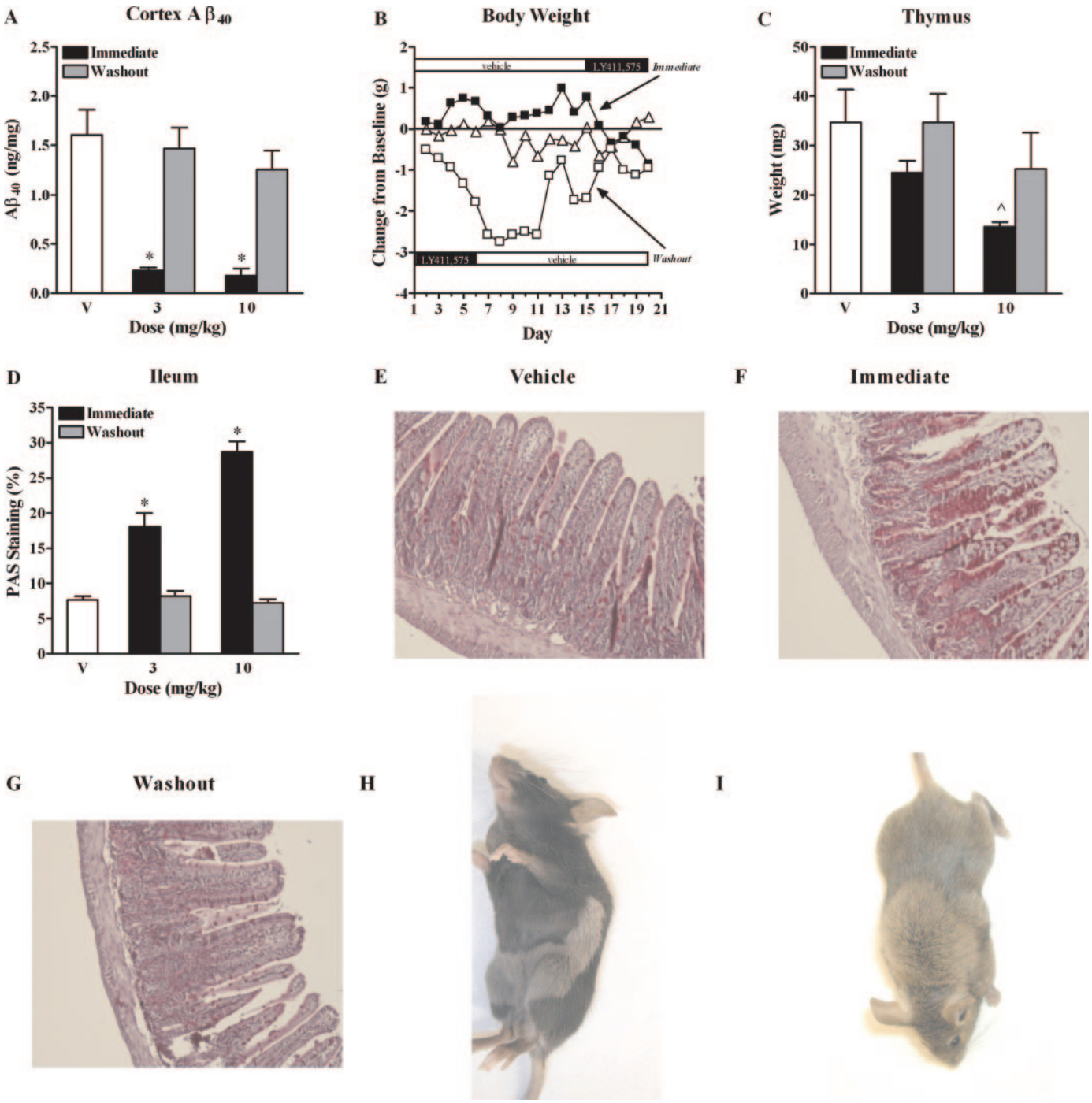

Fig. 5. A, cortical Aβ_{40} levels (n = 4–7/group) in mice treated orally with 3 or 10 mg/kg LY411,575 for 6 days (immediate; black bars) or for 6 days followed by a 14-day washout period (washout; gray bars). B, body weight change from baseline (day 1) for mice treated with vehicle on days 1 to 20 (△), vehicle on days 1 to 14 and 10 mg/kg LY411,575 on days 15–20 (immediate; ■) or 10 mg/kg LY411,575 on days 1 to 6 and vehicle on days 7 to 20 (washout; □). Home cage bedding was changed after weighing and dosing on day 13. C and D, thymus weight (C; n = 4–5) and percentage of ileum villi area covered by PAS stain (D; n = 4–7) in mice treated orally with 3 or 10 mg/kg LY411,575 for 6 days (immediate; black bars) or for 6 days followed by a 14-day washout period (washout; gray bars). E to G, representative PAS-stained sections from the ileum of a mouse treated with vehicle for 20 days (E; 9% PAS staining), 10 mg/kg LY411,575 for 6 days (F; 28%), or 10 mg/kg LY411,575 for 6 days followed by a washout period of 14 days of vehicle treatment (G; 8.5%). H and I, examples of the type of coat color changes (H, lower abdomen and sides; I, upper back) observed in mice treated orally with 1 mg/kg LY411,575 for 20 days (see partial inhibition section for study details). Similar coat color changes were observed in mice from the 3 and 10 mg/kg washout groups. ∗, $p < 0.04$ compared with vehicle (V; white bars) and dose-matched washout groups, Fisher's post hoc t test; ∧, $p < 0.02$ compared with vehicle group only, Fisher's post hoc t test.

level, drugs of this class will have potentially serious side effect liabilities due to reduced processing of other γ-secretase enzyme substrates, such as Notch (e.g., De Strooper et al., 1999; Barten et al., 2006). Consequently, the present experiments were designed to develop an efficient screening paradigm to define the therapeutic window of γ-secretase inhibitors and to investigate means of maximizing the therapeutic window.

TABLE 4
Percentage of inhibition of plasma and cortical $A\beta_{40}$, body weight change from baseline and plasma drug levels (mean ± S.E.M.) following 6 or 20 days of oral dosing with 1 mg/kg LY411,575 in CRND8 mice

Group	$A\beta_{40}$ Inhibition		Body Weight Change	Plasma Levels
	Plasma	Cortex		
	%		*g*	*ng/ml*
Vehicle			+0.3 ± 0.7	
Day 6	96*	44†	+0.3 ± 0.3	1.53 ± 0.31
Day 20	94*	69*	+0.3 ± 0.5	1.29 ± 0.22

* $p < 0.005$ and † $p < 0.10$ vs. vehicle, Dunnett's post hoc *t* test on raw data, not percentage of inhibition.

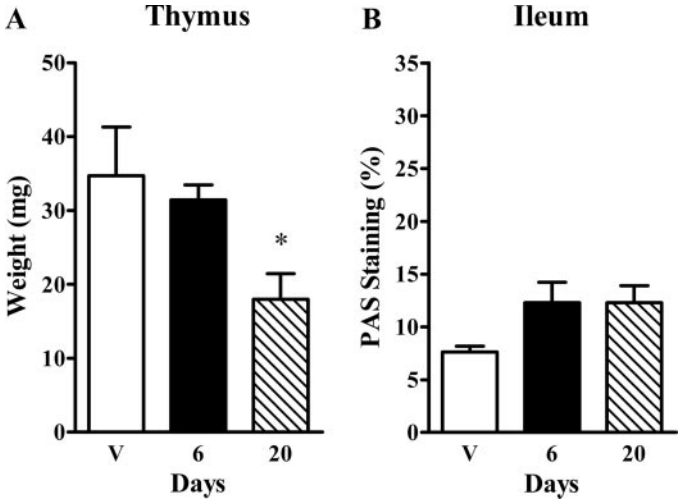

Fig. 6. Effects of 6 (black bars) or 20 (diagonal striped bars) days of oral dosing with 1 mg/kg LY411,575 on thymus weight (A; $n = 5$/group) and percentage of villi area covered by PAS stain in the ileum (B; $n = 6–8$). *, $p < 0.04$ compared with vehicle (white bars; V), Dunnett's post hoc *t* test.

Decreased thymus weight/cellularity and increased intestinal goblet cell hyperplasia were observed in young, preplaque transgenic CRND8 mice after only 3 to 6 days of treatment with the potent γ-secretase inhibitor LY411,575 (May et al., 2001) at a dose that reduced cortical $A\beta_{40}$ by 80 to 90% and at a frequency that provided continuous γ-secretase inhibition (Wong et al., 2004). The less potent diastereoisomer of LY411,575, LY-D (Wong et al., 2004), failed to produce equivalent thymus and intestinal changes at similar plasma exposure, confirming that these LY411,575 effects are due to γ-secretase inhibition. To quantify the extent of intestinal morphological changes observed, which until now has only been described qualitatively, we calculated the percentage of villi area exhibiting PAS staining, which stains mucopolysaccharide-rich goblet cells. Using this measure, the onset of intestinal changes reported here is in agreement with Searfoss et al. (2003) and Milano et al. (2004), who observed intestinal changes after 2 to 4 days of treatment with chemically different γ-secretase inhibitors. In the present studies, the thymus and intestinal changes were robust after 6 days of dosing with LY411,575 in CRND8 mice. This represents a substantial reduction in the duration of dosing needed to observe side effects compared with the 15 days adopted previously (Wong et al., 2004) and consequently increases the efficiency for screening γ-secretase inhibitors both for efficacy and side effect liabilities.

By examining both efficacy and side effects following chronic γ-secretase inhibition in the same animals, we were able to directly assess their relationship and thus estimate a therapeutic index for LY411,575. The oral doses and plasma exposure levels that caused a 50% reduction in thymus weight and statistically significant increases in PAS staining in the intestine were approximately 5-fold higher than those that caused 50% inhibition of $A\beta_{40}$ in the brain. In an effort to reduce intestinal goblet cell hyperplasia and increase this window, LY411,575 was administered by a nonoral (subcutaneous) route to reduce intestinal drug exposure. However, LY411,575 effects on cortical $A\beta_{40}$ levels, the thymus, and the intestine were very similar following oral or subcutaneous dosing, which resulted in similar therapeutic indices in CRND8 mice. Therefore, parenteral administration of γ-secretase inhibitors is not a viable means of increasing the therapeutic window of these drugs.

Since it has been reported that Notch activity is reduced in aged mice (Conboy et al., 2003) and the thymus and intestinal effects of chronic γ-secretase inhibition are most probably mediated by inhibition of Notch processing (Jensen et al., 2000; Fre et al., 2005; van Es et al., 2005), it is plausible that these side effects would be reduced in aged mice. However, the intestinal effects of a high dose of LY411,575 were similar in young (6 weeks) and aged (16–26 months) CRND8 mice. The thymus was too small in the aged group to observe any further reduction in size following LY411,575 treatment. Likewise, thymus involution occurs with normal aging in humans (Bodey et al., 1997), so the reduction of thymus weight/cellularity by γ-secretase inhibitors may not be an issue in aged AD patients. Despite significant reductions in plasma and cortical $A\beta_{40}$ in young mice and plasma $A\beta_{40}$ in aged mice, cortical $A\beta_{40}$ was unchanged in aged mice. Cortical Aβ in 6-week-old CRND8 mice is mainly in a soluble form, and there are no detectable Aβ plaques (Chishti et al., 2001; Hyde et al., 2005), whereas at 18 months of age there are numerous Aβ plaques (Fig. 4) and Aβ is primarily in the insoluble form. Thus, consistent with other reports (May et al., 2001; Lanz et al., 2003; Barten et al., 2005), it seems that γ-secretase inhibitors need to be administered for periods in excess of 6 days, or perhaps at higher doses, to reduce brain Aβ levels when Aβ exists primarily in the insoluble form. Overall, the therapeutic window is not likely to be improved in aged mice.

If the side effects of chronic γ-secretase inhibition were reversible, intermittent dosing might have been suitable for the treatment of AD. After chronic administration of LY411,575 in CRND8 mice, intestinal PAS staining, and to a lesser extent thymus weight, returned to vehicle levels after a 14-day washout, suggesting a return of normal γ-secretase function during this period. Although further studies are necessary to test for subtle histological differences after washout, these studies do suggest that at least certain deleterious effects of chronic γ-secretase inhibition are reversible. Paralleling this, the body weight loss that was observed after chronic treatment with a high dose of LY411,575 was also reversible. Since plasma and cortical $A\beta_{40}$ levels also returned to vehicle-treated levels during the 2-week washout period, additional studies are needed to better understand the balance between maintaining efficacy and avoiding side effects. Overall, these data suggest that an intermittent dosing regimen, similar to that used in cancer chemotherapy,

might be used to balance efficacy and side effects of γ-secretase inhibitors.

Partial inhibition of γ-secretase may also be a way to provide clinical benefit to AD patients without deleterious side effects, even with long-term treatment (Golde and Younkin, 2001), akin to clinical experience with 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors (statins) in the management of hypercholesterolemia (Selkoe, 1999). An extended 20-day treatment in CRND8 mice with a low (1 mg/kg) dose of LY411,575 reduced cortical $A\beta_{40}$ by 69%, yet it did not induce any intestinal changes, although a mild reduction in thymus weight was noted. This suggests that it may be possible to produce a sustained, but partial, level of γ-secretase inhibition without eliciting significant intestinal goblet cell hyperplasia. However, longer term treatment with LY411,575 revealed an additional unexpected drug-related effect, namely, coat color changes. Lightening of fur was first observed in mice after approximately 12 days of dosing a lower dose of LY411,575 and in mice that had been dosed for 6 days with higher doses of LY411,575. However in the latter case, the phenotype emerged a few days after drug administration had ceased. Another group has recently reported similar coat color changes in rats treated chronically with LY411,575 (Pagnozzi et al., 2004). This phenotype may be related to evidence that γ-secretase inhibition reduces melanin synthesis, blocks melanosome pigmentation, and affects trafficking of tyrosinase (involved in the melanin synthesis pathway) (Wang et al., 2006) and that Notch is involved in cell fate determination of hair follicular stem cells (Yamamoto et al., 2003). These findings reflect a cautionary note that other side effects of chronic γ-secretase inhibition may emerge with further testing.

In conclusion, we have shown that mechanism-based side effects can be observed after only a few days of sustained and substantial γ-secretase inhibition in CRND8 mice. The intestinal goblet cell hyperplasia can be readily quantified by PAS staining, and thymus weight provides a reasonably simple measure for cellular changes in this organ. Both efficacy and side effects following chronic dosing can be measured and quantified in the same animals to establish a therapeutic index, which was 3- to 5-fold for LY411,575. Others have suggested that it may be possible to have a larger therapeutic window because side effects were not observed following chronic dosing with structurally diverse γ-secretase inhibitors (e.g., BMS-299897, Milano et al., 2004; Barten et al., 2005). Our 6-day screening paradigm could be used to confirm these findings and allow for direct comparisons among drugs with perhaps different APP versus Notch substrate selectivity at the γ-secretase complex, including certain nonsteroidal anti-inflammatory drugs (Weggen et al., 2003). Most importantly, the data also suggest that partial inhibition of γ-secretase and/or intermittent dosing may be a way to achieve efficacy without deleterious side effects.

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Address correspondence to: Dr. Lynn A. Hyde, Schering Plough Research Institute, K15-2-2600, 2015 Galloping Hill Rd., Kenilworth, NJ 07033. E-mail: lynn.hyde@spcorp.com