Chronic Treatment with the γ-Secretase Inhibitor LY-411,575 Inhibits β-Amyloid Peptide Production and Alters Lymphopoiesis and Intestinal Cell Differentiation*

Received for publication, October 23, 2003, and in revised form, December 19, 2003 Published, JBC Papers in Press, January 6, 2004, DOI 10.1074/jbc.M311652200

Gwendolyn T. Wong‡§, Denise Manfra§, Frederique M. Poulet, Qi Zhang, Hubert Josien**, Thomas Bara***, Laura Engstrom‡, Maria Pinzon-Ortiz‡, Jay S. Fine‡, Hu-Jung J. Lee‡, Lili Zhang‡, Guy A. Higgins‡, and Eric M. Parker‡‡‡
From the Departments of ‡Central Nervous System Research, §Immunology, ***Chemical Research, and §Drug Safety, Schering-Plough Research Institute, Kenilworth, New Jersey 07033

Inhibition of γ-secretase, one of the enzymes responsible for the cleavage of the amyloid precursor protein (APP) to produce the pathogenic β-amyloid (Aβ) peptides, is an attractive approach to the treatment of Alzheimer disease. In addition to APP, however, several other γ-secretase substrates have been identified (e.g. Notch), and altered processing of these substrates by γ-secretase inhibitors could lead to unintended biological consequences. To study the in vivo consequences of γ-secretase inhibition, the γ-secretase inhibitor LY-411,575 was administered to C57BL/6 and TgCRND8 APP transgenic mice for 15 days. Although most tissues were unaffected, doses of LY-411,575 that inhibited Aβ production had marked effects on lymphocyte development and on the intestine. LY-411,575 decreased overall thymic cellularity and impaired intrathymic differentiation at the CD4−CD8−CD44−CD25+ precursor stage. No effects on peripheral T cell populations were noted following LY-411,575 treatment, but evidence for the altered maturation of peripheral B cells was observed. In the intestine, LY-411,575 treatment increased goblet cell number and drastically altered tissue morphology. These effects of LY-411,575 were not seen in mice that were administered LY-D, a diastereoisomer of LY-411,575, which is a very weak γ-secretase inhibitor. These studies show that inhibition of γ-secretase has the expected benefit of reducing Aβ in a murine model of Alzheimer disease but has potentially undesirable biological effects as well, most likely because of the inhibition of Notch processing.

Alzheimer disease (AD) is the third most common cause of death and the leading cause of dementia in the United States. Although the exact cause of AD is still unknown, the etiology of the disease is almost certainly linked to several neuropathological hallmarks observed in the brains of AD victims, particularly extracellular neuritic amyloid plaques and intracellular neurofibrillary tangles (2–4). Although both of these neuropathological lesions probably contribute to progressive neuronal cell death in AD, the proximal lesion appears to be the amyloid plaques and their principal component, the Aβ peptides. A large body of evidence strongly suggests that overproduction, aggregation, and/or plaque deposition of the Aβ peptides, particularly Aβ42, are central to the pathogenesis of AD (reviewed in Ref. 5). In fact, two recent studies of patients immunized against the Aβ42 peptide have provided the first preliminary clinical evidence that Aβ does indeed contribute to the cognitive decline in AD patients (6, 7).

The Aβ peptides are produced by the sequential proteolytic cleavage of the amyloid precursor protein (APP) by β- and γ-secretase. γ-Secretase is a complex composed of at least four proteins, namely presenilins (presenilin 1 or -2), nicastrin, PEN-2, and APH-1 (8). Presenilin 1 and -2 have been proposed to be the novel aspartyl proteases responsible for the catalytic activity of γ-secretase (9, 10). Because of the essential role of γ-secretase in the generation of Aβ peptides, γ-secretase inhibitors may be useful in the treatment of AD. To date, several γ-secretase inhibitors have been identified that lower Aβ production in intact cells and cell-free systems (reviewed in Ref. 11). In addition, two potent γ-secretase inhibitors, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine tert-buty1 ester (DAPT) and LY-411,575, have also been shown to decrease Aβ production after administration to transgenic mice overexpressing human APP (12–14).

It is appreciated now that APP is not the only substrate for γ-secretase. Other proteins that have been shown to be substrates for γ-secretase cleavage include Notch (16) and the Notch ligands Delta1 and Jagged2 (20), ErbB4 (17), CD44 (18), and E-cadherin (19). The cleavage of Notch by γ-secretase has been studied most extensively. Notch plays an evolutionarily conserved role in regulating cell growth and lineage specification particularly during embryonic development (21–23). Notch is activated by several ligands (Delta, Jagged, and Serrate) and is then proteolytically processed by a series of ligand-dependent and -independent cleavages. γ-Secretase catalyzes the terminal cleavage event (S3 cleavage), which releases a fragment known as the Notch intracellular domain (NICD). The NICD fragment then translocates to the nucleus where it acts as a nuclear transcription factor (15, 24). As expected from its role in Notch S3 cleavage, γ-secretase inhibitors have been shown to block NICD production in vitro (25). In vivo, Notch function appears to be critical for the proper differentiation of T and B lymphocytes (reviewed in Ref. 26), and γ-secretase inhibitors reduce the thymocyte number and block thymocyte differen-
activity in membranes prepared from HEK293 cells expressing APP have been described previously (32). Intact HEK293 cells expressing either APP or NAE were treated with various concentrations of LY-411,575 or LY-D for 4 h at 37 °C. In the case of cells expressing NAE, cells were lysed, the cell lysates were separated on a 4–12% NuPAGE gel, and the processed NICD fragment was detected via Western blot with a cleavage site-specific antibody. The inhibition of NICD production was quantified by spot densitometric analysis using FluorChem (Alpha Innotech Corp.). In the case of cells expressing APP, the conditioned medium was collected, centrifuged at 10,000 × g for 5 min to remove cell debris, and stored at −20 °C prior to the determination of Aβ levels. Aβ40 and −42 produced in HEK293 membrane- and cell-based assays, as well as plasma Aβ40 and cortex Aβ40 from TgCRND8 mice, were analyzed without pretreatment using an electrochemiluminescence detection-based immunoassay (32). Plasma Aβ42 was measured by enzyme-linked immunosorbent assay as described previously (33). A commercially available enzyme-linked immunosorbent assay kit (BioSource International) was used to measure cortex Aβ42 according to the manufacturer’s instructions.

Flow Cytometry—Single cell suspensions from the thymus and spleen were prepared from individual mice in RPMI 1640 medium containing 10% fetal calf serum (Invitrogen) by passage through a 100-μm nylon cell strainer (Falcon-BD Biosciences). Peripheral blood was collected as described above, and erythrocytes were lysed (erythrocyte lysis buffer, Sigma). Cells from the thymus, spleen, or blood were enumerated by trypan blue exclusion and then incubated for 20 min at 4 °C in the dark with 5 μg/ml Fe block (Pharmingen) and 300 μg/ml purified mouse IgG in phosphate-buffered saline, 1% bovine serum albumin, 0.1% sodium azide to minimize nonspecific antibody binding. Fluorochrome-conjugated monoclonal antibodies to the following mouse surface markers were then added, and the incubation was continued for 20 min at 4 °C in the dark: CD4 (GK1.5), CD8α (30.4.1), CD8β (53-6.7), CD44 (IM7), CD25 (PC61), CD3 (145-2C11), αβ TCR (H57-597), γδ TCR (GL3), B220 (RA3-6B2), and IgM (B6.80.2) (all obtained from Pharmingen). Matched fluorochrome-conjugated isotype control antibodies were used to determine nonspecific binding. To determine viability, samples were stained subsequently with 5 μg/ml propidium iodide (Calbiochem). Events were acquired on a FACSCalibur instrument (BD Biosciences Immunocytometry Systems) and analyzed with FlowJo software (Stanford University). The number of cells of each phenotype was determined by multiplying the percentage of cells in the population by the total number of thymus or spleen cells, as appropriate.

RESULTS

LY-411,575 Is a Potent γ-Secretase Inhibitor in Vitro and in Vivo—As reported previously (13), LY-411,575 is a very potent γ-secretase inhibitor in vitro as assessed by inhibition of Aβ production (IC50, 0.078 and 0.082 nM in membrane- and cell-based γ-secretase assays, respectively (Fig. 1)). LY-411,575 is also a potent inhibitor of Notch S3 cleavage (Fig. 1, IC50, 0.39 nM). A diastereoisomer of LY-411,575, LY-D, was also synthe-

FIG. 1. Structure and in vitro γ-secretase inhibitory potency of LY-411,575 and LY-D. The arrows identify the two chiral centers at which LY-411,575 and LY-D differ. The potencies of LY-411,575 and LY-D to inhibit Aβ40 or NICD production by isolated membranes or intact cells were determined as described under “Experimental Procedures.”
In vivo effects of γ-secretase inhibition

**Fig. 2. Reduction of plasma (A) and brain (B) Aβ by LY-411,575 but not LY-D.** LY-411,575 (1 and 10 mg/kg (mpk)), LY-D (1 and 10 mg/kg), or vehicle (Veh) were orally administered once/day to TgCRND8 mice for 15 consecutive days. Plasma and brains were harvested from the animals 6 h after the last dose. Plasma and brain Aβ40 (solid bars) and Aβ42 (open bars) were measured as described under “Experimental Procedures.” The data shown are the mean ± S.E. (n, 3–9 animals/group) and were analyzed by one way analysis of variance followed by Dunnett’s post-hoc test (*, p < 0.05 and ***, p < 0.001 compared with vehicle).

**Fig. 3. Time course of the reduction of plasma (A) and brain (B) Aβ by LY-411,575.** LY-411,575 (10 mg/kg) was orally administered to TgCRND8 mice, and plasma and brains were harvested from the animals at the indicated times after dosing. Plasma and brains from vehicle-treated animals (Veh) were harvested 3 h after dosing. Plasma and brain Aβ40 were measured as described under “Experimental Procedures.” The data shown are the mean ± S.E. (n, 4–5 animals/group) and were analyzed by one way analysis of variance followed by Dunnett’s post-hoc test (***, p < 0.001 compared with vehicle).

sized (Fig. 1). Depending on the assay, LY-D is 180–2000-fold less potent than LY-411,575 as an inhibitor of γ-secretase (Fig. 1). In vivo studies demonstrated that both 1 and 10 mg/kg oral doses of LY-411,575 decreased brain and plasma Aβ40 and -42 robustly when chronically administered to TgCRND8 mice (Fig. 2). In contrast, the administration of the less active diastereoisomer LY-D to TgCRND8 mice had no effect on Aβ levels in either the brain or plasma (Fig. 2). The different in vivo activities of LY-411,575 and LY-D are not because of different pharmacokinetic properties, because the two compounds achieved similar plasma levels after oral administration (data not shown). These in vitro and in vivo data demonstrate that LY-411,575 and LY-D are excellent tools to distinguish the mechanism-based effects of γ-secretase inhibition from the compound-related effects that are not due to γ-secretase inhibition.

The time course of the effects of LY-411,575 on plasma and brain Aβ40 levels is shown in Fig. 3. A single oral dose of 10 mg/kg LY-411,575 rapidly decreased plasma Aβ40 with the maximal effect being observed by 1 h after administration (Fig. 3A). The reduction of plasma Aβ was still evident 24 h after the administration of LY-411,575 but returned to base-line levels by 48 h after administration (Fig. 3A). LY-411,575 also substantially decreased guanidine-soluble brain Aβ levels, although the maximal effect was less pronounced, the time required to reach the maximal effect was longer, and the duration of the effect was longer than that seen for reduction of plasma Aβ (Fig. 3B). These data demonstrate that dosing of LY-411,575 once/day is sufficient to maintain continual inhibition of γ-secretase.

**General in Vivo Effects of Chronic γ-Secretase Inhibition—** LY-411,575 and LY-D (1 and 10 mg/kg) were administered to TgCRND8 mice once/day for either 5 or 15 consecutive days. In other experiments, LY-411,575 (1, 3, and 10 mg/kg) was administered to C57BL/6 mice once/day for 15 consecutive days. Similar results were observed in all three experiments. Unless indicated otherwise, therefore, data are presented only for the experiment in which LY-411,575 and LY-D were administered to TgCRND8 mice for 15 days.

Mice receiving 10 mg/kg LY-411,575 for 15 days lost weight (~2 g) compared with mice treated with vehicle (+1.4 g), 1 mg/kg LY-411,575 (+1.4 g), 1 mg/kg LY-D (+2 g), or 10 mg/kg LY-D (+1.5 g). In addition, 20% of the mice dosed with 1 mg/kg LY-411,575 and 40% of the mice dosed with 10 mg/kg LY-411,575 died by day 15 of dosing. In contrast, there was no mortality for mice dosed with vehicle or with either dose of LY-D.

Histopathological analysis demonstrated that neither LY-411,575 nor LY-D had any effect on the majority of the tissues examined, including the brain, liver, kidney, lungs, heart, adrenal glands, and stomach. Bone marrow smears also revealed no abnormalities (data not shown). In contrast, effects of LY-411,575 were observed in the thymus, spleen, and intestine. These observations are discussed individually below.

**Chronic γ-Secretase Inhibition Alters Lymphocyte Development—** Chronic treatment of TgCRND8 mice with LY-411,575 induced a marked atrophy of the cortical zone of the thymus (Fig. 4, compare A with B). This effect was dose-dependent and was consistent with the dose-dependent reduction in the absolute thymocyte cell number caused by LY-411,575 (Fig. 5A). In contrast, LY-D had no effect on thymus histology (Fig. 4, compare A with C) or the absolute number of thymocytes (Fig. 5A). Flow cytometric analysis of intrathymic populations revealed that the number and proportion of thymocytes defined by CD4/
CD8 expression was altered by treatment with LY-411,575 but not by treatment with LY-D. Consistent with the overall decrease in thymocyte number (Fig. 5A), the absolute number of CD4+CD8+ double positive, CD4+CD8− double negative (DN), CD4− single positive, and CD8− single positive cells also decreased in a dose-dependent manner (Fig. 5B). However, the proportion of DN and single positive cells increased, whereas the proportion of double positive cells decreased (Fig. 5C).

A detailed examination of the DN cell population demonstrated that LY-411,575 administration increased the relative percentage of the CD44+CD25− and CD44+CD25− DN thymocytes while causing a commensurate decrease in the proportion of CD44+CD25+ and CD44+CD25+ DN thymocytes (Fig. 5D) indicating a block at a very early stage of intrathymic T cell development. Once again, these effects were not observed in LY-D-treated animals (Fig. 5D). Interestingly, no differences in CD44 mean fluorescence intensity were observed across treatment groups implying that processing of this putative γ-secretase substrate was unaffected by LY-411,575 treatment.

The percentage of thymocytes expressing intermediate or high levels of CD3/−TcR was significantly reduced in accord with the inhibition of CD4/CD8 populations, although no consistent alterations in the percentage or number of γδ-TcR cells were noted. A slight increase in the proportion of thymic B220+ B cells was observed in LY-411,575-treated animals, although this did not reach statistical significance (data not shown).

The effect of LY-411,575 treatment on peripheral leukocyte compartments was also examined. In contrast to the observations made in the thymus, neither LY-411,575 nor LY-D treatment significantly altered T cell populations in the peripheral
spleen cells defined by expression of surface IgM and B220 was determined by flow cytometry as described under “Experimental Procedures.” Data represent the mean ± S.E. (5–6 mice/group) and were analyzed by unpaired Student’s t test (*, p < 0.05 and **, p < 0.01 compared with vehicle).

blood or spleen (data not shown). However, LY-411,575 administration did cause a statistically significant elevation in the percentage and number of immature surface IgM+ B220+ B cells and a corresponding reduction in the percentage and number of mature surface IgM+ B220+ B cells in the spleen (Fig. 6). These observations suggest that LY-411,575 treatment results in a developmental blockade of splenic B cell differentiation. Modest elevations in the number of immature B cells were observed in the blood as well. No remarkable findings on bone marrow cytology, myeloid populations in the blood, or complete blood count analysis were noted (data not shown).

**DISCUSSION**

Several recent reports (12–14) have described potent γ-secretase inhibitors that are active in animal models of Aβ deposition. These studies suggest that γ-secretase inhibitors will be appropriate tools to test the amyloid hypothesis of AD in humans and may ultimately have clinical utility in the treatment of the disease. It is recognized now that in addition to APP, γ-secretase proteolytically processes several other substrates, most notably Notch (10). However, little is actually known about the biological consequences of blocking the processing of the myriad γ-secretase substrates in vivo, particularly in adult organisms. To address this uncertainty, the biological effects of the potent γ-secretase inhibitor LY-411,575 (13) were compared with the biological effects of LY-D, a structurally and pharmacokinetically related diastereoisomer of LY-411,575 that is significantly less potent as a γ-secretase inhibitor. This comparison permits a definitive distinction between the biological effects of γ-secretase inhibition and the biological effects of LY-411,575 that are unrelated to γ-secretase inhibition.

In vitro, LY-411,575 inhibited Aβ and NICD production with similar potencies. It is therefore reasonable to assume that LY-411,575 similarly inhibits APP and Notch processing in vivo. Consistent with the potent inhibition of Aβ production in vitro, chronic treatment of TgCRND8 mice with 1–10 mg/kg LY-411,575 decreased plasma- and guanidine-soluble brain Aβ40 and -42. The inhibitory effect of LY-411,575 on brain Aβ was consistently less pronounced and more delayed than the inhibitory effect of the drug on plasma Aβ. This may reflect the fact that brain Aβ, particularly Aβ42, exists in an oligomeric, aggregated, or otherwise insoluble state and hence is cleared less rapidly than plasma Aβ, which is likely to be largely soluble. In the case of brain Aβ42, this may also reflect the fact that brain Aβ42 levels in TgCRND8 mice are increasing during the course of these experiments, i.e. between 6–8 weeks of age (30). The degree of inhibition of plasma and brain Aβ40 and -42 levels by chronic administration of LY-411,575 was consistent with the degree of inhibition seen in acute (single dose) studies.
account for this discrepancy. Overall, these data suggest that the preceding differentiation step (CD44 gene (34), although these earlier reports localized the block at in vivo proportion of cells in the CD44
mer LY-D. Further analysis of the DN compartment in LY-411,575 treatment induced a profound atrophy of the cortical region of the thymus with a significant depletion of the cortex, a pronounced dose-dependent decrease in overall thymic cellularity, and an overall reduction in each of the major intrathympic populations defined by CD4 and -8. These effects of LY-411,575 are clearly due to γ-secretase inhibition, because they were not seen following chronic treatment with the structurally related diastereoisomer LY-D. Further analysis of the DN compartment in LY-411,575-treated animals revealed a significant increase in the proportion of cells in the CD44+CD25- and CD44+CD25+ subpopulations and a corresponding reduction in the proportion of cells in the CD44+CD25+ and CD44+CD25- subpopulations. These data strongly suggest that LY-411,575 treatment results in a specific developmental blockade of intrathympic differentiation at the step at which CD44+CD25- cells differentiate to CD44+CD25+ cells. These results are generally consistent with the in vitro effects of γ-secretase inhibitors on thymocyte maturation observed in fetal thymic organ cultures (27, 28, 35) and with the in vivo effects seen after inactivation of the Notch1 gene (34), although these earlier reports localized the block at the preceding differentiation step (CD44+CD25- and CD44+CD25+). Differences in the affinity and selectivity of the γ-secretase inhibitor employed or differences between chronic in vivo administration and short term in vitro treatment of fetal thymic organ cultures with γ-secretase inhibitors may account for this discrepancy. Overall, these data suggest that altered Notch activity is responsible for the effects of LY-411,575 on the thymus, although it is not possible to specifically determine which γ-secretase substrate(s) involved in Notch signaling is/are affected (Notch, Jagged, and/or Delta) (36). A contribution of altered E-cadherin (37, 38) or CD44 (39) processing to this altered intrathympic differentiation is possible as well, although no effect of LY-411,575 on CD44 expression in the thymus was observed.

LY-411,575 treatment also exerted profound effects on B cell development. Following LY-411,575 administration, a slight increase in the percentage of B lineage cells in the thymus (as defined by expression of B220) was consistently observed, although this was not statistically significant. This implies that in vivo γ-secretase inhibition may alter B versus T cell lineage decisions within the earliest uncommitted lymphocyte stem cell populations in the thymus, as suggested previously (28, 34, 46). More strikingly, LY-411,575 significantly inhibited splenic B cell maturation and correspondingly increased the number of immature B cells in the blood despite having no overall effect on splenic cellularity or on other spleen cell populations. LY-D treatment did not cause these effects. These data suggest an important role for γ-secretase in B cell development in the spleen. Several recent reports (40–42) suggest that Notch signaling plays a role in marginal zone B cell development in the spleen, although no effect on the peripheral B cell compartment was reported in Notch1-deficient mice (34). Therefore, the elevation in immature B cells in spleen tissues following treatment with LY-411,575 may also be because of inhibition of Notch or Notch ligand processing via γ-secretase.

Chronic treatment of TgCRND8 mice with LY-411,575 also caused profound changes in the gastrointestinal tract. Specifically, LY-411,575 induced a proliferation of mucin-producing goblet cells, a corresponding increase in mucin detected in the lumen of the intestine, and, in one animal, mild crypt necrosis and erosion of the intestinal epithelium with accumulation of cellular debris in the intestinal lumen. These effects of LY-411,575 are clearly because of inhibition of γ-secretase activity, because they were not observed in animals treated with LY-D. It was reported recently (43) that a structurally related γ-secretase inhibitor induced similar effects in rat intestine. The effects of LY-411,575 on the intestine are probably also because of the inhibition of Notch processing by γ-secretase. Indeed, mouse embryos deficient in Hairy/Enhancer of split-1 (Hes-1), which is a transcriptional repressor protein that is intimately involved in Notch signaling, have a very similar phenotype in the gastrointestinal tract (44). It is likely that the decrease in body weight and increase in mortality in TgCRND8 mice treated with LY-411,575 are because of the effects of the compound on the intestine. It also remains possible, however, that subtle alterations in the mucosal immune response might play a role in these findings.

The present study demonstrates the ability of γ-secretase inhibitors to chronically reduce Aβ production. Longer treatment with LY-411,575 (13) and several reports of anti-Aβ42 immunization of mice and humans suggest that chronic reduction of Aβ production also allows clearance of amyloid plaques and improvement of cognitive function (6, 7). Taken together, these data suggest that γ-secretase inhibitors offer substantial promise for the treatment of AD. However, the present study also shows that γ-secretase inhibitors have several potential liabilities that may limit this promise. Some of these potential liabilities may be tolerable. For example, the effect of LY-411,575 on intrathympic T cell development may not be a major issue in the elderly, because the thymus normally atrophies extensively by early adulthood (45). Nevertheless, further assessment of these potential liabilities would be informative. For example, it will be important to measure functional T and B cell responses after γ-secretase inhibition to more fully assess the effect of γ-secretase inhibition on the function of the immune system. In any case, these studies suggest caution in the testing of γ-secretase inhibitors in humans and indicate specific side effects that should be monitored in any future clinical trials.

REFERENCES
In Vivo Effects of γ-Secretase Inhibition


10


Downloaded from http://www.jbc.org/ by guest on November 8, 2017
Chronic Treatment with the γ-Secretase Inhibitor LY-411,575 Inhibits β-Amyloid Peptide Production and Alters Lymphopoiesis and Intestinal Cell Differentiation

Gwendolyn T. Wong, Denise Manfra, Frederique M. Poulet, Qi Zhang, Hubert Josien, Thomas Bara, Laura Engstrom, Maria Pinzon-Ortiz, Jay S. Fine, Hu-Jung J. Lee, Lili Zhang, Guy A. Higgins and Eric M. Parker

doi: 10.1074/jbc.M311652200 originally published online January 6, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M311652200

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 44 references, 17 of which can be accessed free at http://www.jbc.org/content/279/13/12876.full.html#ref-list-1