Articles

L-685,458, an Aspartyl Protease Transition State Mimic, Is a Potent Inhibitor of Amyloid β-Protein Precursor γ-Secretase Activity

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ABSTRACT: Progressive cerebral amyloid β-protein (Aβ) deposition is believed to play a central role in the pathogenesis of Alzheimer's disease (AD). Elevated levels of Aβ(42) peptide formation have been linked to early-onset familial AD-causing gene mutations in the amyloid β-protein precursor (AβPP) and the presenilins. Sequential cleavage of AβPP by the β- and γ-secretases generates the N- and C-termini of the Aβ peptide, making both the β- and γ-secretase enzymes potential therapeutic targets for AD. The identity of the AβPP γ-secretase and the mechanism by which the C-termini of Aβ are formed remain uncertain, although it has been suggested that the presenilins themselves are novel intramembrane-cleaving γ-secretases of the aspartyl protease class [Wolfe, M. S., Xia, W., Ostaszewski, B. L., Diehl, T. S., Kimberly, W. T., and Selkoe, D. J. (1999) *Nature 398*, 513−517]. In this study we report the identification of L-685,458 as a structurally novel inhibitor of AβPP γ-secretase activity, with a similar potency for inhibition of Aβ(42) and Aβ(40) peptides. This compound contains an hydroxyethylene dipeptide isostere which suggests that it could function as a transition state analogue mimic of an aspartyl protease. The preferred stereochemistry of the hydroxyethylene dipeptide isostere was found to be the opposite to that required for inhibition of the HIV-1 aspartyl protease, a factor which may contribute to the observed specificity of this compound. Specific and potent inhibitors of AβPP γ-secretase activity such as L-685,458 will enable important advances toward the identification and elucidation of the mechanism of action of this enigmatic protease.

Amyloid β-protein (Aβ)[1] is the main constituent of the abundant neuritic plaques that are a characteristic hallmark of Alzheimer's disease (AD). It has been postulated that—in its many β-structured oligomeric and protofibrillar forms—Aβ is responsible for, or at least a contributory factor to, the neuronal degeneration that occurs in AD. This hypothesis is supported by the finding that mutations in the amyloid β-protein precursor (AβPP) and presenilin 1 (PS1) and presenilin 2 (PS2) genes, that are causative for familial early-onset AD, alter AβPP processing to result in elevated formation of the Aβ(42) peptide (*1*). This peptide—relative to the predominantly produced Aβ(40) species—is particularly amyloidogenic and appears to form the core of the neuritic plaques. AβPP is cleaved initially by α- or β-secretase to generate membrane-bound C-terminal fragments (C83 and C99, respectively). α-Secretase activity appears to be mediated by the disintegrin and metalloprotease (ADAM) family members TACE and ADAM-10 (*2*). β-Secretase (BACE, Asp-2) has recently been cloned and characterized as a novel membrane-bound aspartyl protease (*3*−*6*). The C83 and C99 cleavage products serve as substrates for the γ-secretase, with Aβ(40) and Aβ(42) being generated from C99. This cleavage is rather unusual in that it occurs at a site predicted to be within the putative transmembrane domain of AβPP. The identity of the enzymatic species that generates Aβ(40) and Aβ(42) peptides—functionally defined as AβPP γ-secretase—remains contentious. It has been claimed to be a single pharmacological entity or to exist as multiple species, belonging to either serine, cysteine, or aspartyl protease classes (*7*−*13*). Alternatively, following the demonstration of an obligatory role of presenilins in Aβ formation (*14*, *15*), it has been suggested that PS1 itself functions as the γ-secretase (*16*).

We have used directed screening in cell culture-based and in vitro assays of AβPP processing to discover structurally novel inhibitors of Aβ peptide formation. We report the

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[1] Abbreviations: Aβ, amyloid β-protein; AD, Alzheimer's disease; AβPP, amyloid β-protein precursor; BACE, β-site AβPP cleaving enzyme; CHO, Chinese hamster ovary; DMSO, dimethyl sulfoxide; EuK, europium cryptate; FBS, fetal bovine serum; FRET, fluorescence resonance energy transfer; HEK, human embryonic kidney; HTRF, homogeneous time-resolved fluorescence; PAGE, polyacrylamide gel electrophoresis; PS, presenilin; SELDI-TOF, surface-enhanced laser desorption/ionization time-of-flight.

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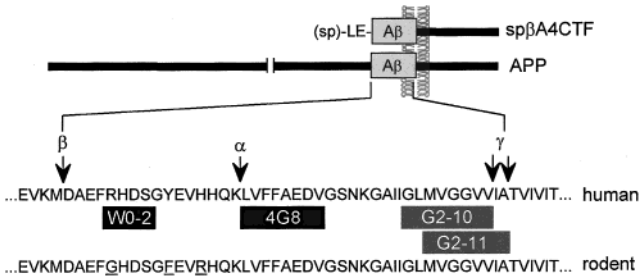


FIGURE 1: Human AβPP constructs and monoclonal antibodies against the amyloid β-protein sequence. The spβA4CT construct is a direct substrate for AβPP γ-secretase, consisting of a signal peptide, the two additional amino acids Leu-Glu followed by the C-terminal 99 amino acids of AβPP (*21*). The monoclonal antibodies shown allow β- and α-secretase-cleaved AβPP C-terminal fragments to be distinguished, as well as identifying Aβ peptides ending at Val-40 and Ala-42.

identification of L-685,458 as a specific inhibitor of AβPP γ-secretase, with a similar potency toward Aβ(40) and Aβ-(42). This compound contains an hydroxyethylene dipeptide isostere which suggests that it functions as a transition state analogue mimic at the catalytic site of an aspartyl protease (*17*). This observation is concordant with claims that AβPP γ-secretase is an aspartyl protease. The potency of L-685,-458 for AβPP γ-secretase and its selectivity over other proteases make it an ideal tool to explore the enzymology of this unusual class of intramembrane-cleaving protease.

EXPERIMENTAL PROCEDURES

Materials. Hybridoma cell clones secreting the monoclonal antibodies G2-10, G2-11, and W0-2 were licensed from the University of Heidelberg (Heidelberg, Germany). The specificity of G2-10 for Aβ ending at Val-40, and G2-11 for Aβ ending at Ala-42, has been described previously (*18*). Samples of protein G-purified antibody were prepared by Cymbus Biotechnology Ltd. (Hampshire, U.K.). Europium cryptate (EuK) labeling of G2-10 and G2-11 was performed at CIS bio international (Bagnols/Ceze, France). Biotinylated and nonbiotinylated monoclonal antibodies 4G8 and 6E10 were purchased from Senetek PLC (Missouri). The epitopes against which these antibodies were raised and their location within the Aβ peptide sequence are shown in Figure 1.

Synthetic Chemistry. {1*S*-Benzyl-4*R*-[1-(1*S*-carbamoyl-2-phenylethylcarbamoyl)-1*S*-3-methylbutylcarbamoyl]-2*R*-hydroxy-5-phenylpentyl}carbamic acid *tert*-butyl ester (L-685,458) was prepared by the coupling of 2*R*-benzyl-5*S*-*tert*-butoxycarbonylamino-4*R*-(*tert*-butyldimethylsilanyloxy)-6-phenylhexanoic acid (*19*) with Leu-Phe-NH_2 followed by deprotection with tetrabutylammonium fluoride. The synthesis of {1*S*-benzyl-4*R*-[1-(1*S*-carbamoyl-2-phenylethylcarbamoyl)-1*S*-3-methylbutylcarbamoyl]-2*S*-hydroxy-5-phenylpentyl}carbamic acid *tert*-butyl ester (L-682,679) has been described previously (*20*). {1*S*-Benzyl-4*R*-[1-(1*S*-carbamoyl-2-phenylethylcarbamoyl)-1*S*-3-methylbutylcarbamoyl]-2-oxo-5-phenylpentyl}carbamic acid *tert*-butyl ester (L-684,-414) was prepared by pyridinium dichromate-mediated oxidation of L-682,679.

Cell Culture. Cell lines stably expressing full-length human AβPP or the truncated human spβA4CTF construct (Figure 1) were propagated under standard conditions using appropriate antibiotic selection. For quantitative assessment of Aβ peptide levels, cells were plated at 30 000/well/100 μL in 96-well microtiter plates and compounds added for 16−20 h, with a final dimethyl sulfoxide (DMSO) concentration of 0.5% (v/v). Replicate sample volumes ranging from 10 to 30 μL were assayed for Aβ peptide levels.

HTRF Immunoassay for Aβ Quantitation. Homogeneous time-resolved fluorescence (HTRF) immunoassay methodology uses fluorescence resonance energy transfer (FRET) between two fluorophores, a donor EuK and a modified allophycocyanine pigment acceptor molecule, XL-665. When in proximity, nonradiative FRET takes place from nitrogen laser-excited EuK to XL-665, resulting in the emission of an amplified long-lived fluorescence signal. The details for Aβ peptide quantitation by this assay will be described elsewhere (M. S. Shearman, E. E. Clarke, unpublished observations). Briefly, in a typical 96-well plate assay, each well contained 0.75 nM antibody-EuK, 1.0 nM antibody-biotin, 2.0 nM SA-XL665, and 0.1−0.2 M potassium fluoride. Samples of conditioned cell culture medium or synthetic peptide standards and culture medium alone were added to give a total assay volume of 200 μL/well. Blank values were determined by the inclusion of 1.0 nM nonbiotinylated antibody in place of the biotinylated antibody. Following mixing, the reaction mixture was left at 4 °C to reach equilibrium binding, and then read on the Discovery HTRF microplate analyzer using the manufacturer's recommended settings.

AβPP γ-Secretase in Vitro Membrane Assay. A total membrane fraction from SHSY5Y spβA4CTF cells was prepared by centrifugation of a broken cell preparation at 120000*g* for 60 min. The pellet was resuspended by homogenization in phosphate-buffered saline, pH 7.3, containing 5% glycerol and 2.5 mM dithiothreitol (DTT), aliquotted, and stored at −80 °C until use. Membranes (5−10 μg of protein) were incubated in 96-well plates for 90 min at 37 °C in 20 mM HEPES, pH 7.3, containing 0.1% bovine serum albumin, 0.25% CHAPSO, 0.5 mM EDTA, 1% glycerol, and 2.5 mM DTT in the presence of vehicle control (0.5% DMSO) or inhibitor. Samples were placed on ice, 100 μL per well of HTRF reagent mixture was added, and Aβ peptide was quantified using the Discovery HTRF microplate analyzer.

Metabolic Radiolabeling Studies. For pulse−chase analysis, SHSY5Y spβA4CTF cells were preincubated for 30 min in methionine-free minimal essential medium containing 5% dialyzed fetal bovine serum (FBS), and then 200 μCi of [^{35}S]-methionine was added to each 10 cm dish for a period of 3.5 h. After washing, the cells were chased in Dulbecco's modified Eagle's medium/F12 supplement containing 10% FBS and 5 mM nonradioactive methionine in the presence of vehicle (0.5% DMSO) or inhibitor, for various time intervals. For steady-state analysis, human embryonic kidney (HEK) 293 cells overexpressing human AβPP695 were preincubated for 50 min in methionine-free minimal essential medium containing 10% dialyzed FBS and 1% glutamine. Vehicle (0.5% DMSO) or inhibitor were added 20 min before the addition of 400 μCi of [^{35}S]methionine for a further 18 h. In both protocols, the conditioned media were harvested and centrifuged for 10 min at 13000*g*. The supernatants were diluted with 0.4 mL of lysis buffer (20 mM HEPES, pH 7.3, containing 1% Nonidet P40, 0.5% deoxycholate, 1 mM EDTA) and the samples precleared with 5 μg of mouse IgG

and 30 μL of a 1:1 slurry of protein G−agarose. Aβ(40) and p3(40) were immunoprecipitated with 10 μg of monoclonal antibody G2-10 or 4G8 and 50 μL of a 1:1 protein G−agarose slurry by incubation overnight at 4 °C. Nonspecifically bound proteins were removed by washing as described (*21*) and immunoprecipitated proteins separated on 10−20% Tris−Tricine precast gradient gels (Novex, San Diego). For immunoprecipitation of membrane-bound spβA4CTF or AβPP695 processing products, cell lysates were prepared by suspending the cells in lysis buffer and incubation for 30 min on ice with repeated mixing. Insoluble material was removed by centrifugation for 10 min at 13000*g*. Preclearing and immunoprecipitation with 10 μg of monoclonal antibody 4G8 were carried out as described above.

Surface-Enhanced Laser Desorption/Ionization Time-of-Flight (SELDI-TOF) Mass Spectrometry. Antibodies were diluted in phosphate-buffered saline to 0.5 μg/μL, and 1.0 μL was applied to an individual spot of the SELDI chip coated with a preactivated surface. Free reactive sites were blocked by incubation for 30 min at room temperature with 1 M ethanolamine hydrochloride, pH 7.5. Conditioned media diluted 2-fold with 25 mM HEPES, pH 7.3, 1 mM EDTA were incubated with an antibody-coupled SELDI chip overnight at 4 °C. Nonspecifically bound peptides were removed by extensive washing. A saturated solution of α-cyano-4-hydroxycinnamic acid in 40% acetonitrile, 0.25% trifluoroacetic acid was diluted 1:5 into the same solvent and 0.5 μL applied to each spot of the chip. After evaporation of the matrix solvent, the samples were analyzed on a SELDI mass analyzer MRS-1 with a linear time-of-flight mass spectrometer (Ciphergen Biosystems Inc., California).

Protease Assays. With the exception of the AβPP γ-secretase assay, counterscreening of L-685,458 against other protease activities was conducted with purified enzymes and commercially available peptide substrates. HIV-1 activity was monitored with DABACYLγ-Abu-Ser-Gln-Asn-Tyr-Pro-Ile-Gln-EDANS (Bachem), human liver cathepsin D (Sigma) at pH 3.5 with H-Pro-Thr-Glu-Phe-*p*-nitro-Phe-Arg-Leu-OH (Bachem), bovine trypsin (type I, Sigma) with Bz-L-Arg-pNA (Bachem), and HepC3 NS3-JK4 with [^{3}H]p164 peptide (Amersham). Inhibition of porcine erythrocyte calpain I and Carica papaya papain (Calbiochem) was assessed using a previously described dye-binding assay procedure with bovine casein as substrate (*22*).

RESULTS

L-685,458 Inhibition of Aβ Peptide Formation Assessed by the HTRF Assay. Directed screening of the Merck Sample Repository based on a compound exemplified in European patent application EP0778266-A1 led to the identification of L-685,458 (Figure 2). L-685,458 dose-dependently inhibits Aβ formation in both Neuro2A and CHO cell lines overexpressing human AβPP695, and in SHSY5Y cells overexpressing the construct spβA4CTF (Table 1). L-685,458 was found not to be cytotoxic to cells at concentrations up to 10 μM, as judged by redox dye measurements of cell viability (data not shown). L-685,458 reduced both Aβ(40) and Aβ-(42) peptide formation in these cells, with the potency for reduction of Aβ(42) being about 2-fold lower (Table 1). The antibody 4G8 was used routinely in the HTRF assay to enable detection of both β- and α-secretase cleaved peptide products, although essentially the same results were obtained either with antibody 6E10 or with antibody W0-2 (Figure 1; data not shown).

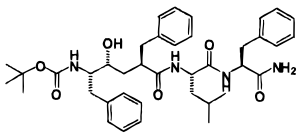
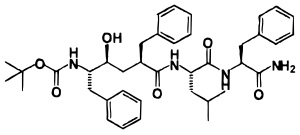
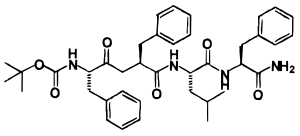
Inhibitor	Structure	IC_{50}, nM
L-685,458		17 ± 8 (15)
L-682,679		> 10 000 (3)
L-684,414		181 ± 1 (2)

FIGURE 2: Structures of the prototypical AβPP γ-secretase inhibitor L-685,458 and two close analogues, and their activity in the SHSY5Y spβA4CTF in vitro membrane assay of Aβ formation. Data are the mean ± SD or mean ± range of *n* determinations.

Table 1: Inhibitor Potency of L-685,458 against Aβ Production in Three Different Cell Lines

cell line	IC_{50} vs Aβ(40)[a]	IC_{50} vs Aβ(42)
Neuro2A h AβPP695	402 ± 108 (5)	775 ± 202 (2)
CHO h AβPP695	113 ± 42 (6)	248 ± 28 (3)
SHSY5Y spβA4CTF	48 ± 17 (14)	67 ± 14 (3)

[a] Values (nM) are the mean ± SD ($n > 2$) or mean ± range ($n = 2$).

L-685,458 Exhibits the Activity Profile of an AβPP γ-Secretase Inhibitor. The finding that L-685,458 is a potent inhibitor of Aβ formation in human neuroblastoma SHSY5Y cells overexpressing the construct spβA4CTF, which serves as a direct substrate for the AβPP γ-secretase enzyme (*21*, *23*), indicated that this compound targets the AβPP γ-secretase activity rather than β-secretase. To confirm this, the metabolic turnover of spβA4CTF in SHSY5Y cells was first investigated by radiolabeling and pulse−chase analysis. In vehicle-treated cells, radiolabeled bands identified as LE-βA4CTF (the expression construct, equivalent to βCTF/C99, but containing the construct-derived additional two amino acids) and p3CTF (αCTF/C83) were seen to disappear with time (Figure 3A). This was accompanied by a time-dependent loss of radiolabeled intracellular LE-Aβ peptide, and the appearance of LE-Aβ and p3 secreted into the cell culture medium (Figure 3B). By comparison, treatment with L-685,-458 resulted in the stabilization of LE-βA4CTF itself, and a marked increase in the formation of the α-secretase-cleaved product p3CTF (Figure 3A). Concomitantly, a reduction in detectable intracellular LE-Aβ (Figure 3A) and a near-complete inhibition of LE-Aβ and p3 secreted into the medium were observed (Figure 3B). The inhibitory action of L-685,458 was verified by conducting metabolic radiolabeling experiments with HEK293 cells overexpressing human AβPP695. Treatment with L-685,458 for 18 h, but not its inactive epimer L-682,679 (see below), resulted in a marked accumulation in the cell lysates of p3CTF, and to a

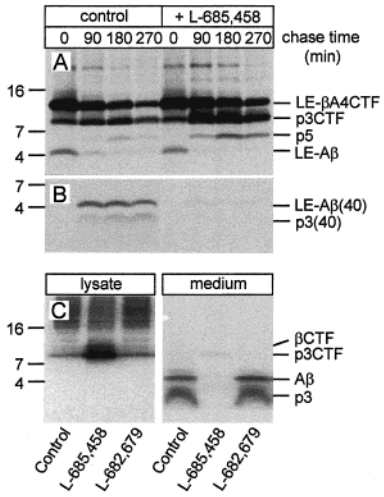


FIGURE 3: Metabolic radiolabeling analysis of AβPP metabolism. SHSY5Y cells stably expressing spβA4CTF were metabolically labeled with [^{35}S]methionine and chased in the presence of vehicle control (0.5% DMSO) or L-685,458 (10 μM) for the time intervals indicated. spβA4CTF processing products were immunoprecipitated from (A) cell lysates using monoclonal antibody 4G8, and (B) cell culture medium using monoclonal antibody G2-10. HEK293 cells stably expressing human AβPP695 were metabolically labeled with [^{35}S]methionine in the presence of vehicle control (0.5% DMSO), L-685,458 (10 μM), or L-682,679 (10 μM) for 18 h. AβPP695 processing products were immunoprecipitated from (C) cell lysates and cell culture medium using monoclonal antibody 4G8. Proteins were separated by SDS−PAGE and visualized by autoradiography. Identification of the different species was based on comigration with the molecular weight standards indicated.

lesser extent βCTF (Figure 3C). As expected, a near-complete inhibition of Aβ and p3 secreted into the medium was also observed (Figure 3C). L-685,458 does not markedly alter the secretion of the N-terminal fragments of AβPP, sAβPPα, and sAβPPβ, generated following α- and β-secretase activity (data not shown). This profile of AβPP processing is consistent with the specific inhibition of AβPP γ-secretase activity (*12*). A novel fragment of approximately 5−6 kDa (p5) also transiently accumulated intracellularly and was stabilized in the presence of L-685,458 inhibition (Figure 3A).

To provide additional confirmation of the HTRF and metabolic labeling results and to obtain a detailed qualitative fingerprint of all Aβ peptide species generated in vehicle- and inhibitor-treated cells, we employed SELDI-TOF mass spectrometry, which combines the covalent immobilization of antibodies to a solid-phase chip with mass spectrometric analysis of captured peptides. Using this technique LE-Aβ-(1−42) and LE-Aβ(1−40) were identified unambiguously in conditioned medium from SHSY5Y spβA4CTF cells (Figure 4A). In addition, two distinct populations of Aβ peptides with intact N-termini but truncated at the C-terminus were also detected. Treatment of the cells with L-685,458 (1 μM) essentially abolished the formation of all Aβ peptide species, with the exception of LE-Aβ(1−16) and LE-Aβ-(1−15), the relative levels of which were increased (Figure 4B). Similar observations to the above have been made in the cell lines overexpressing full-length AβPP which generate Aβ peptides beginning at Asp-1 (data not shown), indicating that the results are not influenced by the use of the truncated AβPP construct.

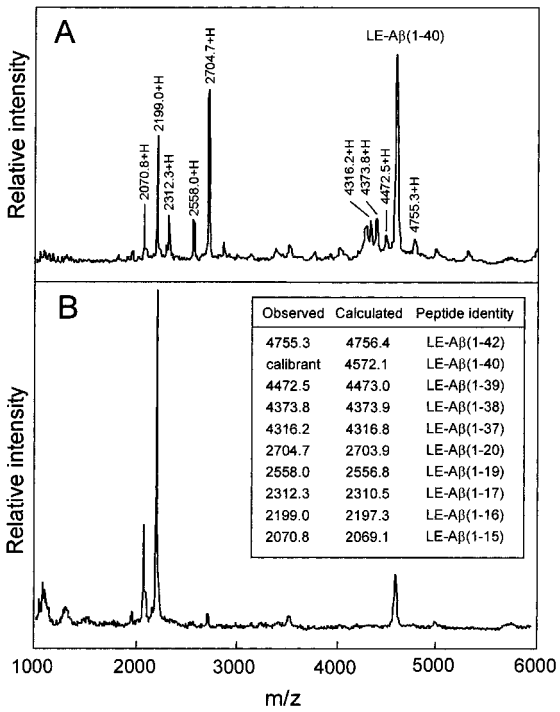


FIGURE 4: Generation of Aβ peptides monitored by SELDI-TOF mass spectrometry. SHSY5Y spβA4CTF cells were treated with (A) vehicle control (0.5% DMSO) or (B) the AβPP γ-secretase inhibitor L-685,458 (1 μM), for 18 h. Aβ peptides were immunocaptured from conditioned media using monoclonal antibody W0-2 (Figure 1) covalently coupled to the SELDI protein chip, as described under Experimental Procedures. Due to the nature of the spβA4CTF construct, all Aβ peptides contain the two additional amino acids Leu-Glu prior to the Asp residue normally found at position 1 of Aβ. Peptides were typically identified using bovine insulin as an external calibrant. Major peaks are indicated in the upper panel, and the confirmed identities of these peaks based on their calculated average masses are given in the inset table of the lower panel. The error for each peptide was <2 Da if the LE-Aβ-(1−40) peak was used for internal calibration. The spectra shown, which are representative examples replicated in at least 3 independent experiments, provide a qualitative rather than quantitative assessment of peptide formation.

Stereoselectivity of the L-685,458 Hydroxyethylene Moiety for AβPP γ-Secretase Inhibition. To confirm the direct binding of L-685,458 to γ-secretase, a SHSY5Y spβA4CTF in vitro γ-secretase membrane assay was used, in which the human γ-secretase enzyme catalyzes the breakdown of the human spβA4CTF endogenously expressed substrate. This assay also allowed us to probe the stereoselectivity at the hydroxyethylene dipeptide isostere by comparison of the activity of L-685,458 against two close structural analogues, L-682,679 and L-684,414 (Figure 2). In agreement with the cell-based data (Table 1), L-685,458 was found to be a potent inhibitor of AβPP γ-secretase activity in this assay (IC_{50} 17 nM; Figure 2). On the other hand, its epimer, L-682,679, a highly potent inhibitor of HIV-1 aspartyl protease activity (*19*), was found to have negligible activity in the AβPP γ-secretase in vitro membrane assay (IC_{50} > 10 000 nM; Figure 2). L-684,414, the ketone derivative, was also found to be active (IC_{50} 181 nM; Figure 2), but less so than L-685,-458, as expected if the hydroxyethylene dipeptide is serving as a transition state mimic.

Selectivity of L-685,458 for AβPP γ-Secretase over Protease Classes. The specificity of L-685,458 was assessed

Table 2: Protease Specificity of L-685,458

enzyme	class	inhibitor potency[a]
AβPP γ-secretase	aspartyl?	17
HIV-1	aspartyl	1000
cathepsin D	aspartyl	1800
trypsin	serine	>1000
HepC3 NS3	serine	>1000
papain	cysteine	2000
calpain I	cysteine	>10000

[a] Values (nM) are IC_{50} or K_i.

by comparison of its activity in the SHSY5Y spβA4CTF in vitro membrane assay against a panel of enzymes representing aspartyl, serine, and cysteine protease classes (Table 2). Of those tested, L-685,458 was clearly most potent against AβPP γ-secretase activity, displaying a selectivity of 50-fold or greater.

DISCUSSION

In this study, we identify L-685,458 as a potent inhibitor of Aβ formation. Subsequent characterization—using a combination of metabolic radiolabeling and SELDI-TOF mass spectrometry studies—showed that L-685,458 is a specific inhibitor of AβPP γ-secretase activity. L-685,458 contains an hydroxyethylene dipeptide isostere, supporting the conclusion—based on the activity of substrate-based difluoroketone peptidomimetic compounds (*11*) and pepstatin A (*13*)—that AβPP γ-secretase is an aspartyl-type protease. The difluoroketone compounds noted above are only weak AβPP γ-secretase inhibitors (IC_{50} > 10 μM) but can be potent inhibitors of cathepsin D (*11*). L-685,458, on the other hand, is a potent inhibitor of AβPP γ-secretase (IC_{50} = 17 nM) and shows greater than 50−100-fold selectivity over other aspartyl proteases tested. A similar or greater selectivity is evident against other classes of protease. By comparison of the activity of L-685,458 and L-682,679, we conclude that this specificity arises, at least in part, from the stereochemistry of the hydroxyethylene dipeptide isostere. For inhibition of AβPP γ-secretase, the preferred stereochemistry of the hydroxyl group is syn relative to the neighboring benzyl group, the opposite to that of the HIV-1 aspartyl protease (*20*). The conclusion that L-685,458 acts as a transition state mimic of an aspartyl protease presumes that it binds to the enzyme in a linear conformation (Figure 5A). An alternative possibility, however, is that the specificity of L-685,458 relative to its epimer L-682,679 arises from the molecules binding to γ-secretase in a manner which mimics the predicted α-helical nature of the AβPP transmembrane domain. In this case, the transition state isostere hydroxyl group in L-685,458 makes key H-bonds to stabilize the helical conformation (Figure 5B). In L-682,679, the epimeric hydroxyl group also forms internal H-bonds, but this now makes a β-turn conformation more energetically favorable than an α-helical one.

Differences in the potency of L-685,458 for inhibiting Aβ formation were observed between clonal cell lines derived from human, mouse, and hamster. This is an empirical observation replicated for many different inhibitors, and may be explained by the relative cell permeability or stability of the inhibitor when in contact with the different cell types. In each cell line, however, L-685,458 inhibited both Aβ-(42) and Aβ(40) formation with similar potencies. Our results favor the existence of a single AβPP γ-secretase activity with broad steric tolerance, but do not exclude the possibility that L-685,458 cross-reacts with two enzymes exhibiting a closely related pharmacology. Data from mutagenesis studies have shown that the cleavage of AβPP by γ-secretase preferentially occurs in the middle of the lipid bilayer, with the length of the lumenal portion of the transmembrane domain determining the site of cleavage (*13*, *23*). The enzyme appears to have a loose amino acid sequence preference around the scissile bond (*13*, *23*, *24*), and cleavage at the Val-Ile and Ala-Thr sites, predicted to be on opposite sides of the transmembrane α-helix domain (*24*), could be achieved by rotation of the substrate and/or enzyme relative to one another. Inhibitor data previously described (*11*, *12*) support the lack of sequence specificity, in that a variety of hydrophobic aliphatic and aromatic residues neighboring the scissile bond appear to be tolerated. In agreement with this, L-685,458, mimicking the sequence Phe-Phe-Leu-Phe, is a potent inhibitor. Whether or not this represents the optimal recognition sequence for inhibition of AβPP γ-secretase remains to be determined.

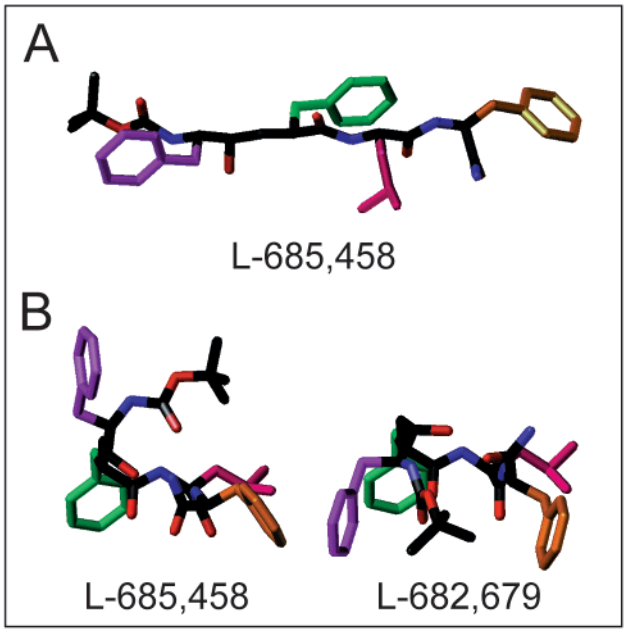


FIGURE 5: Possible conformations of the prototypical AβPP γ-secretase inhibitor L-685,458 and its epimer L-682,679. (A) L-685,458 is shown in an extended conformation with each side chain color-coded for ease of visualization. This conformation is the one normally exhibited by inhibitors of aspartyl proteases. (B) L-685,458 and L-682,679 are shown in alternative conformations, wherein the transition state isostere hydroxyl groups make internal H-bonds to stabilize the conformation.

The speculation that multiple γ-secretase activities exist arose from observations that certain peptide aldehyde inhibitors exhibit a differential effect on Aβ(42) and Aβ(40) formation (*7*, *8*), leading to the suggestion that the two γ-secretases belonged to the serine and cysteine protease classes, respectively (*10*). In contrast to this, using a panel of five structurally diverse peptide aldehyde and difluoroketoamide inhibitors, Durkin and colleagues (*12*) found the same rank order of potency for inhibition of Aβ(42) and Aβ-(40). As the above experiments were carried out in cell-based assays only, the mechanism of action and true molecular targets of these inhibitors could not be specified. A subse-

quent more detailed analysis revealed that for peptide aldehyde and boronates, a good correlation exists between inhibition of Aβ formation and inhibition of the chymotrypsin-like activity of the proteasome (*25*). The exact role of the proteasome in the production and/or secretion of Aβ was not determined in this study, but it draws attention to the complications associated with the use of high concentrations of these compounds as γ-secretase inhibitors in cell-based assays, and casts doubt on the data as evidence of multiple enzymes.

The ability to effectively and selectively block AβPP γ-secretase activity has provided insights into the mechanisms of AβPP processing by cells. First, SELDI-TOF mass spectrometric analysis of human neuroblastoma SHSY5Y spβA4CTF cell culture medium revealed the presence of numerous Aβ peptide species truncated from the C-terminus. Interestingly, the formation of the majority of these peptides was blocked by treatment with L-685,458, and it is likely that the truncated species [other than Aβ(1−16) and Aβ-(1−15)] are derived from Aβ(42) or Aβ(40) following the action of a carboxypeptidase. We interpret the relative increase in generation of Aβ(1−16) (the β- and α-secretase cleavage products) and Aβ(1−15) species in the presence of L-685,458 as resulting from enhanced accessibility of βCTF to the α-secretase. In agreement with this, it has recently been shown that the protein kinase C-dependent α-secretase competes with β-secretase for cleavage of AβPP in the trans-Golgi network (*2*). Pulse−chase analysis revealed a novel fragment of approximately 5 kDa (p5) that transiently accumulated intracellularly and was stabilized in the presence of L-685,458 inhibition (Figure 3A). This fragment could represent an alternative competitive pathway for degradation of AβPP C-terminal fragments, which is enhanced when the activity of γ-secretase is inhibited (*26*).

The properties of L-685,458 are concordant with AβPP γ-secretase being an aspartyl-class protease. Whether or not PS1 and PS2 are aspartyl proteases (*9*, *27*), whose function as AβPP γ-secretases is inhibited by L-685,458, requires further exploration. The recent demonstration that structurally related photoaffinity analogues of L-685,458 bind to PS1 (*28*), and that L-685,458 itself stabilizes full-length PS1 (D. Beher, unpublished observations) supports this contention. In this respect, if AβPP γ-secretase-like activity also participates in the regulated intramembrane proteolysis of proteins such as Notch and Ire1 (*29*), it will be important to determine the effects that AβPP γ-secretase inhibitors may have on these signaling pathways. The marked inhibitory potency and specificity displayed by L-685,458 make it a particularly suitable reagent to address these questions.

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