0026-895X/07/7102-588–601$20.00
MOLECULAR PHARMACOLOGY
Copyright © 2007 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 71:588–601, 2007

Vol. 71, No. 2
24299/3173365
Printed in U.S.A.

Unnatural Amino Acid-Substituted (Hydroxyethyl)urea Peptidomimetics Inhibit γ-Secretase and Promote the Neuronal Differentiation of Neuroblastoma Cells

Yung-Feng Liao, Bo-Jeng Wang, Wen-Ming Hsu, Hsinyu Lee, Chia-Yin Liao, Shin-Ying Wu, Hui-Ting Cheng, and Ming-Kuan Hu

Laboratory of Molecular Neurobiology, Institute of Cellular and Organismic Biology, Academia Sinica, Taipei, Taiwan (Y.-F.L., B.-J.W., S.-Y.W., H.-T.C.); School of Pharmacy, National Defense Medical Center, Taipei, Taiwan (C.-Y.L., M.-K.H.); Department of Surgery, National Taiwan University Hospital and National Taiwan University, Taipei, Taiwan (W.-M.H.); and Department of Life Science and Institute of Zoology, National Taiwan University, Taipei, Taiwan (H.L.)

Received March 9, 2006; accepted November 14, 2006

ABSTRACT

γ-Secretase, exhibiting characteristics of aspartyl protease, mediates the intramembranous proteolysis of β-amyloid precursor protein (APP) and Notch, and it is considered to be a prime pharmacological target in the development of therapeutics for Alzheimer's disease (AD). To identify compounds that block γ-secretase-mediated proteolysis, we used a highly sensitive cell-based reporter gene assay for γ-secretase in which Gal4/VP16-tagged C99-APP was expressed as the immediate substrate of γ-secretase, and Gal4/VP16-tagged APP intracellular domain released by the γ-secretase cleavage then activated the expression of the Gal4-driven luciferase reporter gene. Using this reporter assay, we demonstrated that the newly synthesized (hydroxyethyl)urea peptidomimetics, which contain unnatural amino acid moieties at positions P1′ and/or P3′, can effectively inhibit γ-secretase activity and significantly reduce Aβ production. The γ-secretase-dependent S3 cleavage of Notch was also consistently blocked by these (hydroxyethyl)ureas as evidenced by the decreased generation of the Notch intracellular domain, a prerequisite for the activation of Notch signaling. The inhibition of Notch signaling by active Jia compounds efficiently promotes the neuronal differentiation of neuroblastoma cells, intervening in tumorigenesis and the malignancy of neuroblastomas. Our results suggest that (hydroxyethyl)urea peptidomimetics containing unnatural amino acid substitutions could represent a novel class of γ-secretase inhibitors with enhanced stability, providing the basis for the further development of effective therapeutics for AD and neuroblastomas.

γ-Secretase catalyzes the final proteolytic step in the generation of Aβ (the principal constituent of senile plaques in the AD brain), and it has thus been regarded as a prime therapeutic target for AD. Mounting evidence from pharmacological studies, mutagenesis, affinity labeling, and biochemical isolation strongly suggests that γ-secretase is an aspartyl protease and that the active site of γ-secretase is located at the interface of the presenilin (PS) heterodimer (Wolfe and Haass, 2001). The heterodimeric PS consisting of an ~30-kDa N-terminal fragment (NTF) and a ~20-kDa C-terminal fragment (CTF) is thought to be the active form of PS (Capell et al., 1998). Peptidomimetics that mimic the

This study was supported by National Science Council, Taiwan, grants NSC 93-2320-B-001-037, NSC 94-2320-B-001-004, NSC 94-3112-B-001-001, and NSC 95-3112-B-001-006 (to Y.-F.L.) and NSC 92-2320-B-016-046 (to M.-K.H.) and Academia Sinica (to Y.-F.L.). Proteomic mass spectrometry analyses performed by the Core Facilities for Proteomics Research located at the Institute of Biological Chemistry, Academia Sinica, were supported by National Science Council Grant NSC 91-3112-P-001-009-Y and the Academia Sinica.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.106.024299.

ABBREVIATIONS: AD, Alzheimer's disease; PS, presenilin; NTF, N-terminal fragment; CTF, C-terminal fragment; NICD, Notch intracellular domain; HIV, human immunodeficiency virus; ELISA, enzyme-linked immunosorbent assay; HEK, human embryonic kidney; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; APP, amyloid precursor protein; C99, 99-residue C-terminal fragment of amyloid precursor protein; PBS, phosphate-buffered saline; PLB, passive lysis buffer; DAPT, *N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-*S*-phenylglycine *t*-butyl ester; GV, Gal4/VP16 DNA binding domain; GAP-43, growth-associated protein 43; PAGE, polyacrylamide gel electrophoresis; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBST, phosphate-buffer saline/Tween 20; Aβ, β-amyloid; HRP, horseradish peroxidase; CHAPSO, 3-[(3-cholamidopropyl)dimethyl-ammonio]-2-hydroxy-1-propanesulfonate; siRNA, small interfering RNA; RNAi, RNA interference; ESI, electrospray ionization; MS, mass spectrometry, MS/MS, tandem mass spectrometry; LC, liquid chromatography; OD, optical density; Chy, cyclohexylmethyl; RA, retinoic acid; L-685–458, (2*R*-(2*R**,4*R**,5*S**))*N*-(5-(((1,1-dimethylethoxy)carbonyl)amino)-4-hydroxy-1-oxo-6-phenyl-2-(phenylmethyl)hexyl)-L-leucyl-L-phenylalaninamide.

transition state of aspartyl protease catalysis have been derived from sequences around the γ-cleavage site of APP and are shown to block γ-secretase activity, indicating that γ-secretase is an aspartyl protease (Wolfe et al., 1999a). Two highly conserved aspartate residues residing within the sixth and seventh transmembrane domains of presenilins are required for γ-secretase activity (Wolfe et al., 1999b), suggesting that the active site of this novel aspartyl protease might be located at the heterodimeric interface. A number of compounds designed to interact with the protease active site have been shown to bind directly to both PS subunits (Esler et al., 2000; Li et al., 2000b). Together, these findings strongly support the notion that PS heterodimers constitute the active site of γ-secretase.

Biochemical and genetic analyses further reveal that PSs are part of a multimeric γ-secretase complex whose constituents include a heterodimeric PS, a mature glycosylated nicastrin (NCT), Aph-1, and Pen-2 (Iwatsubo, 2004). Compelling evidence has shown that the full spectrum of γ-secretase activity can be reconstituted by the coexpression of human PS, nicastrin, Aph-1, and Pen-2 in yeast (Edbauer et al., 2003), providing definitive proof for the minimal required constituents of a functional γ-secretase.

The γ-secretase not only mediates the proteolysis of APP but also is critical for the processing of Notch receptor. The Notch signaling pathway is essential for cell fate decisions during development (Mumm and Kopan, 2000). This pathway is initiated by the binding of the Delta-Serrate-Lag2 ligand family, followed by the shedding of extracellular domain (S2 cleavage) mediated by the ADAM family members. The resultant C-terminal membrane-tethered fragment of Notch, termed Notch extracellular membrane truncation (NEXT), is then cleaved within the transmembrane domain (S3 cleavage) by γ-secretase to release the Notch intracellular domain (NICD). Once released from membrane, NICD bounds to mammalian CBF1/RBP$_j$, *Drosophila melanogaster* Suppressor of hairless, and *Caenorhabditis elegans* Lag-1 DNA-binding proteins and converts them from transcriptional repressors to activators, resulting in the expression of Notch downstream target genes. The inhibition of γ-secretase would likewise block NICD production and reduce Notch signaling (Wolfe et al., 1999b, 2002).

(Hydroxyethyl)urea peptidomimetics have recently been identified as a new class of transition state analog inhibitors that mimic the transition state of aspartyl protease catalysis and block γ-secretase much more efficiently than any of the difluoro ketones or difluoro alcohols (Wolfe et al., 2002). Highly potent and selective inhibitors containing hydroxyethyl isostere have also been developed and used to block such aspartyl proteases as renin and HIV protease (Greenlee, 1990; Huff, 1991). Five HIV protease inhibitors currently available as approved treatments for AIDS all contain the hydroxyethyl isostere (Flexner, 1998), and the core structure of (hydroxyethyl)ureas is known to have apparent low toxicity, a prerequisite for clinical applications. (Hydroxyethyl)-urea peptidomimetics have been developed for the affinity isolation and characterization of γ-secretase, and for probing the active site of this protease (Esler et al., 2004). These compounds can be generated through systemic replacements in five positions (P2, P1′, P2′, P3′, and P4′) with small, medium, and large hydrophobic L-amino acids (Wolfe et al., 2002). Furthermore, (hydroxyethyl)urea peptidomimetics systematically altered at positions P2-P3′ either with hydrophobic L- or D-amino acids are shown to effectively block γ-secretase activity (Bakshi and Wolfe, 2004; Esler et al., 2004). These studies not only substantiate the loose sequence specificity of γ-secretase but also raise the possibility that γ-secretase could be targeted by transition state analog inhibitors encompassing unnatural amino acids that can confer peptidomimetics extraordinary biological stability along with enhanced biological activity and proteolytic resistance. Herein, we report the development of (hydroxyethyl)urea peptidomimetics containing unnatural amino acid substitutions that can effectively block γ-secretase activity for lowering Aβ production and attenuating Notch signaling so as to promote the neuronal differentiation of neuroblastoma cells. Our studies provide the proof-of-concept for the use of unnatural amino acids as building blocks in the generation of biologically stable γ-secretase inhibitors for AD treatments and the promotion of neuronal differentiation.

Materials and Methods

Reagents. The BCA protein assay reagent kit and SuperSignal West Pico and SuperSignal West Dura reagents were purchased from Pierce Chemical (Rockford, IL). The rabbit anti-Notch(Val1744) antibody was from Cell Signaling Technology Inc. (Danvers, MA). Horseradish peroxidase-conjugated anti-rabbit IgG was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Horseradish peroxidase-conjugated anti-mouse IgG and ECL Western Blotting detection reagents were from GE Healthcare (Little Chalfont, Buckinghamshire, UK). The FuGENE6 transfection reagent, Expand long template polymerase chain reaction system, and polymerase chain reaction nucleotide mix were from Roche Applied Science (Indianapolis, IN). Dual luciferase assay reagents, Steady-Glo luciferase assay reagents, and the pRL-TK vector were from Promega (Madison, WI). Human Aβ40 and Aβ42 colorimetric ELISA kits were from BioSource International (Camarillo, CA). All other reagents were of at least reagent grade and were obtained from standard suppliers.

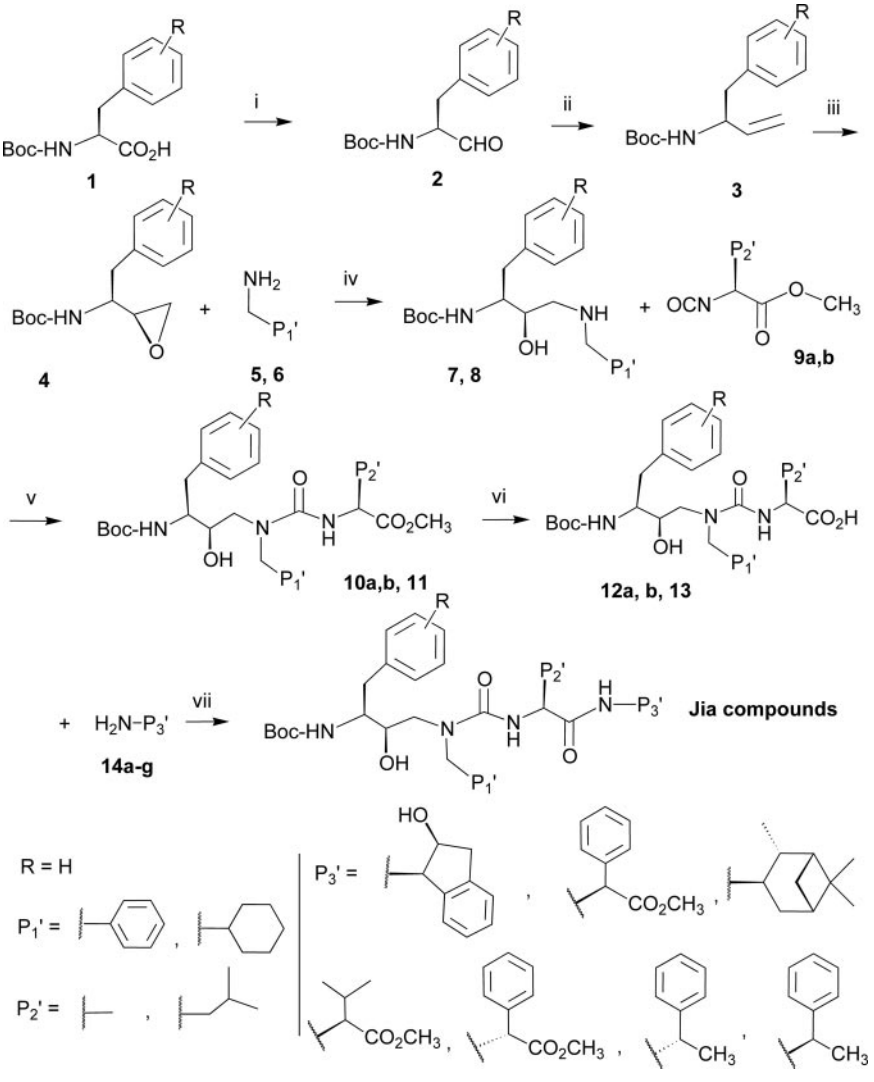
Synthesis of (Hydroxyethyl)ureas. The (hydroxyethyl)urea peptidomimetics containing unnatural amino acid moieties were synthesized using methods described previously with some modifications (Getman et al., 1993) (Scheme 1). In brief, a commercially available Boc-protected amino acid derivative **1**, which fits the desired structural feature of the P1 residue, was reduced to α-aminoaldehyde **2** via the Weinreb amide and then transferred to the corresponding epoxide **4** through alkene **3**. The epoxide was ring-opened by treatment with benzylamine or cyclohexylmethylamine under heated reflux to give the amino alcohols **7** and **8**. These intermediates were condensed with isocyanates **9a** and **9b** (in turn obtained from α-amino methyl esters and phosgene) at room temperature to provide the (hydroxyethyl)urea P1-P2′ isosteres **10a**, **10b**, and **11**. C-Terminal incorporation of P3′ residues was accomplished by hydrolysis of the methyl ester with LiOH and subsequent coupling of P3′ residues **14a**∼**g** with the HATU coupling reagent in DMF to yield the desired (hydroxyethyl)urea isosteres (see Table 1 as Jia compounds). In this synthesis scheme, the epoxide was treated with a variety of alkylamines that came up with P1-P1′ isosteres in which the P1′ site was modified with nitrogen at the backbone and an unnatural cyclohexyl side chain that is not seen in essential amino acids. Moreover, the selected amines and unnatural amino acid derivatives **14a**∼**g** were incorporated to the P1-P2′ fragments to yield entire P1-P3′ (hydroxyethyl)ureas. Through this synthesis scheme, a collection of (hydroxyethyl)urea peptidomimetics that contained unnatural amino acid moieties mainly distributed either at the P1′ and/or P3′ positions was generated. The characterization and the purity of each compound were confirmed by ^{1}H NMR spectros-

copy, high-resolution mass spectroscopy, and high-pressure liquid chromatography.

Cell Culture and Cell Lines. Human embryonic kidney (HEK)293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 0.1 mg/ml penicillin and streptomycin. T-REx293 cells were purchased from Invitrogen and cultured in DMEM supplemented with 10% FBS and 5 μg/ml blasticidin. The generation of stably transfected cell lines, T16, T20, N7, and γ-30 has been described previously (Kimberly et al., 2003; Liao et al., 2004; Bakshi et al., 2005). Cells of the SH-SY5Y human neuroblastoma cell line were grown in DMEM/Ham's F-12 supplemented with 10% FBS. Cells were incubated in a humidified incubator at 37°C in 5% CO_2.

Cell-Based γ-Secretase Assays. The generation of these stable cell lines has been reported previously (Liao et al., 2004). To examine the inhibitory effects of the compounds specifically on γ-secretase, T16 or T20 cells were trypsinized and washed with serum-free DMEM before plating onto 12-well microplates in 1 ml/well DMEM supplemented with 10% FBS at 5×10^5 cells/well. After incubation at 37°C overnight, cells were treated with 10 μM concentrations of individual (hydroxyethyl)urea peptidomimetics in culture medium containing 1% DMSO and 1 μg/ml tetracycline, the inducer of APP695-Gal4/VP16 and the 99-residue C-terminal fragment of APP-Gal4/VP16 (C99-GV) expressions, and they were incubated at 37°C for 24 h or various intervals as specified. For the dose-response studies, various concentrations of (hydroxyethyl)urea peptidomimetics were included in the medium as specified. Cells incubated with the culture medium containing 1 μg/ml tetracycline and 1% DMSO were used to define the basal level of γ-secretase activity, whereas cells treated with DMSO-containing medium without tetracycline were used to estimate the nonspecific background emission of the luciferase signal. To terminate the reactions, cells were harvested with PBS containing 20 mM EDTA and lysed in 100 μl of 1× passive lysis buffer (PLB; Promega). Cell debris was removed by centrifugation at 13,200*g* for 5 min, and luciferase activity in clarified lysates was determined by mixing 20 μl of lysates and 20 μl of Steady-Glo luciferase assay reagent in a 96-well LumiNunc microplate (Nalge Nunc, International, Rochester, NY). After incubation at room temperature for 5 min, emitted luminescence in individual microwells was determined by an MLX Microplate luminometer (Dynex Technologies, Chantilly, VA) and subsequently normalized by the protein content of the lysates. The protein content of clarified lysates was determined using the BCA protein assay kit (Pierce Chemical) following the manufacturer's instructions. The normalized luciferase signal emitted by T20 cells in regular culture medium without tetracycline was referred as 1-fold of activation. Known γ-secretase inhibitors, such as compound E and DAPT (Seiffert et al., 2000; Dovey et al., 2001), were included as positive controls of γ-secretase inhibition.

To examine compound inhibition of γ-secretase-dependent S3 cleavage of Notch, we generated a HEK293-derived stable line (N7) that was constitutively expressing NΔE (Liao et al., 2004). The NΔE



Scheme 1. Reagents and conditions: i, EDC, HOBt, HNMe(OMe) · HCl, then LAH. ii, $MePPh_3Br$, 35% KH, HMDS. iii, *m*-CPBA. iv, isopropanol. v, room temperature, overnight. vi, LiOH. vii, HATU, DIEA.

was a Notch mutant protein lacking its extracellular domain but retaining its membrane-spanning region. The recombinant NΔE was thus expressed as a membrane-tethered protein that can be cleaved directly by γ-secretase independently of its ligand activation and that served as an alternative substrate for the measurement of γ-secretase activity (Kopan et al., 1996). N7 cells were plated onto 12-well microplates in 1 ml/well DMEM supplemented with 10% FBS at 5×10^5 cells/well. After incubation at 37°C overnight, cells were treated with compounds at 10 μM as described above and incubated at 37°C for 24 h. Treated cells were harvested using PBS containing 20 mM EDTA and dissolved in 50 μl of 1× PLB, followed by centrifugation at 13,200*g* for 5 min to remove cell debris. The protein concentrations of clarified supernatants were determined using a BCA protein assay reagent kit, and cell extracts containing equivalent amounts of proteins were resolved by SDS-PAGE and analyzed by Western blotting using an anti-Notch(Val1744) polyclonal antibody as described below.

Cell Viability Assay. T20 cells (5×10^4/100 μl/well) were seeded onto the wells of 96-well microplates in culture medium containing respective compounds at 10 μM and incubated at 37°C for 24 h. Viable cells were determined using the CellTiter 96 AQueous nonradioactive cell proliferation assay (Promega) as specified by the manufacturer's instructions. In brief, after the addition of the combined 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt/phenazine methosulfate solution (20 μl/well), microplates were incubated for 3 h at 37°C. The conversion of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt into formazan in viable cells was quantitated by the absorbance at 490 nm using a Synergy HT ELISA plate reader (Bio-Tek Instruments, Winooski, VT). The number of living cells in culture was directly proportional to the absorbance at 490 nm. Viable cells in culture medium containing vehicle alone (1% DMSO, control) were referred to as 100% viability. The background absorbance shown at 0 cells/well was subtracted from these data.

β-Amyloid ELISA. To determine the inhibitory effects of compounds on Aβ production, T20 or γ-30 cells (5×10^5 cells/well) were seeded onto 12-well tissue culture plates and treated with various amounts of compounds in serum-free DMEM with (for T20) or without (for γ-30) 1 μg/ml tetracycline, followed by incubation at 37°C for 24 h. Conditioned media were harvested, clarified by centrifugation, supplemented with the Complete protease inhibitor cocktail, and stored at −80°C until ready for the assay. Levels of secreted Aβ40 and Aβ42 in conditioned media were determined using quantitative human Aβ40 and Aβ42 sandwich ELISA kits (BioSource International) as described in the manufacturer's instructions. Contents of Aβ40 and Aβ42 in the conditioned media of T20 and γ-30 cells treated with regular culture medium alone were defined as the blank for the quantitation of Aβ production.

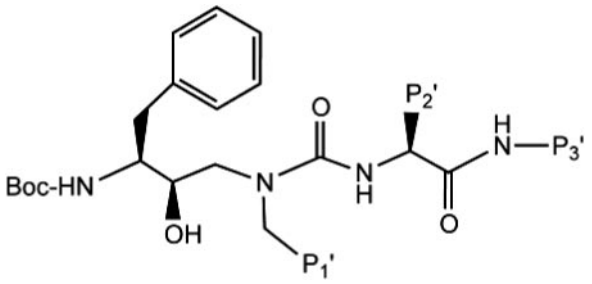
Compound-Induced Neuronal Differentiation of Neuroblastoma Cells. SH-SY5Y human neuroblastoma cells were seeded onto six-well microplates (5×10^5 cells/well) and incubated at 37°C overnight. Adherent SH-SY5Y cells were then treated with 10 μM respective Jia compounds or vehicle alone (0.1% DMSO) in DMEM/Ham's F-12 medium containing 10% FBS, followed by incubation at 37°C for 24 h or various intervals as specified. Compound-treated SH-SY5Y cells were harvested and lysed by PLB (Promega) containing the Complete protease inhibitor cocktail. Clarified lysates containing equivalent amounts of proteins were resolved by SDS-PAGE. The expression levels of calreticulin and GAP-43, two markers for the neuronal differentiation of neuroblastoma cells (Grynfeld et al., 2000; Hsu et al., 2005), were visualized by Western blotting using specific antibodies. The level of GAPDH was also determined as a protein load control.

To quantify neurite outgrowth of neuroblastoma cells induced by γ-secretase inhibitors, 50 or more SH-SY5Y cells whose dendritic trees were relatively isolated and did not have discontinuities in their dendritic trees were chosen for quantification of neurite length. Image for Windows was the morphometric program used to measure the neurite length.

TABLE 1

Structures of (hydroxyethyl)urea isosteres and their IC_{50} values for the inhibition of γ -secretase

IC_{50} values for luciferase were determined by the cell-based reporter gene assay for γ -secretase; those for Aβ40 were determined by the quantitative Aβ40 ELISA kit (BioSource International); and those for NICD were determined by the generation of NICD. DAPT and compound E, two previously identified γ-secretase inhibitors, were included as positive controls



Compound	P_1'	AA_1 (-HN-CH(P_2')-CO-)	-HN-P_3'	IC_{50}		
				Luciferase	Aβ40	NICD
					μM	
Jia040	Phenyl	Ala	(1*R*,2*S*)-AI	N.D.	N.D.	N.D.
Jia046	Phenyl	Leu	(L)-Phg-OMe	0.946	0.704	3.965
Jia047	Phenyl	Leu	(1*R*,2*R*,3*R*,5*S*)-IPCA	5.459	2.566	11.188
Jia097[a]	Phenyl	Leu	(L)-Val-OMe	N.D.	N.D.	N.D.
Jia101	Phenyl	Leu	(D)-Phg-OMe	3.159	1.651	2.018
Jia104	Phenyl	Leu	(*S*)-(−)-1-PEA	4.513	2.462	2.520
Jia105	Phenyl	Leu	(*R*)-(+)-1-PEA	3.149	2.485	4.117
Jia138	Cyclohexyl	Leu	(L)-Phg-OMe	0.172	0.074	0.235
Jia142	Cyclohexyl	Leu	(*R*)-(+)-1-PEA	0.991	0.875	1.073
Jia143	Cyclohexyl	Leu	(L)-Val-OMe	4.863	1.177	3.192
DAPT[b]				0.478	0.794	0.039
Compound E[b]				0.013	0.007	0.001

N.D., not determined; AI, 1-amino-2-indanol; IPCA, isopinocampheylamine; Phg-OMe, phenylglycine methyl ester; 1-PEA: 1-phenylethylamine

[a] Jia097 was the known inhibitor III-31-C.

SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analysis. Clarified cell extracts containing equivalent amounts of proteins were mixed with equal volumes of 2× SDS sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and 5% β-mercaptoethanol) and boiled at 100°C for 5 min. Denatured proteins were resolved in Tris-glycine polyacrylamide gels (10 or 12%). Separated proteins were transferred electrophoretically to polyvinylidene difluoride membranes (Immobilon-P; Millipore Corporation, Billerica, MA). Membranes were then treated with blocking buffer [5% nonfat dry milk and 0.1% Tween 20 in PBS (PBST)] at room temperature for 1 h, followed by a brief rinse with PBST. Blocked membranes were probed with appropriate dilutions of primary antibody in PBST at room temperature for 1 h. The unbound primary antibody was removed by extensive washes with PBST. Thereafter, washed membranes were incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibody in PBST at room temperature for 1 h. After extensive washes with PBST, antibody-reacted proteins were visualized by chemiluminescence using SuperSignal West Dura and Pico reagents (Pierce Chemical). The antibodies used and their dilutions were as follows: anti-Notch(Val1744), 1:1000; HRP-conjugated anti-mouse IgG (GE Healthcare), 1:10,000; and HRP-conjugated anti-rabbit IgG (Santa Cruz Biotechnology, Inc.), 1:1000.

Preparation and Detergent Solubilization of Microsome Membrane. Freshly harvested γ-30 cells that overexpressed all four γ-secretase components (Kimberly et al., 2003) were resuspended and homogenized in a HEPES buffer (50 mM HEPES, 5 mM $MgCl_2$, 5 mM $CaCl_2$, and 150 mM NaCl). After removal of cell debris and nuclei, the supernatant solution was centrifuged at 100,000*g* for 60 min. The microsome-enriched pellets were solubilized in a CHAPSO-containing buffer (50 mM HEPES, 5 mM $MgCl_2$, 5 mM $CaCl_2$, and 1% CHAPSO) at 4°C for 60 min. The ensuing supernatant solution was defined as solubilized γ-secretase.

Preparation of Jia138-Conjugated Affinity Resins and Affinity Chromatography of γ-Secretase. The Jia138-conjugated affinity resins were prepared by a previously described protocol (Esler et al., 2002). In brief, the methyl esters of Jia138 were hydrolyzed by using LiOH in aqueous dioxane. The carboxylic acids were coupled in DMSO by using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (3 equivalents) to the primary amine of a six-atom hydrophilic linker present on the agarose resin Affi-Gel 102 (Bio-Rad Laboratories, Hercules, CA). The conjugation mixtures were incubated at room temperature overnight with continuous gentle inversion, followed by extensive washes with DMSO. The affinity resins were subsequently maintained in aqueous buffer.

The solubilized γ-secretase was incubated with Jia138-conjugated resins for 2 h at room temperature with gentle rocking, and the resin was washed twice with a CHAPSO-containing buffer (50 mM HEPES, pH 7.0, 150 mM NaCl, and 1% CHAPSO). The bound proteins were then released by SDS-PAGE sample buffer and analyzed by SDS-PAGE and Western blotting as specified above.

Transfection of Small Interfering RNAs Targeting Notch1. The chemically synthesized siRNA duplex oligoribonucleotides against human Notch1 (siNotch1, accession no. NM_017617) and a nonspecific scrambled siRNA were purchased from Ambion (Austin, TX). The target sequences for siNotch1 were 5′-CGA CGC AUG CAU CAG CAA C-3′ (sense strand) and 5′-GUU GCU GAU GCA UGC GUC G-3′ (antisense strand). siRNA oligoribonucleotides (50 or 100 pmol) were transiently transfected into SH-SY5Y or N7 cells using the Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. After transfection with siRNAs in DMEM supplemented with 10% FBS at 37°C for 6 h, transfected cells were incubated with fresh culture medium at 37°C for an additional 24 h or as specified. Transfected cells were harvested by PBS containing 20 mM EDTA, and clarified lysates were prepared by using 1× PLB containing Complete protease inhibitor cocktail. Protein contents of cell lysates were determined with a BCA protein assay reagent kit. A nonspecific oligoribonucleotide that is not homologous to any known genes was used as a negative control to rule out nonspecific cellular events caused by the introduction of the siRNAs into cells. The levels of calreticulin and NICD in transfected cells were examined by Western blotting using anti-Notch1(Val1744) as described above. The efficiency of the Notch1-targeting RNAi knockdown was determined by visualizing the endogenous full-length Notch1 (~300 kDa) and NICD (~110 kDa) in SH-SY5Y and N7 cells, whereas the production of exogenous NΔE-derived NICD (~50 kDa) in N7 cells was not affected by RNAi.

Mass Spectrometry Analysis. Purified peptidomimetics DAPT and Jia142 were dissolved in 50% acetonitrile and 0.1% formic acid to a final concentration of 1 mM and incubated at various temperatures for 2 days. Treated compounds were subjected to one-dimensional LC-nano-ESI-MS/MS analysis performed on an integrated nano-LC-MS/MS system (Micromass, a division of Waters, Bedford, MA) composed of a three-pumping Micromass/Waters CapLC system with an autosampler, a stream select module configured for precolumn plus analytical capillary column, and a Micromass Q-Tof Ultima API mass spectrometer fitted with nano-LC sprayer, operated under MassLynx 4.0 control. Injected samples were first trapped and desalted isocratically on an LC-Packings PepMap C18 μ-Precolumn Cartridge (5 μm, 300 μm i.d. × 5 mm; Dionex, Sunnyvale, CA) for 2 min with 0.1% formic acid delivered by the auxillary pump at 30 μl/min after which the peptides were eluted off from the precolumn and separated on an analytical C18 capillary column (15 cm × 75 μm i.d., packed with 5 μm, Zorbax 300 SB C18 particles; Micro-Tech Scientific, Vista, CA) connected inline to the mass spectrometer, at 300 nl/min using a 40-min fast gradient of 5 to 80% acetonitrile in 0.1% formic acid. Online nano-ESI-MS survey scan was fully automated and synchronized with the nano-LC runs under the full software control of MassLynx 4.0. Before online analysis, the nano-LC sprayer and Z-spray source parameters were tuned and optimized with glufibrinopeptide B.

Results

Synthesis of (Hydroxyethyl)urea Peptidomimetics Containing Unnatural Amino Acids. The incorporation of transition-state mimicking moieties into the substrate-based substructure has recently been used to develop aspartyl protease inhibitors (e.g., HIV protease inhibitors; Huff, 1991). This approach using substrate-based modifications has also been successfully applied to the generation of potent and selective γ-secretase inhibitors and is efficient for probing the active site of this aspartyl protease. Previous studies showed that the hydroxyethylene peptidomimetic L-685,458 and the (hydroxyethyl)urea III-31-C can both inhibit Aβ production with IC_{50} values of approximately 10 nM in the cell-free assays and 200 nM in cell-based systems (Shearman et al., 2000; Kornilova et al., 2003). It is intriguing that the P1-P1′ residues of both inhibitors consisted of Phe-Phe isosteres, whereas APP substrate-based hydroxyethylene analogs with Ala-Thr and Val-Ile isosteres showed no inhibitory effects on the γ-secretase-mediated formation of Aβ42 (Nadin et al., 2003). These results suggested that inhibitors such as L-685,458 and III-31-C were functioning as direct transition state analogs of the APP γ-cleavage site and that the Phe-Phe isosteres at positions P1-P1′ are critical for their binding and inhibition of γ-secretase. We thus sought to determine whether (hydroxyethyl)ureas isosteres with modifications especially at positions P1′-P3′ could allow us to delineate the structure-activity relationships of these (hydroxyethyl)ureas to the binding pocket of the γ-secretase active site.

To incorporate unnatural amino acids into newly synthesized (hydroxyethyl)urea peptidomimetics, we introduced a cyclohexyl residue into the P1′ position. The treatment of

epoxide **4** with a variety of alkylamines altered the P1-P1′ isosteres in which the P1′ site was modified with nitrogen at the backbone and unnatural side chains that are not seen in essential amino acids (Scheme 1). Alternatively, the selected amines and unnatural phenylglycine analogs were included in the P3′ position of certain (hydroxyethyl)ureas. Therefore, two series of (hydroxyethyl)urea peptidomimetics were generated. One series of (hydroxyethyl)urea isosteres, including Jia040, Jia046, Jia047, Jia097, and Jia101, carried a phenyl group at their P1′ site, whereas the other series, including Jia138 and Jia143, carried a cyclohexyl group. Subsequently, more lipophilic analogs were prepared by the introduction of decarboxylated phenylglycine residues [e.g., (+)- and (−)-1-phenylethylamines] into the P3′ position (Jia 104, Jia105, and Jia142) to optimize the relative activities and probe the stereochemical diversity of the binding sites. The effects of these synthesized compounds after purification on the inhibition of γ-secretase activity were examined using the specific cell-based γ-secretase assays as described.

Inhibition of γ-Secretase-Mediated Cleavage of APP by (Hydroxyethyl)urea Peptidomimetics Containing Unnatural Amino Acid Moieties. To determine the effects of (hydroxyethyl)urea peptidomimetics containing unnatural amino acid moieties on the inhibition of γ-secretase-mediated cleavage of APP, two highly efficient and quantitative cell-based assays, one assay specifically measuring the β/γ-cleavages of APP (AP-GL-T16) as a whole and the other assay the γ-cleavage of C99 (C99-GL-T20) alone, were used. The generation and validation of both cell-based assays were described previously (Liao et al., 2004; Bakshi et al., 2005). The effects of a series of (hydroxyethyl)urea pipeptidomimetics containing unnatural amino acid moieties (Jia compounds) are shown in Fig. 1. These Jia compounds were structurally related to two well characterized nontransition state analog inhibitors of γ-secretase, compound E and DAPT (Seiffert et al., 2000; Dovey et al., 2001). Initial screening of these Jia compounds showed that compounds Jia046, Jia047, Jia101, Jia104, and Jia105 at 10 μM exhibited potent inhibition of both APP proteolysis and γ-secretase, comparable with the levels displayed by compound E and DAPT (Fig. 1, A and B). The comparable potency of these effective Jia compounds to those of compound E and DAPT suggested that these Jia compounds could directly target γ-secretase. Most of these Jia compounds, with only the exception of Jia105, presented no significant cellular toxicity (Fig. 1C). All five effective Jia compounds also inhibited γ-secretase activity in dose-dependent manners (Fig. 2A). The production of secreted Aβ40 in conditioned media of compound-treated cells was consistently reduced in a similar manner (Fig. 2B). The approximate IC_{50} values of active Jia compounds obtained from our cell-based γ-secretase assay and quantitative Aβ40 ELISA are listed in Table 1. Our data suggested that these active (hydroxyethyl)urea isosteres containing unnatural amino acid moieties could target the γ-secretase-mediated cleavage of APP and effectively inhibit Aβ production. These inhibitory effects were not due to nonspecific inhibition of luciferase as demonstrated by the relatively unaffected luciferase signal upon treatments with respective Jia compound in a control cell line constitutively expressing the luciferase reporter gene (data not shown), substantiating the observed inhibition of γ-secretase activity by these active Jia compounds.

Three additional unnatural amino acid-containing derivatives of (hydroxyethyl)urea isosteres (Jia138, Jia142, and Jia143), whose P1′ sites were occupied by a cyclohexyl group instead of a phenyl group as in previous Jia compounds, were subsequently generated and analyzed for their inhibition of γ-secretase. We found that the replacement of a phenyl group at the P1′ position in Jia046 with a cyclohexyl group in Jia138 further improved the efficacy of the inhibition of γ-secretase in a dose-dependent manner (Fig. 3; Table 1). Nevertheless, the similar replacement in Jia097 and Jia105 with a cyclohexyl group in Jia143 and Jia142, respectively, also increased their inhibitory potencies. Our data suggested that the marginally bulky moiety occupying the P1′ site of these (hydroxyethyl)urea isosteres plays a critical role in determining the efficacy of γ-secretase inhibition by these compounds.

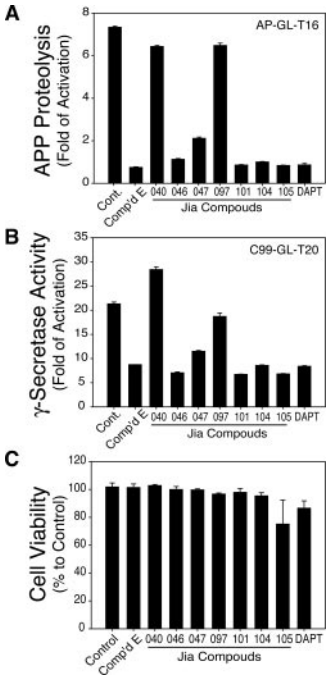


Fig. 1. Screening of (hydroxyethyl)urea peptidomimetics containing unnatural amino acids (Jia compounds) on the inhibition of γ-secretase activity. A, effects of Jia compounds on the secretase-mediated proteolysis of APP using AP-GL-T16 (T16) cells that were stably cotransfected with APP-GV and Gal4-driven luciferase reporter gene (Gal4-Luc). B, effects of Jia compounds on the γ-cleavage of APP using C99-GL-T20 (T20) cells that were stably cotransfected with the membrane-tethered C99 C-terminally tagged with C99-GV and Gal4-Luc. Cells (5×10^5 cells/well in 12-well microplates) were treated with DMEM containing 10% FBS and 1 μg/ml tetracycline in the presence or absence of individual (hydroxyethyl)urea isosteres (10 μM Jia compounds) at 37°C for 24 h. Cells in the control experiments were treated with vehicle alone (1% DMSO). Treatments with compound E and DAPT, two potent γ-secretase inhibitors, were included as positive controls. γ-Secretase activity in clarified lysates was determined as described under *Materials and Methods*. Background luminescence emitted by T16 or T20 cells treated with culture medium in the absence of tetracycline is referred to as 1-fold of activation. C, viability of host cells in the presence of Jia compounds. T20 cells (5×10^4/100 μl/well) were seeded onto the wells of 96-well microplates in culture medium containing 10 μM respective compounds or 1% DMSO alone (Control), and they were incubated at 37°C for 24 h. Viable cells were determined by the CellTiter 96 AQueous nonradioactive cell proliferation assay kit as described by the manufacturer. Viable cells in culture medium without compounds (Control) are referred to as 100% viability. Two known γ-secretase inhibitors, compound E and DAPT, were included in these experiments for comparison. Results from a representative experiment are expressed as the mean ± S.D. of triplicate measurements.

It is noteworthy that four active Jia compounds (Jia047, Jia101, Jia104, and Jia105) at lower concentrations (1 μM) induced an approximate 3-fold rise in the level of Aβ42 production (Fig. 4), but they inhibited Aβ42 generation at 10 μM, whereas there was no significant elevation in Aβ40 production by any of the active Jia compounds at the concentrations examined (Fig. 2). Similar effects of active Jia compounds were seen when either T20 cells overexpressing C99 or γ-30 cells stably transfected with APPswe, PS1, Aph-1, and Pen-2 were treated with these compounds. The elevation of Aβ42 at subinhibitory concentrations was previously reported for a set of difluoro ketone peptidomimetic γ-secretase inhibitors (Wolfe et al., 1999a). How these active Jia compounds can differentially affect γ-secretase-dependent production of Aβ40 and Aβ42 remains to be defined.

γ-Secretase-Mediated S3 Cleavage of Notch Is Blocked by Effective Jia Compounds. We next examined whether these γ-secretase-inhibiting Jia compounds also affect the γ-secretase-mediated S3 cleavage of Notch. To do so, a stable cell line (N7) constitutively expressing NΔE that acted as the direct substrate of γ-secretase-catalyzed proteolysis independent of ligand activation was treated with various amounts of respective Jia compounds. The efficiency of the N7 stable cell line as an alternative cell-based γ-secretase assay was described previously (Liao et al., 2004). By using Western blotting analyses with the anti-Notch(Val1744) antibody, these effective Jia compounds (Jia046, Jia047, Jia101, Jia104, Jia105, Jia138, Jia142, and Jia143) were shown to significantly block γ-secretase-mediated S3 cleavage of NΔE in N7 cells, resulting in complete ablation of NICD production (Fig. 5A). We found that these effective Jia compounds consistently exhibited dose-dependent inhibition of γ-secretase-catalyzed S3 cleavage of NΔE (Fig. 5B). Our findings demonstrated that the efficacies of these active Jia compounds as shown by their IC_{50} values for blocking the γ-secretase-mediated S3 cleavage of NΔE were comparable with those for the γ-cleavage of APP (Table 1). The present data suggested that these novel (hydroxyethyl)urea isosteres containing unnatural amino acid moieties can target γ-secretase independent of substrate selection.

In Vitro Stability of (Hydroxyethyl)urea Peptidomimetics Is Dramatically Enhanced by the Replacement of Phenylethylamine at P3′ Site. To examine whether the unnatural amino acid substitution in Jia compounds can prolong the half-life of these peptidomimetics in culture con-

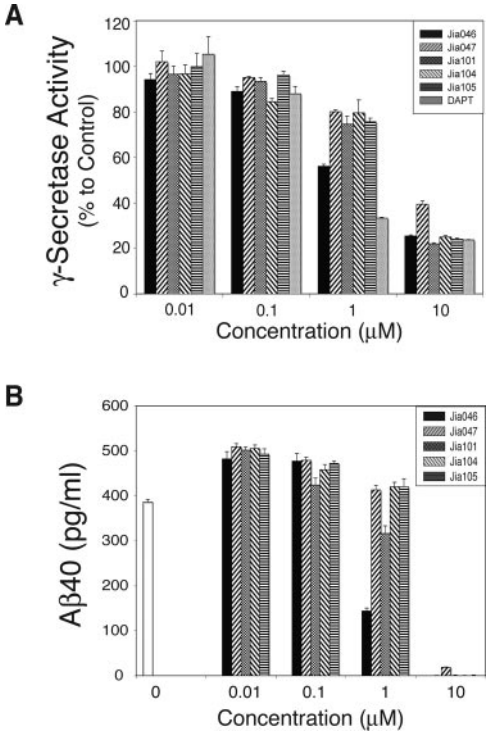


Fig. 2. Dose response of Jia compounds on the inhibition of γ-secretase activity and Aβ production. T20 cells (5×10^4 cells/well) in 96-well microplates were treated with various concentrations of respective Jia compounds in DMEM containing 10% FBS and 1 μg/ml tetracycline for 24 h at 37°C. Clarified cell lysates and conditioned media were subjected to the luciferase reporter gene assay for γ-secretase (A) and the quantitation of secreted Aβ40 (B), respectively. Basal levels of γ-secretase activity and Aβ40 production in the presence of vehicle alone (1% DMSO; Control) were determined. Treatments of DAPT were included for the comparison of inhibitory potency. Data are shown as the mean ± S.D. of triplicate measurements from a representative experiment.

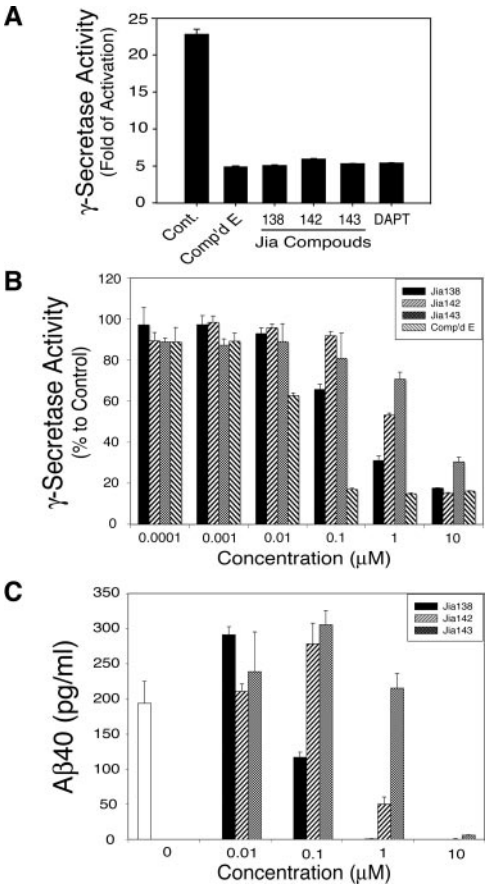


Fig. 3. Inhibition of γ-secretase by (hydroxyethyl)urea peptidomimetics containing a cyclohexylmethyl moiety at the P1′ site. A, T20 cells (5×10^4 cells/well) in 96-well microplates were treated with 10 μM respective Jia compounds (Jia138, Jia142, or Jia143) in DMEM containing 10% FBS and 1 μg/ml tetracycline for 24 h at 37°C. B, T20 cells were treated with various amounts of Jia138, Jia142, and Jia143 for 24 h at 37°C to determine the dose-dependent effects on the inhibition of γ-secretase. γ-Secretase activities in compound-treated cells were determined by the Steady-Glo luciferase reporter gene assay as specified by the manufacturer. C, secreted Aβ40 in the conditioned media of compound-treated cells was quantitated by an Aβ40 sandwich ELISA kit. Basal levels of γ-secretase activity and Aβ40 production in the presence of vehicle alone (1% DMSO; Control) were also determined. Treatments of DAPT were included for the comparison of inhibitory potency. Data are shown as the mean ± S.D. of triplicate measurements from a representative experiment.

dition, Jia compounds (Jia046, Jia097, Jia138, and Jia142) were added into culture media of T20 cells whose γ-secretase activity was then monitored daily for a span of 8 days. Whereas Jia046 and Jia138 exhibited an approximate half-life of 6 to 8 days that is comparable with the stability of DAPT and compound E, the potency of Jia142 can be fully sustained for at least 8 days (Fig. 6A). Using UV-spectrometry, we found that both Jia142 and DAPT have a characteristic absorbance peak at 244 nm (Fig. 6B). We reasoned that the peak absorbance at 244 nm (OD_{244}) could be a biophysical property of both compounds and that alteration in OD_{244} of these peptidomimetics could be indicative of their conformational integrity and chemical stability. Our data demonstrated that the incubations at room temperature for 4 and 24 h both result in a 25% reduction in the OD_{244} of Jia142, whereas the OD_{244} of DAPT significantly decreases 50 and 70% after incubations at room temperature for 4 and 24 h, respectively (Fig. 6C). Using mass spectrometry (MS) analyses, we further confirmed that Jia142 (637.4 Da) is thermally more stable than DAPT (433.2 Da). The MS spectra of untreated Jia142 (Jia142-Control) exhibited a corresponding peak intensity of 2.2×10^5 at 637.4 *m/z* that was diminished to 2.0×10^5 (Jia142–37°C) or 7.7×10^4 (Jia142–50°C) after incubation at 37°C or 50°C for 2 days, whereas those of untreated DAPT (DAPT-Control) displayed a corresponding peak intensity of 6×10^4 at 433.2 *m/z* that is reduced to 4×10^4 (DAPT-37°C) or 5000 (DAPT-50°C) after incubation at 37 or 50°C for 2 days (Fig. 6D). Our data revealed that the abundance of intact Jia142 after incubation at 37 or 50°C for 2 days is reduced to 86 or 33%, whereas only 66 or 8% of DAPT are retained after respective treatments (Fig. 6D, bottom). Together, our findings clearly demonstrated that the substitution of a cyclohexyl moiety at P1′ site and a phenylethylamine at P3′ site could dramatically enhance the in vitro stability of (hydroxyethyl)urea peptidomimetics.

The Affinity Precipitation of γ-Secretase by Jia138. To establish the mode of action of active Jia compounds in the inhibition of γ-secretase, we examined whether Jia138 can directly target to γ-secretase. Jia138 was covalently linked to the free primary amine of a six-atom hydrophilic spacer on an agarose affinity resin (Affi-Gel 102; Bio-Rad). Immobilized Jia138 was then used to precipitate PS1 and nicastrin from a CHAPSO-solubilized microsome preparation of γ-30 cells. Solubilized γ-secretase complexes were incubated with the Jia138-conjugated affinity resin in batch format for 2 h at room temperature. Protein-bound resins were washed with a CHAPSO-containing buffer, and affinity-precipitated pro-

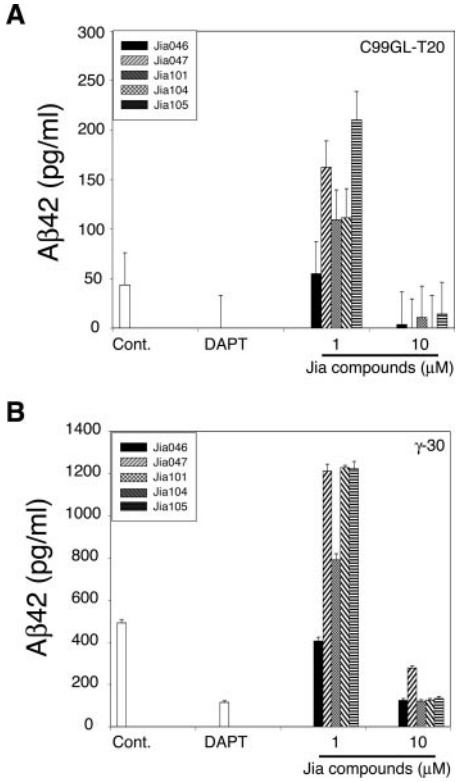


Fig. 4. Effects of active (hydroxyethyl)urea peptidomimetics on Aβ42 production. Conditioned media of T20 (A) or γ-30 (B) cells that were treated with active Jia compounds for 24 h at 37°C were harvested, and secreted Aβ42 was determined by an Aβ42 sandwich ELISA kit. Basal levels of Aβ42 production in the presence of vehicle alone (1% DMSO; Cont.) were also determined. Treatments of DAPT were performed to show the complete inhibition of Aβ42 production in these cells. Data are shown as the mean ± S.D. of triplicate measurements from a representative experiment.

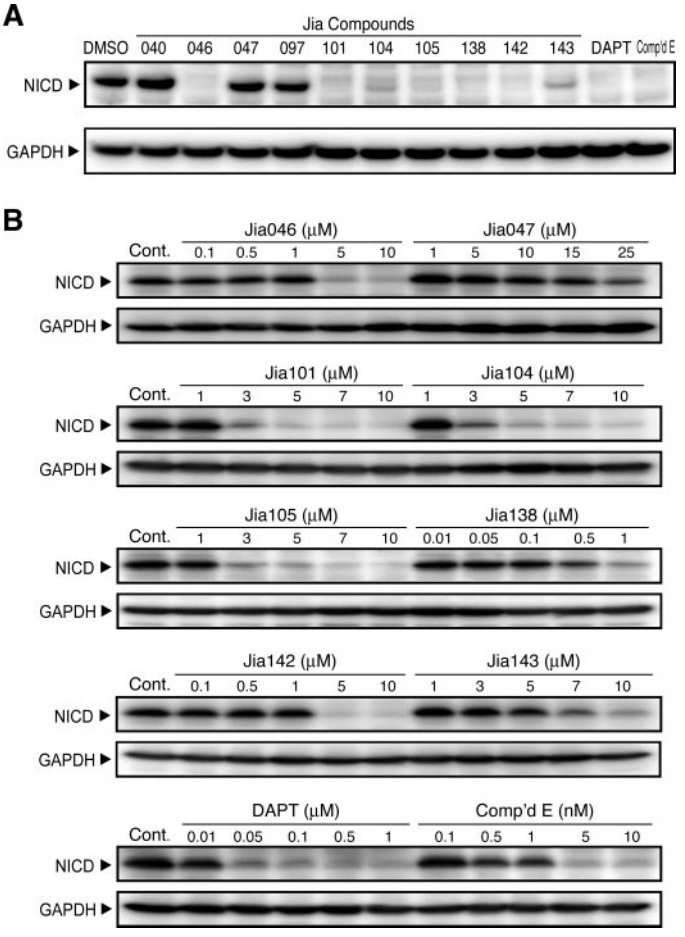


Fig. 5. Inhibition of γ-secretase-mediated S3 cleavage of Notch by the novel (hydroxyethyl)urea peptidomimetics of Jia compounds. Screening of Jia compounds (A) and dose-dependent response of Jia compounds (B) for the inhibition of γ-secretase-mediated S3 cleavage of Notch. HEK293 cells stably transfected with NΔE (N7) in 12-well microplates (5×10^5 cells/well) were treated with 10 μM (A) or various concentrations (B) of individual compounds in DMEM containing 10% FBS for 24 h at 37°C. Clarified lysates of treated cells containing equivalent amounts of proteins were resolved by SDS-PAGE and analyzed by Western blotting using anti-Notch(Val1744). Vehicle alone (1% DMSO) was included as control. Treatment with DAPT was included for comparison.

teins were analyzed by SDS-PAGE and Western blotting. We found that PS1 and nicastrin can be effectively retained on the Jia138-immobilized matrix, but not on an unconjugated control matrix (Fig. 7A). Proteins isolated by unconjugated or Jia138-conjugated resin visualized by silver staining consistently revealed that only Jia138-conjugated resin can pull down γ-secretase components (Fig. 7B). Using various amounts of solubilized proteins, we further demonstrated that γ-secretase can bind to Jia138 resin in a dose-dependent manner (Fig. 7C). These results clearly suggested that Jia138, and probably other active Jia compounds as well, can exert a specific inhibitor-active site interaction that is required for the binding of PS1 and nicastrin. Our findings also confirmed that the formation of heterodimeric PS1 (PS1-NTF and PS1-CTF) is prerequisite for the constitution of functional γ-secretase.

γ-Secretase-Blocking (Hydroxyethyl)urea Peptidomimetics Promote Neuronal Differentiation of Neuroblastoma Cells. We next sought to determine whether these γ-secretase-inhibitory Jia compounds can promote neuronal differentiation of neuroblastomas through the blocking of Notch activation that is required for maintaining neuroblastoma cells in an undifferentiated state. Recent evidence has suggested that the inhibition of Notch signaling leads to maturation of neuroblastomas, whereas constitutively active Notch signaling can impede the induction of differentiation and neurite formation of neuroblastoma cells (Pahlman et al., 2004). We thus reasoned that these effective Jia compounds can block γ-secretase-dependent Notch signaling and promote neuronal differentiation of neuroblastoma cells. The possible enhancement of neuroblastoma differentiation through the inhibition of γ-secretase was first explored by treating a human neuroblastoma cell line (SH-SY5Y) with various γ-secretase inhibitors. We found that, in 24 h, all seven active Jia compounds examined (Jia046, Jia101, Jia104, Jia105, Jia138, Jia142, and Jia143) could significantly induce the expression of GAP-43 and calreticulin, two differentiation markers of neuroblastoma cells, in SH-SY5Y cells, although to varying degrees (Fig. 8). GAP-43 is the most abundant neuron-specific protein in the growth cones

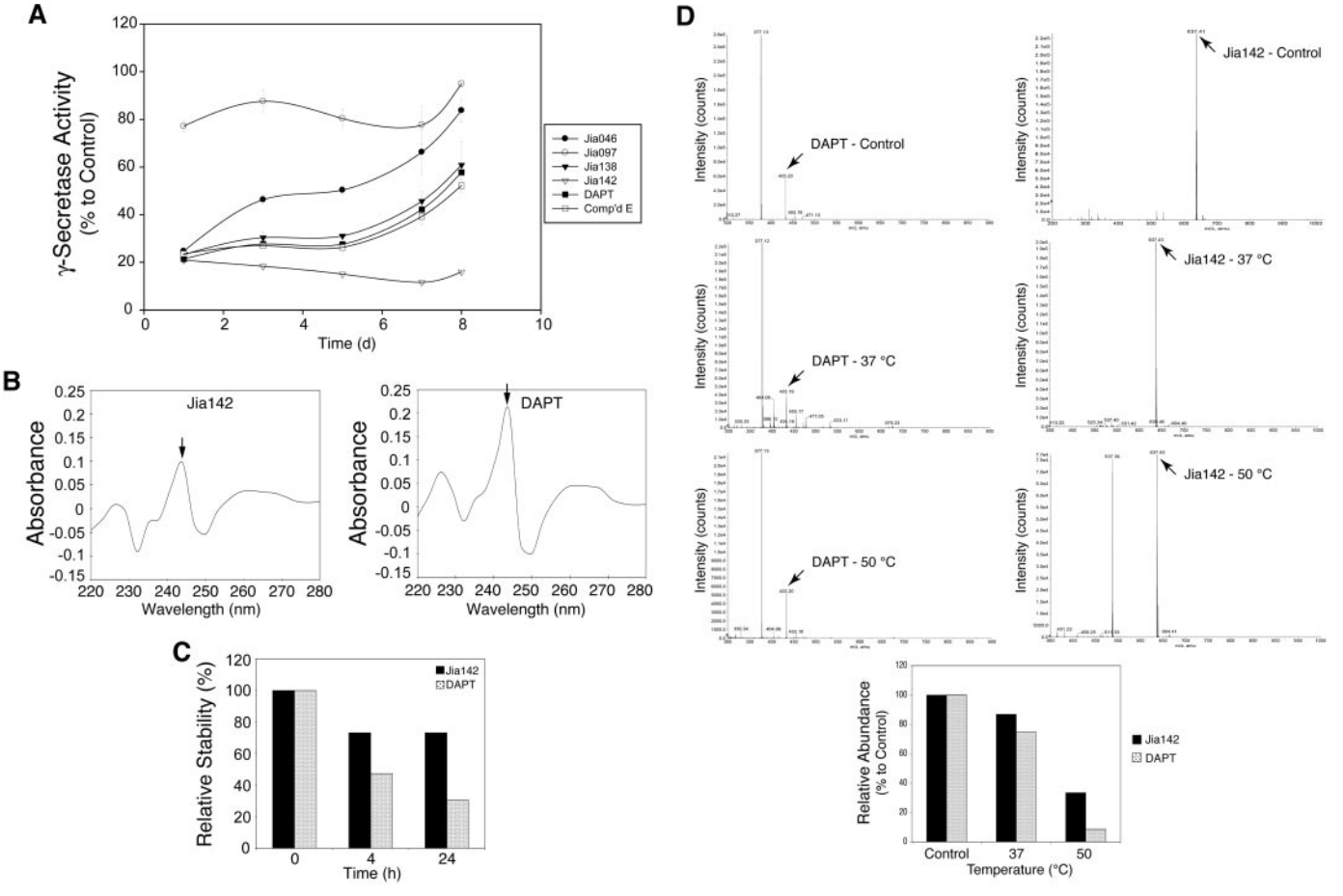


Fig. 6. Stability of unnatural amino acid-substituted (hydroxyethyl)urea peptidomimetics. A, after addition of 10 μM respective Jia compounds (Jia046, Jia097, Jia138, and Jia142) into culture media, γ-secretase activity of treated T20 cells was monitored daily for 8 days. γ-Secretase activity of vehicle-treated (1% DMSO) cells was determined and referred to as 100%. DAPT and compound E were also included for comparison. Data are shown as the mean ± S.D. of triplicate measurements from a representative experiment. B and C, Jia142 and DAPT (1 mM in DMSO) were incubated at room temperature, and UV absorbance spectra of both compounds were obtained at 0, 4, and 24 h. The representative spectra of Jia142 and DAPT at 0 h are shown, and a characteristic peak of absorbance at 244 nm (OD_{244}, arrow) was identified for both compounds (B). The gradual decrease of OD_{244} at various intervals was shown to demonstrate the chemical stability of newly synthesized (hydroxyethyl)urea peptidomimetics at room temperature. The OD_{244} of Jia142 (solid bar) and DAPT (shaded bar) measured at 0 h is referred to as 100% relative stability (C). D, Jia142 and DAPT (1 mM in 50% acetonitrile/0.1% formic acid) were incubated at 37 or 50°C for 2 days and subjected to one-dimensional LC-nano-ESI-MS/MS analysis. The corresponding masses (*m/z*) of DAPT (433.20 Da) and Jia142 (637.42 Da) were identified in the MS spectra (arrow). The intensity of untreated DAPT (DAPT-Control, shaded bar) and Jia142 (Jia142-Control, solid bar) is referred to as 100% relative thermal stability (bottom).

and has been shown to be critical for neuronal differentiation (Mani et al., 2001; Singh et al., 2003). Calreticulin is essential for neurite formation during neuronal differentiation, and its expression is tightly associated with reduced malignancy of neuroblastoma (Hsu et al., 2005). The compound-induced neurite outgrowth of SH-SY5Y cells was readily observed after 72 h of treatment (data not shown) and became prominent after 5 days (Fig. 9A). Quantitative analyses showed that the average neuritic length of compound-treated SH-SY5Y cells is significantly increased, ranging from a 100 to 200% increase (Fig. 9B). To validate the direct link of γ-secretase-mediated cleavage of Notch to the phenotypic changes of neuroblastoma cells, we used an RNAi approach to define whether specific down-regulation of endogenous Notch signaling is sufficient to drive increased expression of neuronal differentiation markers in neuroblastoma cells. The expression of calreticulin in SH-SY5Y cells that were partially depleted of Notch1 by RNAi was consistently significantly increased, but it was unchanged or slightly reduced in those that were transfected with a Notch1-targeting siRNA in conjunction with the expression of a constitutively active NΔE (Fig. 10A). This Notch1-specific siRNA targeted the exon 7 of endogenous human Notch1 gene that encodes part of its extracellular domain and efficiently down-regulated the expression of endogenous Notch1 and its intracellular domain, evidenced by the decreased levels of endogenous full-length Notch1 and NICD (Fig. 10B, top and top middle). The expression of the exogenous NΔE that encoded only the transmembrane and intracellular domains of mouse Notch1 (Kopan et al., 1996) was not affected by the transfection of

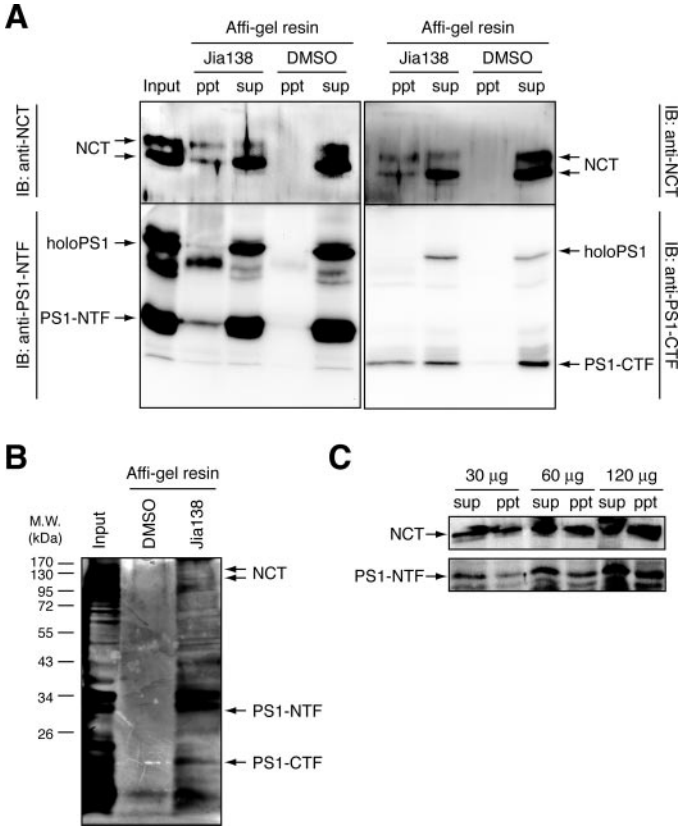


Fig. 7. Affinity precipitation of γ-secretase by Jia138. A, microsome preparation of γ-30 cells was solubilized by a CHAPSO-containing buffer (50 mM HEPES, pH 7.0, 150 mM NaCl, and 1% CHAPSO). Solubilized γ-secretase complexes were incubated with Jia138-conjugated (Jia138) or unconjugated (DMSO) resin for 2 h at room temperature with gentle rocking, and the resin was washed twice with the CHAPSO-containing buffer. The bound proteins were then released by SDS-PAGE sample buffer and analyzed by SDS-PAGE and Western blotting with anti-NCT, and anti-PS1 (PS1-NTF and PS1-CTF). Each boxed area of the duplicate blots was then visualized by anti-NCT, anti-PS1-NTF, and anti-PS1-CTF, respectively. Arrow denotes the antibody-reactive proteins corresponding to NCT, PS1-NTF, or PS1-CTF. Input, total solubilized proteins; ppt, bound proteins; sup, unbound proteins. B, proteins eluted from Jia138 resin or unconjugated (DMSO) resin were resolved by 12% Tris-glycine SDS-PAGE and visualized by silver nitrate. Total solubilized proteins (Input) were also included for comparison. Bands corresponding to NCT (110 and 150 kDa), PS1-NTF (30 kDa), and PS1-CTF (20 kDa) are indicated by arrows. C, various amounts of solubilized γ-secretase complexes (30, 60, and 120 μg) were subject to the precipitation by Jia138 resin. NCT and PS1-NTF that were immobilized onto to the resin (ppt) and those that were remained in the supernatant (sup) were assessed.

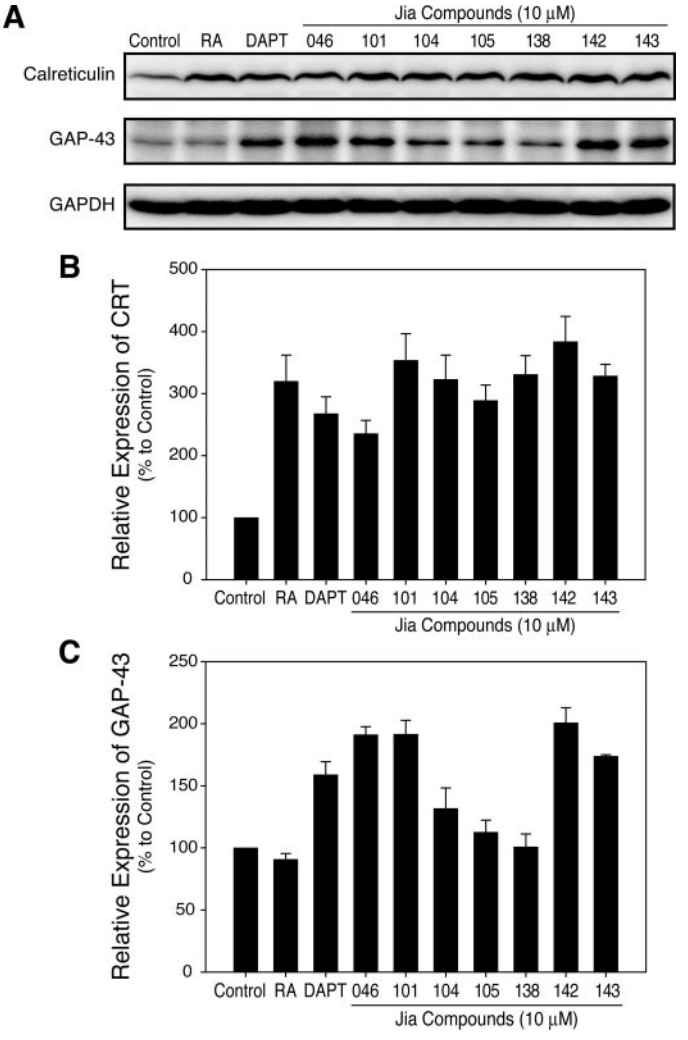


Fig. 8. Effects of active (hydroxyethyl)urea peptidomimetics on the neuronal differentiation of neuroblastoma cells. A, human neuroblastoma SH-SY5Y cells were treated with 10 μM active Jia compounds at 37°C for 24 h. Clarified cell lysates were resolved by SDS-PAGE and analyzed by Western blotting using anti-calreticulin or anti-GAP-43 for the evaluation of induced neuronal differentiation of neuroblastoma cells. GAPDH was visualized to serve as the load control. The levels of calreticulin, GAP-43, and GAPDH were determined by densitometry. The relative expression levels of calreticulin and GAP-43 normalized by those of GAPDH were shown to demonstrate the neuronal differentiation of neuroblastoma cells in the presence of active Jia compounds (B and C). Lysates generated from cells that were treated with 1 μM retinoic acid (RA), 10 μM DAPT, or vehicle alone (0.1% DMSO; Control) were also included for comparison. Data are shown as the mean ± S.D. of triplicate measurements from a representative experiment.

Notch1 siRNA (Fig. 10B, bottom middle). Thus, even the endogenous Notch signaling was significantly down-regulated by this Notch1-siRNA, the expression of exogenous NΔE can still render continuous activation of Notch signaling in transfected SH-SY5Y cells. These results demonstrated that Jia compound-induced neuronal differentiation of neuroblastoma cells was intimately associated with the inhibition of Notch signaling and that the expression of an exogenous NΔE can rescue the phenotype of undifferentiated neuroblastoma cells. These data provided direct evidence strongly suggesting that the attenuation of Notch signaling by γ-secretase inhibitors could effectively initiate the neuronal differentiation of neuroblastoma cells. Together, our findings not only identified a novel class of (hydroxyethyl)urea isosteres that contain unnatural amino acid moieties and exhibit potent inhibition of γ-secretase activity but also provided the direct evidence that the inhibition of Notch signaling by γ-secretase inhibitors is sufficient to induce the differentiation of neuroblastomas.

Discussion

Aβ, the principal constituent of senile plaques in the AD brain, is derived by sequential proteolyses mediated by β- and γ-secretases. Thus, these proteases have been regarded as the prime targets for the development of therapeutics against AD. The present study provides evidence that (hydroxyethyl)urea isosteres containing unnatural amino acid moieties retain their potency toward the inhibition of γ-secretase. This inhibition is verified by using cell lines that overexpress APP695, C99, or NΔE as substrates of γ-secretase-mediated proteolysis. Although the active Jia compounds might not possess as strong a potency as DAPT and compound E do in terms of IC_{50} values, they exhibit comparable levels of potency at submicromolar concentrations to other reported active (hydroxyethyl)urea peptidomimetics in cell-based systems (Bakshi and Wolfe, 2004; Esler et al., 2004). Furthermore, the active (hydroxyethyl)urea peptidomimetics reported here can efficiently promote the neuronal differentiation of neuroblastoma cells, probably through the inhibition of Notch signaling. These findings substantiate the notion that the incorporation of unnatural amino acid moieties, such as phenylethylamine (e.g., Jia142), could stabilize synthetic (hydroxyethyl)urea peptidomimetics without compromising their biological effects and their mode of action. Our results also suggest that the integration of unnatural peptidomimetics can be a valid methodology for further drug development.

Active Jia compounds are selected for their abilities to inhibit γ-secretase-mediated processing of APP695-GV or C99-GV. We have shown that most of the (hydroxyethyl)ureas with a leucyl residue at the P2′ position (Jia046, Jia047, Jia101, Jia104, and Jia105) display excellent potencies for the inhibition of γ-secretase-dependent processing of APP695 and C99, as demonstrated by the reduction in γ-secretase

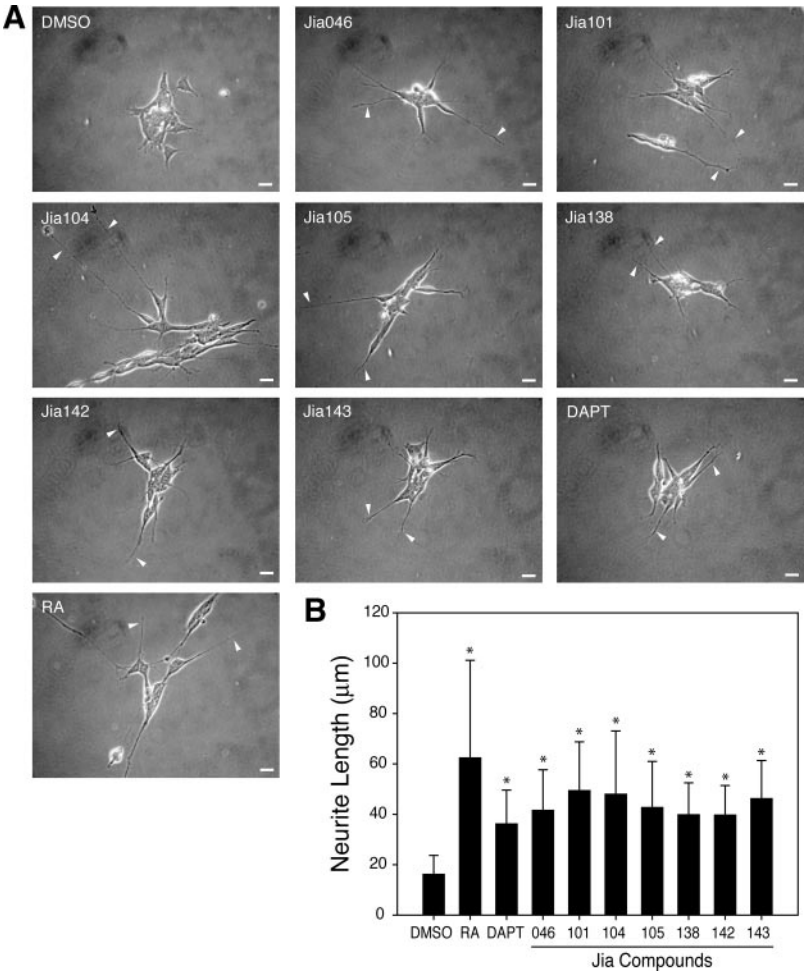


Fig. 9. Neuronal phenotypic changes of neuroblastoma cells induced by active (hydroxyethyl)urea peptidomimetics. A, human neuroblastoma SH-SY5Y cells that were treated with vehicle alone (0.1% DMSO), 1 μM RA, 10 μM DAPT, or 10 μM concentrations of respective Jia compounds were incubated at 37°C for 5 days. SH-SY5Y cells treated with active Jia compounds exhibited significant neurite outgrowth, exemplifying the neuronal differentiation similar to RA-induced phenotypic changes. Phase-contrast images were taken by Sony DSC-W5 digital camera and processed with Adobe Photoshop software (Adobe Systems, Mountain View, CA). Scale bar, 20 μm. B, neuritic length of DMSO- and compound-treated SH-SY5Y cells ($n > 50$ for each treatment) were measured and analyzed by NIH Image as described under *Materials and Methods*. Results are expressed as the average (±S.D.) neuritic length and analyzed by Student's t test. *, $p < 0.001$.

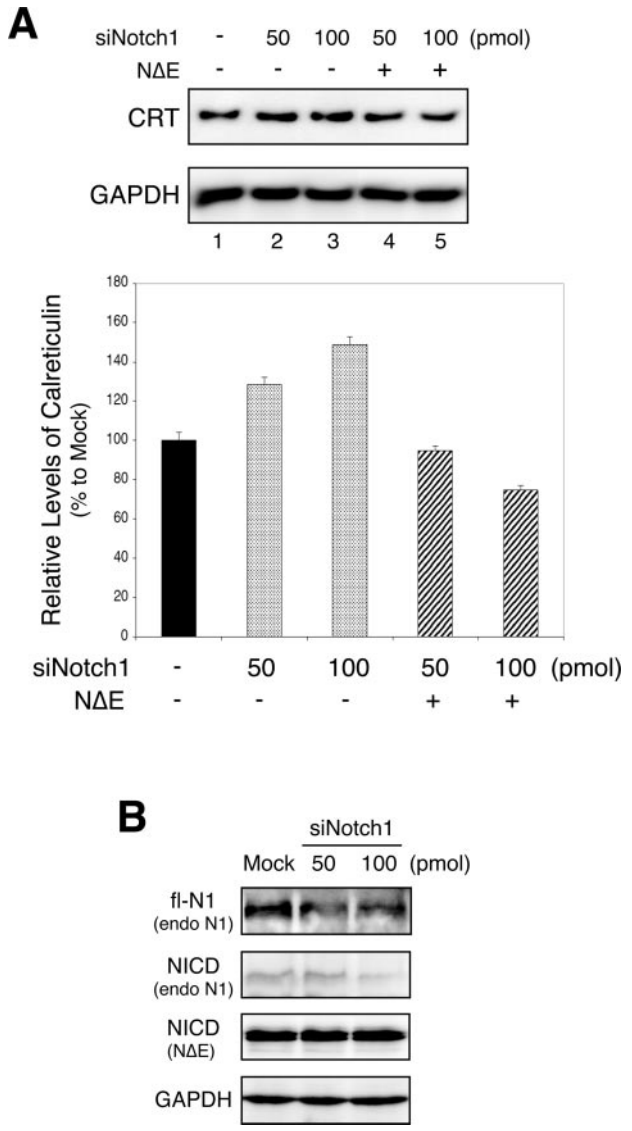


Fig. 10. Expression of calreticulin in neuroblastoma cells in response to the modulation of Notch signaling. A, human neuroblastoma SH-SY5Y cells (5×10^5/well in six-well microplates) were transfected with a siRNA against Notch1 (siNotch1, 50 or 100 pmol) in the presence (striped bar) or absence (shaded bar) of NΔE (1 μg/well) using Lipofectamine 2000, followed by an additional incubation with DMEM containing 10% FBS for at 37°C for 3 days. Clarified cell lysates were analyzed by SDS-PAGE and Western blotting using anti-calreticulin for the evaluation of induced neuronal differentiation of neuroblastoma cells. GAPDH was visualized to serve as the load control. The levels of calreticulin and GAPDH were determined by densitometry. The relative expression levels of calreticulin normalized by those of GAPDH are shown to demonstrate the neuronal differentiation of neuroblastoma cells by the inhibition of Notch signaling (lanes 2 and 3) and the reduced neuronal differentiation by activated Notch signaling (lanes 4 and 5). The relative expression of calreticulin in cells transfected with a nonspecific siRNA and an empty vector (Mock) is referred to as 100% (solid bar). Data are shown as the mean ± S.D. of triplicate measurements from a representative experiment. B, down-regulation of endogenous Notch signaling by a Notch1-targeting siRNA. N7 cells (5×10^5/well in six-well microplates) were transfected with siNotch1 (50 or 100 pmol) using Lipofectamine 2000, followed by an additional incubation with DMEM containing 10% FBS for at 37°C for 2.5 days. Cells transfected with a nonspecific siRNA (Mock) were also included for comparison. Equivalent amounts of protein in clarified cell lysates were analyzed by SDS-PAGE and Western blotting using anti-Notch1(Val1744). Full-length endogenous Notch1 (fl-N1, ~300 kDa, top) and NICD generated from endogenous Notch1 (~110 kDa, top middle) or exogenous NΔE (~50 kDa, bottom middle) were then visualized. GAPDH was visualized to serve as the protein load control (bottom). The data are representative of three independent experiments.

activity down to the basal level. These results are in agreement with previous reports, in which the S2′ active site pocket apparently accommodates moderate isobutyl substituents (Esler et al., 2004). In addition, alterations in the P3′ position reveal that Jia compounds substituted with an unnatural phenylglycine residue exhibit better potencies than the compounds containing a valine residue (Jia097). Consistent with previous studies (Esler et al., 2004), our results suggest that the S3′ pocket presents relatively loose specificity and can accommodate great variation in stereochemistry as shown by the similar potencies between Jia104 and Jia105 or between Jia046 and Jia101. Furthermore, removal of the carboxyl residue at position P3′ (such as Jia 046 changed to Jia104 or Jia 101 changed to Jia105) does not abrogate the inhibitory potency. Therefore, the reduction in the molecular weight and improvement in the lipophilic properties of theses (hydroxyethyl)ureas could be optimized without compromising their pharmaceutical profiles.

The modification of dipeptide isosteres at the P1-P1′ position has also been shown to be critical to γ-secretase inhibition (Nadin et al., 2003). To test this idea, the P1′ residues (Phe) of Jia 097, Jia046, and Jia105 are thus replaced with an isosteric cyclohexylmethyl (Chy) moiety to generate Jia138, Jia142, and Jia143. We show that the Chy-substituted Jia138 exhibits significantly improved efficacy of inhibiting γ-secretase, whereas Jia143 and Jia142 maintained their inhibitory potencies. It is noteworthy that the Chy-substituted Jia138 presents dramatic improvement on the inhibition of Aβ40 production over DAPT (an IC_{50} of 0.074 for Jia138 versus 0.794 for DAPT), whereas its efficacy of inhibiting S3 cleavage of Notch fares less well than DAPT does (with an IC_{50} of 0.235 versus 0.039). Both Jia138 and its Phe-containing analog Jia046 consistently exhibit better potency toward the processing of APP (IC_{50} values of 0.704 μM for Jia046 and 0.074 μM for Jia138 in Aβ40 production) than that of Notch (IC_{50} values of 3.965 μM for Jia046 and 0.235 μM for Jia138 in NICD production). Moreover, all of these active Jia compounds have better potency toward the inhibition of Aβ production than that of NICD production, whereas DAPT and compound E have better potency in blocking NICD production. It is thus likely that the incorporation of selective unnatural amino acids at the P1′ and P3′ sites of (hydroxyethyl)urea peptidomimetics could have greater impact in the γ-secretase-mediated processing of APP than that of Notch. The possibility exists that we can technically drive the transition state analog inhibitors, based on the structural features of Jia138, to preferentially block the Aβ production without tampering with the physiological function of Notch signaling and other γ-secretase substrates. Together, the combination of a Chy moiety at the P1′ site and a phenylglycine analog at the P3′ position might offer the structural scaffold for the subsequent development of γ-secretase inhibitors that selectively block only the pathogenic APP processing.

Our findings also demonstrate that the inhibitory potency of the Chy-containing Jia142, a C-terminal decarboxylated analog of Jia046, can be fully sustained for at least 8 days under cell culture conditions, suggesting that the stability of (hydroxyethyl)urea peptidomimetics could be dramatically improved by the substitution of distinctive unnatural amino acid moiety. Furthermore, these unnatural amino acid-substituted (hydroxyethyl)urea peptidomimetics, as exemplified

by Jia138, can inhibit γ-secretase and bind directly to heterodimeric PSs, resembling the characteristics of previously reported (hydroxyethyl)urea peptidomimetics and hydroxyethylene transition state analogs (Li et al., 2000a,b; Esler et al., 2002). The present data are thus in accordance with the conception that γ-secretase can tolerate unnatural D-amino acids as well as natural L-amino acids in transition state analog inhibitors (Bakshi and Wolfe, 2004; Esler et al., 2004), and they reveal that these unnatural amino acid-substituting (hydroxyethyl)urea peptidomimetics, like other transition state analog inhibitors, could specifically target to the active site of γ-secretase. Our findings also substantiate the model that the formation of heterodimeric PS1 (PS1-NTF and PS1-CTF) is prerequisite for the functional γ-secretase.

These effective Jia compounds significantly blocked the production of Aβ40, but they dramatically augmented the production Aβ42 at a subinhibitory concentration (1 μM). This effect has previously been observed for various γ-secretase inhibitors (Citron et al., 1996; Wolfe et al., 1999a). The mechanism underlying the increased production of Aβ42 by some active Jia compounds at lower concentrations remains unclear. Accumulating evidence has suggested that Aβ42 production mostly occurs in intracellular compartments, including endoplasmic reticulum and Golgi (Cook et al., 1997; Hartmann et al., 1997; Wild-Bode et al., 1997). Given that Jia047, Jia104, and Jia105 all cause dramatic increases in the levels of Aβ42 without affecting the levels of Aβ40 at a 1 μM concentration, our findings are in agreement with the notion that selective inhibition of Aβ40 formation can result in more efficient production of Aβ42 from the accumulated C99 substrate in these intracellular localizations (Wolfe et al., 1999a). Further development of (hydroxyethyl)urea isosteres using the structural scaffold identified in the present study might allow us to selectively target the Aβ42-generating γ-secretase that is localized intracellularly without perturbing Aβ40 production.

The γ-secretase-dependent S3 cleavage of Notch is a prerequisite for its downstream signaling that is essential for a variety of cell fate determination events during development and in adults (Mumm and Kopan, 2000). In addition, the protransforming role of Notch signaling has recently been recognized and well defined in a variety of cancers (Weng and Aster, 2004). The constitutive activation of Notch signaling in neuroblastomas has been shown to block induced differentiation and neurite formation (Grynfeld et al., 2000; Levy et al., 2002), suggesting the oncogenic effect of Notch in neuroblastomas. It is thus plausible that the inhibition of Notch signaling by specific γ-secretase inhibitors might interrupt its oncogenic effect and promote the differentiation of neuroblastoma cells, resulting in a decrease in their malignancy. We demonstrated by biochemical and morphological analyses that all active Jia compounds can block Notch signaling by reducing the generation of NICD and induce the neuronal differentiation of neuroblastoma cells. These data are in accordance with the idea that neurite outgrowth is markedly altered upon the inhibition of γ-secretase (Figueroa et al., 2002) and that activation of Notch signaling results in shrinkage of neuritic processes of cultured neuroblastoma cells (Ishikura et al., 2005). Our findings thus not only validate the efficacy of unnatural amino acid-containing (hydroxyethyl)urea peptidomimetics in biological systems but also demonstrate the beneficial effects of active Jia compounds on the maturation and differentiation of neuroblastoma cells.

Taken together, we demonstrate the identification of a novel class of γ-secretase inhibitors that contain unnatural amino acid moieties as a scaffold structure using various cell-based γ-secretase assays. These active (hydroxyethyl)urea peptidomimetics effectively reduce Aβ production and Notch signaling, and they present beneficial effects on neuroblastomas beyond their capacity for blocking the Aβ-centered pathogenesis of AD. The present evidence further suggests that the substitution of a peptidic moiety with selective unnatural amino acids will not compromise the potency for the inhibition of γ-secretase and could pave the way for the development of synthetic peptidomimetics of γ-secretase inhibitors with great stability in biological systems. The novel structural features of (hydroxyethyl)urea peptidomimetics are thus useful molecular tools for further characterization of this protease, and they could have great implications for the development of anti-AD drugs and therapeutics for neuroblastomas.

Acknowledgments

We thank Dr. Michael Wolfe for providing compound E and DAPT and Dr. Yu-Min Kuo for providing the SH-SY5Y human neuroblastoma cell line. We are also grateful to Drs. Jen-Leih Wu, John Yu, and to Nin-Nin Chuang for generous support. We thank the Core Facility of the Institute of Cellular and Organismic Biology, Academia Sinica, for technical support. We are also indebted to the National Science Council Regional Instruments Center at National Taiwan University and National Chung-Kung University for assistance in high-resolution mass spectrometry.

References

Bakshi P, Liao YF, Gao J, Ni J, Stein R, Yeh LA, and Wolfe MS (2005) A high-throughput screen to identify inhibitors of amyloid {beta}-protein precursor processing. *J Biomol Screen* **10:**1–12.

Bakshi P, and Wolfe MS (2004) Stereochemical analysis of (hydroxyethyl)urea peptidomimetic inhibitors of gamma-secretase. *J Med Chem* **47:**6485–6489.

Capell A, Grunberg J, Pesold B, Diehlmann A, Citron M, Nixon R, Beyreuther K, Selkoe DJ, and Haass C (1998) The proteolytic fragments of the Alzheimer's disease-associated presenilin-1 form heterodimers and occur as a 100–150-kDa molecular mass complex. *J Biol Chem* **273:**3205–3211.

Citron M, Diehl TS, Gordon G, Biere AL, Seubert P, and Selkoe DJ (1996) Evidence that the 42- and 40-amino acid forms of amyloid beta protein are generated from the beta-amyloid precursor protein by different protease activities. *Proc Natl Acad Sci USA* **93:**13170–13175.

Cook DG, Forman MS, Sung JC, Leight S, Kolson DL, Iwatsubo T, Lee VM, and Doms RW (1997) Alzheimer's A beta(1-42) is generated in the endoplasmic reticulum/intermediate compartment of NT2N cells. *Nat Med* **3:**1021–1023.

Dovey HF, John V, Anderson JP, Chen LZ, de Saint Andrieu P, Fang LY, Freedman SB, Folmer B, Goldbach E, Holsztynska EJ, et al. (2001) Functional gamma-secretase inhibitors reduce beta-amyloid peptide levels in brain. *J Neurochem* **76:**173–181.

Edbauer D, Winkler E, Regula JT, Pesold B, Steiner H, and Haass C (2003) Reconstitution of gamma-secretase activity. *Nat Cell Biol* **5:**486–488.

Esler WP, Das C, and Wolfe MS (2004) Probing pockets S2–S4′ of the gamma-secretase active site with (hydroxyethyl)urea peptidomimetics. *Bioorg Med Chem Lett* **14:**1935–1938.

Esler WP, Kimberly WT, Ostaszewski BL, Diehl TS, Moore CL, Tsai JY, Rahmati T, Xia W, Selkoe DJ, and Wolfe MS (2000) Transition-state analogue inhibitors of gamma-secretase bind directly to presenilin-1. *Nat Cell Biol* **2:**428–434.

Esler WP, Kimberly WT, Ostaszewski BL, Ye W, Diehl TS, Selkoe DJ, and Wolfe MS (2002) Activity-dependent isolation of the presenilin-gamma-secretase complex reveals nicastrin and a gamma substrate. *Proc Natl Acad Sci USA* **99:**2720–2725.

Figueroa DJ, Morris JA, Ma L, Kandpal G, Chen E, Li YM, and Austin CP (2002) Presenilin-dependent gamma-secretase activity modulates neurite outgrowth. *Neurobiol Dis* **9:**49–60.

Flexner C (1998) HIV-protease inhibitors. *N Engl J Med* **338:**1281–1292.

Getman DP, DeCrescenzo GA, Heintz RM, Reed KL, Talley JJ, Bryant ML, Clare M, Houseman KA, Marr JJ, Mueller RA, et al. (1993) Discovery of a novel class of potent HIV-1 protease inhibitors containing the (R)-(hydroxyethyl)urea isostere. *J Med Chem* **36:**288–291.

Greenlee WJ (1990) Renin inhibitors. *Med Res Rev* **10:**173–236.

Grynfeld A, Pahlman S, and Axelson H (2000) Induced neuroblastoma cell differentiation, associated with transient HES-1 activity and reduced HASH-1 expression, is inhibited by Notch1. *Int J Cancer* **88:**401–410.

Hartmann T, Bieger SC, Bruhl B, Tienari PJ, Ida N, Allsop D, Roberts GW, Masters CL, Dotti CG, Unsicker K, et al. (1997) Distinct sites of intracellular production for Alzheimer's disease A beta40/42 amyloid peptides. *Nat Med* **3:**1016–1020.
Hsu WM, Hsieh FJ, Jeng YM, Kuo ML, Chen CN, Lai DM, Hsieh LJ, Wang BT, Tsao PN, Lee H, et al. (2005) Calreticulin expression in neuroblastoma–a novel independent prognostic factor. *Ann Oncol* **16:**314–321.
Huff JR (1991) HIV protease: a novel chemotherapeutic target for AIDS. *J Med Chem* **34:**2305–2314.
Ishikura N, Clever JL, Bouzamondo-Bernstein E, Samayoa E, Prusiner SB, Huang EJ, and DeArmond SJ (2005) Notch-1 activation and dendritic atrophy in prion disease. *Proc Natl Acad Sci USA* **102:**886–891.
Iwatsubo T (2004) The gamma-secretase complex: machinery for intramembrane proteolysis. *Curr Opin Neurobiol* **14:**379–383.
Kimberly WT, LaVoie MJ, Ostaszewski BL, Ye W, Wolfe MS, and Selkoe DJ (2003) Gamma-secretase is a membrane protein complex comprised of presenilin, nicastrin, Aph-1, and Pen-2. *Proc Natl Acad Sci USA* **100:**6382–6387.
Kopan R, Schroeter EH, Weintraub H, and Nye JS (1996) Signal transduction by activated mNotch: importance of proteolytic processing and its regulation by the extracellular domain. *Proc Natl Acad Sci USA* **93:**1683–1688.
Kornilova AY, Das C, and Wolfe MS (2003) Differential effects of inhibitors on the gamma-secretase complex. Mechanistic implications. *J Biol Chem* **278:**16470–16473.
Levy OA, Lah JJ, and Levey AI (2002) Notch signaling inhibits PC12 cell neurite outgrowth via RBP-J-dependent and -independent mechanisms. *Dev Neurosci* **24:**79–88.
Li YM, Lai MT, Xu M, Huang Q, DiMuzio-Mower J, Sardana MK, Shi XP, Yin KC, Shafer JA, and Gardell SJ (2000a) Presenilin 1 is linked with gamma-secretase activity in the detergent solubilized state. *Proc Natl Acad Sci USA* **97:**6138–6143.
Li YM, Xu M, Lai MT, Huang Q, Castro JL, DiMuzio-Mower J, Harrison T, Lellis C, Nadin A, Neduvelil JG, et al. (2000b) Photoactivated gamma-secretase inhibitors directed to the active site covalently label presenilin 1. *Nature (Lond)* **405:**689–694.
Liao YF, Wang BJ, Cheng HT, Kuo LH, and Wolfe MS (2004) Tumor necrosis factor-alpha, interleukin-1β, and interferon-γ stimulate γ-secretase-mediated cleavage of amyloid precursor protein through a JNK-dependent MAPK pathway. *J Biol Chem* **279:**49523–49532.
Mani S, Shen Y, Schaefer J, and Meiri KF (2001) Failure to express GAP-43 during neurogenesis affects cell cycle regulation and differentiation of neural precursors and stimulates apoptosis of neurons. *Mol Cell Neurosci* **17:**54–66.
Mumm JS and Kopan R (2000) Notch signaling: from the outside in. *Dev Biol* **228:**151–165.
Nadin A, Owens AP, Castro JL, Harrison T, and Shearman MS (2003) Synthesis and gamma-secretase activity of APP substrate-based hydroxyethylene dipeptide isosteres. *Bioorg Med Chem Lett* **13:**37–41.
Pahlman S, Stockhausen MT, Fredlund E, and Axelson H (2004) Notch signaling in neuroblastoma. *Semin Cancer Biol* **14:**365–373.
Seiffert D, Bradley JD, Rominger CM, Rominger DH, Yang F, Meredith JE Jr, Wang Q, Roach AH, Thompson LA, Spitz SM, et al. (2000) Presenilin-1 and -2 are molecular targets for gamma-secretase inhibitors. *J Biol Chem* **275:**34086–34091.
Shearman MS, Beher D, Clarke EE, Lewis HD, Harrison T, Hunt P, Nadin A, Smith AL, Stevenson G, and Castro JL (2000) L-685,458, an aspartyl protease transition state mimic, is a potent inhibitor of amyloid beta-protein precursor gamma-secretase activity. *Biochemistry* **39:**8698–8704.
Singh US, Pan J, Kao YL, Joshi S, Young KL, and Baker KM (2003) Tissue transglutaminase mediates activation of RhoA and MAP kinase pathways during retinoic acid-induced neuronal differentiation of SH-SY5Y cells. *J Biol Chem* **278:**391–399.
Weng AP and Aster JC (2004) Multiple niches for Notch in cancer: context is everything. *Curr Opin Genet Dev* **14:**48–54.
Wild-Bode C, Yamazaki T, Capell A, Leimer U, Steiner H, Ihara Y, and Haass C (1997) Intracellular generation and accumulation of amyloid β-peptide terminating at amino acid 42. *J Biol Chem* **272:**16085–16088.
Wolfe MS, Esler WP, and Das C (2002) Continuing strategies for inhibiting Alzheimer's gamma-secretase. *J Mol Neurosci* **19:**83–87.
Wolfe MS and Haass C (2001) The Role of presenilins in gamma-secretase activity. *J Biol Chem* **276:**5413–5416.
Wolfe MS, Xia W, Moore CL, Leatherwood DD, Ostaszewski B, Rahmati T, Donkor IO, and Selkoe DJ (1999a) Peptidomimetic probes and molecular modeling suggest that Alzheimer's gamma-secretase is an intramembrane-cleaving aspartyl protease. *Biochemistry* **38:**4720–4727.
Wolfe MS, Xia W, Ostaszewski BL, Diehl TS, Kimberly WT, and Selkoe DJ (1999b) Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and gamma-secretase activity. *Nature (Lond)* **398:**513–517.

Address correspondence to: Dr. Yung-Feng Liao, Institute of Cellular and Organismic Biology, Rm 238, Academia Sinica, 128 Academia Rd. Sec. 2, Taipei 115, Taiwan. E-mail: yliao@sinica.edu.tw