J|A|C|S
COMMUNICATIONS

Published on Web 09/06/2003

Designed Helical Peptides Inhibit an Intramembrane Protease

Chittaranjan Das,[†] Oksana Berezovska,[‡] Thekla S. Diehl,[†] Cedric Genet,[†] Ilya Buldyrev,[†] Jui-Yi Tsai,[†] Bradley T. Hyman,[‡] and Michael S. Wolfe[†,*]

Center for Neurologic Diseases, Harvard Medical School and Brigham and Women's Hospital, Boston, Massachusetts 02115, and Alzheimer's Disease Research Laboratory, Harvard Medical School and Massachusetts General Hospital, Charlestown, Massachusetts 02129

Received July 8, 2003; E-mail: mwolfe@rics.bwh.harvard.edu

γ-Secretase is a novel aspartyl protease that cleaves the transmembrane domain of the amyloid$-\beta$ precursor protein (APP) to produce the amyloid$-\beta$ peptide (Aβ), a process implicated in the pathogenesis of Alzheimer's disease.[1] This enzyme is a multiprotein complex containing the 8-transmembrane presenilin as its presumptive catalytic component and is a founding member of an emerging class of intramembrane proteases.[2] Such proteases resemble their soluble or membrane-tethered counterparts in residues required for catalysis but not with respect to sequence homology. Intramembrane proteases are predicted to have the hydrophilic active site buried in the protein interior, sequestered from the hydrophobic environment of the lipid bilayer.[3] As a corollary, the integral membrane substrate should first interact on the surface of the protease within the membrane before entering the active site. Biochemical evidence supports the existence of such an initial substrate docking site on γ-secretase: an endogenous γ-secretase substrate copurifies with the protease complex from an immobilized transition-state analogue inhibitor.[4] That is, the substrate becomes stalled in the docking site because the immobilized inhibitor occupies the active site.

To further test this idea and identify new inhibitor prototypes, we designed short peptides based on the APP transmembrane domain that would assume a helical conformation, because evidence suggests that the APP transmembrane domain is helical upon initial interaction with the γ-secretase complex.[5,6] To achieve this constraint, we incorporated a well-known helix-inducing residue, α-aminoisobutyric acid (Aib).[7] In the design, APP residues were judiciously swapped with Aib so that, upon helix formation, the Aib residues would be on one face of the helix, and APP residues would be on the other. Further, N-terminal *tert*-butoxycarbonyl, C-terminal methyl ester, and threonine *O*-benzyl protecting groups were retained to enhance cell permeability. These peptides blocked Aβ production from APP-transfected Chinese hamster ovary (CHO) cells in the low μM range (Table 1), with longer peptides showing higher potencies. This effect occurred at the γ-secretase level, as APP γ-secretase substrates were increased in a concentration-dependent manner consistent with effects on Aβ production (see Supporting Infirmation).

Surprisingly, we found that enantiomers of these compounds, with all D-amino acids, were equally or more potent than their L-peptide counterparts. Both enantiomers are helical in solution: the L-peptides are right-handed helices, and the D-peptides are left-handed, as supported by CD (Figure 1a) and 2D NMR (Supporting Infirmation). One of the most potent compounds in this series, D-peptide **11**, was tested in a γ-secretase assay using detergent-solubilized membranes and a recombinant APP-based substrate.[4] Peptide **11** inhibited the production of both the major 40-residue form of Aβ (Aβ40) and the minor 42-residue form (Aβ42) with similar IC_{50} values ($\sim$100 nM, Figure 1b). In contrast, the compound had no effect up to 25 μM on β-secretase in a purified enzyme assay (data not shown). β-Secretase is a membrane-tethered pepsin-like aspartyl protease that generates the N-terminus of Aβ from APP.[8] These findings suggest that the Aib-containing peptides selectively inhibit γ-secretase activity by direct interaction with the protease.

To test whether these peptides inhibit γ-secretase simply by virtue of their hydrophobicity or if conformation is critical, we disrupted the helicity of D-peptide **11**, swapping one of the Aibs with glycine (peptide **12**) or by inverting the α-stereocenters of the fourth and fifth residues (peptide **13**) (Table 2). Either minor modification led to substantial loss in potency. Inversion of the α-stereocenters of the fifth and sixth residues (peptide **14**) led to a $\sim$100-fold loss of potency (and a clear reduction in helicity as determined by 2D NMR, Supporting Infirmation). L-Peptide **5** is substantially less potent than its D-peptide counterpart in the cell-free assay, but a systematic phenylalanine scan of **5** led to the identification of a

Table 1. Aib-Containing Peptides Inhibit γ-secretase Activity in Cells[a]

APP transmembrane residues 707-719:
---ValGlyGlyValValIleAlaThr ↑ Val Ile ↑ ValIle---

Peptides:		IC_{50} (μM)
1	Boc-ValIle*Aib*Thr*ValAib-OMe	12
2	Boc-ValValIle*Aib*Thr*ValAib-OMe	6
3	Boc-*Aib*ValValIle*Aib*Thr*ValAib-OMe	6
4	Boc-Gly*Aib*ValValIle*Aib*Thr*ValAib-OMe	3
5	Boc-ValGly*Aib*ValValIle*Aib*Thr*ValAib-OMe	3
6	Boc-AlaThr*Aib Ile ValAib-OMe	25
7	Boc-Ile AlaThr*Aib Ile ValAib-OMe	15
8	Boc-*Aib*Ile AlaThr*Aib Ile ValAib-OMe	10
9	Boc-Val*Aib*Ile AlaThr*Aib Ile ValAib-OMe	3
10	Boc-GlyVal*Aib*Ile AlaThr*Aib Ile ValAib-OMe	5

[a] Asterisks indicate *O*-benzyl protection of Thr. Arrows: γ-secretase cleavage sites in APP.

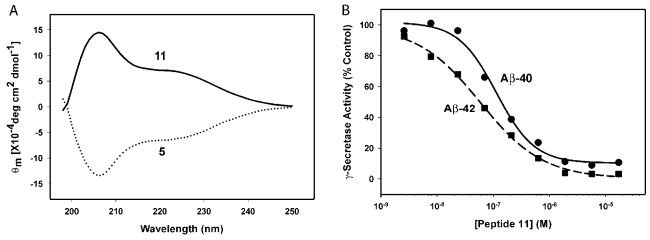

Figure 1. Aib-containing peptides are helical and inhibit detergent-solubilized γ-secretase. (A) CD spectra of enantiomers **5** and **11**. Troughs at 206$-$208 and 220$-$223 nm are characteristic of a right-handed helix, while peaks at these wavelengths are characteristic of a left-handed helix. (B) Helical peptide **11** inhibits the production of both Aβ40 and Aβ42 from solubilized cell membranes and recombinant substrate.

[†] Harvard Medical School and Brigham and Women's Hospital.
[‡] Harvard Medical School and Massachusetts General Hospital.

10.1021/ja037131v CCC: $25.00

Table 2. Disruption of Helicity Reduces Inhibitory Potency toward Detergent-Solubilized γ-Secretase

Peptides:		IC_{50} (nM)
11	Boc-^{D}ValGly*Aib*^{D}Val^{D}Val^{D}Ile*Aib*^{D}Thr*^{D}Val*Aib*-OMe	90
12	Boc-^{D}ValGly*Gly*^{D}Val^{D}Val^{D}Ile*Aib*^{D}Thr*^{D}Val*Aib*-OMe	1,000
13	Boc-^{D}ValGly *Aib*^{L}Val^{L}Val^{D}Ile*Aib*^{D}Thr*^{D}Val*Aib*-OMe	3,000
14	Boc-^{D}ValGly *Aib*^{D}Val^{L}Val^{L}Ile*Aib*^{D}Thr*^{D}Val*Aib*-OMe	9,000
5	Boc- ValGly *Aib* Val Val Ile*Aib* Thr* Val*Aib*-OMe	30,000
15	Boc- ValGly *Aib* Val Val Ile*Aib* Phe Val*Aib*-OMe	30
16	Boc- ValGly *Aib*^{D}Val^{D}Val Ile*Aib* Phe Val*Aib*-OMe	15,000

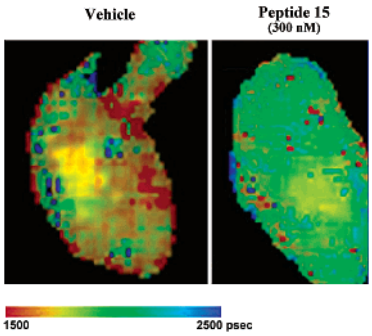

Figure 2. Helical peptides disrupt interaction of γ-secretase substrates with PS1 in cells. Color-coded FLIM shows fluorescence lifetimes, reflecting proximity between PS1 and APP C-terminal fragments. Cell were treated with DMSO vehicle alone (left) or with peptide **15** (right), fixed and immunostained with primary anti-mouse antibodies to the large loop of PS1 (conjugated to a fluorescence donor, FITC) and primary anti-goat antibodies to the APP C-terminus (conjugated to a fluorescence acceptor, CY3). Colorimetric scale shows fluorescence lifetimes. See Supporting Information for a summary table of FLIM assays.

low nM L-peptide inhibitor (peptide **15**). Inversion of two internal stereocenters in this compound (peptide **16**) likewise resulted in a substantial loss of activity. These observations strongly suggest that helical character is critical for inhibitory potency of the Aib-containing peptides.

Further modification of decapeptide **11** led to the discovery of the highly potent **17**, identical to **11** except that 4-benzoyl-D-phenylalanine is the sixth residue. This peptide inhibited γ-secretase proteolysis of APP with an IC_{50} of 10 nM in cell-free assays and 70 nM in cell-based assays. Peptide **17** also potently blocked cleavage of another γ-secretase substrate, the Notch receptor,[1] both in cells and isolated membranes. Peptides **11** and **15**−**17** were tested for their ability to interfere with the interaction between APP γ-secretase substrates and presenilin-1 (PS1) in intact cells using fluorescence lifetime imaging microscopy (FLIM). FLIM relies on the observation that fluorescence lifetimes after excitation of a donor fluorophore are shorter in the presence of a nearby fluorescence resonance energy transfer acceptor. CHO cells stably coexpressing human APP and PS1 were double stained with polyclonal antibody C8 to the APP C-terminus and a monoclonal antibody to the PS1 residues 263−378. Prior to staining, the former antibody was conjugated to acceptor fluorophore Cy3 and the latter to donor fluorophore FITC. Excitation of FITC at 488 nm was followed by image acquisition at 522 nm and analysis to determine fluorescence lifetimes on a pixel-by-pixel basis. We have recently reported this method for studying the interaction of PS1 with APP γ-secretase substrates.[9] At ≤ 2 μM, **11**, **15**, and **17** essentially prevented the fluorescence lifetime decrease of FITC/PS1 antibodies, which results when the Cy8/APP antibodies are <100 Å away (illustrated for **15**, Figure 2). In contrast, helix-disrupted **16** had no effect at 2 μM.

We recently found that a transition-state analogue inhibitor called 31C does not prevent this fluorescence lifetime decrease,[9] i.e., γ-secretase substrates remain associated with PS1 even after treatment with an active site-directed γ-secretase inhibitor. Thus, the helical peptides inhibit γ-secretase by a mechanism different from that of transition-state analogues. We have obtained complementary results using a photoactivatable version of 31C to label PS1: while several other γ-secretase inhibitors prevent this cross-linking, helical peptide **11** does not.[10] These results indicate that the helical peptides bind to a site distinct from the active site. Since the peptides interfere with the interaction between PS1 and APP γ-secretase substrates, binding to the initial substrate docking site on the protease would be their most likely mechanism of inhibition. These new inhibitors could be used as molecular probes to discern where in the protease the initial substrate docking site resides. These findings may also have therapeutic implications: The recent approval of the helical 36-residue L-peptide enfuvirtide for the treatment of AIDS[11] lends credence to the idea that small, hydrophobic, cell-permeable D-peptides have potential for the prevention or treatment of Alzheimer's disease.

Other intramembrane proteases (e.g., the metalloprotease S2P,[12] the serine protease rhomboid,[13] the aspartyl protease signal peptide peptidase[14]) should likewise contain an internal active site sequestered from the hydrophobic environment of the lipid bilayer. We have demonstrated here for the first time the design of inhibitors that take advantage of the unique properties of this newly recognized class of proteases. This strategy may prove to be more widely applicable, providing inhibitors of other intramembrane proteases regardless of the active site residues ultimately responsible for catalysis. Such inhibitors would be important tools for discovering other intramembrane proteases, understanding their biochemical mechanisms, elucidating their biological roles, and identifying new drug prototypes.

Acknowledgment. We thank B. Zheng for Aβ ELISAs and D. Selkoe for the CHO cell line expressing human APP and for antibodies to APP and Aβ. This work was supported by the NIH (M.S.W., B.T.H.), the Alzheimer's Association (M.S.W., B.T.H.), and the Institute for the Study of Aging (M.S.W.).

Supporting Information Available: Experimental procedures, immunoblot of γ-secretase substrates, NMR data for **11** and **14**, and a summary table of FLIM results. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Esler, W. P.; Wolfe, M. S. *Science* **2001**, *293*, 1449−54.
(2) Wolfe, M. S.; Selkoe, D. J. *Science* **2002**, *296*, 2156−7.
(3) Wolfe, M. S.; De Los Angeles, J.; Miller, D. D.; Xia, W.; Selkoe, D. J. *Biochemistry* **1999**, *38*, 11223−30.
(4) Esler, W. P.; Kimberly, W. T.; Ostaszewski, B. L.; Ye, W.; Diehl, T. S.; Selkoe, D. J.; Wolfe, M. S. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 2720−2725.
(5) Lichtenthaler, S. F.; Wang, R.; Grimm, H.; Uljon, S. N.; Masters, C. L.; Beyreuther, K. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 3053−3058.
(6) Wolfe, M. S.; Xia, W.; Moore, C. L.; Leatherwood, D. D.; Ostaszewski, B.; Donkor, I. O.; Selkoe, D. J. *Biochemistry* **1999**, *38*, 4720−7.
(7) Karle, I. L.; Balaram, P. *Biochemistry* **1990**, *29*, 6747−56.
(8) De Strooper, B.; Konig, G. *Nature* **1999**, *402*, 471−2.
(9) Berezovska, O.; Ramdya, P.; Wolfe, M. S.; Bacskai, B.; Hyman, B. T. *J. Neurosci.* **2003**, *23*, 4560−6.
(10) Kornilova, A. Y.; Das, C.; Wolfe, M. S. *J. Biol. Chem.* **2003**, *278*, 16470−3.
(11) LaBonte, J.; Lebbos, J.; Kirkpatrick, P. *Nat. Rev. Drug Discov.* **2003**, *2*, 345−6.
(12) Rawson, R. B.; Zelenski, N. G.; Nijhawan, D.; Ye, J.; Sakai, J.; Hasan, M. T.; Chang, T. Y.; Brown, M. S.; Goldstein, J. L. *Mol. Cell* **1997**, *1*, 47−57.
(13) Urban, S.; Lee, J. R.; Freeman, M. *Cell* **2001**, *107*, 173−82.
(14) Weihofen, A.; Binns, K.; Lemberg, M. K.; Ashman, K.; Martoglio, B. *Science* **2002**, *296*, 2215−8.

JA037131V