Discovery of a Subnanomolar Helical D-Tridecapeptide Inhibitor of γ-Secretase

Frédéric Bihel, Chittaranjan Das,
Michael J. Bowman, and Michael S. Wolfe*

Center for Neurologic Diseases, Harvard Medical School and Brigham & Women's Hospital, 77 Avenue Louis Pasteur, Boston, Massachusetts 02115

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Abstract: γ-Secretase is the second of two proteolytic enzymes responsible for the release of the amyloid β-peptide implicated in the etiology of Alzheimer's disease. Here, we used solid-phase synthesis to generate a new series of helical peptides as γ-secretase inhibitors, one of which, **11**, showed an IC_{50} of 140 pM in a cell-free enzyme assay.

Cerebral deposition of the amyloid β-peptide (Aβ) is an early and invariant step in the pathogenesis of Alzheimer's disease (AD).[1] The 39−43-residue $A\beta^{2-5}$ is formed through proteolytic processing of the integral membrane Aβ precursor protein (APP)[6] by the sequential action of β- and γ-secretases,[7] which consequently have emerged as important therapeutic targets.[8,9] γ-Secretase is a complex aspartyl protease composed of multiple integral membrane proteins, in particular presenilin, which appears to be the catalytic component.[10] This enzyme belongs to a new class of intramembrane proteases that hydrolyze the transmembrane domain of their substrates and are thought to have their active sites buried within the boundaries of the lipid bilayer.[11] According to this model, the hydrophilic active site, containing water and two aspartates, should be in the interior of the complex to avoid unfavorable interactions with hydrophobic lipid hydrocarbon chains. As a consequence, the substrate is predicted to first dock on the outer surface of the complex before moving to the internal active site. Indeed, an endogenous γ-secretase substrate copurifies with the protein complex from an immobilized transition-state analogue inhibitor, supporting the existence of such an initial docking site on γ-secretase.[12]

On the basis of this model, we designed a set of novel γ-secretase inhibitors.[13] These prototypes are hexa- to decapeptides, mimicking the primary and secondary structures of the APP transmembrane domain cleaved by γ-secretase. Evidence suggests that this transmembrane domain adopts an α-helical conformation upon initial interaction with γ-secretase.[14,15] To achieve this conformational constraint, selected APP residues were swapped with α-amino-isobutyric acid (Aib; α-methylalanine), which is well-known to favor helical conformations in peptides.[16] First generation peptides blocked Aβ production from APP-transfected Chinese hamster ovary (CHO) cells in the low micromolar range, with nona- and decapeptides being the most potent.[13] This effect occurred at the γ-secretase level, as intracellular levels of APP γ-secretase substrates were appropriately increased. Surprisingly, the enantiomers of these compounds, only composed of achiral and D-amino acids, were equally or more potent than their L-peptide counterparts. The helical character of these peptides is critical for their activity: inverting the α-stereocenters of internal residues disrupted the helical conformation and dramatically reduced the potency of the compounds. Biochemical and cellular experiments suggest that these compounds indeed interact with the substrate docking site.[13]

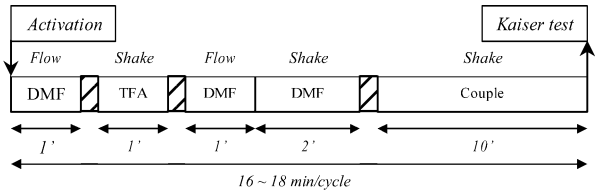

Figure 1. Manual solid-phase synthesis. The hatched regions indicate a drainage step where excess solvent and/or reactant components are removed from the peptide-resin by filtration.

Here we describe a new series of extended helical L- and D-peptides, containing between 10 and 16 amino acids and designed from the APP transmembrane domain, to evaluate the relationship between peptide length and inhibitory activity. We reasoned that longer peptides might be more effective due to increased helical character and a larger surface area for interaction with the protease. One of these compounds displayed remarkably high potency, with an IC_{50} of 140 pM.

The peptides in our original report[13] contained *N*-Boc and *O*-methyl ester termini and were synthesized following a standard liquid-phase procedure with HATU as the coupling reagent in the presence of diisopropylethylamine (DIEA) in DMF.[13] This synthetic strategy was suitable for peptides containing up to 10 amino acids; however, the extension of the peptide chain beyond 10 residues became difficult, particularly during the intermediate purification steps. Despite their potential helicity, the hydrophobic character of these peptides apparently does not favor solubility in common solvents such as dichloromethane or ethyl acetate, leading to a time-consuming synthesis and a dramatic drop-off in yields. To solve this problem, we switched to a solid-phase peptide synthesis (SPPS) using Boc-amino acids. This strategy has the advantage of clean, reliable, and rapid acidolytic N^{α}-Boc deprotection compared with its Fmoc counterpart.[17,18] SPPS was performed using a hydrazinobenzoyl resin, which is stable to acids and bases and can be cleaved with high specificity under mild oxidative conditions.[19] HATU was selected instead of other activators such as HBTU or PyBOP because it leads to superior acylation rates, reduces racemization, and has higher solubility in the common coupling solvent DMF.[17] Each cycle of deprotection, draining, washing, and coupling was optimized to be complete in less than 20 min (Figure 1). After chain assembly, cleavage from the solid-phase was accomplished by treating with copper(II) acetate in a mixture pyridine/methanol. After purification by preparative HPLC, the identity and purity of the final compounds were verified by mass spectroscopy and analytical HPLC, respectively. The helical conformation

* To whom correspondence should be adressed. E-mail: mwolfe@rics.bwh.harvard.edu.

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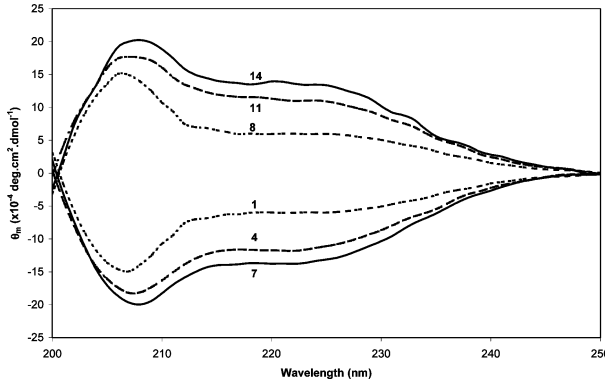

Figure 2. CD spectra of D- and L-peptide enantiomeric pairs containing 10, 13, and 16 amino acids. Troughs at 206−208 and 220−223 nm are characteristic of a right-handed helix, while peaks at these wavelengths are characteristic of a left-handed helix. The molar ellipticity per amino acid increases with the peptide length.

of this peptide family has been previously demonstrated by circular dichroism (CD) and 2D NMR.[13] We noted that, according to CD, the helical character increases with the length of the peptide as expected (Figure 2).

Compounds were tested in a γ-secretase assay using detergent-solubilized membranes from HeLa cells and a recombinant APP-based substrate (Table 1).[12] In the L-series, compound **3** was the most potent, with an IC_{50} of 360 nM, a ~100-fold increase in potency over the reference L-decapeptide **I** ($IC_{50} = 30\ \mu M$). Moreover, the γ-secretase inhibition appears to show dependence on length. Extending the peptide up to 12 amino acids enhanced γ-secretase inhibition, but further extensions resulted in stepwise decreases in inhibitory activity. Extension of the C-terminus of reference L-decapeptide I with Val-Ile-Aib improved potency, as the resultant peptide **4** is almost 40 times more active, with an IC_{50} of 780 nM.

As expected, the D-series was much more potent than the L-series. Starting from the reference D-decapeptide **II** (IC_{50} = 90 nM), we tested the enantiomers of the helical peptide L-series. These D-peptides all showed very potent activity, with IC_{50} values generally in the 10−30 nM range. Surprisingly, the activity did not change much with the length of the peptide in most cases. However, compound **11** was extremely potent, with an IC_{50} of 140 pM, 100 times more active than the other analogues of the D-series. The structural and/or conformational differences responsible for this dramatic increase in potency remain unclear but are under investigation. **11** was also tested in a cell-based assay from APP-transfected CHO cells,[15] in which it showed an IC_{50} of 9 ± 1 nM. This 40-fold loss of activity compared to the cell-free assay may be due to the need to enter into and/or cross cell membranes or some susceptibility to metabolic degradation.

The present results demonstrate that helical peptides can be highly potent γ-secretase inhibitors. Compound **11**, with a subnanomolar IC_{50}, appears to be among the best γ-secretase inhibitors yet reported and represents a potential drug lead. Indeed, the helical conformation along with the D-configuration should increase the metabolic stability of these peptides, which is essential from the perspective of drug development. Recently, enfuvirtide (Fuzeon), a helical peptide that blocks HIV entry into cells, was approved for the treatment of AIDS,[20] supporting the contention that this new class of compounds could have therapeutic potential in other areas. In contrast to enfuvirtide, a 36-residue amphipathic L-peptide that must be given by injection, these helical γ-secretase inhibitors are much shorter, very hydrophobic D-peptides that are clearly capable of crossing biological barriers, based on their ability to inhibit Aβ production in cell culture. Whether these compounds can enter the brain and lower Aβ is an important issue worthy of future study.

In any event, the subnanolar helical peptide inhibitor reported here is an appropriate starting point for the development of affinity labeling reagents suitable for identifying the component(s) that comprise(s) the initial substrate docking site of the γ-secretase complex. Such efforts are underway in our laboratory.

Table 1. Aib-Containing Peptides and Their Inhibitory Potency toward γ-Secretase[a]

L-peptides[b,c]	APP transmembrane residues 704-720 : ----GLMVGGVVIAT V I VIT----- ↑ ↑	Cell free Assay IC_{50} (nM)[e]
I	Boc-VG*U*VVI*U*T*V*U*-OMe	30,000[f]
1	Boc-VVI*U*T*V*U*VI*U*-OMe	10,000 ± 1,000
2	Boc-*U*VVI*U*T*V*U*VI*U*-OMe	3,100 ± 500
3	Boc-G*U*VVI*U*T*V*U*VI*U*-OMe	360 ± 30
4	Boc-VG*U*VVI*U*T*V*U*VI*U*-OMe	780 ± 140
5	Boc-*U*VG*U*VVI*U*T*V*U*VI*U*-OMe	910 ± 170
6	Boc-L*U*VG*U*VVI*U*T*V*U*VI*U*-OMe	4,700 ± 500
7	Boc-GL*U*VG*U*VVI*U*T*V*U*VI*U*-OMe	11,000 ± 3,500
D-peptides[b,d]		
II	Boc-VG*U*VVI*U*T*V*U*-OMe	90[f]
8	Boc-VVI*U*T*V*U*VI*U*-OMe	25 ± 3
9	Boc-*U*VVI*U*T*V*U*VI*U*-OMe	28 ± 19
10	Boc-G*U*VVI*U*T*V*U*VI*U*-OMe	12 ± 4
11	Boc-VG*U*VVI*U*T*V*U*VI*U*-OMe	0.14 ± 0.07
12	Boc-*U*VG*U*VVI*U*T*V*U*VI*U*-OMe	14 ± 10
13	Boc-L*U*VG*U*VVI*U*T*V*U*VI*U*-OMe	16 ± 7
14	Boc-GL*U*VG*U*VVI*U*T*V*U*VI*U*-OMe	26 ± 21

[a] Arrows indicate γ-secretase cleavage sites in APP; *U* corresponds to amino acid Aib. [b] Asterisks indicate *O*-benzyl protection of threonine. [c] Every α-stereocenter has the L-configuration. [d] Every α-stereocenter has the D-configuration. [e] Each value was calculated from at least three independent experiments and represents the concentration required for 50% inhibition of Aβ_{40} production. [f] Ref 13.

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Supporting Information Available: Synthesis, HPLC, and MS characterization of the discussed compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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