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Probing pockets S2–S4′ of the γ-secretase active site with (hydroxyethyl)urea peptidomimetics

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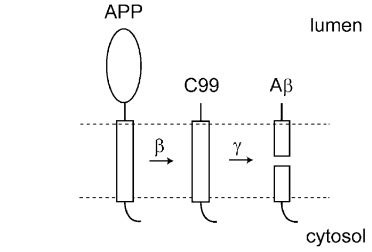
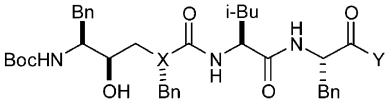
Abstract—(Hydroxyethyl)urea peptidomimetics are potent inhibitors of γ-secretase that are accessible in a few synthetic steps. Systematic alteration of P2–P4′ revealed that the corresponding S2–S4′ active site pockets accommodate a variety of substituents, consistent with the fact that this protease cleaves a variety of single-pass membrane proteins; however, phenylalanine is not well tolerated at P2′. A compound spanning P2–P3′ was identified as a low nM inhibitor of γ-secretase activity both in cells and under cell-free conditions.

The production and self-association of the 38–43 residue amyloid-β peptide (Aβ) are essential steps in the pathogenesis of Alzheimer's disease (AD) and are thus considered appropriate points for therapeutic intervention.[1] In particular, the two proteases, β- and γ-secretases, responsible for cutting Aβ out of its precursor protein (APP) have emerged as principal targets.[2] β-Secretase is a membrane-anchored aspartyl protease in the pepsin family and generates the N-terminus of Aβ (Fig. 1, top).[3] Knockout of β-secretase eliminates Aβ production in mice with little or no apparent developmental defects,[4] suggesting the enzyme is a suitable therapeutic target. Moreover, the co-crystallization of β-secretase with a transition-state analogue inhibitor[5] has made this protease amenable to structure-based design.

Although γ-secretase is also considered a key target, its biology has proven to be much more complicated. This protease somehow accomplishes a hydrolysis in the middle of the APP transmembrane domain to generate the C-terminus of Aβ (Fig. 1, top). AD-causing mutations in presenilin-1 and-2 (PS1 and PS2) affect the site of γ-secretase cleavage in APP to overproduce the more deleterious 42-residue form of Aβ.[6] Knockout of the PS genes eliminates γ-secretase activity[7] but also causes embryonic lethality in mice.[8] Transition-state analogue inhibitors first suggested that γ-secretase is an aspartyl protease[9,10] and led to the identification of two conserved transmembrane aspartates in PS essential for activity and the suggestion that PS is the catalytic component of the protease.[11] Affinity reagents based on these inhibitors label PS, strongly supporting this hypothesis.[12,13] γ-Secretase has recently been discovered to be a complex of three other membrane proteins in addition to PS.[14] This enzyme is a founding member of an emerging family of proteases with membrane-embedded active sites.[15]





1 X = CH, Y = NH_2
2 X = N, Y = OMe

Figure 1. Top: Proteolysis of APP by β- and γ-secretases produce Aβ. Bottom: Structure of hydroxyethylene (**1**) and (hydroxyethyl)urea (**2**) peptidomimetic inhibitors of γ-secretase.

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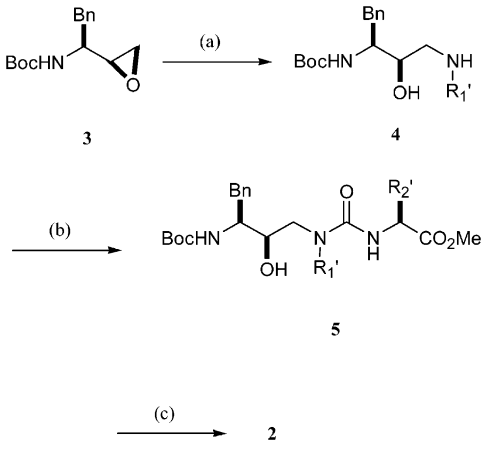
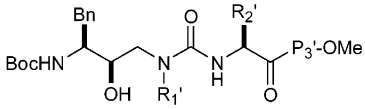
Small molecule inhibitors have been indispensable tools for identifying γ-secretase, elucidating its mechanism of action, understanding its biological roles, and determining its potential as a therapeutic target. Inhibitors used as molecular probes include difluoro ketones and alcohols,[9,16] hydroxyethylenes,[10] benzodiazepines,[17] and (more recently) helical peptides that mimic the conformation of the transmembrane substrate.[18] The hydroxyethylene peptidomimetics (e.g., **1**, Fig. 1, bottom) have been particularly useful for probing the enzyme active site.[12,19] However, the synthesis of the dipeptide isostere building blocks is cumbersome.[19] To simplify the synthesis and access a variety of related analogues, we replaced the chiral carbon atom in P1′ with an achiral nitrogen. The resulting series of (hydroxyethyl)urea peptidomimetics (e.g., **2**, Fig. 1) has provided potent inhibitors as well as feedback about the nature of the S2–S4′ pockets of γ-secretase. In particular, we systematically altered positions P2, P1′, P2′, P3′, and P4′ with small, medium, and large hydrophobic residues (alanine, valine, leucine, and phenylalanine) to explore the steric limits of the corresponding pockets (Scheme 1).

We previously reported a series of difluoro ketone peptidomimetics varied in the P1 position, which provided evidence for a large S1 pocket in the active site of γ-secretase.[20] This, in combination with the report of **1** by Shearman and colleagues,[10] led us to begin the synthesis with the commercially available epoxide **3**, which would provide analogues with the benzyl substituent (i.e., phenylalanine side chain) in P1. Following the method of Getman et al., who developed (hydroxyethyl)urea peptidomimetics as inhibitors of HIV protease,[21] this epoxide was opened with several different alkylamines in high yield (80–99%) by refluxing in isopropanol for 16 h. The amino alcohols **4** were then treated with isocyanates (in turn obtained from α-amino methyl esters and phosgene[22]) to yield the (hydroxyethyl)urea P1–P2′ isosteres **5** in near quantitative yields. C-terminal extension to incorporate P3′ and P4′ (e.g., **2** and **18**, Tables 1 and 2, respectively) was accomplished by hydrolysis of the methyl ester with lithium hydroxide and subsequent coupling of α-amino esters with HATU in the presence of diisopropylethylamine (DIPEA) in DMF. N-terminal extension to P2 (e.g, **23**, Table 2) was accomplished by removal of the *N*-butoxycarbonyl (Boc) group with TFA followed by coupling with Boc-protected amino acids, again using HATU and DIPEA in DMF.



Scheme 1. Reagents and conditions: (a) $R_{1'}$-NH_2, *i*-PrOH, reflux; (b) OCN-CH($R_{2'}$)CO_2Me; (c) aq LiOH, THF; H_2N-CH($R_{3'}$)CO_2Me, HATU, DIPEA, DMF.

Table 1. Structures of (hydroxyethyl)ureas varied at P1′–P3′ and their abilities to lower Aβ production from APP-transfected CHO cells



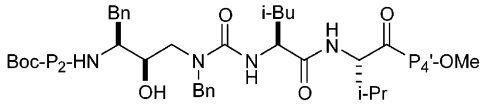
Compd	R1′	R2′	P3′	IC_{50} (μM)[a]
6	Me	*i*-Bu	Phe	3.1
7	*i*-Pr	*i*-Bu	Phe	22
8	*i*-Bu	*i*-Bu	Phe	0.40
9	Bn	*i*-Bu	Phe	0.40
10	Bn	Me	Phe	0.14
11	Bn	*i*-Pr	Phe	0.40
12	Bn	*i*-Bu	Phe	0.39
13	Bn	Bn	Phe	18
14	Bn	*i*-Bu	Ala	2.0
15	Bn	*i*-Bu	Val	0.22
16	Bn	*i*-Bu	Leu	0.65
2	Bn	*i*-Bu	Phe	0.39
17	Bn	*i*-Bu	—	8.0

[a] Values are means of three experiments.

These compounds were tested for their ability to block Aβ production from Chinese hamster ovary (CHO) cells stably transfected with human APP.[9,20,23] Reduction of Aβ from cells by these particular compounds reflects inhibition of γ-secretase, not β-secretase, for several reasons. First, the length and amino acid identity of these peptidomimetics is already known to strongly disfavor inhibition of β-secretase.[24] Second, the identification of β-secretase inhibitors that work in cell culture has been quite challenging.[25] Third, selected compounds in this series elevate APP γ-secretase substrates in cells (data not shown) and block γ-secretase activity in cell-free assays (see below). (Hydroxyethyl)ureas varied in the P1′ position showed better potency with larger substituents (leucine and phenylalanine side chains; that is, **8** and **9**), with IC_{50} values of 400 nM, suggesting a large complementary S1′ pocket. In contrast, phenylalanine was clearly disfavored in the P2′ position (**13**), by nearly two orders of magnitude compared with alanine, valine, and leucine (**10**–**12**). Thus, the S2′ active site pocket apparently cannot accommodate the larger benzyl substituent. Alterations of the P3′ position revealed a slight preference for the valine residue (**15**, $IC_{50} = 220$ nM), and removal of P3′ altogether resulted in substantial loss of potency (**17**, IC_{50} ~8000 nM). The phenylalanine and leucine residues were well tolerated (**2** and **16**), suggesting that the S3′ pocket is also relatively large and can accommodate a variety of residues.

Surprisingly, extension to the P4′ position did not lead to any gain of potency compared to no residue at all (Table 2, **15** versus **18**–**22**). These results suggest either that S4′ is a very large pocket or that the P4′ residues are not interacting with the protease. In contrast,

Table 2. Structures of (hydroxyethyl)ureas varied at P2 and P4′ and their abilities to lower Aβ production from APP-transfected CHO cells



Compd	P2	P4′	IC_{50} (μM)[a]
15	—	—	0.22
18	—	Ala	0.67
19	—	Val	0.53
20	—	Leu	0.54
21	—	Ile	0.27
22	—	Phe	0.25
23	Ala	—	0.15
24	Val	—	0.07
25	Leu	—	0.23
26	Phe	—	0.50

[a] Values are means of three experiments.

extension of the N-terminus into the P2 position resulted in a substantial increase in potency, with installation of the valine substituent (**24**) leading to a compound with a 70 nM IC_{50} value in this cell-based assay. This compound was further tested in a cell-free γ-secretase assay, which involves detergent-solubilized membrane preparations from HeLa cells and a recombinant APP-based substrate called C100-Flag.[26,27] Compound **24** (Boc-VFΨFLV-OMe) could block the generation of the proteolytic products AICD-Flag (Fig. 2) and Aβ (not shown) with an IC_{50} of about 1 nM.

We also prepared compounds with inverted alcohol stereochemistry for selected inhibitors (**2**, **15**, and **16**). The 2*R*,3*S* diastereomeric epoxide (**27**) was not commercially available but could be prepared in several steps from L-phenylalanine (Scheme 2) according to the method of Luly et al.[28] The diastereomeric (hydroxyethyl)ureas displayed an approximately 15- to 20-fold loss of potency. These results are largely consistent with the findings of Shearman and colleagues, who observed that the same stereochemical preference for hydroxyethylene peptidomimetics,[10] although the loss of potency on inversion of the alcohol stereocenter was much more dramatic (several orders of magnitude) for these compounds.

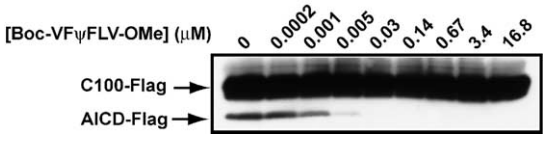
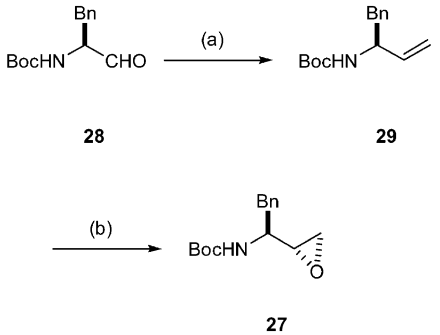


Figure 2. Ability of **24** (Boc-VFΨFLV-OMe) to inhibit γ-secretase activity in a cell-free assay. Conversion of recombinant substrate C100-Flag to AICD-Flag was visualize by western blot with M2 anti-Flag antibodies.



Scheme 2. Reagents and conditions: (a) CH_3PPh_3Br, $KN(SiMe_3)_2$; (b) *m*-CPBA.

In conclusion, (hydroxyethyl)urea peptidomimetics are readily accessible and convenient tools for studying γ-secretase. Indeed, we have already utilized such compounds for the affinity isolation and characterization of the protease complex,[27] and for testing the ability of other inhibitors to affect the γ-secretase active site.[29] These inhibitors reveal a remarkable lack of clear specificity in the S2 through S4′ pockets. Our findings are consistent with the discovery that this protease can cleave a variety of integral membrane proteins (e.g., the APP and Notch families, Notch ligands, the CD44 and Erb-B4 receptors, E-cadherins).[30] However, the phenylalanine residue appears to be strongly disfavored in the P2′ position, consistent with a previous report that artificial V715F mutagenesis of APP, two residues from the cleavage site generating the 42-residue Aβ, lowers production of this Aβ variant.[31] Similarly, mass spectral analysis of the Notch counterpart of Aβ identified several products resulting from transmembrane proteolysis, but not the fragment that would have been generated from phenylalanine in the P2′ position.[32] These observations validate the use of (hydroxyethyl)urea peptidomimetics as probes for the topography of the γ-secretase active site.

Acknowledgements

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