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Alternative Pathways for Production of Beta-Amyloid Peptides of Alzheimer's Disease

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Abstract

This highlight article describes three Alzheimer's disease (AD) presentations made at the 5th General Meeting of the International Proteolysis Society that address enzymatic mechanisms that produce neurotoxic beta-amyloid (A β) peptides. One group described the poor kinetic properties of the BACE 1 β -secretase for cleaving the wild-type β -secretase site in the APP found in most AD patients. They demonstrated that cathepsin D displays BACE 1-like specificity, is 280-fold more abundant in human brain than BACE 1, and pepstatin A inhibits cleavage of β -secretase site peptides by brain extracts and cathepsin D, but not by BACE 1. Nevertheless, as BACE 1 and cathepsin D show poor activity towards the wild type β -secretase site, they suggested continuing the search for additional β -secretase candidate(s). The second group reported that cathepsin B is such an alternative β -secretase candidate possessing excellent kinetic efficiency and specificity for cleaving the wild-type β -secretase site. Significantly, they demonstrated that inhibitors of cathepsin B improved memory function with reduced amyloid plaque neuropathology and decreased brain A β (40/42) and β -secretase activity in AD animal models expressing APP containing the wild-type β -secretase site. The third group addressed isoaspartate and pyroglutamate (pGlu) posttranslational modifications of A β that are present in AD brains, with evidence that cathepsin B, but not BACE 1, efficiently cleaves the wild-type β -secretase site containing isoaspartate. They also found that cyclization of N-terminal Glu by glutaminyl cyclase generates pGluA β (3-40/42) peptides that are highly amyloidogenic. These presentations suggested that cathepsin B and glutaminyl cyclase are potential new AD therapeutic targets.

Keywords

Alzheimer's disease; beta-amyloid; beta-secretase; cathepsins; glutaminyl cyclase; inhibitors

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Introduction

This article reports on presentations made by three independent groups led by Schechter, Hook, and Demuth on Alzheimer's disease (AD) enzyme drug targets reported at the 5th General Meeting of the International Proteolysis Society (IPS) held in Patras, Greece on October 20-24, 2007. Together these studies challenge the current hypothesis that BACE 1 is the predominant β -secretase responsible for the processing of the amyloid precursor protein. These presentations showed that (a) cathepsin D displays BACE 1-like specificity and is much more abundant than BACE 1 in the brain, yet since both enzymes cleave the wild-type (WT) β -site sequence very slowly it is necessary to search for additional β -secretase(s), (b) cathepsin B displays highly efficient processing of the WT β -secretase site of APP expressed in the majority of AD patients, and (c) the production by cathepsin B and glutamyl cyclase of posttranslationally modified forms of A β consisting of isoAsp1-A β and the highly amyloidogenic pGluA β (3-40/42). The authors of this paper represent each of the three research groups.

Beta-Amyloid of Alzheimer's Disease

Abnormal accumulation of neurotoxic A β peptides is a significant factor in the development of AD, and thought to be the likely cause of memory and cognitive loss in this disease condition (Iversen *et al.*, 1995; Sisodia, 1999; Selkoe, 2001; Gandy *et al.*, 2003). Neuropathological examination of brains from AD patients reveals the accumulation of secreted A β peptides in extracellular amyloid plaques that are involved in neuronal loss in brain regions (hippocampus and cortex) responsible for memory and cognitive functions. The majority of AD patients are afflicted with sporadic AD that is not linked to genetic mutations (Blennow *et al.*, 2006; Turner, 2006). A smaller portion of AD patients possess familial forms of AD with inherited genetic mutations, especially those mutations in APP and presenilins that result in increased A β production and memory deficit when overexpressed in transgenic mouse models of AD (Dodart *et al.*, 2002; Masliah and Rockenstein, 2000; Price and Sisodia, 1998). Notably, abnormal accumulation of A β occurs in sporadic and familial AD patients and, therefore, A β represents a biochemical hallmark of the disease condition. Clearly, strategies to reduce A β production will facilitate development of therapeutic agents for this devastating disease for which currently there is no effective drug treatment.

β -Amyloid (A β) production by proteolytic processing of APP

A β peptides are generated in neuronal secretory vesicles by proteolytic cleavage of the amyloid precursor protein (APP) by proteases, called β -secretase and γ -secretase that cleave at the N-terminus and variant C-termini of A β within APP, respectively, resulting in A β of 40 or 42 amino acids (A β 40 and A β 42, respectively) (Figure 1). Because the N-termini of A β 40 and A β 42 are identical, development of inhibitors that reduce cleavage of APP at the β -secretase site are likely to be effective for reducing A β peptide forms with alleviation of neurodegeneration and memory deficit. The APP in the vast majority of AD patients possesses the wild-type β -secretase site sequence.

Production of β -amyloid peptides involves cleavage of the β -secretase site that is located in the soluble intracellular environment, while cleavage at the γ -secretase site occurs within a transmembrane domain of the APP precursor protein (Selkoe, 2004; Haass and Selkoe, 2007). Proteolytic processing of the γ -secretase site, within the transmembrane domain, has been demonstrated in detail by studies of the presenilin γ -secretase complex in membranes (Selkoe and Wolfe, 2007). Significantly, APP in the regulated secretory vesicles (RSV) APP is present as a fully soluble protein (Tezapsidis *et al.*, 1998), with secretion of full-length APP from RSV (Efthimiopoulos *et al.*, 1996). These studies have in large part been conducted in studies of APP and β -amyloid production in the constitutive secretory pathway.

Importantly, the major portion of secreted, extracellular β -amyloid peptides is produced in the regulated secretory pathway (Tezapsidis *et al.*, 1998). In the regulated secretory vesicles (RSV), APP is also present as a fully soluble protein. The full-length soluble APP, thus, has β - and γ -secretase sites accessible for proteolytic cleavage. The approach by the Hook laboratory for examining APP processing in the RSV for producing the majority of extracellular β -amyloid peptide is unique in the field (Hook *et al.*, 2002, 2005), since most studies have examined β -amyloid peptide in the constitutive secretory pathway. The secretory vesicles of the regulated secretory pathway are distinct from those of the constitutive secretory pathway (Lodish *et al.*, 1999). Clearly, in the RSV, the soluble and transmembrane orientation of APP allows its proteolytic processing by β - and γ -secretases.

According to the amyloid hypothesis, proteolytic processing of human brain APP (Selkoe, 2001), preferentially generates A β 1-40 and to a minor degree A β 1-42. Both peptides have been believed to initiate aggregation and later deposition in the neuritic plaques characteristic for AD (Findeis, 2007). In contrast several studies recently clarified that A β 1-40 has neuroprotective functions (McGowan *et al.*, 2005; Kim *et al.*, 2007). In addition, the post mortem analysis of brain samples from sporadic Alzheimer and Down syndrome patients revealed many more A β species whose profiles differ from that in commonly used AD animal models (Roher *et al.*, 1993; Kuo *et al.*, 1997; Saido 1998).

Most abundant among these APP-derived peptides are the isoAsp1-A β and the pGlu3-A β species (Roher *et al.*, 1993; Saido 1998; Shimizu *et al.*, 2000). The isoAsp-A β and also pGlu-A β can make up more than 50% of all amyloid, depending on the site of deposition or on the soluble A β fraction of the brain (Roher *et al.*, 1993; Piccini *et al.*, 2005). In contrast to all other A β species, pGlu-A β aggregates much faster (He and Barrow, 1999) and also seeds the oligomerization and fibrillation process of the full-length A β species (Schilling *et al.*, 2006).

Abundant accumulation of A β species also occurs in brains of cognitive normal elderly patients, without signs of neuronal dysfunction. However, the most prominent species found in these people are the amyloid peptides A β 1-40 and A β 1-42 but not the pGlu3-A β species (Piccini *et al.*, 2005). The proposal that the BACE 1 product A β 1-40 is neuroprotective, combined with the high abundance of N-truncated and pyroglutamated A β peptides, which correlate to deposition markers and the severity of dementia, supports the hypothesis that there may be different pathways of APP turnover which trigger formation of different A β peptide species (Kim *et al.*, 2007; Maeda *et al.*, 2007).

For these reasons, the β -secretase activity that cleaves the wild-type β -secretase site in APP must be determined for effective β -secretase inhibitors to be developed for the majority of the AD population.

Enzymatic and kinetic properties of proteases for cleaving the wild-type β -secretase site of APP: cleavage-site specificity of proteases

The specificity for peptide substrate cleavage by proteases involves a large active site divided into subsites, using the model and nomenclature of Schechter & Berger (Schechter and Berger, 1967). This model describes interactions of the protease with the substrate amino acids flanking the cleavage site (Figure 2a). The amino acids forming the cleaved peptide bond are termed P1-↓P1', with residues at the N-terminal and C-terminal sides of the cleavage site indicated as P1, P2... and P1', P2'..., respectively. The protease active site is viewed as a series of subsites (S1, S2... and S1', S2'...) each accommodating one amino acid residue of the substrate. Protease subsites interact with the polypeptide backbone and with side chains of amino acids of the substrate.

Multiple points of interactions in several subsites are essential for obtaining the high association constants necessary for efficient biological function. Within the large active site certain subsites (close or remote from the catalytic site) play a major role in determining specificity (Schechter and Berger, 1967; Schechter, 2005). For example, the S1 subsite of trypsin has a clear preference for binding to basic P1 residues consisting of lysine or arginine (Halfon *et al.*, 2004). In papain (Schechter and Berger, 1968) and the cysteine cathepsins (Rawlings and Barrett, 2004), specificity is determined mainly by the hydrophobic S2 subsite that prefers binding to hydrophobic P2 residues. The specificity and biological activity of caspases is determined by both S1 and S4 remote from the catalytic site (Nicholson and Thornberry, 1997). Generally, the P1 to P3 residues are important for protease selectivity for cleavage sites, based on interaction with S1 to S3 subsites of the protease. Therefore, cleavage site-specific assays often incorporate the native P1 to P3 residues, or longer extensions of the peptide substrate.

Evaluation of the P2-P1 residues at the Wild-type and Swedish mutant β -secretase cleavage sites

At the wild-type β -secretase site of APP, the -P3-P2-P1-↓P1'-P2'-P3'- residues are Val-Lys-Met-↓Asp-Ala-Glu- (Figure 2b). This wild-type β -secretase site is expressed by the majority of AD patients, and, therefore, represents a key cleavage event for production of neurotoxic A β . Interestingly, a mutant β -secretase site expressed in an extended Swedish family (Citron *et al.*, 1992) results in elevated production of A β in these patients, and transgenic mice expressing the human Swedish mutant APP show elevated levels of A β (Hsiao *et al.*, 1996). The Swedish mutant P2-P1 residues consist of Asn-Leu that substitute for the wild-type P2-P1 residues of Lys-Met. It is important to realize that the amino acids at the Swedish mutant compared to the wild-type β -secretases site differ in chemical properties. A change in the chemical properties of the amino acid side chains may define distinct substrate preferences by the protease.

Notably, the wild-type Lys (lysine) residue at the P2 position is a positively charged hydrophilic residue, but the Swedish mutant Asn (asparagine) residue is an uncharged amino acid. The charged and uncharged properties of Lys and Asn, respectively, at the P2 position may be recognized by different proteases. The P2 residue that interacts with the S2 subsite of proteases is known to be an important determinant of substrate specificity for many proteases, such as the cysteine cathepsins (Rawlings and Barrett, 2004). Based on the different charged property of the P2 residue of the wild-type site compared to uncharged P2 residue of the Swedish mutant β -secretase site, it is possible that different proteases may cleave the wild-type β -secretase site compared to the Swedish mutant β -secretase site.

Kinetic properties of protease enzymes for polypeptide substrates

Kinetic properties of enzymes are studied by measuring their catalytic efficiency (Berg *et al.*, 2002; Voet and Voet, 2004). The ratio $k_{\text{cat}}/K_{\text{m}}$ represents the rate of hydrolysis at infinite dilution of the substrate (Schechter, 1970). In general, higher values of $k_{\text{cat}}/K_{\text{m}}$ indicate greater enzymatic performance towards the substrate (Berg *et al.*, 2002; Voet and Voet, 2004). Active proteases usually have $k_{\text{cat}}/K_{\text{m}}$ values that range between tens of thousands to a few millions.

These kinetic properties should be assessed in studies of proteases with β -secretase activity to identify those that possess the greatest efficiency for β -secretase cleaving activity. Because cleavage of a peptide bond may occur by several proteases, inhibition of the protease with the highest catalytic efficiency is likely to be most effective for reducing cellular A β .

Overview of the Presentations

The presentations by the Schechter, Hook, and Demuth groups together implicate new β -secretase mechanisms and posttranslational modifying enzymatic mechanisms for the production of A β peptide forms. The Schechter group demonstrates that human cathepsin D displays BACE 1-like β -secretase specificity, that cathepsin D is more abundant than BACE 1 in the brain, and that β -secretase activity observed in brain extracts at pH 3.8 is mainly due to cathepsin D, which indicates the need to search for additional β -secretase candidate(s) (Schechter and Ziv, 2008).

The Hook group showed that cathepsin B efficiently cleaves the wild-type β -secretase site (Hook *et al.*, 2005), and cathepsin B is colocalized with A β peptides in regulated secretory vesicles (Hook *et al.*, 2005) that provide the majority of extracellular A β that accumulates in AD. Moreover, inhibitors of cathepsin B improve memory, with reduction of brain A β and amyloid plaques, in the London APP mouse model of AD expressing the wild-type β -secretase site of APP (Hook *et al.*, 2008). These inhibitors also reduced A β in the guinea pig animal model that expresses APP containing the wild-type β -secretase site (Hook *et al.*, 2007b; Hook *et al.*, 2007a). These findings support cathepsin B as a new β -secretase target for drugs to reduce A β that contributes to memory deficit in AD.

The Demuth group discussed the production of posttranslationally modified A β forms in AD brain, consisting of isoaspartate (isoAsp) A β , isoA β (40/42), and N-terminal truncated peptides including the pyroglutamate (pGlu) modified form of truncated A β , pGluA β (3/11-40/42). Evidence was provided that cathepsin B, but not BACE 1, efficiently cleaves the wild-type β -secretase site containing isoAsp and that glutaminyl cyclase catalyzes the formation of pGlu to produce pGluA β (3/11-40/42). These findings independently confirmed cathepsin B as a possible drug target to reduce A β production, and also suggests glutaminyl cyclase as a new drug target for inhibiting the production of pGluA β (3/11-40/42) peptide forms.

Properties of BACE 1 and Cathepsin D Indicate the Need to Search for Additional β -Secretase Candidate(s) (presented by I. Schechter)

BACE 1 fulfills many criteria for the β -secretase that cleaves at the β -site of APP (Lin *et al.*, 2000; Vassar *et al.*, 1999; Wolfe, 2006), yet it has some problems that are described below (Schechter and Ziv, 2008).

Poor kinetic properties of BACE 1 for the wild-type β -secretase site

BACE 1 cleaves the Swedish mutant β -secretase site sequence of APP much better than the wild type β -secretase sequence present in the worldwide AD population. BACE 1 activity for the wild-type β -site is extremely low, cleaving it with very low k_{cat}/K_m values reported as $40 \text{ M}^{-1}\text{s}^{-1}$ (Lin *et al.*, 2000) and $62 \text{ M}^{-1}\text{s}^{-1}$ (Shi *et al.*, 2001). Proteases acting on relevant substrates usually have k_{cat}/K_m values that range between tens of thousands to a few millions (Dunn and Hung, 2000).

BACE 1 is a membrane-bound enzyme and the kinetic studies described above (Lin *et al.*, 2000, Shi *et al.*, 2001) were performed *in vitro* using soluble BACE 1. It has been argued that enzymes anchored in the cell membrane might exhibit reduced activity when freed in solution. TACE is a membrane-bound enzyme like BACE 1, and *in vivo* it cleaves the precursor TNF- α that is a membrane-bound protein like APP. Yet *in vitro* TACE cleaves a peptide spanning the cleavage site of the precursor TNF- α with k_{cat}/K_m of $1.8 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ (Moss *et al.*, 1997), which shows that solubilized TACE retains its enzymatic efficiency. Furthermore, mapping of the active site of BACE 1 led to synthesis of an excellent peptide substrate cleaved by BACE 1 with k_{cat}/K_m of $3.42 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ (Turner *et al.*, 2001). This peptide differs from

the wild-type β -secretase site octapeptide sequence (P4-P4') in 7 out of 8 amino acid residues. These findings suggest that BACE 1 acts on yet unknown substrate(s), and that its cleavage of the wild-type β -secretase site of APP may be a physiological aberration.

Gene knockout experiments in rodents and corresponding genetic defects in human may reveal different phenotype(s)

Several BACE 1 knockout studies conducted in mice expressing human APP show that the absence of BACE 1 reduces A β formation (Cai *et al.*, 2001; Luo *et al.*, 2001, Roberds *et al.*, 2001). These important findings support BACE 1 as a β -secretase. Yet studies in other systems show that gene knockout experiments in rodents may show different phenotypes compared to the corresponding genetic phenotype in the human disease condition (Jacks, 1996). For example, nine different tumor suppressor genes associated with human tumors were mutated in mouse and rat. Two mutated genes represented the human phenotype, five mutated genes showed a different spectrum of tumors, and two mutated genes did not show any tumors in rodents (Jacks, 1996). These studies show that genetic phenotypes in animal models may or may not represent the human disease phenotype.

Considering the above issues, regarding the poor activity of BACE 1 and knockout of BACE 1 in the context of knockout of other genes, we searched for other β -secretase candidate(s) in the brain.

Cathepsin D displays BACE 1-like specificity as β -secretase: their properties indicate the need to search for additional β -secretase candidate(s)

The Schechter group searched for β -secretase in brain utilizing assays of β -secretase activity with peptide substrates containing the wild-type and mutant Swedish β -secretase site sequences. Beta-secretase activity was purified from brain extracts (bovine) and identified as cathepsin D. Although early studies implicated cathepsin D in Alzheimer's disease (Ladror *et al.*, 1994; Cataldo *et al.*, 1995), the finding that knockout of the cathepsin D gene in mice did not affect A β formation (Saftig *et al.*, 1996) abolished interest in cathepsin D. However, in consideration of the relevance or irrelevance of animal gene knockout studies to the human genetic disease phenotype, further studies of cathepsin D were warranted. Thus, we studied the properties of cathepsin D compared to BACE 1 that were not examined in prior studies (Schechter and Ziv, 2008).

The kinetic constants k_{cat}/K_m for cleaving β -secretase sites of peptides with the Swedish mutant and wild-type sequences by cathepsin D and BACE 1 were determined (Table 1). Results showed that human BACE 1 and cathepsin D have similar kinetic constants, cleaving the Swedish mutant β -secretase site more efficiently than the wild-type sequence. Gel filtration experiments of brain extracts revealed β -secretase activity at the position of cathepsin D (42 kDa) but not at that of BACE 1 (72 kDa). Pepstatin A at 20 nM completely inhibits the cleavage of peptide substrates with the Swedish mutant β -site by both cathepsin D and brain extracts, while BACE 1 activity on the same peptides is not inhibited by one thousand fold higher concentration of pepstatin A (20 μ M). Quantitative Western blots show that in human brain cathepsin D is about 280-fold more abundant than BACE 1. Therefore, it is conceivable that β -secretase activity observed in brain extracts may be primarily due to cathepsin D and not BACE 1.

Nevertheless, since both BACE 1 and cathepsin D show poor kinetic activity towards the wild-type β -secretase site sequence (Table 1), it is necessary to continue the search for additional β -secretase candidate(s) for cleaving the wild-type β -secretase site (Schechter and Ziv, 2008).

Cathepsin B as a β -Secretase of the Wild-Type β -Secretase Site and Cathepsin B Inhibitors as Potential Alzheimer's Disease Therapeutics (presented by V. & G. Hook)

The Hook group reported that cathepsin B may represent such a novel β -secretase for wild-type β -secretase site cleavage. Moreover, they showed that inhibitors of cathepsin B are efficacious to improve memory in animal models expressing APP containing the wild-type β -secretase site and suggest that such inhibitors may have potential as AD therapeutics.

A β production in the regulated secretory pathway of neurons

Neurons possess two distinct secretory pathways consisting of the regulated secretory pathway for activity-dependent secretion, and the constitutive secretory pathway for continuous basal secretion (Thomas, 2002; Fugere and Day, 2005; Seidah *et al.*, 1994; Zhou *et al.*, 1999) (Figure 1). Different proteolytic mechanisms occur in the two secretory pathways (Gensberg *et al.*, 1998; Lodish *et al.*, 1999; Gumbiner and Kelly, 1982; Docherty and Steiner, 1982; Gainer *et al.*, 1985; Benjannet *et al.*, 1997; Hook *et al.*, 2004; Thomas, 2002) and thus, it is important to define the primary secretory pathway(s) involved in A β production for elucidation of the major proteases that generate secreted A β , which accumulates in amyloid plaques of AD brains. Such studies indicate that regulated secretion of A β from brain neurons *in vivo* produces most of the secreted A β (Farber *et al.*, 1995; Cirrito *et al.*, 2005; Efthimiopoulos *et al.*, 1996; Hook *et al.*, 2002; Nitsch *et al.*, 1993; Nitsch *et al.*, 1992; Hook *et al.*, 2005; Jolly-Tornetta *et al.*, 1998). Therefore, β -secretase was studied in regulated secretory vesicles that contain A β .

Cleavage site specific assay for cleaving the wild-type β -secretase site leads to identification of cathepsin B in regulated secretory vesicles for A β production

In search of endogenous protease activity for cleaving the wild-type β -secretase site, a cleavage site-specific assay was developed with the Z-Val-Lys-Met- $\downarrow$ MCA substrate that mimics the P1 to P3 residues of the wild-type β -secretase site. This peptide-MCA substrate monitors β -secretase activity by proteolytic production of fluorescent MCA (methylcoumarinamine). The Z-Val-Lys-Met- $\downarrow$ MCA cleaving activity in regulated secretory vesicles, purified from neuronal-like chromaffin cells, showed a pH optimum of approximately pH 5.5 to 6.0 that is consistent with the acidic intravesicular environment (Hook *et al.*, 2002). Moreover, its activity required reducing conditions, which mimics the *in vivo* reducing environment of secretory vesicles (Hook *et al.*, 2002). The Z-Val-Lys-Met- $\downarrow$ MCA cleaving activity was represented by cysteine protease activity, since it was inhibited by E64c (Hook *et al.*, 2002).

The Z-Val-Lys-Met- $\downarrow$ MCA cleaving activity was purified from the regulated secretory vesicles (RSV) by a series of chromatographic purification steps. Identification of the enzyme was facilitated by use of the activity-based probe, DCG-04, that detects active cysteine proteases by irreversible binding to the active site (detected by avidin-biotin) (Hook *et al.*, 2005). Peptide sequencing of the purified enzyme revealed its identity as the cysteine protease cathepsin B (Hook *et al.*, 2005). Immunoelectron microscopy confirmed the localization of cathepsin B within regulated secretory vesicles (Hook *et al.*, 2005). In contrast with the known function of cathepsin B in lysosomes for protein degradation (Mort, 2004), these findings indicate the secretory vesicle as a new subcellular site for a biological function of cathepsin B.

Notably, inhibition of cathepsin B with cysteine protease inhibitors substantially reduced A β production in the isolated RSV (Hook *et al.*, 2005). A β 40 production was reduced by the highly selective cathepsin B inhibitor CA074 (Towatari *et al.*, 1991), as well as by the general cysteine protease inhibitor E64c (Towatari *et al.*, 1991). Treatment of chromaffin cells in primary culture with CA074Me, the-cell permeable form of CA074, also resulted in reduced amounts

of A β produced in the regulated secretory pathway (but not the constitutive secretory pathway) (Hook *et al.*, 2005). In addition, the inhibitor lowered cellular levels of CTF β derived from APP cleavage by β -secretase, suggesting that β -secretase activity was inhibited. These findings indicate cathepsin B as a novel β -secretase in the regulated secretory pathway for production of secreted A β .

Kinetic properties of cathepsin B demonstrate its preference for the wild-type β -secretase site, rather than the Swedish mutant β -secretase site

Kinetic studies demonstrated the high activity and preference of cathepsin B for cleaving the wild-type β -secretase site, but not the Swedish mutant β -secretase site (Table 2) (Hook *et al.*, 2008). The k_{cat}/K_m value for cathepsin B cleavage of the wild-type β -secretase substrate is $3.17 \times 10^5 \text{ M}^{-1}\text{sec}^{-1}$, indicating that cathepsin B efficiently cleaves the wild-type β -secretase substrate with high turnover of substrate to product.

However, cathepsin B showed very low activity with the Swedish mutant β -secretase substrate Z-Val-Asn-Leu-DMCA (mutant residues underlined). This finding is in marked contrast to BACE 1, which shows a preference for the Swedish mutant and not the wild-type β -secretase substrate. These biochemical kinetic studies demonstrate the high preference of cathepsin B for the wild-type β -secretase site, rather than the Swedish mutant site of APP. Significantly, the wild-type β -secretase site is relevant to the majority of the AD population, which expresses wild-type APP.

Animal models that express the wild-type β -secretase site of APP for inhibitor studies

The guinea pig and a transgenic mouse model of AD expressing human APP with the wild-type were used to evaluate the effects of cathepsin B inhibitors on A β production. The guinea pig (gp) expresses APP with the wild-type β -secretase site that generates A β , which is identical to the primary sequence of human A β (Johnstone *et al.*, 1991; Beck *et al.*, 2003; Beck *et al.*, 1997). Transgenic AD mice expressing human APP containing the wild-type β -secretase site, with the London mutation near the γ -secretase site (London APP mice), overproduce A β in amyloid plaques and develop memory deficit (Moechars *et al.*, 1999; Dewachter *et al.*, 2000). The preference of cathepsin B for cleaving the wild-type β -secretase site predicted that cathepsin B inhibitors would be effective in these animal models expressing APP containing the wild-type β -secretase site, but not those expressing APP with the Swedish mutant β -secretase site. This prediction was tested with animal models expressing wild-type and Swedish mutant β -secretase sites of APP (Hook *et al.*, 2007b; Hook *et al.*, 2007a; Hook *et al.*, 2008).

Inhibitors of cathepsin B reduce A β in guinea pig brains

In vivo studies in guinea pigs demonstrated significant reduction of A β in brain by inhibitors of cathepsin B consisting of CA074Me (prodrug form of the selective cathepsin B inhibitor, CA074), E64d (the ester prodrug of its biologically active acid form, E64c) (Tamai *et al.*, 1986), and acetyl-L-leucyl-L-valyl-L-lysinal (Ac-LVK-CHO) (McConnell *et al.*, 1993). Administration (icv) of E64d or CA074Me resulted in substantial reductions of A β 40 and A β 42 by 50-70% after 7 or 30 days of treatment (Figure 3), and similar reductions of these A β peptides resulted after treatment with Ac-LVK-CHO (Hook *et al.*, 2007b; Hook *et al.*, 2007a). The inhibitor treatments also reduced CTF β , suggesting that the inhibitors reduced β -secretase activity. Moreover, the inhibitor treatment reduced A β levels in brain synaptosomes, which are enriched with RSV, thus, indicating inhibition of A β production in the regulated secretory pathway of brain neurons (Hook *et al.*, 2007b). These data demonstrate the *in vivo* effectiveness of cathepsin B inhibitors to reduce brain A β levels produced by cleavage of the wild-type β -secretase site of APP in the regulated secretory pathway.

Inhibitors of cathepsin B improve memory and reduce A β in transgenic AD mice that express the wild-type, but not the Swedish mutant, β -secretase site of APP

In the London APP transgenic mouse model of AD that expresses human APP with the wild-type β -secretase site, administration of E64d or CA074Me for 30 days significantly improved the memory deficit (measured by the Morris water maze test) and reduced brain amyloid plaque (Hook *et al.*, 2008). These inhibitors improved memory by about 50%, which approached the normal level of memory function in non-transgenic age matched controls (Figure 4a). Each inhibitor treatment also reduced brain amyloid plaque load by about 50% and reduced brain A β by about 40-50% (Figure 4b). Furthermore, the reduction of CTF β by these inhibitors indicated inhibition of β -secretase activity.

In contrast, inhibitors of cathepsin B had no effect in a transgenic AD mouse model expressing the Swedish mutant β -secretase site of APP (Hook *et al.*, 2008). In London APP mice that also express the Swedish mutation (Swedish/London APP), treatment with E64d or CA074Me had no effects on memory, brain amyloid plaque, brain A β , or CTF β . The *in vivo* effects of these cathepsin B inhibitors mirrored their *in vitro* cleavage specificity for the wild-type β -secretase site compared to the Swedish mutant β -secretase site. These data support the hypothesis that cathepsin B possesses selectivity for cleaving the wild-type β -secretase site, rather than the Swedish mutant site of APP, for production of neurotoxic A β peptides. Importantly, cathepsin B cleavage of the wild-type β -secretase site is relevant to the majority of the AD population that express wild-type APP.

The function of cathepsin B as β -secretase is not precluded by BACE 1 knockout mouse results

Results presented by the Hook group, combined with knowledge in the field, suggest that cathepsin B and BACE 1 both function as β -secretase enzymes. While BACE 1 has been proposed to be the major β -secretase based on BACE 1 knockout experiments (Cai *et al.*, 2001; Luo *et al.*, 2001; Roberds *et al.*, 2001; Vassar, 2004), detailed assessment of the BACE 1 knockout studies indicate that they do not preclude cathepsin B as a β -secretase for the wild-type β -secretase site in the regulated secretory pathway.

Roberds *et al.* describes that BACE 1 knockout mice lack β -secretase activity but the assays in which the β -secretase activity was assayed contained E64, a potent inhibitor of cysteine proteases, which precluded detection of cathepsin B β -secretase activity in the assay (Roberds *et al.*, 2001). Thus, Roberds *et al.* does not rule out cathepsin B as a β -secretase.

Luo *et al.* found that knocking out BACE 1 in mice expressing human APP containing the Swedish mutant β -secretase site resulted in no A β or C99 production (CTF β) (Luo *et al.*, 2001). However, this study did not evaluate wild-type β -secretase site cleavage. Thus, Luo *et al.* does not rule out cathepsin B cleavage of APP containing the wild-type β -secretase site.

Cai *et al.* found that 'conditioned media' lacked A β from embryonic brain neurons of BACE 1 knockout animals expressing APP containing the wild-type β -secretase site (Cai *et al.*, 2001). However, the 'conditioned media' reflected only constitutive secretion because the neurons were not stimulated with a triggering agent and, therefore, no regulated secretion occurred. Thus, Cai *et al.* does not exclude cathepsin B β -secretase activity in the regulated secretory pathway.

Taken together, the knockout BACE 1 experiments do not address cysteine protease β -secretase cleavage of APP containing the wild-type β -secretase site in the regulated secretory pathway. Therefore, cathepsin B β -secretase activity can be reconciled with BACE 1 knockout data because that data does not rule out cathepsin B as a possible β -secretase.

Interestingly, a recent study examined the role of BACE 1 on endogenous *in vivo* levels of A β in brain to address mechanisms for A β production in sporadic AD, where there is no overexpression of APP (Hirata-Fukae et al., 2008). In adult mice, overexpression of BACE 1, by approximately 12-fold greater than that expressed in wild-type mice, did not result in elevated endogenous A β . Thus, factors other than BACE 1 must be involved in A β production in adult and aging brain, and such factors may provide therapeutic targets. These findings are consistent with the proposal for cathepsin B as a candidate β -secretase for production of A β in adult conditions (Hook et al., 2007a, b; Hook et al., 2008).

Inhibitors of cathepsin B as potential therapeutic agents for Alzheimer's disease

Key findings from studies of cathepsin B in regulated secretory vesicles for the cleavage of the wild-type β -secretase site of APP indicate cathepsin B as a newly identified drug target for AD. The notable efficacy of the CA074Me and E64d inhibitors to improve memory and reduce A β in an AD animal model expressing the wild-type β -secretase APP site present in the majority of AD patients provides support for these inhibitors as potential AD therapeutic agents.

β -secretase and Glutaminyl Cyclase Generation of Post-translationally modified IsoAspartate and Pyroglutamate Forms of A β (presented by H.-U. Demuth)

Analyses of the peptide forms of A β in amyloid plaques of AD brains have identified post-translationally modified forms of A β . These modified A β peptides consist of A β with isoaspartate instead of aspartate as the N-terminal residue (IsoAsp-A β (1-40/42)), and the truncated peptide with pyroglutamate (pGlu) of the pGluA β (3/11-40/42) peptides. The Demuth group elucidated the cathepsin B β -secretase and glutaminyl pathway utilized to generate modified A β peptides.

Formation of IsoAsp-A β (1-40/42) involves cathepsin B but not BACE 1

A β peptides containing the modified aspartate residue, isoaspartate (isoAsp), at the N-terminal amino acid of A β (1-40/42) are highly abundant in AD amyloid depositions. They can make up over 50% of all A β found, depending on the particular tissue (Shimizu *et al.*, 2005). The formation of isoAsp within a peptide chain is a chemically spontaneously occurring process during aging of proteins (for a recent review see Shimizu *et al.*, 2005). Aspartic acid (Asp) or asparagine (Asn) residues within a peptide chain can undergo intramolecular cyclization with release of water or ammonia, respectively (Figure 5). The generated metastable succinimide spontaneously decomposes to yield L-isoAsp, L-Asp and D-isoAsp and D-Asp in an approximate ratio of 70:20:5:5 (Vigneswara *et al.*, 2006). The tendency to form the intramolecular succinimide depends markedly on the amino acids located at the C-terminal side of the Asp/Asn residue. It was found that the velocity of isoAsp formation is much faster if small amino acids (e. g. glycine, histidine, serine or alanine) are located at this position (Galletti *et al.*, 1995). The formation of isoAsp introduces an additional methylene group in the peptide backbone, which could alter the structure and function of proteins. Moreover, this change in the peptide backbone could affect the susceptibility of the proteins for cleavage by proteases as demonstrated for prolyl endopeptidase or the proteasome (Tarcza *et al.*, 2000).

To address the question of what β -secretase may cleave the isoAsp β -secretase site of APP, internally quenched fluorescent peptide substrates containing isoAsp at the wild-type and Swedish mutant β -secretase site (RE(Edans)EVKM(or **NL**) **D**(or **isoD**) AEFK(Dabcyl)R-NH₂) were assessed for cleavage by BACE 1 and cathepsin B. Interestingly, cathepsin B showed excellent catalytic efficiency to cleave *in vitro* the isoAsp wild-type β -secretase peptide with a k_{cat}/K_m value of $1.83 \times 10^4 \text{ M}^{-1}\text{sec}^{-1}$, but, in contrast, BACE 1 did not cleave that

substrate at all (Table 3). Moreover, the cathepsin B inhibitor, Ac-LVK-CHO, inhibited cleavage of the wild-type β -secretase substrate by cell extracts, whereas the BACE 1 inhibitor KTEEISEVN(Statine)VAEF had no effect. These data suggest the possibility that cathepsin B, rather than BACE 1, produces isoAsp-A β in AD patients by cleaving the wild-type β -secretase site containing isoAsp (Böhme *et al.*, 2008).

Further evaluation of β -secretase activities utilized longer, more structured peptides, consisting of unlabelled 27 amino acid long peptides Ac-EEISEVKM(NL)D(isoD)AEFRHDSGYEVHHQKLVF-NH₂ covering the β cleavage site from amino acid -8 to 19 (A β numbering, corresponding to APP 664-690) were synthesized. Hydrolysis of these peptides by BACE-1, cathepsin B and SH-SY5Y cell extracts was analyzed by MALDI-TOF mass spectrometry (Demuth *et al.*, 2007; Böhme *et al.*, 2008). The incubation of the peptides with BACE-1 for up to 24 hours supported the findings obtained with the 12 amino acids long internally quenched fluorescent peptide substrates. A cleavage pattern characteristic for BACE-1 (prior to Asp1) could only be observed in the case of the SW peptide (-L↓D-). This hydrolysis was abolished during coincubation of peptide, BACE-1 and the specific BACE-1 inhibitor. However, the isoAsp-containing 27 amino acid long peptides are not cleaved by BACE-1 but in contrast, cathepsin B hydrolyses the isoAsp containing substrates even more readily than the L-Asp containing variants. Incubating the peptides with neuroblastoma cell extract resulted in a cleavage pattern that was basically the same as with the purified enzymes, and substrate cleavage could be blocked by the cathepsin B inhibitor Ac-LVK-CHO (Böhme *et al.*, 2008). Similar as with the 12 amino acid long peptides, cathepsin B cleavage occurred within the longer peptides preferentially after position P2 of the substrates which corresponds to penultimate amino acid of the Asp or isoAsp-linkage (i.e. in WT at -K↓MDA- or -K↓MisoDA- and in the Swedish sequences at -N↓LDA- or -N↓LisoDA-). This cleavage pattern clearly differentiates the cathepsin B mediated hydrolysis of APP-like substrates from the known BACE-1 mediated APP hydrolysis. The data clearly corroborate the results obtained by Hook *et al.*, 2005 and by Schechter and Ziv, 2008. However, in contrast to BACE-1 and cathepsin D, the turnover of longer substrates by cathepsin B takes place not at the common APP 672 cleavage site. Moreover, posttranslational modifications such as the isoAsp formation, which is highly abundant in APP as well as in deposited A β in AD, even augment the unexpected cathepsin B cleavage pattern. In fact, this could be one reason for the different metabolic fate of APP leading to further N-terminal truncated and modified, neurotoxic A β peptide species such as pyroGluA β (Russo *et al.*, 2002; Cynis *et al.*, 2006; Cynis *et al.*, 2007).

Truncated pyroGluA β (3-40/42) peptide forms in senile plaques of AD brains

Initial studies of A β peptide forms in AD brains identified A β (1-40) and the C-terminally extended A β (1-42) peptide forms of β -amyloid in amyloid plaques (Saido *et al.*, 1996; Saido *et al.*, 1995). In addition to differences in C-termini of A β forms, N-terminal peptide heterogeneity represents a significant portion of A β in AD brains. Interestingly, a greater number of plaques positive with antibodies directed to pGluA β (3-40/42) was observed in AD compared to normal age-matched brains. These data suggest that the deposition of pGluA β (3-40/42) may precede that of A β (1-40/42).

Notable results were obtained from analyses of the seeding of A β peptides to form oligomers, showing that the pGlu-modified A β peptides accelerated initial acceleration of aggregate formation by up to 250-fold compared to unmodified A β (Schilling *et al.*, 2006). Furthermore, results suggested that pGlu-modified A β peptides promote seeding of mixed A β peptide aggregates. These biophysical characteristics of pGlu-amyloid peptides may be crucial for the initial development of AD.

Glutaminyl cyclase (QC) mediates N-terminal pyroglutamate cyclization to generate pGluA β (3-40/42)

N-terminal pyroglutamate (pGlu) modification of A β (3-40/42) by glutaminyl cyclase (QC) is predicted to generate pGluA β (3-40/42) (Figure 6). Inhibition of glutaminyl cyclase reduces formation of pGluA β (3-42), thus, revealing the importance of QC activity for maturation of cellular pGlu-containing A β peptides (Schilling *et al.*, 2004; Cynis *et al.*, 2006).

Recent *in vivo* studies showed that administration of a QC inhibitor prevented the formation of pGlu-containing A β peptides in brains of rats, as well as in a transgenic mouse model of AD. Importantly, the QC inhibitor improved memory during reduction of pGluA β peptides. These findings implicate the importance of QC in catalyzing the production of neurotoxic pGlu-modified A β peptides (Schilling *et al.*, 2007; Schilling *et al.*, 2008).

Evidence that formation of pGlu-A β peptides prefers utilization of the β -secretase pathway derived from wild-type APP, rather than the Swedish mutant APP

Because formation of N-terminally truncated pGluA β (3-42) may be a key event in the development of AD, it is important to understand cellular mechanisms that generate these forms of A β from APP. Recent experiments have compared the formation of pGluA β (3-40/42) and A β (1-40/42) from wild-type and Swedish mutant APP forms by expression in cells (HEK293 cell line). Results showed that a larger amount of pGluA β (3-40/42) was produced from wild-type human APP and human London APP, both of which express the wild-type β -secretase site. However, a larger amount of A β (1-40/42) was generated from Swedish mutant APP or Swedish/London APP, both of which possess the Swedish mutant β -secretase site. Furthermore, production of pGluA β (3-40/42) from wild-type APP was not reduced by an inhibitor of BACE 1. These findings suggest that a β -secretase other than BACE 1 participates in the formation of pGluA β (3-40/42) from wild-type APP. Based on the studies of this article describing a role for cathepsin B in A β production utilizing the wild-type β -secretase site of APP, it will be of interest in future studies to examine the possibility that cathepsin B may be involved in the production of pGlu forms of A β (Cynis *et al.*, 2007; Cynis *et al.*, 2008).

Summary

This paper summarizes the work presented by three independent groups at the 5th General Meeting of the IPS on potential AD drug targets. The combined data support the existence of β -secretases other than BACE 1 and provide evidence for novel enzymatic pathways mediated by cathepsin B as β -secretase and glutaminyl cyclase production of pGlu-modified forms of A β . BACE 1 and the more abundant cathepsin D cleave the wild-type β -secretase site with low efficacy. Notably, cathepsin B specifically and efficiently cleaves the wild-type β -secretase site present in the majority of AD patients. Subsequent to such a cathepsin B β -secretase step, glutaminyl cyclase modification of truncated A β (3-40/42) can generate modified pGluA β (3-40/42). These findings provide evidence for cathepsin B and glutaminyl cyclase as potential new drug targets to inhibit production of multiple A β peptide forms in Alzheimer's disease.

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A β Production in Regulated and Constitutive Secretory Pathways of Neurons

Figure 1. A β production from APP in neurons via regulated and constitutive secretory pathways A β peptides are generated by proteolytic processing of the amyloid precursor protein (APP) in secretory vesicles that undergo axonal transport from the neuronal cell body to nerve terminals, where A β is secreted. Extracellular A β peptides accumulate as oligomers and aggregates in amyloid plaques, and cause loss of memory in Alzheimer's disease. Cleavage at the N-terminus of A β within APP is achieved by proteases known as β -secretases, and cleavage at the C-termini of A β within APP is achieved by γ -secretases. Proteolytic processing by β - and γ -secretases results in A β peptides of 40 and 42 residues, known as A β (1-40) and A β (1-42). In addition, APP can be modified to contain isoaspartate (isoAsp) residues, which generates A β containing isoAsp, isoA β (40/42). Moreover, A β can be truncated and the terminal glutamate residue modified to pyroglutamate (pGlu) to form pGluA β (3-40/42). The β -secretases that produce these various forms of A β and the enzyme which generates pGlu, are the subject of this report.

(a) Schechter and Berger Nomenclature: Protease and Peptide Substrate Interactions

(b) Wild-Type β -Secretase Cleavage Site: P3 to P3' Residues

Figure 2. Model of protease and peptide substrate interactions: application to the wild-type β -secretase cleavage site

a. Schechter and Berger model and nomenclature for protease active site interactions with peptide substrate. The active site of the enzyme is composed of several subsites. The scheme shows an active site of six subsites, termed S1 to S3 and S1' to S3'. Subsites are located on both sides of the catalytic site and are numbered from this point in either direction. The positions of amino acid residues of the hexapeptide substrate are counted from the point of cleavage and thus have the same numbering as the subsites they occupy (P1 to P3 and P1' to P3'). Cleavage always occurs between P1 and P1' (Schechter and Berger, 1967).

b. P3 to P3' residues at the wild-type β -secretase cleavage site. Cleavage of the wild-type site β -secretase site of APP occurs between Met-↓Asp which represent the P1-P1' residues. The Val-Lys-Met residues represent the P3, P2, and P1 residues, respectively; the Asp-Ala-Glu residues are the P1', P2', and P3' residues, respectively.

Inhibitors of Cathepsin B Reduce A β in Guinea Pig Brain

Figure 3. *In vivo* administration of CA074Me, E64d or Ac-LVK-CHO reduces brain A β and CTF β in the guinea pig

E64d or CA074Me were administered by constant icv infusion into guinea pig brains by ALZET minipumps for 7 days or 30 days. Brain extracts were prepared and total A β (A β 40 and A β 42) were measured by ELISA assays.

(a) **E64d reduces brain A β levels.** E64d treatment for 7 or 30 days reduced the total A β by 31% and 55% relative to control, respectively (diagonal striped bars). Results are shown as mean $\pm$ SEM (pg/mg protein), with * indicating a significant difference from controls ($p < 0.05$, student's t-test).

(b) **CA074Me reduces brain A β levels.** *In vivo* CA074Me treatment for 7 or 30 days reduced total A β by 57% and 62% relative to control, respectively (solid bars). After 7 days of treatment, CA074Me significantly reduced total A β . Data are shown as mean $\pm$ SEM (pg/mg protein),

with statistical significance with * indicating a significant difference from controls ($p < 0.05$, student's t-test).

Inhibitors of Cathepsin B Improve Memory in the London/APP Mouse Model of AD

a Morris Water Maze Test for Memory

b Amyloid Plaque Load

Figure 4. *In vivo* administration of CA074Me or E64d improves memory and reduces brain plaque in transgenic mice expressing human APP containing the wild-type β -secretase site

a. Inhibitors of cathepsin B improve memory deficit in the London APP mouse model of AD. The CA074Me and E64d inhibitors of cathepsin B were administered (icv) to the London APP mice that express human APP with the wild-type β -secretase site and a mutation near the γ -secretase site. The constant icv administration into the brain utilized Alzet minipumps for 28 days. Mice were then evaluated for the status of memory by the Morris water maze test; the latency period measures the time required for the animal to swim to a submerged platform after training to learn of the platform location, with shorter times reflecting improved memory. The mean latency times and percent standard errors (% SEM) are shown for the control (vehicle

treated), CA074Me-treated, and E64d-treated London APP animals. The statistical significance between the means for the London APP animals of each treated group and the control is indicated (***) $p < 0.0001$, student's t-test). The mean latency period for untreated wild-type, strain-matched, non-transgenic mice is shown as a dashed line. Thus, the CA074Me and E64d treatments significantly shortened the latency period by 42% and 53%, respectively, relative to the London APP controls and restored about 68% and 86%, respectively, of the memory of wild-type non-transgenic mice.

b. Inhibitors of cathepsin B reduce amyloid plaque load in the London APP mice. Reduction of total amyloid plaque load occurred in brains after treatment of London APP mice with CA074Me or E64d inhibitors. After treatment with these inhibitors (as described in previous paragraph), amyloid plaques in brain were revealed by anti-A β immunohistochemistry of brain sections. Quantitation of relative amyloid plaque load in the entire brain section was achieved by computer image analyses, expressed as percent of the tissue area. The relative amyloid loads are displayed as the mean (% tissue area) and SEM. Statistical significance between each inhibitor-treated group and control is indicated (***) $p < 0.0001$, student's t-test). Inhibitor treatments reduce the plaque load by about 45%.

Formation of Peptidyl-isoAsp-Peptides

Figure 5. Scheme of Peptidyl-isoAspartyl-Peptide formation

The spontaneous occurring chemical rearrangement of asparagine or aspartate residues depends on the structure of the adjacent C-terminal amino acid. It proceeds very fast if Asp is followed by small aliphatic amino acids such as Gly, Ser or Ala (half-times of minutes). The majority of the isoAsp-residues have been found on the protein surface, since the more rigid protein core prevents this intramolecular reaction. The enzyme Peptidyl-isoAspartyl Methyltransferase can convert only within a peptide chain isoAsp back into Asp (Clarke, S., 2003).

Glutaminyl Cyclase Catalyzed Formation of pGlu-Peptides from N-Gln or N-Glu-Precursors

Figure 6. Pyroglutamate modification of A β peptides by glutaminyl cyclase

The N-terminal glutamate residue of A β (3-40/42) peptide undergoes modification to pyroglutamate (pGlu) by glutaminyl cyclase to generate pGluA β (3-40/42) peptide forms of A β . Glutaminyl cyclase catalyzes the formation of pGlu-peptides from N-Glu- or N-Gln-peptide precursors. The resultant modified pGluA β (3-40/42) peptides represent a significant portion of A β peptides of amyloid plaques in Alzheimer's disease brains (Roher *et al.*, 1993; Saido *et al.*, 1995; Saido *et al.*, 1996; Schilling *et al.*, 2004).

Table 1

The $k_{\text{cat}}/K_{\text{m}}$ values for cleavage of the β -secretase sites of Swedish mutant (SW) and wild-type (WT) peptide substrates by human cathepsin D and human BACE 1

Enzyme	Substrate ¹	$k_{\text{cat}}/K_{\text{m}}$ ² ($\text{M}^{-1}\text{s}^{-1}$)	Ratio SW/WT
<i>10-Residue Peptide Substrate</i>			
<i>Swedish mutant β-secretase site substrate:</i>			
Cathepsin D	SEVNL-↓DAEFR	5,300	180
BACE 1	SEVNL-↓DAEFR	3,310	71
<i>Wild-type β-secretase site substrate:</i>			
Cathepsin D	SEVKM-↓DAEFR	29.5	
BACE 1	SEVKM-↓DAEFR	46.6	
<i>14-Residue Peptide Substrate:</i>			
<i>Swedish mutant β-secretase site substrate:</i>			
Cathepsin D	EEISEVNL-↓DAEFRG	28,500	212
BACE 1	EEISEVNL-↓DAEFRG	10,300	182
<i>Wild-type β-secretase site substrate:</i>			
Cathepsin D	EEISEVKM-↓DAEFRG	134	
BACE 1	EEISEVKM-↓DAEFRG	78.5	

¹Peptide substrates with the Swedish mutant (SW) β -secretase site or the wild-type (WT) β -secretase site are shown with their amino acid sequence. The underlined NL residues indicate the Swedish mutations. Cleavage of these peptides at the SW or WT sites (indicated by ↓) were assessed for $k_{\text{cat}}/K_{\text{m}}$ values.

²The $k_{\text{cat}}/K_{\text{m}}$ values for Cathepsin D and average values for BACE 1 are from Schechter and Ziv (2008).

Table 2Kinetic properties of cathepsin B and BACE 1 for cleaving the wild-type β -secretase site of APP.

Protease	Specific Activity (pmol AMC/min/ μ g)		Catalytic Efficiency
	Wild-type (WT) site Z-V-K-M- <u>L</u> MCA	Swedish mutant site Z-V-N- <u>L</u> - <u>L</u> MCA	$k_{\text{cat}}/K_{\text{m}}$ ($\text{M}^{-1}\text{s}^{-1}$) WT β -secretase site
Cathepsin B	547	0.2	3.17×10^5
BACE 1	0	0.01	$[63.5]^I$

The peptide-MCA substrates that mimic the wild-type and Swedish mutant β -secretase cleavage sites are represented by Z-V-K-M-LMCA and Z-V-N-L-LMCA (mutant residues are underlined), respectively. Purified cathepsin B and BACE 1 were assessed for specific activities for cleaving the representative substrates in cleavage site specific assays for the wild-type and Swedish mutant β -secretase sites. The kinetic catalytic efficiency of cathepsin B for cleaving the representative wild-type β -secretase substrate (Z-V-K-M-LMCA) was assessed by its $k_{\text{cat}}/K_{\text{m}}$ value.

^I The average $k_{\text{cat}}/K_{\text{m}}$ value for cleaving WT peptide substrates by BACE 1, calculated from data in Table 1.

Table 3

Kinetic parameters of BACE 1 and cathepsin B hydrolysis of peptides containing the wild-type (WT) β -secretase cleavage site, with and without the chemical isoAsp modification (WTiso)

#	Substrate	BACE-1		Cathepsin B	
		K_m [μ M]	k_{cat} [s^{-1}]	K_m [μ M]	k_{cat} [s^{-1}]
1	I _{WT}	> 1	< 0.1	36.7	0.017
2	WTiso	no cleavage	no cleavage	23.1	0.423

I_{WT}The wild-type β -secretase peptide substrate was RE(Dans)VKMDAEFK(Dabcy)R-NH₂, and the isoAsp- substrate had isoD exchanged for D. Major cleavage by cathepsin B occurred at VK↓MDAE and VK↓MisoDAE in the APP WT derived substrates.