REVIEW ARTICLE

Cell biology, regulation and inhibition of β-secretase (BACE-1)

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Since the discovery of the β-secretase responsible for initiating the Alzheimer's amyloid cascade as a novel membrane-bound aspartic proteinase, termed 'β-site amyloid precursor protein cleaving enzyme', 'aspartyl protease-2' or 'membrane-anchored aspartic proteinase of the pepsin family-2', huge efforts have been devoted to an understanding of its biology and structure in the subsequent decade. This has paid off in many respects, as it has been cloned, its structure solved, novel physiological substrates of the enzyme discovered, and numerous inhibitors of its activity developed in a relatively short space of time. The inhibition of β-secretase activity *in vivo* remains one of the most viable strategies for the treatment of Alzheimer's disease, although progress in getting inhibitors to the clinic has been slow, partly as a consequence of its aspartic proteinase character, which poses considerable problems for the production of potent, selective and brain-accessible compounds. This review reflects on the development of β-secretase biology and chemistry to date, highlighting the diverse and innovative strategies applied to the modulation of its activity at the molecular and cellular levels.

The proteinase originally termed 'β-secretase', catalyses the initial step in the amyloidogenic metabolism of the large transmembrane amyloid precursor protein (APP), releasing a soluble APPβ (sAPPβ) ectodomain and simultaneously generating a membrane-bound, C-terminal fragment consisting of 99 amino acids (CTF99) [1]. The latter is then further processed by the γ-secretase enzyme complex which, in turn, generates the APP intracellular domain and releases the 39–42-amino-acid amyloid β-peptide (Aβ) [2]. An alternative and protective ('non-amyloidogenic') pathway of APP metabolism is initiated by the metalloproteinase, α-secretase pathway, which predominates in most cell types (Fig. 1). The identification of the Aβ peptide as the main constituent of the extracellular plaques which characterize Alzheimer's disease (AD) [3,4] led to the formulation of the 'amyloid cascade' hypothesis of AD [5]. Interruption of this metabolic cascade at one of several sites could potentially reduce the amyloid burden, and slow or even reverse the devastating consequences of the disease. Hence, the identification of β-secretase and the formulation of potent and selective inhibitors of the enzyme that can cross the blood–brain barrier have been the primary targets of pharmaceutical development for almost two decades. β-Secretase is particularly attractive in this context, as it catalyses the first and rate-limiting step in the pathway. Its deletion in mice has minimal

Abbreviations
AD, Alzheimer's disease; ADAM, a disintegrin and metalloprotease; APP, amyloid precursor protein; Asp-2, aspartyl protease-2; Aβ, amyloid β-peptide; BACE, β-site APP cleaving enzyme; CTF, C-terminal fragment; eIF, eukaryotic initiation factor; ER, endoplasmic reticulum; EST, expressed sequence tag; HEK, human embryonic kidney; memapsin-2, membrane-anchored aspartic proteinase of the pepsin family-2.

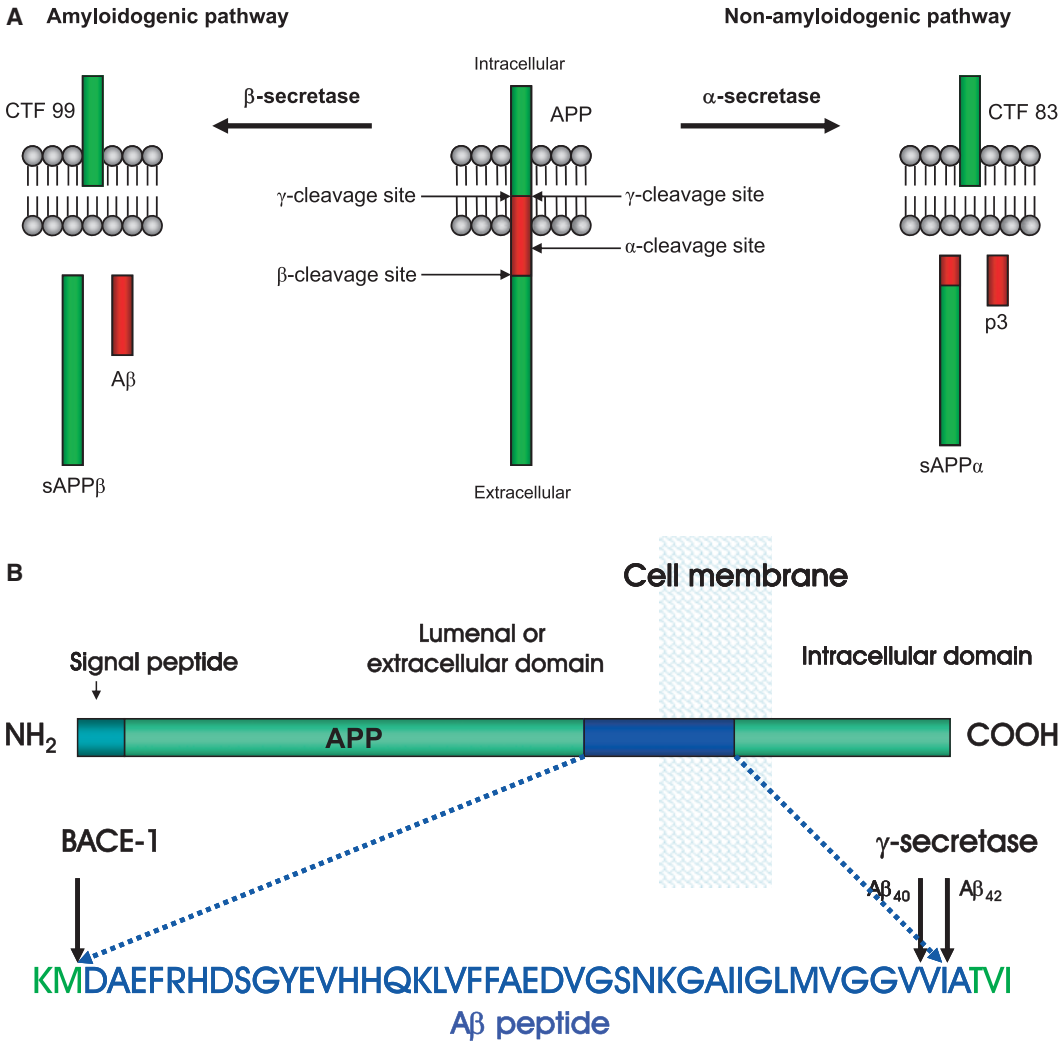


Fig. 1. Processing of APP to form Aβ peptides. (A) Schematic diagram of the alternative processing pathways of APP. The transmembrane APP undergoes two alternative and competing pathways of metabolism. The major and non-amyloidogenic, or α-secretase, pathway precludes the formation of Alzheimer's Aβ peptide. The amyloidogenic, or β-secretase, pathway initiates the formation of Aβ, which is completed by the action of the γ-secretase. α-Secretase has been identified as a zinc metalloproteinase of the ADAMs family, whereas both β- and γ-secretases are membrane-bound aspartic proteinases (see text for full details). (B) Sites of cleavage of APP by β- and γ-secretases to form Aβ peptides. The sites of the juxtamembrane and intramembrane cleavages of transmembrane APP by β- and γ-secretases, respectively, are indicated by arrows. The γ-secretase cleavages are heterogeneous, mainly producing Aβ peptides of 40 and 42 amino acids. The amino acid sequences of Aβ and around the scissile bonds are indicated by the one letter code for amino acids. The sequence shown is the wild-type sequence. The 'Swedish mutant' APP sequence around the β-secretase cleavage site is …NL/DAEF… rather than …KM/DAEF… The development of many BACE-1 inhibitors has used the sequence around the scissile bond in the Swedish mutant as the lead for synthetic chemistry to produce potent and selective compounds.

phenotypic and behavioural consequences [6], although more recent data have suggested subtle phenotypic changes in β-secretase-deficient mice [7], and the enzyme appears to play a role in both peripheral and central myelination. This review article provides current progress in this context, and also highlights alternative strategies to the modulation of β-secretase activity and expression independent of targeting its active site directly (Table 1).

Identification of the β-secretase

The protein responsible for the activity of β-secretase was reported almost simultaneously by a number of independent groups using quite distinct methodologies. It is unique in being a transmembrane aspartic protease of type I topology, in which the N-terminus and catalytic site reside on the lumenal or extracellular side of the membrane. It has variously been named by

Table 1. Potential strategies to inhibit β-secretase processing of APP by BACE-1.

Active site-directed (competitive) inhibition of enzyme activity. Transition state, small-molecule inhibitors; peptidic or non-peptidic
Non-competitive or allosteric inhibition, e.g. targeting protein processing, conformational changes ('flap movement'), distant subsites from scissile bond
Modulation of oligomeric state and hence activity of the enzyme
Modulation of protein–protein interactions affecting localization and/or activity
Modulation of lipid environment of the enzyme
Immunization with BACE-1
Modulation of miRNA regulation of BACE-1

different groups as 'β-site APP cleaving enzyme' (BACE), 'aspartyl protease-2' (Asp-2) or 'membrane-anchored aspartic proteinase of the pepsin family-2' (memapsin-2) [8–12]. Vassar *et al*. [8] originally used an expression cloning strategy to identify genes that altered Aβ production in human embryonic kidney (HEK) cells overexpressing APP containing the amyloidogenic Swedish mutation. This cell line was known to express both the β- and γ-secretases. They isolated a sequence from a clone that produced elevated levels of Aβ and that encoded a novel aspartic protease, which they termed 'BACE' (subsequently BACE-1). A classical biochemical strategy involving affinity chromatographic isolation of the enzyme activity and its subsequent cloning also proved to be highly effective [9]. In another approach, β-secretase was independently identified using expressed sequence tag (EST) databases. Hussain *et al*. [10] screened a proprietary EST database, from which they identified a sequence of interest which they termed Asp-2. Subsequently, they cloned the cDNA, transfected it into HEK cells and observed an increase in the β-cleavage of APP. In an alternative strategy, Yan *et al*. [11] visually inspected the β-cleavage sites within APP, and concluded that the cleavage may be carried out by an aspartic protease. They subsequently searched the database of the newly emerging *Caenorhabditis elegans* genome using the characteristic active site motif for aspartic proteases, D(S/T)G. Using these isolated sequences, they next searched human EST databases, which identified four novel aspartic proteases that they named Asp-1–4. Accordingly, they transfected two of these sequences into HEK cells, and those containing the Asp-2 construct were found to possess β-secretase activity. From the human EST database at the time, Lin *et al*. [12] identified, and subsequently cloned and expressed, two novel human aspartic proteinases which they named memapsin-1 and memapsin-2. All groups succeeded in identifying the same protein as the putative β-secretase (BACE-1, Asp-2, memapsin-2), together with a close homologue (BACE-2, Asp-1, memapsin-1). The localization, specificity and other enzymological properties of BACE-1 most closely fitted the profile of β-secretase. Although BACE-2 is interesting in comparative terms, its precise physiological roles are unclear, and there is no compelling evidence that it plays a direct role in the β-secretase processing of APP. The rest of this article focuses exclusively on BACE-1, although inhibitor development studies must clearly consider compound discrimination between the two activities (and other relevant protease activities).

Molecular cell biology of BACE-1

BACE-1 is synthesized as a proprotein in the endoplasmic reticulum (ER) before it is transported to the trans-Golgi network, where it undergoes maturation [13]. The efficient exit of the enzyme from the ER is determined by the prodomain [13], which is subsequently removed by the proprotein convertase, furin or a furin-like protease [13–15]. This process is not required for its activation as pro-BACE can still cleave APP [14]; however, removal of its prodomain increases BACE-1 activity by approximately twofold [16]. Molecular dynamics simulation studies have suggested that the partial catalytic activity of the zymogen could be explained by the high mobility of the prosegment in comparison with that of other zymogens, resulting in the occasional exposure of the catalytic site for access by its substrate, APP [17]. During maturation, BACE-1 also undergoes a number of post-translational modifications during its transport through the cell. The catalytic domain contains four potential *N*-linked glycosylation sites at asparagines 153, 172, 223 and 354, all of which appear to be occupied with some degree of heterogeneity between the bound carbohydrates [18]. The simple carbohydrates added in the ER produce an immature BACE-1 protein of approximately 65 kDa [14]. These sugars are further processed to an endoglycosidase H-resistant, complex form producing the mature 75 kDa species [14,19]. These modifications appear to be important for the maximal catalytic activity of the enzyme, as site-directed mutagenesis of these asparagine residues significantly reduces the proteolytic activity [20]. BACE-1 also contains three disulphide bonds in the catalytic domain between cysteines 216–420, 278–443 and 330–380 [18], which are important for the correct folding, and hence proteolytic activity, of the enzyme [21]. Within the membrane, BACE-1 probably functions as a dimer, as may the APP molecule [22,23]. The dimerization of BACE-1 could facilitate the binding and cleavage of physiological substrates, as the

purified native BACE-1 dimer revealed a higher affinity and turnover rate in comparison with the soluble BACE-1 ectodomain, which exists as a monomer [22,23]. Understanding the oligomeric states and nature of the molecular interactions between the secretases and their protein substrates could allow the development of secretase inhibitors which specifically bind to the contact sites of dimers and hence inhibit Aβ formation. In addition, serine 498 is phosphorylated by casein kinase 1, which appears to determine its subsequent subcellular location [24]. Both the wild-type, phosphorylated BACE-1 and an unphosphorylatable mutant localize to early endosomes, but only the phosphorylated form is recycled back to the membrane [24]. Adjacent to serine 498 within the extreme C-terminus of BACE-1, there is also a dileucine motif. This sequence has been shown previously in a variety of proteins to determine their trafficking from the cell surface to the endosomal and lysosomal compartments [25]. Mutation of the dileucine motif [26] resulted in increased levels of BACE-1 at the cell surface, consistent with decreased internalization to endosomes. The cytoplasmic domain also contains several cysteine residues which are subject to palmitoylation [13]. This modification may function to anchor the protein to the membrane, as mutation of these cysteine residues increases the release of the BACE-1 ectodomain into the medium [13]. The stability and turnover of BACE-1, like that of the low-density lipoprotein receptor, is regulated by reversible acetylation of seven lysine residues in its lumenal (N-terminal) domain, this event occurring in the ER and serving as a 'quality control' step in protein maturation [27,28]. Acetylated BACE-1 can then traffic to the Golgi, where deacetylation of the mature protein can occur. Non-acetylated, immature BACE-1 is degraded in a non-proteasomal, post-ER compartment [27]. The proprotein convertase PCSK9 appears to be involved in the disposal of non-acetylated BACE-1 [28].

BACE-1 is shed from cells through cleavage at its membrane anchor between alanine 429 and valine 430 [29] to generate a soluble BACE-1 ectodomain [13] by an as yet unidentified proteinase activity. Metalloproteinase inhibitors block BACE-1 shedding from cells overexpressing BACE-1 [29,30], from which it was concluded that the BACE-1 'sheddase' is likely to be a member of the 'a disintegrin and metalloprotease' (ADAM) family of proteins [31]. Shedding is a process by which many integral membrane proteins, such as angiotensin-converting enzyme and tumour necrosis factor-α, are cleaved to release a large soluble ectodomain by a protease referred to as a 'sheddase' or 'secretase' [31,32]. The physiological role of soluble BACE-1, if any, and its potential to modulate the amyloidogenic processing of APP still remain contentious. Hussain *et al.* [30] showed that the inhibition of BACE-1 shedding using metalloprotease inhibitors had no effect on the β-cleavage of APP. In contrast, the activation of protein kinase C, which is known to upregulate the shedding of BACE-1 [30], has been shown by a number of groups to decrease Aβ production in cell lines [33,34], primary cells [34] and mouse brain [35]. However, this decrease may largely reflect the upregulation of the competing α-secretase pathway. Soluble BACE-1 is still able to process APP, as Benjannet *et al.* [14] clearly showed that the overexpression of soluble BACE-1 resulted in a dramatic increase in the production of Aβ, and so membrane anchorage in the vicinity of its substrate is not essential.

Expression and localization of BACE

BACE-1 mRNA [8,9,11] and enzyme activity [9] levels are highest in the brain, with lower expression in peripheral tissues, consistent with its role as an APP β-secretase. Surprisingly, significant BACE-1 mRNA has also been detected in the pancreas [8,9,11], although the enzyme activity is very low in this tissue [9]. In the brain, BACE-1 is largely expressed by neurons, with seemingly little produced by glial cells [8,10,36–38]. However, in animal models of chronic gliosis and in brains of AD patients, BACE-1 expression can be detected in reactive astrocytes, suggesting that astrocyte activation may play a role in the development of AD (for a review, [39]). Hence, targeting astrocyte activation could be a viable strategy in the treatment of AD for this and other reasons.

Evidence that BACE-1 is the sole β-secretase activity in the brain (at least in transgenic mouse models) was provided by the observations that BACE-1 knockout mice completely lacked both β-secretase enzyme activity and the product of β-cleavage, CTF99 [6,40]. In addition, cultured primary neurons from these animals do not secrete detectable levels of Aβ [6,7,40,41]. In support of this view, a commercial BACE-1 inhibitor administered to wild-type mice was shown to decrease the levels of endogenous Aβ compared with those in control animals [42]. Increased levels of BACE-1 activity have been reported in the brains of patients with sporadic AD [36,43,44], and a truncated, soluble form of BACE-1 can be detected by activity assay in cerebrospinal fluid, which may provide a useful biomarker in AD and a source for monitoring the efficacy of drug candidates [45].

Elevated BACE-1 levels have been reported in the cerebrospinal fluid of patients with mild cognitive impairment [46]. Nevertheless, some studies have shown that other proteases could contribute to the β-secretase activity in brain against the wild-type β-secretase APP site, e.g. cathepsins B and D, and that cathepsin inhibitors may be therapeutically useful in AD [47,48]. A recent study of the effect of glutaminyl cyclase inhibition on AD-like pathology in mouse and *Drosophila* disease models also indirectly suggests the occurrence of a very low abundant but pathologically relevant β-secretase activity distinct from BACE-1 [49].

The precise subcellular location(s) at which BACE-1 cleaves APP is still controversial. BACE-1 undergoes recycling and is transported to the cell surface from where it is internalized. The enzyme has been found, through co-localization studies, to be associated with the Golgi apparatus [8,14,19,24] and endosomal compartments [8,14,50,51] from where the Aβ product may be routed to multivesicular bodies and then secreted via exosomes [52]. Specialized membrane domains, referred to as lipid rafts, have also been proposed as the location for β-cleavage [53–55]. The direct targeting of BACE-1 to lipid rafts by the addition of a glycosyl-phosphatidylinositol anchor has been shown to upregulate both sAPPβ and Aβ production in SH-SY5Y cells [56]. In addition, the disruption of lipid rafts by the depletion of cellular cholesterol levels has been shown to decrease Aβ production in both cells [56–58] and *in vivo* [58,59], whilst animals fed a diet high in cholesterol showed enhanced accumulation of Aβ [59]. Interestingly, data presented in [53] suggest that these differing concepts regarding the location of β-cleavage of APP can be reconciled. Using antibody co-patching, evidence was provided to suggest that BACE-1 and APP in lipid rafts come together during endocytosis into endosomes where β-cleavage occurs. Not all studies are consistent with the elevation of cellular cholesterol enhancing amyloid peptide formation, and an optimal level of neuronal membrane cholesterol may be critical as, under some conditions, loss of membrane cholesterol can potentiate amyloid peptide synthesis [60]. Palmitoylation-deficient mutants of BACE-1, which are not raft-localized, can still cleave APP, suggesting that β-site processing can take place in both raft and non-raft microdomains [61]. Chronic treatment with statins as inhibitors of cholesterol biosynthesis (and hence lipid raft stability) has, in some studies, been reported to reduce the risk of developing AD, although the literature is conflicting (for example, [62]). Indeed, any effect of statins on amyloid production may relate to the inhibition of protein isoprenylation, rather than any direct effect on cholesterol levels [63]. A specific inhibitor of cholesterol biosynthesis, BM15.766, does however reduce the expression of β-secretase, and consequently the production of amyloid-β, at least *in vitro* [64].

BACE-1 activity itself is highly sensitive to its lipid environment and is stimulated by glycosphingolipids, glycerophospholipids and sterols [65]. Glycosaminoglycans may also act as allosteric modulators of BACE-1 activity, as heparan sulphate specifically inhibits the BACE-1 cleavage of APP, but not that by α-secretase [66]. Heparin itself has a complex mode of action by activating the partially active BACE-1 zymogen at low concentrations, but promoting autocatalytic cleavage and hence inhibition of the protease domain at higher concentrations [67,68]. Hence, in total, these studies suggest that modulation of the subcellular site(s) of APP processing may represent a potential therapeutic strategy in the treatment of AD [69]. In this context, APP may normally be segregated from BACE-1 in distinct membrane domains through its interaction with X11/Munc18 [70] proteins, but neuronal activity, coupled with the phosphorylation of Munc18, appears to influence the movement of APP into BACE-1-containing membrane domains, a process referred to as 'membrane microdomain switching' [71]. A variety of BACE-interacting proteins have been reported that might influence enzyme localization and/or activity, for example reticulon/NOGO proteins, which can inhibit the access of BACE-1 to its substrate APP [72,73]. A conserved C-terminal QID sequence among reticulon family members is involved in the interaction with the BACE-1 cytoplasmic domain [74]. The cellular form of the prion protein also negatively regulates β-secretase cleavage of APP, probably through its raft interaction with glycosaminoglycans [75]. Hence, the cellular form of the prion protein may normally suppress Aβ formation through its inhibition of BACE-1 [76]. Small-molecule mimics of such modulating interactions could provide novel BACE-1-inhibiting therapeutics.

Regulation of BACE-1 expression

A variety of physiological stressors and signalling pathways have been found to regulate BACE-1 and may be a factor in the reported increased BACE-1 protein levels and enzyme activity in AD brains [36,43,44], although BACE-1 transcript levels generally appear unchanged in AD brains [77,78]. Hypoxia and ischaemia are important risk factors for AD, and chronic hypoxia in the neuroblastoma line SH-SY5Y promotes amyloidogenic processing of APP [79]. Hypoxia-inducible factor-1α binds to the

BACE-1 promoter, and several studies have reported the upregulation of BACE-1 mRNA both *in vitro* and *in vivo* following hypoxia [80–82]. Oxidative stress can stimulate BACE-1 expression in cells through the *c-jun* N-terminal kinase pathway in a mechanism which requires the presence of presenilin [83]. The lipid peroxidation product 4-hydroxynonenal also upregulates BACE-1 expression through the stress-activated protein kinase pathway [84]. The activation of cyclin-dependent kinase 5 also leads to increased levels of BACE-1 mRNA and protein *in vivo* and *in vitro*, and the BACE-1 promoter contains a cyclin-dependent kinase 5-responsive region [85]. Other stressors that can cause the activation of BACE-1 expression include traumatic brain injury, a strong risk factor for AD [86], and infection of neuronal cells with herpes simplex virus 1 [87]. Herpes simplex virus 1 is also a risk factor for AD, particularly when in association with the ε4 allele of the apolipoprotein E4 gene [88], and the viral DNA is localized within amyloid plaques in AD brains [89].

Post-transcriptional mechanisms have a major influence on BACE-1 levels, and BACE-1 translation is regulated at multiple stages, consistent with the presence of a long and highly conserved transcript leader [90,91]. In particular, the 5′-UTR represses the rate of BACE-1 translation [92], and alternative splicing of the transcript leader can influence the rate of translation in a tissue-dependent manner [90]. A detailed mutagenesis analysis suggested that the GC-rich region of the 5′-UTR acts as a 'translation barrier' [92]. The presence of several upstream ATGs also strongly reduces the translation of the main open reading frame, which implies that BACE-1 translation might increase in conditions that favour phosphorylation of the translation eukaryotic initiation factor-2α (eIF2α) [90]. More recent studies have shown that cellular energy deprivation (glucose deprivation in cell culture) produces a post-transcriptional increase in BACE-1 levels, which is indeed mediated through increased eIF2α phosphorylation [92]. These observations *in vitro* correlated with *in vivo* studies in AD transgenic (Tg2576) mice, in which chronic energy inhibition with 2-deoxyglucose or 3-nitropropionic acid was shown to increase eIF2α phosphorylation, BACE-1 levels and amyloidogenesis [93]. Thus, a common mechanism by which stress (e.g. hypoxia/ischaemia, viral infection, etc.) can influence BACE-1 levels may be through the regulation of translation initiation at the level of eIF2α. BACE-1 protein stability can also be influenced by the lysosomal and proteasomal pathways [94] and through its lysine acetylation status [27,28].

Substrates of BACE-1

Like most proteases, BACE-1 is not uniquely specific to one substrate, and APP may not even be the primary substrate of the enzyme, except where mutations in the enzyme or in APP render it far more effective in this reaction. Hence, in addition to APP, BACE-1 is also involved in the proteolytic processing of a number of other proteins. The amyloid precursor-like proteins 1 and 2, which are closely related to and structurally similar to APP, are also processed by BACE-1 [95], as are the APPε product (the ε-secretase-derived N-terminal product of APP) [96] and Aβ itself, which is cleaved at the 34/35 site [97]. Additional substrates include the sialyltransferase ST6Gal I [98], the cell adhesion protein P-selectin glycoprotein ligand-1 [99], the low-density lipoprotein receptor-related protein [100] and the β-subunits of voltage-gated sodium channels [101]. Recently, using BACE-1 knockout mice, Willem *et al*. [102] have suggested a role for BACE-1 in the myelination of peripheral nerves through the processing of type III neuregulin 1, and the enzyme also appears to modulate myelination in the central nervous system [103]. However, inhibition of BACE-1 *in vivo* in adult mice expressing human wild-type APP lowered brain Aβ levels and increased sAPPα, but did not affect neuregulin processing [104]. Given the diversity of the BACE-1 substrates so far identified, there are probably considerably more to discover. In order to validate BACE-1 as a realistic therapeutic target, it is important that the manifestations of inhibiting these alternative activities are understood, particularly in the adult and aging animal.

Inhibitors of aspartic proteinases

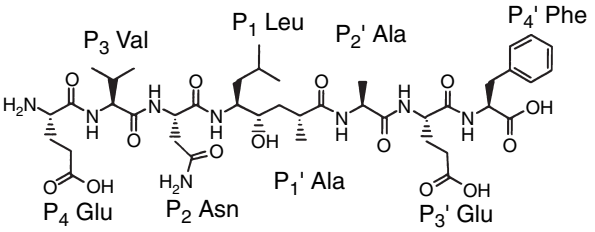
Aspartic proteinases are endopeptidases which use two aspartic acid residues to catalyse the hydrolysis of a peptide bond. These aspartic acid residues in the active site bind and activate a water molecule, which, in turn, acts as a nucleophile to attack the scissile bond at the cleavage site of its substrate. Of the various clans of aspartic proteases, BACE-1 belongs to the same clan as pepsin, although it is only very weakly inhibited (IC_{50} = 0.3 mM) by the statine-based transition state inhibitor of pepsin, pepstatin. The statine moiety of pepstatin represents a tetrahedral, hydroxymethylene isostere of the scissile peptide bond, and hence mimics the putative transition state intermediate of the catalytic reaction. This mode of inhibition has been generally applied to the development of BACE-1 inhibitors (see below). Members of the pepsin family are only found in eukaryotes and are most active at an

acidic pH (approximately pH 4 for BACE-1, although it rapidly and irreversibly loses activity at pH 3.5 or lower). Most are synthesized as proproteins with a signal domain targeting them to the secretory pathway. The crystal structures of several of the members of this family have revealed a bilobed structure, in which each lobe contributes one of the aspartic acid residues which makes up the catalytic pair at the active site (for a review, [105]). The two lobes are structurally similar and appear to have evolved from a gene duplication event [106].

Towards the development of BACE-1 inhibitors

Ever since the elucidation of the metabolic pathway leading to the formation of Aβ (Fig. 1), the β-secretase has been a primary target for inhibitor design in AD therapy. Considerable efforts have been directed towards the identification of low-molecular-mass, specific and stable non-peptide analogues as BACE-1 inhibitors that can lead to the development of a successful therapeutic. Such compounds must be of high potency, stable to hydrolysis, deliver low toxicity and be able to cross the blood–brain barrier. Approaches to the discovery of novel BACE-1 inhibitors have involved understanding the substrate specificity of the enzyme, coupled with structure-based design and high-throughput screening *in vitro* and *in silico*. To date, the screening of extensive libraries for non-peptide-based BACE-1 inhibitors has resulted in the discovery of relatively few, generally low-affinity, compounds, indicating that this is not an easy protein target to inhibit effectively *in vivo*. This is partly because of the extended substrate-binding site requirements [107], a problem also seen with other aspartic proteinase targets. The crystal structure of the protease domain of BACE-1 complexed to an eight-residue, peptide-based inhibitor (OM99-2) was determined shortly after the enzyme was identified [108]. The design strategy for OM99-2 was based on comparisons of the amino acid sequences around the scissile bond in the wild-type APP (–EVKM/DAEF–), which is a relatively poor substrate for BACE-1, and the very efficiently hydrolysed, Swedish mutant APP (–EVNL/DAEF–), with a 60-fold higher k_{cat}/K_m relative to the wild-type. The residues of the inhibitor in the S_1–S_4 subsites were unchanged from the Swedish mutant sequence (EVNL), but those at the S_1'–S_2' subsites were changed from Asp–Ala to Ala–Val, as the key specificity of BACE-1 appeared to reside mainly at the S_1' site, where small side-chains, such as alanine, are highly preferred over aspartic acid. The aim was also to reduce the polarity and increase the lipophilicity of the inhibitor to aid penetration across the blood–brain barrier. This peptide backbone was used to generate a typical aspartic proteinase inhibitor by converting the P_1–P_1' peptide bond to a hydroxyethylene transition state isostere, leading to the compound OM99-2 (EVNL*AAEF, where * indicates the isostere), which is shown in Fig. 2.

The structural solution of BACE-1 [108] revealed a bilobed structure with the same general folding pattern as other known aspartic proteases, such as pepsin, including high conservation of the hydrogen-bonding structure around the active site (Fig. 3). However, there are important structural differences between BACE-1 and pepsin. The most significant differences are four insertions, which considerably increase the molecular boundary of BACE compared with pepsin, and a 35-residue C-terminal extension in the C-lobe which contains two of the disulphide bonds unique to BACE-1. The large, active site cleft which contains the two catalytic aspartate residues is located between the two lobes and appears to be more open and accessible than that of pepsin.



OM99-2

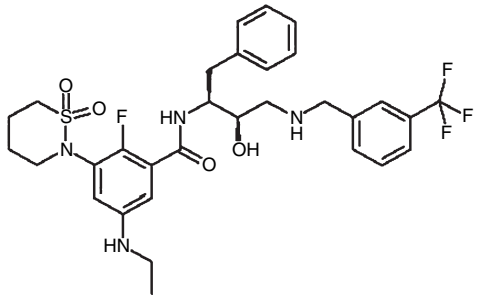


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Fig. 2. BACE-1: from peptide-based to non-peptidic BACE-1 inhibitors. Examples of two BACE-1 inhibitors: the first reported compound OM99-2 (reproduced from [108] with permission of the American Association for the Advancement of Science) and a recently described orally active, non-peptidic inhibitor GSK 188909 (reproduced from [117] with permission of the International Society for Neurochemistry). In OM99-2, the constituent amino acids and their subsite designations are indicated. The hydroxyethylene transition state isostere is between P_1-Leu and P_1'-Ala. Figure reproduced from Hussain *et al.* [117] by kind permission.

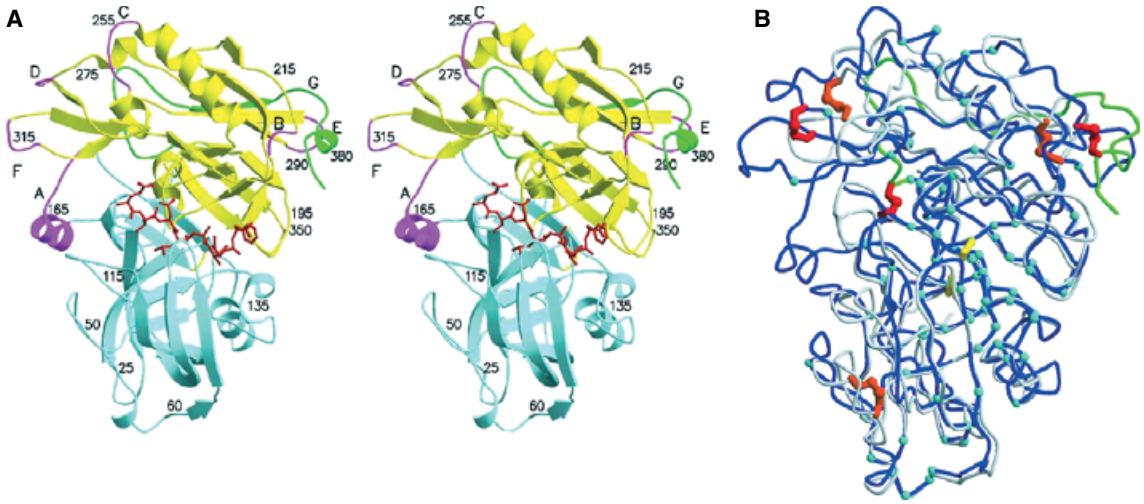

Fig. 3. The crystal structure of BACE-1 complexed to the peptide-based inhibitor OM99-2. (A) Stereoview of the polypeptide backbone of BACE-1 is shown as a ribbon diagram. The N-lobe and C-lobe of the bilobed aspartic proteinase structure are shown in blue and yellow, respectively. The inhibitor bound between the lobes is shown in red. (B) The chain tracings of human BACE-1 (dark blue) and human pepsin (grey) are compared. The light blue balls represent identical residues which are topologically equivalent. The disulphide bonds are shown in red for BACE-1 and orange for pepsin. The C-terminal extension in BACE-1 is shown in green and the active site aspartic acid residues are shown in yellow. Reproduced from [108] with permission of the American Association for the Advancement of Science. Figure reproduced from Hong *et al.* [108] by kind permission.

More extensive studies of the specificity requirements of BACE-1 have subsequently been carried out, establishing that the enzyme has a relatively loose substrate specificity, which has been defined in detail by Turner *et al.* [109]. A peptide containing the sequence of the eight most favoured residues around the scissile bond [–EIDLMVLD–] is the most efficient known substrate of the enzyme [109]. A variety of other short peptides have typically been used in BACE-1 assays, usually as fluorogenic substrates incorporating a fluorophore and a quencher, mimicking the sequence around the β-secretase cleavage site in APP or in the Swedish mutated form (for example, [110]). However, caution should always be used in interpreting data from small peptide substrates as they lack many of the subsite and other interactions of the genuine protein substrate. Nevertheless, such studies have led to the development of novel BACE-1 inhibitors, usually transition state analogues incorporating the hydroxyethylene transition state isostere, or statine, residue typical of many aspartic proteinase inhibitors. Refinement of OM99-2 [111] led to the development of OM00-3 (Glu-Leu-Asp-Leu*Ala-Val-Glu-Phe), the most potent inhibitor known to date with a K_i value of 0.3 nM. The cell permeability and blood–brain barrier penetrance of such compounds are, however, often a problem compounded by active P-glycoprotein-mediated efflux, leading to poor inhibition constants *in vivo*. Ideally, such compounds should be < 500 Da for passive barrier penetration. An alternative is to permit facilitated penetration. The cell permeability problem has been overcome, in one successful example, by the incorporation of a penetratin sequence to the inhibitor, considerably enhancing the cell potency [112]. The inhibitor itself [JMV1195; EVN(statine)AEF-NH_2] represents one of the statine-based peptidomimetic BACE-1 inhibitors [109], again modelled on the Swedish mutant peptide sequence. In another approach, a series of isonicotinamides derived from traditional aspartic proteinase transition state isostere inhibitors has been optimized to yield low-nanomolar inhibitors with sufficient penetration across the blood–brain barrier to demonstrate β-amyloid reduction in a murine model [113]. Hence, structure-based approaches to inhibitor design against BACE-1 are now beginning to yield potential therapeutic compounds. Recent disclosures of the crystal structures of BACE-1 with lower M_r inhibitors have provided further insights into active site interactions, producing more potent and selective, cell-permeable compounds, including both peptidomimetic and non-peptidic compounds [114–116]. For example, using a rational drug design approach, Hussain *et al.* [117] identified GSK188909 (Fig. 2) as a small-molecule ($M_r \sim 600$), potent and selective non-peptidic inhibitor able to block Aβ formation in transgenic mice when co-administered with a P-glycoprotein inhibitor.

BACE-1 inhibition cannot be considered in isolation from that of BACE-2. Detailed studies on BACE-2

specificity [118] indicate that it is broad-based and, not surprisingly, rather similar to BACE-1, consistent with several BACE-1 inhibitors also inhibiting BACE-2. Nevertheless, in a separate study, inhibitors with isophthalamide derivatives as the P_2–P_3 ligands showed good selectivity between BACE-1 and BACE-2, nanomolar potency *in vitro* and in cell-based studies, and a significant reduction in Aβ40 levels *in vivo* in transgenic mice after intraperitoneal administration [119]. Relatively few detailed kinetic and mechanistic studies have been carried out on BACE-1 inhibition. A notable exception is provided by Marcinkeviciene *et al*. [120], in which steady state and stopped flow kinetics of BACE-1 inhibition by a statine-based inhibitor [Ac-KTEEISEVN(statine)VAEF-COOH] were carried out. These studies revealed a two-step mechanism involving an initial low-affinity binding, followed by a tightening up of the binding, induced either by a conformational change ('flap movement') or displacement of a catalytic water molecule. The scene is now set for the refinement of existing molecules and the exploration of their efficacy further in animal models. The ability of an orally administered BACE-1 inhibitor to reduce cerebrospinal fluid and plasma Aβ levels in a non-human primate (rhesus monkey) has recently been reported [121] and, at long last, clinical trials of BACE-1 inhibitor drug candidates are being initiated almost a decade on from the original cloning of the enzyme. This has largely been because of the problems inherent in the design of potent and selective aspartic proteinase inhibitors sufficiently small to penetrate the blood–brain barrier. The BACE-1 inhibitor CTS-2166 has entered a phase I study in healthy volunteers, and the drug was reported to be well tolerated and effective in lowering plasma Aβ levels [122], and further trials are ongoing.

Future approaches and therapeutic potential of β-secretase inhibition

The development of clinically successful BACE-1 inhibitors has been hampered by a number of factors, including effective inhibitor design, selectivity and stability, brain access and potential toxicity. Combination therapies employing BACE inhibition with other strategies may provide a more versatile treatment in AD, and other novel strategies are also currently being explored. An innovative and promising recent experimental approach has been to attempt to immunize transgenic AD mice with BACE-1, which resulted in a significant reduction in brain Aβ levels and an improvement in cognitive function, without any reported evidence of inflammatory responses [123]. The rationale for this study was that immunization with BACE-1 could produce a proportion of brain-penetrant antibodies, which, in turn, bind to neuronal cell surface BACE-1 molecules. Internalization of the BACE-1 antibody complexes then results in inhibition of the enzyme activity within endosomes, and hence of Aβ production. Recent studies have also shown that microRNAs (miRNAs) can bind to the 3′-UTR of BACE-1 mRNA, and hence regulate BACE-1 levels. Loss of specific miRNAs (e.g. miR-107, 298, 328 and the cluster miR-29a/b-1) during AD progression could contribute to increases in BACE-1 and Aβ levels [124–126], but exploiting miRNAs therapeutically is currently very challenging. Only time will tell which of these diverse approaches to the modulation of β-secretase activity of BACE-1, directly or indirectly, is likely to have the potential to reach the clinic.

Conclusions

Almost 10 years since BACE-1 was unequivocally identified, it still remains a promising, indeed probably the most viable, target for therapy in AD, although some have urged caution in adopting this approach [7]. Although much has been learned about the structure and action of the enzyme, there are still many unanswered questions relating to its true physiological roles, its locations and the physiological consequences of its inhibition *in vivo*. Targeting aspartic proteinases is not a trivial exercise and there remains considerable scope for innovative design and application of BACE-1 inhibitors, but their efficacy and safety still remain to be demonstrated, particularly in the chronic treatment regimes that would be required. Alternative strategies that seek to manipulate the location, lipid environment, antigenicity, transcriptional regulation or processing of the enzyme may also be strategically useful, as described above. The importance of the problem demands both an imaginative and thorough approach to rational drug design and application.

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