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Review article

Overview of BACE1 structure and effect of genetic polymorphisms and protein isoforms on enzyme activity

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Abbreviations
AD – Alzheimer's disease
APP – amyloid precursor protein
BACE1 – β-site APP cleaving enzyme 1

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Abstract

One of the major hallmarks of Alzheimer's disease (AD), deposition of amyloid plaques in the brain, arises from the proteolytic processing of the amyloid precursor protein (APP), a process in which the rate-limiting step is carried by β-secretase (BACE1). Due to this essential role in the development of AD, BACE1 has become a widely studied drug target for the development of new drugs aimed at blocking amyloid β peptide formation and aggregation through inhibition of BACE1 activity. The development of the first BACE1 inhibitors allowed the determination of its crystal structure and description of its active site, confirming the existence of eleven well-defined subsites able to accommodate a range of substrates, which enabled BACE1 to participate in a number of biological processes. For this reason, the study of BACE1 structure and determination of specific structural motifs responsible for the accommodation of known substrates could enable defining drugs with very specific targets, as well as provide insight into the mode of action of BACE1 itself. This review will focus on the overall structure of BACE1 and its active site, as well as on the effect of genetic polymorphisms and isoforms on its activity.

INTRODUCTION

The β-site APP cleaving enzyme 1, commonly known as BACE1 (EC 3.4.23.46), is a type I transmembrane protein that belongs to the group of aspartic proteases *(1)*. These enzymes share a structural similarity and are characterized by two catalytically active aspartates, one in the *N*- and one in the *C*-terminal domain *(2)*. BACE1 is expressed in most cell types at low levels and the highest concentrations of this enzyme are found in neurons *(3)*, especially in axonal presynaptic terminals *(4)*. On the contrary, the mRNA of its homolog, BACE2, is expressed at very low levels throughout the brain's regions *(5, 6)*. BACE1 cleavage of its substrates results in the release of an extracellular fragment which can further interact with molecules on the surface of the same or adjacent cell and thereby facilitate signal transduction or cell-cell interactions *(3)*. In such a manner, BACE1 is a sheddase that processes its substrates through ectodomain shedding. The best known BACE1 substrate is the amyloid precursor protein (APP) to which it owes its name, but besides APP, there are a number of other reported substrates for BACE1 such as voltage-gated sodium channel β subunit, β-galactoside alpha-2,6-sialyltransferase 1 (ST6Gal-I), Seizure protein 6 (Sez6) and its homolog seizure 6-like protein (Sez6L), P-selection glycoprotein ligand-a (PSGL-

1), Jagged 1 and 2, interleukin-1 receptor II (IL-1R2), pro-inflammatory cytokine receptor gp130, cell adhesion molecules L1 and close homolog of L1 (CHL1) and neuregulin 1 (NRG1) *(7-16)*. Being an aspartic protease, BACE1 requires low pH for optimal activity and is primarily active in early endosomes where it is trafficked from the cell membrane *via* the endocytic pathway *(17-19)*. Proteolytic cleavage of APP catalyzed by BACE1 results in the formation of soluble β-fragment sAPPβ and membrane-bound C-terminal fragment (CTFβ, C99). Further cleavage of C99 fragment by γ-secretase releases amyloid β peptides of different lengths, the most important being Aβ42 (constituted of 42 amino acids), as the main component of senile plaques found in brains of people suffering from Alzheimer's disease *(20)*. Since proteolysis of APP by BACE1 is the first enzymatic reaction *en route* from APP to senile plaques, as well as the rate-limiting step in the formation of said plaques *(21)*, BACE1 became a central point of numerous studies looking for a potential inhibitor that would prevent APP cleavage and Aβ aggregation *(22)*. In this review, we focus on the overall structure of BACE1 and its active site, structure-activity relationship and biological implications of genetic polymorphisms. Our aim is to provide a summarized insight into the structural functionality of the BACE1 enzyme.

HYSTORY OF BACE1 ENZYME IDENTIFICATION

Ideas of the notion that there may exist an enzyme responsible for initiating amyloid β peptide generation rose from mid-twentieth century findings that people with Down syndrome (DS) develop a neuropathology similar to that of Alzheimer's disease (AD) - accumulation of amyloid plaques and neurofibrillary tangles of the tau protein in the brain *(23)*. The presence of an extra copy of chromosome 21 in people with DS causes a genetic disbalance and disturbed expression of proteins coded from genes on chromosome 21 *(24)*. In the 1980s, Glenner and Wong found that amyloid beta peptides (Aβ) in cerebrovascular amyloid fibrils of people with AD and DS are structurally almost identical, which suggested that amyloid plaques in both AD and DS could be the result of the same gene product *(25, 26)*. The hypothesis was confirmed in the 1990s when it was discovered that patients with hereditary cerebral hemorrhage with amyloidosis of the Dutch Type (characterized by amyloid deposition in cerebral vessel walls) and patients with familial AD (FAD), carry point mutations in the *APP* gene located on chromosome 21 *(27, 28)*. These findings triggered the search for an enzyme responsible for the β-cleavage of the amyloid precursor protein (APP). In 1999, five independent groups reported the identification of a novel aspartic protease. Using different approaches and/or methods, all of the groups reported an enzyme that cleaves the luminal domain of the APP at the β-site and causes a release of Aβ peptides and named them β-secretase *(19)*, memapsin-2 (membrane-anchored aspartyl protease) *(29),* Asp2 (aspartic protease) *(30, 31)* or BACE (β-site APP-cleaving enzyme) *(32)*. Sinha *et al.* purified the enzyme from human brain samples using affinity-purification with immobilized P10 – P4' StatVal inhibitor peptide. The *N*-terminal sequence of the protein was revealed after the Edman degradation of purified enzymes and was used for isolation of complementary DNA clone from human neurons and human fetal brain. This cDNA was subsequently transfected and co-expressed with APP in 293T cells, where it was shown to cleave APP at the β-site *(19)*. By inspection of the cDNA expression library from the HEK293 cell line, Vassar *et al.* identified the enzyme by expression cloning of cDNAs for identification of genes/proteins that modulate Aβ production. Of the cDNA pools showing increased Aβ production, the clone which shared sequence similarities with pepsin-like aspartic proteases encoded a novel enzyme termed BACE. The overexpression of this enzyme in HEK293 cells resulted in increased β-secretase activity and Aβ production, confirming that the novel enzyme is indeed β-secretase *(32)*. Lin *et al.* and Hussain *et al.* used the expressed sequence tag (EST) database to identify and clone cDNAs based on a sequence homology with known aspartic proteases and observed upregulation of APP cleavage in cells transfected with BACE1 cDNA *(29, 30)*. Yan *et al.* searched the database of predicted *C. elegans* proteins and vertebrate EST database to identify novel human aspartic protease candidates with active-site motifs resembling that of cathepsin D, an aspartic protease with the ability to cleave synthetic BACE1 substrates. The research resulted in four proteases named Asp *(1-4)*. To assess which candidate protease acts as a β-secretase, they probed the APP processing with a panel of antisense oligomers targeting these novel enzymes of interest in different cell lines and observed changes in Aβ peptide release. As only Asp2 targeting oligomers decreased the release of Aβ peptides in the medium, Asp2 was revealed as β-secretase, which was further confirmed through measurement of proteolytic activity of Asp2 purified from Chinese hamster ovary cells against synthetic APP peptide substrates *(31)*. Because since its discovery BACE1 has become the focus of many studies in the search for a drug that can prevent BACE1-mediated APP cleavage and reduce the extent of Aβ aggregation, numerous studies have been conducted focusing on the structure, localization, and distribution of this enzyme in humans.

BIOSYNTHESIS AND POSTTRANSLATIONAL MODIFICATIONS OF BACE1

BACE1 is a transmembrane type I protein constituted of 501 amino acids (Figure 1) *(1)*. The first 45 amino acids are *N*-terminal signal sequence (21 aa) and pro-peptide domain (24 aa) which are co-translationally and post-

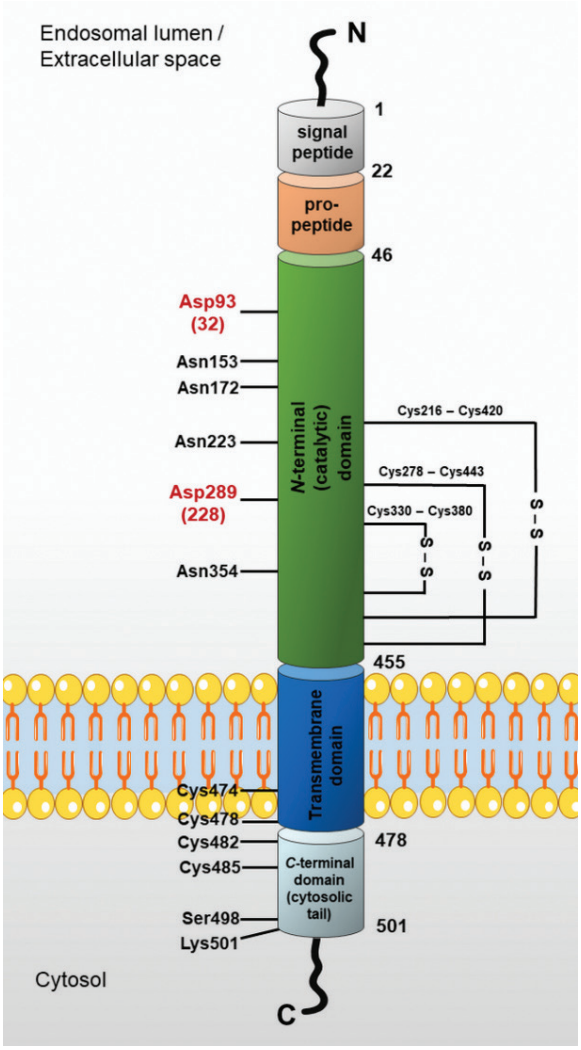

Figure 1. *Structure of the BACE1 enzyme. The primary structure consists of 501 amino acids comprising 5 subdomains: signal peptide and pro-peptide (both are cleaved off in the process of protein maturation), N-terminal (catalytic) domain, transmembrane domain and C-terminal (cytosolic) domain. Amino acids that are post translationally modified are shown. Catalytic aspartates (in red) are numbered as in whole, newly synthetized proteins and as in mature protein (inside parenthesis). Modified from (3).*

translationally cleaved off by signal peptidase and proprotein convertase, respectively *(33)*. Amino acids 46 – 455 form the *N*-terminus of a mature β-secretase oriented towards extracellular space when the enzyme is associated with a cell membrane or towards the lumen when in early endosomes. The rest of the enzyme is comprised of a transmembrane domain (455 – 478 aa) and short cytosolic *C*-terminal domain (478 – 501 aa) *(33, 34)*.

Biosynthesis of BACE1 takes place in the endoplasmic reticulum (ER). As is the case with other aspartic proteases, BACE1 is firstly synthetized in its proenzyme form, which gains its activity after removal of pre- and pro-domains. Interestingly, BACE1 shows activity already in its proβ-secretase form but, after the pro-domain is cleaved off, its activity rises. This means that the optimal enzymatic activity of BACE1, beside post-translational modifications, depends on its maturation phase and pro-domain cleaving enzymes *(33, 35)*. Mature BACE1 is post-translationally modified through glycosylation, palmitoylation, phosphorylation and ubiquitination in ER *(33, 35-37)*. Glycosylation takes place in both the ER and the Golgi apparatus (GA) and is an important factor for achieving active protein conformation and consequently optimal catalytic activity. Four *N*-glycosylation sites, Asn153, Asn172, Asn223 and Asn354, are found in the vicinity of the active site. The palmitoylation of cysteines Cys474, Cys478, Cys482 and Cys485 in the transmembrane and cytoplasmatic domain of BACE1 has an important role in its localization and enhancing the enzyme's ability for association with lipid rafts in the cell membrane *(33, 38)*. *In vivo*, BACE1 is susceptible to homodimerization but, for the process to occur, a prior association of BACE1 and the cell membrane is required *(39, 40)* meaning that palmitoylation indirectly mediates the homodimerization process. As homodimerization takes place in ER and *cis*-Golgi before enzyme maturation, through enzyme localization and homodimerization, palmitoylation is deemed responsible for higher BACE1 affinity towards certain substrates *(40)*. Phosphorylation of Ser498 is mediated by casein kinase 1 (CK-1) and is important for the regulation of the intracellular transport of BACE1. A phosphorylated enzyme is efficiently retrieved from early endosomes and further targeted to late endosomes, *trans*-Golgi network and secretory pathways, while the non-phosphorylated enzyme is retained within early endosomes. Both phosphorylated and non-phosphorylated enzymes are subjected to endocytosis into early endosomes *(36)*. Besides phosphorylation, ubiquitination also assists the subcellular trafficking of BACE1 since it regulates BACE1 degradation by aiding its transport towards lysosomes *(37)*. The lack of ubiquitination results in postponed BACE1 degradation and accumulation of active enzymes in early/late endosomes, cell membranes and lysosomes as well.

BACE1 ACTIVE SITE: STRUCTURE DETERMINATION THROUGH DEVELOPMENT OF INHIBITORS

As a member of the aspartic protease family, BACE1 shares an overall structure similarity with pepsin, an aspartic protease that was one of the first enzymes to be crystalized *(2)*. BACE1 has a bilobal overall structure like pepsin, consisting of two lobes (named *N*- and *C*-terminal) each providing one catalytically active aspartate. Both catalytically active Asp residues are located in the active site cleft positioned between *N*- and *C*-terminal lobes (Figure 2) *(41)*. In the years following the discovery of BACE1, its catalytic active site was described using

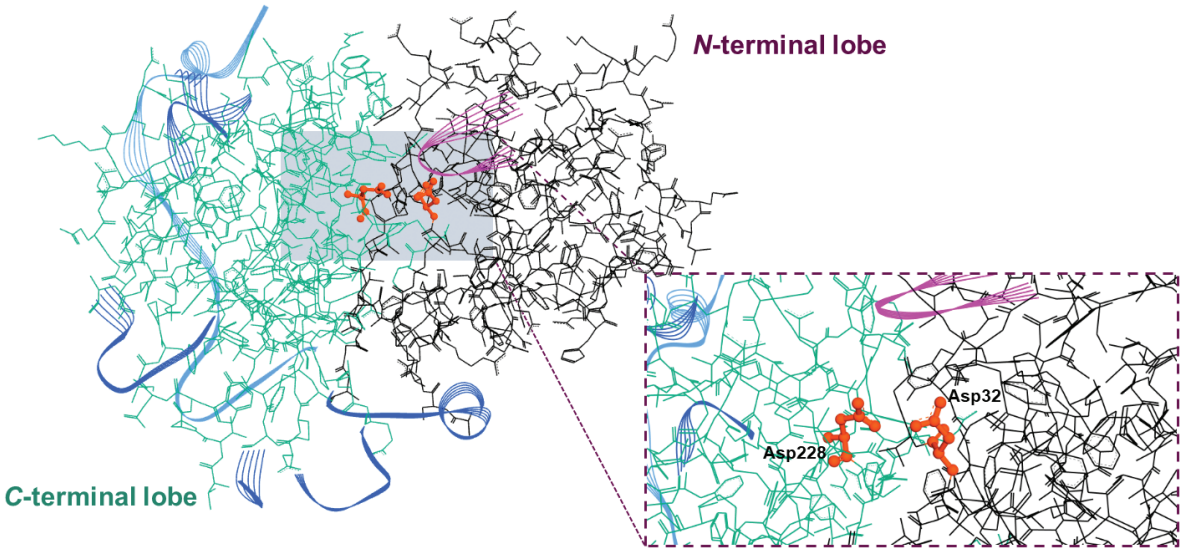

Figure 2. *3D structure of BACE1 (PDB ID: 1FKN (41)) with close-up view of the active site. The* N*-terminal lobe (1 – 180 aa) is shown in black, and* C*-terminal lobe (181 – 385 aa) in green. Aspartates comprising catalytic dyad are in orange, and the flap region (Val69 – Lys75) is in magenta. Insertions are shown in blue (Gly158 – Leu167; Lys218 – Asn221; Ala251 – Pro258; Trp270 – Gly273; Glu290 – Ser295; Asp311 – Asp317), while COOH-terminal extension is in light blue (Cys359 – Thr393).*

different transition-state analogs developed to resemble its natural substrate, APP.

Unlike pepsin, BACE1 is characterized by the presence of six insertions and a long COOH-terminal extension in the *C*-terminal lobe. These structural motifs enlarge the molecular surface of BACE1 resulting in a more open active site with easier access for the substrate, compared to that of pepsin (Figure 3) *(41)*. Six cysteines of the *N*-terminal domain form intramolecular disulfide bonds (Cys216 – Cys420, Cys278 – Cys443 and Cys330 – Cys380) *(43)*. Two of these disulfide bonds (216/420 and 278/443), which connect the ends of COOH-terminal extension to the *C*-terminal lobe *(41, 44)*, are typical for BACE1 as they are not seen in other aspartic proteases like pepsin and cathepsin D, but they are not crucial for BACE1 maturation and processing activity *(44)*. The human BACE1 active site is constituted of eleven subsites: S1 – S7 termed *N*-terminal subsites and S1' – S4' termed *C*-terminal subsites. Three (S5 – S7) are found in the area enlarged by the above-mentioned additional motifs and which preferentially interact with hydrophobic side chains of substrate (45). Eight subsites (S4 – S4') show no strict preference for substrate side chains, although *N*-terminal subsites (S1– S4) preferentially interact with fewer different inhibitor amino acid residues than *C*-terminal subsites (S1' – S4'). The most pronounced stringency for specific amino acid side chains shows subsites surrounding the scissile bond of substrate, S1 and S1' *(46)*. An important difference between BACE1 and pepsin is the presence of hydrophilic active site subsites in BACE1: S2 formed by Ser325, Ser327, Tyr71, Thr72, Gln73, Gly230, Thr231 and Arg235, and S4 formed by Gly11, Gln73, Thr232 and Arg307 *(41)*. Another significant distinction is the different conformation of hydrophobic S1 (Leu30, Asp32, Tyr71, Gln73, Phe108, Asp228 and Gly230) and S3 (Gly11, Gln12, Gly13, Leu30, Ile110, Gly230, Thr231, Thr232) subsites compared to pepsin *(41, 47)*. These subsites represent unique structural motifs which should be taken into consideration while designing BACE1-specific inhibitors.

BACE1 cleaves its substrates following the general two-step acid/base mechanism of hydrolysis of the peptide bond employed by other aspartic proteases: (1) nucleophilic attack of an activated water molecule on the carbonyl group of the substrate resulting in the formation of the gem-diol intermediate and (2) cleavage of the peptide bond (Figure 4) *(48, 49)*. In the first step, Asp228

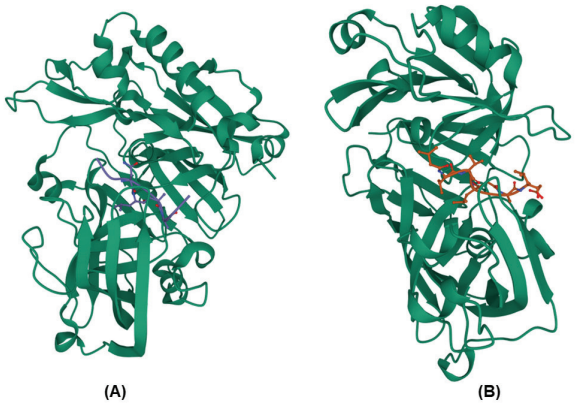

Figure 3. *Human BACE1 in complex with inhibitor OM99-2 (blue) (panel A; PDB ID: 1FKN (41)) and human pepsin in complex pepstatin (orange) (panel B; PDB ID: 1PSO (42)).*

serves as a base that deprotonates active site water and activates it for a nucleophilic attack on the carbonyl carbon atom of the scissile bond, while at the same time Asp32 transfers a proton to the carbonyl group, serving as an acid. This step, resulting in the formation of the gem-diol intermediate, due to an energetic barrier of approximately 22 kcal/mol determined for wild-type (WT) APP, represents the rate-limiting step of the entire mechanism *(49)*. In the next step, Asp228 now acts as an acid and protonates the amide group of the scissile bond, while Asp32, acting as a base, takes the proton from the gem-diol. This results in the cleavage of the peptide bond, formation of products and regeneration of enzyme *(48, 49)*. Moreover, the binding of substrates and inhibitors in the active site of aspartic proteases is dependent on the β-hairpin, also known as flap, positioning over the active site cleft, whose movement controls the access of substrate/inhibitor to the active site and its hydrolysis. The movement of the flap changes the conformation of the enzyme from closed (substrate/inhibitor are bound on enzyme) to open (unbound state of the enzyme). A vital role in this conformation change is played by Tyr71: in the closed-flap conformation, Tyr71 interacts with Trp76 *via* hydrogen bonds and forms multiple interactions with P1 and P2' inhibitor subsites, while in open-flap conformation these interactions are omitted and replaced by a hydrogen bond with Lys107 *(50)*. A computational modeling study by Barman *et al.* analyzed BACE1 substrate specificity, and the mechanisms of cleavage and catalysis for WT APP and Swedish-type (SW) APP *(49)*. SW APP is an APP variant with Lys670Asn and Met671Leu mutations which allow more efficient BACE1 catalyzed hydrolysis of SW over the WT APP *(29, 51)*. Molecular dynamics (MD) simulations revealed that one of the reasons for different rates of hydrolysis for these two substrates is flap positioning, which is more closed when SW APP binds in the active site compared to WT APP binding. This difference in flap position is a result of interactions within the active site upon substrate binding: P1'-Asp of SW APP interact with BACE1 Thr72 positioned at the tip of the flap, indirectly interacting with Gln73 of the flap through a bridging water molecule, while P1'-Asp of WT APP interacts with Thr72 only through bridging water *(49)*. Moreover, SW APP forms more hydrogen bonds within the BACE1 active site compared to WT APP, among which the P4-Glu interaction with Arg307 of BACE1 was shown to be important for BACE1 catalytic activity and is not present in the WT APP-BACE1 complex *(49)*. Arg235, which has been implicated to have a role in substrate recognition (cited in *49*), forms a hydrogen bond with the P2-Asn of SW APP. This interaction is not formed with WT APP, where the P2 position is occupied by positively charged Lys. Altogether, the described differences in the structure and binding patterns point to higher BACE1 affinity for SW APP compared to WT APP *(49)*.

As the BACE1 active site is very spacious, allowing the formation of numerous interactions of substrates/inhibitors with amino acids that form it, a more detailed structural analysis of BACE1 active site will be further presented based on the inhibition and crystallization of the enzyme in complex with inhibitors OM99-2, OM00-3, OM03-4 and P10-P4'StatVal.

OM99-2

The first time the crystal structure of BACE1 was determined was in complex with inhibitor OM99-2 (Figure 3) *(41)*. OM99-2 is an eight-residue transition-state analog of BACE1-catalyzed hydrolysis of SW APP where the hydroxyethylene isoster between P1-Leu and P1'-Ala mimics the structure of transition-state *(41)*. Compared to SW APP (…Glu-Val-Asn-Leu/Asp-Ala-Glu-Phe…; where „/“ denotes the position of the scissile bond) the only change in OM99-2 was done at position P1' which was changed from Asp to Ala *(52)*. OM99-2 showed very high inhibition potency for human recombinant BACE1 with K_i being 1.6 nM. Its binding to the BACE1 active site revealed the presence of eight catalytic subsites (S1 – S4') with the proteolytically active Asp32 and Asp228 in the center of the cleft *(41)*. The P1, P2, P3 and P4 residues of the inhibitor interact with BACE1 subsites S1, S2 S3, S4 located on the *N*-terminal end of the enzyme active

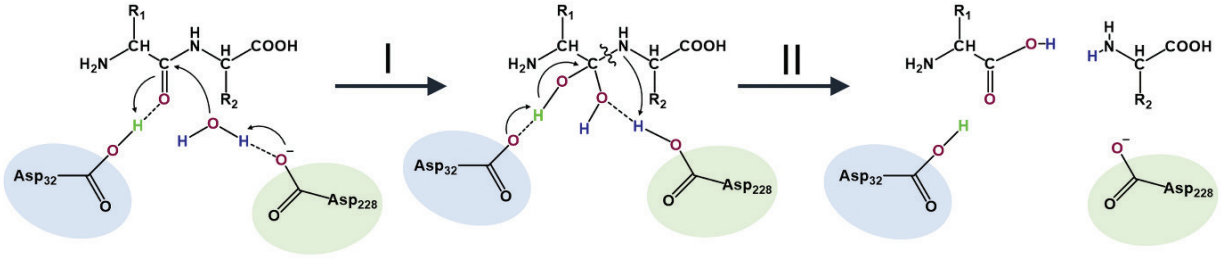

Figure 4. *The mechanism of general substrate hydrolysis by BACE1 (modified from ref. 49). In the first step (I), Asp228 deprotonates and activates water molecule for a nucleophilic attack on carbonyl carbon atom of substrate, while Asp32 donates proton to the carbonyl group, forming the gem-diol intermediate. In the second step (II), Asp228 protonates the amide group of the substrate's scissile bond, while Asp32 deprotonates the gem-diol intermediate, which results in the breakdown of the peptide bond and formation of products.*

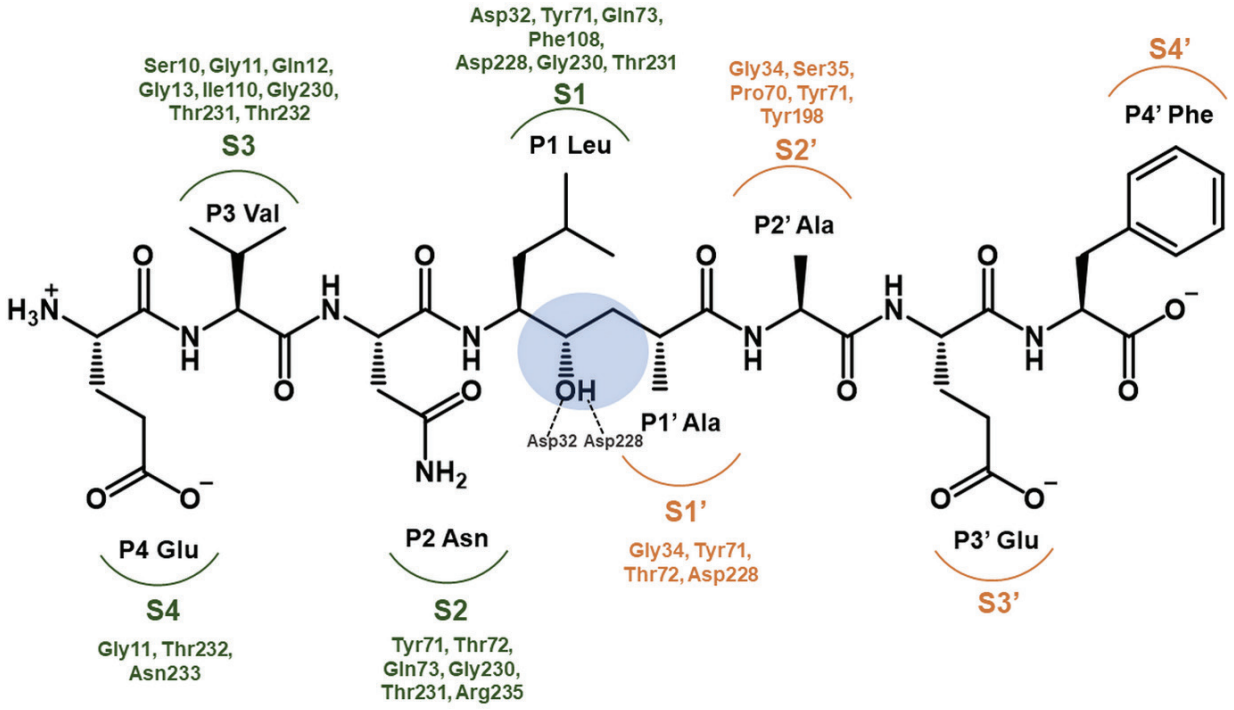

Figure 5. *Structure of OM99-2 inhibitor and its interactions with active site subsites of BACE1. N-terminal subsites (S1 – S4) and amino acids which interact with P1 – P4 inhibitor positions are shown in green, and C-terminal subsites (S1' – S4') and amino acids which interact with P1' – P4' inhibitor positions are shown in orange. The hydroxyethylene moiety of OM99-2 is marked with blue (modified from ref. 41 and 47). The ionization state of the inhibitor at pH 4.5 was determined utilizing corresponding protocols implemented in Biovia Discovery Studio Client v21 (53).*

site, while P1', P2', P3' and P4' interact with S1', S2', S3' and S4' positioned on its *C*-terminal end (Figure 5).

The hydroxyl group of the hydroxyethylene isostere interacts with catalytic aspartates through multiple hydrogen bonds (Figure 6). OM99-2 is bonded to the BACE1 active site in a way that its P4-P1' residues are in extended conformation, and at P2' the chain turns and directs the rest of the inhibitor towards the enzyme surface, causing weaker interactions with BACE1 subunits S3' and S4'. Such accommodation potentially points to a way of directing long substrates out of the active site of BACE1 *(41)*. S2 and S4 subsites accommodated P2-Asn and P4-Glu, respectively, with P4-Glu being close to Arg235 (S2) and Arg307 (S4). These contacts were proven to be important for OM99-2 binding to BACE1, as deleting P4-Glu residue to construct a shorter inhibitor OM99-1 decreased its inhibitory potency 10-fold *(41, 52)*. Subsites S1 and S3 are of hydrophobic character and accommodate P1-Leu and P3-Val. P1-Leu interacts with Phe108 and Tyr71, the latter interaction being especially significant as Tyr71 is an important element of the flap movement *(41)*. The presented findings made the fundamental basis for the further development of new BACE1 inhibitors, such as OM00-3, which allowed the determination of S3' and S4' subsite residues *(47)*.

OM00-3

Shortly after OM99-2, a new more potent BACE1 inhibitor OM00-3 (K_i = 0.3 nM) was synthetized (Figure 7) *(47)*. Like OM99-2, this inhibitor is also a transition-state analog with similar backbone conformation (P3 – P2') as seen in OM99-2 and hydroxyethylene moiety between P1-Leu and P1'-Ala position (Glu-Leu-Asp-Leu*Ala-Val-Glu-Phe; hydroxyethylene isostere denoted with *). The difference in the accommodation of those two inhibitors in the BACE1 active site lies in their interactions with the S2, S3 and S4 subunits of the active site. The S4 subunit was shown to involve Gly11, Gln73, Thr232 and Arg307, the latter forming favorable ion bonds with P4-Glu from OM00-3, which is not the case with OM99-2. In OM00-3 there is Asp on the P2 position which does not form favorable interactions with P4-Glu as does P2-Asn in OM99-2, thus allowing P4-Glu to interact with the S4 pocket, pointing to P2-Asp as the preferred residue at that position in OM00-3. Multiple interactions between S4 residues and P4-Glu of OM00-3 show that this active site pocket contributes greatly to inhibitor binding. The Leu30 of BACE1 S3 subsite interacts with P1 and P3 leucines, which is omitted in OM99-2 where P3 is Val. The electron density analysis of OM00-3-BACE1 complex allowed for the identification of

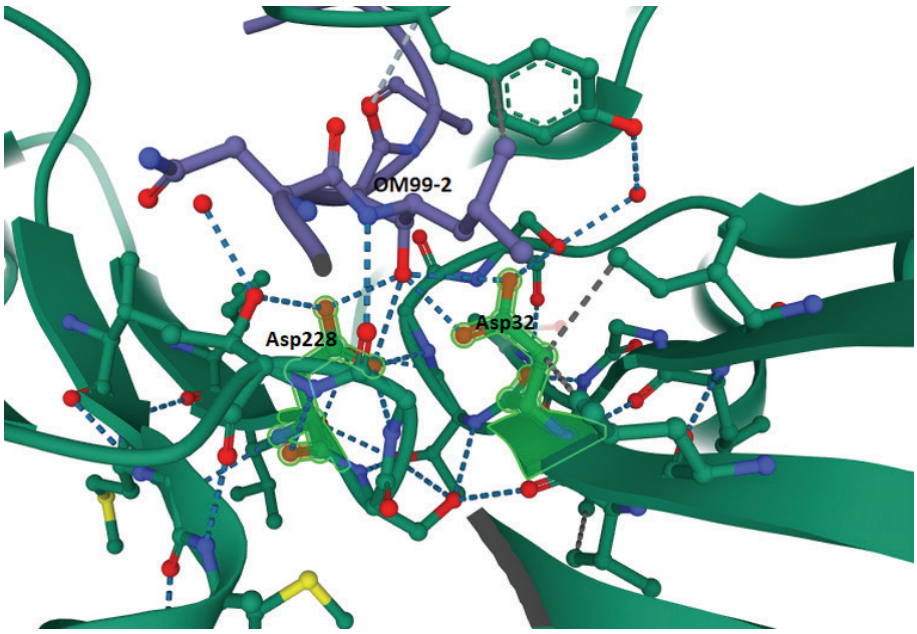

Figure 6. *Hydrogen bond network between BACE1 active site residues and inhibitor OM99-2 (PDB ID: 1FKN (41)).*

amino acids forming the S3' (Pro70, Tyr71, Arg128, and Tyr198) and S4' subsites (Glu125, Ile126, Trp197, Tyr198) based on their interaction with residues Glu and Phe at the P3' and P4' positions, respectively. The higher preference for binding of OM00-3 over OM99-2 can be ascribed to Val at the P2' position of OM00-3, which has more favorable contacts with active site residues than Ala at the same position in OM99-2, which contributes to the positioning of P3' and P4' residues to the enzyme active site *(47)*.

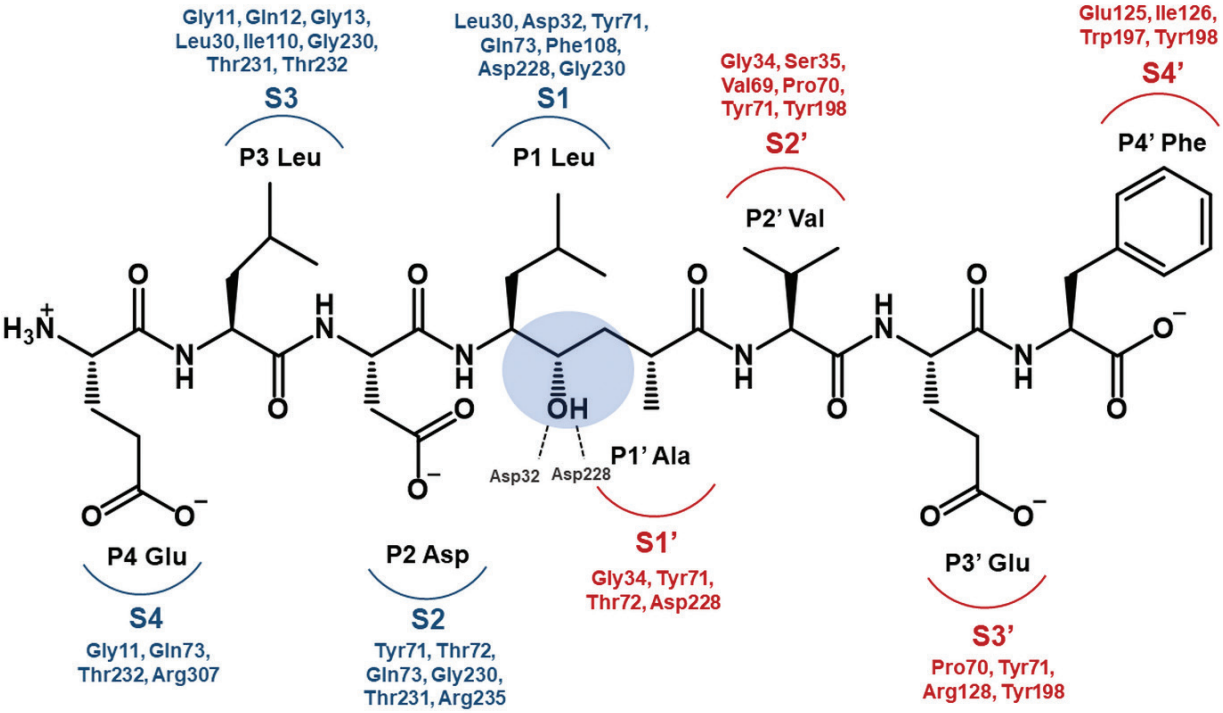

Figure 7. *Structure of OM00-3 inhibitor and its interactions with active site subsites of BACE1. N-terminal subsites (S1 – S4) and amino acids which interact with P1 – P4 inhibitor positions are shown in dark blue, and C-terminal subsites (S1' – S4') and amino acids which interact with P1' – P4' inhibitor positions are red. The hydroxyethylene moiety of OM99-2 is shown in light blue (modified from ref. 47). The ionization state of the inhibitor at pH 4.5 was determined utilizing corresponding protocols implemented in Biovia Discovery Studio Client v21 (53).*

P10-P4'StatVal and OM03-4

The crystal structure of the BACE1/P10-P4'StatVal enzyme – inhibitor complex was determined to investigate the possibility of extra active site subsites existing beyond S4 (Figure 8A) *(45)*. In this inhibitor, statine (P1-P1') occupies the S1 and S1' subsites of enzyme active site and mimics the transition state of BACE1-catalyzed hydrolysis. Electron density analysis showed that residues at the P7 – P4' positions of inhibitor (Glu-Ile-Ser-Glu-Val-Asn-(statine)-Val-Ala-Glu) are placed in the enzyme active site, which was not the case for positions of P10-P8 (Lys-Thr-Glu) and P5' (Phe), suggesting that P7 and P4' residues interact with the ends of the binding cleft and that the residues beyond these two positions cannot realize significant interactions with the enzyme active site (Figure 9A.). The residue preference of subsites S5 – S7 was determined by varying residual compositions on the P5 – P7 positions. The rest of the substrate was composed of BACE1 preferred residues at the P4 – P4'positions (Glu-Val-Asp-Leu-Ala-Ala-Glu-Phe; as determined from OM99-2 and OM00-3 interactions with BACE1 (46)), P5 – P9 (Thr-Glu-Glu-Ile-Ser; as corresponding residues from APP, with variations made at P5 – P7 to obtain a mixture of different substrates) and P10-Arg. It was shown that enzyme subsites S5 – S7 generally prefer hydrophobic residues, especially Trp. To further investigate the role of S5 – S7 subsites in catalysis by BACE1, the OM03-4 inhibitor was synthetized, with Trp at positions P6 and P7 (Figure 8B). This molecule inhibited BACE1 with a K_i 10 times lower than that of OM00-3, proving that the S5 – S7 subsites are a part of active site cleft and interact with OM03-4 (Figure 9B). The P8 and P9 residues were not observed to interact with the active site, while for P7 weak interactions were observed confirming that P7 binds to an end of the active site cleft, that being the S7 pocket. Given the importance of P5 – P7 residues for successful binding and catalysis of substrates, as shown with OM03-4, it is interesting to note that the APP protein, as BACE1's best known physiological substrate, does not possess the most preferred residues at the said positions *(45)*.

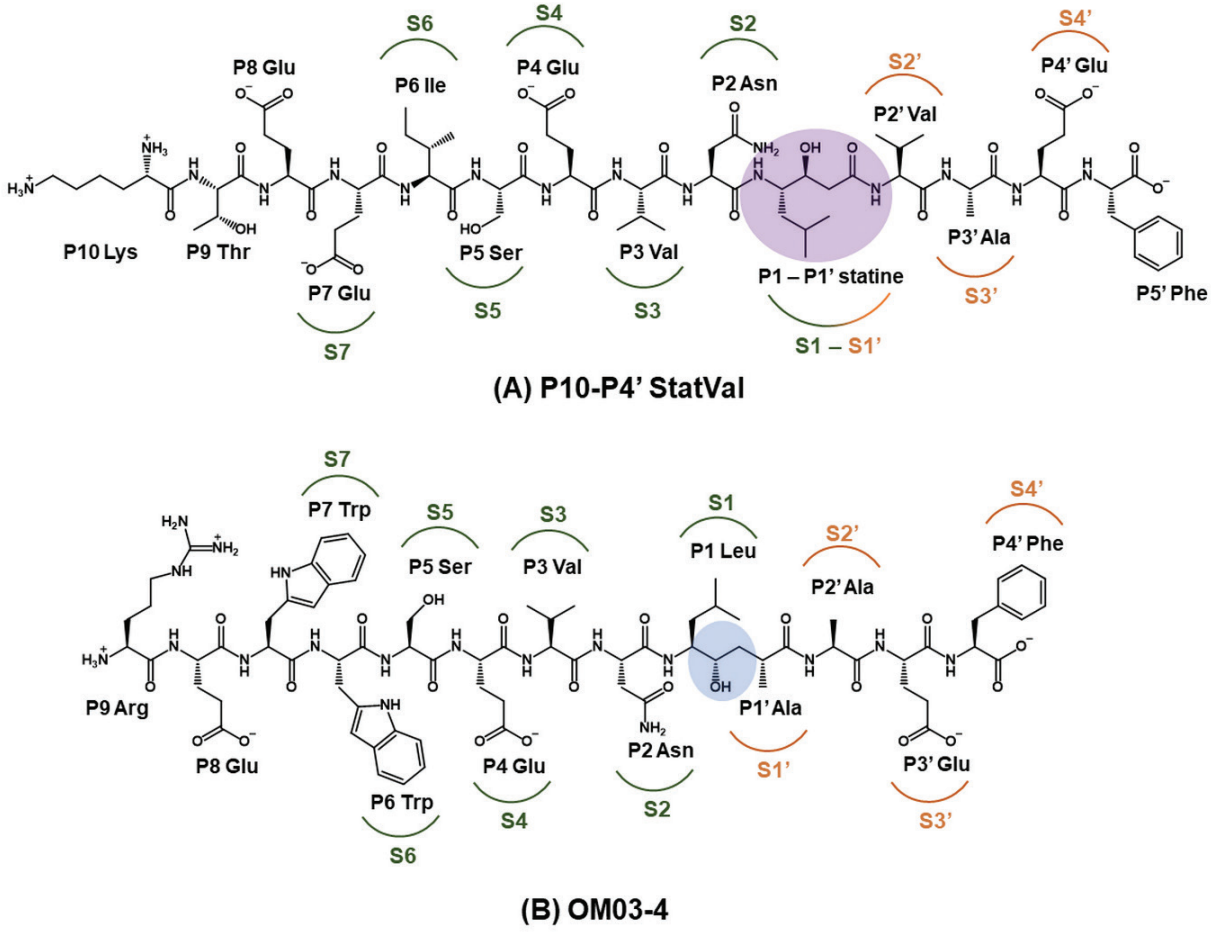

Figure 8. *Structures of inhibitors P10-P4' StatVal (**A**) and OM03-4 (**B**). The subsites of BACE1 active site they interact with are shown (N-terminal subsites in green, C-terminal subsites in orange). Statine moiety of P10-P4' StatVal inhibitor is shown in purple, while hydroxyethylene moiety of OM03-4 is in blue (modified from ref. 45). The ionization state of the inhibitor at pH 4.5 was determined utilizing corresponding protocols implemented in Biovia Discovery Studio Client v21 (53).*

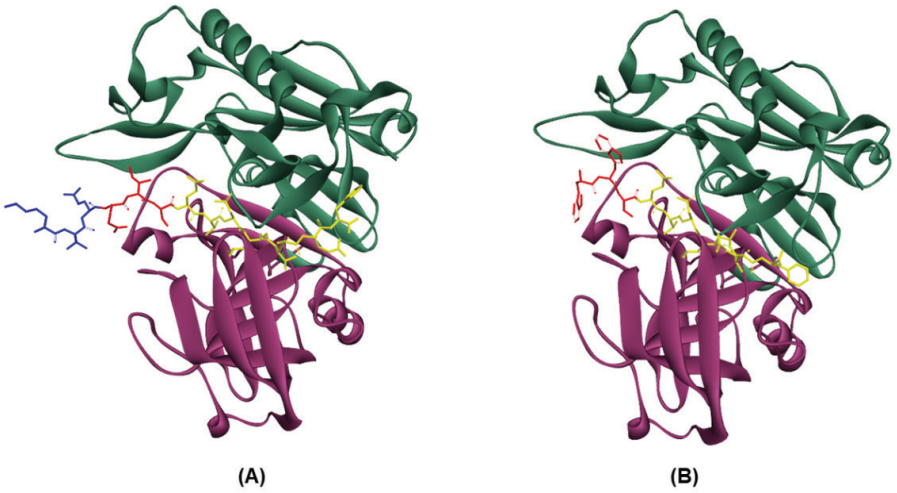

Figure 9. *Crystal structure of BACE1 complexed with P10-P4'StatVal (panel **A**; PDB code: 1XN3 (45)) and OM03-4 (panel **B**; PDB code: 1XN2 (45)). N-terminus of enzyme is shown purple, while C-terminus in green. P4 – P4' positions of inhibitors are shown in yellow, while P5 – P7 is red in both inhibitors. Additional residues of P10-P4'StatVal inhibitor (P8 – P10) are shown in blue.*

GENETIC POLYMORPHISMS OF BACE1

With BACE1 having a crucial role in the formation of amyloid plaques and development of AD, numerous studies have been conducted to examine the expression of *BACE1* gene in AD and healthy subjects, as well as to look for potential mutations within the gene that could be associated with altered BACE1 activity. Fukumoto *et al.* and Yang *et al.* both found elevated BACE1 expression in the brains of patients with sporadic AD. Furthermore, this elevated enzyme expression was translated to increased BACE1 activity in AD brains compared to healthy ones *(54, 44)*. In line with this, Li *et al.* observed that the amyloid load in sporadic AD brains positively correlates with an increase in BACE1 levels and activity *(56)*.

When it comes to mutations in the *BACE1* gene sequence, all of the known polymorphisms are silent mutations, meaning that they do not cause a change in the amino acid sequence of the enzyme, which is why it is hard to elucidate their effect on enzymatic activity. The single nucleotide polymorphism (SNP) occurring in exon 5 has been most extensively studied. This mutation, known as rs638405 or Val262, causes a silent G/C substitution without altering the amino acid sequence *(57)*. Although no association with sporadic AD has been observed at allelic and genotypic level for G/C substitutions (codon 262) *(57)*, Nowotny *et al.* detected a weak association between the GG genotype (exon 5, codon 244) and sporadic AD among elderly Caucasian carriers of $\varepsilon 4$ allele of apolipoprotein E (APOE $\varepsilon 4$) *(58)*, which has been known as the primary genetic risk factor for sporadic AD *(59)*. The same association has been observed in Spanish *(60)*, Swiss *(61)* and German studies *(62)*. On the other hand, two studies conducted in the Chinese Han population yielded opposing results. While both found that the polymorphism in exon 5 correlates with sporadic AD, Shi *et al.* reported a higher frequency of the C-allele in the control group (healthy individuals) and both APOE $\varepsilon 4$ carrier and non-carrier AD subjects, while Kan *et al.* observed increased G-allele frequency and GG-genotype only among APOE $\varepsilon 4$ non-carriers *(63, 64)*. Furthermore, a meta-analysis combining four different Chinese studies and a Korean study found an association between the CC-genotype and APOE $\varepsilon 4$-associated AD *(65)*. However, a large study among the Northern Irish population (901 individuals) found no genetic susceptibility for AD among the subjects and no influence on platelet membrane BACE1 activity from the detected polymorphisms *(66)*. Similarly, a Swedish study found no influence of rs638405 SNP on either BACE1 activity or Aβ peptide levels in the cerebrospinal fluid *(67)*. An interesting study by Tsai *et al.* investigated the association between *BACE1* rs638405 and grey matter volume and cognitive functions in healthy subjects. The results showed that G homozygotes exhibited larger grey matter volumes in the cerebellum compared to C-allele carriers, but no differences in cognitive functions were observed. Nevertheless, the difference in grey matter volume suggested that *BACE1* polymorphisms might influence brain morphology and neurodegeneration *(68)*. Besides AD, rs638405 has been linked to the risk of developing sporadic Creutzfeldt-Jakob disease (prion disease) in an age-dependent manner: it seems that the rs638405 polymorphism is more relevant in early-onset patients in which a possible genetic interaction between *BACE1* and *PRNP*, a major susceptibility marker for human prion disease, was observed *(69)*. A Norwegian study investigated the possible association of *BACE1* rs638405 polymorphism and risk for development of Parkinson's disease (PD) due to the occurrence of Aβ pathology in this disorder. The results showed a

significant association between *BACE1* rs638405 and PD, suggesting that this polymorphism could be a genetic risk factor for the development of PD *(70)*.

When it comes to the *BACE1* promoter region, which plays an essential role in the protein expression, Zhou *et al.* identified the C-1040G polymorphism in sporadic AD and healthy subjects (Indiana, USA), but failed to find any significant difference in genotype and allele frequency in the promoter region of *BACE1* between AD and healthy subjects, as well as no connection between AD cases and previously reported polymorphisms in exon 5 and intron 5 regions *(71)*. On the other hand, Wang *et al.* reported two polymorphisms in the *BACE1* promoter region in Chinese Hans, rs4938369 (-918G/A) and rs3017608 (-2014T/C), respectively, and found that -918GG carriers had a 1.67-fold higher risk for development of sporadic AD compared with -918AA and GA carriers. Moreover, the -918G/-2014T haplotype showed significantly higher transcriptional activity in neural and non-neural cell lines compared to -918A/-2014C, suggesting that this haplotype could cause over-expression of the BACE1 enzyme, meaning that certain polymorphisms in the promoter region could be susceptibility gene mutations, increasing the risk of sporadic AD *(72)*.

With regard to other *BACE1* polymorphisms, SNP rs490460, located at intron 5, has been associated with risk of schizophrenia in the Iranian population *(73)*. The implications for BACE1 activity being related to schizophrenia come from BACE1-dependent processing of neuregulin 1 (NRG1) *(16)* whose gene is a known candidate for susceptibility to schizophrenia *(74)*. The Iranian study found a significant association between rs490460 T allele and risk of schizophrenia *(72)*. Moreover, Dong *et al.* associated the rs490460-TT genotype with a higher p-tau/Aβ_{42} ratio in Chinese Han AD patients *(75)*, suggesting an altered Aβ metabolism which is interconnected with the (hyper)phosphorylation of the tau protein *(76)*. A SNP rs535860 (AT and AT + TT), located in the *BACE1* 3' untranslated region, was associated with focal seizures in the Chinese Han population, but a significant difference between patients and controls was seen only in males, not females *(77)*. Considering that BACE1 cleaves several proteins associated with seizures and epilepsy (Sez6, Sez6L, NRG1, VGSC-β), the connection between *BACE1* polymorphisms and these conditions could be plausible. All mentioned genetic polymorphisms are briefly described in Table 1.

BACE1 ISOFORMS

To this day, six isoforms of BACE1 enzyme resulting from alternative splicing have been reported, with the protein consisting of 501 amino acids being considered canonical *(78)*. Tanahashi and Tabira and Ehehalt *et al.* identified BACE1 isoforms lacking 25 (BACE476), 44 (BACE457) and 69 (BACE432) amino acids *(79, 80)*. Splice variants BACE457 and BACE432 lack two of four *N*-gylcosylation sites, which could affect their posttranslational modifications. Moreover, Tanahashi and Tabira found that BACE457 and BACE476 show lower protease activity towards APP in HEK293 cells, while Ehehalt found these isoforms to be inactive under physiological conditions in N2a cells *(79, 80)*. Furthermore, the Ehehalt group found BACE476 and BACE457 to be solely localized in ER, suggesting that they are either misfolded and unable to exit ER, or properly folded ER residents *(80)*. These three splice variants, together with canonical BACE1, were reported to be expressed in most brain regions, although differently: canonical BACE1 and BACE457 were highly expressed in the human frontal and temporal cortex, sites of amyloid deposition in AD,

Table 1. *Overview of BACE1 genetic polymorphisms and their biological effects.*

Polymorphism	Location	Effect
rs638405	exon 5	GG-genotype associated with sporadic AD in Caucasian APOE *e4* carriers *(58, 60-62)* and Asian APOE *e4* non-carriers *(64)*. CC-genotype associated with sporadic AD in Asian APOE *e4* carriers and/or non-carriers *(63, 65)*. No genetic susceptibility for AD in Northern Irish population *(66)*. Risk for developing sporadic Creutzfeldt-Jakob disease (age-dependent) *(69)*. Risk for development of PD *(70)*.
C-1040G	promoter region	No significant difference in genotype and allele frequency between AD and healthy subjects *(71)*.
rs4938369 (-918G/A)	promoter region	Higher risk for development of sporadic AD in Chinese Han -918GG carriers *(72)*.
rs3017608 (-2014T/C)	promoter region	-918G/-2014T haplotype shows significantly higher transcriptional activity in neural and non-neural cell lines *(72)*.
rs490460	intron 5	Significant association between rs490460 T allele and risk of schizophrenia *(73)*. Higher p-tau/Ab_{42} ratio in Chinese Han AD patients with rs490460-TT genotype *(75)*.
rs535860 (AT and AT + TT)	*BACE1* 3' untranslated region	Significant association with focal seizures in the male Chinese Han population *(77)*.

Table 2. *Overview of BACE1 isoforms and their activity and expression profiles.*

Isoform	Activity / Expression
BACE476	Lower protease activity towards APP in HEK293 cells *(79)*. Low protease *in vitro* activity *(84)*. Inactive under physiological conditions in N2a cells *(80)*. Low expression in the human frontal and temporal cortex *(81)*.
BACE457	Lower protease activity towards APP in HEK293 cells *(79)*. Low protease *in vitro* activity *(84)*. Inactive under physiological conditions in N2a cells *(80)*. Highly expressed in the human frontal and temporal cortex *(81)*.
BACE432	Low expression in the human frontal and temporal cortex *(81)*. Low protease *in vitro* activity *(84)*.
BACE127	No protease activity in cells *(83)*. Splicing induced by treatment of human neuroblastoma cells with protein synthesis inhibitors *(83)*.
BACE455	Increased presence in pancreas *(84)*. Low protease *in vitro* activity *(84)*.

while BACE432 and BACE476 were expressed at low levels at the said regions *(81)*. Canonical BACE1 is expressed significantly more than the other three splice variants in the young and old transgenic mouse model of AD, tg2576, while in wild type mice the expression of all isoforms is more balanced. Moreover, canonical BACE1 was also found to be twice as highly expressed in tg2576 compared to wild type. The BACE1 mRNA levels in tg2576 were unchanged, meaning that different mechanisms could drive the increased expression of enzymatically most active isoform in the transgene brain, compared to wild type brain *(82)*. Tanahashi and Tabira also identified the unusual short BACE127 isoform lacking one of two conserved aspartic protease active sites, a transmembrane domain and a *C*-terminal cytosolic tail. This variant showed no effect on Aβ secretion when expressed in cells. Low expression of BACE127 mRNA was detected in brains of AD and non-AD subjects *(83)*. Lastly, Mowrer and Wolfe identified the BACE455 isoform, which occurs as a consequence of exon 4 skipping. They also found that all 6 isoforms are present in both the human brain and pancreas to some degree, with the pancreas showing an increased presence of BACE476, BACE455 and BACE432, suggesting that BACE1 splicing could depend on the cellular environment *(84)*. All BACE1 isoforms are briefly described in Table 2.

CONCLUSION

Given its crucial role in the formation of amyloid plaques, one of the major pathological hallmarks of Alzheimer's disease, BACE1 remains a highly researched enzyme. Understanding its structure, especially active site, is essential for the development of potential drugs which could show inhibitory action against this enzyme. The spacious active site of BACE1, consisting of eleven defined subsites, allows it to accommodate a broad spectrum of substrates. This can present difficulties for drug design, as it is important not to completely abolish BACE1 activity due to its other physiological roles besides APP processing, as well as to assure the selectivity against other aspartic proteases to avoid adverse effects due to non-selective inhibition. Investigating the structural motifs characteristic for aspartic protease and BACE1 activity, such as the flap positioning over the active site and amino acids involved in its movement (Ty71), and exploring the possibilities of hydrogen bond network formation with and around catalytic aspartates is an important prerequisite prior to target-directed drug design and synthesis. As all known genetic polymorphisms of the *BACE1* gene are silent mutations that do not alter the amino acid sequence of the protein, no effects on the mechanism of action have been observed. Nevertheless, the existence of several isoforms of enzymes with different activities and expression profile in the human organism call for a further investigation of their occurrence in healthy and AD subjects.

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