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REVIEW ARTICLE

Is It the Twilight of BACE1 Inhibitors?

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Abstract: β-secretase (BACE1) has been regarded as a prime target for the development of amyloid beta (Aβ) lowering drugs in the therapy of Alzheimer´s disease (AD). Although the enzyme was discovered in 1991 and helped to formulate the Aβ hypothesis as one of the very important features of AD etiopathogenesis, progress in AD treatment utilizing BACE1 inhibitors has remained limited. Moreover, in the last years, major pharmaceutical companies have discontinued clinical trials of five BACE1 inhibitors that had been strongly perceived as prospective. In our review, the Aβ hypothesis, the enzyme, its functions, and selected substrates are described. BACE1 inhibitors are classified into four generations. Those that underwent clinical trials displayed adverse effects, including weight loss, skin rashes, worsening of neuropsychiatric symptoms, *etc*. Some inhibitors could not establish a statistically significant risk-benefit ratio, or even scored worse than placebo. We still believe that drugs targeting BACE1 may still hide some potential, but a different approach to BACE1 inhibition or a shift of focus to modulation of its trafficking and/or post-translational modification should now be followed.

Keywords: β-secretase, amyloid beta, Alzheimer's disease, substrates, inhibitors, clinical trials.

1. INTRODUCTION

Alzheimer's disease (AD) is characterized by the progressive decline of cognitive functions, manifested in abnormalities of speech, social behavior, and memory. The disease is fatal, with maximal survival of 10 years following the diagnosis. The symptoms of AD may result from the formation of insoluble amyloid plaques in the brain, which consists of extracellular deposits of insoluble amyloid-β (Aβ) [1-3]. Aβ was first isolated and described in 1987 [4, 5]. Aβ is derived from an amyloid precursor protein (APP), which is a type I transmembrane protein with a large extracellular domain and a short cytoplasmic region. Several different APP isoforms exist as a result of alternative splicing, ranging in length from 695 to 770 amino acid residues [6]. APP is produced in large amounts in neurons. However, it is typically very quickly metabolized. Norstrom listed six different enzymes able to cleave APP, including α-, β-, δ-, η- and θ-secretase and meprin β [7]. In AD, APP is cleaved alternatively through the sequential action of integral membrane ß- and γ-secretase in endosomal compartments, releasing Aβ from the APP [8, 9]. In the initial cleavage, ß-secretase (BACE1) splits APP and produces a ~100 kDa soluble N-terminal APP ectodomain (APPsβ) and a 12 kDa membrane-tethered C-terminal fragment with 99 or 89 amino acid residues (C99/C89), according to whether it cleaves at Asp^{1} or Glu^{11} of the APP. Under physiological conditions, BACE1 predominantly cleaves APP at the Glu^{11} site and the result is the non-amyloidogenic form C89, which results in truncated Aβ production (Fig. **1**).

Zhang and his colleague demonstrated that the APP Swedish mutation strongly shifted the BACE1 primary cleavage site from Glu^{11} to Asp^{1}, resulting in a higher C99/C89 ratio. This well-known double mutation was first identified in a Swedish family and results in a substitution of two amino acids, Lys^{595} to $Asn^{595}$and Met^{596} to Leu^{596}. The replacements are responsible for three to six times higher production of Aβ [10-15]. The C99 or C89 fragment is subsequently processed by γ-secretase. The γ-secretase is a complex which consists of four components - presenilins (PS1 and PS2), presenilin enhancer 2 (PEN-2), anterior pharynx-defective 1 (APH-1), and nicastrin. It cleaves C99 in the transmembrane region, liberating APP intracellular domain (AICD) and Aβ peptide [16-18]. Various isoforms of Aβ fragments, ranging in size from 37 to 49 residues, are caused by γ-secretase cleavage of C99 at multiple sites and by further processing of the fragments. The amyloid fibrils in AD predominantly consist of 40 and 42 amino acids

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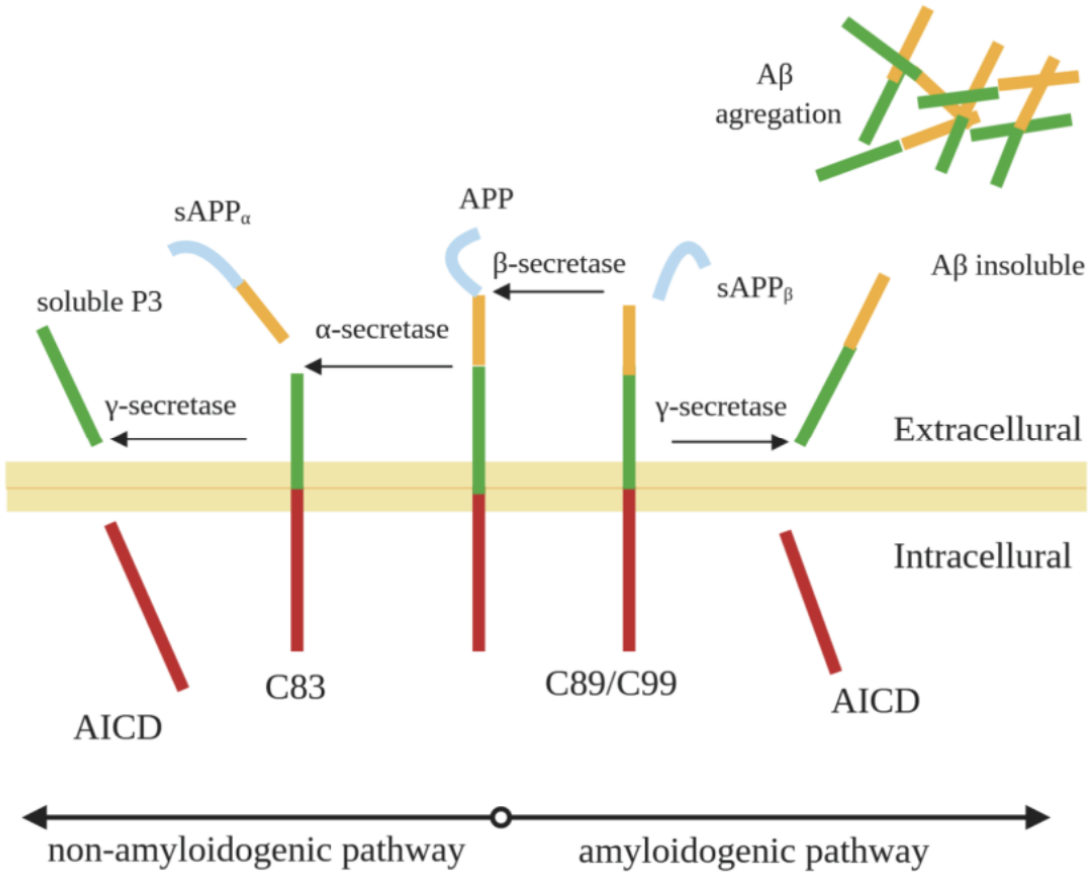

Fig. (1). Proteolytic processing of APP. (Left) Non-amyloidogenic pathway: proteolytic cleavage by α- and γ-secretases precludes amyloid β (Aβ) generation. This pathway is initiated by α-secretase and produces the soluble amino-terminal ectodomain of amyloid precursor protein (sAPPα) and the α-carboxyl-terminal fragment C83. C83 can be further cleaved by γ-secretase, producing the short fragment P3, also known as amyloid β (peptide $Aβ_{17–40/42}$) and APP intracellular domain (AICD). (Right) Amyloidogenic pathway: sequential proteolytic cleavage through β- and γ-secretases is responsible for the generation of Aβ. β-secretase produces the secreted sAPPβ and the β-carboxyl-terminal fragment C99. C99 can be cleaved by γ-secretase, giving rise to Aβ and AICD. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

($Aβ1_{40}$/$Aβ1_{42}$) that exhibit aggregative ability and neurotoxicity. The $Aβ_{42}$ plays a key role in the pathogenesis of AD as its aggregative ability and neurotoxicity are substantially higher than in $Aβ_{40}$ [19, 20]. Genetic variations in the APP amino acid sequence in the proximity of the BACE1 cleavage site may affect the activity of the enzyme. Studies of APP have identified 24 mutations and duplications in nine families of various ethnic origins (French, Dutch, Japanese, and Swedish) that significantly contribute to the pathogenesis of AD. In contrast, a rare A673T variant seems to protect against the development of the disease. BACE1 cleaves 50-fold less for such threonine substitution than for alanine in the same position. Thus, understanding of APP processing appears fundamental for the development of a therapeutic strategy aimed at reducing Aβ levels in AD patients [21-25].

2. BACE

Beta-secretase was discovered independently by five research teams in 1991. All of them simultaneously reported a new integral membrane aspartyl protease. These findings initiated a large number of studies focused on this enzyme [26-30]. Beta-secretase exists in two major forms, BACE1 containing 501 amino acids (EC 3.4.23.46) and BACE2 containing 518 amino acids (EC 3.4.23.45). Both forms show approximately 75% sequence homology. BACE1 is known to cleave APP, and mature BACE1 is found on the cell surface and in endosomes, but not in the endoplasmic reticulum (ER) or lysosomes. There is still some lack of clarity regarding the major subcellular compartment where APP is cleft by BACE1, leading to Aβ production. On the other hand, the role of its homolog BACE2 is still unidentified. The results of biochemical and morphological analyses confirmed a physiological role, such as the regulation of glucose homeostasis, as well as the amyloidogenic role of BACE2 in pigment cells [31]. The BACE1 gene includes a ~30 kilobase (kb) region within human chromosome band 11q23.2 – 11q23.3 and consists of 9 exons and 8 introns. The gene encoding BACE2 has been located on the long arm of chromosome 21 at 21q22.3. Trisomy of chromosome 21 is associated with Down syndrome (DS). As adults with DS age, they are at high risk for AD and virtually all of them have sufficient senile plaques and neurofibrillary tangles for neuropathological diagnosis of AD by the age of 40. Adults with Down's syndrome who were also diagnosed for AD-type dementia dis-

played increased levels of BACE2 protein in the frontal lobe, suppressing the overexpression of APP, the gene for which is also located on chromosome 21, and by that prevent the progressive impairment in DS. However, the role of BACE2 in amyloidogenesis is not completely known [32-34].

2.1. BACE1 Structure and Localization

BACE1 is a ~75 kDa integral membrane protein showing approximately 30% sequence homology with other pepsin family members. BACE1 has a low pH optimum. Therefore, the enzyme is predominantly active in acidic intracellular compartments (*e.g.* endosomes, trans-Golgi) with its active site oriented to the lumen of vesicles. BACE1 is present in many tissues but mainly in the brain and pancreas [35, 36]. In the brain, the highest expression is found in substantia nigra, locus coeruleus and medulla oblongata. Immunopositive structures are neurons and, to a much lesser extent, resting glia [37, 38].

BACE1 expression is up-regulated by cellular stress, *e.g.* energy deprivation, hypoxia and ischemia, and oxidative stress [39, 40]. A region of DNA that initiates BACE1 transcription lacks typical CAAT and TATA boxes and contains GC-rich sequences and four GATA sites. The promoter contains a several transcription factor binding sites cAMP response element-binding protein (CREB) [41], hepatocyte nuclear factor-3 (HNF-3) [42], nuclear factor- κB (NF-κB) [43], specificity protein 1 (Sp1) [44], Yin Yang 1 (YY1) [45], and signal transducer and activator of transcription (STAT) [46]. While CREB is considered a negative regulator, HN-3, NF-κB, Sp1, YY1, and STAT are positive regulators. It is noteworthy that the overexpression of Sp1 protein not only supports BACE1 expression but also increases Aβ levels [47]. Single nucleotide polymorphisms do not seem to affect BACE1 transcription. Zhou and colleagues examined potential polymorphism in the promoter region from 472 AD cases and control individuals and did not find any genetic association with AD [48]. On the other hand, epigenetic factors broaden the complexity of BACE1 regulation. Decreased methylation of CG sites in the BACE1 promoter or increased acetylation of histone H3 in the same region elevates BACE1 expression [49, 50]. On the contrary, several miRNAs, including, for instance, miR-16-5p, miR-19b-3p [51] and miRNA-31 [52] (for more details see reviews [53, 54]), have been identified as negative regulators of BACE1 expression, whereas BACE1-antisense lncRNA stabilizes BACE1 RNA and promotes APP cleavage.

Full-length BACE1 is a 501 amino acid zymogen. The enzyme consists of an N-terminal signal peptide (residues 1-21), followed by a pro-domain (residues 22-45), and a protease domain (residues 46-460) with two active motifs characteristic for aspartyl proteases: DTGS (residues 93-96) and DSGT (residues 289-292). Both aspartic residues are important for enzymatic activity. If they are mutated, the enzyme becomes inactive [55, 56]. BACE1 also contains a single transmembrane domain near its C-terminus (residues 461-477) and a short cytosolic domain (residues 478-501; Fig. **2**). The transmembrane domain is required for BACE1 activity within the cell and it is necessary for the generation of intracellular C99 in the Golgi compartment (GC). Regulation of subcellular localization of BACE1 may be another option to control C99 production [57, 58].

In addition to the full-length isoform, the enzyme has three alternatively spliced isoforms, which have been isolated from the human brain: $BACE1_{457}$, $BACE1_{432}$, $BACE1_{476}$. These isoforms are deficient in amino acid and glycosyl residues. However, all three variants retain both catalytic aspartyl motifs and demonstrate secretase activity [59-61].

BACE1 is synthesized directly into the ER, as with all other aspartyl proteases. Subsequently, several post-translational modifications take place in the ER and the GC [62]. BACE1 is exposed to simple glycosylation on four asparagine residues (Asn_{153}, Asn_{172}, Asn_{223}, and Asn_{354}) and short-term acetylation on seven lysine residues (Lys_{126}, Lys_{275}, Lys_{279}, Lys_{285}, Lys_{299}, Lys_{300}, and Lys_{307}) within the lumen of ER. Subsequent deacetylation of BACE1 occurs in the lumen of GC [38, 63, 64]. Further addition of complex carbohydrates and removal of the BACE1 prodomain by furin convertases occur in the GC [65, 66]. The fully maturated BACE1 is phosphorylated on Ser_{498} by casein kinase 1. Phosphorylation/dephosphorylation affects the subcellular localization of the enzyme. Non-phosphorylatable BACE1 is retained within early endosomes. Phosphorylated BACE1 is

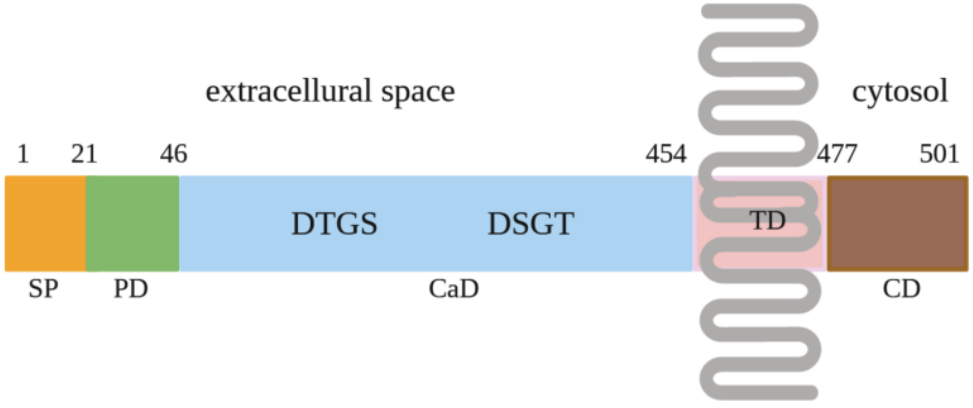

Fig. (2). Structural organization of BACE1. SP - signal peptide, PD - prodomain, CaD - catalytic domain, TD - transmembrane domain, CD - cytoplasmatic domain, DTGS and DSGT - active sites. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

reinternalized from the cell surface to early endosomes and could be recycled back to the cell surface upon dephosphorylation [67, 68]. BACE1 also undergoes S-palmitoylation on four cysteine residues located at the junction of the transmembrane (Cys_{474}) and cytosolic domains (Cys_{478}, Cys_{482} and Cys_{485}). S-palmitoylation of membrane proteins plays an important functional role in protein-protein interactions, folding, trafficking, and association with a lipid membrane [69, 70]. Recent studies identified lipid rafts as important sites for the generation and accumulation of Aβ. Lipids rafts are membrane microdomains enriched with cholesterol and sphingolipids. BACE1 and γ-secretase complexes are partially and mainly localized in lipid rafts, respectively. Decreased levels of cholesterol and sphingolipids, both of which are necessary constituents of lipid rafts, correlate with reduced β-cleavage [71-73].

Intracellular localization and trafficking of BACE1 can be modified by several factors. This includes Golgi-localized, γ-ear containing, ADP-ribosylation factor binding proteins (GGAs), reticulons/Nogo proteins (RTNs) and sorting nexins (SNXs). The GGA family of multi-domain coat proteins was first described in 2000 [74]. The family contains three members: GGA1, GGA2, and GGA3. Decreased levels of GGA3 bring about an increase in BACE protein levels with the sorting of BACE to lysosomes where it is degraded. This mechanism is ubiquitin-dependent. GGA1 proteins have a particular cargo-sorting function in endosomal/Golgi compartments. GGA1 interact with BACE1 and are responsible for transporting of the enzyme between the late Golgi and early endosomes. The increase in GGA1 levels correlates with increased intracellular APPβ level, whereas levels of extracellular APPs and Aβ decrease. On the other hand, depletion of cellular GGA3 proteins increases levels of BACE1 and its activity during ischemia and in AD brain (Schema of BACE1 trafficking and interrelationship with GGA is described in Fig. **3**) [75-78].

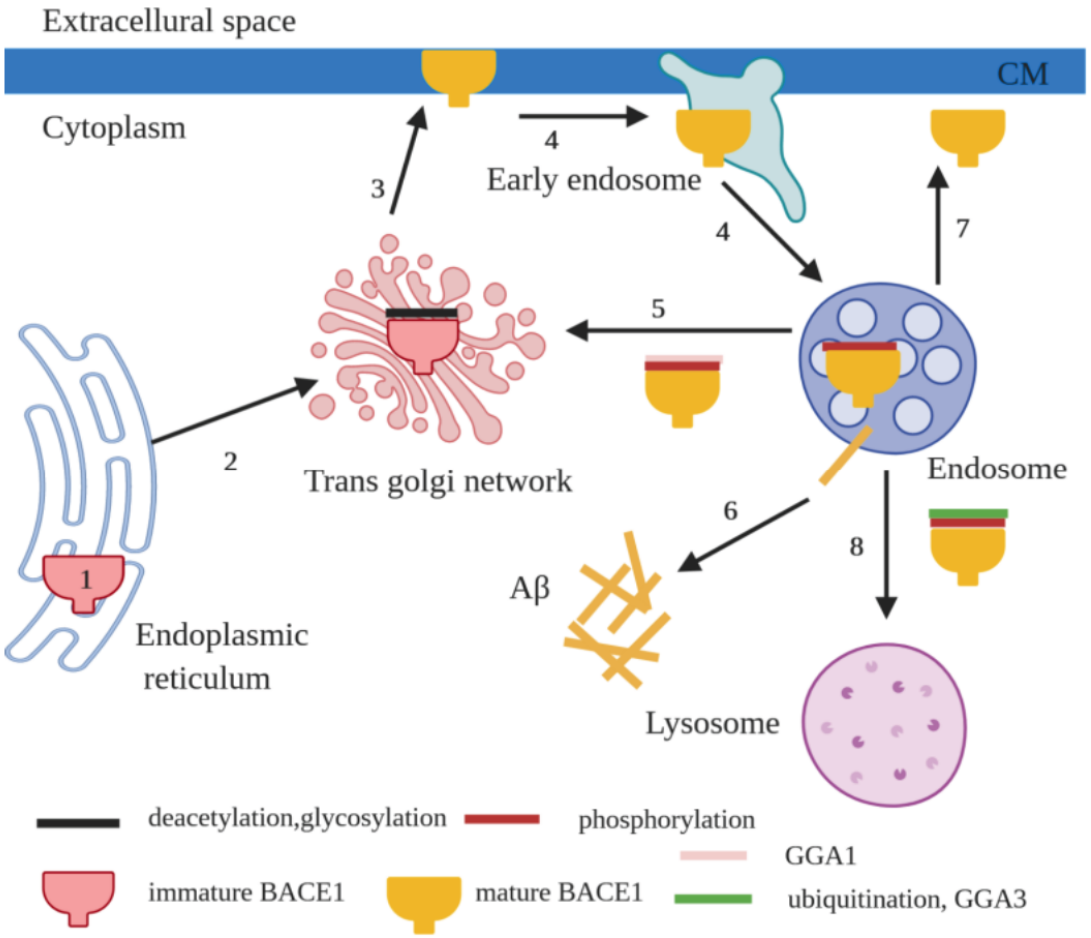

Fig. (3). Trafficking of BACE1. (**1**) In the endoplasmic reticulum, BACE1 is initially synthesized as a zymogen and subjected to N-glycosylation, palmitoylation, transient acetylation, and disulfide bridge formation. (**2**) Full maturation takes place in the Golgi compartment, where complex glycosylation and removal of the prodomain by furin prohormone protein convertases lead to the 75 kDa form. (**3**) Newly synthesized BACE1 is transported from the trans-Golgi network (TGN) to the cytoplasmic membrane (CM). (**4**) BACE1 can be reinternalized from CM to endosomes. (**5**) BACE1 binds with GGA1 *via* an acid-cluster-dileucine (ACDL) motif. The binding regulates the transport of enzymes from endosomes to TGN. (**6**) Amyloid precursor protein (APP) is cleaved by BACE1 in the endosomes, producing amyloid β (Aβ). (**7**) Phosphorylation at ser498 regulates BACE1 trafficking. Phosphorylated BACE1 is intercepted from the cell surface to early endosomes. The non-phosphorylated BACE1 recycles back to CM. (**8**) Lastly, mono-ubiquitination at lysine 501 and binding with GGA3 promote lysosomal degradation. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Regarding the RTNs family, RTN3 and RTN4-B/C were identified as interacting with BACE1. They negatively modulate BACE1 activity and reduce its ability to produce Aβ in the brain [79]. Murayama *et al.* demonstrated that overexpression of these RTNs resulted in a 30-50% reduction of $A\beta1_{40}$ and $A\beta1_{42}$ secretion from HEK293 cells expressing APP with the Swedish mutation [80]. Finally, SNXs belong to a large family of proteins containing a conserved PX domain. Many members of this family have been shown to regulate protein sorting in early endosomes. For instance, the downregulation of SNX12 increases endocytosis of BACE1 and decreases the level of this enzyme on the cell surface. SNX6 was identified as another negative regulator of BACE1. Okada and his colleagues confirmed that SNX6 negatively regulates the retrograde trafficking of BACE1 from the cell surface through the endosomal structure to the perinuclear space, thereby regulating Aβ biogenesis. Consequently, inhibition of BACE1 by regulating SNXs might be a novel approach in the treatment of AD [81, 82].

2.2. BACE1 Substrates

Many potential substrates of BACE1 have been identified during the last decade with advances in proteomics. Hemming and his colleagues performed quantitative proteomic analysis of two human epithelial cell lines stably expressing BACE1 and identified 68 putative BACE1 substrates. The majority of them were of type I transmembrane topology (with N-terminus extracellular orientation), one was of type II (with C-terminus extracellular orientation), and three were glycosylphosphatidylinositol (GPI)-linked proteins. BACE1 substrates have been associated with diverse functions, including synaptic processes, cell signaling, and immune responses. Confirmation of the physiological roles of BACE1 substrates *in vitro* as well as *in vivo* may help to understand the complexity of BACE1 functions and may reveal how much its inhibition affects other essential biological processes [83-85].

One of the physiological roles of BACE1 is associated with the proteolytic processing of Neuregulin-1 (Nrg1). Nrg1 is a cell adhesion molecule regulating axon myelination in the peripheral nervous system *via* ErbB receptors. Mutant mice lacking BACE1 display severe hypomyelination of peripheral nerves similar to that seen in mice lacking Nrg1/ErbB signaling in Schwann cells. Nrg1 is also an important candidate gene with a very strong association with schizophrenia. In other tissues, its signaling has been linked to cardiogenesis and the development of the mammary gland [86-90].

Neural cell adhesion protein close homolog of L1 (CHL1) is another BACE1 substrate [91]. The cleavage of CHL1 by BACE1 regulates the balance between growth cone extension and collapse *via* the axon guidance molecule semaphorin 3A, regulating correct axonal targeting [92]. This process is critical, particularly during embryogenesis. In the adult brain, CHL1 deficiency may disrupt the organization of axonal pathways in the hippocampus, an important structure for learning and memory [93].

Besides APP, BACE1 processes its paralogs amyloid precursor-like protein 1 (APLP1) and amyloid precursor-like protein 2 (APLP2). APLP1 and APLP2 are type I transmembrane proteins that undergo cleavage by secretases, including BACE1. Both are metalloproteins with a possible role in synaptogenesis and the subsequent maintaining of synaptic structure. Knockout mice lacking both APLP1 and APLP2 show postnatal lethality and growth deficiency, metabolic stress such as hypoglycemia, and central respiratory problems. On the other hand, their specific role within the CNS and PNS is still poorly understood [94-96].

Another class of BACE1 substrates is represented by β2 subunit of voltage-gated sodium channels (VGSCs). The β2 subunit has an important role in the activation and propagation of electrical membrane potentials. It also modulates cell adhesion and neurite outgrowth *in vitro* [31]. The β2 subunits contribute to myelination and their dysfunction has been associated with neurodegenerative disorders through a variety of mechanisms. Mutations in sodium-channel subunits are associated with epilepsy. Kim and colleagues found that the ablation of BACE1 led to decreased sodium channel levels suggesting that complete blockage of BACE1 is likely to cause side effects through altered sodium current densities [97]. On the other hand, BACE1 inhibitors may be effective in the treatment of epileptic symptoms derived from abnormal neuronal activity in AD patients, but it will be important to find the therapeutic window for inhibiting BACE1 activity and simultaneously maintaining physiological VGSC functions [98].

BACE1 is also involved in the cleavage and secretion of membrane-bound α-2,6-sialyltransferase I (ST6Gal-I). ST6Gal-I is a glycosyltransferase type II membrane protein, which is highly expressed in the liver. Some of the ST6Gal-I soluble forms measured in serum could be considered as a diagnostic marker since their increased levels have been related to inflammation, malignant transformation or liver injury. Kitazume and his colleagues found that the serum level of ST6Gal-I was correlated with the activity of hepatic inflammation in hepatitis C patients [99-101]. Deng and his colleagues investigated the potential role of BACE1 in endothelial cells. The results showed that BACE1 protein levels were dramatically upregulated after TNF-α treatment, thereby resulting in ST6Gal-I cleavage and a dramatic decrease in α-2,6-sialylation in vascular endothelial cells. It suggests that inhibition of BACE1 expression may represent a new approach for treating atherosclerosis [102, 103].

Low-density lipoprotein receptor-related protein (LRP), a multifunctional endocytic and signaling receptor, is a novel BACE1 substrate. LRP is a type I integral membrane protein cycling between the cell membrane and endosomes. LPR interacts with BACE1 on the cell surface in association with lipid rafts. BACE1-LPR interaction releases both secreted LRP and the LRP intracellular domain from the membrane. BACE1 inhibition may, therefore, preserve LRP on the surface of neurons, increasing Aβ clearance. However, other experiments have demonstrated that LRP binds to longer isoforms of APP, containing a Kunitz-type proteinase inhibitor domain. These isoforms are the most abundant forms of APP in the brain and LRP may modulate their processing, leading to increased Aβ production. It is also noteworthy that LPR is also the major apolipoprotein (apoE4) receptor in

neurons. Both apoE4 and a silent polymorphism in exon3 of the LRP gene (C776T) represent a significant genetic risk factor for late-onset AD. Thus, further research is needed to illuminate the LRP role in AD pathogenesis [104-108].

In summary, despite the substantial progress in this field, the normal function of BACE1 and its substrates has not been fully identified. A knowledge of their subcellular localization and a precise definition of their roles are essential for the development of BACE1 inhibitors. Full inhibition of BACE1 will contribute to the accumulation of misfolded BACE1 substrates in the cellular endomembrane system, including ER. Accumulation of misfolded proteins in ER underlies the induction of stress, *i.e*. the status that is already associated with AD pathology [109]. ER stress over-activates three pathways, namely pancreatic ER kinase (PERK), activating transcription factor-6 (ATF-6), and inositol-requiring enzyme-1 (IRE1) pathways, which when continuously activated, contribute to neuronal death by apoptosis and consequently memory deficits [110]. Thus, it seems necessary first to determine the relation between the quality and the quantity of BACE1 inhibition and its impact on physiological functions.

2.3. BACE1 *vs*. BACE2 Selectivity

BACE1 and BACE2 show similar expression in tissues, with one exception. BACE2 has been reported to be expressed in the pancreas, whereas BACE1 is not (Table **1**). BACE1 and BACE2 share 50% homology. Additionally, similarities can be found to proteases such as cathepsin D, cathepsin E, pepsin, and renin. BACE1 was predominantly confirmed as a neuronal protein. The co-expression pattern of the BACEs raises the question of whether BACE2 is a functional substitute for BACE1. Thus, the challenge is to develop a highly selective inhibitor to minimize side effects [111, 112].

Several studies, including those by Fluhrer *et al*. and Yan *et al*., demonstrated that BACE2 does not possess β-secretase activity [113, 114]. On the contrary, BACE2 overexpression suppresses Aβ production in AD transgenic mice and cells [115, 116]. Nevertheless, Wang and colleagues reported that under certain circumstances, BACE2 can become a conditional β-secretase. This is enabled by clusterin (apolipoprotein J) through binding to the juxtamembrane helix of wild-type APP. Both BACE2 and clusterin display increased expression in neurons of aged wild-type mice [117]. Interestingly, β-secretase activity, but not BACE1, increases during aging in human, monkey and mouse brains. Thus, BACE2 could act as β-secretase during this process [118]. BACE2 expression is also up-regulated under inflammatory conditions [119]. Nevertheless, the role of BACE2 in AD pathology needs to be further elucidated. BACE2 is also highly expressed in pancreatic endocrine β-cells. It up-regulates the expression of transmembrane protein 27 (TMEM27). TMEM27 augments β-cell mass and insulin production. According to Esterházy's study, non-selective inhibitors can have a beneficial effect in the treatment of type 2 diabetes [120, 121].

Table 1. Gene expression of human BACE1 and BACE2.

Anatomical Locus	BACE1	BACE2
Cardiovascular system	+	+
Respiratory system	+	+
Haematological system	+	+
Lymphoreticular system	+	+
Alimentary system	+	+
Urogenital system	+	+
Endocrine system	+	+
Musculoskeletal system	+	+
Dermal system	+	+
Nervous system	+	+
Pancreas	N	+

N - no expression is reported in contrast to BACE2 [112].

On the other hand, BACE2 is expressed in pigment cell-specific melanocytes playing a pivotal role in the melanogenesis of the hair follicle. This could be the underlying mechanism of irreversible hair depigmentation observed during clinical trials with several non-selective BACE1 inhibitors [122]. Altogether, it seems necessary to assess the relation between the extent of inhibition and beneficial as well as adverse effects to evaluate the possible contribution of non-selective inhibitors to AD therapy.

2.4. Off-targeting

Off-targets are most likely responsible for other reported adverse effects. For instance, the novel BACE1 inhibitor series exhibited a potential cardiovascular risk associated with QT prolongation. The interesting structural motifs have been based on amidine or guanidine core structures. This has been related to an interaction with the human Ether-A-Go-Go ion channel (hERG), which is responsible for the rapid component of delayed rectifier potassium current in the heart [123, 124]. Inhibition of the R-subunit of IKr channels is a critical element in the development of new drugs and *in vitro* methods have been developed to assess hERG activity of BACE1 inhibitors. AMG-8718 is an example of a substance with high activity against BACE1 and a reduced affinity to hERG [125]. However, the lack of hERG channel affinity does not always mean the absence of delayed cardiac repolarization under clinical settings [126]. It is noteworthy that $A\beta_{1-40}$ and $A\beta_{1-42}$ are expressed in the heart of AD patients, and compounds showing anti-BACE-1 activity and no hERG affinity may prevent heart failure [127, 128]. In this case, the application of BACE1 inhibitors may not only be a benefit for AD treatment but may also positively influence cardiovascular status.

Another important off-target belongs to the cathepsin family. Zuhl *et al*. associated the ocular toxicity of BACE1 inhibitors with cathepsin D off-targeting and found that quantification of cathepsin D inhibition is predictive of the toxic effect *in vivo* [129]. It is noteworthy that cathepsin D deficient mice develop seizures and retinal atrophy associated with blindness [130], while in humans, it is related to

early blindness and progressive psychomotor impairment [131]. Cathepsin E deficiency, on the other hand, is related to atopic dermatitis, an inflammatory skin disease [132]. Skin rashes have been consistently reported as BACE1 inhibitors´ side effects during clinical trials [133].

Finally, gastrointestinal undesired effects, including weight loss, were reported in clinical studies of Verubecestat [134]. Although no mechanistic explanation has yet been demonstrated, we may hypothesize that it may be linked to the similarity between BACE1 and the digestive enzyme pepsin.

3. BACE INHIBITORS

The current AD treatment strategy is based on four drugs. Three of them, including donepezil (Aricept), rivastigmine (Exolon) and galantamine (Reminyl), are acetylcholinesterase inhibitors decreasing the breakdown of the neurotransmitter acetylcholine. Only 20-30% of patients respond positively to treatment with cholinesterase inhibitors. In the case of intolerance of or a contraindication for acetylcholinesterase inhibitors, the fourth drug, NMDA antagonist memantine (Ebixa) is prescribed [135-138].

The first studies targeting amyloid processing were focused on γ-secretase. The use of γ-secretase inhibitors soon became problematic as the complex appears to have multiple substrates, including Notch, the protein which regulates cell proliferation, differentiation, and growth. Therefore, it is not surprising that hematological disorders, gastrointestinal symptoms, skin reactions, and hair color changes were observed in patients during clinical trials [139, 140].

Consequently, the focus of AD therapy research turned to BACE1 [141-143]. Four distinct generations of BACE1 inhibitors are recognized (Table **2**) based on molecular size and selectivity to BACE1, BACE2 and other proteases.

The ***first generation*** of BACE1 inhibitors is represented by large hydrophobic substrate binding-site polypeptides. Although peptidomimetic inhibitors were highly potent *in vitro*, these enzyme inhibitors had low oral bioavailability, a short half-life, metabolic instability, and poor ability to penetrate the blood-brain barrier (BBB) [144-146]. Peptidomimetics were also prone to P-glycoprotein efflux [147]. As an example, OM99-2 is an eight-residue inhibitor with a molecular weight of 893 g/mol. The compound showed very potent BACE1 inhibitory activity with a K_i of 1.7 nM but failed in clinical trials. Hong *et al*. elucidated the crystal structure of the BACE1/OM99-2 complex [148]. Understanding of the interactions between the inhibitor and the active sites of BACE1 represented a major advance in the development of subsequent generations of highly selective inhibitors [149, 150]. For this reason, it is still being used as a reference compound in biological assays, in docking and molecular dynamics simulations, and the development of electrochemical biosensor assays for monitoring of BACE1 activity [151-154].

Non-selective inhibitors based on small molecules represent the ***second generation***. **LY2811376** was the first non-peptidic BACE1 inhibitor tested in humans. Early experiments showed 63-fold selectivity over cathepsin D. However, later study by Ellis *et al*. considered pH-depend binding behavior and demonstrated that LY2811376 is only 6 times more selective [155]. Eli Lilly began phase I clinical trial in 61 healthy volunteers in 2008 to assess the single-dose effects of the drug on the body, including cerebrospinal fluid (CSF) (for study details see NCT00838084). The published results proved safety, tolerability, and good BBB penetration. The doses of 30 and 90 mg displayed a significant reduction of Aβ1-40/42 in the CSF. In parallel to the phase I trial in healthy participants, a 3-month toxicology study was conducted in rats to prepare for longer clinical exposures. In this model, the drug caused cytoplasmic accumulations of autofluorescent material in the retinal epithelium, neurons and glial cells at doses ≥30 mg/kg [156], discontinuing clinical tests. Subsequently, several structurally distinct BACE1 inhibitors have been withdrawn from development due to ocular toxicity. This includes AMG-8718 introduced as a perspective drug in 2014 [125]. In 2015, Filden *et al*. reported an increase in autofluorescent granules in the retinal pigment epithelium, which led to a significant loss of photoreceptor cells and retinal thinning in rats [157].

The second generation of BACE1 inhibitors was generally unable to achieve satisfactory results since BACE2 and cathepsin D active sites show high homology with BACE1 and induced severe side effects [158]. The shift of selectivity from cathepsin D towards BACE enzymes was solved with the development of potent ***third-generation*** small-molecule BACE1 inhibitors. These compounds exhibit satisfactory pharmacokinetics and robust cerebral Aβ reduction in preclinical animal models [113].

However, in 2018 (Table **3**), clinical trials with two third-generation substances, including **verubestat** (MK-8931) and **lanabecestat** (AZD3293/LY3314814), were terminated.

Verubecestat was developed by Merck and the drug entered the final clinical trial in 2013. Altogether, 1958 volunteers from 90 different countries entered the study. The

Table 2. Generations of BACE1 inhibitors.

Generation	Group	Representatives
First	oligopeptides	OM99-2
Second	non-specific small molecule inhibitors	LY2811376
Third	non-selective BACE small molecule inhibitors	MK-8931 AZD3293
Fourth	BACE1 preferential small molecule inhibitors	CNP250 E2609

Table 3. Latest II/III phase clinical trials with BACE1 inhibitors.

Compound	Company	Phase Trial	Population	Start Date	Stop Date	Expected Completion Date
Verubecestat (MK-8931)	Merck	III	Prodromal AD	10/2013	3/2018	5/2019
Lanabecestat (LY3314814)	Eli Lilly	II/III	Early AD	5/2016	6/2018	6/2019
Atabecestat (JNJ-54861911)	Janssen	IIb/III	Prodromal and pathophysiology (asymptomatic) AD	10/2015	1/2019	5/2023
Umibecestat (CNP520)	Novartis, Amgen, and Banner	II/III	Unimpaired two APOE4 genes	4/2015	6/2019	8/2024
Elenbecestat (E2609)	Eisai and Biogen	III	Early-stage AD	11/2016	9/2019	6/2020

Table 4. IC_{50} or K_i of the third and fourth generation of BACE inhibitors.

Compound	IC_{50} or K_i (nM)		
	BACE1	BACE2	CatD
Verubecestat (MK-8931)[a]	2.2	0.38	>100,000
Lanabecestat (LY3314814)[b]	0.6	0.9	16,100
Atabecestat (JNJ-54861911)[c,d]	1.0-2.6	-	-
Umibecestat (CNP520)[d]	11.0	30.0	205,000
Elenbecestat[e] (E2609)	-	-	-

[a][166], [b][167], [c][168], [d][169], [e]IC50 values of BACE1, BACE2 and CatD are not available.

drug was administered at doses of 12 and 40 mg for 260 weeks. (for study details see NCT01953601) [159]. Although Egan *et al*. demonstrated that verubestat reduces amyloid levels in the brain and cerebrospinal fluid, cognition and daily function were worse among patients who received verubecestat than among those who received a placebo [160]. This study was therefore discontinued on the grounds of futility. Additionally, there was an average weight loss of 1.6 kg, changes in hair color were frequent, and rashes were almost twice as common in the verubecestat group than with placebo [161]. At present, Verubestat is utilized only in biochemical assays as a reference compound.

Lanabecestat was developed by a British-Swedish company AstraZeneca. AstraZeneca joined Eli Lilly and started a phase II/III clinical trial with 2218 participants with early AD in 2014, a phase III trial with 1722 participants with mild AD dementia in 2016 and a phase III trial with 421 participants with Early Alzheimer's Disease Dementia. These multi-center, randomized, double-blind, placebo-controlled studies were evaluating the disease-modifying potential of Lanabecestat at daily doses 20 and 50 mg for 24-156 weeks. The treatment was well tolerated and did not show any cognitive or functional decline (for study details see NCT02245737, NCT02783573, NCT02972658). Nevertheless, both studies were terminated early after futility analysis. Besides, they reported weight loss, hair color changes and depigmentation of the skin [162].

Atabecestat (JNJ-54861911) was another substance discontinued in 2018. The compound cannot be precisely classified into the third or the fourth generation because only BACE1 affinity was published (Tab. 4) and classification cannot be assigned based on reported adverse effects. The compound was developed by Janssen Pharmaceutica in collaboration with Shionogi. Clinical trial phase IIb/III aimed at asymptomatic subjects at risk for developing AD. Overall, 557 participants were administered with placebo, 5 mg or 25 mg of Atabecestat once daily up to 54 months (for study details see NCT02569398). The trial was terminated as a result of liver enzyme elevations pointing to hepatotoxicity. It was concluded that the benefit/risk ratio was insufficiently favorable to continue its development for patients who have late-onset preclinical stage Alzheimer's disease [163-165].

Due to the nature of the above-mentioned adverse effects of the third-generation inhibitors and the possible involvement of BACE2 in their mechanism-of-action, specific small molecules of inhibitors preferentially inhibiting BACE1 were developed (Table **4**). Thus, compounds such as **umibecestat** (CNP250) and **elenbecestat** (E2609), can be considered ***the fourth generation***.

According to Neuman *et al*., **umibecestat** is ~3-, ~20,000- and ~6,000-fold more selective for BACE1 than for BACE2, CatD, and CatE, respectively [169]. Another important feature is that the drug can decrease Aβ concentration irrespective of *APOE4 status, which was demonstrated in the brain of APOE4-transgenic* mice and the CSF of humans during phase IIa clinical trial [170, 171]. In 2015, Novartis with Banner Alzheimer's Institute, ran Phase II/III study. This study enrolled 480 participants to match placebo,

Table 5. Reduction of Aβ in CSF depending on the daily dose.

Compound	Dose (mg)	Reduction Aβ (%)	Dose (mg)	Reduction Aβ (%)	Dose (mg)	Reduction Aβ (%)
Verubecestat (MK-8931)[a]	**12**	50-75	**40**	80-90	-	-
Lanabecestat (LY3314814)[b]	**15**	63	**50**	79	-	-
Atabecestat (JNJ-54861911)[c]	**5**	50	**30**	80-85	**50**	90
Umibecestat (CNP520)[d]	**15**	95	**50**	95	-	-
Elenbecestat (E2609)[e]	**25**	43.6	**50**	59.4	**100**	71,3

[a][189], [b][190], [c][163], [d][191], [e][188].

CAD106 immunotherapy and 50 mg umibecestat administered for the duration of treatment (for study details see NCT02565511). Unfortunately, the trial was terminated as the participants taking umibecestat deteriorated on the repeatable battery for the assessment of neuropsychological status (RBANS) cognitive test, showed more pronounced brain atrophy, and lost more weight than did people on placebo [172].

Elenbecestat is another member of the fourth generation of BACE1 inhibitors [173]. Eisai company reported that elenbecestat binds BACE1 and BACE2 with an affinity of 19 and 67 nM, respectively, amounting to a 3.5-fold preference for BACE1 over BACE2 [174]. The results of an 18-month long phase II clinical study revealed that the drug was generally safe, well-tolerated and may have moderating effects for patients who had mild to moderate cognitive impairment (for study details see NCT02322021) [173]. In 2016, Eisai initiated two large multinational phase III clinical trials including 2199 patients with early AD. Both studies tested the effects of Elenbecestat at a daily dose of 50 mg for 24 months (for study details see NCT02956486). But again, the trial was prematurely terminated. Detailed data of the study have not yet been published [175, 176]. It seems that hopes placed on the higher BACE1 selectivity were overoptimistic and the studies were stopped due to an unfavorable risk/benefit ratio.

Above mentioned representatives of the third and the fourth generation were the only BACE1 inhibitors that entered phase III of clinical trials. Recent years additionally closed another two clinical studies. Pfizer introduced **PF-06751979**, a substance displaying broad selectivity to BACE1 over BACE2 and related aspartyl proteases. This indicates a classification into the fourth generation, which is also supported by data from a 9-month toxicology study, revealing no hair coat color changes in dogs [177]. Phase I was conducted in 2016-2017 (for study details see NCT02509117, NCT02793232). However, early 2017 Pfizer announced stopping the development of BACE1 assets. **LY3202626** was developed by Eli Lilly. It is ~1.4- and ~23,000-fold more selective for BACE1 than for BACE2 and CatD, respectively [178]. Due to the relatively low difference in inhibition between both BACE enzymes, the substance could be considered a member of the third generation. The results of the phase I indicated that the drug was safe, effectively penetrated trough BBB and reduced Aβ40 in CSF of healthy volunteers by 50%, 90%, 100% at doses 1, 6 and 26 mg, respectively (for study details see NCT03023826, NCT02555449) [179]. The phase II (2016-2018) aimed at participants with mild AD dementia and tested daily doses of 3 and 12 mg for 52 weeks (for study details see NCT02791191). The study was halted early after an interim analysis showed a statistically low probability of success. Despite both companies backed away from BACE1, the research in this field is still ongoing [180, 181]. Novel compounds have been synthesized and tested towards BACE1 activity, including selective or multi-target drugs, natural compounds, and their derivatives [182-186].

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Almost thirty years after the cloning and identification of β-secretase, we have been unable to find a suitable drug for the treatment of AD. The failure of recent clinical trials raises the question of whether or not to continue therapeutic targeting of BACE1. No benefits and reported undesirable side effects could indicate the first. However, BACE1 inhibitors reduce Aβ brain load and associated inflammation [185]. According to Alzforum, people on the drug also scored better than placebo on language tests [133]. Thus, could there be a compromise stopping at least the progress of the disease?

So far, Aβ concentration in CSF has been used as a biochemical marker of substance efficacy, a parameter that reflects AD pathology in the brain [187]. As first mentioned by Koelsch [21] and as shown in the following table (Table **5**), rather higher dose administration and target >50% reduction of Aβ was preferred in clinical studies. Such inhibition could be associated with the risk of side effects due to the large number of physiological substrates processed by BACE1. A lower level of Aβ inhibition was demonstrated in the case of elenbecestat administered at a dose of 25 mg, but only a 50 mg dose entered phase III clinical trial [188]. Additionally, atabecestat was tested at 1 and 3 mg. Both doses displayed trends slightly exceeding placebo but no substantial $A\beta_{1-40}$ reduction was observed [163]. Therefore, the most relevant question in this context is whether a possible therapeutic window exists. If so, it needs to be specified and the dose of BACE1 inhibitors must be adjusted accordingly.

Determination of the therapeutic window will be the challenge. The recent trials of BACE1 inhibitors were proposed based on *in vivo* experiments aimed at reducing the Aβ level in CNS by 50-90%. Although the results indicated a benefit in the mouse brain, human trials did not confirm this finding. Hence, mice do not appear to be an appropriate

model. It may be necessary to use higher mammalian models with a predisposition to AD. Among presently established models, non-human primates (NHP) are the most closely related to humans. Their CNS anatomy, neurobiology, immune system, and AD-related pathologies share higher similarities with those of humans than other species [192, 193]. But despite intensive research, a model resembling sporadic later onset form of AD has not yet been developed. There are also ethical issues and high costs of maintaining, restricting wider use of NHPs [194, 195]. On the other hand, genetically modified primate that develops AD within a reasonable time frame would be a sensible model before embarking on clinical trials [193]. Such a model, together with BACE1 activity, reduced to a certain percentage could resolve the issue regarding the therapeutical window [196, 197]. Furthermore, conditional inhibition of BACE1 expression may be required to avoid compensatory mechanisms upregulating this protein [198, 199]. Higher mammals can also help in the investigation of the effects of the long-term BACE1 inhibition and determine whether the predicted positive effects of Aβ reduction can outweigh any deleterious effects on synapses and cognitive function in the long-term perspective [161].

Another therapeutic strategy could be focused on modulation of BACE1 trafficking into the extracellular space to spare intracellular functions. This process is regulated *via* post-translational modifications and several interaction proteins [200-203]. The physiological role and function of BACE1 post-translational modifications and interactions are still not fully understood and could potentially cause unwanted side effects as well as the full inhibition of BACE1 enzymatic activity. Puzzo *et al*. also showed that low picomolar concentrations of $A\beta_{42}$ monomers and oligomers increase hippocampal long-term potentiation and stimulate synaptic plasticity [204]. Thus, the utilization of inhibitors of BACE1 post-translational modifications and/or interactions might be again limited by a therapeutic window to avoid complete loss of Aβ that could lead to synaptic deficits. On the other hand, such inhibitors could represent an entirely ***new generation*** of BACE1 modulators but their design must be based on a deeper knowledge of BACE1 biology.

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CONFLICT OF INTEREST

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