Modulation of γ- and β-Secretases as early prevention against Alzheimer's Disease

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Summary

The genetic evidence implying Aβ in the initial stage of Alzheimer's disease (AD) is unequivocal. However, the long biochemical and cellular prodromal phases of the disease suggest that dementia is the result of a series of molecular and cellular cascades which nature and connections remain unknown. Therefore, it is unlikely that treatments directed at Aβ will have major clinical effects in the later stages of the disease. We discuss here the two major candidate therapeutic targets to lower Aβ in a preventive mode, i.e. γ- and β-secretase; the rationale behind these two targets; and the current state of the field.

Introduction

Despite the current widespread criticism on the amyloid hypothesis for Alzheimer's disease (AD), drug targets in this pathway remain by far the most rational ones in the field (1). Apart from small molecules targeting the Aβ producing β- and γ-secretases, only antibodies against Tau and Aβ are in advanced clinical development. The hope is that antibodies will counteract Tau- and Aβ-aggregation, spreading, and/or toxicity in the human brain (2, 3). However, it remains uncertain which epitopes or conformations of Aβ and Tau should be targeted to modulate the disease process. In addition, Aβ-immunotherapies like bapineuzemab and aducanemab tested in clinical trials focus on removing amyloid deposits (4, 5). However, this strategy may have little chance of success, as damage that occurred as a consequence of toxic Aβ species might turn out to be irreversible despite removal of the plaques. Furthermore, the efficiency of brain penetration of these potential drugs poses serious concerns. The lack of knowledge with regard to mechanisms, causal relationships and the different phases of the disorder (6) is probably the major hurdle for advancement at the therapeutic front and makes it very difficult to learn from the failures of the clinical trials in the field (7).

In the absence of mechanistic understanding, the best rationale for the selection of a drug target is clinical genetic evidence linking it to disease. The PSEN/γ-secretases provide unique targets with more than two hundred mutations linking them directly to familial AD (8). It is remarkable that the pharmaceutical industry has largely given up on these targets after the first trials. Could alternative strategies, now that we have a much better understanding of these proteases, circumvent the major side effects encountered with the broad-spectrum γ-secretase inhibitors tested in the clinic(9)?

The other secretase, β-secretase or BACE1, is now the favorite of the companies, with at least 5 clinical trials in phase III testing the hypothesis that reduction of total Aβ levels is sufficient to slow down pathological progression from early to later phases (10). Of notice, the therapeutic strategy of BACE1 inhibition in AD is entirely based on a purely quantitative interpretation of the amyloid hypothesis (6, 11), in which higher Aβ production is pathogenic. However, biochemical evaluation of pathogenic PSEN

and APP FAD mutations suggests that relative increases in longer Aβ species (which have an extended hydrophobic carboxy-terminus) are more disease relevant than an absolute increment in total Aβ load (12–14).

AD dementia only sets in after decades of biochemical stress caused by accumulation of abnormally folded Aβ and Tau. This 'cellular' phase of AD is characterized by synaptic alterations, astrogliosis, vascular modifications, and, likely very important, microglia reactions (6). Once the cellular response is fully installed, neuronal loss and dementia occur. Thus, anti-Aβ therapy will likely only be effective if used in early, preclinical, phases of the disease, i.e. at the moment that biochemical stress is initiated but when irreversible cellular reactions have not yet occurred.

We briefly update about research going on in the γ- and β-secretases field while focusing on relevance for therapeutic developments.

γ-Secretases

γ-Secretases are multimeric aspartyl proteases complexes with Presenilin (PSEN, catalytic subunit), Nicastrin (NCT), Presenilin-enhancer 2 (PEN2) and the Anterior pharynx defective 1 (APH1) as essential components. In the human genome, two genes coding for PSEN (*PSEN1* and *PSEN2*) and two others for APH1 (*APH1A* and *APH1B*) generate a family of structurally similar complexes (15–17), which however differ in kinetic properties (18–20), subcellular localizations (21, 22), cellular/tissue expression (23, 24) and physiological functions (19, 25–27).

High-resolution 3D-structures of the PSEN1/APH1A γ-secretase (28, 29) reveal a horse-shoe like membrane-associated core and a prominent extracellular NCT ectodomain that establishes several interactions with the membrane core. Intriguingly, the 3D-structures depict PSEN with a loose and flexible, likely metastable, fold that co-exists in several conformations (29). The functional relevance of this conformational heterogeneity is poorly understood, but FRET-based and low resolution structural studies show relatively large conformational changes in the complex that might contribute to allosteric modulation of its activity (30–33). For instance, inhibitor binding triggers widespread conformational changes that compact γ-secretase architecture, blocking substrate access to the active site and turning γ-secretase less sensitive to detergent dissociation (34, 35). However, the molecular mechanisms underlying these conformational rearrangements remain unknown and a better understanding of the nature and dynamics of these mechanisms is likely required to advance our knowledge about how these complex proteases recognize and cleave substrates in the membrane.

Substrate recruitment and cleavage

Close to 90 type-1 membrane proteins are cleaved by γ-secretases, indicating their involvement in a wide variety of physiological processes, such as cell fate determination, cell adhesion and migration,

neurite outgrowth, axon guidance, formation of synapses, etc. (36). Unbiased proteomic-based profiling (37) indicates that only a small subpopulation of the type 1 membrane proteins (38) are substrates for these complexes. The mechanistic basis of γ-secretase-substrate specificity/selectivity is unknown. However, the notion that global structural and dynamic features of both enzyme and substrate are determinant for regulated intramembrane proteolysis is emerging (39, 40). Furthermore, different layers of regulation seem to apply, as substrate recruitment may be influenced not only at the level of Enzyme-Substrate (E-S) interaction but also by cellular context (21, 22).

A sequential turnover mechanism distinguishes γ-secretases from more classical proteases. The first endoproteolytic (ε-) cleavage releases soluble intracellular domains (ICDs) from the membrane and generates a *de novo* substrate, which remains in the active site. The N-terminal *de novo* substrate is subject to successive carboxypeptidase-like γ-cleavages, until the release of an N-terminal peptide stops the sequential process (**Figure 1**). Ihara and colleagues demonstrated elegantly that endoproteolytic (ε-) cleavage of APP-C99 releases the intracellular (ICD) from the membrane and generates, depending on the position of the ε-cleavage, either Aβ49 or Aβ48. Acting as *de novo* Aβ substrates, long Aβ49 (or Aβ48) peptides are further processed along two major product lines: Aβ49-> Aβ46-> Aβ43-> **Aβ40**-> Aβ37 or Aβ48-> Aβ45-> **Aβ42**-> Aβ38 (41, 42). While ICD of γ-secretase substrates like APP or Notch or others may participate in a variety of signaling pathways (43), the N-terminal products (such as Aβ) are degraded by diverse proteolytic systems. Aβ has however the unfortunate property to adapt aggregation-prone conformations that become resistant to proteolysis.

The processing of APP by γ-secretase involves the sequential formation of distinct E-S complexes, each containing shortened de novo Aβn substrates. The stability of each E-S complex determines its dissociation-probability and thereby the length of Aβ peptides released. Of note, conditions that negatively affect the stabilities of the E-S complexes decrease γ-secretase processivity and promote the generation of longer, more hydrophobic Aβ peptides. Conditions that stabilize E-S interaction enhance enzyme processivity and promote the generation of short Aβ (44). In agreement, γ-secretase modulators (GSMs) slow down the dissociation of Aβ42 from γ-secretase, whereas FAD-linked PSEN1 mutants act opposite (45).

FAD-linked mutations place long Aβ central to disease

More than 200 mutations in PSEN/γ-secretase and about 20 in APP (enzyme and substrate) cause early onset FAD in an autosomal dominant manner and provide direct insight into AD pathogenesis. Note that apart from the age of onset, familial and sporadic cases present the same symptoms and progress in similar ways, although FAD is accompanied often by additional phenotypes (8).

FAD linked pathogenic mechanisms have been discussed in terms of Loss-of-function (LOF) or Gain of function (GOF) triggered by the mutated genes. The LOF hypothesis proposes that mutant-induced

impaired endopeptidase activity compromises γ-secretase mediated cell signaling processes and leads to neurodegeneration (46, 47). The proposal was prompted by data showing that conditional double knockout mice lacking PSEN1 and PSEN2 in the adult cerebral cortex develop neurodegeneration in an age dependent manner, in the absence of Aβ (48). How full γ-secretase inactivation triggers neurodegeneration actually represents an intriguing question for γ-secretase biology (49). However, it is unlikely that full silencing of these proteases is an AD relevant mechanism (49), as no single FAD-PSEN variant fully inactivates γ-secretase (14, 50, 51) and, importantly, FAD patients carry one normal PSEN allele in addition to the disease allele.

A second hypothesis states that increments in Aβ42 production underlie AD pathogenicity. *In vivo* kinetic estimations of Aβ production in FAD patients seem to support this (52). Elevated Aβ42/Aβ40 has been widely associated with both PSEN and APP pathogenic mutants and is used as a hallmark of AD. However, elevation in the Aβ42/Aβ40 ratio in many FAD patients is actually due to lower Aβ40, rather than higher Aβ42 levels (13, 52). Some PSEN mutations even lower Aβ42 production, generating instead mainly Aβ43 (14, 51, 53). Thus, gain of toxic Aβ42 function is actually not consistently seen among PSEN mutation carriers.

Recently, Sun et al. analyzed Aβ generation by 138 FAD mutants of PSEN/γ-secretase in cell-free assays, finding no correlation between clinical age of onset and Aβ42/Aβ40 ratio. Based on this result, they question the amyloid cascade hypothesis and support instead the LOF hypothesis (50). In our view, this observation confirms that the focus on Aβ42 alone is too simplistic and that Aβ peptides longer than Aβ42, generated by several FAD-linked PSEN/γ-secretase complexes, should be considered to play critical pathogenic roles.

Comprehensive kinetic, cell-based and in vivo studies demonstrate that pathogenic mutations in PSEN/γ-secretase invariably negatively affect γ-secretase processivity (γ-secretase dysfunction). This effectively shifts Aβ profiles towards Aβ42 and Aβ43, but possibly also longer Aβ at the expense of shorter Aβ40 and Aβ38 (12–14, 53-55). The invariant link between FAD-PSEN mutations and impaired γ-secretase processivity strongly supports the hypothesis that increased production of longer over shorter Aβ peptides causes neurodegeneration in AD.

Finally, a key point in this discussion are the pathogenic mutations in APP. These mutations are unlikely to cause LOF of PSEN. With the exception of the Swedish variant, they are located in the APP-C99 part of the protein, which is the actual substrate of γ-secretases. Thus, AD-linked mutations mark enzyme (E) and substrate (S) and destabilize Enzyme-Aβ interactions from one (E) or the other side (S) of the 'E-S complexes' resulting in premature release of long Aβ peptides (44).

γ-Secretase therapies: inhibitors, modulators and the future: stabilizers.

The failure of several clinical trials with general γ-secretase inhibitors (GSI) decreased substantially the interest in these enzymes as therapeutic targets. Current knowledge indicates that lack of fundamental understanding of the biology and mechanisms of these proteases primed the clinical tests for failure. New investigations suggest that it is time to reconsider the situation (9).

The development of GSI was based on the same quantitative interpretation of the amyloid hypothesis as the BACE-1 inhibitors. GSIs block indiscriminately all different γ-secretase complexes. It is however extremely difficult (although likely not impossible, see (9)) to obtain with such general approach a safe therapeutic window lowering Aβ levels significantly without affecting important signaling pathways. An alternative approach to achieve selective inhibition of Aβ generation is blocking the binding of APP to an exosite in γ-secretase (56). Such protein-protein interactions are however difficult to target with small compounds. Selective γ-secretase complex-specific inhibition is another possible approach (9). Proof of concept comes from the PSEN1-selective inhibitor MRK560 that effectively cures amyloid plaque formation in mice, while no classical GSI related side effects are seen (57).

Interesting is the carboxypeptidase-modulatory approach (GSM) focused on selective lowering of Aβ42 production (58). A number of potent but hydrophobic compounds, some of which have shown hepatotoxicity, were identified but only few companies (Pfizer, Forum and Torey Pines) (59) have continued investing in this interesting approach. Pfizer has active clinical trials (modulator PF-06648671). How these drugs decrease the production of Aβ42 remains unclear.

A third approach is based on the hypothesis that generation of long Aβ peptides (≥42) and not only Aβ42, drives AD pathogenesis. Our recent findings show how clinical mutations in PSEN negatively impact the stabilities of different E-Aβn complexes and result in premature release of long Aβ peptides (44). Based on this data, we propose that the reverse could also happen and that compound-mediated stabilization of γ-secretase/Aβn complexes should shift Aβ profiles towards the generation of short non-amyloidogenic peptides. The γ-secretase stabilizers (GSS) postulated here would keep the γ-secretase(s) in a conformation that retains the *de novo* generated Aβn in the catalytic site until it is processed to short Aβ40- or Aβ38-peptides. GSS should not interfere with the endoproteolytic cleavage, thus maintaining Notch and other important signaling events.

BACE1 as a therapeutic target in AD

BACE1 (beta-site amyloid precursor protein cleaving enzyme 1) is an aspartyl protease and belongs to the peptidase A1 family (pepsins) which includes, among others, Cathepsin D (60). BACE1, and its close homologue BACE2, are, in contrast to the other members, linked via a membrane-spanning domain to the cell membranes (61). BACE1 provides the first cleavage in APP, releasing APP ectodomain (sAPPβ) and generating a C-terminal membrane-anchored fragment of APP, the substrate for the γ-secretases.

BACE2 can cleave APP at the β-site, but cuts also at other sites (62), thus acting more like an α-secretase and promoting the generation of shorter Aβ-like peptides, the role of which is unknown. BACE1 is abundant in brain, and in neurons in particular (63, 64), and is the primary β-secretase responsible for Aβ production (65) as shown in numerous mouse knock out studies (66-69) and recently in several trials with β-secretase inhibitors in humans (70-71).

Unlike APP and PSEN1, the genetics underpinning β-secretase as a drug target in AD are relatively weak. No AD-linked genetic mutations or even SNPs have been found in the *BACE1* gene. The Swedish mutation increases β-secretase processing of APP. Recently an additional APP-A673T mutation was discovered in mentally healthy, aged members of the Icelandic population. This mutation resides adjacent to the β-cleavage site in APP and results in a 40% reduction of Aβ generation *in vitro* (72). However, the effect on Aβ production is only 28% in heterozygous carriers (73). This mutation also affects the aggregation properties of Aβ (74, 75) and induces alternative Bace1 cleavage at the β' site in APP, which might be protective (76). Thus although widely cited as genetic evidence supporting the notion that reducing β-secretase cleavage would benefit patients, the argument remains clearly circumstantial. The main argument in favor for BACE1 lowering strategies is the assumption that lowering total Aβ levels in the brain is sufficient to stall AD. As discussed above, the genetic evidence suggests however that the quality (hydrophobicity) of the Aβ peptides is more important for disease. Furthermore, very little attention is given to the fact that BACE1 inhibition leads to compensatory processing of APP, resulting in p3 fragments (77) and alternative "η-peptides" (78) whose potential contribution to neurodegenerative processes has been hardly investigated.

BACE1 function and its substrates

Since its discovery, BACE1 was promoted as the most promising therapeutic target for AD. Initial claims that BACE1 KO mice displayed no abnormalities (66, 67) turned out however to be premature. Subsequent more detailed examinations revealed a complex phenotype of BACE1-deficient mice. They tend to be smaller and less exploratory than WT mice, and are prone to die in the first few weeks after birth (68, 69); they also exhibit neurochemical deficits (68) and problems with myelination (79, 80). BACE1 cuts other proteins besides the members of the APP family (81, 82). The processing of neuregulin-1 by BACE1 and its role in myelination and muscle spindle formation has been well established (79, 80, 83, 84) but several dozens of other substrates have been uncovered in primary cultures of neurons (85, 86), and mouse CSF (87). For example, Seizure protein 6 (Sez6), involved in dendritic arborization, is almost exclusively cleaved by BACE1 in the CNS, and consequently, BACE1 inhibition impairs synaptic plasticity through action on Sez6 (88–90). The cell adhesion molecules L1 and CHL1 (86) might be responsible for axon guidance defects seen in BACE1 KO mice (91–93), and CHL1 cleavage is part of the growth cone collapse in response to Sema3A (27). Moreover, lack of BACE1

activity might cause retinal pathology (94), schizophrenia-like phenotypes and epileptic-like seizures (95, 96). The good news is that BACE1 inhibitors currently tested in the clinic appear to be remarkably safe. Some compounds are now in human for more than 2 years. It is unclear whether species-specific elements play in this difference between mouse and men. Many of the phenotypes detected in mice were found after genetic knock out of BACE1 expression, which is a more drastic interference than pharmacological inhibition. Moreover, several deficits are developmental and therefore may be less relevant to the adult organism.

BACE1 therapy – off-target effects through cross-inhibition of BACE2

All compounds currently in clinical trials cross-inhibit the homologue BACE2. The most consistent side effect seen in pre-clinical studies is depigmentation explained by unwanted cross-inhibition of BACE2 dependent processing of the melanocyte protein in pigment cells (PMEL) (97). Depigmentation was observed in several strains of BACE2 KO animals (69) and in inhibitor studies in mice, rats (98, 70), Beagle dogs (99) and Dutch belted rabbits (70), showing the conservation of this feature across species. Nonetheless, the effect was not seen in monkeys treated for 9 months (70) or in humans in Phase I clinical trials of two separate inhibitors (70, 99).

BACE2 cleaves the substrate TMEM27 (transmembrane protein 27) in β-cells of the pancreas. TMEM27 is involved in insulin production and its inhibition in insulin-resistant mice improves β-cell mass and function (100). Accordingly, BACE2 inhibition was proposed as a promising target for treatment of type 2 diabetes (101). Given the frequent co-occurrence of AD and diabetes, it remains to be seen whether BACE2 cross-inhibition is a problem or in fact a positive addition to the therapy. It is clear, however, that, as AD therapies target the brain, BACE2 function in the brain urgently needs more attention.

BACE1 therapy – opportunities/overcoming set-backs

Having learnt from clinical failures of previous Aβ-therapies (9), development of BACE1 inhibitors is progressing cautiously, paying close attention to potential side effects. Many companies have potent and brain-penetrant small molecules in their pipeline; among the promising leaders are AZD3293 (or LY3314814) from AstraZeneca and Eli Lilly&Co., CNP520 from Amgen and Novartis Pharmaceuticals Corporation, E2609 from Biogen and Eisai Co., Ltd., JNJ-54861911 from Janssen and Shionogi Pharma, and Merck's verubecestat (or MK-8931), having all reached Phase III clinical trials. The most advanced is verubecestat that reduces Aβ in brain, plasma, and CSF in preclinical animal studies and in CSF of healthy controls (102), with other compounds showing similar results (71, 103, 104). All compounds achieve high selectivity for BACE1 over cathepsin-D, although commonly used cell-free assays are not fully predictive for selectivity versus cathepsin-D (105). This might have been the pitfall of LY2811376,

discontinued because of ocular toxicity (105). Verubecestat has achieved a >45,000-fold selectivity over cathepsin-D and cathepsin-E, which is likely the reason for its favorable safety profile even after 2 years of testing. In contrast, it is almost 6-fold more selective for BACE2 over BACE1, explaining the common depigmentation side effects (70). CNP520 from Amgen and Novartis partnership is 3-fold selective for BACE1 over BACE2 and, unlike an earlier Novartis compound NB-360, does not cause coat discoloration in mice (98). It is clear that BACE1 inhibitors are still in their first wave of development, and we can expect selective second-generation compounds. Of notice, highly selective inhibitory antibodies (106, 107) were developed for BACE1, and these might be useful in combination with Aβ-antibody therapies.

Unfortunately, despite the progress made, Merck decided to stop the EPOCH trial of verubecestat in mild to moderate AD ahead of time due to lack of positive effects (108). Although disappointing, it is yet another lesson for the field re-iterating the importance of starting Aβ therapy at very early stages of AD. When significant synapse and neuronal loss responsible for the cognitive deficits has occurred, BACE1 inhibitors alone will not undo the damage. Other BACE1 inhibitors currently in trials could meet a similar fate, especially AZD3293 tested in a large trial of mild AD patients. Fortunately, most compounds are tested in early AD, although the question is what will be early enough. For instance, the second Phase III verubecestat trial APECS will test its efficacy in prodromal AD patients, and will hopefully bring some positive news in 2019. Similarly, AZD3293 is tested in patients with less severe pathology (probable AD with a biomarker evidence and MMSE of 10 to 26). Amgen and Novartis are running a secondary prevention trial with their CNP520 compound in cognitively normal, homozygous ApoE4 carriers and Janssen started the EARLY trial of JNJ-54861911 in asymptomatic people at risk of developing AD, as defined by PET scan, family history, ApoE4 genotype or biomarker evidence. Both compounds are to finish trials in 2023.

Conclusion

Given the multifaceted nature of AD it is unrealistic to expect that BACE1 or γ-secretase inhibitors are the panacea for such complex and multifactorial condition. It may even be unrealistic to expect triumph of a single Aβ therapy altogether, especially in stages of the disease when neurodegeneration has occurred and cognitive decline has set in. This was seen again recently with the disappointing failure of the solanezumab immunotherapy (109).

Strategies like inhibitors or modulators of the two proteases involved in Aβ production might however turn out excellent preventative therapies for people at risk of developing AD, not unlike statins for atherosclerosis. In addition, synergistic effects of BACE inhibitors and immunotherapy are likely to be explored further in future clinical studies (110).

As discussed, a word of caution with BACE inhibitors is necessary, as AD genetics tell us that longer Aβ peptides, and not higher Aβ levels, cause AD. Therefore, it remains important to consider γ-secretases s therapeutic targets and especially stabilization seems the way to go.

Advances in diagnostic tools, including biomarkers, novel imaging techniques, and genetics, will allow identifying in the future patients at risk. Hopefully this early diagnosis will be accompanied by a good panel of safe and efficient Aβ therapies ready to prevent disease in these people.

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Figure legend

Figure 1: The first ε-endopeptidase cut releases a soluble intracellular domain, which may translocate to the nucleus to regulate gene expression; the remaining N-terminal transmembrane domain (TMD) fragment is then successively cut (carboxypeptidase-like γ-cleavages). Each γ-cleavage releases a short C-terminal (tri- or tetra-)peptide until the secretion of an N-terminal fragment terminates the sequence (41)). Importantly, the efficiency of the ε-endopeptidase (substrate molecules cut per unit of time) determines ICD product levels and inhibition of the ε-cut blocks Notch mediated signaling. The carboxypeptidase-like efficiency (processivity), or number of cuts per substrate, defines the length of N-terminal γ-secretase products. Decreasing its efficiency leads to production of longer aggregation-prone Aβ peptides from the amyloid precursor protein (APP).