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Chapter 10

Dropping the BACE: Beta Secretase (BACE1) as an Alzheimer's Disease Intervention Target

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1. Introduction

The β-site amyloid precursor protein cleaving enzyme 1 (BACE1) is an important regulator for the production of amyloid plaques, a characteristic of the Alzheimer's disease (AD) brain. The proteolytic cleavage of the amyloid precursor protein (APP), by BACE1, produces an insoluble amyloid-β (Aβ) fragment which has the ability to aggregate and migrate onto the dendrites and cell body of neuronal cells, initiating chronic immune responses of inflammation and microglia activation.

The cleavage of Aβ fragments trigger a feedback system that complements production numbers. This increases Aβ loading to such an extent that it exceeds the defences required for natural elimination. The fragments aggregate, developing into an insoluble plaque that has the ability to effect normal functioning by causing dysfunction. Without early identification and effective inhibition of this pathogenic pathway, the disease is anticipated to become more widespread with an aging population, in which AD is most prevalent.

Attempts to inhibit BACE1 have been relatively fruitless with most therapeutic trials being aborted in the early stages. A number of obstacles can be detrimental to the ability of an inhibitor like solubility, bioavailability, potency and effectiveness. In addition to the complexity, there are also a number of substrates cleaved by BACE1 which are important in other pathways like voltage gated sodium channels and axon myelination [1, 2]. This can create adverse reactions beyond the reduction of plaques.

Inhibitors of BACE1 have, so far, looked at active-site mimics that diverge from small and large molecules to short peptidic structures and expression modulators. Other approaches look to utilize technology with modelling software to determine the BACE1 3D structure and formulate an effective inhibitor via domain analysis. The underlying issue is translat-

ing an analytically based, site-directed mimic into a potential inhibitor with pharmaceutical integrity. This process is generally thwarted by the blood brain barrier (BBB), a specialized endothelium that separates systemic blood flow from the central nervous system (CNS). The aim is to fabricate an inhibitor that can pass through the BBB whilst maintaining structure and function.

The importance of BACE1 and the influence it has on AD, has been investigated thoroughly since it was first identified in 1999. The enzyme is an intricate part of Aβ cleavage and plaque formation instigating that inhibition could be the mechanism for relief in AD. In this chapter, we will summarize the structure and function of BACE1 and identify past attempts at its inhibition. The identification of important features regarding the protein-enzyme cleavage of APP will aid the understanding of the process and help theorize future perspectives of research.

2. Neurodegenerative disorders

Neurodegenerative disorders cover a wide range of brain conditions relating to the damage or death of neuronal cells [3]. Clinical characterisation suggests a regression in structure and function of the brain and central nervous system which is usually the final stage of a preceding period of neuronal dysfunction [4]. Specifically, dementia is of particular significance because of the devastating influence it bares on an ageing population and as the life expectancy of the general public increases, disease rates are predicted to escalate accordingly.

The burden of disease in relation to AD, the most predominant form of dementia, was calculated to be 26.6 million cases worldwide in 2006 [5]. This figure implicated 34% of the population over the age of 65 and 45% over the age of 85. The World Health Organization revised this figure in 2010 to incorporate dementia as a whole because of the difficulty to diagnose the varieties of neurodegenerative disorders [6]. The worldwide incidence was tallied at approximately 35.6 million with an estimated 7.7 million new cases annually instigating a new case arising every 4 seconds. The significance of this in regards to AD is that it covers 60-70% of dementia cases [6]. These figures could be inflated further because most cases go undiagnosed due to the requirement of post-mortem autopsy for confirmation.

The ripple effect of dementia extends far from those affected and into patient support networks. The worldwide costs are estimated to be US$604 billion annually and as the number of affected increases this number is expected to follow suit [6]. The chronic onset of the disease indicates an eventual requirement for long term care. The financial burdens relating to carers, as the disease evolves and symptoms increase with severity, the eventual requirement for long term formal care is inevitable [7]. AD patients specifically require increased supervision after diagnosis because of the increased risk of developing associated diseases like cardiovascular disease, diabetes, and bone weakening [8].

AD is largely defined by chronic symptoms of progressive neuronal and synaptic apoptosis in the cerebral cortex and subcortical regions of the brain [4]. The direct consequence is cel-

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lular atrophy, which includes degeneration in the temporal and parietal lobes, parts of the frontal cortex and cingulate gyrus [9, 10]. Progression of brain atrophy to well-defined brain structures results in symptoms of delusions, hallucinations, agitation, depression, anxiety, elation, apathy, disinhibition, irritability, aberrant motor behaviour and sleep disorders [11]. As the chronic nature of the disease progresses, the number of symptoms increases, becoming severely debilitating. Eventually, death results after long-term stress and reduction of brain structure and function [9].

Currently there are a variety of different intervention strategies being investigated to remedy these debilitating pathologies and focus to reduce the cause of neuronal cell death. Upwards of 100 irregular protein changes occur in a brain with AD, including hyperexpression, fragmentation and phosphorylation [12]. A number of these irregular protein modifications could be a result of degenerating neurons causing further damage to associated structures of the central nervous system [12]. The overall progression of AD is however, not restricted to the dysfunction of one mechanism. The number of different symptoms result from a conglomerate of sporadic cellular events that, as a consequence, stimulate the disease. The trigger or initial changes are not entirely clear and do not occur simultaneously which adds to the ambiguity of the disease.

Histopathological review of AD patients highlighted amyloid plaques and neurofibrillary tangles (NFT) as the two significant characteristics of cerebral regression identifying them as targets for the prevention of neuronal cell death [4, 13]. Investigation into the onset of NFTs implicate the tau protein as the leading cause [14]. The tau protein becomes hyperphosphorylated and releases from the intracellular microtubules decreasing structure and function and causing axons to become dishevelled and dedifferentiated. These subsequent microtubules bind together to become an insoluble fibre decreasing the ability of neurons to transmit action potentials along the axon and neurotransmitters across the synapse [15]. Alleviation from this facet of the disease is yet to be elucidated.

Amyloid plaque formation is characterized by an accumulation of amyloid-β (Aβ) sub-units on the cell membrane of neurons that, as a result, cause a decrease in cellular function [16]. The progressive damage of amyloid plaques cause neuronal apoptosis by activating the complement cascade and stimulating the membrane attack complex [17]. In developed cases of AD, where there is a high concentration of Aβ, significant damage to the physical structure of the brain can be a result of the immune response. The activation of the complement cascade involves microglia and astrocytes, which have both protective and destructive attributes. The eventual outcome is apoptosis because of the overwhelming immune response, which cannot be hindered by anti-inflammatory drugs [18]. The aggregations of Aβ deposits are neurotoxic, insoluble and become vastly distributed around the brain as they increase in numbers [19]. AD needs to be proactively prevented before amyloid plaque formation gains the ability to manipulate regular brain function and cause irreversible brain damage.

Closer investigation of the AD brain will encounter a plethora of imperative neuronal cell functions failures like synaptic failure [20], depletion of neurotransmitters [21], mitochondrial dysfunction [22], decreased cholesterol metabolism [23], reduction of neurotrophin [24] and axonal transport deficiencies [25] which can be classified as associated effects of the dis-

ease. These associated deficiencies become apparent with the onset of plaque formation, which precedes tau deconstruction [26]. The repercussions of tau mutations are a characteristic of frontotemporal dementia (FTD) and Parkinson's like defects rather than AD, which makes plaque formation the focus of this chapter [27].

3. Amyloid plaque formation

Amyloid plaques were originally purified in the early 80s and examined to contain peptides of approximately 40 amino acids that aggregated as oligomers, later to be generically named amyloid-beta (Aβ) [28]. Gene cloning and cDNA analysis of these monomers lead to the realization that the origins of this peptide remained part of a larger precursor protein [29]. This protein was later identified as the 695 amino acid, membrane bound cell receptor, amyloid precursor protein (APP), which contained the Aβ sequence in the extracellular domain [30]. It is the proteolytic cleavage, by that of multiple secretases, which releases the Aβ product.

Proteolytic processing of APP occurs by one of two pathways, the amyloidogenic (pathogenic) or the non-amyloidogenic (non-pathogenic). Transport to the membrane via endosomes is chaperoned by the intracellular adaptor protein, sorting nexin 17 (SNX17), where it becomes available for processing by the secretases [31]. The determinant of amyloidogenesis depends on the initial proteolytic cleavage in the extracellular space by either α- or β- secretase to create a soluble or insoluble fragment [19]. Cleavage of APP by the α-secretase, a protein investigated as part of the disintigrin and metalloprotease (ADAM) family, cleaves APP at the α-site releasing a soluble fragment sAPPα into the extracellular space [32]. This leaves the C-terminal fragment of 83 amino acid residues (C-83), which is abruptly cleaved by γ-secretase in the intramembrane space. This creates two subsequent fragments of the APP intracellular domain (AICD), released in the cytosol, and p3 which is released into the extracellular space (Figure 1).

The amyloidogenic pathway also utilises APP as a target. This pathway is much like that of the non-amyloidogenic pathway but instead α-secretase is substituted for β-secretase or beta site APP converting enzyme 1 (BACE1). This enzyme also cleaves APP in the extracellular space at the β-site, which is 18 amino acids towards the *N*-terminal, releasing a much shorter soluble APP-β (sAPPβ) fragment [33, 34]. The remaining C-terminal fragment of 99 amino acid residues (C-99) remains membrane bound until cleaved by γ-secretase. This releases two fragments of AICD and Aβ (Figure 1).

The non-amyloidogenic pathway has the ability to nullify the amyloidogenic pathway by simply having α-secretase cleave APP prior to that of BACE1. Since the α-site of APP is situated between that of β- and γ-, the shorter p3 fragment is produced instead of Aβ. The question over competition for the cleavage of APP is still debated but there is evidence suggesting that α-secretase nullification does not increase BACE1 activity *in vitro* [35]. The subsequent fragments of sAPPα and AICD from the non-amyloidogenic pathway suggest a purpose from the subsequent cleavage of APP. The sAPPα has shown to have neurotrophic

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and neuroprotective properties as well as promoting neurite expansion, synapse production and cell adhesion, while AICD has a role in p53 expression and caspase 3 activation, both associated with cell death, and in maintaining cellular actin dynamics [36-38]. This indicates that the non-amyloidogenic cleavage of APP is imperative to the maintenance of neuronal growth and function.

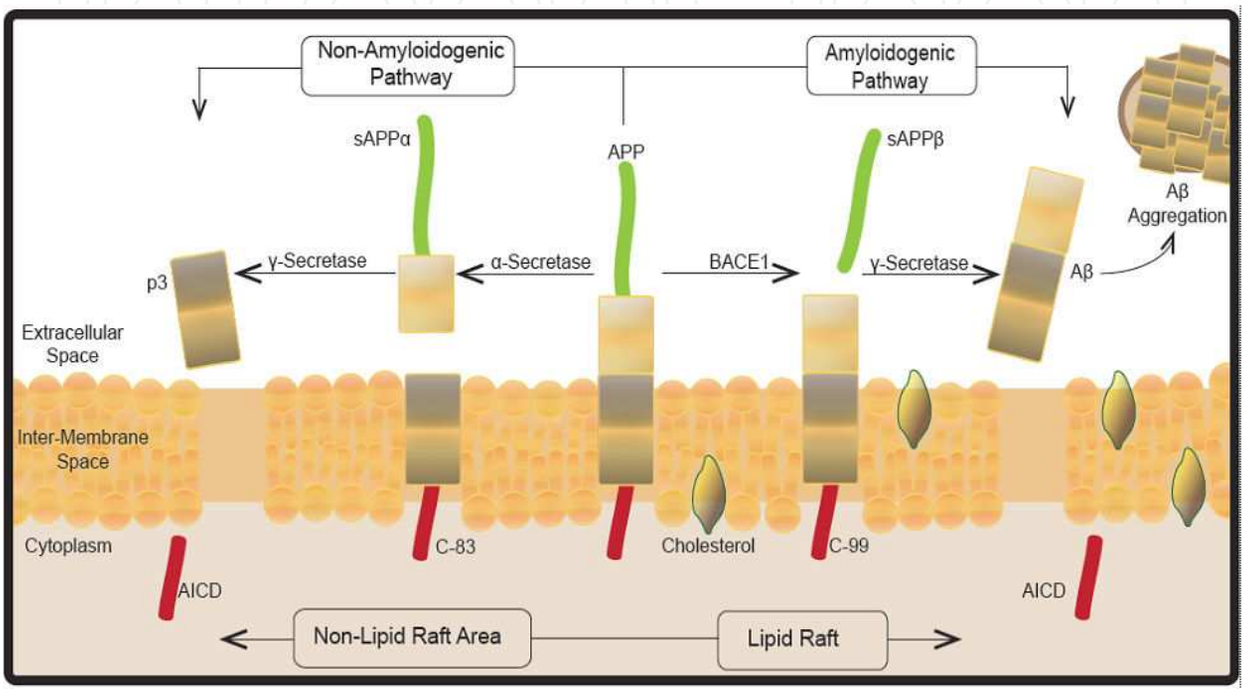

Figure 1. The cleavage of APP, depending whether by BACE1 or α-secretase, results in the production of an amyloid plaque. The non-amyloidogenic pathway (non-pathogenic) begins with α-secretase, which releases sAPPα externally from the endosome or cell membrane. The resulting C83 fragment is cleaved by γ-secretase in the intermembrane space releasing both the AICD and p3 fragments. The release of p3 into the extracellular space is not associated with plaque formation. Alternatively, the amyloidogenic (pathogenic) pathway begins with BACE1, instead of α-secretase, and cleaves APP in a lipid raft region of the membrane. The sAPPβ released is considerably shorter than sAPPα and is still released externally. The γ-secretase cleaves APP, the same as in the non-amyloidogenic pathway, but releases the 38-42 amino acid Aβ, and AICD. The externally released fragment, Aβ, has the capability to form an amyloid plaque by aggregation.

The underlying difference between the two processes is the release of the Aβ and p3 fragments. The p3 is a shorter bi-product of the overall APP cleavage that has no known function. However, the p3 fragment does not have the same ability to form stable oligomeric intermediates like that of Aβ, which poses no threat to synaptic function instigating that it is not the cause of amyloid plaques and is the reason for being the non-amyloidogenic pathway [39, 40]. This suggests that the longer Aβ peptide is the main neurotoxic fragment established from the APP fragment. The cleaved product becomes a peptide of 40 amino acid residues but this can be varied between 38 and 42 depending on where the γ-secretase cleaves APP.

3.1. Amyloid-β

The γ-secretase complex is composed of four proteins: either one of presenilin 1 (PS1) or presenilin 2 (PS2), nicastrin (NCT), anterior pharynx-defective phenotype 1 (Aph-1) and presenilin enhancer 2 (Pen-2) [41]. The complex formation is initiated with a sub complex that forms between NCT and Aph-1 which then binds to one of the PS proteins [42]. Pen-2 is used to activate the complex by selective binding [43]. Either PS can be used in this process, with a 67% amino acid homology, including two separate aspartate residues, asp257 and asp385, considered essential to catalytic ability, both have the ability to cleave APP [44, 45]. Consequently, the decrease in availability of the γ-secretase components results in a reduction of overall functionality, which can be targeted for use when considering inhibitors and the vulnerabilities of this complex [46]. Mutation to either the presenilins, whether it be PS1 or PS2, can be seen as a potential threat to the production of $A\beta_{40\text{-}42}$, as one can compensate for the other.

The γ-secretase, being the consistent piece of the APP cleavage puzzle, is actually responsible for the diseased state in which plaques are formed. There are upwards of 100 missense mutations identified in the presenilins, mostly creating the difference in cleaved fragments of APP [47]. The Aβ peptide created will be influenced with a varying overall length of between 38-42 amino acids [19]. The majority of these mutations influence a higher number of Aβ42 peptides, which have been found to be more amyloidogenic and neurotoxic [48, 49]. The wild-type presenilin generates Aβ40, which is considered to be less neurotoxic even though it is present in amyloid plaques. γ-secretase cuts downstream from the transmembrane domain into the ε-site of APP and therefore slightly shortens the AICD cleaving domain [50]. Aβ42 provides the basis for oligomerisation, fibrillation and plaque generation, even with Aβ40 being found with a limited ability to protect neurons in mouse models [51]. The ratio of Aβ42/Aβ40 increases after the AD age of onset but is still relatively low. However, with a binding affinity to Aβ40, fibrils can, as a consequence, bind together, meaning there does not need to be a high concentration to be effective [52].

The Aβ fragments are not always cleaved to the extracellular space either. There are a number of conformations that Aβ fragments can take including monomers, which are found free in neurons as a consequence of APP cleavage. Studies have also shown that fragments can form the shape of α-helices, random coils and even as β-sheets in a neutual pH to add to the complexity [53-56]. These individual fragments maintain the ability to block synaptic neurotransmitter transfer and instigate an apoptotic response via activation of the p53 promoter leading to cell death [57]. Alternatively, soluble Aβ oligomers have been referred to as Aβ-derived diffusible ligands (ADDLs) that have the ability to aggregate into protofibrils, spherical structures of 7-10 nm wide, that can have the ability to interrupt nerve signal transduction leading to cell death [58]. Initial investigations into the Aβ fibrils showed there was a toxic response from the neurons in which they were attached [59].

To target the Aβ fibrils, gene knockout studies of PS1 have found adverse reactions relating to formation of the axial skeleton, neurogenesis and neuronal survival causing effected mice to die late in embryogenesis [60]. These reactions however, can be attributed to a

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dysfunction of the Notch signalling pathway because of its involvement with cell proliferation, apoptosis and myelin formation, not to mention it is a substrate of γ-secretase [61]. This indicates that there are a number of associated risks with nullifying γ-secretase that with any inhibition, the complex will invariably prevent the cleavage of two regulatory pathways.

3.2. Neuroinflammation

The increase of intra and extracellular Aβ has a direct effect on the complement system and the recruitment of microglia, instigating the activation of inflammatory mechanisms, a trademark of stress on an Alzheimer's brain [62]. The microglia, derived from the mesenchyme and transferred to the CNS where proliferation occurs, are classified as the macrophages of the brain and regulate apoptotic cell abundance [63]. Stress incurred, mainly by amyloid plaque formation, triggers an immune response which leads to the activation of astrocytes and microglia to dismantle the ailing cell. The Aβ fragment has the ability to activate this process via the receptor for advanced glycation end products (RAGE), a multi-ligand member of the immunoglobulin family that is increased in production in an AD brain, and by CD40, an inflammatory signalling receptor [64-66]. The general immune response is aimed at eliminating Aβ, removing disease ridden cells and restoring tissue integrity but does more damage when it becomes of a chronic nature. The activation of reactive oxygen species (ROS), prostaglandins, and pro-inflammatory cytokines are a characteristic of the chronic inflammation and neuronal dysfunction in AD [67].

The onset of AD encourages the migration of microglia to the plaque affected areas. Cultured microglia, from elderly human patients, showed this migration as they coupled with Aβ for the purpose of deconstruction [68]. Similar findings were discovered in sections of cortex tested in vitro for migration but growing evidence suggests that once endocytosed, the microglia struggle to breakdown the Aβ, causing stress and functional changes to the cell [68-70]. Additionally, the APP mRNA translation is upregulated in the event of trauma, nerve damage and brain ischemia, which can be beneficial for the release of more AICD, but can also result in the production of more Aβ [71-73]. BACE1 has also been found to be regulated by Aβ42 via the c-Jun N-terminal kinase (JNK) pathway, otherwise known as the stress-activated signalling pathway which is important for the mediation of pro-inflammatory cytokines [74, 75]. This reiterates the important influence that a variety of stressors have when responding to the amyloidogenic pathway and that there are many different pathways that can be involved in the pathogenesis of amyloid plaques.

All aspects of the amyloid plaque progression relates back to the effectiveness of the BACE1 enzyme. Taking into consideration alternative BACE1 substrates, the proteolytic cleavage of APP can be regulated with an inhibitor. The Aβ concentration increases as the disease gets worse but could be diverted with the use of an effective pharmaceutical intervention. The possibilities and implications will not be recognised until BACE1 is inhibited successfully *in vivo*.

4. BACE1

4.1. BACE1 production and natural degradation

The BACE1 enzyme (also called β-secretase, Asp2 or memapsin2) is developed in the endoplasmic reticulum from a 501 amino acid, 60 kDa, immature precursor protein. The transfer from the endoplasmic reticulum to the Golgi is dependent on the prodomain, which is removed by the proprotein convertase, furin [76]. At this stage the immature BACE1 can already cleave APP so is not a true zymogen but the removal of the prodomain can double the proteolytic effectiveness whilst increasing structural stability [77, 78]. The immature BACE1 is deacetylated and transformed in the golgi by post-translational modification into a type 1 transmembrane protein [79]. A bi-lobal structure is formed with two aspartate motifs (D93TG and D289SG in a D-T/S-G-T/S conformation), which forms the active site that stimulates water molecules to hydrolyse APP peptide bonds, a defining characteristic of aspartic proteases [80, 81]. The proteolytic ability of this motif is only limited by site-directed mutagenesis of the aspartate residues [81]. The active site remains an important structure of this enzyme because of its supposed vulnerability. However, although it has been the focus of inhibition studies since its highly contested discovery in 1999, no effective pharmaceutical options have been elucidated since [34, 82-85]. The distinctiveness surrounding BACE1 is exposed when comparing it to other aspartic proteases and the contrasting characteristics of a C-terminal cytosolic tail and a transmembrane domain.

The transfer from the Golgi to the cell membrane is mediated by transport vesicles because BACE1 is membrane bound. The serine and di-leucine residues of BACE1 act as a signal for the Golgi-localized γ-ear-containing ARF binding protein 1 (GGA1), a sorting protein that aids the link between the transporting endosome and the cell membrane [86]. The endosome provides an optimal transport vesicle because of its internal acidic nature (approximately pH 4.5), a fundamental for the conformational shape and functionality of BACE1, but also because it is much more stable environment for transporting proteins [87, 88]. Changes in pH below 4.0 can have a negative effect on Wat1, a molecule considered to be the nucleophile with the ability to attack the carbonyl carbon of a peptide bond in the active site resulting in the loss of functionality. Changes at the other end of the scale, above that of pH 7.0, render the enzyme inactive and unable to cleave a substrate [87] (Figure 2A,B,C).

The active site placement is also vulnerable as it remains exposed to the extracellular space once it makes the transition to the lipid bi-layer of the membrane where it concludes its intracellular transport. BACE1 becomes susceptible to post-translational modification, protein to protein interactions or even inhibitor attachment because of its availability. It is here, however, that the BACE1 enzyme comes into contact with the membrane bound APP [89]. In some instances, the help of increased cellular cholesterol producing lipid rafts helps improve the availability of APP for BACE1. So what is considered a negative of vulnerability of the enzyme actually serves an important purpose in that a specific environment is created for APP to become more available. The lipid rafts are formed as an essential membrane stabilizer of intermediate space but have also controversially, improved signal transduction and intracellular trafficking ability of the cell [90].

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BACE1 is found in a number of tissues throughout the body but majority of expression is found in the brain [91]. The levels of this expression have been of much debate due to conflicting reports separating different tissues in which the samples were taken, animal or human models and the use of controls [92]. The increase in expression could then be argued as a reason for the uncontrollable aggregation of Aβ but reports of little or no increase in expression of BACE1 instigates the influence of another factor is involved [93].

The natural degradation of BACE1 has been found to be involved the ubiquitin proteasome pathway. BACE1 can be transported from endosomes to lysosomes by ubiquitination with help from the sorting proteins, ADP ribosylation factor 6 (ARF6) and GGA3, another member of the GGA family [94-97]. The degradation by lysosomes is still ambiguous with an increase in BACE1 protein available in an AD brain, whilst there is little to no increase in mRNA levels. The failed or impaired lysosome could be a contributing factor to the increase in cellular Aβ or BACE1 could be getting recycled back into endosomes [93] (Figure 2D).

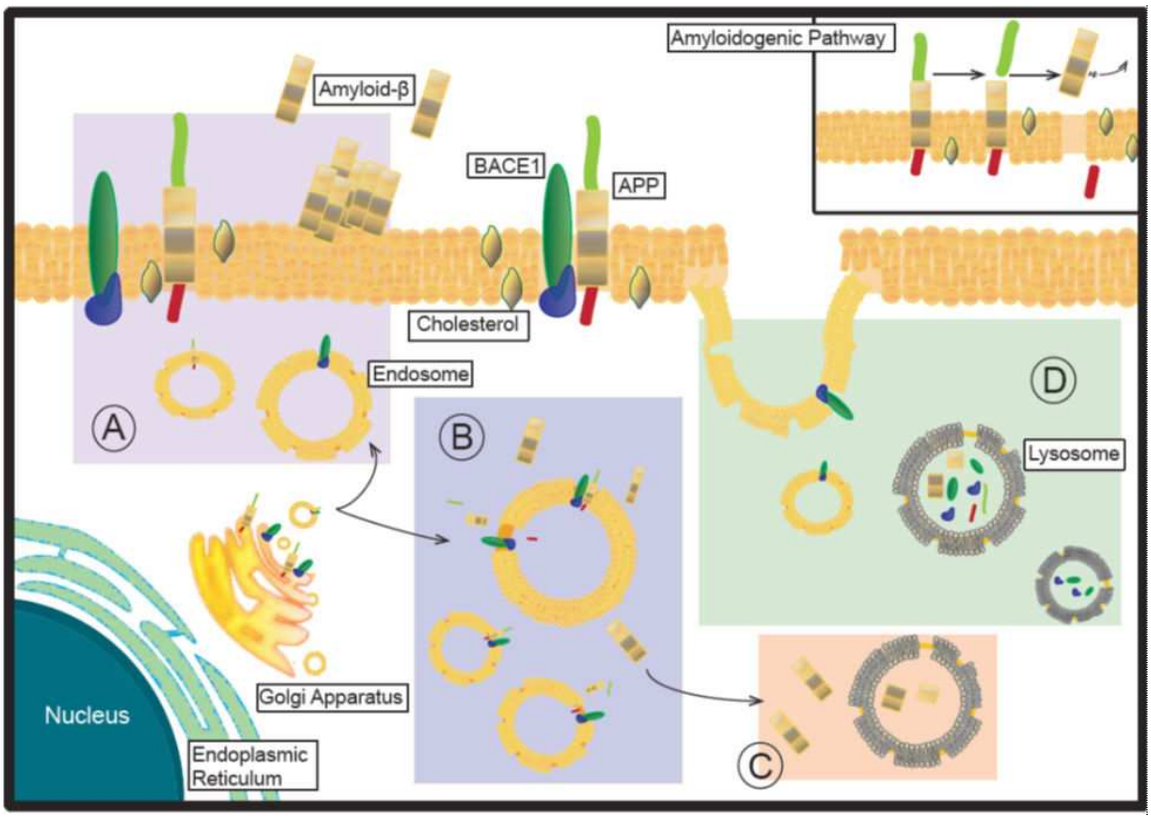

Figure 2. BACE1 Trafficking. BACE1 is constructed in the ER and processed, with the removal of the proBACE1 domain, in the Golgi. A) BACE1 is a membrane bound protease and so is either exocytosed from the Golgi or transported to an endosome with the help of the sorting protein GGA1. From here, the cleavage of APP to produce extracellular Aβ takes place in the lipid raft region of the membrane. The synthesis of Aβ occurs through the amyloidogenic (pathogenic) pathway. B) Processing of APP can otherwise occur whilst present in the endosome and so can release Aβ into the cytosoplasm. C) The Aβ can then either aggregate together or be endocytosed by lysosomes and degraded. D) Once APP proteolysis has occurred, BACE1 is either internalized to endosomes via GGA1 and ARF6 or labelled with ubiquitin and endocytosed. With assistance from the sorting protein, GGA3, the lysosomes will either recycle or degrade BACE1.

Recently, a natural inhibition of BACE1 has been found via the sAPPa fragment produced from the non-amyloidogenic pathway [98]. The sAPPa, once produced from APP, has been found to implicate its own cleavage. The findings showed that sAPPa can bind to BACE1 and interfere with the APP cleavage in a mouse model. This resulted in an overall reduction in Aβ formation under physiological conditions. The consequence of this pathway being impaired in any way, could result in a decrease in BACE1 production thus reducing the cleavage of further units of APP. The sAPPa production or cellular concentration could be a therapeutic intervention strategy in future research.

4.2. BACE2 and closely related proteins

BACE2 is a closely associated β-secretase to BACE1. Sequence analysis show amino acid residue similarities of ~45% and structural comparisons of 75% homology [99]. BACE2 is also classified as an aspartic protease with the ability to cleave APP at the β-site [100]. In vitro studies have also implicated BACE2 as a possible competitor of BACE1 for the cleavage of the APP protein but it is not formally expressed in excess in the brain and does not compensate for the loss of BACE1 in gene knockout models in mice [89, 101]. In fact, over expression of BACE2 has actually been found to reduce Aβ production in primary neuronal cultures derived from APP transgenic mice. This could be caused by the ability of BACE2 to cleave APP before BACE1 or because BACE2 cleaves a longer 79 residue Aβ fragment from APP, which is closer to the cleaved fragment of α-secretase then that of BACE1 [89, 99].

Apart from the sequence homology, BACE2 is not primarily expressed in the brain. It is more commonly expressed in the colon, kidney and pancreas, showing that whilst theoretically still having the ability to increase the pathogenesis of AD it is not considered a threat to Aβ generation [100, 102]. BACE1 is the main amyloidogenic enzyme and still remains the major contributor to amyloid plaque formation but does show some sequence homology with a number other aspartic proteases including renin and cathepsin D. These homologs are generally used for selectivity testing with *in vitro* assays to confirm no unwanted binding occurs.

4.3. Substrates of BACE1

The BACE1 protein is not solely defined to the cleavage of APP and has the ability to cleave other proteins like amyloid like precursor proteins 1 and 2, APPε (which is another closely related *N*-terminal product of APP), neuregulin-1 and -3 involved in axon myelination, β-subunits of voltage gated sodium channels required for neuronal action potentials, P-selectin glycoprotein ligand-1 (PSGL-1), which regulates leukocyte adhesion in the inflammatory process, the interleukin 1 receptor type-II (IL1-R2) and the low density lipoprotein receptor-related protein 1(LLRP1), which is a multifunctional endocytic and signalling receptor [1, 2, 103-106]. With the use of an unbiased, quantitative proteomics method, the identification of 64 new potential substrates were elucidated. The majority of these were type I transmembrane proteins but an added 3 glycophosphatidylinositol anchors and one type II transmembrane protein were also identified, all of which are membrane bound [107]. This suggests that BACE1 has a significant purpose in normal cellular functions but a number of these are

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yet to be characterised in vivo and subsequently cannot be verified as a substrate until this time. With the interaction between BACE1 and APP being widely of interest in the prevention of AD, the actual damage by completely inhibiting the enzyme from normal functioning could prove detrimental in its own right. APP may not even be the primary substrate of the enzyme. Therefore, partial inhibition of BACE1 is all that may be required as an intervention therapy to normalize the enhanced BACE1 activity seen in AD patients.

Alternatively, both of the amyloidogenic creating enzymes, BACE1 and γ-secretase, have alternative purposes relating to other substrates. Gene knockout studies in mouse models produced the reaction of hyperactivity, premature death and seizure like behaviours for BACE1 whilst presenilin -/- mice displayed early neurodegenerative behaviours and an increase of Aβ species which could possibly be due to the large number of substrates in which both are effective [108-111]. The γ-secretase complex is essential for the Notch signalling pathway, which is important for the development of the nervous system and this would also be in deficit if inhibited completely [112]. Likewise, the γ-secretase complex is also required for the non-amyloidogenic pathway, suggesting that the complete inhibition or deletion of these proteins will be detrimental to the homeostatic processing related to normal development and function.

5. Challenges

5.1. Past BACE1 inhibitors

Inhibition of BACE1 has been the focus of many studies since it was discovered. The main interest in this enzyme remains with the obvious involvement in plaque formation but also to the positive intervention target for which it provides. Firstly, mouse models have shown that without BACE1, Aβ is no longer produced, which indicates that if a product can reduce the enzymatic ability of BACE1 then plaques will not be formed [109]. The enzyme has been characterised and 3-D structure produced, which will allow for inhibitor modelling [80]. BACE1 also has the ability to bind to a wide variety of peptidic structures, while specific binding is an attribute there is still access to the active site for potential targeting [113]. BACE1 is a part of the large aspartic protease family, which have had the success of at least two members (renin and HIV protease-1(HIV-1) inhibited with success [114]. This shows confidence that BACE1 is a viable option for inhibition and, if successful, could influence the production of Aβ.

Drug intervention of AD is limited between therapeutic relief of symptoms and the prevention of the underlying etiology of the disease [115]. The treatment to minimise symptoms is the only viable option for sufferers of AD. This method slows the decline of cognition, function and behaviour but only masks the underlying neurodegeneration taking place [116]. The drugs currently available to treat AD are divided into two categories of cholinesterase inhibitors and receptor antagonists [115].

Cholinesterase inhibitors attempt to prevent the metabolism of acetylcholine allowing the neurotransmitter to maintain effect in the neuron [117]. Cholinesterase inhibiting drugs like

donepezil, galantamine and revastigmine are only effective in the early stages of AD by allowing the retention of acetylcholine [115]. By inhibiting the hydrolysis of acetylcholine by cholinesterase, once the neurotransmitter has crossed the synapse, the cholinergic neuron remains active [117]. This action becomes redundant when inadvertent progression of NFTs interfere with the normal signal transmission of the axon [15]. Cholinesterase inhibitors have shown that they can actually increase the amount of phosphorylated tau which can further the progression of AD, minimising the effectiveness of the drug [118, 119].

The other pharmaceutical option is memantine, an uncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist, which prevents binding of the primary excitatory neurotransmitter in the brain, glutamate [120, 121]. As a result, glutamatergic overstimulation can occur causing neuronal cell damage by increased localized calcium stores or excitotoxicity [122]. Both drug options have important side effects and can only offer therapeutic relief of symptoms and are not useful as a long-term intervention strategy.

Therapeutic relief by drugs like donepezil, galantamine and memantine are required to optimize the productivity of the brain whilst the disease progresses and cannot translate to a definite cure. The production of Aβ is the basis of senile plaques and should be investigated as an important therapeutic target. Theories suggest that by inhibiting BACE1 before it cleaves APP, the formation of the beta amyloid residues would be reduced [123, 124]. To prevent this process, inhibition would be paramount between the translation of BACE1 and its binding to APP, before sAPPβ has been cleaved.

Modern drug discovery for a pharmaceutical intervention aims to hinder the BACE1 enzymatic activity by exactly this process. Notable methods look at high-throughput screening (HTS), fragment-based drug discovery (FBDD) and substrate-based inhibitors [125]. However, none of these methods have been successful in therapeutic trials. The more complex approach, generally taken by larger pharmaceutical companies, relate to either the HTS strategy or a FBDD approach. Both processes use a large library of complex compounds in order to find a suitable hit followed by a long modification process to refine it into a suitable chemical. Initial use of HTS sourced complex, high-molecular weight, compounds that were difficult to manipulate. Potential therapeutic candidates showed strong oral availability and good brain permeability but could not provide the standards of potency and selectivity for therapeutic trials [125, 126]

The HTS method has been substituted by the FBDD approach because it uses smaller and more specific compounds [127]. The screening of a fragment library is more appealing because a higher hit ratio is produced and the options show favourable drug properties. The main problem with the hit compounds is again, the low potency and selectivity. Often, fragments that showed promise were too small to be effective and have not provided any real inhibition with the effectiveness required for therapeutic trials.

A large range of inhibitors are being researched including statins, primary, secondary and tertiary amines, hydroethylamines (HEA) of many different conformations, arylamino compounds and acyclic acylguanidines. Most of these compounds seek to act as transition state mimics with a reduced peptidic structure that preclude them from the same scrutiny

as other peptidic structures. To their detriment, hydroxyethylamine derivatives had poor brain exposure, mainly because of p-glycoprotein (PgP) mediated efflux [128-130]. Bristol-Myers Squibb produced a HEA-derivative that contained an indole and a 7-azoindole carboxamide but struggled to maintain brain Aβ levels in vivo [131]. Unfortunately, the inhibitor showed a high potency *in vitro* that had an IC_{50} of 10 nM and a low affinity for BACE2 and cathepsin D.

Another study has looked to hybridize the HEA isotere and replace the sulfonate ester present in one molecule with the sulphonamide of another [132]. The result was a compound that had high potency IC50 of 15 nm to BACE1 that was able to bind to the hydrophobic space of the active site. Unfortunately, this compound was able to bind to BACE2 which minimised the selectivity to BACE1. After problems with HEA structures and brain penetration, Merck decided to use a macrocyclic inhibitor, produced by cross linking the P1 and P3 sidechains of an isophthalamide-based inhibitor. This process helped improve potency but a bolus of 100 mg/kg fed to APP-YAC mouse model could only obtain Aβ decreases of 25% in the first hour and 10% after the third [133].

From another perspective, BACE1 inhibitors have been manufactured from peptide sequences by a substrate-based method. The initial peptide sequences showed promise with high potency and selectivity but did not progress as a viable pharmaceutical target because of the large, unstable products it produced. Branded OM99-2, this peptide was originally used to determine many functions and shapes of BACE1 including the active site. Unfortunately, it was too bulky to cross the BBB but maintained appropriate potency. Revision of this method looked specifically at the structure and function of BACE1 in order to manufacture a peptide sequence that would exploit its weaknesses [134]. It was designed to competitively inhibit the binding regions and shut down the enzymatic properties. The finished product was a long peptide upwards of 18 amino acid residues, which produced a high potency but was not stable enough for use in therapeutic trials.

These studies made it clear that the peptidic structure would fulfil the desired properties required for an inhibitor, the only issue being BBB transport which has motivated the research into small molecules that can freely penetrate the brain. The potential inhibitor needs to be lipophilic enough to permeate the BBB by passive diffusion via the use of a transporter that can maintain entry to the CNS without exiting the same way. Another issue with the peptide inhibitors is the added convolution of ubiquity throughout the body. Since amino acids, peptides and proteins are the building blocks of the human form and function, there is the added complication of anonymity with other proteins and the possibility of unwanted binding causing adverse reactions. If a severe enough reaction to the peptide is apparent, it would be more detrimental then beneficial.

5.2. Crossing the blood brain barrier

The BBB is a network of brain capillaries that regulate the transfer of nutrients and co-factors that are important to the functioning and maintenance of the brain. The conformation begins with the lumen that is lined with a monolayer of capillary endothelial cells, held together with tight gap junctions. This monolayer is complemented by pericytes for the pur-

pose of BBB-specific gene expression and inducing polarisation of the astrocyte end-feet with the surrounding basal lamina [135]. The tight junctions are known to prevent the paracellular diffusion of polar molecules from systemic circulation and brain parenchyma [136]. Complementing the endothelium are a number of compounds that defend against foreign transfer like cytochrome p450 and glutathione S-transferase in conjunction with transporters and the multidrug resistance associated proteins 1 and 2 (Mrp1/2) [137-139].

The defence mechanisms that protect the brain from systemic circulation become a challenging interference when fabricating an inhibitor, especially when it comes to testing the administration of localised drug performance. The molecular weight threshold, which is a relevant property of all membranes, is <450 Da [140]. A molecular model can be used to determine the effect of molecular size on membrane permeability and should be a consideration when investigating an inhibitor [141]. This creates a challenge that goes beyond the production of protein specific intervention strategies. In the case of BACE1, there are a number of different challenges, as previously mentioned, influencing the inhibition of APP cleavage. With the influence of the BBB, the drug will be required to maintain an ability to inhibit BACE1 but also maintain solubility across the tight junctions of vessel epithelium.

The natural functioning of the BBB is to maintain the tight junctions, for which it is idiosyncratic, but in order to transport a required drug, the concept of BBB disruption can be considered. The relaxing of these junctions with hyperosmotic chemicals, for example, could be enough to encourage transport to the brain. The only concern with this method is due to the unfavourable uptake of plasma proteins, like albumin, which is toxic to astrocytes [142]. Consequently, it could also affect drug retention and enhance unwanted migration of otherwise rejected contaminants.

Another drastic, invasive technique involves drilling into the cranium and administering the treatment via intracerebroventricular injection, intracerebral implantation and convection enhanced diffusion. While all are incredibly invasive and risky, the required responses from the drug once applied are generally not that positive [143]. Intracerebroventricular injection specifically has been found to cause haemorrhage after the insertion of a needle into the brain as an adverse reaction but has otherwise has shown good responses [144]. This suggests these invasive techniques are questionable and should be used with extreme caution.

The interesting, non-invasive, concept of lipid carriers has the most potential. The carriers are attached to water soluble inhibitors, that cannot otherwise penetrate the BBB, and turn them lipid soluble [145]. This will allow the transfer into the BBB but once across, the requirement to either shed the carrier or be able to function with it attached in order to maintain functionality, becomes apparent. The naturally occurring, 60 amino acid, galanin-like peptide (GALP) has the ability to cross the BBB where needed with the use of a saturable transport system [146]. The glucose transporter (GLUT1) is another natural carrier system that is used frequently as glucose is the main energy source of the brain [147]. If this method

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can be transformed for BACE1 inhibitors, the transfer of larger, more potent molecules with an increased selectivity, could be achieved.

5.3. Transporters

The ability to transport potential inhibitors across the blood brain barrier can determine the success or failure of the potential therapeutic intervention. The BBB has motivated the research base to investigate small <450 Da molecules that have the potential to pass the barrier with minimal scrutiny. The issue being a lack of potency and selectivity *in vivo* which indicates that a greater effectiveness could be achieved with a larger style inhibitor. The potential for transporters could revolutionize the future of drug production by providing a medium in which the larger more effective inhibitors can cross this barrier.

The earlier transporters aimed to cross the BBB by utilising the already formed channels and receptors. The idea was to couple an inhibitor with the specific ligand of a surface receptor like that of the H1 histone, insulin, insulin-like growth factors or transferrin [148-151]. These systems can also work by binding the inhibitor to antibodies that identify surface epitopes already present in the BBB. The coupling of an 18mer peptide to transferrin receptor specific antibodies were used to infiltrate the BBB via the transferrin receptor for the inhibition of the rev gene of HIV-1 [152]. This study demonstrated a 15-fold increase in transfer across the BBB. This system can be modified to display a number of different biotinylated peptides specifically but could also be manipulated for other effective compounds.

A more advanced method of targeted liposomes progresses from the inhibitor-ligand structure to a colloidal carrier system. The concept still utilizes BBB receptors and channels but is improved with an increase in the concentration of the inhibitor that it can carry. The liposomal system can hold >10,000 drug molecules [153]. The main question regarding this system is the avoidance of opsonins which are members of the complement system and immunoglobulins that cover the colloidal particle and are able to activate phagocytosis. This method has already been used to transfer monoclonal antibodies across the BBB [153]. The negative of this system however, is the vulnerability to macrophages via opsonisation and the lack of selectivity that liposomes have in regards to the brain and BBB. The targeted liposome system has already been used successfully to transfer sodium borocaptate in defence against malignant glioma, which suggests that further research could provide a neurodegenerative diseases template [154].

Alternatively, fullerenes are an allotropic form of carbon that form an arrangement of 60 atoms into the shape of a hollow sphere that is 1 nanometre in diameter and can be coupled with an inhibitor [155]. These fullerene systems have shown promise in the fight against chronic multiple sclerosis [156]. Whilst still in the early stages of research, recovery has been attributed to a reduction in axon loss and demyelination in the spinal region of the CNS in a mouse model. Another study has shown a reduction of infarct size in gerbils and rats with the use of a hexasulfinonated C60 that was administered intravenously [157, 158]. Further

research is required for the use in human studies but there is anticipation for its use against the aggregation of Aβ.

6. Future perspectives

There are a number of exciting directions that future research will take to determine a safe and effective treatment for AD. The regulation of internal pathways for the natural management of BACE1 activity is of key interest as it involves the crossroad from compound based, active site mimics. The recent study identifying the sAPPα as a BACE1 modulator could motivate the investigation of the broader pathway rather than focussing on the enzyme itself [98]. Pathway regulation could reduce side effects and associated pathologies because of the broad spectrum enzymes that envelope amyloidogenesis.

The implication of Aβ signalling in plaque formation is another concept for investigation for the possibility of a feedback system. The promotion of Aβ proliferation could increase the ability for a plaque to form as there is the availability to encourage the process. The application of a potential BACE1 inhibitor is rudimentary to the prevention of this disease because of the potential to bypass the amyloidogenic pathway. With minimal Aβ production the pressure released on the natural inflammatory defences would allow for the metabolism of impeding plaques.

γ-secretase inhibitors have already been progressing to phase III clinical trials which seek to influence the production of Aβ but maintain a number of important consequences that surround this process [159]. The implication of the Notch signalling pathway, important in axon myelination and apoptosis, could be more detrimental than plaque formation because of the regulatory purpose in which it serves [112].

Merck have developed the MK-8931 inhibitor, which is currently undergoing phase II clinical trials [160]. It has already shown an ability to reduce $A\beta_{40}$, $A\beta_{42}$ and sAPPβ levels, is well tolerated *in vivo* and shows minimal adverse reactions, which is encouraging for the <450 Da compounds. Eli Lilly and AstraZeneca are also involved with BACE1 inhibitor clinical trials, which will increase competition once results come to fruition. The opposing mentality behind BACE1 inhibitors, as small <450 Da compounds, could be the answer to years of attempts. The crossing of the barrier is not a major obstacle for this method but show alternative hurdles of selectivity and bioavailability. This has created a race for a pharmaceutically able inhibitor.

Alternatively, the membrane transporters concept for the advantage of allowing the transfer of a range of different inhibitors, in a variety of doses, could produce a platform for a number of different brain based diseases without being overly invasive. The BBB is an ominous obstacle for a number of the earlier peptidic inhibitors, whilst showing a lot of promising pharmaceutical attributes. The combination of the two technologies could provide the difference required for drug penetration and selectivity.

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Once an inhibitor has been potentiated, the focus can move into research looking at plaque clearing and neurogenesis that will aid regeneration of the AD brain. One option is the utilization of stem cells to replace lost brain mass by utilizing multi-potent adult neural stem cells found in the subgranular and subventricular zones. The CNS has the ability to regenerate a number of neuronal cell lines with astrocytes, oligodendrocytes, and functional neurons [161]. By utilizing these cell lines the brain could be "rebuilt" to maintain the structures affected by the plaque formation. However, this would be implicated by numerous ethical challenges.

7. Conclusions

The onset of AD is relatively unknown, but early stages of the disease are met with the accumulation of amyloid plaques. The amyloidogenic pathway, mediated by the proteolytic cleavage of APP, is the major focus for the alleviation of AD. BACE1, the defining enzyme of this process, is responsible for the cleavage of the Aβ fragment. The determinant of fragment length, 38-42 amino acids, is coordinated by γ-secretase. The focus is placed specifically on fragments $A\beta_{40}$ and $A\beta_{42}$ because of the influence they maintain over plaque formation and an increased affinity for aggregation.

Whilst there is an indication that γ-secretase maintains the ability to determine the severity of plaque formation, there are a number of reasons to avoid inhibition, mainly because of its involvement in the Notch pathway and the extreme consequences which brings to light the cost outweighing the means. This is a hallmark of plaque formation as the enzymes involved have a number of substrates in different pathways that do not involve AD. This motivates the notion of regulation, as opposed to complete inhibition, to aid the process alleviating majority of the proteolysis.

The recent association between the cleaved product of APP by α-secretase, sAPPα, having an influence on the amyloidogenic pathway adds another perspective to AD and the mechanisms relating to brain homeostasis. It prompts the idea of maintaining normal processing as opposed to initiating inhibition. The BACE1 cleavage of APP is not a response, or trigger, to AD progression but is fundamental in Aβ production, which implies that normal conservation is a natural process and regulation can be achieved with an external stimulus.

A large obstacle with potential BACE1 inhibitors is the ability to maintain the pharmaceutical properties between *in vitro* and *in vivo* testing, namely the BBB. The transfer between the Tight junctions of brain endothelium and the neuronal cells has defined the way BACE1 inhibitors are engaged. The change from basic peptidic structures to that of small <450 Da chemicals has solved one hurdle but has inadvertently created others. The original *in vitro* studies of peptidic inhibitors maintained positive selectivity for BACE1 but were too bulky for transport. The application of a BACE1 inhibitor, whatever structure it is formed from, will be rudimentary in treating and alleviating the devastating prognosis of AD in the years to come.

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