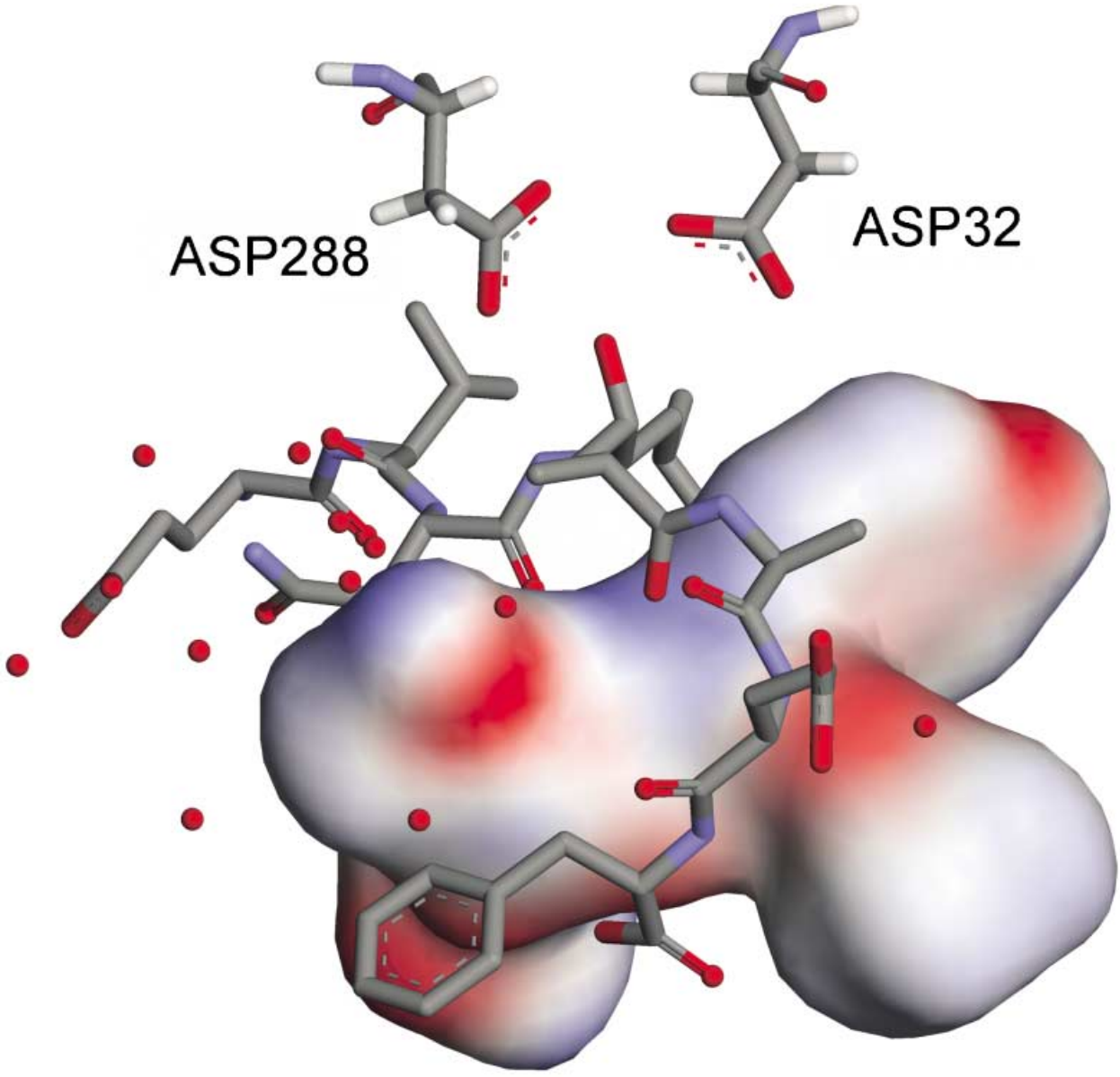

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Aspartic Proteases Involved in Alzheimer's Disease

Boris Schmidt*[a]

Alzheimer's disease afflicts every tenth human aged over 65. Despite the dramatic progress that has been made in understanding the disease, the exact cause of Alzheimer's disease is still unknown. Most gene mutations associated with Alzheimer's disease point at the same culprits: amyloid precursor protein and ultimately amyloid β. The enigmatic proteases α-,β-, and γ-secretase are the three executioners of amyloid precursor protein processing, and disruption of their delicate balance is suspected to result in Alzheimer's disease. Significant progress has been made in the selective control of these proteases, regardless of the availability of structural information. Not even the absence of a robust cell-free assay for γ-secretase could hamper the identification of nonpeptidic inhibitors of this enzyme for long. Within five years, four distinctly different structural moieties were developed and the first drug candidates are in clinical trials. Unfortunately, selective inhibition of amyloid β formation remains a crucial issue because fundamental fragments of the γ-secretase complex are important for other signaling events. This problem makes β-secretase inhibition and α-secretase induction even more appealing.

KEYWORDS:

inhibitors · medicinal chemistry · membrane proteins · peptidomimetics · structure – activity relationships

"Alzheimer's disease is a devastating illness that robs humans of their ability to remember, to think and to understand all the things we cherish most about being human." (P.F. Chapman)

Alzheimer's disease (AD) is an epidemic neurodegenerative disorder that claims millions of victims each year. The ageing of the world population will be accompanied be an increasing disease toll. According to the World Health Organisation, the prevalence of AD amongst people above 60 years of age is 5.5 %, and this figure increases for older people (clinical AD: 16 % 85-year-olds, 22 % 90-year-olds).[1, 2] "The onset of Alzheimer's disease is usually after 65 years of age, though earlier onset is not uncommon. As age advances, the incidence increases rapidly (it roughly doubles every 5 years)."[1] Thus, age is the dominant risk factor and overrules even the positive impacts of nutrition and education.[3–5] The socio-economic impact of AD, the care needed for disabled and chronically wasting patients and the consequences for patients, relatives and caretakers alike, will be a major social and financial issue for the coming decades. "The direct and total costs of this disorder in the United States have been estimated to be US$ 536 million and US$ 1.75 billion, respectively, for the year 2000."[1] Despite all efforts, the exact cause of Alzheimer's disease is still unknown, although a number of factors have been suggested. These factors include the metabolism and regulation of the amyloid precursor protein, plaque-related proteins, tau proteins, and zinc, copper, and aluminium cations.

Acetyl cholinesterase inhibitors and general therapy moderate symptoms at the onset of the disease[6, 7] and improve cognitive function as expressed by the Alzheimer's disease assessment scale (ADAS-COG), but these drugs do not address the severe mortality rate at the final stage of the disease.[8] Promising results were obtained with nonsteroidal antiinflammatory drugs (NSAID), both in vitro and in a prospective, population-based cohort study of 6989 patients.[9, 10] Immunization therapies against amyloid β peptide (Aβ) have high potential and are under investigation by several companies. The most advanced companies in this field, Elan Corp plc. and Wyeth-Ayerst Laboratories, suffered a setback in their joint clinical development of the drug AN-1792 in March 2002. The phase IIa trials were abandoned after observation that four patients in France displayed clinical symptoms consistent with inflammation in the central nervous system (aseptic meningoencephalitis). The gloomy outlook for this therapy brightened a little when Aβ antibodies that address fibrillized Aβ instead of soluble amyloid peptide were applied. These antibodies do not interact with amyloid precursor protein (APP), an interaction that may result in crucial autoimmune responses.[11, 12]

Thus, a causal therapy is still utterly in demand as no existing therapy effectively stops or even cures the disease. The incidence of early-onset Alzheimer's disease in Down syndrome patients indicates chromosome 21 as a likely hotspot for location of Alzheimer's-related genes. Further gene mutations linked to early-onset Alzheimer's disease afflicted several families in London and Sweden and additional polymorphisms that either cause or further AD provided some insight into the biological pathways concerned and the involvement of APP.[13–16] The genetic background of AD is quite heterogeneous and associ-

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ations have been made with genes localized on almost every chromosome. However, replicated or confirmed associations are few: late-onset AD is linked to the ε4 allele of apolipoprotein E (ApoE), but the presence of this allele is neither necessary nor sufficient to cause the disease, although there is a dose-dependent relation to the age of onset.[17] Another cluster of mutations is located on chromosome 14 in the gene encoding for presenilin 1.[18] Mouse models expressing mutated human APP and presenilin 1 display many symptoms of AD, although no model represents the full range of pathologies of the human disease. In particular, the inflammation processes in humans and mice do not adequately relate to each other.[19, 20] The observed loss of neurons is accompanied by formation of plaques consisting of Aβ. A rational approach to a successful, causal therapy must be based on a detailed understanding of Aβ formation and deposition, and the inflammatory consequences. Decisive functions were assigned to the amyloid precursor protein and its degrading aspartic proteases: β-secretase (a beta-site APP-cleaving enzyme, BACE) and the presenilins (PS; also called γ-secretase).

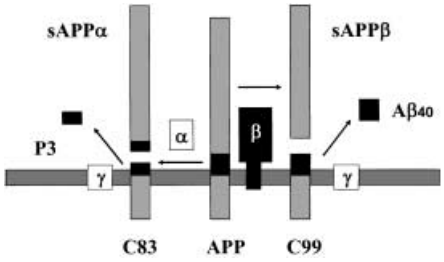
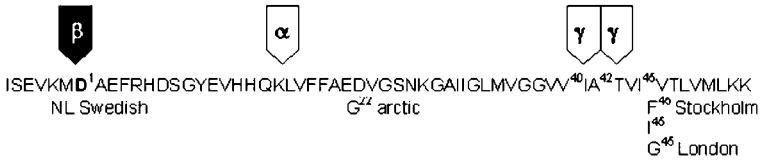


Figure 1. *Processing of APP by secretases.*



Scheme 1. *APP amino acid sequence close to the cleavage sites, and point mutations given with the Aβ numbering.*

A simplified cartoon of APP processing is depicted in Figure 1. APP is up to 771 amino acids long, occurs in three isoforms (APP695, APP751, and APP771), and includes a signaling sequence (a large extramembraneous sequence) and the crucial membrane-spanning domain followed by a short cytoplasmic tail. The nonpathological cleavage occurs between Lys687 and Leu688 (K16L17, respectively, in Scheme 1) catalyzed by an α-secretase that belongs to the 'a disintegrin and metalloprotease' (ADAM) family and is suspected to be TACE or ADAM10. This dominating event leaves just 10% of the APP behind for the β-secretase, produces α-APP, and ultimately leads to formation of the fragments P3 and C83 (Figure 1). The α-secretase is sensitive to membrane cholesterol levels and can thus be modulated.[21] α-Secretase induction was suggested to be partially responsible for the memory-enhancing activity of AIT-082 (**7**, Scheme 2),[22–24] which was in phase I trials in 2001.

The most relevant point mutations for Aβ formation are the double mutation Lys670Asn,Met671Leu (Swedish) and Val717Phe (Stockholm or Indiana), which cause familial Alzheimer's dementia (FAD). The molecular basis of these point mutations is explained by their modulation of the secretases. The rate-limiting β-secretase usually cleaves between the Met671 and Asp672 residues, but prefers the preceding amino acids Asn670 and Leu671 of the Swedish mutation over Lys670Met671. The Val717Phe mutation results in enhanced cleavage after Ala714, which leads to the notorious Aβ_{42}. The released C-terminal fragment C57/C59 interacts in the cytoplasm with an adapter protein, Fe65, and finally induces apoptosis in H4 cells.[25, 26] However, the Fe65 interaction did not protect C57/C59 from rapid degradation in a pulse-chase experiment, with little contribution from endosomal/lysosomal proteolysis. Partial migration of C59 to the nucleus was confirmed in this study.[27]

The C-terminal transmembrane domain of β-secretase is not strictly required for activity, but localization of both enzyme and substrate in the same membrane enhances kinetics and specificity. Crucial for assay development and animal models: BACE1 $-/-$ knockout mice are fertile and healthy but display reduced Aβ levels.[28] Selectivity issues arise not only for other aspartic proteases, but for the homologous BACE2, which displays a less pathogenic APP cleavage pattern and a distinctly different localization.[29–31]

Only through a subsequent proteolysis at Val711 – Ile712 or Ala713 – Thr714 by the intramembrane protease γ-secretase is the Aβ protein released, now 40 – 42 amino acids long, to give Aβ_{40} and Aβ_{42} and the C-terminal fragment C99. Paradoxically, despite being the first reported secretase, the identity of the γ-secretase is still subject to debate and the detailed structure

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remains unknown. The relation of this process to the Notch pathway, which is important in embryonic development, has become less hazy over the years, whilst the importance of cofactors is increasingly obvious. Notch 1, an integral membrane receptor, is processed by proteases upon ligand binding. The intramembraneous cleavage is similar to the APP cleavage and requires PS1. The released intracellular domain migrates to the nucleus, where it finally activates Notch target genes.[32] Cross-over to the Notch pathway hampered all attempts at breeding PS $-/-$ knockout animals, which do not pass the embryonic state, but embryonic stem cells may fill part of the gap.[33] The intracellular trafficking of Notch in human central nervous system (CNS) neurons is reduced by PS1 inhibitors and results in dramatic changes in neurite morphology. It may be that Notch dysregulation causes the neuritic dystrophy observed in AD brain tissue.[34]

Additional proteases may be responsible for γ-secretase-type activity in the early secretory pathway.[35] In spite of being the minor cleavage product, $A\beta_{42}$ is the dominant factor in Aβ deposition and plaque formation. Therefore Aβ formation, deposition, and clearance are highly competitive targets for drug development. Several companies and academic groups have reported cell-free assays in isolated membranes or membrane preparations for this purpose. However, most of these assays lack the robustness necessary for reliable high-throughput screening.[36–39]

To date several reviews on secretase inhibition exist.[40–45] Rapid progress in the field demands a continuous survey, and yet, potent nonpeptidic inhibitors of β-secretase have not been published. Several peptide-based inhibitors were patented or published immediately after J. Tang's disclosure of BACE – inhibitor complex X-ray structures[46, 47] (Figures 2 and 3 show fragments of the homodimeric structure) and were recently reviewed by S. Roggo.[48] Nonpeptidic inhibitors of presenilin were made known through patents by Elan/Eli Lilly, Bristol Myers Squibb, and DuPont. A single original publication appeared for DAPT (phase II drug candidate, Eli Lilly) and its drug-metabolism

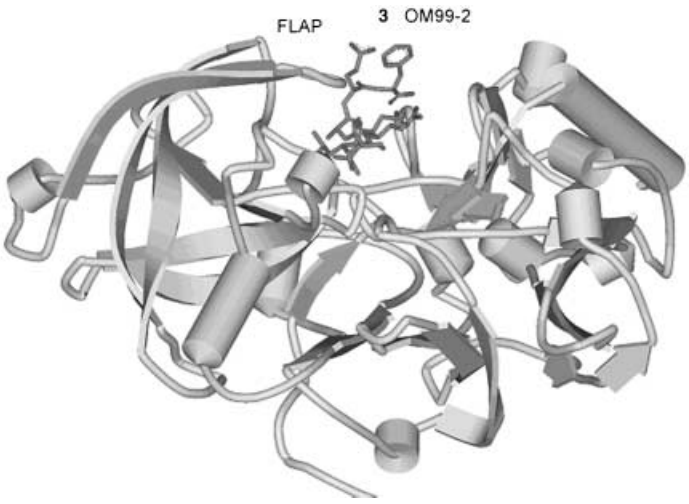


Figure 2. *BACE complexed to Glu-Val-Asn-Ψ(Leu-Ala)-Ala-Glu-Phe (**3**, OM99-2; PDB code: 1FKN).*

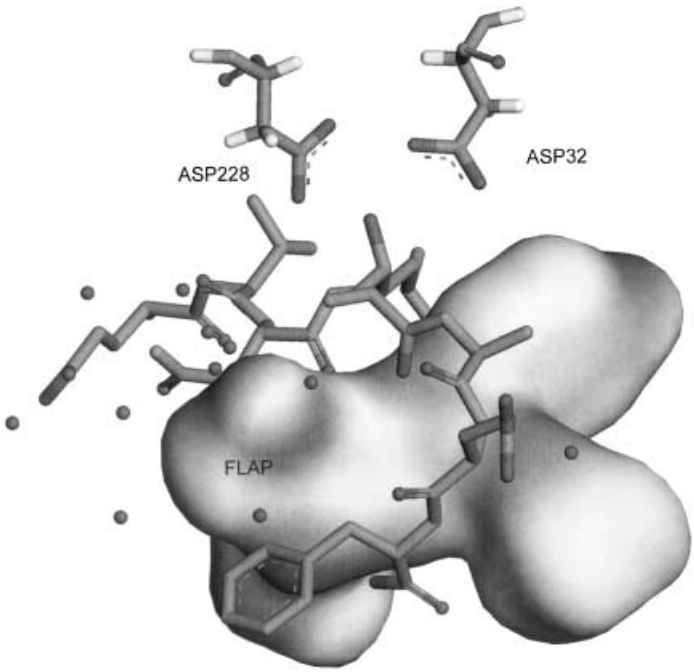


Figure 3. *BACE complexed to Glu-Val-Asn-Ψ(Leu-Ala)-Ala-Glu-Phe (**3**, OM99-2; PDB code: 1FKN).*

pharmacokinetics outside the patent literature. However, as a result of the commercialization of DAPT, it will turn into the standard for other compounds to come. Peptidic presenilin inhibitors,[39, 49] like Merck's L-685,458, which is still the most potent inhibitor known, were patented[50–52] prior to publication in scientific journals.[53]

The well-known beneficial influence of nonsteroidal antiinflammatory drugs on the progress of Alzheimer's disease has been confirmed for some NSAID subtypes. Work carried out by Weggen et al. indicates potential for COX 1 inhibitors (for example, diclofenac, sulindac, indomethacin, ibuprofen, but not the most prominent inhibitor: aspirin) in PS inhibition.[9] Reports on nicastrin, which is a protein linked to familial dementia in the italian town Nicastro, and its co-precipitation with presenilin by presenilin-specific antibodies[54] stimulated the ongoing debate about γ-secretase/PS identity. C. Haass suggests mature nicastrin plays a crucial role in PS1 trafficking from the endoplasmic reticulum to the plasma membrane.[55]

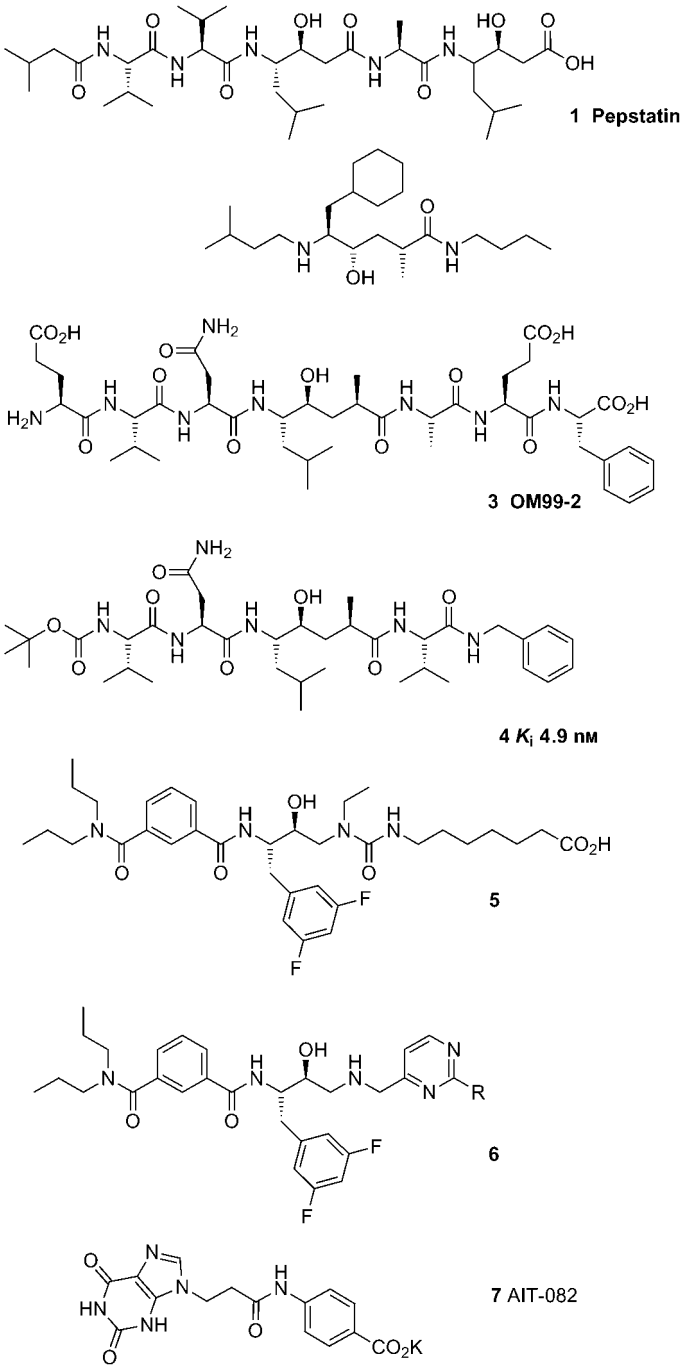
β-Secretase (BACE) was established as an aspartic protease by molecular biology[56] despite the initial lack of selective inhibitors. This enzyme bears all the hallmarks of a typical aspartic protease, which include the flexible flap region that is crucial for substrate docking. The two states of the enzyme, open and closed, contribute to its selectivity and activity.[57] BACE1 is anchored to the membrane by its transmembrane domain (Residues 455 – 480). The catalytic domain is stabilized by three cystine residues in analogy to other aspartic proteases. However, the fully active BACE1 used by Tang for cocrystallization lacks the transmembrane and intracellular domains, and some flexible N-terminal regions were not resolved. The inhibitor is placed in the active site as intended by design (Figure 3); the transition

state analogue hydroxyethylene is coordinated through four hydrogen bonds to the two catalytic aspartate moieties. A further 10 hydrogen bonds are established between the inhibitor, the binding pockets, and the flap region. Despite the analogies between BACE1 and other aspartic proteases, there are significant differences in side-chain preferences. The substrate residues required in the S4 and S3′ positions are hydrophilic and readily accessible by water. The hydrophilic S4′ residue, a phenylalanine, is located at the surface and contributes less to binding, therefore shortened peptidomimetics **4**–**6** (Scheme 2) retain activity. The S1′ position offers space for more than just an alanine residue, as found in the cocrystallized inhibitor **3**; this fact was deduced from the results of ethyl substitution of the hydroxyurea **5**. The importance of the flap region for structural reorganization and activity modulation was concluded from the kinetics of statine-based peptides (hydroxyethylenes), which revealed a two-state mechanism.[58] A detailed analysis of BACE distribution, structure, species variation, and properties was published recently.[48] BACE2 is highly similar to BACE1 but leads to additional hydrolysis close to Phe20 (Scheme 1).

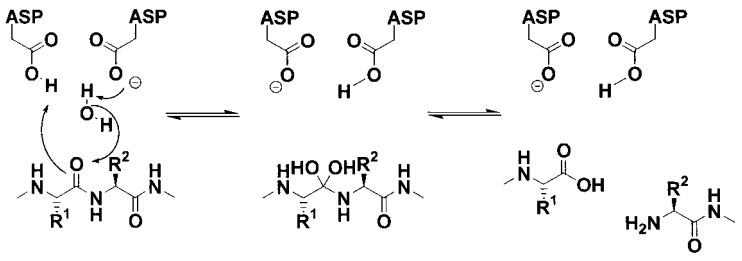
Aspartic proteases hydrolyze the amide bond through a concerted effort by an aspartic acid residue and an aspartate moiety. The aspartic acid residue protonates and activates the amide towards nucleophilic attack, while the aspartate species is required to coordinate and deprotonate water in order to supply a nucleophilic hydroxy anion (Scheme 3). Accordingly, hydroxyethylene isosters of peptidic substrates, which are known to inhibit aspartic proteases, were adapted to the specific requirements of the β-secretase's active site. The peculiarities of the hydrolysis and the stabilization of the tetrahedral transition state by a hydroxyethylene group results in a stable intermediate complex.

Formation of the high-affinity complex of Glu-Val-Asn-Ψ(Leu-Ala)-Ala-Glu-Phe (**3**, OM99-2) and β-secretase results in complete inhibition of β-secretase activity and allows crystallization and structure determination at 1.9-Å resolution (Figure 2 and Figure 3).[46, 59, 60] The subsite specificity was established by measurement of cleavage rates of combinatorial substrate mixtures, which resulted in production of Glu-Leu-Asp-Ψ(Leu-Ala)-Val-Glu-Phe (OM00-3, Figure 3), found to be the most potent known inhibitor (inhibition constant, $K_i = 0.31$ nM) of β-secretase. The second member of the BACE family, BACE2 (also called memapsin 1), causes additional cleavages reminiscent of α-secretase activity.[30, 61]

A second BACE–inhibitor complex structure was revealed by J. Tang (Protein Data Bank (PDB) code: 1M4H) in 2002.[47] The inhibitor OM00-3 was taken from the previous BACE subsite specificity study. The improved binding of this inhibitor, in particular between P2′–P4′ and the S′2–S′4 pockets, resulted in a more linear, extended conformation, which shifts the phenylalanine residue exposed on the surface of the enzyme further out into the solvent (Figure 4). The replacement of the P2′ alanine residue by a valine residue facilitates binding and leads to the reorientation of the P3′ glutamine and P4′ phenylalanine residues.



Scheme 2. *BACE inhibitors and α-secretase inducers.*



Scheme 3. *Mechanism of hydrolysis of an amide bond by aspartic proteases.*

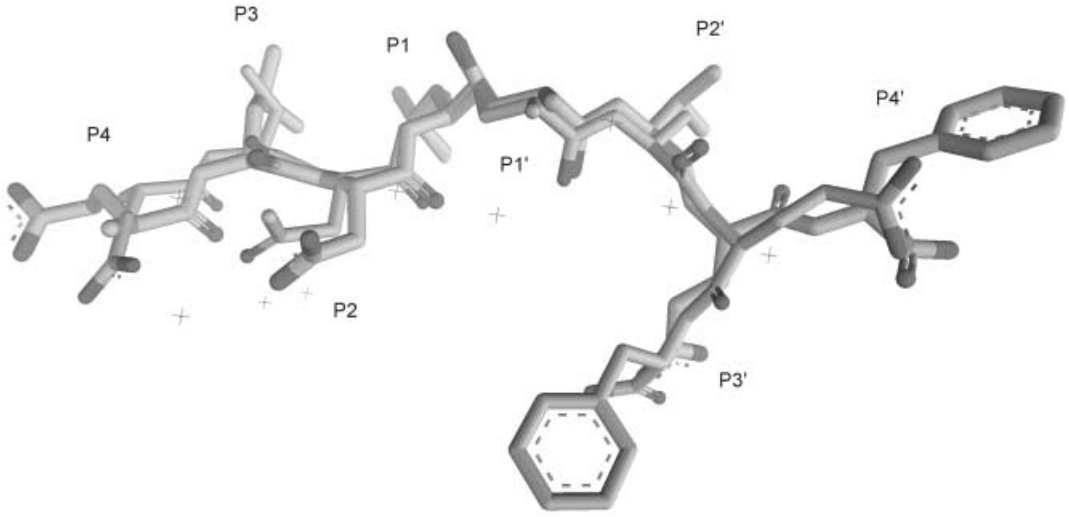


Figure 4. *Overlay of the BACE X-ray structures of the BACE inhibitors OM99-2 and OM00-3.*

The pharmacological evidence compiled for γ-secretase indicates that it has the activity of an aspartic protease that requires at least one additional cofactor.[62] The localization of the active site within the membrane and the occurrance of the cleavage within the membrane anchor of C99 makes γ-secretase quite unique. Currently, there is only one known related enzyme: the signal peptide peptidase, which shares a number of the features and problems described for γ-secretase, but is inserted into the membrane by seven transmembrane helices.[63] Unfortunately, it will not be easy to isolate and purify the intramembrane protease while retaining its activity. Therefore, this protease has so far escaped crystallization and X-ray structure determination. However, mutation analysis of the two conserved aspartate residues of all presenilins supports their key role in γ-secretase activity, at least for an autoproteolytic mechanism that sheds the exon E9. A proposal for the arrangement of the transmembrane helices has been made, but it does not yet explain the observed cleavage pattern.[64] A rudimentary configuration for the eight membrane-spanning domains[16] is depicted in Figure 5.

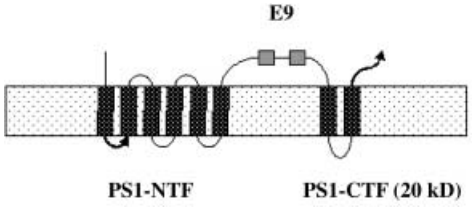
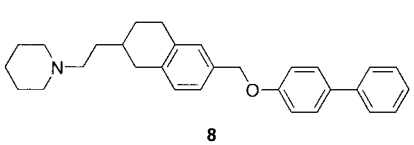


Figure 5. *Schematic representation of presenilin 1 membrane localization.*

β-Secretase Inhibitors

Several inhibitors of β-secretase have been identified in cellular assays but, more often than not, the true nature of the inhibition mechanism was not reported. Broad-spectrum protease inhibitors such as pepstatin (**1**), known aspartic protease inhibitors from renin, and HIV protease programs, as well as cocktails thereof, have little inhibitory effect and gave misleading results. The consequent utilization of the Swedish mutation and the structure–activity relationships of early compounds made by Bristol-Myers Squibb[65] (structure **2**) resulted in the development of OM99-2 (**3**) and its successful cocrystallization with BACE. Activities have been reported for Leu–Ala hydroxyethylene isosters such as **4**, which provide insight into the binding mode. However, these compounds do not really invite drug development because the obstacles for Alzheimer therapy are even higher in comparison to those for the inhibition of renin and HIV protease.[59, 66] Significant efforts were made to reduce the molecular weight and the flexibility of the lead structure. Inclusion of a valine instead of an alanine residue improved binding to the pocket S2′. This replacement and the omission of the small contributions from P4-Glu- and P4′-Phe-binding were first steps taken on a bumpy road towards optimization. The Elan compounds (**5**, **6**) have lost a good part of their peptidic heritage, which is mandatory to obtain sufficient oral absorption and blood–brain barrier penetration.[67–69]

Despite all efforts made by pharmaceutical companies and academic groups, nonpeptidic lead structures for BACE inhibition are very scarce. Takeda reported the tetraline **8** (Scheme 4), which is not an obvious scaffold for protease inhibition and is likely to originate from high-throughput screening efforts.[70] The activity is poor ($IC_{50} = 1$ μM) and the mode of action is insecure. Latifolin (**9**), isolated from the heartwood of *Dalbergia sissoo*, was found to inhibit Aβ synthesis with an IC_{50} value of 180 μM, again a rather weak and insecure activity.[71] However, steady assay



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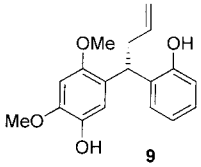

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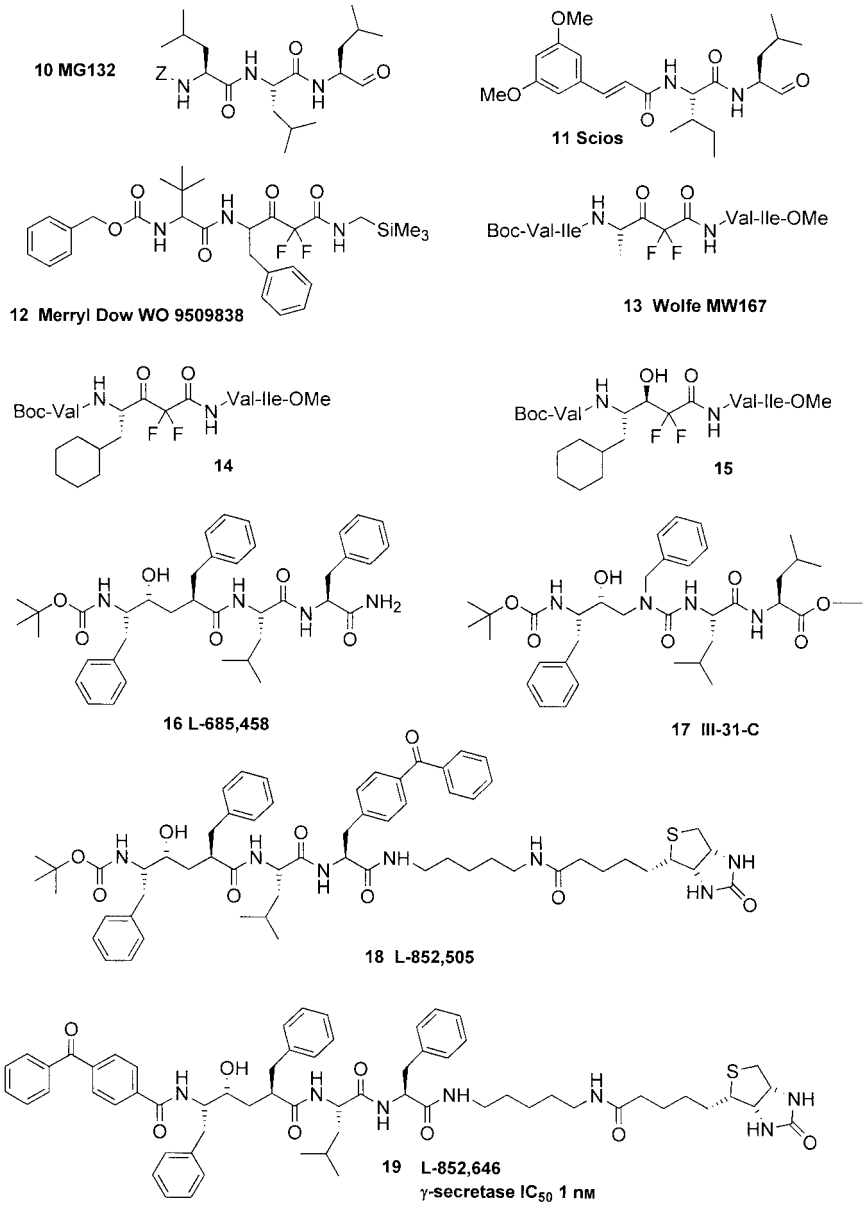
Scheme 4. *Nonpeptidic BACE inhibitors.*

development will provide further nonpeptidic leads. For example, protease-cleavable luciferases, which can be used in protease determination and screening for protease inhibitors, were suggested for use with the BACE sequence,[72] but the reported method needs improvement.

γ-Secretase Inhibitors

The active site of γ-secretase is still subject to debate, but replacement of both Asp257 and Asp385 within the transmembrane regions of PS1 (and the analogue replacements in PS2) inhibits γ-secretase activity.[73, 74] However, single replacement of Asp257 or formation of a deletion construct that lacks the Asp257-containing transmembrane domain allows the protein to retain some activity, which sheds a little doubt on the classical model of the transition state.[75] Furthermore, these Asp257 modifications significantly inhibit the Notch pathway.

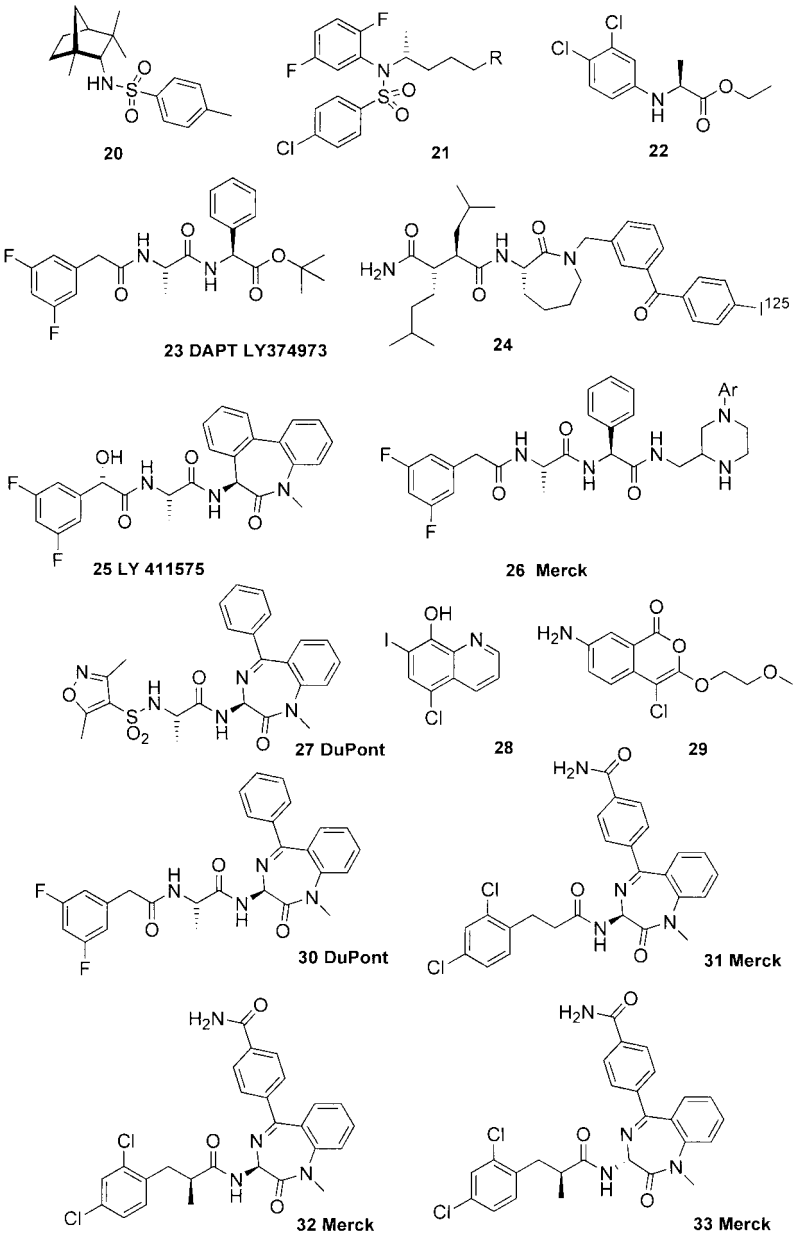
Several peptidic aldehydes have been reported as inhibitors of either γ-secretase or β-secretase, or both. These were taken from previous protease programmes or resulted from combinatorial chemistry experiments. Common to both series are lipophilic di- and tripeptides with bulky N-terminal protection groups, for example, Z-LLL-CHO (**10**, MG132; Scheme 5), Z-YIL-CHO, Boc-GVV-CHO. However, the general lack of specificity of these aldehydes and their inhibition of serine and cysteine proteases makes the data interpretation rather cumbersome. Indirect mechanisms that occur through general protease inhibition result in complex concentration – activity relationships. In fact, Z-LLL-CHO (**10**, MG132) blocks the maturation of the amyloid precursor protein.[76] Improved N termini for the peptide aldehydes were identified by Scios researchers, who reported a range of substituted cinnamic acid amides (for example, **11**, $IC_{50} = 10\ \mu M$).[77] Some of the drawbacks of peptide aldehydes were avoided by using difluoroketones (**12**), initially pioneered by Merryl Dow,[78] whose weak inhibitory activity highlighted the promiscuous nature of the enzyme. Accordingly and somewhat later, M. S. Wolfe reported the small impact of amino acid variation in derivatives of MW167 (**13** – **15**).[79, 80] Clearly, this structural motif serves only as a tool for assay development and labeling.[81–86] Difluoroketones were used to block endoproteolysis of PS1 and to distinguish γ-secretase and the Notch receptor, which share a cleavage site in the transmembrane region and several other features.[38, 87, 88] The low impact of different difluoroketones on the Aβ40/42 ratio supports the point mutation analysis and confirms the results of the phenylalanine scan of the transmembrane region of APP carried out by K. Beyreuther et al.[64] The observed pattern of these phenylalanine introductions and their outcome in terms of Aβ40/42 ratio strongly support an α-helical presentation of the C99 fragment to the γ-secretase. If confirmed, this result will make the γ-secretase quite unique amongst the aspartic proteases as there is only one report of a proteolysis with such an α-helical substrate. First reports[89] of α-helix induction by incorporation of α-aminoisobutyric acid (Aib) are not at all convincing. An extended C99 fragment will bring γ-secretase back in line with all other aspartic proteases, which prefer nonhelical orientations or unfold their substrate into an extended geometry prior to proteolysis.[90] Accordingly, α-helix mimetics of the scission site lack activity.[89]



Scheme 5. *γ-Secretase inhibitors. Z = benzyloxycarbonyl; Boc =* tert*-butoxycarbonyl.*

A giant leap forward was obtained by the serendipitous identification of Merck's L-685,458 (**16**). This compound was taken from a previous protease programme that included its diastereomeric hydroxyethylene, the corresponding ketone, and the parent tetrapeptide, the latter of which was readily cleaved in the assay. The all-lipophilic sequence with three phenylalanine residues was somewhat anticipated, as several studies[64, 77, 79] had indicated the presence of large lipophilic binding pockets (P2, P1, P1′, P2′, and even P4′ and P7′) in proximity to the cleavage site. Inversion of the hydroxyethylene moiety reduces the inhibition 270-fold. This result established the β- and γ-secretase preferences for hydroxyethylene isosters and allows their differentiation by control of the absolute stereochemistry. Labeling studies were conducted with different nonradioactive probes by linking biotin and photoreactive fragments N- or C-terminally to the core structure to give L-852,505 (**18**) and L-852,646 (**19**). The biotin was introduced to facilitate the isolation and identification of the irreversibly labeled adducts through their streptavidin-enzyme-linked conjugates. Both photoreactive benzophenone attachments allowed the compound (L-852,646 (**19**), L-852,505 (**18**)) to retain potent inhibition activity ($IC_{50} < 1$ nM for γ-secretase). Photolysis in the presence of solubilized γ-secretase provided a protein of 20 kD (from L-852,505 (**18**)) after isolation on a biotin-specific streptavidin – agarose gel, followed by partial digestion. This 20-kD fragment was shown to be the C-terminal fragment of presenilin 1 (PS1-CTF) by identification with specific PS1-CTF antibodies. Binding to wild-type PS1 was not observed in a control experiment, yet binding to the deletion construct PS1ΔE9, which lacks the cytosolic E9 loop (Figure 5), did occur.[39] Additional experiments supported the idea that PS1ΔE9 is part of the catalytically active complex but lacks activity on its own. It is still questionable whether L-685,458 competes with C99 for the active site or inhibits the assembly of the active γ-secretase at an earlier stage, for example, the endoproteolytic[91] processing of PS1. Useful information resulted from the photolysis of L-852,646 in the presence of solubilized γ-secretase, which leads to isolation of a 34-kD fragment, assigned as an N-terminal fragment. A similar transition-state motif, the hydroxyethylurea[92] was utilized for an activity-based affinity purification. Immobilization of III-31-C (**17**, $IC_{50} <$ 300 nM) on affi-gel 102 by exchange of the methyl ester for a hydrophilic amide linkage allowed isolation and identification of PS1-CTF, PS1-NTF, and nicastrin from solubilized γ-secretase preparations.[93] Disappointingly, all strategies to free active γ-secretase from the affinity gel failed. This problem is partially due to the good binding affinity of the III-31-C core to the target protein complex and partially due to the deep and narrow binding site, both of which demand strong denaturing conditions to break up the binding interactions. The co-precipitation of the inhibited γ-secretase with its substrates C83 and C99 gave rise to speculations about additional binding sites where the substrate is recognized prior to transfer to the active site. These speculations are in accordance with the observed promiscuous nature of the cleavage as they assign the specific recognition to other complex domains.

In comparison to the β-secretase, for which detailed structural information and the enzyme kinetics are at hand, very little is known about the more complex γ-secretase. The diversity of selective, nonpeptidic γ-secretase inhibitors is once again in favor of high-throughput screening over de novo design. Elan's development of DAPT (**23**; Scheme 6) originated from an *N*-dichlorophenylalanine ester **22**, which displayed an IC_{50} greater



Scheme 6. *Further γ-secretase inhibitors.*

than 30 μM in a cellular screen. After several rounds of refinement and hundreds of compounds, activity peaked for the dipeptide mimetic DAPT (**23**) featuring the nonnatural amino acid phenylglycine, which is crucial for activity: in human embryonic kidney cells, $IC_{50} = 20$ nM. Another key contribution stems from the difluorophenylacetic acid, whose presence results in a very strong structure – activity relationship.[94–96] A broad variation of the difluorophenyl moiety confirmed the demand for small electron-withdrawing substituents. Branched esters displayed similar activity, and despite speculations about the labile nature of tertiary butyl ester, which may be cleaved at the low pH value of the gut, DAPT is not a prodrug. Replacements of the ester by amides are somewhat tolerated, but reduction to primary alcohols results in a drop of activity by a factor of 10^2.[97] Subcutaneous application of DAPT to mice at a dose of 100 mg kg^{-1} resulted in a 50% reduction of cortical Aβ levels after 3 h. A 40% Aβ reduction was observed at a dosage of 100 mg kg^{-1} given orally, again after 3 h, but no brain levels of DAPT were reported for the latter study. Interestingly, there were no signs of in vivo toxicity, although at higher levels (100 – 1000 times higher) DAPT does effect the Notch pathway.[98] Inhibition of Notch signaling by DAPT was reported for drosophila and zebra fish embryos.[99] Further development of the compound under the auspices of Eli Lilly included stereoselective placement of the hydroxy group and locking of the spatial arrangement of two phenyl rings in a caprolactam conformation to result in **25** (LY411575, $IC_{50} < 1$ nM in HEK cells), which is still the gold standard in the field. This compound halved plasma and cortical Aβ levels in young mice even at an oral dosage of 1 mg kg^{-1}. A commercial derivative that lacks the hydroxy group of the difluoromandelic acid is very potent but still interferes with Notch in drosophila and zebra fish.[100, 101] The fully decorated **25** may be void of Notch signaling and any direct toxicity, but indicative long-term studies have not yet been reported [102] and the community eagerly awaits the outcome of early clinical trials. Curiously, the Aβ lowering abilities of DAPT-like compounds are not affected by their sometimes poor blood – brain barrier (BBB) penetration. Thus inhibition of APP processing in the periphery or enhanced clearance of peripheral Aβ by neprelysin[103–105] and other degrading enzymes[106] may hold a key for causal treatment. The trafficking of Aβ over the BBB, or more precisely, through the endothelial layer, was ruled out from blood to brain, but not vice versa.[107–109] Monoclonal antibodies directed specifically at peripheral Aβ affect the Aβ equilibrium between the CNS and plasma. This initially unrelated observation may ease the problems associated with blood – brain barrier penetration of the drugs to come.

After several failures with peptidic structures such as **2**, which all caused toxicity problems in development, Bristol Myers Squibb and Merck[110] published close to 1000 derivatives of 4-chloro-*N*-(2,5-difluorophenyl)-benzenesulfonamides **21**. 500 of these compounds were reported to be very good inhibitors of γ-secretase activity. One of the activity clusters centres around the core structure **21**, with wide variation of the substituent R to modulate bioavailability. Less active sulfonamides (**20**, $IC_{50} = 2$ μmol) that feature similar bicycloalkane skeletons were reported by Amgen and Merck & Co. These compounds share the arylsulfonamide moiety with **21** but lack the crucial *N*-alkyl extension.[51, 111]

DuPont's hybrid structure **24**[112, 113] bears the signatures of a dipeptide-based structure – activity relationship for activity on γ-secretase and is reminiscent of its lead, which was taken from a matrix metalloproteinase programme. Removal of the central amide bond of the parent dipeptide, replacement of the hydroxamic acid by an amide, and introduction of a caprolactam provided good activity and removed some of the problems associated with dipeptide lead structures. The potent compounds (IC_{50}: 20 – 90 nM) are related to DAPT-like compounds and are hybrids of the two series. The difluorophenacyl caprolactam derivative **30**, which stems from a Scios/DuPont cooperation, proved to be the most potent compound ($IC_{50} = 0.3$ nM).[113] "Hot" labeling by photoactivation of I^{125}-benzophenone specifically cross-linked the inhibitor to three cell-membrane proteins, which include the N-terminal fragment (NTF) and CTF of PS1.

The membranes of PS1 knockout embryos lacked these proteins in a gene-dose-related manner. DuPont Pharmaceuticals, which was taken over by Bristol-Myers Squibb, went on to elaborate the caprolactam motif and described a large number of derivatives in a patent family. Some effort was dedicated to modifying the N terminus in order to avoid patent infringement of Elan patents and resulted in the oxazolylsulfonamide **27**.[114, 115] Another straightforward attempt to bypass Elan claims is obvious in a recent Merck series, which is generalized as compound **26.** Moreover, this series is claimed to be inactive with respect to Notch signaling.[116] Several other compounds exist or are claimed to have been made that are hybrid structures of **23** and **24**; a common feature is an aza-caprolactam, which places the phenyl groups in a defined, twisted arrangement. Additional residues can be attached to the lactam amide and the glycine residue is commonly exchanged for small spirocyclic amino acids or amide excision peptidomimetics. Unfortunately, activities for these compounds have not been reported yet. The claimed activity[117, 118] of the chloroisocoumarin **29** for Notch signaling and γ-secretase inhibition stimulated several groups to look at this dubious activity in more detail. The poor selectivity, combined with the inherent general protease properties of this chemically labile compound rendered it to be an indirect inhibitor of γ-secretase.[119] A benzodiazepine core serves as a scaffold and allows wide variation, as seen in the 2,4-dichlorinated compound **31** ($IC_{50} = 33$ nM), which resulted from Merck Sharp & Dohme's whole-cell γ-secretase screening effort.[120, 121] The structure – activity relationship of the dihydrocinnamic acid's halogen substitution pattern was reported recently.[122] Activity is somewhat improved for the 2,3- and 3,4-dichlorinated isomers ($IC_{50} = 22$ nM and 12 nM, respectively). Stereoselective synthesis provided compounds **32** ($IC_{50} = 1.9$ nM) and **33** ($IC_{50} = 500$ nM) and insight into the stereochemical preferences. Unfortunately, the activity on Notch signaling has not been reported yet.

Outlook

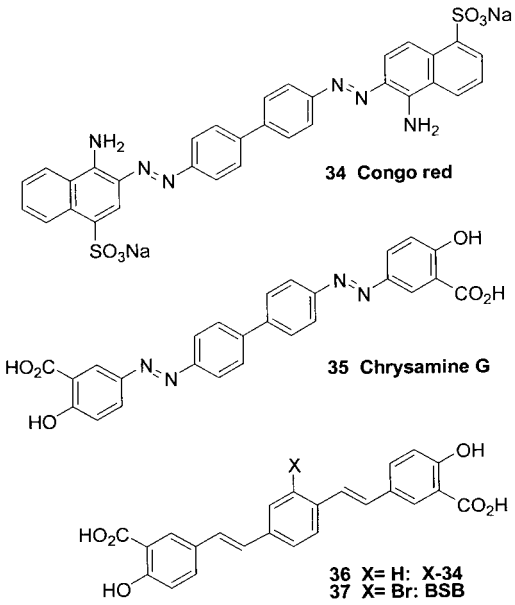
Despite the tremendous progress in the field, a robust cell-free γ-secretase assay is still in demand. Substrate optimization helps to

enhance the kinetics and feasibility of protease assays. Such optimization was recently reported for β-secretase,[123] but is still lacking for γ-secretase. Unfortunately, this process will not be trivial because of the specificities of the intramembrane location of the rather promiscuous enzyme γ-secretase. Alternative approaches may be based on the cofactors of the presenilin complex or α-secretase modulation, as an enhanced cleavage by α-secretase may result in reduced Aβ production. Cholesterol-lowering drugs of the statine class[124] were shown to reduce Alzheimer's disease dementia. This effect may be explained by the sensitivity of α-secretase for cholesterol levels in the membrane.[125, 126] Promising immunotherapy with Aβ suffered from a severe setback due to the negative outcome of clinical trials.[127] Metal chelators have frequently been investigated to reduce copper levels in brain tissue, which is thought to be partially responsible for Aβ toxicity. However, in most in vitro studies, the copper concentrations required to observe an effect were orders of magnitude higher than those found in vivo. Recent studies in 20 patients with clioquinol (**28**), a metal chelator that crosses the blood–brain barrier readily and has good affinity for zinc and copper ions, indicated interesting results. However, the lack of a control group in the study leaves ample room for other explanations, for example, inflammation stimulus.[128, 129] Considering the multitude of approaches and the tremendous efforts underway, the chances of finding a cure within the lifetime of the author and readers are high.

Markers for AD

Several genes and small molecules have previously been linked to the age of onset and the severity of Alzheimer's disease.[130, 131] Genetic factors are well established and reviewed, for example, the ApoE ε4 allele, which is a risk factor but not a deterministic gene.[132] Currently, all these genes and their derived products (for example, inflammatory proteins[133]) lack the features required for robust diagnosis or medical imaging. Yet new candidates emerge at a steady pace: the concentration of the iron transport protein p97 is increased in the serum of AD patients, but the underlying mechanism of the correlation is unknown.[134, 135] Unfortunately, most of the promising small molecules require sampling of cerebrospinal fluid,[136] which will be a major obstacle in clinical trials.[137–151] Levels of the α7 nicotinic acetylcholine receptor (α7-nAChR) correlate[152] to Alzheimer's disease, but the reliable sampling of indicative cells (for example, olfactory neuroblasts) remains an issue.

Congo red (Scheme 7, **34**), which has been known to stain amyloid deposits for several decades,[153–155] still stimulates the ongoing search for small biomarkers of Aβ levels, although the detailed mechanism of Congo red binding is still subject of investigations.[156–163] Specifically designed compounds address the issue of improved brain penetration and radioactive compounds have been explored for noninvasive imaging, that is, positron emission tomography and single-photon computed tomography. A bipyridyl technetium complex was reported by P. T. Lansbury as early as 1996, but the baton was not taken up by other groups.[164] Chrysamine-G (**35**)[156–158, 164] overcomes some of the drawbacks of Congo red, particularly in terms of



Scheme 7. *Amyloid markers.*

BBB penetration. Furthermore, it shows a promising inhibition of Aβ derived toxicity. X-34 (**36**)[165–167] and its derivative BSB (**37**)[168–171] were radio labeled and profiled in transgenic *Caenorhabditis elegans* and relevant rodent models. Their pharmacokinetic properties and their indicative value justify a second general abbreviation, recently assigned as BSB: beta sheet breakers. Finally, a very promising and assayable candidate was identified recently: the plasma homocysteine level.[172]

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