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Developing β -secretase inhibitors for treatment of Alzheimer's disease

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Abstract

β -Secretase (memapsin 2; BACE1) is the first protease in the processing of amyloid precursor protein leading to the production of amyloid- β ($A\beta$) in the brain. It is believed that high levels of brain $A\beta$ are responsible for the pathogenesis of Alzheimer's disease (AD). Therefore, β -secretase is a major therapeutic target for the development of inhibitor drugs. During the past decade, steady progress has been made in the evolution of β -secretase inhibitors toward better drug properties. Recent inhibitors are potent, selective and have been shown to penetrate the blood-brain barrier to inhibit $A\beta$ level in the brains of experimental animals. Moreover, continuous administration of a β -secretase inhibitor was shown to rescue age-related cognitive decline in transgenic AD mice. A small number of β -secretase inhibitors have also entered early phase clinical trials. These developments offer some optimism for the clinical development of a disease-modifying drug for AD.

Introduction

It is generally recognized that an excess level of amyloid- β ($A\beta$) in the brain over a long time period is a leading factor in the pathogenesis of Alzheimer's Disease (AD) (Selkoe and Schenk, 2008). Logically, clinical intervention to reduce $A\beta$ levels in the brain, has been an attractive approach for the development of therapeutics for this disease. The homeostasis of brain $A\beta$ level is a consequence of its production, efflux out of the brain, degradation and possibly formation of insoluble aggregates in AD brains. In theory, each of these factors can be clinically manipulated to achieve a reduction of $A\beta$ level. However, current technologies do not provide for effective manipulation of efflux or degradation of $A\beta$ in the brain. On the other hand, inhibition of $A\beta$ production is much more appealing. $A\beta$ is generated in neurons from amyloid precursor protein (APP) by the activities of two aspartic proteases, β -secretase (memapsin 2, BACE1) and γ -secretase. Considering past success in the development of inhibitor drugs of other aspartic proteases, such as HIV protease for treating AIDS and renin for treating hypertension, it is not surprising that the inhibitors for both of these $A\beta$ -generating proteases have been extensively investigated in recent years.

γ -Secretase inhibitor drugs have been actively pursued over the years and several compounds have been brought to human trials. A major obstacle of γ -secretase inhibitors is their toxicity (Wolfe, 2008). γ -Secretase has many physiological functions in the regulation of cell growth and catabolism of proteolytic fragments of membrane proteins, including APP fragments produced by α - and β -secretases. Some of the toxicity of γ -secretase inhibitors

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may have come from the lack of compensatory pathways for these important physiological functions. At present, it is not clear if the function of γ -secretase in A β production can be specifically inhibited without interfering with other important functions of this protease.

The development of β -secretase inhibitor drugs, however, has presented a different set of problems. On one hand, it is devoid of the function-based problems seen for γ -Secretase inhibitors. Elimination of β -secretase activity by gene deletion essentially abolished the production of A β , yet brought about only minor phenotypic abnormality in mice (Cai et al., 2001; Luo et al., 2001; Roberds et al., 2001; Ohno et al., 2004;). This suggests that the activity of β -secretase can be attenuated by inhibitor drugs without serious physiological consequences. On the other hand, the development of β -secretase inhibitors with desirable drug properties has been very challenging and slow in coming. Twelve years after the cloning and identification of β -secretase, only a few compounds have been tested in early stages of clinical trials. The progress in this area has been hampered by both the stringent requirements of a drug to treat a brain disorder and the uncompromising nature of the active site of the protease making it very challenging to manipulate inhibitor structures necessary for better drug properties. Nevertheless, significant progress has been made which renders optimism for the future. In this article, we review the major developments and outlook for the future.

β -Secretase as a drug target

Since the cloning of β -secretase over a decade ago (see a separate article in this volume), its structure and catalytic properties have been thoroughly investigated. β -Secretase is a type I transmembrane protein and its catalytic domain is an aspartic protease with a pair of active-site aspartyl residues. These and other structural features that are important for catalysis in the active site of β -secretase (Hong et al., 2000) are nearly identical to other aspartic proteases of the pepsin family. β -Secretase has an elongated substrate-binding site that can bind up to 11 substrate residues (Turner et al., 2001; Turner et al., 2005). The amino acid preference in these subsites are somewhat broad (Turner et al., 2001; Li et al., 2010), suggesting that different side chains of peptidic inhibitors can be accommodated. Many of the central subsites, such as P1 and P1', prefer hydrophobic side chains. This preference can be exploited in designing inhibitors with good lipophilicity which is important for membrane penetration.

Evolution of β -Secretase inhibitors

Since the catalytic apparatus of β -secretase is virtually the same as those in HIV protease and renin, it was assumed from the beginning that the principles of inhibitor design for other aspartic protease drugs may be employed for the development of β -secretase inhibitors. From the precedence of drug development for HIV protease and renin, it is likely that successful β -secretase inhibitor drugs will mimic the conformation of substrates at transition state. The resulting transition-state inhibitors typically exhibit high potency (Ghosh, Gemma and Tang, 2008). β -Secretase hydrolyzes APP and generates A β primarily within the endosomes of brain neurons. Therefore, a clinically effective β -secretase inhibitor must have the ability to penetrate the blood-brain barrier (BBB) and the neuronal membranes. The upper limit of molecular size that cross BBB is around 550 Da. In addition, such inhibitors should possess good drug-like ADME properties. For developing selectivity, potency of the inhibitors against β -secretase is often compared to that against two other human aspartic proteases: memapsin 1 (BACE2), as it is the closest homolog of β -secretase, and against cathepsin D, the most abundant aspartic protease in human cells. Since the discovery of β -Secretase, both academic and industrial laboratories have devoted much effort toward the development of drug-like β -secretase inhibitors. More than 400 publications and patents

focusing on β -secretase inhibitors have now appeared in the last eight years (Ghosh, 2010). Herein, we will attempt to summarize the major developments in this field.

Pseudopeptide β -secretase inhibitors

The first highly potent inhibitor OM99-2 (Ghosh et al., 2000) was created based upon β -secretase substrate where the scissile peptide bond was replaced by a Leu-Ala hydroxyethylene transition-state isostere. The X-ray crystal structures of β -secretase complexes of OM99-2 (Hong et al., 2000) provided detailed information on the extensive interactions in each of the eight subsites of the protease. Subsequently, replacement of Leu-Ala with a statine derivative led to a variety of potent β -secretase inhibitors. A statine-derived cell permeable β -secretase inhibitor **1** is shown in Figure 1. The active diastereomer ($IC_{50} = 0.12 \mu M$) displayed β -secretase selective inhibition and was effective in inhibiting A β formation in transfected human embryonic kidney (HEK-293) cells ($EC_{50} = 4.0 \mu M$) (Hom et al., 2003).

Kimura and co-workers (2005) have reported inhibitor **2** ($IC_{50} = 8.2 \text{ nM}$) containing a phenylnorstatine moiety as the TS-isostere. Subsequently, they reported phenylnorstatine-based compounds **3** and **4** as potent β -secretase inhibitors (**3**, $IC_{50} = 4.8 \text{ nM}$; **4**, $IC_{50} = 1.2 \text{ nM}$), in which tetrazole rings were demonstrated to be an appropriate bioisosteric replacement for the carboxylic acids at both P4 and P1' positions (Kimura et al., 2006). In an attempt to develop pharmaceutically useful compounds, the same group started an investigation of bioisosteres of the acidic tetrazole ring. Introduction of a 5-fluoroorotyl group at the P4 position and *L*-cyclohexylalanine residue at the P2 position resulted in inhibitor **5**, which maintained optimal enzyme inhibitory activity ($IC_{50} = 5.6 \text{ nM}$) while displaying 84% β -secretase inhibition in cultured cells at a concentration of 100 $\mu M/L$ (Hamada et al., 2006).

Larhed and co-workers reported a series of new *tert*-alcohol containing β -secretase inhibitors utilizing α -phenylnorstatine or α -benzylnorstatine as the central core. The most potent inhibitor, **6** ($IC_{50} = 0.19 \mu M$), was co-crystallized with the β -secretase. A novel binding mode for this class of inhibitors was identified, in which the N-terminal amine and not the *tert*-hydroxy group served as the TS-isostere (Wangsell et al., 2011). Larhed and co-workers, used a masked *tert*-hydroxy central core in combination with a substituted isophthalamide containing an inverted amide bond, designed potent inhibitor **7** ($IC_{50} = 0.23 \mu M$). This inhibitor was selective towards cathepsin D, but showed low cell permeability. The epimers at the quaternary center were equally potent suggesting that in this series of inhibitors the absolute stereochemistry of that carbon is of minor importance (Wangsell et al., 2009).

Hydroxyethylene (HE)-based inhibitors

The HE transition state isostere was investigated as a scaffold to provide potent, small molecule inhibitors of β -secretase. Compound **8** (Figure 3) with *N*-terminal isophthalamide compound **8** proved to be a potent inhibitor ($IC_{50} = 30 \text{ nM}$ and HEK-293 $EC_{50} = 3000 \text{ nM}$) leading to enhanced cell penetration (Hom et al., 2004). Samuelsson and co-workers (Bjorklund et al., 2010) explored the insertion of an oxygen atom in P1 position of the HE-based peptidomimetic inhibitors. The most potent inhibitor **9** exhibited a good β -secretase IC_{50} value of 3.1 nM and an IC_{50} value of 160 nM in the cell-based assay (Wangsell et al., 2010). Compound **10** ($IC_{50} = 0.32 \text{ nM}$) showed to be a potent and cathepsin D selective β -secretase inhibitor.

Furthermore, compound **11** displayed an IC₅₀ value of 140 nM showing that the interactions in the S2' and S3' pocket as well as the amide linkage are of key importance for potency (Meredith et al., 2010).

Hydroxyethylamine (HEA)-based inhibitors

Maillard and co-workers reported a series of HEA-based inhibitors combining the isophthalamide moiety with an HEA isostere bearing the *R* stereochemistry at the TS hydroxyl and the 3,5-difluorophenyl fragment as the P1 aryl group. Compound **12** (Figure 3, IC₅₀ = 20 nM and EC₅₀ = 15 nM) represents a potent and cell permeable peptidomimetic inhibitor of the human β -secretase (Maillard et al., 2007). Similarly, high enzymatic potency was observed for inhibitor **13** in which the C-5 position of the isophthalamide ring was functionalized with a polar primary amide in order to increase affinity for β -secretase and selectivity over cathepsin D (Kortum et al., 2007).

Poor metabolic stability due to microsomal *N*-debenzylation and *N*-depropylation (Freskos et al., 1007) prompted to the replacement of the isophthalate *N*-terminus by acyclic sulfones. The authors first identified the racemic Cbz-derivative **14** as a lead compound endowed with good enzymatic activity, but more potent against cathepsin D (IC₅₀ = 67 nM) (Freskos et al., 2007). Subsequently, structure based design resulted in the synthesis of derivative **15** with highly improved enzymatic inhibitory activity (IC₅₀ = 2 nM) and cellular potency (1 nM). The X-ray crystal structure of **15**-bound β -secretase highlighted a close association between the pyridyl nitrogen and the Arg235 in the S2 site. The authors suggested that selectivity (cathepsin D IC₅₀ = 474 nM) could be due to the higher lipophilicity of the cathepsin D S2 pocket (Freskos et al., 1007a).

Inhibitor **16** (GSK188909) was described as an orally bioavailable β -secretase inhibitor capable of lowering brain A β in APP transgenic mice (Hussain et al., 2007) and the studies which led to the discovery of this orally active hydroxyethylamino isostere-based inhibitor have been reported (Clarke et al., 2008; Clarke et al., 2008a; Beswick et al., 2008). GSK188909 inhibited β -secretase activity with an IC₅₀ of 5 nM, while showing good selectivity with respect to BACE-2, renin and cathepsin D. It caused a decrease in A β ₄₀ and A β ₄₂ production in cell based assays expressing both wild type and Swedish variant APP sequences (IC₅₀ = 5 and 30 nM, respectively).

Subsequently, molecular modeling suggested that the key nonprimed side interactions of **16** could be mimicked by a tricyclic indole derivative. It was reasoned that constraining the active conformation in this way would make binding to the protein much more efficient while also potentially reducing or eliminating the propensity for *N*-dealkylation of aniline moiety. They examined a variety of heteroaryl P2' groups, representative compound **17** with a 4-pyranyl amine (IC₅₀ = 20 nM, EC₅₀ = 16 nM) showed improved permeability (*b/p* = 0.37) and clearance, resulting in an increased bioavailability in rats and dogs (Charrier et al., 2008). Ghosh et al. reported a series of potent and selective inhibitors incorporating HEA isostere. Representative compound **18** (GRL-8234) has exhibited excellent *in vitro* potency (K_i = 1.8 nM, Cell IC₅₀ = 1 nM) and modest selectivity against cathepsin D (IC₅₀ = 79 nM) and BACE1 (IC₅₀ = 138 nM). This compound inhibited A β production in mice (Ghosh 2008). An IP administration of **18** at a dose of 8 mg/kg to Tg 2576 mice resulted in 65% reduction of A β production in plasma after 3h. Furthermore, GRL-8234 was shown to rescue age-related cognitive decline in APP transgenic mice (Chang 2011).

Iserloh et al. (2008) investigated conformationally constrained versions of the HEA motif found in many aspartyl protease inhibitors. They developed 4-benzyloxypyrrolidine and 4-phenoxy-pyrrolidine containing inhibitors **19** and **20** shown in Figure 4. These inhibitors exhibited good *in vitro* potency (5 nM and 3 nM, respectively) although with low cellular

activity (150 nM and 165 nM respectively) and modest selectivity against other human aspartyl proteases. In addition, both inhibitors exhibited insufficient pharmacokinetic properties in rats as evidenced by low plasma levels following oral dosing (Iserloh et al., 2008). Subsequently replacement of the *N,N*-dipropylamide present in **19** and **20** with a 2-(*R*)-methoxymethylpyrrolidine amide resulted in compound **21** with a marked improvement in cellular potency ($K_i = 0.7$ nM, cell $IC_{50} = 21$ nM), while maintaining good selectivity over related human aspartyl proteases such as cathepsin D, cathepsin E and renin (Iserloh et al., 2008a).

Based upon X-ray crystallography and molecular modeling, a series of novel, potent piperazinone and imidazolidinone-based peptidomimetic β -secretase inhibitors were developed. Piperazinones in particular are tolerant of a wide diversity of modifications to their non-prime side. Compound **22** ($IC_{50} = 3$ nM, cell $IC_{50} = 300$ nM), produced modest inhibition of peripheral $A\beta_{40}$ in a transgenic mouse model with a single dose (Cumming et al., 2008).

The *m*-tolyl sulfonamide moiety, combined with the optimal methoxymethyl pyrrolidine isophthalamide group on the non-prime side, led to the extremely potent inhibitor **23** ($K_i = 0.8$ nM, cell $IC_{50} = 7$ nM). This inhibitor has resulted in a robust and persistent lowering of peripheral $A\beta$ in a transgenic mouse model following a single subcutaneous dose. However, this compound, as with many peptidomimetics, is a substrate for Pgp, and this liability limits its oral bioavailability, brain penetration, and ultimately its central efficacy (Cumming et al., 2009).

Sealy et al. reported β -secretase inhibitor **24** ($IC_{50} = 47$ nM and cell $ED_{50} = 17$ nM). The X-ray crystal structure of β -secretase with **24** illustrated that difluoroaryl, cyclohexyl, and *tert*-butyl substituents occupy the S1, S1', and S2' pockets, respectively. Compound **24** did not possess good pharmacokinetic parameters (Sealy et al., 2009). Further work led to highly permeable HEAs with very low levels of Pgp liability which translated into an orally efficacious inhibitor in the wildtype preclinical guinea pig animal model. Compound **25** has shown cell IC_{50} of 26 nM and only 230 nM in the biochemical assay (Truong et al., 2010).

Carbinamine-based inhibitors

Rajapakse and co-workers (2006) described a series of tertiary carbinamine-derived inhibitors in which the primary amine is reported to interact with the catalytic Asp of β -secretase. Inhibitor **26** (Figure 5) displayed high potency in enzymatic and cellular assays ($IC_{50} = 12$ and 65 nM, respectively) and good selectivity toward both renin and cathepsin D, while showing only moderate selectivity towards BACE-2 enzyme ($IC_{50} = 620$ nM). A series of interesting inhibitors based on a 2,6-diamino-isonicotinamide core coupled to a truncated reduced amino isostere as the aspartate binding element has been developed. Compound **27** (Stauffer et al., 2007), displayed cellular IC_{50} of 49 nM and in vivo activity in transgenic mice expressing human wild-type APP. After i.v. administration of a 50 mg/kg dose of inhibitor **27**, a maximal reduction of $A\beta_{40}$ (34%) at 3 h from dosing was observed and the concentration of drug in the brain was 1.9 μ M (Stanton et al., 2007). The combination of the isonicotinic core containing a P3 methylcyclopropyl group with the oxadiazolyl tertiary carbinamine resulted in compound **28**. It turned out to be a very potent inhibitor with good functional activity ($IC_{50} = 0.4$ nM; sAPP β _NF $IC_{50} = 40$ nM) along with minimal Pgp susceptibility suggesting good potential for brain penetration (BA/AB = 1.9, $P_{app} = 22 \times 10^{-6}$ cm.s $^{-1}$) (Nantermet et al., 2009). However, significant pharmacokinetic liabilities were associated with **28** as it exhibited poor oral bioavailability in multiple species. IP dosing in transgenic mice at high doses showed reduction of brain $A\beta$ levels. The co-crystal structure of **28** with β -secretase revealed that the inhibitor occupied the S1-S3 sites. The benzyl group occupied the S1 pocket. Optimization of the P1 substituent was

subsequently explored. Incorporation of a 4-fluoro substituent gave compound **29** a twofold improvement in both *in vitro* and cell-based assays as compared to **28**. Unfortunately, the modest improvement of *in vitro* potency did not result in a superior pharmacodynamic response for compound **29** (Zhu et al., 2010).

Macrocyclic inhibitors

The macrocyclization strategy is an established method to pre-organize and stabilize bioactive conformations. Highlighting the open nature of the S1–S3 subsites, many macrocyclic β -secretase inhibitors have appeared in the literature (Ghosh 2005). The close spatial proximity between the P1 aryl group and the P3 methyl of carbinamine-based inhibitors suggested the possibility of increasing potency by stabilizing the bioactive conformation as well as the potential of improving the physicochemical liabilities of the acyclic series with the preparation of macrocyclicethers and macrolactones.

As shown in Figure 6, macrolactone **30** exhibited good potency ($IC_{50} = 2$ nM; sAPP β _NF $IC_{50} = 5$ nM), and was found to be not only hydrolytically stable at physiological pH but also stable in rat and human plasma and in microsomal preparations in the absence of NADPH (Lindsley et al., 2007). Inhibitor **31** incorporated an isophthalamide scaffold coupled to a reduced amide isostere. This compound presented an enzyme IC_{50} of 4 nM, a cellular IC_{50} of 76 nM and, most importantly, improved membrane permeability and reduced Pgp susceptibility. When i.v. administered in a mouse model at a dose of 100 mg/kg, it produced a decrease in A β_{40} levels of 25% in brain extracts (Stachel et al., 2006). A series of macrocyclic peptidic β -secretase inhibitors was recently designed by Lerchner and co-workers. Representative compound **32** has shown an IC_{50} of 9 nM. The introduction of the cyclopropyl into HEA-bearing β -secretase inhibitors led to a loss of selectivity over the closely related aspartyl proteases cathepsins D and E (Lerchner et al., 2010).

Non-peptidomimetic β -secretase inhibitors

One of the major issues in the design of protease inhibitors is the transition from peptidomimetics to small molecules. This task has been proven to be quite challenging. In the case of β -secretase, the relatively large size of the aspartic peptidase active site and the need for efficient brain penetration created additional challenges for the design of actual nonpeptidic inhibitors (Durham et al., 2006). A number of drug screening approaches have been used to identify novel non-peptidomimetic β -secretase inhibitors and a wide variety of scaffolds have been proposed in recent years.

Acylguanidine-based inhibitors

A low molecular weight acylguanidine inhibitor was discovered by using high-throughput screening (HTS) at Wyeth. Optimization of the hit using structure-based design led to the design of compound **33** (Figure 7, $IC_{50} = 110$ nM). The X-ray structure of β -secretase complexed with a closely related analogue of **33** revealed that the *N*-acylguanidine moiety forms hydrogen bonding interactions with the key catalytic aspartates while the substituents on the acylguanidine nitrogen extend into the S1' pocket, forming hydrogen-bonding interactions with Arg235 and Thr329 via bridging water molecules. The *p*-propyloxyphenyl group extends from the S1 to the S3 pocket with minimal strain and the pyrrole ring forms a Π -stacking interaction with the flap Tyr71. Moreover, the crystal structure revealed that the inhibitor stabilizes the enzyme in an open conformation. This is different to most peptidomimetic inhibitors which bind to β -secretase in a closed-flap form (Cole et al., 2006).

Preliminary SAR investigations as well as the bioisosteric replacement of the guanidyl functionality resulted in an only modest improvement of β -secretase inhibitory potency. However, poor selectivity over BACE-2 enzyme and poor permeability, as assessed in a

Caco-2 drug transport model, remain the main drawbacks for this class of compounds (Cole et al., 2008; Jennings et al., 2008).

Aminoimidazole and aminohydantoin-based inhibitors

Aminoimidazole-based inhibitor **34** was derived from the addition of either a pyridine or a pyrimidine ring on a previously identified lead at Wyeth. The extension towards the S3 region of the β -secretase binding pocket led to an IC_{50} value for β -secretase of 20 nM. Furthermore, **34** showed cellular activity of 90 nM, and more than 100-fold selectivity over the other structurally related aspartyl proteases BACE-2, cathepsin D, renin, and pepsin. Acute oral administration of the *R* isomer of **34** at 30 mg/kg resulted in a significant 71% reduction of plasma $A\beta_{40}$ measured at the 6 h time point in a Tg2576 mouse model ($p < 0.001$) (Malamas et al., 2009). An optimized derivative **35** (*S* was the active enantiomer), displayed an IC_{50} value for β -secretase of 10 nM, cellular EC_{50} activity of 20 nM, and more than 80-fold selectivity over the other tested aspartyl proteases. Acute oral administration of **35** at 100 mg/kg resulted in a 69% reduction of plasma $A\beta_{40}$ at 8 h in a Tg2576 mouse ($p < 0.001$) (Malamas et al., 2010).

Aminoquinazoline-based inhibitors

Aminoquinazoline **36** in Figure 8, ($K_i = 0.9 \mu M$) resulted from the screening of a vast library of compounds. The X-ray structure of **36** bound to β -secretase revealed that the aminoquinazoline moiety binds to the catalytic Asp, while the lateral chain adopts a hairpin conformation in which the cyclohexyl ring occupies the S1 site stabilizing the enzyme in an open conformation. Structure-based optimization of compound **36** led to the discovery of compound **37** showing remarkable potency ($K_i = 11$ nM). This inhibitor exhibited a moderate selectivity over cathepsin D and renin. It is a substrate for Pgp, as indirectly evaluated by the efflux ratio calculations in the Caco-2 model. The compound also lowered $A\beta$ levels in plasma by 40–70% in rats after oral administration (30 mg/kg) (Baxter et al., 2007). Ghosh et. al. designed a series of inhibitors incorporating specific heterocycles to interact with residues in the active site (Ghosh, 2010). A representative example is compound **38** ($K_i = 103$ nM).

Miscellaneous non-peptidic scaffolds

Using virtual screening, Mok and co-coworkers discovered a novel non-peptidic inhibitor (Figure 9) of β -secretase based on an isatin motif (compound **39**, $IC_{50} = 2.4 \mu M$). The relatively poor solubility of the present series of compounds coupled with the presence of the nitro and the phenolic functionalities are the main drawbacks for this series of compounds (Moka et al., 2009). Virtual screening in combination with bioassay also resulted in identification of multiple novel non-peptide inhibitors. The most potent molecule, compound **40** ($IC_{50} = 2.8 \mu M$) has a benzothiazole ring which docks into the S1 pocket of the enzyme and spans the interaction through almost all the subsites of β -secretase.

An *in silico* virtual screening of the commercial database SPECS chemical library led to the identification of compound **41** as a sub-micromolar inhibitor ($IC_{50} = 0.53 \mu M$) with low cytotoxicity (Xu et al., 2010a). Starting from peptidomimetic β -secretase inhibitors, Hanessian and co-workers developed a series of inhibitors where the P2 amino acid, including the P2/P3 peptide bond was replaced with a rigid 3-aminomethyl cyclohexane carboxylic acid. Compound **42** was the most active compound of the series ($IC_{50} = 2.5$ nM), with a 50-fold selectivity over BACE-2 and cathepsin D and good cellular activity. Co-crystallization revealed an unexpected binding mode with the P3/P4 amide bond placed into the S3 pocket resulting in a new hydrogen bond interaction pattern. Unfortunately, testing of compound **42** in an *in vitro* model (MDCK cells stably transfected with the gene for the human Pgp transporter), resulted in a high efflux ratio ($BA/AB = 97$). Therefore, the

modification of the P2/P3 amide region did not overcome this challenging problem (Hanessian et al., 2010).

Recently, Sasaki et al. found that amentoflavone-type biflavonoids have significant neuroprotective effects and β -secretase inhibitory activity. Among these compounds, 2,3-dihydroamentoflavone **43** and 2,3-dihydro-6-methylginkgetin **44** exhibited potent inhibitory effects with IC_{50} values of 0.75 and 0.35 μ M, respectively (Sasaki et al., 2010). Macchia and coworkers, using a HTS fluorescence assay, identified compound **45** (IC_{50} = 0.50 μ M) as a promising new lead (Asso et al., 2008).

Multi- target inhibitors

A multi-target-directed ligand (MTDL), the goal of which is to enhance efficacy and improve safety, is rationally designed to hit multiple targets for a particular disease. The complexity and multiple etiologies of AD render the MTDL approach a potentially effective strategy for AD treatment. In the last few years, many MTDLs have been reported with improved pharmacological profiles, such as β -secretase inhibitors bearing acetylcholinesterase (AChE) inhibitory activity or metal chelating properties.

Melchiorre and co-workers (Cavalli et al., 2007) designed compound **46**, incorporating a 1,4-benzoquinone functionality as a radical scavenger into the polyamine skeleton series of cholinergic derivatives. The biological profile of **46** was then explored in detail by means of both *in vitro* and *in vivo* assays to assess its therapeutic potential for combating AD. Compound **46** was found to be a potent inhibitor of the activity of AChE (IC_{50} = 1.55 nM) and it also inhibited β -secretase activity in a concentration-dependent manner (IC_{50} = 108 nM). Moreover, when tested at an equimolar concentration (50 μ M) with $A\beta_{42}$, **46** was able to inhibit fibril formation ($95.5 \pm 0.4\%$) and it also showed activity against the formation of reactive oxygen species (ROS).

Recently, a series of dual inhibitors of acetylcholinesterase (AChE) and β -secretase were designed based on the MTDL strategy. Among them, inhibitor **47** exhibited modest dual potency in an enzyme inhibitory potency assay (β -secretase: IC_{50} = 0.567 μ M; AChE: IC_{50} = 1.83 μ M), and also showed good inhibitory effects on $A\beta$ production of APP transfected HEK293 cells (IC_{50} = 98.7 nM) (Zhu et al., 2009). Considering the crucial roles of β -secretase and metal ions in AD pathology, Huang and colleagues designed a series of novel 1,3-diphenylurea derivatives by hybridizing the metal chelator LR-90 with a β -secretase inhibitor. Compound **48** (IC_{50} = 27.85 μ M) was the most effective inhibitor. All compounds in this series showed the ability to chelate metal ions (Huang et al., 2010).

Efficacy studies on animal models

Early studies demonstrating the ability β -secretase inhibitors to lower $A\beta$ *in vivo* were carried out with transgenic mice Tg2576. Inhibitors were injected into young mice of 4 – 6 months of age and plasma $A\beta$ was measured at various time points. Since $A\beta$ is produced in young Tg2576 mice from APP with Swedish mutations (APP^{sw}) in the brain and is rapidly transported to the plasma by efflux, such assays gave a quick indication on the overall efficacy of inhibitors to guide structural evolution. A number of inhibitors were shown to effectively lower plasma $A\beta$ (Ghosh et al., 2007). The first β -secretase inhibitor shown to transit the BBB and lower brain $A\beta$ was a peptidic inhibitor based on the first potent inhibitor, OM99-2 (K_i = 1.7 nM, Ghosh et al., 2000) conjugated to an oligo-D-arginine for membrane penetration (Chang et al., 2004). $A\beta$ reduction in mice has also been shown for other β -secretase inhibitors by direct intracranial (Asai et al., 2006; Nishitomi et al., 2006; Sankaranarayanan et al., 2008) and other forms of administration (Stachel et al., 2006; Hussain et al., 2007; Stanton et al., 2007). In addition, $A\beta$ reduction by β -secretase inhibitors

in cerebrospinal fluid and plasma has been shown in a primate model (Sankaranarayanan et al., 2009). Overall, the evidence from many laboratories supported the contention that β -secretase inhibitors reduce A β in the brain, cerebrospinal fluid and plasma in various animal models.

A more challenging efficacy question is whether A β reduction from β -secretase inhibitors would slow the AD-like cognitive decline in an animal model. The best characterized model available for such study is the transgenic AD mice. AD mice share some early human AD features including excess production of brain A β , a decline of cognitive performance with age, and the development of amyloid plaques in the brain at middle to late age. Although AD mice do not progress to dementia as in human AD, for testing an amyloid reduction therapy using β -secretase inhibitors, the AD syndromes in these mice can produce useful insights to the clinical treatment of AD in the future. Since no definitive efficacy data is yet available in human trials, it would be informative to learn if these AD-like syndromes can be rescued in AD mice by partial amyloid reduction using a β -secretase inhibitor.

A prerequisite to such a study is the availability of a brain penetrating β -secretase inhibitor that can be administered over a long period of time. Chang *et al.* (2010) reported recently that a β -secretase inhibitor GRL-8234 ($K_i = 1.8$ nM; IC_{50} about 1 nM in cellular assays) (Ghosh et al., 2008) effectively entered the brain of Tg2576 mice and reduced brain A β . In Tg2576 mice, a slow cognitive decline starts at about 6 months of age and continues through life and amyloid plaques start to appear in the brain at about 10–12 months of age. The age-related cognitive decline in this strain of AD mice (Lesne et al., 2006) has shown good correlation with the overproduction of A β in the brain and is the most stringent efficacy criteria available in the AD mouse model. In the studies of Chang *et al.*, mice were continuously infused with the inhibitor, which reduced brain and plasma A β by about 50% of the controls. In three separate experiments, in which the starting age of the mice ranged from 5.5 to 9 months, differences in the cognitive performance in Morris' Water Maze between the treated and the control mice were seen after a treatment period ranging from about 4 to 7 months. The length of time for treatment to demonstrate the cognitive rescue was likely due to the slow decline in cognitive function in this strain of mice (Lesne et al., 2006). Inhibitor infusion of shorter periods or starting treatment at an older age (18 months) failed to show the benefit of cognitive rescue.

These results demonstrated that a reduction of A β production of about 50% was sufficient to rescue the cognitive decline in this model, implying that the cognitive decline can be significantly improved in spite of some excess of brain A β . Whether this can be extended to AD remains to be shown in human trials. Other encouraging observations include the absence of overt toxicity associated with treatment over a 7-month experimental period and no significant accumulation of APP, the substrate of β -secretase, in the brain of the inhibitor treated mice. The latter observation suggested that the activity of α secretase was sufficient to dispose the excess APP from treatment.

The effect of another β -secretase inhibitor TAK-070 on Tg2576 mice has also been studied (Fukumoto et al., 2010). TAK-070 (IC_{50} about 1 – 1.5 μ M in cellular assays) was reported to moderately reduce A β . Improvement in cognitive performance by TAK-070 was observed after 9 days of administration of the inhibitor in 5 month old Tg2576 mice. Since the age-related cognitive decline in Tg2576 does not start until after these mice are 6 months old and can not be accurately measured in 9 days (Westerman et al., 2002; Lesne et al., 2006), it is not clear if the observed cognitive improvement was a result of rescue of cognitive decline or a consequence of other pharmacological effects.

Clinical trials

Clinical trials for drug candidates of β -secretase inhibitors are essential not only for developing AD therapy but also for substantiating the amyloid hypothesis and the concept of amyloid reduction therapy. Unfortunately, only a few β -secretase inhibitors have entered clinical trials to date and not many results have been released to the public. The first publicly announced Phase I clinical trial on a β -secretase inhibitor CMT-21166 was conducted by CoMentis (Koelsch, 2008; Hey et al., 2008; Hsu, 2010). This inhibitor has good solution potency (K_i vs. β -secretase = 2.5 nM), cellular potency (IC_{50} is about 3 nM), and selectivity (K_i values vs. memapsin 1 and cathepsin D are about 100 fold that vs. β -secretase). A six-week administration of CMT-21166 (structure not revealed) to 13-month old transgenic AD mice at 4 mg/kg reduced brain A β by about one-third. Chronic dosing of this inhibitor to mice produced no detectable change on myelination in peripheral nerves.

Phase I clinical trials on CTS-21166 have been carried out on healthy young males and evaluated for safety and preliminary A β responses. Subjects in the first trial were infused once with either vehicle solvent or vehicle containing the inhibitor at six different concentrations up to 225 mg. A dosage dependent reduction of plasma A β was observed immediately and reach nadir of about 80% inhibition for the highest dosages at 3 h. Significant inhibition of plasma A β persisted beyond 72 h. The recovery of plasma A β to the pre-infusion level was nearly complete by 144 h after the inhibitor administration. Significantly, no rebound of A β over the baseline level was seen during the recovery period even though the inhibitor concentration in the serum was nearly depleted at 72 h post-infusion. These observations suggest that an effective alternative pathway for APP degradation, possibly by α -secretase, had prevented the accumulation of APP during the inhibition period of β -secretase, a conclusion consistent with the mice experiments (Chang et al., 2010) described above. Similar results were obtained from a second Phase I trial on subjects receiving oral liquid solution of 200 mg CTS-21166.

Although the suppression of plasma A β may have resulted from the inhibition of β -secretase both inside and outside the brain, for a compound that penetrates the BBB, the inhibition of A β production would likely be significant in the brain. Potentially, the inhibition of peripherally generated A β may also be beneficial for drawing out brain A β by the so called 'sink effect'. As a whole, the limited preliminary results on efficacy were encouraging for the future therapeutic development of β -secretase inhibitors.

Conclusions and outlooks

The development of an effective and safe β -secretase inhibitor, as a disease-modifying Alzheimer treatment, is a critical necessity in interrupting the progress of this devastating disease. Toward this end, the structural evolution of β -secretase inhibitors is an essential path. The availability of three-dimensional structural information for β -secretase in complex with a variety of compounds has enabled great advances in development of new classes of inhibitors. In addition to the critically important SARs gleaned from the early peptidic inhibitors, past experience with comparable enzymes (renin, HIV protease), has facilitated the design of a variety of peptidomimetic inhibitors. On the other hand, the high homology among aspartyl proteases has also provided challenges for selectivity over cathepsin D, renin and especially BACE-2. Additionally, given the brain localization of the target, inhibitors require low molecular weight and a reduced susceptibility to Pgp-mediated efflux in order to cross the BBB. To effectively fulfill this aim, in recent years there has been an obvious shift toward the exploration of non-peptidic inhibitors/scaffolds. Modifications of peptide-based inhibitors have led to compounds with little resemblance to the original peptidomimetics now showing improved drug-like profiles. Furthermore, HTS and fragment-based screening

have generated a broad range of promising lead structures showing potential to overcome ADME hurdles generally associated with peptidomimetic β -secretase inhibitors.

The progress in structural evolution of β -secretase inhibitors has led to relatively small, non-peptidic, potent and selective compounds. Several recent inhibitors are able to cross the BBB and reach the brain. In transgenic mice, a β -secretase inhibitor has been administered on long-term basis to significantly suppress A β level and rescue the age-related cognitive decline. In Phase I clinical trials, a β -secretase inhibitor has been shown to reduce human plasma A β . Clearly, the hope for the next step would be to develop inhibitors with better pharmaceutical properties and to carry out well designed efficacy trials to determine if they can rescue cognitive decline in AD patients. Positive results in these clinical trials would provide the long-awaited proof for the 'amyloid reduction strategy' in AD therapy and will be one step closer to the ultimate effective treatment of the disease.

The next step in clinical trials for β -secretase inhibitors however, will be a challenging undertaking. Since no successful disease-modifying efficacy has previously been carried out for AD therapy, initial Phase II/III trials for β -secretase inhibitors will be entering uncharted territory. It is unlikely that past trial design for cognitive-enhancing drugs would be useful as guides for the trials of β -secretase inhibitors since the basis of their treatment is completely different. At present, there are no FDA-approved biomarkers for AD clinical trials and the only acceptable efficacy end-point is the improvement of cognitive decline. Thus, in efficacy trials for β -secretase inhibitors, treatments must slow or stop cognitive decline over sufficient length of time to produce statistically significant differences in cognitive performance of the treated and the control groups. Since cognitive performance declines slowly in AD, a long efficacy trial with options to be extended would have a better chance of observing the benefit of treatments. Another obvious consideration is the stage of AD in patients selected for trial. It seems reasonable to suggest that efficacy of β -secretase inhibitors has a best chance to be observed in treatment of early stage AD patients (Tang and Ghosh, 2010). In late stage patients, secondary pathology not directly induced by A β , such as brain inflammation, may provide a high background cognitive influence and obscure the benefit of A β reduction. Such argument is supported by the observation that in old transgenic mice, the cognitive rescue was not observed with a β -secretase inhibitor in spite of significant reduction of brain A β (Chang et al., 2010).

So far, the most promising β -secretase inhibitors appear to have emerged through the structure based design cycles of transition-state analogues. The overall progress in the field permits some optimism that structure-based design approach will lead to better compounds. It seems probable that β -secretase may ultimately fulfill its promise as a valuable therapeutic target for treatment of Alzheimer's Disease.

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Figure 1.
Statine, phenylnorstatine and *tert*-hydroxy-based inhibitors (compounds 1–7).

Figure 2.
Hydroxyethylene (HE)-based inhibitors (compounds **8–11**).

Figure 3.
Hydroxyethylamine (HEA)-based inhibitors (compounds **12–18**).

Figure 4.
Diverse Hydroxyethylamine (HEA)-based inhibitors (compounds 19–25).

Figure 5.
Carbinamine-based inhibitors (compounds **26–29**).

Figure 6.
Macrocyclic inhibitors (compounds 30–32).

Figure 7.
Acylguanidine, aminoimidazole and aminohydantoin-based inhibitors (33–35).

Figure 8.
Aminoquinazoline-based inhibitors (compounds 36–38)

Figure 9.
Miscellaneous non-peptidic scaffolds (compounds **39–45**).

Figure 10.
Multi target inhibitors (compounds **46–48**).