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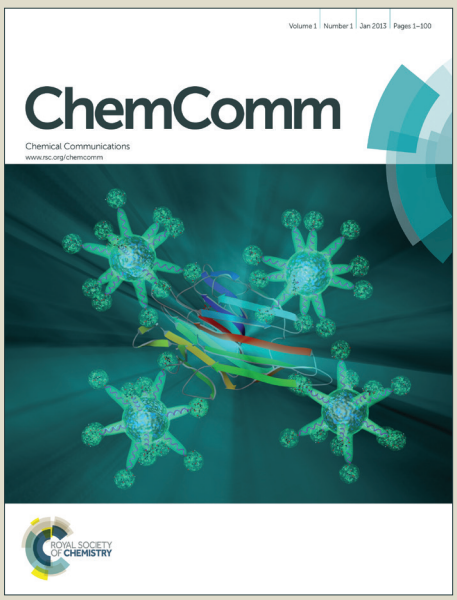

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Function and toxicity of amyloid beta and recent therapeutic interventions targeting amyloid beta in Alzheimer's disease

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Amyloidogenesis has been implicated in a broad spectrum of diseases in which amyloid protein is invariably misfolded and deposited in cells and organs. Alzheimer's disease is one of the most devastating ailments among amyloidogenesis induced dementia. Amyloid beta (Aβ) peptide derived from amyloid precursor protein (APP) is misfolded and deposited as plaques in the brain, which are said to be the hallmark of Alzheimer's disease. In normal brains physiological concentration of Aβ peptide has been indicated to be involved in modulating neurogenesis and synaptic plasticity. However, excess Aβ production, its aggregation and deposition deleteriously affect a large number of biologically important pathways leading to neuronal cell death. Targeting Aβ production, Aβ aggregation or its clearance from the brain has been active areas of research for preventing or curing AD. Our Feature Article intends to detail the aggregation mechanism, physiological role of Aβ peptide, elaborate its toxic effects, and outline the different classes of molecules designed in the last two years to inhibit amyloidogenic APP processing, Aβ oligomerization or fibrillogenesis and to modulate different pathways for active clearance of Aβ from the brain

Introduction

Understanding the mechanism of protein misfolding and aggregation has been a prime subject of research as this process is identified in the main pathological event involved in several diseases like Alzheimer's disease (AD), Parkinson disease (PD), Huntington disease (HD), Type II diabetes (T2D) and prion-related disorders among others.[1-3] AD is the most prevalent form of neurodegenerative diseases causing progressive attrition of cognition, task performance ability, mood, speech, behavior and memory.[4] Age is still considered the most important risk factor in AD with the elderly being more likely to develop the disease.[5,6] A recent report from the National Center for Health Statistics (NCHS), USA suggests that deaths caused by diseases like ischemic heart disease, brain stroke, AIDS (acquired immune deficiency syndrome) and cancer have decreased significantly while deaths caused by AD is in constant rise.[7] Alarmingly, in the next decade AD is likely to become particularly devastating for the poor and developing countries, severely affecting their public health and economy.[8] For this reason both scientific and clinical research is putting tremendous efforts to understand and cure the disease. AD involves two major types of misfolded protein aggregates: intracellular aggregates of the microtubule-associated tau protein (called neurofibrillary tangles - NFT) and extracellular peptide aggregates known as senile plaques, mainly composed of amyloid β (Aβ) peptides.[9] Aβ peptides are

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transmembrane peptides produced by incorrect processing of the integral membrane protein called amyloid precursor protein (APP).[10] The physiological function of Aβ peptide in normal brain is not completely understood, although it is said to be playing a vital role in neurogenesis and modulation of synaptic plasticity.[11] Excess production or dysfunctioning of Aβ clearing pathways leads to their deposition which deleteriously affects a large number of vital pathways like lipid metabolism, intracellular signaling cascades, autophagy regulation, neurotransmitter release and synaptic function ultimately resulting in neuronal death.[12] Aβ toxicity is attributed to the toxic oligomeric and fibrillar species formed through its aggregation (amyloid cascade hypothesis); therefore, targeting fibrillogenesis of Aβ or activating pathways like autophagy or activating neuronal signaling for maintaining neuronal homeostasis that are blocked by toxic Aβ aggregates have been taken up as therapeutic stratagies for preventing or curing AD.[13] The molecular pathways which drive the formation of Aβ oligomers and fibrils, and their interdependence for existence is not well understood. Recent studies indicate that once certain concentration of fibrils is deposited, they in turn catalyse the formation of toxic oligomeric species from soluble Aβ monomers and the process is termed as secondary nucleation.[14] Therefore, targeting secondary nucleation mechanism also could be an effective therapeutic strategy. Molecules ranging from peptides to small synthetic and natural compounds have been extensively studied, and proven to be helpful in modulating the Aβ aggregation. Metal ions (Cu^{2+}, Zn^{2+} and Fe^{2+}) have been reported to play a key role in accelerating and stabilizing Aβ oligomers (toxic form of Aβ). Therefore, targeting metal ions using metal chelators is a promising therapeutic tool in decreasing Aβ toxicity.[15,16] Targeting the proteolytic enzymes (β- and γ-secretase) involved in the APP processing (amyloidogenic pathway) is a potential strategy in decreasing the amyloid deposits in the brain.[17] Developing Aβ-specific antibody, upregulating Aβ clearing pathways and activating autophagy are few other therapeutic routes for tackling AD.[18,19]

In this Feature Article we cover literature from the past two years, discussing functional and therapeutic aspects of Aβ in AD. Further, we refer the reader to many primary research and review articles to gain a detailed overview of the past research findings for continuity and their understanding. We first briefly summarize the APP processing, recent developments in understanding the mechanism of Aβ aggregation and their structure. This is followed by a brief discussion on the physiological role of Aβ and its mechanism of toxicity. Finally therapeutic molecules that directly or indirectly interfere with the Aβ-induced toxicity is discussed in detail.

Malabika Chakrabarti

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Aβ processing

Aβ is the key component involved in the progression of AD. In this section, we explain its origin, recent developments in understanding the aggregation mechanism and its structure. A certain concentration (picomolar) of Aβ is always present in normal human brains and recent literature accentuates its role in neural plasticity, synapse formation, metal sequestration and homeostasis.[11] Aβ peptides with sequence length ranging from 36-43 are derived from the proteolytic cleavage of an integral membrane protein called APP (Fig. 1). APP is a type 1 transmembrane glycoprotein (695 amino acids) expressed on both intra- and extracellular membranes and has various physiological functions.[20] Production of Aβ in the amyloidogenic pathway involves sequential cleavage of APP by proteolytic enzymes β-secretase (β-site APP-cleaving enzyme 1 - BACE 1, which is an integral membrane protease) and γ-secretase (membrane-bound enzyme).[21] In the case of non-amyloidogenic pathway, a third secretase (α-secretase) cleaves within the Aβ sequence and prevents its production (Fig. 1). In amyloidogenesis, APP is first cleaved by β-secretase producing

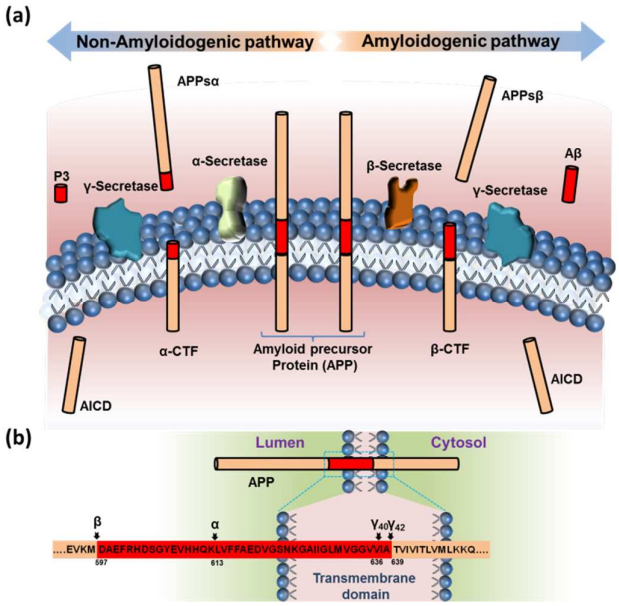

Fig. 1 Proteolytic processing of APP. (a) In non-amyloidogenic pathway cleavage occurs when α-secretase acts on APP to liberate APPsα and α-CTF, the latter being cleaved by γ-secretase to generate P3 and intracellular C-terminal fragment (AICD) (left). Amyloidogenic cleavage by β-secretase liberates APPsβ and the residual peptide (β-CTF) is cleaved by γ-secretase to produce Aβ and AICD (right). (b) The schematic structure of APP is shown with the Aβdomain shaded in red and enlarged. The major sites of cleavage by α, β and γ-secretase are indicated along with Aβ numbered from N-terminal.

APPsβ and β-CTF (β C-terminal fragment). Successive action by γ-secretase on the transmembrane domain produces Aβ and AICD (amyloid precursor protein intracellular domain) (Fig. 1).[10] In non-amyloidogenic pathway, α-secretase cleaves the ectodomain of APP resulting in the formation of the APPsα fragment and α-CTF. Then the γ-secretase cleaves the α-CTF releasing the so-called P3 peptide and AICD (Fig. 1). The imperative role of BACE 1 and γ-secretase in Aβ production make them obvious therapeutic targets for AD.[17,22]

Aβ aggregation. Unraveling the structure and mechanistic insights on the higher order Aβ aggregates has been an important area of research because of its relevance to AD. Nearly 80% of the Aβ in normal human brain constitutes of Aβ40 whereas in the diseased condition excess Aβ42 is produced and predominantly accumulated as amyloid plaques. The Aβ42 has severe neurotoxicity and possesses faster aggregation kinetics compared to Aβ40.[23] A native unfolded state of Aβ shows slow transformation into a partially folded state (β-sheet).[24] The rate of transformation is slower therefore, an initial lag in aggregation is observed. The external addition of higher-order aggregates (seeding effect) results in faster aggregation, suggesting a nucleation growth mechanism.[25] Further, partially folded units associate with each other through hydrophobic interaction and hydrogen bonding to form paranucleus which then self-associate to form higher-order structures called protofibrils (Fig. 2a). The protofibrils are further self-assembled through the elongation phase to form long fibrillar aggregates.[25] To understand the elongation mechanism Stultz *et al*. recently performed a detailed molecular dynamic (MD) simulation suggesting that during elongation the N-terminal associates with the core Aβ fibril through intermolecular hydrogen bonding (β1) (Fig. 2b).[26] This is followed by the formation of the bent β-hairpin structure and association of β2 strand of the monomer with β1 strand through intramolecular hydrogen bonding; this unit finally associates with the full Aβ fibril. β-Hairpin stabilization enhances aggregation rate and appears to be one of the possible targets for designing drugs. The Aβ fibril formation includes oligomers as the intermediate state and oligomers existence depends on the Aβ monomer concentration (primary nucleation). Recently, Knowles *et al*. have shown that existence of the Aβ oligomers depends on the concentration of both monomers and fibrils.[14] Initially, Aβ monomers undergo aggregation to form fibrils through primary nucleation with

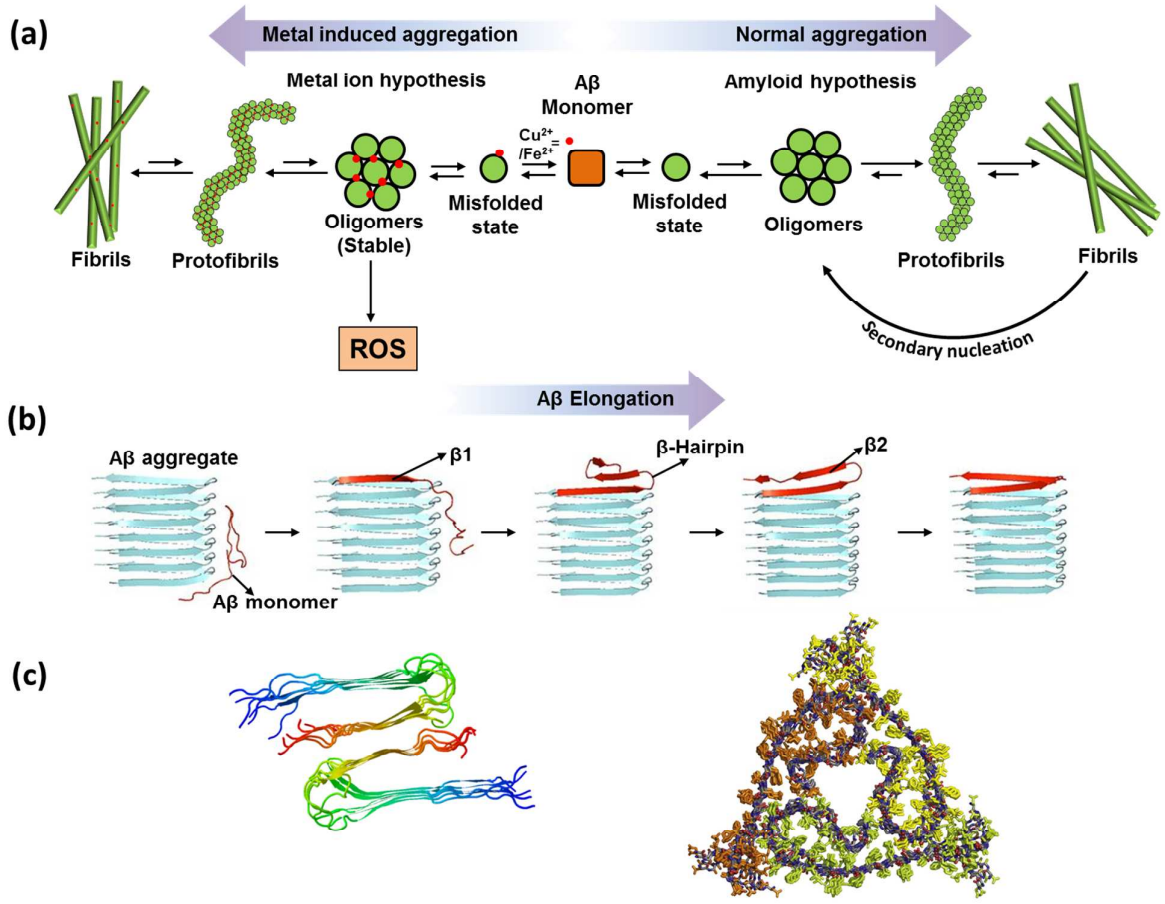

Fig. 2 Aβ aggregation and structure. **(a)** Schematic illustration for Aβ peptide aggregation in normal pathway representing amyloid cascade hypothesis (right) and metal induced Aβ aggregation to stable toxic oligomers and fibrils representing metal ion hypothesis (left). **(b)** Schematic for different energy minimized states for Aβ peptide interacting with Aβ aggregate during elongation process. Reproduced with permission from American Chemical Society (ACS) from ref. 26. **(c)**. High-resolution structural model of Aβ40 with two-fold symmetry about the fibril growth axis (left), developed previously from ssNMR and electron microscopy measurements and fibrils seeded from Alzheimer's patient tissue were analysed by ssNMR to obtain a structural model with three-fold symmetry (right).Reproduced with permission from Cell press from ref. 36.

oligomers as the intermediates. Once a certain concentration of the fibrils is reached (10 nM) they catalyse the formation of the oligomers on their surface (secondary nucleation). Thus, a new concept has been introduced which implies that the Aβ oligomer formation is initially guided by primary nucleation and subsequently enriched by secondary nucleation on the surface of the Aβ fibrils.

Introducing single or multiple mutations at critical sites of the Aβ peptide has only minor effects on the fibril formation.[27] C-terminal elongation of Aβ40 to Aβ42 and Aβ43 has shown greater influence on the aggregation rates and toxicity. Structural analysis of Aβ aggregates has been hindered by its non-crystalline and insoluble nature. In this context, solid state nuclear magnetic resonance (ssNMR) has become one of the most effective and sought after method to study the structural aspects of Aβ aggregates. Tycko *et al.* proposed one of the first structural model for Aβ40 aggregates using ssNMR.[28] Aβ40 aggregates show U-shaped β-sheet confirmation where stretches of amino acid residues 12-24 and 30-40 are involved in intramolecular β-sheet formation. While the residues in the stretch of 1-10 are in a disordered state and the loop region corresponding to 23-30 is involved in the salt bridge formation between D23 and K28. In the Aβ40 fibrils hydrophobic C-terminal face (30-40) of individual cross β-sheet units face towards each other to form dimer units which further interact to form higher order fibrils (striated-ribbon morphology in TEM). Wetzel *et al*. and Shirasawan *et al.* studied proline substitution mutants of Aβ40 and Aβ42 respectively, to understand the secondary structure of amyloid fibrils and its influence on aggregation.[29,30] Notably, the structure proposed by Wetzel *et al*. is in good aggrement with the structural model proposed by Tycko *et al*.[28,29] Hydrogen-deuterium (H/D) exchange NMR experiments of Aβ40 showed that residues 16-36 are inaccessible by solvent and might be involved in the formation of β-sheet structure of fibrils.[31] Riek *et al.* presented a new structural model of Aβ42 protofilaments where odd numbered amino acid residues of β1 (10-20) interact with even numbered residues of β2 (31-42) in Aβ42 unit.[32] This folding leads to unpaired β strands at the end of Aβ42 unit, they can further interact with similar subunits to form protofilaments. Overall, distinctive intermolecular interactions explain the unidirectionality and sequence selectivity in Aβ fibril growth. Tycko *et al.* analysed Aβ40 samples which showed twisted fibrillar morophology in TEM using ssNMR and obtained a three-fold symmetric structural model for the fibrils. This model completely differs from their previously proposed model for Aβ40 emphasising two-fold symmetry.[33] Nesi *et al.* analysed uniformly [^{13}C,^{15}N]-enriched Aβ40 aggregates sample and obtained a structure which showed deviation in intramolecular and dimeric (between two Aβ40) interactions compared to previously reported structural models of Tycko *et al*.[34] These structural variations highlight the polymorphic nature of Aβ at atomic resolution and the extent of structural variation among Aβ aggregates also depending on the method of preparation. Reif *et al*. have proposed that Aβ fibrils consist of assymetric peptide dimer as a basic structural subunit and interations among these subunits during the fibrillizaton process lead to formation of polymorphic Aβ fibrils.[35] Recently, Tycko *et al*. extracted Aβ aggregates from two AD patients, and analyzed them by ssNMR spectroscopy and electron microscopy.[36] Fibrils extracted from individual patients and *in vitro* formed fibrils have shown structural and morphological dissimilarities. Individual brains constituted similar-structured aggregates signifying single point nucleation of Aβ and consecutive migration in the brain. The Aβ fibrils gave a three-fold symmetric structural model with C-terminal residues buried in the fibril core, indicating that the use of C-terminal-specific monoclonal antibodies as therapeutic targets or as a tool for detection would be an unreliable strategy (Fig. 2c). A recent study by Bockmann *et al.* using ssNMR showed that N-terminal of Aβ peptide in fibrillar aggregates is rigid which was generally considered flexible.[37]

A native unfolded Aβ peptide can also undergo off-pathway self-association to form stable oligomeric aggregates.[38] The oligomers are shown to be the most toxic form of Aβ and they are considered to exist in the lag phase of the fibril formation.[25] These oligomers can act as nucleation centers in the brain for the formation of new oligomers and higher-ordered aggregates as well. Metal (Cu^{2+}) coordination with Aβ has shown enhanced aggregation rates, stabilization of oligomeric state and reactive oxygen species (ROS) generation (metal ion hypothesis) (Fig. 2a).[15] Aβ oligomers are highly unstable and determining their structure is difficult. Maiti *et al*. used a combination of rapid fluorescence and slower two-dimensional ssNMR technique to understand the Aβ oligomeric structure.[39] Hydrophobic regions (residues 10–21 and 30–40) attain a conformation similar to the fibrils, while the turn region (residues 22–29, involving a salt bridge) and the N-terminal (residues 1–9 are more flexible in oligomers) are different. Oligomers exhibit structural similarity with corresponding protofibrils whereas protofibrils share less similarity with their fibrils.[40] This indicates the possible conversion of intramolecular hydrogen bonds to intermolecular hydrogen bonds during structural transition from Aβ protofibrils to the corresponding matured fibrils. This mechanistic and structural analysis of toxic oligomers and matured aggregates provides a handle in designing potential molecules to modulate the Aβ aggregation.

Aβ function

The toxic effects of Aβ have been explored widely but a few studies in the past decade have also highlighted its physiological roles in maintaining a healthy nervous system in a concentration-dependent manner.[41] The physiological processes positively influenced by Aβ are neurogenesis, synaptic plasticity, memory formation, calcium homeostasis, metal sequestration and antioxidant property (Fig. 3).[11] In this section we summarize the recent progress in understanding physiological functions of Aβ in different aspects of the brain development.

Neurogenesis. It is the process of formation and differentiation of neurons. Neuronal stem cells (NSCs) or progenitor cells serve as the origin of this process and

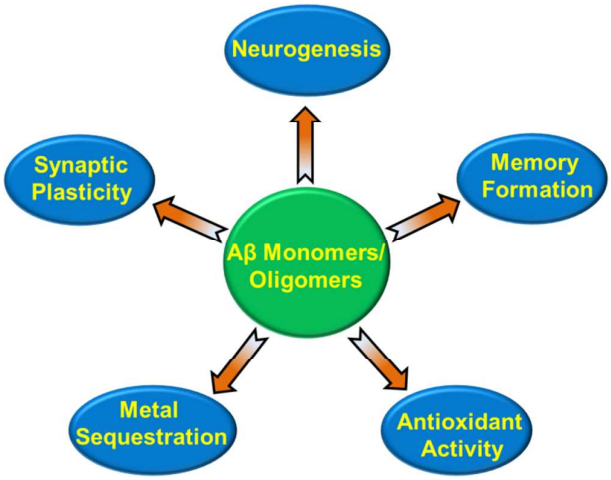

Fig. 3 Aβ physiological functions.

generate two different types of cells in the nervous system (neurons and glial cells) in response to different neurotrophins (nerve growth factor, brain derived growth factor, neurotrophin-3, neurotrophin-4).[42] Aβ40 has been shown to promote NSCs proliferation and neurogenesis whereas Aβ42 favors gliogenesis of NSCs.[43] Aβ40 and Aβ42 also exert neuroprotective effects and enhance cell viability in the absence of growth factors, neurotrophins and excitotoxic conditions.[44] Current studies are focused on identifying the biochemical pathways involved in the induction of neurogenesis by Aβ isoforms. A recent study has shown the effect of Aβ on the proliferation of NSCs in a mouse model that acquired AD on ageing.[45] Soluble form of Aβ increases NSCs proliferation before the onset of AD and the reason seems to be the activation of the PI3K-Akt pathway. Aβ40 increases the expression of the $GABA_A$ α6 subunit by activating $p75^{NTR}$ and modulates the MEK/ERK pathway where both are crucial for neuronal maturation.[46] Neurogenesis influenced by the Aβ is also related to other important intracellular processes like autophagy. Low micromolar levels of soluble Aβ increase autophagy throughout the NSCs differentiation process in an ROS-independent manner.[43] Use of autophagy inhibitors such as 3-MA significantly increases the cell death indicating that activation of autophagy by soluble Aβ might operate as an NSCs survival response.

Synaptic plasticity and memory formation. Strengthening and weakening of synapses in response to neurotransmitters is termed as synaptic plasticity.[47] It plays a vital role in learning and memory formation.[48] Long-term potentiation (LTP) is the enhanced level of neuronal transmission over a prolonged time period and it is involved in memory formation by strengthening the synaptic junction over time. Loss of synaptic plasticity and LTP is prevalent in AD due to increased concentration of Aβ and the positive effect is assumed to be through the interaction of Aβ with α7 nicotinic cholinergic receptor present on neurons.[49] The N-terminal fragment generated by the action of α-secretases showed an induced post-tetanic potentiation and LTP in mouse hippocampal slices. This activity was attributed to N-terminal metal binding residues YEVHHQ.[50] The Hippocampal region in the brain is important for the consolidation of memory. Administration of picomolar concentration of Aβ improved memory formation whereas inhibition of endogenous Aβ production reduced the retention of memory.[51,52] Memory formation is modulated via nicotinic acetylcholine receptor interaction with the Aβ.

Metal sequestration and antioxidant activity. Transition metals like copper, zinc or iron can take part in different biochemical redox reactions and produce ROS. Aβ plays an agonistic role in scavenging ROS when present in lower concentration.[53] Histidine residues (H6, H13 and H14) at the N-terminal of Aβ chelates metal ions and prevents them from participating in any redox reaction or ROS production while the methionine (position 35) residue at the C-terminal has radical scavenging property.[54] A recent study has confirmed the antioxidant property of Aβ42 in a cell-free system.[55] Hydroxyl radicals and H_2O_2 were formed in mitochondria diseased (cancer, AD etc.) conditions which could be replicated *in vitro* using $FeSO_4$ and ascorbate. Applying different concentrations of Aβ42 aggregates to the *in vitro* system reduced the production of ROS. This effect was also observed in mitochondria of a rat brain. Chelation of iron with Aβ at low concentrations resulted in a reduced ROS generation.

Aβ toxicity

The progression of AD and its mechanism of toxicity is not very well understood. In order to address this researchers have put forward various hypotheses; these primarily include the amyloid hypothesis, oligomer cascade hypothesis, Tau hypothesis, metal ion hypothesis, and oxidative stress hypothesis.[13,15] Although each hypothesis has been backed by sufficient conceptual proof, the amyloid hypothesis is the most renowned and widely accepted till date. This hypothesis suggests that excess Aβ production, aggregation and its deposition in the brain as plaques is the prime reason for the progression of AD. However, the oligomer cascade hypothesis indicate that Aβ oligomers are the highly toxic species compared to fully grown fibrils and could be a trigger for AD.[56] Each hypothesis deals with separate aspects of toxicity involved in AD whereas it is remarkable to note that Aβ has been implicated as the key player in all the proposed hypotheses. This has understandably raised immense interest in comprehending its origin and means of toxicity.[57] In the following sections, different means of Aβ toxicity have been discussed (Fig. 4).

Oxidative stress. Accumulation of Aβ peptide coordinated with redox active metals has been hypothesized to induce oxidative stress.[58] However, the exact causative mechanism for stress generation by Aβ is still a matter of debate. Redox-active copper ions have been found accumulated in amyloid plaques which form Cu-Aβ complexes and catalyse the production of ROS. Aβ has the ability to reduce Cu^{2+} to Cu^{+} or Fe^{3+} to Fe^{2+} and these reduced metal ions can react with O_2 to produce superoxide anion which further takes up $2H^{+}$ ions and generates H_2O_2. H_2O_2 may react with another reduced metal

ion to produce the toxic HO· radicals through Fenton reaction (Fig. 4).[59] These radical species are said to be involved in lipid and protein peroxidation and finally leading to neuronal death. Metal reduction in the Fenton cycle is said to be mediated by methionine (M35) whose sulfide group has the ability to oxidize and easily donate electrons, although recent studies indicate that absence of M35 does not prevent Aβ toxicity.[60,61] Aβ has also been known to induce T10 intermolecular crosslinking to promote the formation of toxic oligomeric species.[62] The N-terminal region of Aβ has a metal binding domain and amino acid residues D1, H6, H13 and H14 are proposed to be involved in metal chelation. Cu^{2+} coordinates through D1, H6 and H13 or H14 while Cu^{+} coordinates with H13 and H14. Large structural rearrangement occurs in the copper coordination during electron transfer (redox reaction) indicating the presence of an intermediate state involved in ROS generation. Recently, Collin *et al.* showed the existence of the intermediate transition state through mass spectroscopy where copper coordinates with A1, H13 and H14 residues whereas H6 has to break its bond with Cu^{2+} to initiate the redox reaction.[63] ^{13}C and ^{15}N NMR studies have supported the contention that such major rearrangement in the copper binding site occurs during the redox cycle of ROS production.[64] Metal binding elongates the lag phase of fibril formation or stabilizes the oligomeric state.[65] Cu^{2+} with Aβ forms toxic oligomeric species whereas Zn^{2+} forms amorphous non-fibrillar aggregates with reduced neurotoxicity. The impact of Zn^{2+} on Aβ-Cu complex in altering ROS production is minimal.[66] Matured Aβ aggregates are said to be less toxic but Cu^{2+}-induced Aβ fibrillar aggregates retain their redox activity and are able to produce hydroxyl radical from H_2O_2.[67]

Synaptic dysfunction. Synaptic loss is better correlated with cognitive impairment of AD rather than with the amount of Aβ plaques.[68] Disturbances of synaptic transmission occur long before the development of the hallmark Aβ deposits. Therefore the mechanism by which Aβ disturb the synaptic transmission is not fully understood. Aβ oligomers bind to neural receptors on the synaptic cleft and hinder their function, thus leading to synaptic dysfunction and cognitive decline.[68] Aβ oligomers and not their fibrillar aggregates are considered to be responsible for synaptic dysfunction. Aβ oligomers bind to essential synaptic receptors like glutamate receptors (GluR), NMDA receptor (N-methyl-D-aspartate receptor) AMPA receptor (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor) and deregulate synaptic plasticity, memory formation and learning (Fig. 4).[48] Aβ has high affinity binding to the α7- nicotinic receptors which has a significant role in the internalization and intracellular accumulation of Aβ in neuronal cells.[69] Small ubiquitin-like modifier (SUMO) molecules acting through post-translational modification are required for normal synaptic and cognitive function. SUMOylation is involved in long-term potentiation (LTP) and hippocampal-dependent learning. The Aβ oligomers are involved in impairment and inhibition of SUMOylation

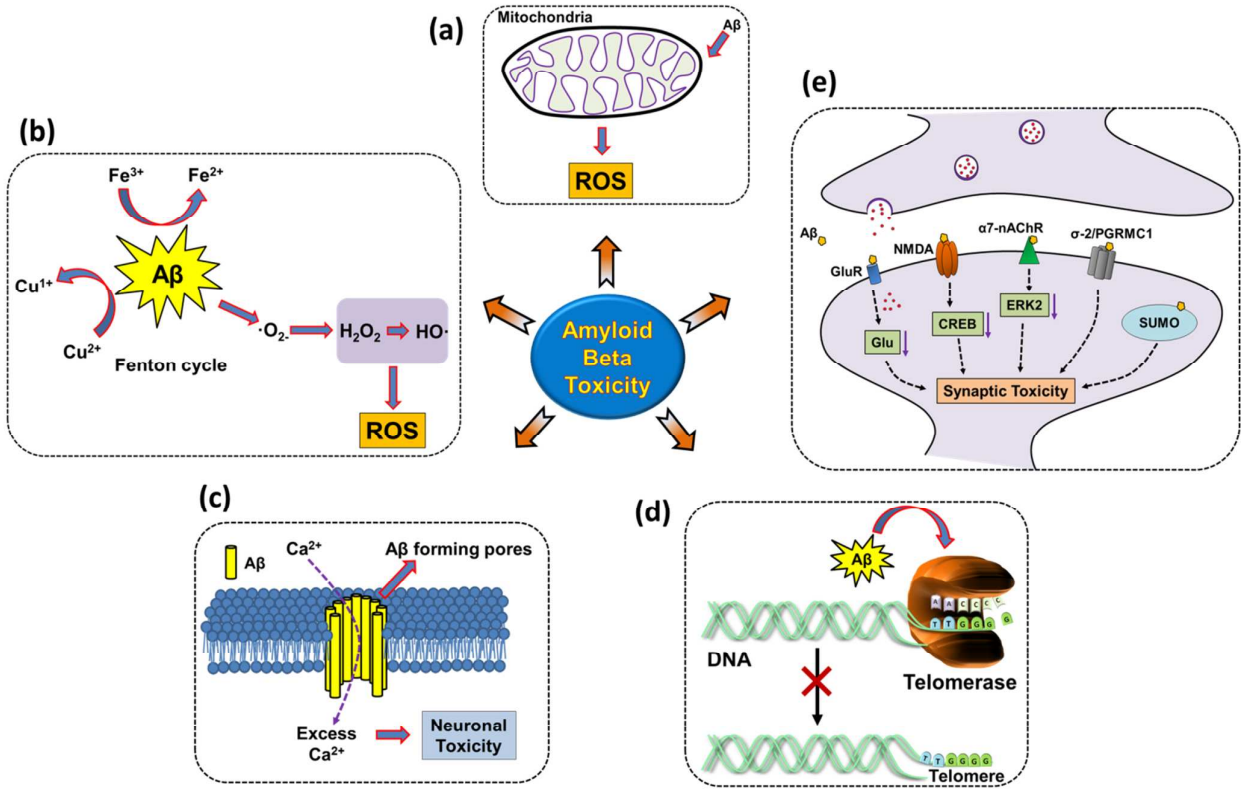

Fig. 4 Aβ toxicity. **(a)** Aβ cause mitochondrial dysfunction leading to ROS generation. **(b)** Oxidative stress caused by Aβ oligomers. **(c)** Cell membrane disruption by Aβ aggregates. **(d)** Telomerase inhibition. **(e)** Aβ interfere with signalling pathways causing synaptic toxicity.

leading to cognitive decline.[70] Catalano *et al.* have shown that sigma-2/PGRMC1 (progesterone receptor membrane component 1) is involved in Aβ oligomer binding and inducing toxicity. It indicates that sigma-2/PGRMC1 plays a key role in pathogenesis of AD and can act as a disease modifying therapeutic target.[71]

Membrane interaction. Interaction of Aβ aggregates with the cellular membrane leads to pore formation causing the abnormal flow of ions, in and out of the neuronal cells. These pores facilitate Ca^{2+} entry disturbing its active regulation which leads to cellular damage and neuronal death (Fig. 4).[72,73] The mechanism of pore formation in the cell membrane by the Aβ oligomers is similar to that of antibacterial agents killing bacteria through pore formation in the bacterial membrane by different mechanisms.[74]

Telomerase dysfunction. Telomerase is a ribonucleoprotein enzyme that adds DNA sequence repeats (TTAGGG) to the 3' end of DNA strands in the telomere regions which are found at the ends of eukaryotic chromosomes and are essential for cell survival. Telomere length is related to biological aging and its shortening is observed in many age-related diseases.[75] Recent studies have revealed that telomere shortening can play an important role in the AD pathological process. Upregulation of telomerase activity is also being considered as a therapeutic strategy in treating the AD. Recently, Qu *et al*. found that the Aβ aggregates could inhibit telomerase activity both in *vitro* and *in vivo*. Aβ oligomers bind to the DNA-telomerase complex (DNA-RNA complex) and block the elongation of telomeric DNA. Telomerase inhibition might be one of the reasons for Aβ cytotoxicity (Fig. 4).[76]

Apoptosis. It is the process of programmed cell death and is implicated in neuronal loss in AD patients. It is triggered by the shutdown of mitochondrial function and the mechanism behind it is still not fully understood (Fig. 4).[77] Recent studies show that Aβ induce the activation of IκB α/NF-κB pathway which decreases the expression of cytochrome c oxidase subunit (COXIII) and inhibits COX activity leading to mitochondria dysfunction.[78] In another report, high levels of apoptosis signal-regulating kinase 1–interacting (ASK1-interacting) protein-1 (AIP1) was observed in the brain of AD Tg2576 mice.[79] Interaction of Aβ with AIP1 initiates a cascade of pathways inducing apoptosis in the neuronal cells. Khanday *et al.* reported that the Aβ causes the phosphorylation of MKK6 at S207, T211 and Y219 residues.[80] Then phosphorylated MKK6 interacts with P66sch and forms MKK6-P66shc complex which is involved in phosphorylation of p66shc at S36 and ROS production triggering apoptosis in cells and finally leading to cell death. Further efforts are required to understand the exact mechanistic role of Aβ in initiating the apoptotic pathway and subsequent neuronal death.

Aβ therapy

The prominent role played by Aβ in AD makes it an obvious therapeutic target. In this section various therapeutic strategies and recent developments in preventing Aβ-induced toxicity will be discussed (Fig. 5).

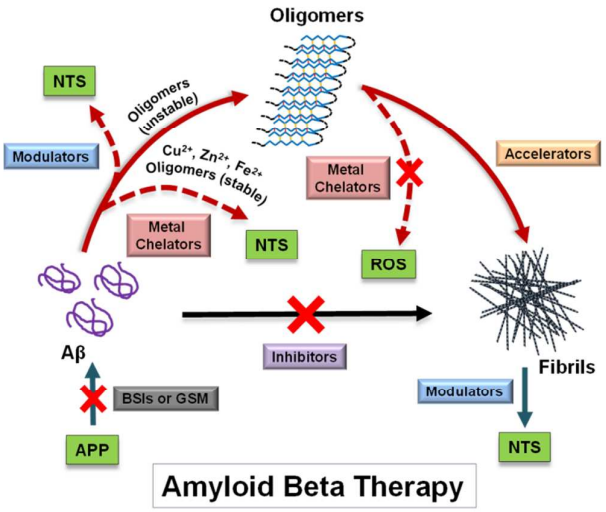

Fig. 5 Aβ therapy. Schematic illustration of different therapeutic targets which are explored to prevent Aβ toxicity. NTS (nontoxic species), BSIs (β-secretase inhibitors), GSM (γ-secretase modulators).

Modulators of Aβ aggregation

The amyloid hypothesis suggests a direct correlation of AD with Aβ aggregates; hence targeting Aβ aggregation is considered an effective therapeutic strategy. Aβ peptide switches from a non-toxic α-helical state to a toxic β-sheet conformation. Molecules that can (i) block the β-sheet formation (ii) prevent the fibrillogenesis (iii) dissolve Aβ aggregates to non-toxic species (iv) destabilize Aβ oligomers (v) accelerate conversion of Aβ oligomers to Aβ aggregates are considered as modulators of Aβ aggregation.[14] This section has been organized into peptide-based modulators and small molecule-based modulators depending upon their chemical structure.

Peptide-based modulators. Peptide-based inhibitors (sequence of 5-15 natural or unnatural amino acids) are a minor class of molecules designed upon the understanding of β-sheet-driven self-assembly involved in the aggregation of Aβ peptide. The hydrophobic core (KLVFF) of the Aβ plays a central role in the initiation of Aβ aggregation and it act as a recognition unit for their elongation to fibrillar aggregates.[81] For the past two decades KLVFF has been the basis of designing most of the peptide-based modulators of the Aβ aggregation. Hydrophobic peptides like KLVFF and LPFFD have been screened, and reported to be effective in the inhibition of Aβ fibrillogenesis both *in vitro* and *in vivo*.[82,83] Recently, Xu *et al*. developed a recognition unit based peptide H102 (HKQLPFFEED) which could inhibit the Aβ aggregation, decrease expression of p-tau, inflammatory and apoptosis factors, and enhance cognitive ability in mice. H102 undergoes rapid metabolism by proteolytic enzymes therefore, further chemical modifications are necessary to enhance its proteolytic stability.[84] Sugar-based pentapeptides of Ala-Val or

Val-Leu with D-glycopyranosyl derivatives showed inhibition of Aβ oligomers (**1**) and acceleration of oligomer conversion to less toxic fibrillar aggregates in the case of benzyl-protected sugar moiety of inhibitor (**2**) (Fig.6). Peptide-based drugs are very specific and effective but their poor bioavailability and protease stability hinders their use as potential therapeutic agents.[85]

Several modification strategies such as incorporating unnatural amino acids (peptidomimics), functionalising the N- or C- terminal with various organic moieties and cyclisation of modified peptides have been reported in the literature to enhance bioavailability, protease stability and therapeutic values. Similar modifications have been adopted in developing peptide-based modulators of the Aβ aggregation in AD. N-terminal ferrocene-tagged water soluble β-sheet peptide (Fe-$KLVFFK_6$, Fe = Ferrocene) showed disruption and inhibition of toxic oligomeric, and fibrillar Aβ aggregates.[86] A retro-inverted peptide R1-OR2 (Ac-rGffvlkGr-NH_2) tagged to TAT protein R1-OR2-TAT (Ac-rGffvlkGrrrrqrrkkrGy-NH_2) reduced the expression of Aβ oligomers and fibrils in APPswe/δE9 transgenic mice. This inhibitor was protease stable and TAT peptide helped in crossing the BBB, thus increasing its bioavailability.[87]

Peptoids are an eminent class of peptidomimetics made of N-methyl glycine units which can be effectively used in designing Aβ inhibitors and are protease-resistant. Once bound to Aβ they can inhibit the elongation process as the peptoid backbone lacks amide protons.[88] Servoss *et al.* designed a peptoid-based mimic of KLVFF, JPT1 (**3**) which exhibited an α-helix state in solution and had an enhanced aromatic interaction with the Aβ. JPT1 showed a decrease in lag time of Aβ40 fibrillar aggregation and reduction in the level of Aβ40 fibrillar aggregates when compared to its parent peptide KLVFF.[89] Bhubaneswar *et al*. introduced *ortho*-aminobenzoic acid (Ant) in a known Aβ inhibitor to obtain [Ac-L(Ant)FFD-NH_2] (**4**) as a modulator for both inhibition and dissolution of Aβ40 aggregates.[90] We developed an optimized peptidomimetic inhibitors for Aβ aggregation based on KLVFFA. To enhance the binding affinities of inhibitors we incorporated multiple hydrogen bond donor-acceptor moieties at the N-terminal and modified the backbone by introducing N-methylglycine units (Sr = sarcosine) at alternate positions of the recognition unit to retain its recognition property and also to enhance its blood serum stability (**5**: Thymine-SrL(Sr)F(Sr)A-NH_2, **6**: Thymine-K(Sr)V(Sr)F(Sr)-NH_2). These inhibitors showed good activity in both inhibition and dissolution of Aβ42 aggregates.[91] The efficacy of **5** and **6** was studied in a yeast cell model displaying Aβ42 toxicity and the peptidomimetic (**5** and **6**) succeeded in rescuing the yeast cells from Aβ42 toxicity by clearing the Aβ aggregates from the cell through upregulation of autophagy. To understand the role of **5** and **6** in dissolving the Aβ42 and activating autophagy. An autophagy-defective mutant (Δatg1 mutant) was used to perform growth analysis which showed no rescue in yeast cells. These results indicate that the inhibitors not only dissolve the aggregates but also clear them from cells by initiating autophagy which is generally downregulated in the case of AD. On-bead peptoid library screening by Bezprozvanny *et al.* resulted in selective and efficient binders IAM2 (**7**) and $(IAM2)_2$ (K_d = 60 nM) for the Aβ42 peptide. IAM2 and $(IAM2)_2$ showed moderate inhibition and neuroprotective behavior in hippocampal neurons treated with Aβ42 aggregates.[92]

In recent years cyclic peptides (CP) have emerged as a new class of powerful and specific amyloid modulators.[93] CP are

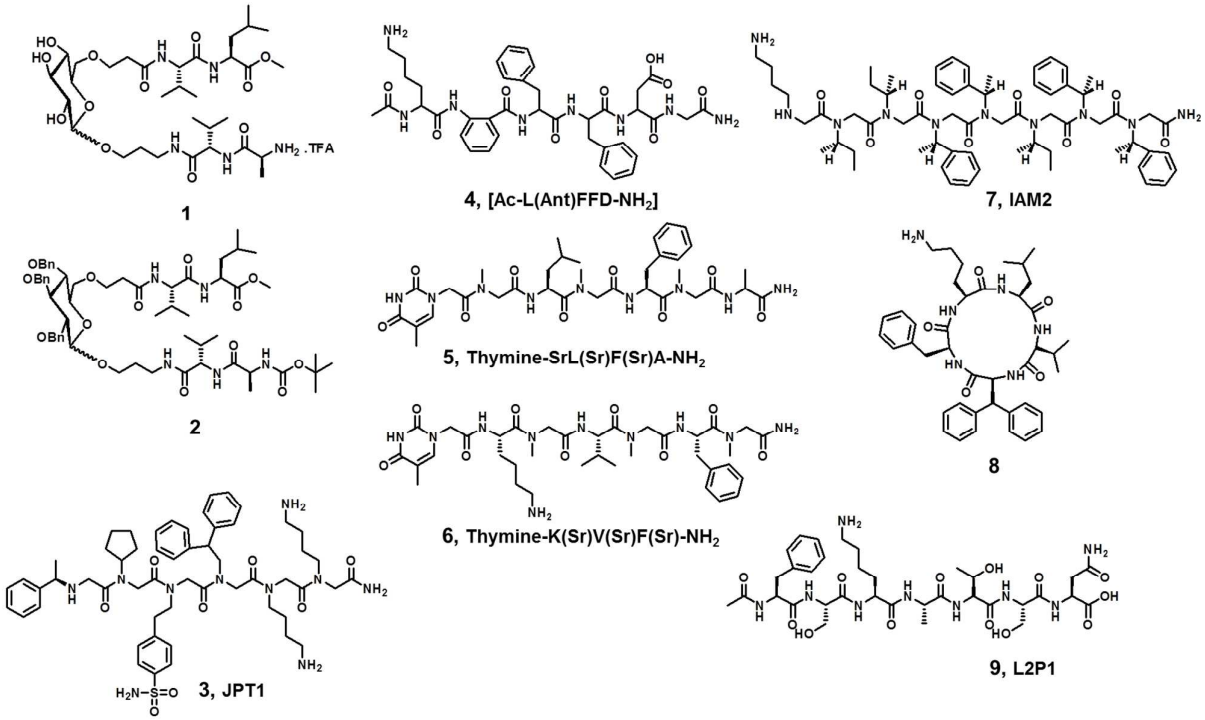

Fig. 6 Peptide-based modulators of Aβ aggregation.

metabolized slowly than their non-cyclic analogs and hence display higher bioavailability. Kanai *et al*. showed that cyclisation of the recognition moiety (cyclo-D-[KLVFF]) enhanced its inhibition efficiency by three-fold compared to its linear analog. Further, by understanding the structure activity relationship (SAR) of phenyl group in the inhibition of Aβ aggregates, a phenyl group at β-position of F4 (**8**) was introduced leading to enhanced inhibition efficiency and neuroprotective property. A natural CP such as rapamycin is known to modulate AD by upregulating the autophagy process and clearing protein aggregates. Therefore, designing conformational mimics for the Aβ aggregates based on natural CP is a promising approach. Abrahams *et al.* modified the structure of a natural cyclic antibiotic gramicidin S by exchanging hydrophobic and hydrophilic moieties, and introducing an alkyl chain in the place of aromatic amino acid to generate an amphiphilic inhibitor. The Inhibitor showed significant inhibition of Aβ40 amyloid formation *in vitro* and could also dissolve preformed amyloid aggregates. Molecular docking studies suggest that inhibitors adopt β-sheet conformation and bind to Aβ40 through β-sheet interaction.[94] Recently, Mason *et al*. developed a new screening method called protein-fragment complementation assay (PCA) for obtaining selective inhibitors for Aβ aggregation. Around 16,000 peptide sequences were screened to identify KAT (Ac-GAKATLM), L2P1 (Ac-FSKATSN, **9**) and L2P2 (Ac-PVKATTA) molecules capable of binding to Aβ42, and showed efficient inhibition and reversal of fibrillar Aβ42 aggregates *in vitro*.[95] Similar selection process was used to identify peptide modulators that could disrupt the disulfide bridge formed in mutated Aβ42 (A21C/A31C; $Aβ_{42cc}$) which induces β-hair loop structures and also shows epitopes resembling the oligomeric state. During library screening, cys_{1521} (Ac-QKVLLFA-NH_2) binding to first β-strand, and cys_{2935} (Ac-AGKATGL-NH_2) and cys_{3642} (Ac-RWGVVWG-NH_2) binding to second β-strand were obtained; a combination of these two peptides enhanced their inhibition and reversal property towards fibrillar Aβ42 aggregates.[96]

Up to now we have discussed inhibitor development based on the recognition unit or by selecting a high affinity inhibitor by different screening techniques. There is a third strategy in which proteins naturally interacting with Aβ *in vivo* have been explored. Laminin and gelsoline are naturally occurring proteins that can complex with Aβ peptide and form non-toxic species exhibiting reduced neurotoxicity.[97] Transthyretin (TTR) is a homotetrameric plasma protein (55 KDa) and plays an important role in neutralizing Aβ toxicity by forming a stable complex with the Aβ peptide. Cecchi *et al*. showed that human-TTR was able to suppress toxicity caused by Aβ oligomers in human neuroblastoma and rat primary neuronal cells.[98] Further, Murphy *et al*. reported that TTR binds to Aβ through two binding domains - strand G (IAALLSPYSYS) of the inner β-sheet and strand E (DTKSYWKALG) of the helix and loop region.[99] Instead of using the entire TTR, their binding domains were used and modified through varying sequences to obtain G16 (PRRYT IAALLSPYSWS) as an efficient Aβ binding peptide. G16 (short fibril forming peptide) prevents aggregation of Aβ monomers while transforming Aβ oligomers to large, nontoxic globular aggregates.

In conclusion, although the serum stability and BBB crossing issues of peptide-based inhibitors can be overcome by using peptidomimetics but their inhibition efficiencies are still low compared to small molecule-based inhibitors. Selectivity and biocompatibility are key factors that have kept researchers active in developing peptide-based disease modifying therapeutics for AD.

Small molecule-based modulators. Modulation of Aβ aggregation using small molecules has been reported to be a highly efficient approach.[100] Most of the modulators control the fibrillogenesis of Aβ through blocking hydrophobic interactions. Natural products like curcumin (**10**) (IC_{50} ~ 13.3 μM), resveratrol (**11**) (IC_{50} ~ 15.1 μM), and epigallocatechin-3-gallate (**12**) (EGCG) (IC_{50} ~ 2.4 ± 0.4 μM) have been shown to be effective in decreasing the load of Aβ plaques in the brain through their antioxidant and aggregation inhibition properties.[101] Curcumin and resveratrol inhibit Aβ aggregation by binding to the N-terminal of the low molecular weight Aβ oligomers and prevent the formation of the more toxic, high molecular weight oligomers. Recently, Tooyama *et al*. performed structural modification in curcumin by introducing alkyl (propyl) ester and its corresponding acid at the C_4-position to obtain FMeC1 (**13a**) and FMeC2 (**13b**), respectively (Fig.7).[102] APPswe/PS1dE9 double transgenic mice fed with **13a** for 6 months showed reduction in the insoluble Aβ deposits and reduced cognitive deficits as compared to the animals receiving curcumin or **13b** in their diet. EGCG a polyphenolic antioxidant flavonoid and a key bioactive ingredient of green tea is known for its beneficial effects ranging from anti-inflammatory to neuroprotective nature. Wanker *et al*. showed that EGCG (in phase III clinical trial) is not just an antioxidant but also perturbs the aggregation propensity of Aβ by binding to its monomeric state and rendering it into non-toxic species.[103] The same research group also reported orcein, a natural product-based molecule (**14**) which accelerates the formation of Aβ42 fibrillar aggregates from highly toxic Aβ42 oligomeric species. Orcein was found to bind parallel to the long axis of Aβ42 aggregates targeting the hydrophobic region of spherical oligomeric Aβ42 species. Once bound, it nucleated the formation of less toxic higher order Aβ42 fibrillar aggregates.[104] Brazilin (**15**) a natural product obtained from *Caesalpinia sappan* inhibited Aβ42 aggregation and also remodeled the Aβ42 aggregates to prevent them from acting as secondary nucleation centers for further fibrillogenesis.[105] Molecular docking studies suggest that brazilin binds to Aβ42 through hydrophobic interactions and interferes with the intermolecular salt bridge of D23-K28 via hydrogen bonding to induce a pathway for the formation of non-toxic aggregates. Therefore targeting salt bridge formation (D23-K28) in the loop region of the Aβ can modulate Aβ aggregation and is a promising therapeutic strategy.[106] Inhibition efficiency of brazilin (IC_{50} of 1.5 ± 0.3 μM) is reported to be higher than that of well-known natural products like curcumin, resveratrol and EGCG. Zheng *et al.* reported tanshinone (TS1, **16**) obtained from the Chinese herb danshen, as an efficient and natural

inhibitor of Aβ aggregation.[107] These natural products could inhibit Aβ42 fibrillogenesis and also dissolve preformed Aβ42 fibrillar aggregates. MD simulation suggests that **16** binds to the C-terminal hydrophobic groves of Aβ42 and prevents the lateral association of Aβ42 to form toxic oligomeric species. Gazit *et al*. reported 1,4-naphthoquinon-2-yl-L-tryptophan (NQTrp) as an Aβ aggregation inhibitor. Recently, a detailed MD simulation of NQTrp and its analogs was performed to understand the mechanism of inhibition, binding modes, and to design efficient and improved inhibitors.[108] Based on the multiple target ligand approach Li *et al*. designed an inhibitor (E)-5-(4-hydroxystyryl)quinoline-8-ol (**17**) which is a combination of clioquinol, a well-known inhibitor for both normal and copper-induced Aβ fibrillar aggregates toxicity, and resveratrol.[109] Inhibitor **17** showed higher inhibition and dissolution efficiency for copper-guided Aβ42 aggregates, antioxidant property and BBB permeability compared to its constituent elements clioquinol and resveratrol.

Takahashi *et al*. synthesised a small library of thiophene-based organic dyes, which are generally used in dye-sensitized solar cells. This set of compounds was tested against Aβ aggregation to identify lead compounds with good inhibition efficiency towards Aβ aggregation.[110] Further, Matthias *et al*. also developed thiophene-based bis(hydroxphenyl)thiophene inhibitors.[111] The dual target inhibitor **18** inhibited the fibrillogenesis of Aβ40 (IC_{50} ~ 33 to 11 μM) as well as the enzyme tau kinase Dyrk1A which is mainly involved in tau phosphorylation (IC_{50} ~ 11 to 8 μM). A series of 2-pyridyl-benzimidazole-based Ir(III) (**19**), Ru(II) and Pt(II) metal complexes were synthesized and demonstrated to rescue primary cortical neuronal cells from Aβ toxicity.[112] Recently, an enantioselective triple helical dinuclear metallosupramolecular complex was designed as a first chiral Aβ inhibitor.[113] It was based on the fact that Aβ is made of L–amino acids and creates a chiral surface on aggregates. Interestingly one of the enantiomeric metallosupramolecular complexes showed high inhibition and dissolution efficiency towards Aβ40 aggregates. The inhibitor bound to an α/β-discordant stretch of Aβ13-23 observed in the early stages of Aβ aggregation and inhibited further aggregation. These studies open up new vistas for understanding and designing highly efficient Aβ aggregation modulators.

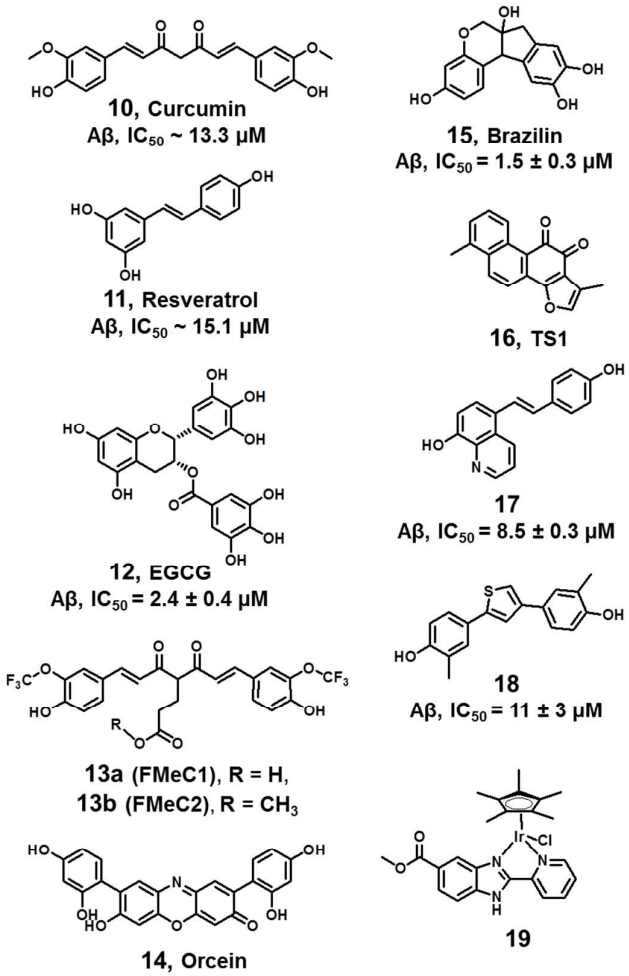

Fig. 7 Small molecule-based modulators of Aβ aggregation.

Metal chelators

The metal ion hypothesis is a well-received pathway and contributes substantially to the neuropathogenesis involved in AD. Presence of high concentrations of metal ions such as Cu^{2+}, Zn^{2+} and Fe^{2+} coordinated with the Aβ peptide in senile plaques indicates their strong involvement in Aβ aggregation and its toxicity.[114,115] Metal binding to the Aβ stabilizes the toxic oligomeric form which is indeed involved in ROS generation and causes synaptic breakdown, finally leading to neuronal cell death.[15] Sequestration of physiologically important metal ions by Aβ disturbs metal ion homeostasis in the brain.[116] Disruption of Aβ-metal interactions via metal chelators has been tried in order to reduce neurotoxicity initiated by Aβ-metal complex and to refurbish metal ion homeostasis in the brain.[117] Desferrioxamine B was the first metal chelator used to dissolve metal-directed Aβ aggregates and enhance cognitive ability in a mouse model. However, its use was constrained by its poor BBB permeability and fast *in vivo* degradation in combination with other adverse side effects. In the past few years 8-hydroxyquinoline-based molecules have gained tremendous interest as metal chelators.[118] In this context, Lindquist *et al*. have shown that the 8-hydroxyquinoline-based molecule clioquinol (**20**) exhibited a prominent role in perturbing the aggregation of Aβ in the presence and absence of metal, and also restored the endocystic function in a yeast model of AD (Fig.8).[119] However the presence of mutagenic di-iodo form of clioquinol (impurity) barred its usage and it also failed in phase II clinical trials.[120] Later PBT2 (**21**), a clioquinol derivative lacking the iodine atom is in phase II trials.[121]

An acetohydrazone (**22**) and thiosemicarbazone (**23**) derivative of 8-hydroxyquinoline have shown Cu^{2+} and Zn^{2+} sequestration from Aβ-Cu^{2+}/Zn^{2+} oligomers while the metal-free aggregation pathway of Aβ was unaffected. **22** (TEAC =

~1.5) and **23** (TEAC = ~1.2) showed decent antioxidant property in Trolox equivalent antioxidant capacity (TEAC) assay.[118] Li *et al*. designed a series of MFLs (Multifunctional ligands) based on clioquinol and the well-known antioxidant resveratrol (**24** and **25**).[122] Ligands **24** (Aβ, IC_{50} = 7.56 μM) and **25** (Aβ, IC_{50} = 6.51 μM) showed remarkable efficiency towards Aβ. The docking studies show that these ligands interact mainly at the C-terminal of Aβ peptide through hydrogen bonding and hydrophobic interactions. Ligands **24** and **25** inhibit and dissolve preformed Aβ aggregates in case of both self- and metal-mediated aggregation. Oxygen radical absorbance capacity (ORAC-FL) assay showed good antioxidant property for **24** (4.72 ± 0.14) and **25** (4.70 ± 0.57), and enhanced BBB permeability compared to their parent molecules. They also displayed excellent inhibition of monoamine oxidase (MAO) and moderate inhibition of acetylcholinesterase (AChE). Similarly, 6-chlorotacrine an AChE inhibitor derivatized with Cu^{2+} and Zn^{2+} chelating ligands led to the hybrid 6-chlorotacrine–metal–Aβ modulator (**26**) with inhibition and dissolution ability for both self- and metal-guided Aβ aggregation and AChE inhibition.[123] Molecular modeling of Aβ40-TcAChE complex with ligand showed its close proximity for H6, H13 and H14 residues of Aβ40. Lim *et al*. designed a bifunctional ligand L2NO (**27**) derived from Aβ imaging agent *p*-stilbene (recognition moiety) and clioquinol (chelation moiety).[124] **27** showed preferential and efficient inhibition activity in case of Cu^{2+}-directed Aβ40 aggregation over Zn^{2+}-mediated or self-aggregated Aβ40. L2NO interacts with almost all amino acid residues of Aβ40 while showing stronger interaction with E11, V18, F20, and M35 and induce global conformational change in the Aβ40 aggregates (2D NMR). Phenanthrene and *p*-stilbene-derived MFL, L2-b (**28**) targets Aβ40-Cu^{2+}/Zn^{2+} complex, a process which was thoroughly analysed by ion mobility mass spectroscopy (IM-MS).[125] **28** acts by metal chelation, dissolving toxic Aβ40 oligomeric species and also as an antioxidant (TEAC = 2.3 ± 0.2). *In vivo* studies have indicated that **28** can cross BBB, prevent cognitive decline and decreases the Aβ load in the brain of 5XFAD mice model for AD. L2-b was further modified by linking it to 2-[(8-quinolinylamino)methyl]phenol to obtain ligand ML (**29**).[126] Ligand **29** accommodates copper in the distorted square planar form preventing the copper redox cycle and has strong binding affinity with both Aβ40 monomers (binds to F4, R5, V12, and Q15 amino acid residues of Aβ) and its aggregates (quinoline ring interact with F19 and dimethylamino group with I32 and L34 residues through van der Waals interactions). The docking studies showed that ligand **29** binds between the steric zipper of Aβ40 distorting hydrogen bonding and its elongation to toxic aggregates. It also modulates toxic oligomer formation, exhibits superior antioxidant property (TEAC = 0.86 ± 0.10) and displays neuroprotective nature in the Aβ40/Aβ40-metal treated neuroblastoma cells. DPP2, a diphenylpropynone derivative has also shown good chelation and inhibition activity but its cytotoxicity hindered its use. Thereafter, a series of DPP2 derivatives were designed which showed better inhibition efficiency, higher binding affinity and reduce cytotoxicity compared to DPP2.[127] On similar lines, Mirica *et al*. designed MFL L1 and L2 with its core structure derived from ThT, o-vanillin (Aβ recognition) and N-(2-pyridylmethyl)amine (metal chelating moiety). ThT bound to Aβ aggregates was displaced by L1 (135 ± 25 nM) and L2 (36 ± 6 nM) showing their high binding affinity.[128] Inhibition and dissolution studies of Aβ showed prevention of fibril formation. Generally dissolution of fibrillar aggregates by inhibitors or metal chelators leads to the formation of non-toxic species. However, in the above case neurotoxic oligomeric species were obtained. Therefore MFLs should be carefully designed and studied *in vivo* to illustrate their non-toxic nature in both presence and absence of Aβ aggregates.

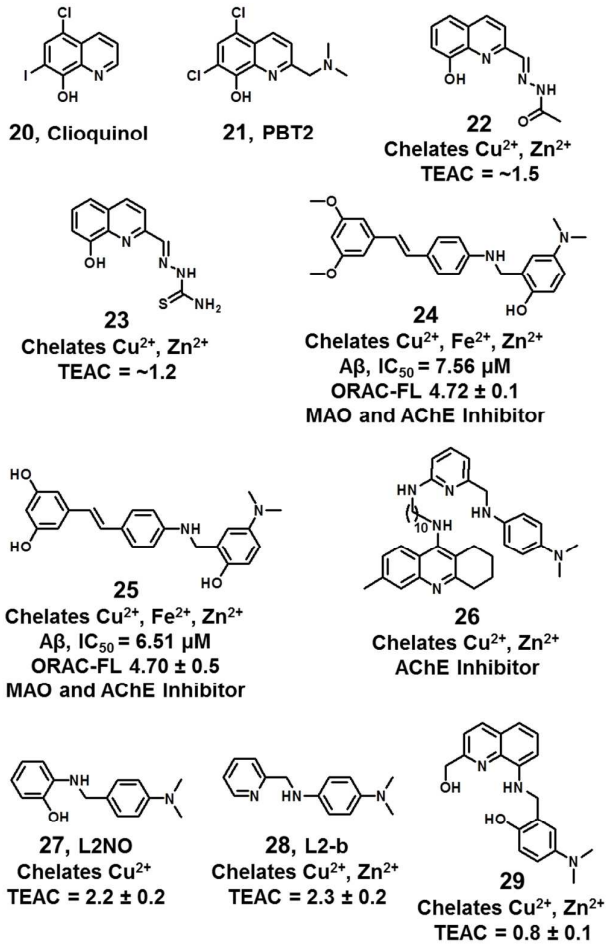

Fig. 8 Metal chelators for inhibiting metal guided Aβ aggregation and toxicity.

Enzyme inhibitors

There are large number of pathways guided by enzymes that affect the production and clearance of Aβ. In this section we focus on enzyme inhibitors of β-secretase and γ-secretase which are involved in APP processing to produce Aβ.

β-Secretase inhibitors. BACE1 (beta-site APP-cleaving enzyme 1) is a 501 amino acid type-I transmembrane aspartic protease. It controls nerve axonal myelination and muscle spindle

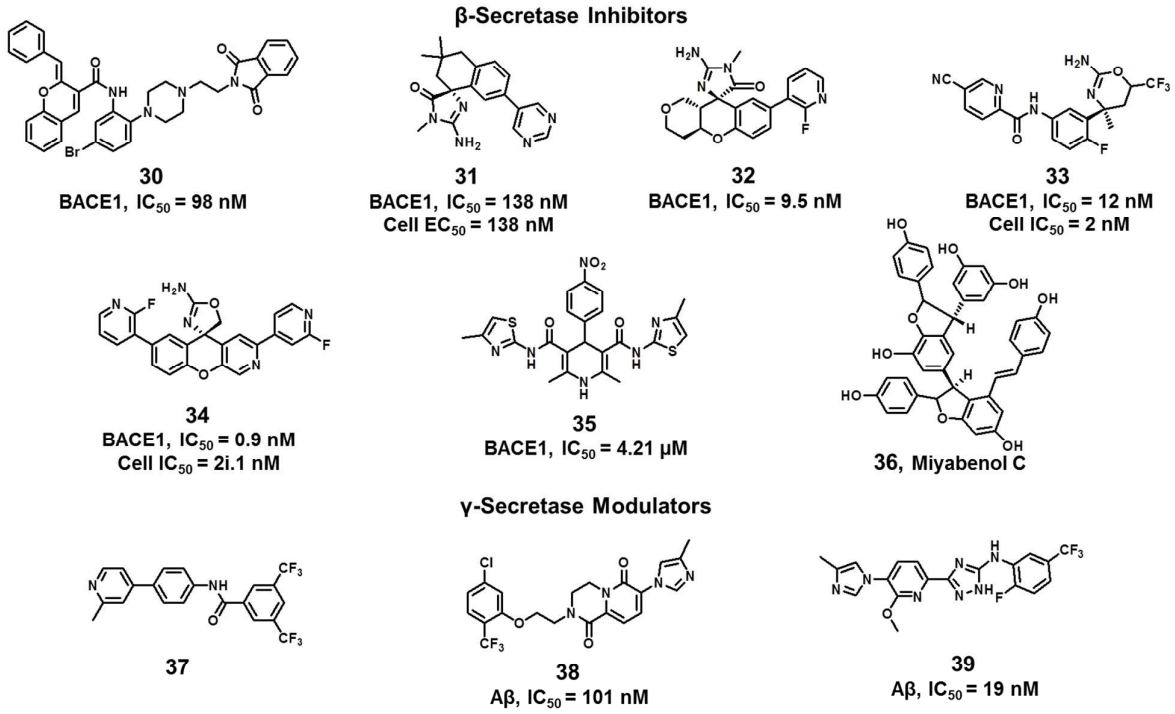

Fig. 9 α and β-Secretase inhibitors.

formation via proteolytic processing of neuregulin 1. BACE1 cleavage of APP mostly occurs in endosomes with optimum protease activity at lower pH (pH = 5).[129] *In vivo* (mice) testing of β-secretase inhibitors (BSIs) have shown least effect on normal physiological fuctions implicating that BSIs use may have minimum side effects. In recent times, LY2886721, MK-8931 and E-2609 have completed phase 1 clinical trials and entered phase II trials.[130,131] This promising approach inspires further efforts to develop efficient BSIs. A series of molecules containing 4-bromophenyl piperazine coupled to phenylimino-2H-chrome-3-carboxamide moiety were designed based on docking studies of the lead compound. The study showed π-π interaction of BSIs with side chain of F108 of the flap pocket. Further, modification of piperazine ring of the lead compound with series of heteroaromatic moieties was found to enhance their interaction with G34, G230, T231 or T232.[132] Molecule **30** (BACE1, IC_{50} = 98 nM) displayed remarkable inhibition property due to hydrogen bond interaction between piperazine N4, phthalimide (C=O) and D228, T232, R235, G230 sites, respectively (Fig.9). Hunt *et al*. have designed a spirocyclin-based efficient and selective inhibitor (R)-50 (**31**) (BACE1, IC_{50} = 138 nM, cell IC_{50} = 53 nM).[133] *In vivo* studies in different pharmacological models of rat, guinea pig and monkey showed a >50% decrease in CSF Aβ40 levels. Similarly, spirocyclic acyl guanidine based molecules were designed and screened for inhibition of BACE1 activity. The lead compound **32** with 2-fluoropyridin-3-yl as a substituent showed > 50% reduction in the Aβ levels in *in vivo* studies as a consequence of inhibition of BACE1 activity (IC_{50} = 9.5 nM).[134]

1,3-Oxazine is a moderate BSI which showed enhanced BACE1 inhibition once derivatised with fluorine moiety. Further, Hilbert *et al*. substituted oxazine with a CF_3 to obtain an efficient inhibitor (**33**, hBACE1, IC_{50} = 12 nM and cell IC_{50} = 2 nM) with high BBB crossing ability.[135] The inhibitor was able to reduce the Aβ40 and Aβ42 expression levels in a rat model at a low dosage of 1 mg/kg with a long-lasting inhibition effect. 2-Aminoxazoline is a promising BACE1 inhibitor shown to reduce Aβ levels *in vivo;* however it's cross-binding to hERG channels cause adverse effects by QTc elongation. To overcome this problem substitution was performed on aminoxazoline xanthine to obtain a efficient molecule (**34**, BACE1, IC_{50} = 0.9 nM and cell IC_{50} = 21.1 nM). **34** showed reduced hERG binding affinity, improved permeability and reduced levels of Aβ *in vivo* (CSF and brain).[136,137] Miri *et al*. designed a series of molecules based on 3,5-*bis*-N (aryl/heteroaryl) carbamoyl-4-aryl-1,4-dihydro pyridine to obtain a BSI (**35**, IC_{50} = 4.21 μM) with negligible calcium channel blocking affinity.[138] Molecular docking and DFT *ab initio* studies demonstrated the important role of two carbonyl and amide NH groups of the inhibitor in forming key hydrogen bonds with D228, G230, R235 and T232 residues, and hydrophobic interaction with V332. The natural product miyabenol C (**36**) a resveratrol trimer has showed selective and effective inhibition of BACE1 *in vitro* (N2a695 cells, N2a cells stably expressing human APP695) and have also shown reduced and enhanced levels of Aβ and sαAPP, respectively in *in vivo* (APP/PS1 mice) model.[139]

γ-Secretase inhibitors. γ-Secretase is an intramembrane aspartyl protease composed of the subunits presenilins (PS),

nicastrin, anterior pharynx defective 1 (APH-1), and presenilin enhancer 2 (PEN-2). Mutations in PS account for overproduction of amyloidogenic Aβ42 and the majority of inherited forms responsible for early onset of AD. The role of γ-secretase in sequential cleavage of APP in the production of Aβ makes it an appealing therapeutic target for AD. Direct use of γ-secretase inhibitors has shown detrimental side effects as the enzyme is involved in many other critical functions like lymphocyte development and cell differentiation, among others. The concept of γ-secretase modulators (GSMs) was introduced to selectively modulate the APP cleaving site and prevent the production of neurotoxic Aβ42 while still maintaining normal Aβ40 concentration in the brain.[22] Wood *et al.* performed high throughput screening to obtain sulfonamide based molecules as a novel GSM scaffold. Further, they performed structural optimization on the lead molecule to obtain a better modulator (**37**).[140] **37** showed improved cell potency, enhanced PKDM (pharmacokinetics and drug metabolism) and reduced Aβ42 (IC_{50} = 0.26 ± 0.10 μM) production in an *in vivo* model. Recently, Pettersson *et al*. introduced pyridopiperazine-1,6-dione ring into their previously designed GSM to enhance its ADME (absorption, distribution, metabolism and excretion) parameters like clearance, permeability and MDR efflux ratio.[141] The outcome of such modification was molecule **38** which has shown reduced Aβ42 levels (IC_{50} = 101 nM) in guinea pig at 30 mg/kg dosed orally and this activity was linked to its binding to presenilin N-terminal fragment of γ-secretase complex.[142] Based on a less potent GSM core structure an efficient anilinotriazole (**39**) GSM was optimized through varying spacer link between the triazole ring and substituted aromatic ring. **39** displayed enhanced efficiency in reducing the Aβ42 levels (IC_{50} = 19 nM) in both *in vitro* and *in vivo* models and had a superior ADME profile compared to its lead moiety.[143] Most of the recent works focus on designing GSMs, but more efforts are needed to understand their mechanism of action on γ-secretase that will provide us with essential information for designing efficient GSMs.

Immunotherapy

Immunotherapy is a therapeutic tool where antibodies are raised in a living organism against a specific antigen. Antibodies are very specific and selective toward their targets making them efficient therapeutic tools for treating many pathological diseases. Most Aβ-directed immunotherapies are based on non-selective antibody that can bind to different forms of Aβ (monomers, oligomers and fibrils). Immunotherapy in AD patients is constrained by its poor brain penetration levels and non-specific binding to Aβ monomers and fibrils (high concentration in AD patient) over low concentration oligomers which are relevant in inducing toxicity.[18] Immunotherapy becomes more relevant when oligomer-specific antibody is developed. Recently, Krafft *et al*. reported a selective, high affinity humanized antibody engineered into an IgG2 (ACU-193) for Aβ oligomeric (IC_{50} = 17 nM) species. ACU-193 showed dose-dependent pharmacokinetics, biodistribution and the brain penetration in various AD models.[144] Similarly Pradier *et al*. developed SAR228810 humanized antibody engineered into an IgG4 which bound higher molecular weight soluble Aβ oligomers and fibrils with high affinity over monomeric Aβ and lower molecular weight soluble Aβ oligomers.[145]

Aβ homeostasis

Aβ concentration in the normal brain is maintained by a regulatory pathway involving Apolipoprotein E (apoE), ABCA1, ABCG1, low density lipoprotein receptor-related protein-1 (LRP-1), P-glycoprotein (P-gp) and LRP-2. The APOE4 allele of apoE is the greatest genetic risk factor for AD compared to its other isoforms (APOE2 and APOE3).[19] In AD apoE/Aβ complex forming probability is reduced, thus enhancing the concentration of Aβ in the brain. The apoE4 is less lipidated which results in reduced stability and lower levels of apoE4/Aβ complex and causes increased oligomeric Aβ levels. Thus, increasing the lipidation of apoE may reduce Aβ.[146] The apoE expression is transcriptionally regulated by peroxisome proliferator–activated receptor gamma (PPARγ) and liver X receptors (LXRs) which form heterodimers with retinoid X receptors (RXRs). Landreth *et al*. used bexarotene as the agonist for RXRs and observed the over-expression of apoE, ABCA1, ABCG1 (lipid transporters) and reduction in Aβ levels (>50%), leading to rapid improvement in cognitive, social, and olfactory deficits in a mice model.[147] Recently, Holdzman *et al*. proposed that Aβ clearance by apoE was mediated through LRP1 and other interacting receptors or transporters but not completely through direct binding to Aβ. Neprilysin (NEP) and neprilysin 2 (NEP2) are endopeptidases that are involved in clearing Aβ by peptidase activity, and increasing expression of these peptidases using virus transfection can reduce Aβ load in the brain.[148] Minami *et al*. pursued lentivirus-mediated over-expression of progranulin (PGRN) protein (involved in neurotrophic and inflammatory processes) and lowered Aβ plaque load in AD mice.[149] The authors speculated that PGRN increases phagocytosis of Aβ aggregates and also activates various pathways which are involved in reducing Aβ load and its toxicity. Hippocampal PGRN showed a dose-dependent inhibitory effect of PGRN on plaque deposition. Further, inhibition of RhoA, a Rho GTPase family member is involved in modulating Aβ production. Principal downstream effectors of RhoA are protein kinase ROCK1 and ROCK2. Recent studies show that knockdown of ROCKI (increased) and ROCK2 (decreased) showed variation in Aβ levels in the mice brain. Inhibition of ROCK2 using small molecule SR3677 suppressed β-site APP cleaving enzyme 1 (BACE1) enzymatic action and diminished production of Aβ in AD mouse brain.[150] These recent studies indicate that manipulating Aβ clearing pathways can also be a useful strategy in reducing the Aβ levels and its toxicity.[151]

Autophagy activators

Autophagy is an intracellular degradation pathway involved in clearance of damaged organelles, misfolded proteins and recycling of cytosolic components during starvation condition.[152] In AD defective delivery of autophagosomes to

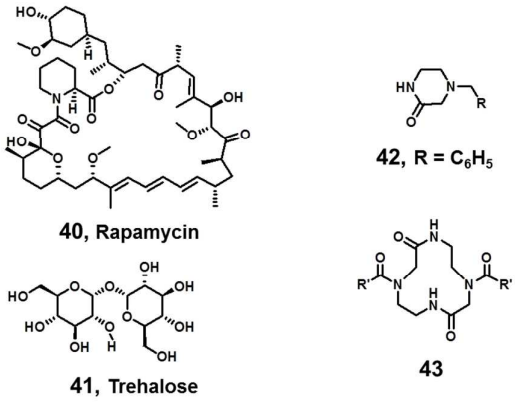

Fig. 10 Autophagy activators.

lysosomes has been observed indicating down-regulation of autophagy.[153,154] Galvan *et al*. showed that inhibition of mTOR (regulate autophagy) by rapamycin (**40**) prevented AD-like cognitive deficits and lowered levels of Aβ42 in the PDAPP transgenic mouse model. These data indicate that inhibition of mTOR pathway may reduce Aβ42 levels and hence, is a therapeutic target for AD (Fig. 10).[155] Trehalose (**41**), a natural alpha-linked disaccharide has showed improvement in cognitive and learning ability, and Aβ deposit in hippocampus was reduced through upregulation of the autophagy process.[156] Glycogen synthase kinase-3 (GSK) impairs lysosomal acidification and hinders autophagy. Treatment with L803-mts a GSK inhibitor restored lysosomal acidification in 5XFAD mice brains. Inhibition of GSK enables clearance of Aβ and reactivation of mTOR.[157] Recently, we have designed a series of six (**42**) and twelve (**43**) membered cyclic hybrid peptoids, of which molecule 4a (**42**) showed enhanced autophagic degradation of cargo in a live yeast cell model.[158] Such upregulation of autophagy using small molecules is a promising approach for the elimination of misfolded protein aggregates. Therefore designing molecules that can upregulate autophagy for degrading deleterious Aβ aggregates is a useful and promising therapeutic strategy.

Conclusions

The critical role of Aβ aggregates in AD has generated an immense interest in the scientific community to understand its role in neurodegeneration. In this Feature Article, we highlighted recent research activities and outcomes on various facets of Aβ concerning its structural aspects, mechanism of aggregation, positive physiological function, toxicity induced by its aggregates and its significance as a therapeutic target in AD. Alzheimer's patients show a similar type of Aβ aggregates throughout the brain but possess dissimilarities within *in vitro* aggregates, a fact which indicates that Aβ aggregation is a very complex process and that it's *in vivo* mechanism is different from *in vitro* observations. Therefore, further efforts are required to understand the structure and mechanism of Aβ aggregation in the brain. Secondary nucleation is highly interesting phenomena that might help in understanding the mechanism of oligomers formation, their relation with Aβ fibrils and therefore targeting secondary nucleation mechanism could be one of the effective therapeutic strategies. Although Aβ is considered toxic, there is always a certain concentration of Aβ peptide (picomolar) sustained in the brain and CSF supporting the fact that Aβ has a positive effect on different regulatory aspects of neuronal function. Aβ oligomers are considered to play a crucial role in the pathogenesis of AD by inducing oxidative stress, synaptic dysfunction, cell membrane disruption, mitochondria dysregulation and apoptosis. Understanding the exact mechanism of toxicity induced by either oligomers or fibrillar aggregates or both together is still a matter of debate. Several hypotheses have been proposed in current literature all of which concur that in some way or the other Aβ aggregation plays an important role in AD. Therefore modulation of Aβ aggregation using peptides and small molecule-based inhibitors is a promising approach. Peptide modulators are selective, specific and moderately efficient in inhibiting Aβ aggregation but low BBB permeability and bioavailability has hindered their use as therapeutics. Small molecules are ideal for modulating Aβ aggregation and BBB permeability but most of the reported molecules exhibit lack of selectivity towards Aβ aggregates. Therefore designing a hybrid of peptide mimics with small molecules can be considered as an efficient strategy as these modulators can selectively bind to the target, efficiently inhibit Aβ aggregation and also exhibit enhanced bioavailability. Using only metal chelators for sequestration of metal ions from metal-induced Aβ oligomers is not sufficient to reduce Aβ toxicity. Multifunctional ligands (MFLs) which can interfere with other aspects of aggregation in addition to metal chelation could be an interesting approach. Secretases are good targets and currently being pursued seriously but their adverse effects on physiological function may affect their success. Masking the cleavage site on APP where β- or γ-secretase act can be an effective strategy. Activating or overexpressing proteins (apoE, ABCA1, ABCG1 and LRP) or activating pathways (autophagy) that maintain Aβ concentration inside the brain are recent strategies which have created enormous interest in the scientific community. To date multiple inhibitors have entered clinical trials but with moderate to poor success rate. In fact most of the clinical trial results still remain inconclusive. Deciphering the basic mechanisms of action of anti-amyloid compounds remains to be studied in detail and the understanding of the exact interaction between inhibitors and amyloidogenic proteins will be of critical importance. Yet more important is the potential to develop new therapies, as most of the current therapeutic strategies have failed to live up to the expectation. Characterizing the cytotoxicity pathways is another significant challenge in this field and an improved understanding of these will be of critical importance for optimizing the therapeutic action of inhibitors of amyloid formation.

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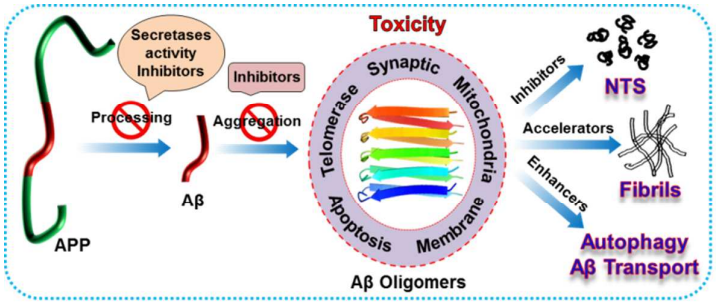

Our Feature Article detail the physiological role of amyloid beta (Aβ), elaborate its toxic effects and outline therapeutic molecules designed in the last two years targeting different aspects of Aβ for preventing AD.