Review Article

Toxic species in amyloid disorders: Oligomers or mature fibrils

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Abstract

Protein aggregation is the hallmark of several neurodegenerative disorders. These protein aggregation (fibrillization) disorders are also known as amyloid disorders. The mechanism of protein aggregation involves conformation switch of the native protein, oligomer formation leading to protofibrils and finally mature fibrils. Mature fibrils have long been considered as the cause of disease pathogenesis; however, recent evidences suggest oligomeric intermediates formed during fibrillization to be toxic. In this review, we have tried to address the ongoing debate for these toxic amyloid species. We did an extensive literature search and collated information from Pubmed (http://www.ncbi.nlm.nih.gov) and Google search using various permutations and combinations of the following keywords: Neurodegeneration, amyloid disorders, protein aggregation, fibrils, oligomers, toxicity, Alzheimer's Disease, Parkinson's Disease. We describe different instances showing the toxicity of mature fibrils as well as oligomers in Alzheimer's Disease and Parkinson's Disease. Distinct structural framework and morphology of amyloid oligomers suggests difference in toxic effect between oligomers and fibrils. We highlight the difference in structure and proposed toxicity pathways for fibrils and oligomers. We also highlight the evidences indicating that intermediary oligomeric species can act as potential diagnostic biomarker. Since the formation of these toxic species follow a common structural switch among various amyloid disorders, the protein aggregation events can be targeted for developing broad-range therapeutics. The therapeutic trials based on the understanding of different protein conformers (monomers, oligomers, protofibrils and fibrils) in amyloid cascade are also described.

Key Words

Amyloid disorders, fibrils, neurodegenerative disorders, oligomers, protein aggregation

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Introduction

Protein misfolding and aberrant self-assembly in an infinitely propagating fashion-forming large molecular weight aggregates is a key pathognomonic feature of various seemingly unrelated neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington disease (HD) and Prion disease.[1] The accumulation of these insoluble fibrous protein aggregates termed as amyloid in various organs/tissues causes their degeneration and loss of function.

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The fibrillar deposits are commonly known as amyloid plaques or neurofibrillary tangles in AD, Lewy bodies in PD, and nuclear inclusions in HD. The aggregating proteins are different in different neurodegenerative disorders. Further, the occurrence of these diseases can be sporadic or hereditary and the aggregates can localize intracellularly or extracellularly [Table 1]. For past many decades, the field of amyloid disorders has been a major concern in medicine as these are progressive disorders and result in irreversible neurodegeneration. The amyloids demonstrated as anomalous tissue deposits, were initially misidentified as starch but later determined to be composed of protein.[2] Histologically, these deposits exhibit affinity for Congo red dye with concomitant apple-green birefringence under plane polarized light,[3] which has long been considered as a gold standard in diagnosis of amyloid disorders. In the mid-20th century, with the advancement in high resolution structural techniques such as electron microscopy, X-ray diffraction, NMR (nuclear magnetic resonance) it became evident that the amyloid deposits consists of bundles of unbranched fibrils[4] and the constituent proteins were rich in highly ordered cross-β structures with β-strands

running perpendicular to fibrillar axis.[5-7] These fibrils have an indefinite length and diameters of ~2-20 nm. Moreover, these β-sheets have different arrangements in different amyloid proteins. For instance, parallel in-register β-sheets as in α-synuclein, β-microglobulin, β-amyloid ($A\beta_{1-40}$ and $A\beta_{1-42}$), amylin, human prion protein[8-12] and antiparallel β-sheets as in $A\beta_{16-22}$ and $A\beta_{11-25}$ fragments, C terminus of $A\beta_{34-42}$, amylin and huntingtin.[13-15] X-ray studies have revealed that mature fibrils have 'steric zipper structure' formed by interlocking of emanating side chains, like the teeth of a zipper.[16]

Protein Aggregation Model

Although various triggering factors (including increased temperature, mechanical stress, extreme pH, oxidative stress, glycation or mutation) have been implicated for protein misfolding, the basic mechanism of fibril formation is same in all amyloid diseases as shown in Figure 1. According to the fibril formation model, native proteins misfold and undergo conformational change. When these misfolded proteins reach a critical concentration, oligomers are formed that result in protofibrils and finally culminate in mature fibrils [Figure 1a]. The oligomeric intermediates are referred to as prefibrillar or on-pathway oligomers. In addition to these prefibrillar oligomers, off-pathway oligomers are formed which do not end up in fibrils [Figure 1b]. Further, the mature fibrils can shear or fragment to form fibrillar oligomers (originated from fibrils) that can again aggregate to form mature fibrils [Figure 1c]. The molecular structure of these transient species is not clearly defined but they exhibit spherical or annular morphology where annular oligomers are doughnut or ring shaped assemblies.[17] In addition, FTIR (fourier-transform infrared spectroscopy) and NMR studies showed that oligomers lack the parallel in-register β-sheet structure present in fibrillar form but they contain the β-loop-β secondary and tertiary folds as in fibrils.[18-20] However, these oligomers formed by various amyloid proteins (associated with different neurodegenerative disorders) have common immunological epitopes that are recognized by conformational antibodies, A11 (spherical) and αAPF (annular). Interestingly, A11 and αAPF antibodies do not bind to mature fibrils or oligomers formed by fragmentation of fibrils/protofibrils (fibrillar oligomers).[17,21] The mature fibrils and the fibrillar oligomers share generic epitopes that are recognized by another conformational antibody OC. OC antibody does not bind prefibrillar oligomers suggesting that prefibrillar and fibrillar oligomers are structurally different.[22]

Recently, a new dimension has been added to this already existing fibrillization model. Cohen *et al.*, 2013 have *in vitro* demonstrated secondary nucleation model for fibril formation using $A\beta_{42}$ peptide. This model suggests that mature fibrils catalyze the secondary nucleation reaction by forming diffusible oligomers from the monomers [Figure 1d]. This reaction depends on the concentration of both native protein (monomers) and fibrils unlike the primary nucleation reaction which depends only on protein monomer concentration.[23]

Toxicity of Protein Aggregation

According to the original amyloid cascade hypothesis (ACH) proposed in 1991, mutations in three genes: APP, Presenilin 1 and 2 were thought to be the initiating events in the abnormal accumulation of Aβ peptides (mature fibrils) leading to cell death and dementia in AD. Earlier researchers used to believe that large fibril deposits were the primary cause of neuronal damage and disease pathogenesis. Since protein aggregation

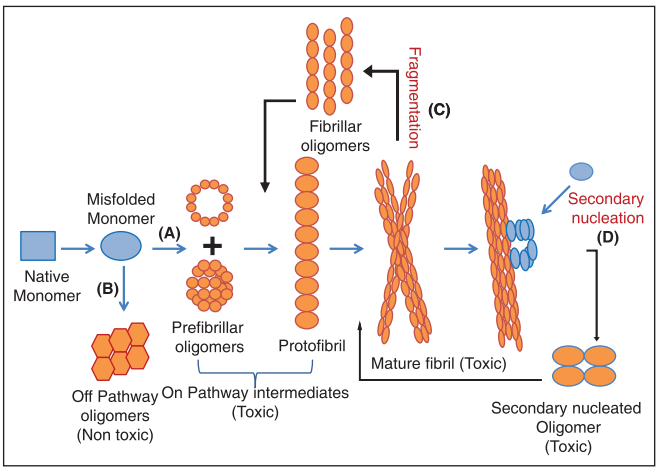

Figure 1: Schematic illustration of protein aggregation in amyloid disorders. Four different aggregation pathways are described here. (a) Native proteins misfold and undergo conformational change to form protofibrils and mature fibrils, (b) Misfolded monomers form off-pathway oligomers which do not end up in fibrils, (c) Mature fibrils undergo shearing or fragmentation to form fibrillar oligomers (originated from fibrils) that again aggregate to form mature fibrils, (d) Mature fibrils act as a template for oligomerization and catalyzes the secondary nucleation reaction by forming diffusible oligomers from the monomers

Table 1: Proteins causing amyloid diseases and location of filamentous lesions

Disease	Aggregating proteins	Location	Affected tissues (region)
Alzheimer's disease (AD)	β-Amyloid (amyloid plaques), Tau (neurofibrillary tangles)	Extracellular Intracytoplasmic intraneuronal	Brain (Cerebral cortex)
Huntington disease (HD)	Huntingtin	Intranuclear neuronal	Whole Brain most vulnerable part is neostriatum)
Spinocerebral ataxia (SCA)	Ataxin	Intranuclear neuronal	Brain (Cerebellum and Spinocerebellar)
Prion diseases	Prion	Extracellular	Brain (grey matter) and Peripheral nervous system
Transthyretin amyloidosis	Transthyretin	Extracellular/Intracellular	Peripheral nervous system, heart, kidney, eye
Parkinson's Disease (PD)	α-Synuclein	Intracytoplasmic	Brain (Substantia nigra, Brain stem)
Amyotrophic Lateral Sclerosis (ALS)	SOD1, FUS, TDP-43, ubiquitin positive proteins	Intraneuronal	Motor neurons

is a dynamic process and involves formation of transient intermediate structures as well as dissociation of mature fibrils, it has been challenging to define the real culprit leading to toxicity. However over the years, no linear correlation between dementia/cognitive decline and Aβ deposition in the brain was observed. Further, increasing number of recent studies suggests intermediate oligomers formed during fibrillization to be more toxic species than mature fibrils. Instead in some instances, formation of mature fibrils has been proposed to be protective by acting as harmless reservoir of toxic oligomers. Hence, the original ACH has been modified to include the intermediary species formed during aggregation/fibrillization process and is commonly known as oligomer hypothesis. In the following sections, we intent to review the ongoing debate of toxicity demonstrated by fibrils and oligomers and define the current status of amyloid pathology.

Toxicity of Oligomers versus Mature Fibrils

Presence of amyloid plaque rich in Aβ fibrils in brains of AD patients supported the close link of protein aggregation with neurodegeneration. Initial studies postulated amyloid fibrils to be the primary cause of cell death and disease pathogenesis. It was demonstrated by independent approaches that application of extracellular Aβ fibrils induced AD-like changes. In cultured neurons, Aβ fibrils increased frequency of action potentials and membrane depolarization and reduced the cell viability.[24] Injection of Aβ fibrils in primary hippocampal neurons in rat-impaired synaptic transmission, induced cognitive/memory decline and caused neuronal cell death.[25] However, there have been conflicting reports to define the correlation between amyloid plaque burden and severity of neuronal loss and other AD-symptoms. Hey *et al*., 2012 demonstrated that oligomeric Aβ infused into left ventricles of brain showed significant impairment of learning and memory functions while there was no significant effect observed due to fibrils.[26] Moreover, in a cell culture study Aβ oligomers were found to suppress synaptic plasticity by specifically inhibiting presynaptic P/Q calcium currents unlike protein monomers and fibrils.[27] Most of the earlier transgenic AD animal models exhibited plaque formation as the dominant pathology feature of AD with little neurotoxicity but a very recent AD transgenic mice supports that neurotoxicity is due to Aβ oligomers (oligomer hypothesis). In this transgenic mouse with mutation in presenilin gene ($PS1_{V97L}$-Tg), Aβ oligomers accumulated in the neurons and exhibited memory and synaptic dysfunction and tau hyperphosphorylation. No extracellular amyloid plaques were observed in this transgenic mice.[28] Interestingly, Aβ oligomers extracted from the cerebral cortex of AD patients when administered causes memory deficits and disrupts synaptic plasticity in normal rat.[29] Later on, during the analysis of fractionated brain homogenate from patients with AD it was observed that levels of Aβ oligomers is associated with the loss of synaptic markers such as the synaptic vesicle protein VAMP2, and the post-synaptic protein PSD95.[30,31]

In case of PD, where α-synuclein (α-syn, the protein associated with PD) is considered as a major component of protein inclusions (Lewy bodies or Lewy neurites), a detailed attempt has been made to study the process of aggregate formation for identifying the toxic species responsible for neuronal dysfunction and cell death. Specific α-synucleinopathies were achieved in transgenic mouse models by expressing human wild type and mutant α-syn in brain regions. The mutant α-syn form inclusions containing fibrils showed behavioral impairment in these transgenic models such as locomotor dysfunction and cognitive decline leading to death.[32,33] Later, it has been shown that α-syn fibrils decrease cell survival in cell culture system by inducing the activation of caspase-3 pathway leading to apoptotic cell death.[34] In yet other studies, soluble oligomeric α-syn have been proposed as the main toxic species instead of α-syn fibrils. In an *in vivo* study using murine rat model, it was found that unlike fibrils, lentiviral injected α-syn oligomers increases loss of nigral tyrosine hydroxylase (TH)-positive neurons and showed strong reactivity with membrane, thus exhibiting its neuronal toxicity through membrane disruption.[35] Furthermore, overexpression of α-syn mutant that has high propensity to form oligomers but not fibrils, in three established PD models: Mammalian neurons, *Caenorhabditis elegans* and *Drosophila melanogaster* resulted in lower mitochondrial dehydrogenase activity and degeneration of dopaminergic and other neurons.[36] In this study, it was evident that α-syn variants with impaired ability to form fibril correlates better with increased toxicity and neurodegeneration in PD.

The reasons for the differential toxicity of amyloid oligomers and fibrils can be explained by their structural arrangements:

1. In oligomers hydrophobic surfaces are exposed in β-sheets while they are hidden inside the interacting stacks in fibrils[10]
2. Oligomers are smaller in size so can easily diffuse in tissues as compared to longer fibrils[37]
3. The more number of open active ends present in case of oligomers than fibrils allow improved interaction of oligomers with cellular targets.
4. Oligomers are highly unstable disordered structures whereas fibrils are stable organized molecules.

Although the toxicity studies reviewed here portrays only part of the advancing knowledge on the ongoing debate, it may be sufficient to clearly conclude that there is a link between protein aggregation and disease pathogenesis. However, there is a need of development of *in vivo* imaging techniques to dissect the process of protein aggregation and provide definite clinical correlation of disease stage or severity with functional proteins, oligomeric intermediates and mature fibrils.

Cellular Mechanism of Toxicity

The similarities in amyloid structures (oligomer and mature fibrils) of various amyloid proteins irrespective of its sequence, suggest a common shared mechanism of toxicity. Since amyloid proteins are cytosolic and/or extracellular, interaction of higher molecular weight amyloid structures with molecules on the plasma membrane can be the primary target for toxicity. There are two primary mechanisms proposed, which are proximal to the bifurcated downstream events in the causal cascade:

Membrane interaction

Mature fibrils interact with monosialotetrahexosylganglioside (GM1)-rich membrane domains whereas oligomeric species are proposed to interact with glutamatergic receptors, voltage-gated

channels and GM1-rich domains on the membrane to initiate the neurotoxic cascade.[38-40] $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides interact with GM1 and adopts an alternate conformation depending on the protein density.[41,42] Lower protein to ganglioside ratio prefers α-helix rich structure, whereas higher ratio facilitates β-sheet forms, which culminates into toxic fibrils. Various studies showed that during the accumulation of fibrils on GM1 cluster-membrane, no oligomers were detected, accounting that cell death is only due to fibril-induced disruption of cell membranes.[43,44] Instead, recent studies demonstrating interaction of neuritic cell membrane with $A\beta_{1-40}$ oligomers and fibrils, oligomers were observed to damage the lipid bilayer by stimulating phospholipid composition and negative net charge of the membranes.[45] α-synuclein oligomers were shown to interact with large and small unilamellar negatively charged vesicles on membrane and transiently altering the membrane permeability.[46]

Perturbation of calcium homeostasis

Calcium homeostasis perturbation was found to be a ubiquitous toxicity mechanism for soluble oligomers whereas no detectable effect was observed for fibrils.[47,48] There are conflicting reports about the mechanism by which calcium homeostasis gets disturb. (i) Amyloid ion channel formation: Demuro and many other researches tried to check whether this Ca^{2+} dysfunction is due to the pre-existing ion channel or oligomers creates altogether different channels in the membrane. Cobalt which is known to block the Ca^{2+} channel didn't affect the Ca^{2+} influx, supporting the new channel hypothesis.[48] (ii) Lipid bilayer conductance: In contrary to the channel hypothesis, Sokolov, in his study found that the increase in membrane conductance is due to the thinning of the lipid bilayer and lowering the dielectric barrier for ion translocation rather than channel formation.[49] Numerous studies have shown that amyloid oligomers increase the lipid bilayer conductance where as there was no observable effect on lipid bilayer in case of fibrils.[50,51]

Irrespective of the precise mechanism of membrane conductance, elevated intracellular Ca^{2+} levels is proposed as the central mechanism of toxicity since it regulates key downstream pathological events. Membrane permeabilization and calcium dyshomeostasis may initiate various downstream events including transmembrane signaling processes, mitochondrial dysfunction, reactive oxygen species production and apoptotic pathway leading to toxicity.[52-58] Both amyloid fibrils and oligomers stimulate these downstream cascades of events but with unique signaling responses. Oligomers stimulate inflammatory response through NF-κB and differentially activate microglia through increasing levels of phosphorylated Lyn and SyK kinase as well as p38 MAP kinase, whereas fibrils stimulate greater amount of Keratinocyte chemoattractant chemokine and active phosphorylated form of ERK (extracellular signal-regulated kinase).[26,59]

Intermediary Oligomers as Potential Biomarker

Although the debate between the correlation of oligomers or mature fibrils with disease severity in amyloid disorders is ongoing, Aβ and α-synuclein oligomer count in CSF (cerebrospinal fluid) or plasma has been shown to be potential diagnostic biomarker for AD and PD. In all these studies the oligomers were found to be elevated in the body fluid samples of patients as compared to controls.[60,61] Thus, the thrust is now towards the development of sensitive and specific assays to detect oligomeric amyloid species. Among the few assays developed, Aβ-PMCA (protein misfolding cyclic amplification assay) and monoclonal single antibody sandwich ELISA assay for Aβ and time-resolved Forster resonance energy transfer (TR-FRET)-based immunoassays for α-synuclein oligomer detection are most sensitive.[62-65] These oligomeric assays are specific and sensitive to Aβ oligomers and do not recognize APP (amyloid precursor protein), monomeric Aβ and other non-Aβ-peptide oligomers. Though, these studies suggest Aβ oligomer as a promising biomarker but the number of patient sample is small. Further research on larger cohorts using highly sensitivity and rigorous standard assays is needed before considering the analysis of oligomers as a routine diagnostic assay for the clinical evaluation. Moreover, there is also a need to develop similar oligomeric-based assays for other amyloid proteins.

Therapeutics Under Clinical Trials

At present, there are limited approved drugs for amyloid diseases. Moreover, effects and benefits of these drugs are mainly symptomatic and marginal rather than targeting the underlying cause of the amyloid formation. However, in recent years, the understanding of different protein conformers (monomers, oligomers, protofibrils and fibrils) in amyloid cascade has lead to development of new strategies for therapeutics and diagnostics.[66]

Broadly two major strategies are being explored for development of drugs for amyloid disorders-1) Small molecules as therapeutic agent: Numerous small molecules are being studied that can target different conformers in amyloid cascade and prevent amyloid formation [Table 2]. Their interventions can be at different steps of amyloid formation. a) Molecules that inhibit formation of oligomers or fibrils: EGCG, a flavonoid present in green tea is under phase 3 trials.[67] Recently, Keampferol-3-O-rhamnoside has been found as a promising molecule in abrogating Aβ toxicity by modulating monomers and remodeling oligomers and fibrils to non-toxic oligomers (Sharoar *et al*., 2012).[68] b) Molecules that destabilize oligomers and fibrils: Two proteases plasmin and cathepsin B destabilize Aβ oligomers and fibrils.[69,70] Rifampicin disaggregates the alpha-synuclein fibrils and inhibits their fibrillization.[71] Scyllo-inositol now in phase 2 trial, effectively impedes Aβ aggregation by accelerating aggregate dissociation. We have also recently shown that curcumin, a component in turmeric has the potential to modulate fibril formation as well as dissociate the preformed huntingtin (htt) aggregates in a unicellular eukaryote model organism.[72] 2) Immunotherapy: Therapeutic interventions based on both active (stimulation of immune response by injecting amyloid peptides) and passive (direct administration of anti-Aβ monoclonal antibodies) immunization are under different phases of clinical trial for many amyloid diseases. Here, we briefly summarize the outcomes of clinical trials for AD [Table 3]. Initial clinical trials for active immunization with full length Aβ vaccines were discontinued due to severe side effects. Smaller fragments of Aβ under trial appear to be more promising. CAD-106, Aβ 1-6

peptides has successfully completed the phase 2 trial for mild AD patients without any side effects.[73] Passive immunotherapy using antibodies against Aβ monomers, oligomers and fibrils are under trial and show promising results [Table 3]. Among

Table 2: Small molecules in clinical trials

Small molecules	Mode of action	Dementia stage	Trial status	Outcome
Tramiprosate	Aβ aggregate dissociation	Mild to Moderate	Phase 3, terminated	poor CNS penetration and the weak potency
Scyllo-inositol	Aβ aggregate dissociation	Mild to Moderate	Phase 2, complete	No report
Epigallocatechin-3-gallate (EGCG)	Inhibiting fibrillization of Aβ, htt, α-syn	Early stage	Phase 3, ongoing	–
TRx0237 (methylene blue)	Tau aggregates dissociation	Mild to Moderate	Phase 3, ongoing	–
Curcumin, a natural polyphenol	Inhibit fibrillization, Aggregate dissociation	Mild to Moderate	Phase 2, complete	No reports
Valproate	Inhibit tau aggregation	Mild to moderate	Phase 3, complete	no effects on cognition and functional status
Davunetide	Inhibit tau aggregation	MCI	Phase 2, complete	Showed benefits
BMS-241027	Inhibit tau aggregation	Mild	Phase 1, complete	Safety profile

Table 3: Active and passive immunotherapies in different phases of Clinical trials

Therapeutic molecules	Details	Dementia stage	Trial status	Outcome of trial	Remarks
Active immunotherapy: Aβ peptide-antigens as the therapeutic agent					
AN1792	Full length $Aβ_{42}$ with QS-21 adjuvant, Presented as T-cell epitope	Mild to moderate	Phase 2a, complete	6% of patients developed meningo-encephalitis	Follow up patients showed reduced plaque density as well as reduced phosphorylated tau and cognitive score
Affitope AD02	Six amino acid sequence of N-terminus Aβ, Presented as B-cell epitope	Mild to moderate	Phase 2, ongoing	Not much effective	AD04, a placebo formulation of AD02 showing beneficial effects than AD02
CAD-106	Multiple copies of Aβ1- 6 expressed from the virus Qβ, Presented as B-cell epitope	Mild	Phase 2, complete	Safe and Effective	
UB 311	Aβ 1-14, Presented as B-cell epitopes	Mild to moderate	Phase 1, complete	Reduced plaque burden and improved cognitive decline	
V950	Aβ N-terminal conjugated to ISCO-MATRIX (Aluminum containing adjuvant), Presented as B-cell epitopes	Mild to moderate	Phase 1, complete	Favorable safety profile	
ACC-001 (Vanutide cridificar)	Aβ 1-7 conjugated to inactivated diphtheria toxin	Mild to moderate	Phase 2, complete	Discontinued due to adverse effects	Sequel to famous AN-1792 Vaccine Trial
Passive immunotherapy: Antibodies against Aβ epitopes as therapeutic agent					
Bapineuzumab (AAB-001)	Aβ 1-5 epitope Binds fibrils/ plaques	Mild to moderate	Phase 3, complete	Vasogenic cerebral edema and microhemorrhage	Side effects mainly in ApoEε4 carriers patients
Solanezumab	Aβ 16-24 epitope Binds monomer and oligomers	Mild	Phase 3, ongoing	Slowed cognitive decline	ApoE4 allele carriers and non-carriers
Gantenerumab	Aβ 1-11 Binds plaques	Prodromal* and Mild to moderate	Phase 3, ongoing	No report till date	
Crenezumab	Aβ 12-23 Binds monomer, Oligomer and fibrils	Mild to moderate	Phase 2, ongoing	No report till date	
Ponezumab	Aβ 33-40 Binds monomer and plaques	Mild to moderate	Phase 2, Discontinued	Adequate safety profile but no effect on the Aβ burden	In phase 2 trial for cerebral amyloid angiopathy (CAA)
BAN2401	mAb against Aβ, Binds protofibrils	MCI/ Mild to moderate	Phase 2; ongoing	No report till date	
BIIB037	mAb against Aβ, Binds fibrils	Prodromal and mild	Phase 1, ongoing	No report till date	

these, Solanezumab targeting Aβ oligomer and Gantenerumab against Aβ fibrils/ plaques are now under phase 3 trial.[74,75] Since in AD pathophysiology both Aβ and tau proteins are involved, combined antibody (IVIG) therapy targeting these amyloid proteins is also under trial.[76]

Concluding Remarks

As the average life span has increased globally, the probability of getting age-related neurodegenerative diseases, including amyloid disorders has increased. Amyloid disorders are a major concern as they cause irreversible degeneration and are usually fatal. Despite complexity of the process of protein aggregation, the progress made in the field of amyloid biology in the past few years is really commendable. Today, we understand the mechanistic and structural aspect of protein aggregation disorders. This knowledge is being exploited to define the toxic species that can be clearly associated with disease pathogenesis. It is incredible how the transient oligomeric species formed during protein aggregation can be used as a biomarker. Highly specific and sensitive assays are being developed for the detection for amyloid oligomers from either CSF or plasma. This can significantly improve early diagnosis, which can lead to better management and treatment of the disease. Though further studies are warranted to establish the oligomeric species as the real culprit, tremendous efforts toward development of strategies to inhibit protein aggregation offers hope for cure for amyloid disorders in near future.

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