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Inhibition of protein aggregation and amyloid formation by small molecules

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For decades, drug after drug has failed to slow the progression of Alzheimer's disease in human trials. How compounds reducing fibril formation *in vitro* and toxicity in transgenic mice and flies bind to the Aβ toxic oligomers, is unknown. This account reviews recent drugs mainly targeting Aβ, how they were identified and report their successes from *in vitro* and *in vivo* experimental studies and their current status in clinical trials. We then focus on recent *in vitro* and simulation results on how inhibitors interact with Aβ monomers and oligomers, highly desirable knowledge for predicting new efficient drugs. We conclude with a perspective on the future of the inhibition of amyloid formation by small molecules.

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Introduction

Amyloid plaques are a central pathological feature of Alzheimer's disease (AD) and largely consist of Aβ peptides of 39–43 amino acids. Any step to interfere with the production of Aβ or its self-assembly is a potential treatment for preventing or delaying the onset of sporadic and familial AD [1]. The mechanisms by which the self-assembly of Aβ1-42 (DAEFRHDSGY^{10}EVHHQKLVF-F^{20}AEDVGSNKGA^{30}IIGLMVGGVV^{40}IA) leads to toxicity, are not well understood. This is a hard structural biology problem because all transient intermediates and oligomers preceding fibrils comprise a very large number of conformations [2•]. They also vary with peptide length, familial AD mutations, external conditions, sample preparations, and whether Aβs are synthetic or AD-brain derived, leading to polymorphisms from monomers to fibrils [3••]. Plaques also contain truncated Aβs, such as Aβ5-42 and Aβ1-26, post-translational modifications, such as N-terminal pyroglutamate, and Aβ can interact with many protein receptors [4••]. This review focuses on where we stand on the design of small compounds targeting mainly against AD. Bad clinical news from two recent antibodies can be found elsewhere [5].

In vitro and *in vivo* studies of amyloid binding molecules

Many molecules have been screened against Aβ aggregation and toxicity. The fragments 17–21 (Central Hydrophobic Core, CHC), 32–37 and 37–42 have been often used as starting points for developing inhibitors, since they are the β-strand-forming self-recognition elements (SREs), though the N-terminal has also been considered. The SREs can be modified to prevent self-aggregation and make them more drug-like, since L-peptides are hydrolyzed rapidly and too polar and flexible to make good drugs. Figure 1 shows the chemical structure of some inhibitors. They are presented, as they are first mentioned in the text. We found difficult to classify them by physical–chemical properties, inhibitory effects, or other properties.

D-Peptides

A major advantage of D-peptides instead of L-peptides is that they are much poorer substrates for proteolytic enzymes. Jagota designed D-(PGKLVYA) and D-(KKLVFFARRRRA) to inhibit Aβ aggregation and extend the lifetime of a transgenic *Caenorhabditis elegans* model expressing Aβ1-42 [6]. Computer aided design was also used to develop D-peptide inhibitors targeting tau [7]. None of these inhibitors have strong binding to their amyloid targets with binding constants of 1–10 μM.

Retro-inverso (RI) peptides

Using D-amino acids and flipping the NH and CO groups in peptide bonds retain the same spatial positioning of the side chains compared to L-peptides and preserve the desired 3D structure. RI-OR2 (H_2N-rGklvffGr-Ac), where lower case letters denote D-amino acids and all peptide bonds are flipped, retains the KLVFF SRE with terminal Arg to improve solubility and prevent self-aggregation [8]. Addition of the HIV protein transduction domain TAT to RI-OR2, to give RI-OR2-TAT (Ac-rGffvlkGrrrrqrrkkrGy-NH_2), facilitates crossing the blood–brain-barrier (BBB). RI-OR2-TAT reduces Aβ

aggregation and plaque levels, reduces activation of microglia and oxidative damage, and increases the number of young neurons in the dentate gyrus. Surface plasmon resonance reports a low K_d of 58–125 nM between RI-OR2-TAT and Aβ42 fibrils [9•].

N-methyl peptides

N-methylation of an SRE has one side able to bind to the target by hydrogen bonding (H-bond), while the other has an N-methyl in place of backbone H, thus preventing H-bonds. Selectively N-methylated soluble IAPP (islet amyloid polypeptide peptide) mimics nanomolar inhibitors of cytotoxic self-assembly of both IAPP and Aβ1-40 [10].

Molecular tweezers

CLR01 inhibits aggregation of Aβ, tau, α-synuclein, IAPP, transthyretin (TTR), a prion protein fragment, β_2-microglobulin, insulin and calcitonin. CLR01 binds to Lys16 in Aβ and sequesters it into non-toxic oligomers [11]. CLR01 preferentially binds to the Lys10 or Lys12 in α-synuclein (out of 15 Lys in total), favoring the monomeric state [12].

Alzhemed and Rember

Alzhemed was stopped after human trials and Methylene Blue variants [13•] are undergoing human trials.

SEN compounds

SEN304, D-[(chGly)-(Tyr)-(chGly)-(chGly)-(mLeu)]-NH_2 with one N-methylated residue, inhibits Aβ aggregation and toxicity. Biophysical assays, toxicity assays in cell culture (MTT and lactate dehydrogenase in human SH-SHY5Y cells, mouse neuronal cell death and synaptophysin) and long-term potentiation (LTP) showed SEN304 binds directly to Aβ1-42 and redirects toxic oligomers into non-toxic forms with different morphologies. SEN304 does not work in the manner that was expected (by blocking Aβ aggregation) — instead it induced aggregation, and removed toxic oligomers [14•]. SEN1269, based on RS-0406 and discovered by high-throughput screening was modified to give SEN1576, which can be given orally, binds to monomeric Aβ1-42, protects neuronal cells exposed to Aβ1-42, reduces deficits in LTP and improves behavior following injection of Aβ oligomers to normal rats [15].

Polyphenols

Numerous polyphenols, usually from natural products such as turmeric, grapes, red wine and green tea, have been reported to inhibit Aβ aggregation and toxicity and show promise *in vivo*, including curcumin [16], resveratrol [17] and ε-Viniferin glucoside (EVG) [18]. In particular, data from both epidemiology and animal studies suggest that drinking tea decreases the incidence of dementia and Parkinson's [19]. The major tea polyphenol, epigallocatechin gallate (EGCG), shows neuroprotective activity against neurological disorders [20], inhibits the Aβ [21] and α-synuclein oligomer toxicity [22••] and is undergoing phase II–III clinical tests.

Using virtual screening, 18,000 compounds of the CDS-Zinc bank and the Flat compound set were ranked according to their binding energies to Aβ16-21 fibril. BAF31 significantly reduces toxicity in cells by preventing fibril fragmentation [23••]. Simulations revealed however that inhibitors can block lateral association of layers [24], suggesting that BAF31 may also prevent protofibrils association. In addition, the complex and highly dynamical behavior of an inhibitor on human amylin fibrils was revealed using isotope-edited IR, indicating the limitations of the static amyloid fibrils/inhibitor structure [25••]. Finally, the mixture of the natural orcein compound with a substance, using a membrane-essay, accelerated Aβ1-42 fibrils but reduced toxicity [26].

Quinone derivatives

These are NQTrp, 1,4-naphthoquinon-2-yl-L-tryptophan, and variants. NQTrp reduces the aggregation of Aβ1-42 and toxicity toward a cultured neuronal cell line and a transgenic AD Drosophila model with a high molar ratio of NQTrp [27].

Other molecules

The α-sheet has all its peptide groups orientated in the same direction. Using molecular dynamics (MD) simulations, α1 and α3 of 23 and 21 amino acids, with alternating D-amino acids and L-amino acids, were designed to be a linear hairpin having high α-sheet and a cyclic amide backbone, respectively. Both peptides reduce Aβ1-42 and TTR aggregation and toxicity at molar ratio of at least 10:1, but crossing BBB is highly unlikely [28]. Pande [29] and Arai [30••] also designed two compounds.

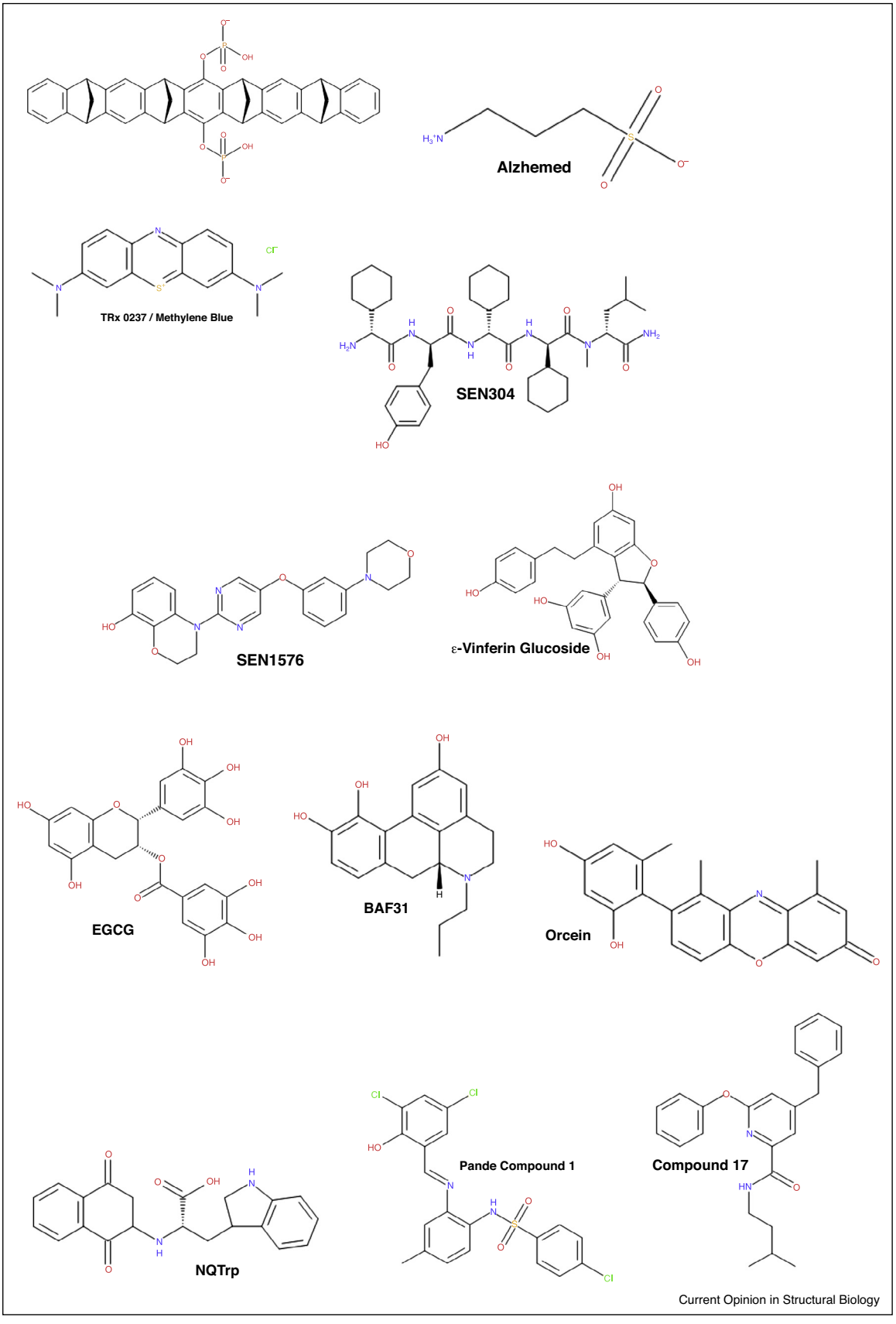
3D structures of drug/Aβ oligomers from *in vitro* and *in silico* experiments

Because Aβ or any amyloid peptide has a high propensity to associate/disassociate and explore a heterogeneous ensemble of conformations prior to primary nucleus formation, we only have low-resolution structural data on Aβ monomer and oligomers, with or without inhibitors.

Segal proposed a 3D structure of NQTrp/Aβ12-28 monomer based on NMR resonance shifts and medium-range NOEs [27]. With a higher molar ratio of EVG/Aβ1-40, the NMR spectrum in DMSO also showed a low number of medium NOEs [31]. In both systems the absence of long-range NOEs in Aβ and intermolecular NOEs indicates the very high conformational variability of Aβ monomer with a small molecule.

Using isothermal titration calorimetry (ISC) at different EGCG and salt concentrations, Sun showed the interactions between Aβ1-42 and EGCG are mainly H-bonds in the region 1–16 and hydrophobic in the region 17–42 [32].

Figure 1


Alzhemed
TRx 0237 / Methylene Blue
SEN304
SEN1576
ε-Vinferin Glucoside
EGCG
BAF31
Orcein
NQTrp
Pande Compound 1
Compound 17
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Experimentally, EGCG has been reported to generate unstructured, off-pathway Aβ1-42 oligomers [21]. By using NMR, electron microscopy and a 10-fold molar excess of EGCG, spherical EGCG-induced Aβ1-40 oligomers were identified with residues 1–20 disordered, a D23-K28 salt bridge and G29-V36 in a β-strand. EGCG interacts with CHC preventing the formation of β-strand at CHC [33•].

Replica exchange molecular dynamics (REMD) simulations of Aβ1-42 dimer with OPLS/TIP3P was performed in the absence and 5 five excess of EGCG as used experimentally starting from dispersed, random coil monomers [34]. Upon EGCG binding, the Aβ dimer collision cross-sections are increased by 8%, the β-content is reduced in 1–16, the CHC and 39–42, resulting in a less-fibrillar state, while β-strand formation in 30–36 is not impacted, consistent with NMR [33•]. EGCG is buried in the interface between Aβ peptides and binds to the hydrophobic CHC and C-terminal regions and to the N-terminal amino acids through H-bonds, consistent with ITC experiments [32]. Despite a molar ratio of 2:10, there is, however at 300 K, 5% of Aβ1-42 monomers free of any interaction with EGCG [34].

Free monomers have been discussed in many simulations using a molar ratio of 1:1. These include REMD of Aβ1-28 monomer (population of 20–25%) [35•] and dimer (10%, in preparation) with NQTrp in explicit solvent. Two MD in implicit solvent showed NQTrp interacts 71% of the time with Aβ12-28 monomer at a concentration of 2.5 mM [36], and carnosine (β-alanyl-L-histidine) was in contact with Aβ1-42 monomer for only 20% of the time (and 10% with Aβ1-28) at a concentration of 5 mM [37].

A common feature of all simulations appears to be an increase in α-helix and decrease in β-strand at the CHC upon inhibitor binding [35•]. The structural differences between Aβ and Aβ/inhibitor emphasize the limitations of simulations of amyloid systems followed by docking of an inhibitor to determine its real binding sites even if the identified hot spots are somewhat consistent with what we expect from intuitive arguments. Using REMD of Aβ1-42 monomer in water, fragment mapping calculations and docking, Zhu identified potential binding pockets for curcumin and Congo Red consisting of CHC, but with F4/I31/M35 and hydrophilic residues [38]. REMD of Aβ17-42 trimer followed by docking shows that curcumin, EGCG, 2002-H20, resveratrol and NQTrp drugs often bind to the side chains of F19/F20 and the main chain atoms of F19-E22, but other residues contribute [39].

Compounds interacting with REMD-predicted dimer or trimer structures have a much higher binding affinity than for the same oligomers in the fibril state [39]. Drugs interacting with Aβ monomer, dimer and trimer show many binding sites with small occupancies and contact surfaces. For example, Zhu suggests 35 binding pockets for Aβ1-42 monomer/curcumin [38]. Zhang suggests 100 pockets for Aβ1-42 dimer/2NQTrp and even within a transient pocket there are multiple binding modes [40•]. While the Aβ12-28 or Aβ16-21/22 fragments can rank compounds, results are not transposable to Aβ40/42 because both the N-terminal and C-terminal contribute to binding. It is also necessary that the simulations use physiological conditions because salt concentration changes the population of interactions between charged residues.

Conclusions

Most compounds designed as 'aggregation/toxicity inhibitors' work by accelerating aggregation, removing toxic oligomers or reducing fibril fragmentation, but it not always clear at which stages of oligomerization they are effective. Each of these compounds may act to lower adverse consequences of Aβ deposition, by decreasing the concentration of Aβ oligomers or by rendering them less toxic, perhaps by blocking a binding site. Binding constants for aggregation inhibitors are generally quite weak and it is challenging to get a K_d lower than the Aβ concentration. Blocking the transition from monomer to dimers seems out of reach using small compounds owing to the intrinsic disorder of Aβ. Oligomers are probably the best targets for a drug, but there are many different

(Figure 1 Legend continued) Chemical structures of inhibitors. We show the molecular tweezer CLR01, the Alzhemed and Trx 0237 compounds, SEN304, SEN1576, EVG, EGCG, BAF31, orcein, NQTrp, Pande compound **1** (4-chloro-*N*-[2-[(E)-(3,5-dichloro-2-hydroxyphenyl)methylidene-amino]-4methylphenyl]benzene sulfonamide), and the non-peptidic compound 17 designed by Anai. Molecular tweezers are designed to bind tightly to a Lys side chain, by wrapping aromatic rings around the methylene groups of Lys and forming electrostatic interactions to the terminal amine. Papers on the efficacy of Methylene Blue are sparse and report mixed results. TRx 0237 (LMTX™) is a second-generation tau protein aggregation inhibitor. It is a replacement formulation for Rember, a formulation of methylthioninium chloride, which is widely used for the treatment for malaria and methemoglobinemia. SEN1576 compound was derived from SEN1269, which had better cell penetration ADME properties than RS-0406, and was protective against Aβ-induced cell death and synaptotoxicity, and prevented cognitive deficits induced by Aβ oligomers in rodents. Aβ1-42Nle35p37, with norleucine at position 35 and D-Pro at position 37, does not form amyloids and displays short-range NOEs in its monomeric form. The Pande's compound was designed as follows. Ligand-based drug design was performed using the structure of Aβ42Nle35p37 monomer, the top 100 best hits from the Maybrige Screening were tested in a single ThT aggregation assay and a compound was identified with effects on inhibition similar to the modified Aβ1-42 peptide with an IC50 of 13 μM [29]. Arai performed rational design of a non-peptidic inhibitor (compound **17**) based on the Aβ16-20 pharmacophore under the assumption that intermolecular side chain and main chain interactions must be optimal and minimal, respectively [30••].

oligomers that are toxic to different levels and it is very hard to target them all. In addition, there are multiple sources of toxicity and the solution might be to use many compounds simultaneously.

While structure determination of small oligomers of Aβ/inhibitor by combining simulations, solution NMR and electron-spray ionization mass-spectrometry [41•], would represent a real breakthrough, in-cell NMR experiments on amyloid peptides alone and with inhibitors are critical because crowding can change the stability and equilibrium ensembles [42]. It is also important to take into account that the population of brain Aβ oligomers changes with aging [43••], and understand why the A2T and A2V mutations protect from AD [44••,45]. With the human gamma-secretase complex structure at 4.5 Å [46••], it might be also possible to control Aβ production without side-effects, offering new opportunities against AD. Finally, with the recent design of a novel 3D human neural cell culture system recapitulating amyloid-β and tau pathology, we have in hands now more precise models [47].

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