Inhibitors of protein aggregation and toxicity

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Abstract

The aggregation of numerous peptides or proteins has been linked to the onset of disease, including Aβ (amyloid β-peptide) in AD (Alzheimer’s disease), asyn (α-synuclein) in Parkinson’s disease and amylin in Type 2 diabetes. Diverse amyloidogenic proteins can often be cut down to an SRE (self-recognition element) of as few as five residues that retains the ability to aggregate. SREs can be used as starting points for aggregation inhibitors. In particular, N-methylated SREs can bind to a target on one side, but have hydrogen-bonding blocked on their methylated face, interfering with further assembly. We applied this strategy to develop Aβ toxicity inhibitors. Our compounds, and a range of compounds from the literature, were compared under the same conditions, using biophysical and toxicity assays. Two N-methylated α-peptide inhibitors with unnatural side chains were the most effective and can reverse Aβ-induced inhibition of LTP (long-term potentiation) at concentrations as low as 10 nM. An SRE in asyn (VAQKTV) was identified using solid-state NMR. When VAQKTV was N-methylated, it was able to disrupt asyn aggregation. N-methylated derivatives of the SRE of amylin are also able to inhibit amylin aggregation.

Introduction

Amyloid fibrils are found as deposits of proteinaceous aggregates in patients with a range of diseases including BSE (bovine spongiform encephalopathy), vCJD (variant Creutzfeld–Jakob disease), AD (Alzheimer’s disease), PD (Parkinson’s disease) and Type 2 diabetes [1,2]. The precursor protein is specific to each disease. It converts from its soluble native state into insoluble amyloid fibrils with a polypeptide conformation referred to as ‘cross-β structure’, in which β-strands form ordered β-pleated sheets with the direction of the strands being perpendicular to the long axis of the fibre. Formation of amyloid fibrils often involves the conversion of α-helix structure into β-sheet, but is probably not a simple two-state process and occurs via partially folded intermediates that form either through partial unfolding of the native protein or partial folding of the unfolded protein. The intermediate then undergoes misfolding to form the extended cross-β-sheet structure. These β-sheet structures can aggregate further into protofibrils and fibrils, which are the insoluble species that form the tissue deposits implicated in neurodegenerative diseases.

For all diseases linked to amyloid formation, existing therapies, at best, offer alleviation of symptoms for a limited time, with no cure possible. A promising strategy, in principle, is to treat the diseases and conditions arising from unwanted amyloid formation by interfering with protein or peptide aggregation, since this targets the first event in pathogenesis directly [11,12]. Most work has targeted Aβ, whose aggregation is probably the initial event in the onset of AD. Numerous small molecules have recently been reported as inhibitors of Aβ aggregation, including rifampicin [13], (-)-5,8-dihydroxy-3R-methyl-2R-(dipropylamino)-1,2,3,4-tetrahydronaphthalene [14], type IV collagen [15], melatonin [16], danuomycin [17], glycosaminoglycans [18], fullerene [19], apomorphine derivatives [20], 3-indole propionic acid [21], nordihydroguaiaretic acid, tannic acid,

Key words: α-synuclein, Alzheimer’s disease, β-amyloid, Parkinson’s disease.

Abbreviations used: Aβ, amyloid β-peptide; AD, Alzheimer’s disease; asyn, α-synuclein; AAPP, islet amyloid polypeptide; NAC, non-amyloid component; LTP, long-term potentiation; MTI, 3-[(4S)-dimethylthiazol-2-yl]-2,5-diphenyl-2H-tetrazolium bromide; NMR, non-amyloid component, PD, Parkinson’s disease; SRE, self-recognition element.

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Figure 1 | Examples of inhibitors of Aβ aggregation

DAPH, 4,5-dianilinophthalimide.

Curcumin

SEN606

DAPH

Clioquinol

AZD-103

SE304

RS-0406

iAβSp

Alzhemed

PPI-1019

quercetin [22], phenylazo benzenesulfonamides [23], amphiphilic surfactants [24], curcumin [25], oligomeric acylated aminopyrazoles [26], cyclohexanehexol (inositol) stereoisomers [27], polyphenols [28], 3-amino-1-propanesulfonic acid (Alzhemed or Tramiposate) [29], Salvinolic acid B [30], Δ9-tetrahydrocannabinol [31] and Congo Red linked to a synthetic ligand for the FK506-binding protein chaperone [32]. Examples of these compounds are shown in Figure 1. The diversity in their structures is striking, suggesting that these molecules act by binding to multiple binding sites in Aβ or by various modes of action. This is in contrast with most drugs that may at least have some resemblance to the substrate of an enzyme, e.g. if they are all binding within the same site.

Several groups have shown that peptides or peptidomimetics can inhibit Aβ aggregation [33]. In particular, we, and other groups, have incorporated N-methylated amino acids into peptides to generate inhibitors of amyloidosis. Inhibitors are first developed by N-methylating the SRE of the target peptide. After N-methylation, one side presents an unchanged hydrogen-bonding ‘complementary’ face to the amyloid target, with the other having one or more N-methyl groups in place of backbone amide groups, thus presenting a ‘blocking’ face. The N-methyl group also induces the desired β-structure at the N-methylated site, unlike proline. We first showed that N-methyl derivatives of Aβ-(25–35), an aggregation-prone and toxic fragment of Aβ, was able to prevent Aβ-(25–35) aggregation and inhibit toxicity in PC12 cells [34]. The location of the N-methyl group was critical, with some of the most effective inhibitors surprisingly altering fibril morphology, rather than preventing Aβ-(25–35) aggregation, as anticipated. Meredith and co-workers investigated N-methylated peptides of a region corresponding to residues 16–22 and later 16–20 of the amyloid SRE [35,36]. They prevented Aβ fibrils from forming and broke down pre-formed fibrils. Multiple N-methylations must be on the same side of the peptide, i.e. at alternate positions, so that the peptide retains an unblocked binding face when in a β-conformation. Cruz et al. [37] used N-methylated inhibitors based on Aβ-(36–40) (VGGVV). We optimized the structure of an inhibitor of Aβ aggregation and toxicity by starting with the KLVFF SRE and varying the side-chain identity, chirality, N-terminal acetylation, C-terminal amidation and location of N-methyl groups [38]. After four rounds of optimization, the most active compound was found to be D-[chGly-(Tyr)-(chGly)-(chGly)-(mLeu)]-NH2 (where ch is cyclohexyl and m is N-methyl), where every group had changed from the starting sequence, although retaining the pentapeptide framework. D-Peptides were clearly more effective than L-peptides, for reasons that remain unclear. Inhibitors that had the N-methyl group replaced with an amide group were highly insoluble and often toxic, precluding the direct measurement of the effect of the replacement. This lack of solubility may appear surprising, since a change from N-methyl group to amide group ought to increase hydrophilicity. In this case, however, it appears that peptides come out of solution by hydrogen-bonding to each other. Hence, the presence of amide groups is key for lack of aqueous solubility. We found variable results from methylating at different positions and with different side-chain substitutions. The rationalization of these data will probably require structure determination or modelling of an Aβ/inhibitor complex. PPI-1019 D-[His-[mLeu]-Val-Phe-Phe-Leu]-NH2 is another effective N-methylated peptide inhibitor of Aβ aggregation and
Inhibition of 10 μM Aβ-(1–42) aggregation

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 using the thioflavin T assay (μM)</th>
<th>IC50 using the MTT assay (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IAβ5p</td>
<td>None*</td>
<td>None*</td>
</tr>
<tr>
<td>PPI-1019</td>
<td>1.6</td>
<td>10</td>
</tr>
<tr>
<td>SEN304</td>
<td>1.8</td>
<td>2.9</td>
</tr>
<tr>
<td>SEN606</td>
<td>1.3</td>
<td>4.2</td>
</tr>
<tr>
<td>RS-0406</td>
<td>5.1</td>
<td>8.3</td>
</tr>
<tr>
<td>3-APS</td>
<td>None*</td>
<td>None*</td>
</tr>
<tr>
<td>AZD-103</td>
<td>None*</td>
<td>None*</td>
</tr>
<tr>
<td>DAPH</td>
<td>2.9</td>
<td>Small reduction in toxicity above 10 μM. Toxic above 30 μM.</td>
</tr>
<tr>
<td>Curcumin</td>
<td>2.6</td>
<td>None. Toxic above 30 μM.</td>
</tr>
<tr>
<td>Cloquimol</td>
<td>8.6</td>
<td>None. Toxic above 50 μM.</td>
</tr>
</tbody>
</table>

*No inhibition observed up to 50 μM inhibitor concentration.

toxicity [39]. It is fundamentally different from other N-methylated peptides, however, as the methylation of the unacetylated N-terminus means that an amine is methylated, rather than an amide, which would be the case at other positions. A singly methylated amine still has two hydrogen-bond donors, so its mode of action may well be different from peptides having methylated amides, where hydrogen-bond donation is eliminated. N-methylation of the N-terminal amine in Aβ-(25–35) has no effect, for example, whereas N-methylation at other sites prevents the peptide from being amyloidogenic [34].

Results with Aβ are notoriously unreliable, with aggregation being highly sensitive to conditions and minor contaminants. This makes it difficult to compare the efficacy of Aβ aggregation inhibitors in the literature, even if the same assay is used. We therefore sought to compare a range of inhibitors under the same conditions to allow more accurate comparisons to be made. We compared ten compounds using the thioflavin T assay commonly used to monitor Aβ aggregation. Effective inhibitors should cause a decrease in thioflavin fluorescence as their concentration is increased, with the concentration that gives 50% fluorescence (IC50) giving a quantitative indication of effectiveness (Table 1). Three inhibitors (Alzhemed, AZD-103 and iAβ5p) showed no indication of activity under these conditions, at concentrations of up to 50 μM. This suggests that these compounds are not strong inhibitors of Aβ aggregation, even if they are active in vitro, and hence may have a different mode of action. We do note, however, that Alzhemed and iAβ5p have been dropped from drug development.

Apparent activity from the thioflavin T assay is not always supported by cell toxicity data. The most widely used toxicity assay with Aβ is the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay [40]. Table 1 shows some MTT data for the same ten compounds using SHY5Y cells after a 24 h aggregation of 10 μM Aβ-(1–42).

The N-methylated peptides SEN304, SEN606 and PPI-1019 all show a reduction in cell toxicity at concentrations similar to that used in the thioflavin T assays. Some compounds show little or no activity with this assay, in apparent contradiction to the thioflavin T data. DAPH (4,5-dianilinophthalimide) and curcumin even cause cell death at concentrations above 30 μM. This illustrates that it is dangerous to rely on a small number of assays for Aβ aggregation. All have their flaws, even when simply assaying activity in vitro. The relevance of any assay to a human patient remains to be seen.

More sophisticated assays can potentially bridge the gap between biophysical assays and using animal models for disease. LTP (long-term potentiation) is a long-term improvement in communication between two neurons resulting from electrically stimulating them simultaneously. LTP is considered to be one of the major cellular mechanisms underlying learning and memory. Its strong inhibition by Aβ is consistent with the loss of short-term memory that is diagnostic of AD (Figure 2). SEN304 is able to reverse inhibition of LTP by Aβ-(1–40) at remarkably low stoichiometry; the effects of 1 μM Aβ-(1–40) can be prevented by just 1 nM SEN304. This contrasts with the concentrations and stoichiometries required to show efficacy by biophysical and cell toxicity assays. This may be because the Aβ species responsible for inhibition of LTP could be present as only a small fraction of the total Aβ. Only a low inhibitor concentration would then be required to bind to this toxic structure. If many more Aβ species bind thioflavin T, then a higher inhibitor concentration could be necessary to detect activity using this assay.

![Figure 2](image-url)
Asyn
Lewy bodies are a form of neurodegenerative lesion with a dense core of insoluble filamentous proteins mainly composed of asyn [41]. In aqueous solution, in vitro asyn is predominantly unfolded [42], but it can misfold into amyloid fibrils [43]. The aggregation of asyn is a probable key event in the onset of several neurodegenerative diseases, including PD and the Lewy body variant of AD. A central hydrophobic region (residues 61–95) identified as the NAC (non-amyloid component) of AD plaques [44] is believed to be responsible for aggregation, with various residues within the NAC region suggested as the SREs, including residues 66–74 [44], 68–78 [45] and 71–82 [46].

We applied the same strategy of N-methylating an SRE to discover an inhibitor of asyn aggregation, starting with the peptide 71–82 (VTGVTAVAQKTV) [4]. This peptide is a good starting point because (i) its residues constitute a critical SRE of native asyn [46], (ii) the peptide alone readily aggregates to form insoluble amyloid fibrils of similar morphology to those of the parent protein [47], and (iii) in the presence of full-length asyn, the peptide binds to and co-fibrillizes with the aggregating parent protein [46]. High-resolution \(^{13}\)C solid-state NMR was used to examine how the structure of asyn-(71–82) differs before and after aggregation, to specify which amino acid residues undergo a transformation from the disordered random state into the highly ordered \(\beta\)-sheet conformation associated with amyloid. Site-specific structural information about monomeric soluble asyn-(71–82) and insoluble fibrils was obtained from measurements of peak widths and chemical shifts from two-dimensional \(^{13}\)C dipolar-assisted rotational resonance spectra of the peptide monomer in aqueous solution at \(-10^\circ\)C and then, at the same temperature, for an aqueous suspension of fibrils formed after 4 weeks of incubation. Peak width and chemical shift changes were large in residues 75–78, but small in residues 71–74, suggesting that amyloid formation is restricted to the C-terminal end of residues 71–82. We then compared the peptides asyn-(71–76) and asyn-(77–82) to compare directly the two halves of asyn-(71–82). Congo Red binding, electron microscopy and dynamic light scattering clearly showed that asyn-(77–82) was amyloidogenic, whereas asyn-(71–76) was not. The SRE in asyn can thus be narrowed down to only six residues, despite aggregation in vivo taking place with the full 140-residue protein. Asyn-(77–82) was N-methylated in several locations to try to develop an inhibitor of full-length asyn aggregation. VAQKTmV was effective, as judged by thioflavin T fluorescence, electron microscopy and dynamic light scattering. As with the A\(\beta\) inhibitors, the location of the N-methyl group was critical, because VmAQKTV had no effect on asyn aggregation.

Amylin (IAPP)
The aggregation of the 37-residue peptide hormone amylin (IAPP) is associated with the onset of Type 2 diabetes. Kapurniotu and colleagues have also used N-methylated peptides to inhibit the aggregation of IAPP [48]. Although the doubly N-methylated full-length IAPP analogue ([N-(N-Me)G24,(N-Me)I26]-IAPP (IAPP-G1)) binds IAPP with low nanomolar affinity and completely blocks IAPP cytotoxic self-assembly and fibrillogenesis, it is much larger than most drugs [48]. Much smaller inhibitors can be developed from the IAPP SRE sequence NFGLAIL. NF(N-Me)GA(N-Me)GL is able to bind with high affinity to full-length hIAPP (human IAPP), to inhibit its fibrillogenesis and to inhibit hIAPP-mediated apoptotic \(\beta\)-cell death [49].

Conclusion
Diseases caused by the misfolding and aggregation of peptides and proteins are a massive and growing health problem. Interfering with aggregation is therefore a possible strategy to develop new drugs that are needed to treat diseases such as AD, PD and Type 2 diabetes. Only a small part of an amyloidogenic protein appears to be responsible for aggregation, namely the SRE. N-methylation of SREs offers a general solution to discovering aggregation inhibitors. We, and other groups, have shown that this can be successfully done for A\(\beta\), asyn and IAPP.

Acknowledgements
We thank Neurosolutions for performing the LTP assay.

Funding
This work received financial support via a research fellowship to J.M. [grant number RF2006/3] and an equipment grant [grant number 2003B] from the Alzheimer’s Research Trust. The Wellcome Trust is acknowledged for funding the cost of the peptide synthesizer [grant number 075314]. We thank The Wellcome Trust and BTG for funding Senexis. H.A. thanks the Biotechnology and Biological Sciences Research Council for funding a CASE (Co-operative Awards in Science and Engineering) Ph.D. studentship.

References

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