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Probing the efficacy of peptide-based inhibitors against acid- and zinc-promoted oligomerization of amyloid-β peptide via single-oligomer spectroscopy

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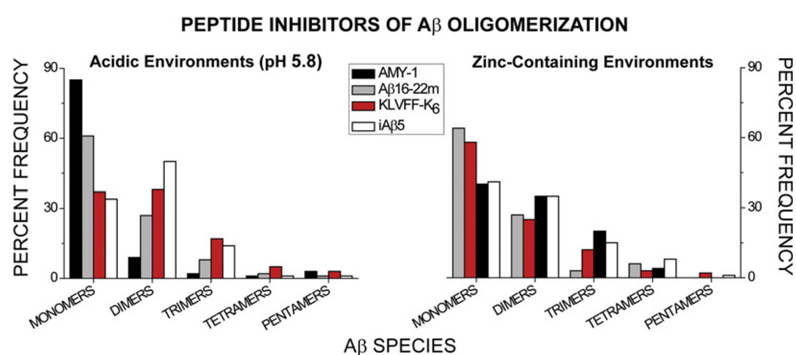
HIGHLIGHTS

- ► Four fibrillogenesis inhibitors reduce zinc- and acid-promoted oligomerization.
- ► β-structured inhibitors with blocking groups best prevent association at pH 5.8.
- ► With zinc, multiple strategies give equal inhibition; oligomers may be looser.
- ► N-methylated Aβ16-22m is most effective across both sample environments.

GRAPHICAL ABSTRACT



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ABSTRACT

One avenue for prevention and treatment of Alzheimer's disease involves inhibiting the aggregation of amyloid-β peptide (Aβ). Given the deleterious effects reported for Aβ dimers and trimers, it is important to investigate inhibition of the earliest association steps. We have employed quantized photobleaching of dye-labeled Aβ peptides to characterize four peptide-based inhibitors of fibrillogenesis and/or cytotoxicity, assessing their ability to inhibit association in the smallest oligomers ($n=2$–5). Inhibitors were tested at acidic pH and in the presence of zinc, conditions that may promote oligomerization in vivo. Distributions of peptide species were constructed by examining dozens of surface-tethered monomers and oligomers, one at a time. Results show that all four inhibitors shift the distribution of Aβ species toward monomers; however, efficacies vary for each compound and sample environment. Collectively, these studies highlight promising design strategies for future oligomerization inhibitors, affording insight into oligomer structures and inhibition mechanisms in two physiologically significant environments.

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1. Introduction

Amyloid-β (Aβ) is a peptide of 39–43 amino acids that is formed through specific proteolytic cleavage of the transmembrane amyloid precursor protein (APP) [1] and normally circulates in brain plasma and cerebrospinal fluid at pico- to nanomolar concentrations [2]. Although Aβ monomers may be protective to neurons [3], numerous factors have been shown to promote the peptide's self-association into neurotoxic oligomers and aggregates implicated in Alzheimer's disease (AD; for a review, see ref. [4]). Among the multitude of aggregated species, Aβ fibrils exhibiting cross-β sheet structure [5, 6] have historically garnered the greatest attention, due to their prevalence in the senile plaques characteristic of AD brain [7-9]. Inhibiting Aβ fibrillogenesis has been a primary strategy in Alzheimer's drug development. A variety of small molecules [10, 11] and peptides [12] have been

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reported to inhibit fibril formation and/or reduce neurotoxicity of Aβ aggregates. Successful peptide inhibitors include block copolymers containing recognition and disrupting elements [13, 14], β-sheet breakers employing Pro residues [15, 16], *N*-methylated sequences [17-19], derivatives disubstituted at the α-carbon [20] and sequences containing D-amino acids [19, 21, 22].

Over the last decade, focus has shifted somewhat from the inhibition of fibrillogenesis, as several key results have linked small Aβ oligomers to the etiology of AD [23]. The accumulation of SDS-stable dimers and trimers [24-27] in the brain is strongly correlated with synapse loss, cognitive impairment and increased severity of end-stage AD [24, 25, 28]. Direct application of cell-derived dimers, trimers and tetramers results in impaired long-term potentiation (LTP) [29-31], short-term memory deficits [32] and synapse loss [33] in rodents. Additionally, Aβ dimers isolated from the cortex of human AD brain [34] and found in human cerebrospinal fluid [35] inhibit LTP in vivo. Collectively, these findings illustrate the crucial role of small Aβ oligomers in Alzheimer's progression and provide clear incentive to investigate inhibition of Aβ association in its earliest stages.

Toward that goal, we selected four successful inhibitors of fibrillogenesis and/or neurotoxicity, each with a distinct inhibition strategy (Fig. 1) [14, 15, 17, 20], and examined their abilities to prevent or reverse association in the smallest oligomers ($n = 2$–5). Since oligomers and fibrils have been shown to possess different structures and form through distinct pathways [36], we were interested to determine which compound(s) would most successfully inhibit the earliest association steps. All four inhibitors employ recognition sequences similar to Aβ's central hydrophobic region (amino acids 16–21; see Fig. 1A) and bind to the full-length peptide via a combination of hydrophobic side-chain interactions and backbone hydrogen bonds [37-40], the atomic-level details of which are not known. KLVFF-K_6 (Fig. 1B) contains residues 16–20 of Aβ with a lysine hexamer as a disrupting element. Murphy and coworkers reported that it significantly alters aggregation kinetics and aggregate morphology while reducing Aβ cytotoxicity [14, 41, 42]; Moss et al. found that it inhibits monomer aggregation [43]. AMY-1 (Fig. 1C) is a peptide analog of KLVFF-K_6 containing alternating α,α-disubstituted amino acids (ααAA). The Hammer group reported that equimolar concentrations of AMY-1 are highly effective in inhibiting Aβ fibrillogenesis: while the L-amino acids on one face of the inhibitor permit hydrogen-bonding to Aβ, steric effects of the ααAA on the other face effectively prevent continued association [20]. Aβ16-22m (Fig. 1D) also functions by blocking binding of Aβ on one face of the Aβ-inhibitor complex, as *N*-methyl amino acids on one side of the inhibitor lack the amide hydrogens used in hydrogen bonding and β-sheet formation [17]. Meredith and colleagues reported that Aβ16-22m dramatically inhibits Aβ fibrillogenesis and successfully dissociates preformed fibrils [17]. Successful inhibition of fibril formation and dissociation of preformed fibrils have also been reported for iAβ5 (LPFFD, Fig. 1E), developed by Soto and coworkers; in addition, this inhibitor was shown to prevent Aβ neurotoxicity [15]. iAβ5 is designed around residues 17–21 of Aβ: Asp is used in place of Ala-21 for improved solubility, and Pro is substituted in place of Val-18, harnessing the ability of proline to prevent and disrupt β-sheet formation [15, 38].

We tested each of the four inhibitors in Fig. 1 against samples of the earliest oligomers, prepared under acidic conditions (pH 5.8) and at physiological pH in the presence of Zn^{2+}. These sample environments have been shown to promote Aβ aggregation [44-49] (and oligomerization [50]) in vitro and are also relevant to association in vivo. The acidic conditions mimic those of early endosomes where Aβ is produced [51, 52] and the zinc concentrations used are within the range observed in the brain [53-55], where zinc release during neurotransmission (at concentrations up to 300 μM [54, 55]) may cause Aβ oligomers to target synapses [56]. Single-molecule fluorescence spectroscopy was used to determine distributions of monomers and oligomers present under the various sample conditions, using a method we have reported previously [50] (which was further substantiated by the work of Steel and colleagues [57]). For these measurements, we used Aβ40 – the most prevalent form of Aβ in vivo [58] – that is covalently labeled at the N-terminus with carboxyfluorescein (FAM), and at the C-terminus with Lys-Biotin; this construct is abbreviated FAβB. The peptide is tethered to functionalized cover slips via biotin-streptavidin binding; a dense base layer of polyethylene glycol provides a non-fouling surface, preventing adsorption to the glass [50, 59]. Localized FAβB monomers and oligomers are interrogated, one at a time, through laser excitation. The quantized photobleaching of individual dye molecules permits quantification of the number of associated peptides [50, 57, 60]. By investigating dozens of peptide species, one by one, we have characterized the distributions of monomers and oligomers present under our conditions of interest. We report that all four inhibitors are successful in reducing association in the smallest oligomers, shifting the distribution of FAβB species toward monomers. The variations in efficacy observed for the different compounds in acidic versus zinc-containing samples suggest successful strategies for preventing or reversing the earliest Aβ association steps and afford new insight into oligomer structures and inhibition mechanisms in two physiologically significant environments.

2. Experimental methods

2.1. Materials

FAM-Aβ40-Lys-Biotin (FAβB) and iAβ5 (LPFFD) were purchased from AnaSpec, Inc. (Fremont, CA); the remaining three inhibitors (i.e., KLVFF-K_6, AMY-1 and Aβ16-22m) were obtained from the Hammer research group, formerly of Louisiana State University. All peptides were stored at −20 °C upon receipt. Zinc(II) chloride ($ZnCl_2$), sodium chloride (NaCl), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and phosphate buffered saline (PBS) powder were obtained from Fisher. Solutions were prepared in distilled, deionized water (18 MΩ) from a Millipore system. Functionalized cover slips (STREP-01) were purchased from MicroSurfaces, Inc. (Austin, TX). These are pre-coated with a dense layer of polyethylene glycol (PEG), a small percentage of which is functionalized with biotin (surface density ~10^{10}/cm^2); the biotin moieties are further bound to streptavidin, each of which has 2–3 accessible sites for binding of biotin-labeled FAβB [61]. Packaged cover slips were stored, vacuum-sealed, at ≤−20 °C; after opening, unused cover slips were kept in a vacuum desiccator at room temperature, according to the manufacturer's instructions.

2.2. Sample preparation

Phosphate buffered saline (PBS, 1X: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.47 mM KH_2PO_4) and HEPES/NaCl (100 mM HEPES, 50 mM NaCl) buffer solutions were prepared in distilled, deionized water; pH was adjusted as needed with HCl and/or NaOH. Lyophilized FAβB was added to prepared buffer after thawing for 30 min; these peptide stock solutions were made at ~1 μM concentrations, as determined from spectrophotometry (Beer's Law), using the molar absorptivity of the FAM dye. Additional solutes (e.g., $ZnCl_2$, inhibitor compounds) were added at this point. Samples were then incubated at room temperature for 1 h, after which they were diluted to final FAβB concentrations of approximately 60 pM.

Functionalized cover slips were prepared for use by dragging the bottom, uncoated sides across methanol-moistened lens tissues and edging the top, coated surfaces with Mylar frames (McMaster-Carr), to prevent sample loss. Samples (30–40 μL) were added to the centers of prepared cover slips already in place on the microscope, forming small pools of solution (area ~1 cm^2), and left for 30 min to permit tethering of the biotin-labeled peptides to the streptavidin on the cover slips. At ~60 pM, assuming 100% binding of FAβB monomers to

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