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Clioquinol and other Hydroxyquinoline Derivatives Inhibit A β (1-42) Oligomer Assembly

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Abstract

Soluble oligomeric amyloid- β (A β) species are toxic to many cell types and are a putative etiological factor in Alzheimer's disease. The NINDS-Custom Collection of 1040 drugs and biologically active compounds was robotically screened for inhibitors of A β oligomer formation with a single-site biotinylated A β (1-42) oligomer assembly assay. Several quinoline-like compounds were identified with IC₅₀'s < 10 μ M, including the antiprotozoal clioquinol that has been reported to have effects on metal ion metabolism. The 2-OH, 4-OH, and 6-OH quinolines do not block A β oligomer formation up to a concentration of 100 μ M. Analogs of clioquinol have shown activity in reducing A β levels and improving behavioral deficits in mouse models of A β pathology. The inhibitory effects of clioquinol and other 8-OH quinoline derivatives on oligomer formation *in vitro* are unrelated to their chelating activity. Crosslinking studies suggest that clioquinol acts at the stage of trimer formation. These preliminary data may suggest that 8-OH quinolines have the potential for suppressing A β oligomer formation which should be considered when assessing the effects of these compounds in animal models and clinical trials.

Keywords

amyloid; crosslinking; PICUP; biotin; ELISA; robotic screening

Introduction

The amyloid- β (A β) peptide has a high propensity to assemble into multimeric states of unusual stability. In humans with Alzheimer's disease (AD), A β fibrils associate into amyloid plaque structures to form the classical Alzheimer's disease pathology along with neurofibrillary tangles. Soluble oligomeric assemblies of A β peptide, of which some are precursors for fibril

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and plaque formation, are not detected by standard histological evaluation. Unlike oligomeric native proteins, the A β oligomers are considered misfolded structures due to their stable cross- β sheet secondary structure that renders them resistant to proteolytic degradation. Formation of A β oligomers follows an ordered assembly process and has been studied in some detail by Bitan [5] and Glabe [12] (Figure 1). The two extra C-terminal amino acid residues of the A β (1-42) peptide confer a high propensity to form stable oligomeric species at nM concentrations of A β (1-42). This concentration approaches the levels of A β oligomers found in the brain and cerebral spinal fluid (CSF) of AD subjects [11,13] and cell culture media [33] that block long-term potentiation in culture. The A β (1-40) peptide is much less prone to form oligomers and when formed, the A β (1-40) oligomers differ structurally from A β (1-42) oligomers and are much less stable [2,37,39]. Mutagenesis of the C-terminal residues of A β (1-42) indicates a structural role for this part of the peptide in oligomer assembly [5]. *In vitro* incubation of synthetic A β (1-42) peptide produces a series of rapidly exchanging unstable low-n oligomers culminating in a proportion of relatively stable 12-24-mers that can associate to higher order species. These soluble misfolded oligomers of the Alzheimer's A β peptide are significantly more toxic to neurons and other cell types than are monomers or fibrils [12]. A number of studies have suggested that these soluble oligomers may be an etiologic agent in AD. Therapeutic approaches, such as immunization and inhibition of the secretase enzymes that produce A β from its precursor protein, β APP, indirectly target oligomers by reducing levels of the monomeric peptide from which it is formed. Since A β monomer may have positive effects in the brain [31], it may be difficult to completely inhibit A β production or to clear oligomers from the brain once they have formed. Therefore, an alternative approach may be to directly block oligomer formation.

High affinity binding of a small molecule to a conformationally flexible peptide, such as A β , is difficult to achieve, although certain peptides designed to interact with β -strand edges specifically block β -sheet formation [9,18]. An ordered peptide assembly mechanism implies the existence of multimeric intermediates that may have sufficient surface area to interact with a small molecule modulator. An empirical approach targeting low abundance intermediates of fibril formation by assaying for sub-stoichiometric inhibition has yielded inhibitors of A β fibrillation [24,25,34].

Oligomers of synthetic A β (1-42) peptide comprised of 10-12 monomers or more are stable to spontaneous dissociation and have been detected *in vitro* and *in vivo* [21,28]. Further assembly into protofibrils and fibrils can occur at higher peptide concentrations [10,12,17,19]. Spontaneous peptide nucleation, the sensitivity of a nucleated assembly process to container surface properties and the air-water interface, and effects of the shear forces of agitation have hampered the search for inhibitors of oligomer formation. An oligomer-conformation-specific antibody has been used *in vitro* to identify compounds that prevent the formation of immunoreactive oligomers from synthetic peptide [27]. Interestingly, compounds that were either selective for the inhibition of fibril formation or for oligomer formation were identified. Other compounds inhibited both fibril and oligomer formation. Therefore, small molecules and conformation-specific antibodies [12] *in vitro* can distinguish between intermediates in both fibril and stable soluble oligomer formation.

The single-site biotin-avidin A β (1-42) oligomer assay [23] (illustrated in Figure 2) was developed to facilitate the rapid screening of small molecule compound libraries. Substituting the biotin-avidin system for antibodies reduces the impact of potential artifactual interaction of compounds with β -sheet regions of the antibodies and reduces reagent costs. The biotin-avidin interaction is also familiar to screeners, and few library compounds interfere with the binding. The sensitivity of the single-site biotin-avidin A β oligomer assay allows the IC₅₀ determinations to be measured in the nanomolar range using a total of 10 nM bioA β (1-42). At this low concentration of A β (1-42), the confounding of potency with compound stoichiometry

at higher concentrations of peptide is now avoided. This single-site biotin-avidin assay was used to screen the NINDS-Custom Collection (NINDS-CC) of 1040 drugs and biologically active compounds (<http://www.msdiscovery.com>). Most of the compounds were inactive in our assay, but we have identified a series of 8-hydroxyquinolines that inhibit A β oligomer formation. These compounds may be of interest because clioquinol (an 8-hydroxyquinoline) and derivatives are currently being tested as therapeutics for AD based on their ability to complex metal ions [6,30]. We find that in our *in vitro* system, these compounds inhibit A β (1-42) oligomer formation, apparently independent of metals, such as zinc. The larger and more chemically diverse ApexScreen 5040 library (TimTec) is also being assayed by the single-site method.

Methods and Materials

A detailed description of the materials and the robotic screening procedure is provided as Supplemental Material.

Results

The NINDS-CC contains a significant number of disinfectants, detergent-like molecules, alkyl amphiphiles, hydrophobic cyclic peptides, polyketides, and other high molecular weight natural products. Since detergent-like and micellar molecules can non-selectively interfere with or even stimulate A β oligomer formation, any hits with these types of structures were excluded from further analysis. One particular series of small molecule inhibitors was interesting because of its homology to clioquinol, a compound shown to be active in animal models of A β pathology [1] and has been in clinical trials for AD [30,32]. This chemical series is of the hydroxyquinoline (HQ) class shown in Table I. The highest concentration used was 100 μ M. This concentration was significantly lower than the reported mM aqueous solubility of these compounds [8,16]. The compounds were first dissolved in DMSO and then diluted into aqueous buffers to give a solution with no detectable precipitates. Clioquinol was chosen as a representative HQ oligomer assembly inhibitor for further study.

Because the biotinyl single-site assay detects stable oligomers that are > 30 kDa, size exclusion chromatography was used to verify that oligomer formation was prevented. The amount of A β detected in the void volume of the Sephadex G75 column was drastically reduced by clioquinol (data not shown). This indicated that there were very few high molecular weight oligomers present. Separate experiments were performed to control for the possibility that clioquinol or any of the other HQ compounds produced apparent inhibition by interacting with the assay components. The compounds did not prevent the interaction of preformed oligomers with NeutravidinTM or with Streptavidin-HRP (data not shown).

Incubation of preformed bio42 oligomers with the HQ compounds in Table I for long periods of time (Figure 3) did not significantly reduce the amount of oligomers. A possible exception may be the incubation of 6-HQ at 28 hours. The amount of preformed oligomers recovered with those inhibitors was similar to the amount of oligomers recovered when incubated in the presence of vehicle (3% DMSO). The lack of dissociation of preformed oligomers suggests that inhibition may occur early in the assembly process.

Early stages of oligomer assembly were probed by crosslinking studies. Bitan, et. al. [3,4] had reported that synthetic peptide at nM concentrations did not accumulate the small (low-n-mer) intermediates on the pathway to stable A β oligomers. Hence, these small intermediates are rarely observed by Sephadex G75 chromatography because they rapidly dissociate and exchange with monomer [3,4]. These small intermediates could be trapped with a crosslinking agent [4,26]. Crosslinking agents with two different chemical reactivities and different lengths

were employed to avoid potential interference by the compounds. The photochemical zero-length PICUP reaction exploits the free radical-catalyzed intermolecular reaction of Y10 in A β with an adjacent nucleophilic residue on another A β molecule [4]. Tween 20 (0.1% v/v) inhibits stable oligomer formation measured by single-site assay or size exclusion chromatography [22]. It completely inhibits crosslinking of dimer and higher multimer formation from monomer, but it does not inhibit crosslinking of preformed oligomers. Clioquinol appears to inhibit trimer formation, and has little effect on dimer crosslinking (Figure 4). This lack of effect on dimer crosslinking may suggest that the aromatic OH of clioquinol is not significantly interfering with the crosslinking reaction.

A second crosslinking agent with different chemistry corroborated the observations of clioquinol inhibition of oligomer assembly with the free radical-based PICUP reaction. The bis-aldehyde glutaraldehyde agent reacts with amino groups and multimerizes in solution to span longer distances between reactive residues [26]. Similar results to the PICUP measurements were observed with glutaraldehyde. These findings support the interpretation that clioquinol and other hydroxyquinolines may be destabilizing the formation of trimeric and higher A β intermediates. Additional experiments and different techniques are required to further probe this finding since crosslinking is a covalent reaction that could introduce artifacts in the analysis.

Discussion

8-Hydroxyquinoline derivatives in the NINDS-CC were effective inhibitors of *in vitro* bio42 oligomer formation. The fact that one of these 8-HQ compounds had already shown some activity in animal models of AD pathology and in humans [1,32] increased the importance of investigating this class of compounds for potential anti-oligomer activity. Clioquinol originally was an antiprotozoal agent that was discontinued for that indication because of reported severe neurologic side effects, paralysis and blindness, particularly at high doses [7]. The results from a small pilot phase II clinical trial of clioquinol in 36 AD subjects indicated a decrease relative to placebo control in the rate of decline of the most severely affected AD cases in the ADAS cognitive test [32]. In addition, PBT-1 (clioquinol) and PBT-2 (a clioquinol analog modified to alleviate the toxicity issues) have shown some efficacy in the Tg2576 mouse model of A β pathology by mitigating behavioral deficits and other measures of the phenotype [1]. A recent trial of PBT2 revealed no safety issues and showed improvement in two measures of executive function [20].

The mode of action of these agents is proposed to be metal ion (primarily Zn and Cu) chelation by the compound [6,30]. Clioquinol has also been shown to bind more avidly to the metallated form of A β peptide [29]. The metals hypothesis of Alzheimer's disease has recently been refined to extend beyond removal of metal ions from the A β peptide [6,30,38]. Zinc is known to aggregate proteins, in particular, A β [14]. The arrangement of histidine residues 6, 13, and 14 in A β leads to metal ion binding to the peptide, particularly with redox active metals (copper, iron) that are implicated in oxidative chemistry [35,36]. We excluded the likelihood that trace levels of transition metal ions effected the oligomerization of bio42 in our system since neither inclusion of 10 mM EDTA, 1 mM of 1,10-phenanthroline nor the presence of 100 μ M ZnCl₂ affected oligomerization or oligomer detection in the assembly or capture reactions (data not shown). The lack of effect of added zinc on oligomer formation could be due to the low (10 nM) peptide concentration. Small oligomers (<30 kDa) are also not detected in the single-site assay configuration.

In summary, our results suggest that, in addition to any effects on zinc, copper, or other metal ion-dependent mechanisms, there are intrinsic effects of the hydroxyquinolines on A β oligomerization that deserve further study. In addition, the responses in animal models and in

the clinical trial of related compounds suggest that the observed effects of these types of compounds may also include effects on oligomer formation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Clioquinol	5-chloro-7-iodo-8-hydroxyquinoline
Broxyquinoline	5, 7-dibromo-8-hydroxyquinoline
Chloroxine	5, 7-dichloro-8-hydroxyquinoline
Iodoquinol	5, 7-diiodo-8-hydroxyquinoline
Aβ	Alzheimer's β -amyloid peptide
CSF	cerebrospinal fluid
HRP	horseradish peroxidase
DMSO	dimethylsulfoxide
ELISA	enzyme-linked immunosorbant assay
PICUP	Photo-induced crosslinking of unmodified proteins

Figure 1. A β (1-42) Oligomer Assembly

A β (1-42) self-associates in aqueous solution to form a population of rapidly interconverting small oligomers that can further associate more stable dodecamer-size species. These larger species are further stabilized to larger stable oligomers that persist in solution and can be assayed by the single-site ELISA technique. The rapidly equilibrating intermediates can be trapped by crosslinking.

Figure 2. Principle of the Single-Site Bio42 Oligomer Screening Assay

Single-biotinylated A β (1-42) monomers and oligomers bind to a NeutrAvidin-coated plate. After washing, only oligomers have a free biotin available to react with a Streptavidin-HRP detection reagent. Compounds that block A β assembly reduce the signal due to oligomers compared to a vehicle-treated control.

Figure 3. Effect of 8-OH Quinoline Analogs on Pre-formed bio42 Oligomers

Oligomers preformed at 6.6 nM bio42 peptide were incubated in the presence of 0.1% v/v Tween 20 to prevent re-association of monomers and 100 μ M of the 8-OH quinoline analogs for 4 hours (open bars) or 28 hours (hatched bars) as described in METHODS. The remaining oligomers were determined by single-site assay. The solid line is the oligomer content of the DMSO vehicle control at 4 hours. The dashed line is the oligomer content of the DMSO control at 28 hours. #1 = clioquinol; #2 = 5, 7-dibromo-8-hydroxyquinoline; #3 = 5, 7-diiodo-8-hydroxyquinoline; #4 = 8-hydroxyquinoline; #5 = 5, 7-dichloro-8-hydroxyquinoline; #6 = 5-chloro-8-hydroxyquinoline; #7 = 5, 7-dimethyl-8-hydroxyquinoline; #8 = 2-hydroxyquinoline; #9 = 4-hydroxyquinoline; #10 = 6-hydroxyquinoline.

Figure 4. Clioquinol Interferes with bio42 Metastable Trimer Formation

The effect of clioquinol on the early stages of oligomer assembly was assessed by initiating bio42 oligomer formation with 30 nM peptide in the presence of the PICUP reagents and photocrosslinking with an electronic flash as described in METHODS. Samples were subjected to SDS-PAGE on a 12% NuPAGE gel in the Mes buffer system, blotted to nitrocellulose, reacted with SA-HRP, and the bio42 detected by ECL as described in METHODS. The monomer (M), dimer (D), and trimer (T) bands are marked with arrows.

Table I
Potencies of Hydroxyquinoline Oligomer Formation Inhibitors

Compound	R1	R2	R3	IC ₅₀ (μM)
8-hydroxyquinoline *	H	H	OH	>30
Clioquinol *	Cl	I	OH	1.4
Broxyquinoline *	Br	Br	OH	1.7
Chloroxine *	Cl	Cl	OH	1.4
Iodoquinol	I	I	OH	1.2
5-chloro-8-hydroxyquinoline	Cl	H	OH	2.2
5, 7-dimethyl-8-hydroxyquinoline	CH ₃	CH ₃	OH	20
ST054891	CH ₂ OH	H	OH	11
Chloroacetoxyquinoline	Cl	H	OAc	21

* Compounds in the NINDS-Custom Collection

2-OH, 4-OH, and 6-OH quinoline were inactive at 100 μM