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Cannabidiol-Mediated Sequestration of Alzheimer's Amyloid-β Peptides in ADDL Oligomers

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Abstract

Cannabidiol (CBD), one of the most studied phytocannabinoids, is non-psychotropic and can induce protective effects on the central nervous system against acute and chronic brain injury. Interestingly, CBD inhibits processes relating to amyloid beta (Aβ)-induced neurotoxicity in mouse models of Alzheimer's disease, though the detailed molecular mechanism underlying the CBD neurotoxicity modulation is not fully understood. In this study, using atomic force microscopy, we find that CBD promotes the aggregation of Aβ peptides, enhancing the formation of Aβ oligomers, also known as Aβ-derived diffusible ligands (ADDLs). The CBD-mediated sequestration of Aβ monomers in soluble ADDLs could reduce neurotoxicity. This study highlights a possible role of CBD in modulating the formation of ADDL aggregates and provides insight into potentially neuroprotective properties of CBD in Alzheimer's disease.

Keywords

Cannabidiol, Amyloid, Alzheimer's Amyloid-β Peptides, Aβ-Derived Diffusible Ligands, Atomic Force Microscopy, Amyloid Peptide Sequestration

1. Introduction

Alzheimer's disease (AD), a chronic neurodegenerative disease leading to dementia, affects tens of millions of people worldwide and is often associated with comorbidities including hypertension and diabetes mellitus [1]. The hallmarks of AD, reflecting the breakdown of neural system integrity and function, are the formation of beta-amyloid (Aβ) peptide plaques, neurofibrillary tangles comprising hyperphosphorylated tau proteins, and ultimately the occurrence of neurodegeneration and brain atrophy [2] [3] [4]. While the molecular origins of AD pathogenesis remain poorly understood, the amyloid cascade hypothesis is one of the most popular explanations [3] [5], as reflected by recent treatment strategies and clinical trials [3] [6] [7]. Aβ is a protein fragment consisting of 39 - 43 amino acid residues cleaved from the type-1 transmembrane protein amyloid precursor protein by enzymes β-secretase and γ-secretase [8]. In AD, due to increased production or decreased clearance, the expression levels of Aβ increase. The Aβ peptide can form soluble neurotoxic oligomeric species leading to the formation of protofibrils, fibrils, and ultimately, large aggregates. This process is thought to be partly responsible for the neurodegenerative damage observed in the post mortem AD brain [3] [8]. A$\beta_{1\text{-}40}$ and A$\beta_{1\text{-}42}$, species known to form toxic fibrils and plaques, [9] [10] are widely studied in AD-related research [3] [11] [12]. Recent studies have shown that soluble Aβ-peptide oligomers, also known as Aβ-derived diffusible ligands (ADDLs) are found in high concentrations in post mortem Alzheimer's brain tissue and are reportedly more toxic than the insoluble Aβ fibrils [6] [8] [13] [14]. These ADDLs may reduce insulin receptor expression or could induce cell autophagy, leading to the death of mature neurons and instigating synaptic failure [12] [15] [16], with increased toxicity correlated with higher concentrations of ADDLs [17].

The high toxicity of ADDLs is reportedly related to the production of reactive oxidative species as well as the breakdown of chemically reactive species, generating oxidative stress and inducing tau hyperphosphorylation and structural changes in the synaptic spine and ectopic redistribution of receptors critical in neuroplasticity and memory [18] [19]. Aβ peptide oligomer toxicity can also be related to their specific interactions with lipid headgroups in cellular membranes, including their ability to perturb membrane integrity, the formation of pores, and, possibly, amyloid channels [9] [10]. Therefore, a protective approach that inhibits binding of Aβ peptide oligomers to mature hippocampal neurons could also protect the synaptic spine receptors from ADDLs. Several studies have been performed with this aim, for example the use of insulin in synergy with rosiglitazone, a drug related to type II diabetes mellitus, and whose purpose is to regulate neuronal and synaptic functions in the hippocampus [20]. The relationship between AD and other diseases, such as type II diabetes, highlights a vicious circle that leads to health and social problems, among other negative implications, hence the importance of studying other mechanisms to reduce ADDL levels, such as the use of non-psychoactive phytocannabinoid derivatives to modulate

ADDL aggregation and, possibly, for sequestration.

Phytocannabinoids are natural compounds extracted from the cannabis plant and have been applied in various clinical settings owing to their antioxidant, neuroprotective, anti-inflammatory, and immunomodulatory benefits [21]. Compared to other cannabinoids, cannabidiol (CBD) [22] lacks cognitive and psychoactive actions, has a better safety profile in humans [21], and stands out as a promising drug for the treatment of AD [23], particularly in light of its ability to modulate oxidative stress and signaling [24]. CBD displays remarkable neuroprotective properties [12] [25] [26] [27], and appears to stimulate synaptic plasticity and facilitate neurogenesis [21]. Recently, CBD has been shown to reverse the acute effects of $A\beta_{1\text{-}42}$ and protect synaptic plasticity in an *in vitro* model of AD [10] [28]. CBD has also been shown to decrease the level of $A\beta_{40}$ in the hippocampus and reverse some AD-relevant deficits in the Aβ*PPxs*1 mouse model [29]. A recent review summarizes the use of cannabinoids as it relates to AD [30].

While findings highlight the therapeutic potential of CBD, whether CBD exerts a direct effect on the aggregation of Aβ remains unclear. To better understand the interactions between CBD and Aβ it is important to investigate the effect CBD has on amyloid peptide aggregation.

Atomic force microscopy (AFM) studies have provided insight into amyloid fibril formation and structure [31] [32] [33] [34] [35], including at early stages of aggregation [36] [37], and into the functional mechanical and electromechanical properties of amyloids [38]-[43] AD and other amyloid-related diseases are associated with the formation of β-sheet-rich structures, attributed to protein misfolding [44]. Amyloid peptide folding and aggregation, as well as fibril formation, can be influenced by various factors including solvent, temperature, pH, and ion concentration, as revealed through simulations [45]-[51] and experiments [52] [53] [54]. Interestingly, a recent molecular dynamics simulations study suggests strong interactions between CBD and short segments of Aβ peptides (*i.e.*, $A\beta_{25\text{-}35}$ and $A\beta_{31\text{-}35}$) [55], motivating our experimental investigation. Fibril inhibition has been observed by AFM with small molecules such as hydroxyindole and polyphenols [56] [57] [58] [59] [60]. Graphene oxide and other nanomaterials have also reportedly inhibited the assembly of peptide-based structures and modified their properties [53] [61]-[68]. A recent review summarizes the role of small molecules and nanomaterials on amyloid aggregation [69]. Here, we use AFM to examine the aggregation of ADDLs incubated in CBD solution *in vitro*. This preliminary study reveals the direct effects of CBD on Aβ aggregation and provides a basis for further understanding the neuroprotective mechanism of CBD and the potential of CBD as a candidate for developing new prophylactic and therapeutic approaches for AD.

2. Materials and Methods

2.1. Preparation of ADDLs

Human synthetic $A\beta_{1\text{-}42}$ peptide, synthesized and purified using reverse phase

HPLC by Dr. James Elliot at the ERI amyloid laboratory (Oxford, CT, USA; as used in other publications) [70] [71] [72] [73] was dissolved in ice-cooled 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) to form a 1 mM solution, which was sonicated for 10 minutes. After leaving this solution at ~20˚C for 1 hour, the HFIP was evaporated with a gentle stream of nitrogen in a fume hood to produce a thin film of $A\beta_{1\text{-}42}$, which was then dissolved in anhydrous DMSO with vortexing to produce a 2.5 mM stock solution. DMSO is widely used to prepare $A\beta_{1\text{-}42}$ stock solutions [70] [71] [72] [73] [74]. The resulting solution was diluted with ice-cooled phenol red-free Ham's nutrient mixture F12 (Ham's F12) to 100 μM, which was vortexed for 15 seconds, incubated at ~20˚C for 16 hours to allow Aβ oligomers to form [13], and then aliquoted and stored at −80˚C. To study the effect of CBD (obtained from STI Pharmaceuticals UK) on aggregation 15 μL of the solution was defrosted and diluted 200 times with Ham's F12 to a final volume of 3 mL. The solution was divided into three parts to which 0.5 μL Ham's F12, 0.5 μL of 10 mM CBD (dissolved in DMSO) or 0.5 μL DMSO were added, respectively, and incubated for 1 hour at ~20℃ before AFM measurements. The final concentration of CBD used was 5 μM.

2.2. Atomic Force Microscopy

Immediately prior to AFM imaging, each sample was diluted 25 times with MilliQ water. A 50 μL droplet of each sample was deposited on a freshly cleaved mica substrate for 15 minutes to adhere the ADDLs on the mica surface. The mica was then washed gently with MilliQ water and finally dried with a gentle steam of nitrogen. All samples were imaged in air using amplitude modulation mode (Cypher S, Asylum Research) with a Si tip (PPP-NCHR, Nanosensors). The tip radius is <10 nm with a nominal resonance frequency of ~330 kHz and a nominal spring constant of ~42 N/m. Each sample was imaged in two or three distinct positions.

3. Results and Discussion

Fibrillogenesis of Aβ is a multi-step self-assembly process. After dissolving in solution, Aβ peptides aggregate to form amyloid oligomers (ADDLs) with the structural transformation from random coils to amyloidogenic β-sheet structures [59] [75]. ADDLs are often classified by their toxicity—one type is generally considered toxic while the other is considered non-toxic [13]. Non-toxic ADDLs can further associate into nucleating cores, followed by a chain-like assembly to yield the intermediate protofibril, which subsequently form amyloid fibrils, and finally, plaques [13] [59] [75].

The effect of CBD on the aggregation of soluble ADDLs (*i.e.*, toxic ADDLs associated from $A\beta_{1\text{-}42}$ in Ham's F12 medium [13]) is investigated in **Figure 1**. CBD was prepared in DMSO due to its insolubility in water. The experimental group was incubated with 0.5 mM CBD (dissolved in DMSO), while an equal volume of Ham's F12 or DMSO was added to the other two samples as controls.

Representative 10 μm × 10 μm AFM topography images obtained for solutions of ADDLs 1 hour after adding Ham's F12, CBD (in DMSO), and DMSO are shown in **Figures 1(a)-(c)**, respectively. The surface roughness (mean and standard deviation from three locations for each sample) was similar for all three samples: Ham's F12 (389 ± 135 pm), CBD (315 ± 140 pm), and DMSO (293 ± 89 pm). Aggregates larger than 300 nm in size were only observed from the solutions incubated in the presence of CBD. Representative 1 μm × 1 μm images (**Figure 1(d)-(f)**) show that ADDLs, and aggregates in the case of CBD, readily adhere to the mica surface. The average circle equivalent diameters of the particles in **Figure 1(d)-(f)** are 16 ± 10 nm, 16 ± 8 nm, 24 ± 15 nm, respectively, excluding the aggregates in **Figure 1(e)**. The aggregate size in **Figure 1(e)** is 521.94 nm in length and 391.79 nm in width, and the circle equivalent diameter is 340.66 nm. The average aggregate size was determined to be 501 ± 364 nm in length and 500 ± 244 nm in width, with a circle equivalent diameter of 578 ± 166 nm. As aggregates did not form in DMSO (**Figure 1(a)**, **Figure 1(f)**), aggregation of ADDLs is attributed to the presence of CBD. Unlike with the DMSO used here, early-stage aggregation studies by AFM of ADDLs prepared in aqueous solutions revealed A$\beta_{1\text{-}42}$ structures [36] [37]. As we compared ADDLs in DMSO and ADDLs with CBD in DMSO separately in our investigation, we can attribute the aggregates to CBD even if the DMSO affects the kinetics of beta-amyloid aggregation. Nevertheless, future work should seek to utilize ADDLs and CBD prepared in aqueous solutions; the CBD used in this work was already dissolved in DMSO and so it was not possible to obtain entirely DMSO-free results.

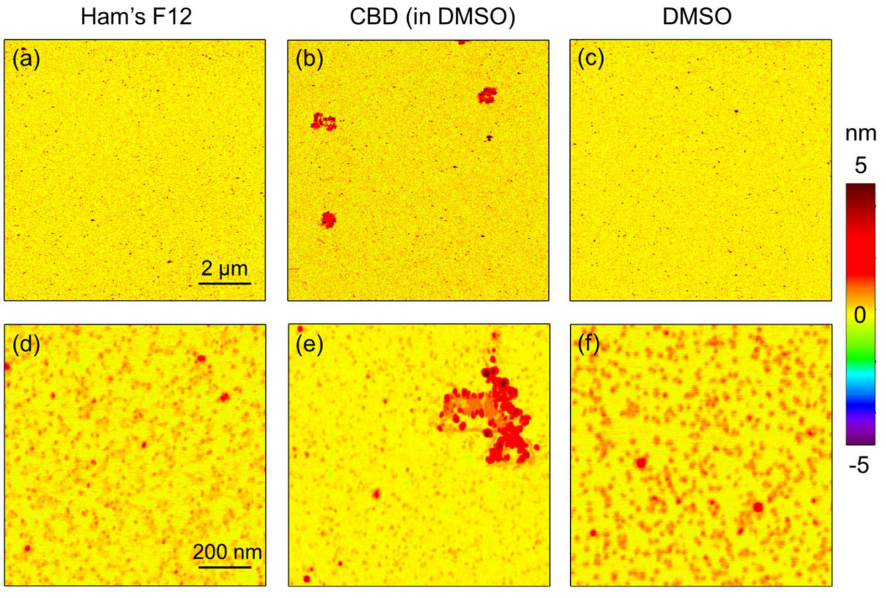

Figure 1. AFM topography images recorded from solutions containing A$\beta_{1\text{-}42}$ ADDLs 1 hour after adding ((a), (d)) Ham's F12, ((b), (e)) CBD (in DMSO), and ((c), (f)) DMSO and following the 15-minute incubation period on mica. Image sizes are ((a)-(c)) 10 μm × 10 μm and ((d)-(f)) 1 μm × 1 μm. Scale bar, 2 μm in upper three images and 200 nm in lower three images. Data are representative of the three locations investigated.

To confirm that the features observed in **Figure 1(b)** and **Figure 1(e)** e correspond to ADDLs, images of the Ham's F12 medium in the absence of A$\beta_{1\text{-}42}$ were also obtained. Following the same sample preparation and using the same concentration of Ham's F12, a roughness of 103 ± 27 pm (two locations) was obtained from the measured topography (**Figure 2(a)**) and particles of comparable size are present on the mica surface (18 ± 6 nm circle equivalent diameter, measured from **Figure 2(e)**). Notably, mica surfaces prepared with CBD in DMSO (**Figure 2(b)**) and DMSO alone (**Figure 2(c)**) in the absence of ADDLs and Ham's F12 have a similar surface with a low roughness surface of 50 ± 12 pm (three locations) and 58 ± 10 pm (two locations), respectively. Few particles can be found in 1 μm × 1 μm areas of these two samples (**Figure 2(f)**, **Figure 2(g)**). Surfaces prepared with Ham's F12 and CBD in DMSO, but in the absence of ADDLs also presented no obvious aggregates on the surface (**Figure 2(d)**). Even though the roughness of this sample (368 ± 47 pm) is higher than the other three, which are below 100 pm, the particle size is similar (18 ± 8 nm circle equivalent diameter, measured from **Figure 2(h)**) with Ham's F12. No aggregates like those appearing in **Figure 1(b)**, **Figure 1(e)** were observed in any of the 10 μm × 10 μm images obtained for surfaces prepared with Ham's F12 and CBD in DMSO. Thus, inferred from the results of **Figure 1** and **Figure 2**, CBD promotes the aggregation of ADDLs and Ham's F12 contributes background particles.

CBD reportedly protects the central nervous system against acute and chronic brain injury by suppressing astrocyte activity and decreasing proinflammatory signaling [76]. While CBD reportedly inhibits processes relating to Aβ-induced neurotoxicity in models of AD [76], our investigation highlights a direct effect of CBD on the aggregation of Aβ protein. The effect of cannabinoid on A$\beta_{1\text{-}42}$ aggregate formation was previously investigated by Janefjord *et al.* by transmission

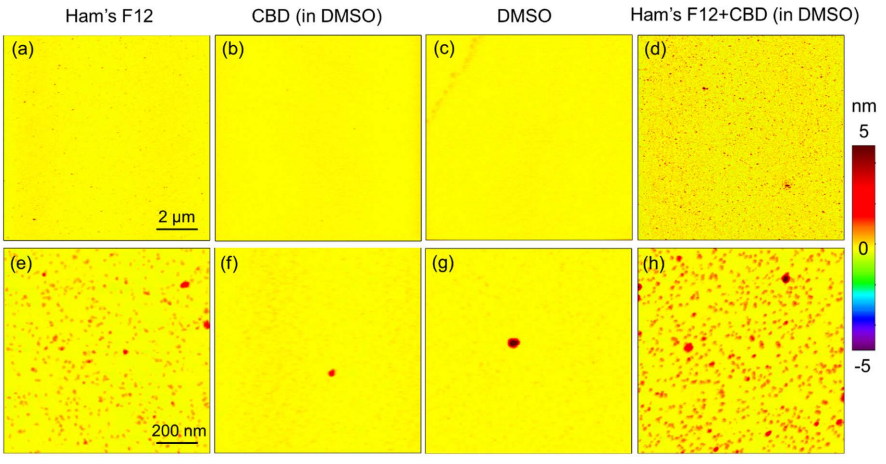

Figure 2. Representative ((a)-(d)) 10 μm × 10 μm and ((e)-(h)) 1 μm × 1 μm AFM topography images of mica surfaces prepared using ((a), (e)) Ham's F12 only, ((b), (f)) CBD in DMSO, ((c), (g)) DMSO only, and ((d), (h)) Ham's F12 and DMSO in CBD. None of the solutions contained A$\beta_{1\text{-}42}$ ADDLs.

electron microscopy [27]. They reported that CBD at a concentration of 10 μM did not alter the morphology of the $A\beta_{1\text{-}42}$ aggregate, whereas other cannabinoid ligands (2-arachidonoyl glycerol and O-1602) caused aggregation.

The aromatic amino acids of Aβ can enhance formation kinetics [57] [77]. As such, substances containing aromatic groups can affect Aβ aggregation and several small aromatic molecules, such as Congo red, resveratrol, catechin, and thioflavin T, have been demonstrated to inhibit the formation of fibrillar assemblies *in vitro* [57] [78] [79] [80] [81] [82] Catechol rings can interfere with the aromatic residues of Aβ by $\pi - \pi$ interactions [59] [77] [83]. Besides, aromatic molecules can remodel toxic ADDLs into multiple less-toxic conformations [77]. In addition to the catechol structure, hydrophobicity of the molecules may increase its affinity (via the repulsion of water) for binding with Aβ, which has a hydrophobic core [81] [84]. In this manner, CBD can alter the structure and morphology of amyloids and their formation kinetics. The results suggest that CBD transformed the (reportedly toxic [13]) ADDLs investigated here possibly into a conformation with reduced toxicity, forming aggregates and potentially preventing them from assembling into the structures that would otherwise form (*i.e.*, mis-aggregation). Nevertheless, given that $A\beta_{1\text{-}42}$ sample preparation is notoriously difficult to reproduce, in future work, experiments on toxicity should be conducted in parallel with AFM studies on the same samples which are supplemented by additional complementary characterizations, e.g., dynamic light scattering and mass spectrometry.

These findings reveal the effect of CBD on Aβ aggregation with implications for fibrillogenesis: CBD changes the conformation of ADDLs, potentially reducing the toxicity of soluble ADDLs. Similar strategies for reducing soluble ADDL toxicity could be pursued further (e.g., amyloid peptide sequestration facilitated by the presence of other natural compounds or engineered nanoparticles), and may help with the development of new prophylactic and therapeutic approaches for AD. The findings are in agreement with recent molecular modeling and simulation results that suggest that CBD can indeed modulate effectively the aggregation propensity of $A\beta_{25\text{-}35}$ and $A\beta_{31\text{-}35}$ [55]. Inspired by recent efforts to use small molecules for sequestration of Aβ as a new drug discovery strategy in AD [85], our study further highlights that AFM investigations of Aβ can be a useful tool to screen other compounds for therapeutic potential for the treatment of AD and other neurodegenerative and amyloid-associated diseases involving oligomeric peptide aggregates.

4. Summary

In this work, CBD is found to promote the aggregation of ADDLs. This preliminary study may contribute to our understanding of the neuroprotective function of CBD, and to the development of new strategies for drug development in Alzheimer's disease based on amyloid peptide sequestration. Future work should explore the effect of CBD concentration, including concentrations used *in vivo*,

the role of pH, as well as the impact of CBD on Aβ fibril formation, including A$\beta_{1\text{-}42}$.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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