

A Molecular Link between the Active Component of Marijuana and Alzheimer's Disease Pathology

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Received June 11, 2006

Abstract: Alzheimer's disease is the leading cause of dementia among the elderly, and with the ever-increasing size of this population, cases of Alzheimer's disease are expected to triple over the next 50 years. Consequently, the development of treatments that slow or halt the disease progression have become imperative to both improve the quality of life for patients and reduce the health care costs attributable to Alzheimer's disease. Here, we demonstrate that the active component of marijuana, Δ^9 -tetrahydrocannabinol (THC), competitively inhibits the enzyme acetylcholinesterase (AChE) as well as prevents AChE-induced amyloid β -peptide ($A\beta$) aggregation, the key pathological marker of Alzheimer's disease. Computational modeling of the THC–AChE interaction revealed that THC binds in the peripheral anionic site of AChE, the critical region involved in amyloidogenesis. Compared to currently approved drugs prescribed for the treatment of Alzheimer's disease, THC is a considerably superior inhibitor of $A\beta$ aggregation, and this study provides a previously unrecognized molecular mechanism through which cannabinoid molecules may directly impact the progression of this debilitating disease.

Keywords: Cannabinoids; Alzheimer's disease; acetylcholinesterase

Introduction

Since the characterization of the *Cannabis sativa* produced cannabinoid, Δ^9 -tetrahydrocannabinol (THC) (Figure 1), in the 1960s,¹ this natural product has been widely explored as an antiemetic, anticonvulsive, anti-inflammatory, and anal-

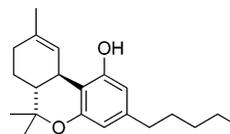


Figure 1. Chemical structure of Δ^9 -tetrahydrocannabinol (THC).

gesic.² In these contexts, efficacy results from THC binding to the family of cannabinoid receptors found primarily on central and peripheral neurons (CB_1) or immune cells (CB_2).³ More recently, a link between the endocannabinoid system and Alzheimer's disease has been discovered⁴ which has provided a new therapeutic target for the treatment of patients suffering from Alzheimer's disease.⁵ New targets for this

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debilitating disease are critical as Alzheimer's disease afflicts over 20 million people worldwide, with the number of diagnosed cases continuing to rise at an exponential rate.^{6,7} These studies have demonstrated the ability of cannabinoids to provide neuroprotection against β -amyloid peptide ($A\beta$) toxicity.^{8–10} Yet, it is important to note that, in these reports, cannabinoids serve as signaling molecules which regulate downstream events implicated in Alzheimer's disease pathology and are not directly implicated as effecting $A\beta$ at a molecular level.

One of the primary neuropathological hallmarks of Alzheimer's disease is deposition of $A\beta$ into amyloid plaques in areas of the brain important for memory and cognition.¹¹ Over the last two decades, the etiology of Alzheimer's disease has been elucidated through extensive biochemical and neurobiological studies, leading to an assortment of possible therapeutic strategies including prevention of downstream neurotoxic events, interference with $A\beta$ metabolism, and reduction of damage from oxidative stress and inflammation.¹² The impairment of the cholinergic system is the most dramatic of the neurotransmitter systems affected by Alzheimer's disease and, as a result, has been thoroughly investigated. Currently, there are four FDA-approved drugs that treat the symptoms of Alzheimer's disease by inhibiting

the active site of acetylcholinesterase (AChE), the enzyme responsible for the degradation of acetylcholine, thereby raising the levels of neurotransmitter in the synaptic cleft.¹³ In addition, AChE has been shown to play a further role in Alzheimer's disease by acting as a molecular chaperone, accelerating the formation of amyloid fibrils in the brain and forming stable complexes with $A\beta$ at a region known as the peripheral anionic binding site (PAS).^{14,15} Evidence supporting this theory was provided by studies demonstrating that the PAS ligand, propidium, is able to prevent amyloid acceleration in vitro, whereas active-site inhibitors had no effect.¹⁶ Due to the association between the AChE PAS and Alzheimer's disease, a number of studies have focused on blocking this allosteric site.¹⁷ Recently, we reported a combined computational and experimental approach to identify compounds containing rigid, aromatic scaffolds hypothesized to disrupt protein–protein interactions.^{18–20} Similarly, THC is highly lipophilic in nature and possesses a fused tricyclic structure. Thus, we hypothesized that this terpenoid also could bind to the allosteric PAS of AChE with concomitant prevention of AChE-promoted $A\beta$ aggregation.

Experimental Section

Docking Procedures. THC was docked to the mouse AChE structure (PDB ID code 1J07) using AutoDock 3.0.5.²¹ Twenty docking runs (100 million energy evaluations each) were run with a 26.25 Å × 18.75 Å × 26.25 Å grid box

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with 0.375 Å grid spacing. This grid box was designed to include regions of both the catalytic site and the peripheral anionic site. Otherwise, standard docking settings were used for the AutoDock calculations, as previously detailed.¹⁸

Acetylcholinesterase Inhibition Studies. All assays were performed using a Cary 50 Bio UV–visible spectrophotometer using an 18-cell changer, and conducted at 37 °C, using a Cary PCB 150 water Peltier system. Solutions of acetylthiocholine iodide (ATCh iodide) and 5,5'-dithiobis(2-nitrobenzoic) acid (DTNB) were prepared according to the method of Ellman et al.²² Stock solutions of acetylcholinesterase from *Electrophorus electricus* were prepared by dissolving commercially available enzyme in 1% gelatin. Prior to use, an aliquot of the gelatin solution was diluted 1:200 in water. For the assay, the solution was diluted until enzyme activity between 0.1 and 0.13 AU/min at 500 μM ACTh iodide was obtained. Compounds were prepared as solutions in methanol.

Assays were performed by mixing AChE, THC, and 340 μM DTNB in 100 mM phosphate buffer, pH 8.0, containing 5% methanol. Solutions were incubated at 37 °C for 5 min before the reaction was initiated by the addition of ATCh iodide (75–300 μM). The increase of absorbance at 412 nm was monitored for 2–5 min. All assays were run in triplicate. Initial rates were determined by subtracting the average observed initial rate from the nonenzymatic reaction.

Linear regression analysis of reciprocal plots of $1/v_0$ versus $1/[S]$ for four THC concentrations was performed using Microsoft Excel software. The slope was plotted against $[I]$ to give K_i values. Propagation of error was performed to determine the error, ΔK_i .

For studies to determine the mutual exclusivity of THC and propidium iodide, experiments were performed identically to THC inhibition studies with a fixed concentration of ACTh iodide (125 μM), and varied concentrations of propidium iodide (0–25 μM) and THC (0–15 μM).

AChE-Induced β-Amyloid Peptide Aggregation in the Presence of AChE Ligands. The aggregation of the β-amyloid peptide was measured using the thioflavin T based fluorometric assay as described by LeVine²³ and Bartolini.¹⁶ Assays were measured using a SpectraMAX Gemini fluorescence plate reader with SOFTmax PRO 2.6.1 software. $A\beta_{1-40}$ stock solutions were prepared in DMSO and HuAChE stocks prepared in distilled water. All stock solutions of $A\beta$ and HuAChE were used immediately after preparation.

In a 96-well plate, triplicate samples of a 20 μL solution of 23 nM $A\beta$, 2.30 μM HuAChE, and various concentrations

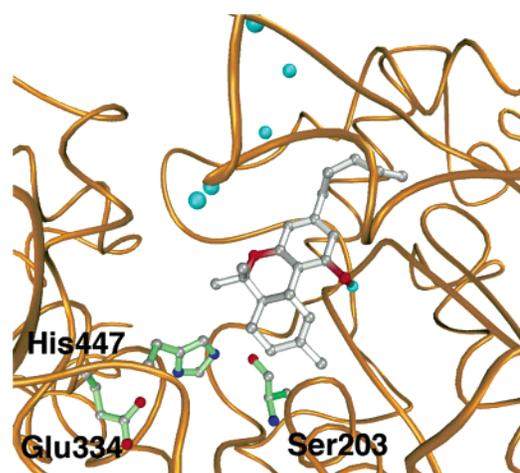


Figure 2. Predicted binding mode of THC (gray) to AChE (orange ribbon). The catalytic triad residues of AChE (green) and water molecules included in the docking calculations (light blue spheres) are shown.

of THC in 0.215 M sodium phosphate buffer, pH 8.0, were prepared. These solutions were incubated at room temperature along with triplicate solutions of $A\beta$ alone, $A\beta$ with AChE, and $A\beta$ with varying concentrations of THC. After 48 h, a 2 μL aliquot was removed from each well, placed in a black-walled, clear-bottomed 96-well plate, and diluted with 50 mM glycine–NaOH buffer, pH 8.5, containing 1.5 μM thioflavin T to a total volume of 200 μL. After incubation for 5 min, the fluorescence was measured using $\lambda_{exc} = 446$ nm and $\lambda_{em} = 490$ nm with excitation and emission slits of 2 nm. The fluorescence emission spectrum was recorded between 450 and 600 nm, with excitation at 446 nm.

The fluorescence intensities were averaged, and the average background fluorescence of buffer only, or buffer and THC, was subtracted. The corrected fluorescence values were plotted with their standard deviation. The equation, $F_i/F_0 \times 100\%$, where F_i is the fluorescence of AChE, $A\beta$, and THC, and F_0 is the fluorescence of AChE and $A\beta$, was used to quantify the extent to which each compound inhibits $A\beta$ aggregation. The Student's *t*-test function of Microsoft Excel was used to determine *p* values and assess statistical significance between reactions.

Control experiments containing AChE, THC, and thioflavin T or AChE and thioflavin T alone were also performed to ensure that any observed fluorescence decrease was not attributable to the molecular rotor properties of thioflavin T upon binding to AChE. For these reactions, all concentrations were identical to those used in the described $A\beta$ aggregation assays (vide supra).

Results and Discussion

THC binding to AChE initially was modeled in silico using AutoDock 3.0.5.²¹ Twenty docking runs with 100 million energy evaluations each were performed with a 26.25 Å × 18.75 Å × 26.25 Å grid box with 0.375 Å grid spacing, which included regions of both the catalytic site and the PAS. Examination of the docking results revealed that THC was

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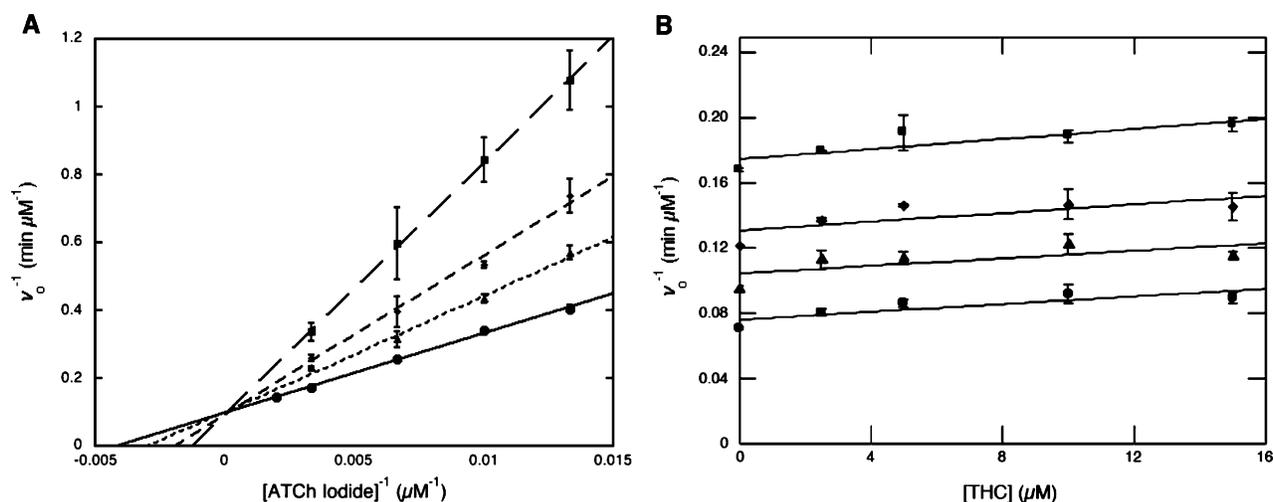


Figure 3. (A) Kinetic analysis of AChE inhibition by THC: 0 (●), 6.25 (▲), 12.5 (◆), and 25.0 μM (■). Steady-state kinetic analysis was performed using acetylthiocholine (75–300 μM) and Ellman's reagent (340 μM) at 37 °C. (B) Dixon plots of $1/v$ versus [THC] at different fixed concentrations of propidium iodide: 0 (●), 6.25 (▲), 12.5 (◆), and 25 μM (■).

predicted to bind to AChE with comparable affinity to the best reported PAS binders, with the primary binding interaction observed between the ABC fused ring of the THC scaffold and the Trp86 indole side chain of AChE (Figure 2). Further interactions were also evident between THC and the backbone carbonyls of Phe123 and Ser125. Encouraged by these results, we tested the ability of THC to inhibit AChE catalytic activity. Steady-state kinetic analysis of THC inhibition revealed that THC competitively inhibits AChE ($K_i = 10.2 \mu\text{M}$) (Figure 3A). This level of inhibition is relatively modest, yet it is important to note that inhibition of acetylcholine cleavage is not a prerequisite for effective reduction of $A\beta$ aggregation; indeed, most PAS binders are moderate AChE inhibitors displaying either noncompetitive or mixed-type inhibition.¹⁶ While THC shows competitive inhibition relative to the substrate, this does not necessitate a direct interaction between THC and the AChE active site. In fact, given the proximity of the PAS to the protein channel leading to the catalytic triad active site, it is possible to block substrate entry into the active site while bound to the PAS, thus preventing the formation of an ESI complex.^{18,24} In order to test this hypothesis, additional kinetic experiments were performed to determine the mutual exclusivity of THC and propidium, a well-characterized purely noncompetitive AChE inhibitor and PAS binder. Dixon plots of v^{-1} versus THC concentration at different fixed concentrations of propidium returned a series of parallel lines, indicating that THC and propidium cannot bind simultaneously to AChE (Figure 3B). Thus, these studies verify our docking results and demonstrate that THC and propidium are mutually exclusive PAS inhibitors. Additionally, recent reports have suggested that the selectivity of a given inhibitor for AChE over butyryl-

cholinesterase (BuChE) can be correlated with the ability of a compound to block AChE-accelerated $A\beta$ aggregation.^{25,26} Kinetic examination of BuChE inhibition revealed a slight reduction in enzymatic activity at high concentrations of THC ($\text{IC}_{50} \geq 100 \mu\text{M}$); however, these experiments were limited by the poor solubility of THC in aqueous solution.

The activity of THC toward the inhibition of $A\beta$ aggregation was then investigated using a thioflavin T (ThT) based fluorometric assay to stain putative $A\beta$ fibrils.²³ Using this assay, we found that THC is an effective inhibitor of the amyloidogenic effect of AChE (Figure 4). In fact, at a concentration of 50 μM , propidium does not fully prevent AChE-induced aggregation ($p = 0.03$, Student's t test), while THC completely blocks the AChE effect on $A\beta$ aggregation, with significantly greater inhibition than propidium ($p = 0.04$, Student's t test), one of the most effective aggregation inhibitors reported to date.¹⁶ However, the observed decrease in fluorescence could also be rationalized as a result of a competition between THC and ThT for the same site on AChE. It has been shown that ThT also can bind to the PAS and that this binding leads to an increase in fluorescence. Presumably, this phenomenon results from ThT serving as a molecular rotor in which fluorescence quantum yield is sensitive to the intrinsic rotational relaxation; thus, when molecular rotation is slowed by protein binding, the quantum

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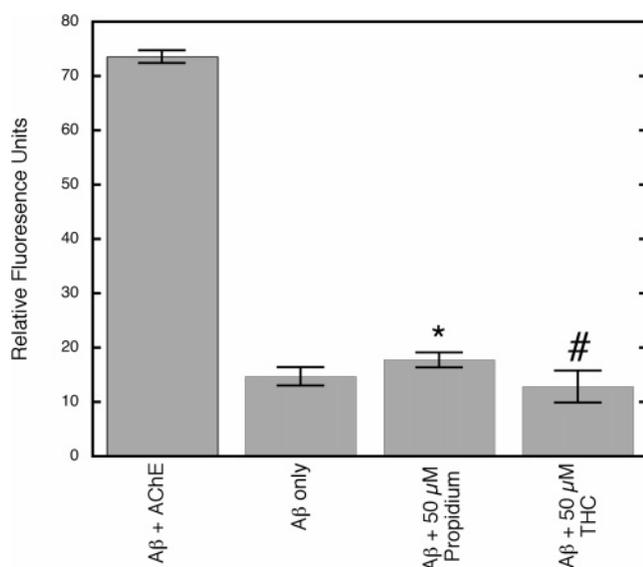


Figure 4. Inhibition of AChE-induced A β aggregation by THC and propidium ((*) $p < 0.05$ versus A β only; (#) $p < 0.05$ versus A β + propidium).

yield of the molecule can increase dramatically.^{27,28} In order to ensure that the observed fluorescence decrease was due to fibril inhibition, control experiments were performed using

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AChE, THC, and ThT. Reactions containing AChE and ThT alone showed the same fluorescence output as those containing AChE, THC, and ThT, providing convincing evidence that any observed reduction in fluorescence can be attributed to fewer A β fibrils.

Conclusion

We have demonstrated that THC competitively inhibits AChE and, furthermore, binds to the AChE PAS and diminishes A β aggregation. In contrast to previous studies aimed at utilizing cannabinoids in Alzheimer's disease therapy,^{8–10} our results provide a mechanism whereby the THC molecule can directly impact Alzheimer's disease pathology. We note that while THC provides an interesting Alzheimer's disease drug lead, it is a psychoactive compound with strong affinity for endogenous cannabinoid receptors. It is noteworthy that THC is a considerably more effective inhibitor of AChE-induced A β deposition than the approved drugs for Alzheimer's disease treatment, donepezil and tacrine, which reduced A β aggregation by only 22% and 7%, respectively, at twice the concentration used in our studies.⁷ Therefore, AChE inhibitors such as THC and its analogues may provide an improved therapeutic for Alzheimer's disease, augmenting acetylcholine levels by preventing neurotransmitter degradation and reducing A β aggregation, thereby simultaneously treating both the symptoms and progression of Alzheimer's disease.

Acknowledgment. This work was supported by the Skaggs Institute for Chemical Biology and a NIH Kirschstein National Research Service Award to L.M.E.

MP060066M