THE POTENTIAL THERAPEUTIC EFFECTS OF **THC** ON **ALZHEIMER'S** DISEASE

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The Potential Therapeutic Effects of THC on Alzheimer's Disease

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Abstract. The purpose of this study was to investigate the potential therapeutic qualities of Δ^9-tetrahydrocannabinol (THC) with respect to slowing or halting the hallmark characteristics of Alzheimer's disease. N2a-variant amyloid-β protein precursor (AβPP) cells were incubated with THC and assayed for amyloid-β (Aβ) levels at the 6-, 24-, and 48-hour time marks. THC was also tested for synergy with caffeine, in respect to the reduction of the Aβ level in N2a/AβPPswe cells. THC was also tested to determine if multiple treatments were beneficial. The MTT assay was performed to test the toxicity of THC. Thioflavin T assays and western blots were performed to test the direct anti-Aβ aggregation significance of THC. Lastly, THC was tested to determine its effects on glycogen synthase kinase-3β (GSK-3β) and related signaling pathways. From the results, we have discovered THC to be effective at lowering Aβ levels in N2a/AβPPswe cells at extremely low concentrations in a dose-dependent manner. However, no additive effect was found by combining caffeine and THC together. We did discover that THC directly interacts with Aβ peptide, thereby inhibiting aggregation. Furthermore, THC was effective at lowering both total GSK-3β levels and phosphorylated GSK-3β in a dose-dependent manner at low concentrations. At the treatment concentrations, no toxicity was observed and the CB1 receptor was not significantly upregulated. Additionally, low doses of THC can enhance mitochondria function and does not inhibit melatonin's enhancement of mitochondria function. These sets of data strongly suggest that THC could be a potential therapeutic treatment option for Alzheimer's disease through multiple functions and pathways.

Keywords: Alzheimer's disease, amyloid-β peptide, cannabinoid, CB1 receptor, CB2 receptor, delta(9)-tetrahydrocannabinol, neurodegeneration

INTRODUCTION

In 2011 alone, 15 million family members have provided more than 17.4 billion hours of care to diagnosed Alzheimer's disease (AD) patients. That care translates into more than $210 billion of AD-related services [1]. This disease translates into an enormous burden on caregivers, as well as the health care system, both medically and economically. To date, there have been no effective treatments developed to cure or delay the progression of AD [2, 3]. By 2050, an estimated 11 to 16 million Americans will be living with the disease [1, 4].

AD pathology can be divided into two categories, familial inherited AD and sporadic AD. The histopathologies of early onset familial AD and late onset sporadic AD are indistinguishable. Both forms of AD are characterized by extracellular amyloid-β (Aβ) peptide, and by amyloid plaques and tau-containing neurofibrillary tangles [3]. The misfolded structure of the Aβ peptides generates a characteristic tendency for their aggregation [5]. It has long been believed

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that $A\beta_{1-40}$ ($A\beta_{40}$) and $A\beta_{1-42}$ ($A\beta_{42}$) aggregates are the constituents of the insoluble plaques that are characteristic of AD. This disease is also associated with neuroinflammation, excitotoxicity, and oxidative stress [6, 7]. However, the continuous aggregation of Aβ peptides along with hyperphosphorylation of the tau protein inside the cell, causing neurofibrillary tangle formation, are generally accepted as the major etiological factors of the neuronal cell death associated with the progression of AD [8–10].

Recent studies have also suggested that glycogen synthase kinase 3 (GSK-3) has a key role in the pathogenesis of both sporadic and familial AD [11, 12]. It has been reported that GSK-3β induces hyperphosphorylation of tau [13–17]. Moreover, overexpression of GSK-3 in Tet/GSK-3β mice reveal pathological symptoms that correspond to AD pathology with respect to spatial learning deficits, reactive astrocytosis, increased Aβ production, and plaque associated inflammation, as well as tau hyperphosphorylation resulting in Aβ-mediated neuronal death [18]. Additionally, chronic lithium (GSK-3 inhibitor) treatment in double transgenic mice overexpressing GSK-3β and tau has shown to prevent tau hyperphosphorylation and neurofibrillary tangle formation [19]. Some reports have also indicated that GSK-3α plays a role in regulating amyloid-β protein precursor (AβPPP) cleavage, resulting in increased Aβ production [20, 21]. It has also been shown that the Aβ load in mouse brain can be robustly ameliorated by the inhibition of GSK-3β [22].

Along with past research suggesting an involvement of GSK-3 in the pathogenesis of AD, there have also been recent studies suggesting the intricate involvement of the cannabinoid system in AD. It was reported that the cannabinoid system can limit the neurodegenerative processes that drive the progression of the disease, and may provide a new avenue for disease control [23]. Currently the complete pathway and mechanism of action of the cannabinoid system are unknown, however, studies have been conducted to determine the involvement of the cannabinoid 1 (CB_1) and cannabinoid 2 (CB_2) receptors in AD brain [6]. The CB_1 receptor is abundant in the brain and contributes to learning, memory, and cognitive processes which are interrupted early in the course of AD [24]. To the contrary, CB_2 receptor expression is more limited and has been anatomically found in neurons within the brainstem [25], cerebellum [26], and microglia [27]. Recent research has also investigated the propensity of endocannabinoid receptor sub-types 1 (CB_1) and 2 (CB_2) to elicit a neuroprotective and anti-inflammatory effect on the brain when stimulated by endocannabinoids [28]. Postmortem studies of AD brains have detected increased expression of CB_1 and CB_2 receptors on microglia within the plaque, while CB_1 expression is reduced in neurons more remote from the plaque [29]. It is also noted that the endocannabinoid metabolizing enzyme, fatty acid amide hydrolase, is upregulated in the plaque [30]. There is also an increase in expression of anandamide metabolites, such as arachidonic acid, in the vicinity of the plaque [30]. These findings may indirectly suggest that the increase in CB_1 and CB_2 receptors may be to offset the lack of activity with their ligands due to increased metabolic activity of fatty acid amide hydrolase. These alterations in the cannabinoid system suggest an involvement of endogenous cannabinoids in the pathogenesis of AD or that this system may be altered by the pathophysiology of the disease [6]. Understanding that microglial activation is reserved in all cases of AD, it is important to identify that endogenous cannabinoids prevent Aβ-induced microglial activation both *in vitro* and *in vivo* [31]. These receptors are known to experience time dependent and brain region specific alterations during neurodegenerative and neuroinflammatory disorders to attempt to counteract excitotoxicity and inflammation [32].

Endocannabinoid receptors, CB_1 and CB_2, have been reported to interact with the endocannabinoid molecules: 2-arachidonoyl glycerol and anandamide. However, it has also been reported that CB_1 and CB_2 also react interact with Δ^9-tetrahydrocannabinol (THC) isolated from the *Cannabis sativa* plant [33]. Furthermore, early reports indicate that Dronabinol, an oil-based solution of Δ^9-THC, improves the disturbed behavior and stimulates appetite in AD patients [34], and alleviates nocturnal agitation in severely demented patients [35]. Accumulated evidence also suggests antioxidants having anti-inflammatory and neuroprotective roles [23].

It has also been shown that THC can decrease the level of Aβ-induced increases in reactive oxygen species, decreases in mitochondrial membrane potential, and caspase (a protein that is intimately involved in the regulation of apoptosis) activation, as well as protect human neurons from oligomeric Aβ-induced toxicity [36]. While it is understood that cannabinoids are active against inflammation, our research investigated the neuroprotective properties of THC, the active component of marijuana. Here we evaluated: 1) the effects of THC against Aβ expression in N2a/AβPPswe cells against the effects of caffeine, a reported Aβ expression suppressor [37]; 2) the direct effects of THC against Aβ aggregation, one pathological marker of AD; 3) the mechanism behind the anti-pathological properties of THC on AD; 4) the toxicity of THC and caffeine individually; and 5) the effects of THC on GSK-3β and other related signal pathways in N2a/AβPPswe cells.

MATERIALS AND METHODS

Drugs used in this study

THC solution was purchased from Sigma (T4764-1ML Sigma Aldrich); caffeine was purchased from Sigma (C0750-100G, Sigma Aldrich); melatonin was purchased from Sigma (M5250-5G, Sigma Aldrich).

ELISA for detection of total Aβ in protein samples

50 μl of goat anti-PWT1-42 antibody solution was added to the sample and incubated overnight, followed by a 1-hour incubation with 0.1% I-block buffer. The tissue culture supernatant was diluted 1:10 with diluent buffer containing a protease inhibitor. Standards (1000, 500, 250, 125, 62.5, 31.25 pg/ml) were prepared by serial dilution. The plate was washed and 50 μl of sample or standard was added with triplication. 50 μl of both Biosource 40/42 (HS) (primary antibody) Aβ and a standard solution was added to each well and incubated for 3 hours followed by 5× wash with PBST. 100 μl prepared secondary antibody (1:350 anti-rabbit HRP) was added and incubated at 37°C for 45 minutes on a shaker. The plate was washed; TMB substrate was added (100 μl) and incubated for 10–30 minutes in the dark. The reaction was halted by adding 100 μl stop solution for detection at 450 nm. A 4 parameter regression was used for the standard.

Cell culture and drug treatment

N2a/AβPPswe cells, N2a cells stably expressing human AβPP carrying the K670N/M671L Swedish mutation (AβPPswe), were grown in Dulbecco's modified Eagle medium containing 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 400 μg/mL G418 (Invitrogen), at 37°C in the presence of 5% CO_2. N2a/AβPPswe cells were diluted with medium to a concentration of 2×10^5/ml, and plated into the each well in 3 ml. 2 ml of trypsin was incubated at room temperature, or 37°C. When most of the cells began to float, trypsin was decanted and 5 ml of fresh pre-warmed medium was added. Pipetting was performed more than 30 times to ensure cells were separated into individual cells. One drop of medium was put into 1.5 ml tubes for counting; 10 μl of trypan blue and 10 μl of medium of cells were added and applied to cytometer for counting. The rule was total number of cells of all for diagonal blocks/4 X 2 X 10000 = number of cells/ml. The proper amount of cell medium and fresh medium was added into new flasks according to the ratio of dilution. Pipetting was performed 10 times to homogenize cells. 3 ml of cells were seeded into medium into each 6 well plate. When one pipette was used up, the cells were mixed in the flask before using them for the next pipette. Compounds for screening were resolved in DMSO, at 1000 fold to the final concentration in the well. Pipetting of 10 μl solution, then addition into 990 μl medium was performed; mixing followed. 12 hours after cells were plated, 400 μl of compounds were added into 3.6 ml medium. The medium was then removed from the six-wells. 3 ml of medium with 1% DMSO was added to well 1; in well 2, 3 ml melatonin solution was added. In well 3, 4, 5, and 6, compound solutions of 3 ml were added.

MTT assay

Cells were plated in 96-well tissue culture plate at 10,000 cells/well, 100 μl/well. 100 μl THC solution was added at 2× concentrations in each well. Control groups are: 1) cells without THC treatment, cells and fresh medium only; and 2) blank, wells with medium without cells. All wells were replicated. Wells were incubated for 36 hours. Cell proliferation kit (Roche 11465007001) was then applied for toxicity assay according to the standard protocol. 10 μl of MTT reagent was first added to each well and incubated at 37°C for 4 hours. Then 100 μl of solubilization solution was added to each well. These were incubated overnight and optical density (OD) values were read at 575 nm. The percentage of cell viability was calculated as: Cell viability% = (OD – OD blank) / (OD control – OD blank)

Western blot for anti-aggregation assay

HFIP pretreated $A\beta_{1-40}$ peptide were obtained from Biomer Technology, California. $A\beta_{1-40}$ peptide solution was prepared in Ham's F-12 solution to concentrations of 200 μM as stock. In the 15 μl aggregation system: 1) THC at final concentration of 25 nM, 2.5 nM, or 0.25 nM; and 2) 1.5 μl peptide stock solution was added. Then 15 μl with F-12 medium was made. Aggregation was allowed for 48 hours at 37°C. After incubation, isomers of Aβ peptide were separated by 12% Tris-Tricine gel electrophoresis at 100 V for 180–210 minutes, a temperatures under 4°C. The protein was transferred to PVDF membrane with semi-dry transfer at 200 mA for 70 minutes. For western blot detection, the membrane was blocked with 1.5% BSA solution in PBST solution (0.5%), then incubated in 1st Antibody: 6E10 (Signet) 1 mg/ml, diluted by 1:1000 dilution in blocking buffer. It was then washed 3 times with 1× PBST solution, and then incubated with second antibody (anti-mouse IgG-HRP sigma A9044. 1:5000 diluted in blocking buffer). After membrane was developed, film with bands were scanned, followed by analysis of gel-quantification software (QuantityOne, from Bio-rad).

Thioflavin T fluorescence assay

HFIP pretreated $A\beta_{1\text{-}40}$ peptide was obtained from Biomer Technology, California. In thioflavin T (ThT) solutions (1.6 μg/ml dissolved in 20 mM Tris-HCL), THC solution was prepared at concentration of 250, 25, 2.5, and 0.25 nM. THC solution was added containing ThT buffer into black 96 well plates. Unaggregated Aβ peptide solution was thawed, diluted, and immediately added to wells, making the final concentration of $A\beta_{1\text{-}40}$ at 1 μM. Control groups were setup as: 1) aggregation control; 2) control with ThT buffer only; and 3) Tris-HCl buffer only. Plate was mixed and fluorescence was read at 482 nm with excitation 440 nm with Biotek All-in-One plate reader. Fluorescence was screened for 2 hours with 5-minute intervals.

Western blot for total and phosphorylated GSK-3β, total tau and phosphorylated tau, and β-actin

Followed by THC treatment in tissue culture, N2a/AβPPswe cell lysate were collected, quantified, and aliquoted. Using 12% Tris-Glycine gel system (Biorad), protein were separated by electrophoresis and semi-dry transferred to PVDF membrane. GSK-3β and β-actin antibodies were used as primary antibody. After adding secondary antibody, the membranes were exposed using ECL substrate (Pierce). After membrane was developed, film with bands were scanned, followed by analysis of gel-quantification software (QuantityOne, from Biorad).

Mitochondria isolation and respiratory measurements

The respiratory function of isolated mitochondria was measured using a miniature Clark type oxygen electrode (Strathkelvin Instruments, MT200A chamber, Glasgow, UK). Detail method is published in Dragicevic et al. [38].

Statistical analysis and graphs

All data were analyzed with one-way ANOVA and *post hoc* analysis was conducted with Turkey's group analysis and $p < 0.05$ was considered as statistical significance (GraphPad 6.0). All graphs were graphed with GraphPad 6.0 software.

RESULTS

THC can decrease Aβ level in N2a/AβPPswe

ELISA assay was performed for $A\beta_{40}$ levels in N2a/AβPPswe cells 6 hours after cells were treated at different concentrations individually with THC, and caffeine—a reported compound to lower serum $A\beta_{40}$ levels in a mouse model [39]—showed a significant reduction in $A\beta_{40}$ levels of THC and caffeine versus the control (Fig. 1A). However, 24 hours after treatment of N2a/AβPPswe cells, $A\beta_{40}$ concentrations were measured again in the THC treated cells versus the control. An increasing difference in $A\beta_{40}$ concentrations were noted in both THC treated cells and caffeine treated cells in a dose-dependent manner (Fig. 1B). The assay was performed again, 48 hours after treatment of N2a/AβPPswe cells with THC versus the control at each concentration of the drugs originally used. THC-treated N2a/AβPPswe cells significantly differed more in $A\beta_{40}$ concentrations versus the control then at the 6- and 24-hour time point. The significant difference was conserved and greater over each increasing dose of THC and caffeine administered versus the control (Fig. 1C). These data suggest THC's and caffeine's inherent anti-$A\beta_{40}$ properties are time and dose dependent in N2a/AβPPswe cell models. This data also reveals that THC may delay of halt the progression of AD by inhibiting the production of $A\beta_{40}$ peptide in the central nervous system.

Synergy between THC and caffeine on $A\beta_{40}$ concentration in N2a/AβPPswe cells

THC and caffeine were assayed for a synergistic effect on $A\beta_{40}$ concentration in N2a/AβPPswe cells (Fig. 2). However, no synergistic properties of THC and caffeine are seen as there is no significant difference in the concentration of $A\beta_{40}$ in N2a/AβPPswe cells solely treated with THC as compared to cells

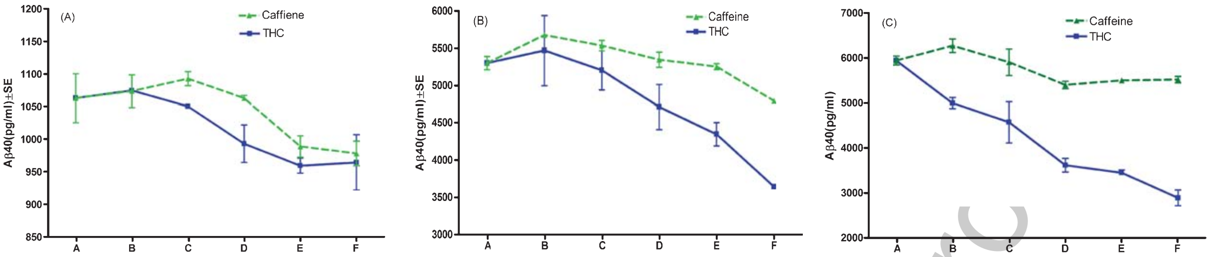

Fig. 1. (A) Aβ_{40} (pg/ml) *in vitro* measured 6 hours from incubation in N2a/AβPPswe cells. Three groups of cells were assayed: 1) those that were not treated with THC; 2) those that were treated with THC; and 3) those that were treated with caffeine. Treatment in both the THC group and in the caffeine group resulted in a dose-dependent decrease in Aβ_{40} concentration after 6 hours. There are no significant differences among all groups ($p > 0.05$). The concentrations of THC from A to F are 0 nM, 0.25 nM, 2.5 nM, 25 nM, 250 nM, and 2500 nM respectively, and concentrations of caffeine from A to F are 0 μM, 0.625 μM, 1.25 μM, 2.5 μM, 5 μM, and 10 μM, respectively, (B) Aβ_{40} (pg/ml) *in vitro* measured 24 hours from incubation in N2a/AβPPswe cells. A dose-dependent decrease in concentration of Aβ_{40} was still observed. THC: A, B, C, versus F are $p < 0.05$ and all other groups in comparison are $p > 0.05$. Caffeine: A versus B, B versus E, and all other groups versus F are $p < 0.05$. The concentrations of THC from A to F are 0, 0.25 nM, 2.5 nM, 25 nM, 250 nM, and 2500 nM, respectively, and concentrations of caffeine from A to F are 0, 0.625 μM, 1.25 μM, 2.5 μM, 5 μM, and 10 μM, respectively, (C) Aβ_{40} (pg/ml) *in vitro* measured 48 hours from incubation in N2a/AβPPswe cells. A dose-dependent decrease in Aβ_{40} (pg/ml) in conserved. THC groups: $p > 0.05$ for A versus B, and all other groups are $p < 0.05$. Caffeine groups: $p < 0.05$ for B versus D, and all other comparisons between groups are $p > 0.05$. The concentrations of THC from A to F are 0, 0.25 nM, 2.5 nM, 25 nM, 250 nM, and 2500 nM, respectively, and concentrations of caffeine from A to F are 0, 0.625 μM, 1.25 μM, 2.5 μM, 5 μM, and 10 μM, respectively.

treated with 2.5 μM caffeine and THC at various concentrations.

Repeated treatment can continuously decrease Aβ production

Our data also illustrates N2a/AβPPswe cells treated with THC twice, 24 hours apart from each treatment, showed a significant decrease in Aβ_{40} concentration compared to cells treated once (Fig. 3A). While the decrease in Aβ_{40} expression is not observed at concentration close to 10 μM, they are seen at 25 μM and greater suggesting multiple treatments may be efficacious in reducing Aβ_{40} concentration in N2a/AβPPswe cells and animal models.

Cell toxicity detection of THC on N2a/AβPPswe cells

THC was also measured for toxicity versus the caffeine and the untreated N2a/AβPPswe cells, which served as the control. The MTT assay showed no significant difference from the control for toxicity as compared to each concentration of THC and caffeine administer suggesting THC and caffeine lack toxicity to the cells at each concentration assayed (Fig. 3B).

THC can inhibit Aβ_{40} aggregation as shown by ThT assay and western blot

The ThT assay was to exhibit the direct interaction THC has with Aβ demonstrates that as the concentration of THC added to the assay was increased, the intensity of fluorescence in Aβ decreased. This data suggests that Aβ peptide directly binds to THC and prevents the uptake of fluorescence (Fig. 4A). Moreover, our lab performed an additional ELISA assay to confirm that the interaction of the Aβ peptide with THC did not shield amino acids 1–10, the major B-cell epitope [40] (Fig. 4B). There is no significant difference in absorbance at each concentration of THC, indicating that at each concentration of THC the Aβ antibodies were able to bind with equal distribution and affinity.

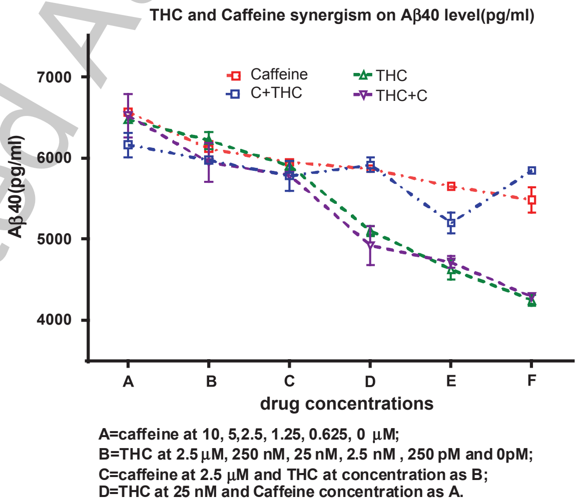

Fig. 2. Aβ_{40} (pg/ml) concentration in N2a/AβPPswe cells at various drug concentrations among groups. Treatment with both THC and caffeine resulted in a dose-dependent decrease in Aβ_{40} concentration. However, no synergistic effect was observed.

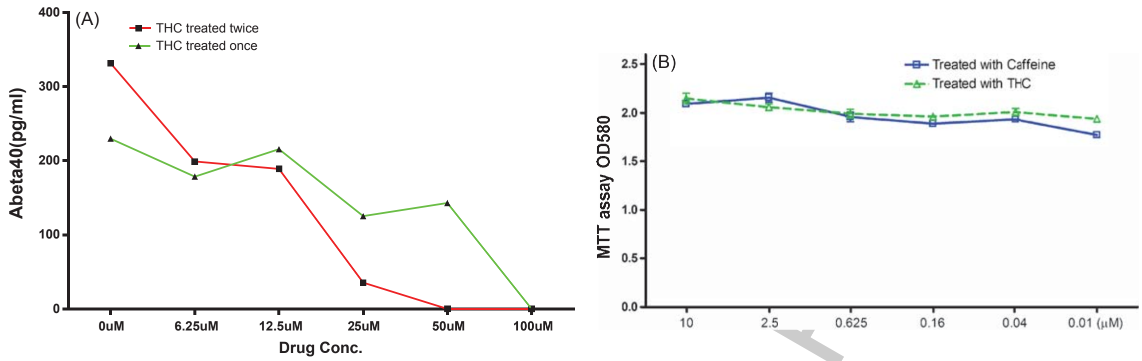

Fig. 3. (A) Aβ_{40} (pg/ml) concentration N2a/AβPPswe cells treated with THC, as well as the Aβ_{40} (pg/ml) concentration of N2a/AβPPswe cells treated with THC twice, 24 hours apart. The number of treatments has shown to decrease the concentration of Aβ_{40} (pg/ml), (B) This shows the data obtained from the reduction of MTT at different concentrations of THC versus the different concentration of caffeine. Untreated N2a/AβPPswe cells were also assayed to compare with the MTT reduction of N2a/AβPPswe cells treated with THC and caffeine at different concentrations.

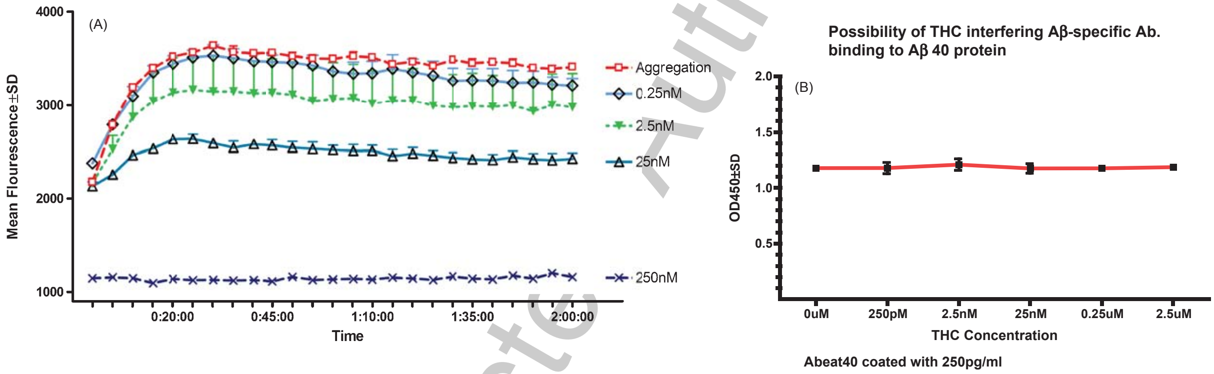

Fig. 4. (A) ThT assay measuring the fluorescence of Thioflavin T which binds to β-sheet structure of Aβ aggregation. With addition of THC, dose-dependent decreases in intensity of fluorescence indicates THC directly interferes with the binding of ThT to Aβ peptide. (B) THC incubated with Aβ peptide to determine the occurrence of THC interference with the major B cell epitope. No identified interference was observed at each increasing concentration of THC.

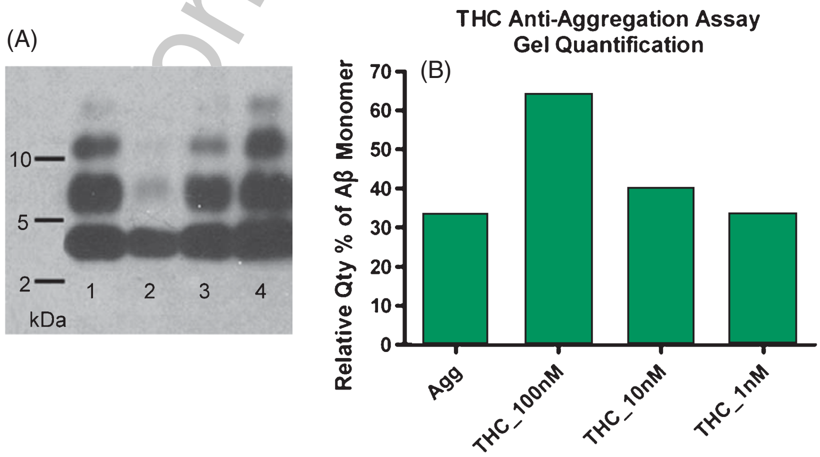

Fig. 5. (A) Polyacrylamide gel from a western blot indicating the concentration of aggregated Aβ peptide with and without the treatment of THC at various concentrations. Groups: 1: Aggregation control; 2: THC 100 nM; 3. THC 10 nM; 4: THC 1 nM, (B) THC anti-aggregation assay gel quantification indicating the relative percent monomeric Aβ.

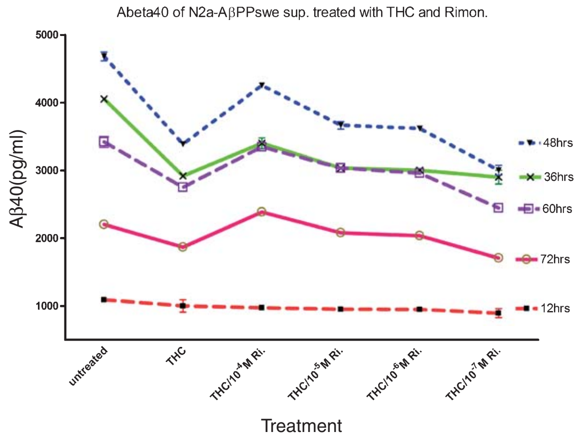

Fig. 6. ELISA assay elucidating a possible mechanism through which THC functions to decrease the synthesis of Aβ in N2a/AβPPswe cells. Aβ level increases at 36 hours and reaches its peak level at 48 hours. Follow this mark; it then starts decreasing at 60 hours. The drug treatment benefit time is seen at 36 hours and last to 48 hours (the best window time). THC can significantly lower Aβ and this function can be partially blocked by CB1 antagonist Rimon at 10^{-4} M. However, inhibition function is lost at 10^{-7} M.

Therefore, we can postulate that THC's direct interaction with the Aβ peptide will not dampen an immune response to clear the Aβ peptide.

Further analysis with western blot was performed measuring the anti-aggregation properties of THC with Aβ peptide. At each increasing concentration of THC, a higher relative % of Aβ monomer was observed correlating with a lower intensity of aggregated Aβ peptide. This data suggests the direct interaction of THC with Aβ peptide and its ability to bind to the peptide and inhibit aggregation (Fig. 5A,B).

CB1 receptor antagonist can partially rescue Aβ level inhibited by THC

An ELISA was performed to determine the mechanism of THC in supporting the reduction of Aβ in N2a/AβPPswe cells. A known inhibitor of the CB1 receptor, rimonabant, was mixed with THC at different concentrations. Untreated N2a/AβPPswe cell Aβ concentrations were used as a control. It was noted that a dose dependent increase in Aβ was observed as the concentration of the inhibitor was increased. A time dependent effect of the inhibitor was also witnessed as the assay was repeated at the 12-, 36-, 48-, 60-, and 72-hour mark (Fig. 6). Due to increasing Aβ concentrations as the inhibitor concentration is increased, this suggests that THC partially functions through the CB1 receptor to mediate the synthesis of Aβ. The RT-PCR results for CB1 receptor expression level showed that there is no significant upregulation by THC to CB1 receptor (data not shown).

THC can inhibit total GSK-3β and phosphorylated GSK-3β (pGSK-3β) production

The western blot assay performed to examine the effect of THC on GSK-3β exhibits a dose-dependent decrease in GSK-3β. β-actin, a housekeeping gene, was used as a control to indicate that GSK-3β was expressed at a constant rate and that the changes in intensity are not related to the change in expression amount. As shown in Fig. 7A-D, this data suggests that THC is efficacious in modulating and ameliorating the expression of GSK-3β and could decrease neuronal apoptosis by down regulating GSK-3β.

THC can inhibit phosphorylated (pTau) production, but not affect AβPP production

We detected pTau and AβPP levels among different treatment conditions. THC can lower pTau expression level with dose-dependent administration, but we did not see the differences in AβPP levels detected with 6E10 antibody (Fig. 8A-F).

THC can enhance mitochondrial function but will not interfere with melatonin's enhancement of the mitochondria

Isolated mitochondria from N2a/AβPPswe cells showed higher oxygen utilization when treated with THC. When combined with melatonin, the function of the mitochondria is not altered (Fig. 9A, B).

DISCUSSION

Advances in therapeutics to prevent AD, or delay the progression, are currently being made. Recent research has shown caffeine and coffee are effective in limiting cognitive impairment and AD pathology in the transgenic mouse model by lowering brain Aβ levels, which are thought to be central to the pathogenesis of AD [41]. Similarly, the current study shows the *in vitro* anti-Aβ activity of caffeine, and of another naturally occurring compound, THC.

N2a/AβPPswe cells were incubated separately with various concentrations of caffeine, melatonin, and THC. The relative anti-Aβ effect of THC was observed to increase in a time dependent manner. A dose-dependent decrease in Aβ concentration was noticed at lower concentrations of THC, as compared to caf-

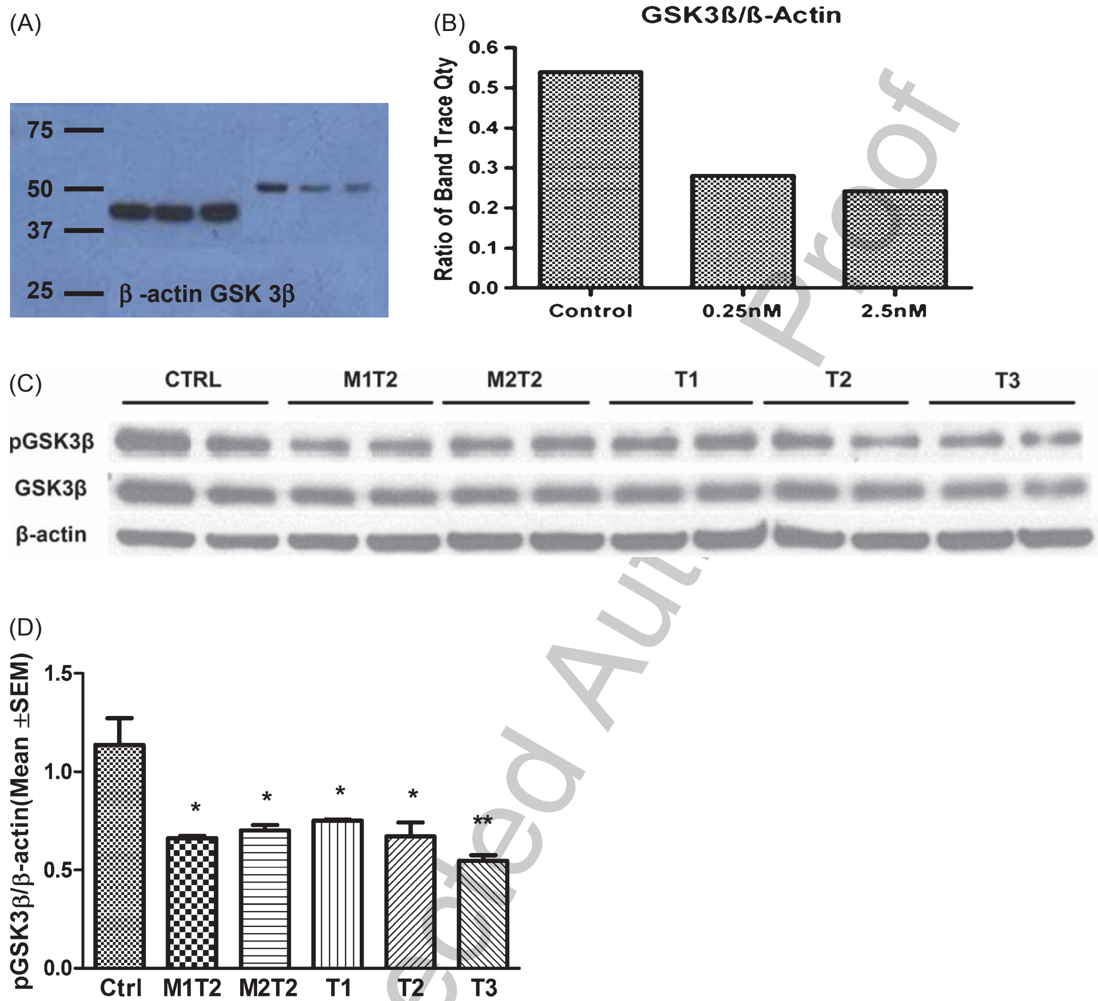

Fig. 7. (A) A western blot performed to determine the effects of THC on GSK-3β in N2a/AβPPswe. β-actin was used as a control to indicate that the expression rate was constant. The left indicator is molecular weight. Lane 1, 2, and 3 are β-actin level and lane 4, 5, and 6 are GSK-3β expression. 1 and 4 are cell controls, 2 and 5 are cells treated with 2.5 nM THC, and lane 3 and 6 are cells treated with 0.25 nM THC. THC can inhibit GSK-3β level at 2.4 nM concentration, (B) Graph representing the expression decrease in GSK-3β in a dose-dependent manner by using β-actin to obtain a value for the ratio of expressed GSK-3β. As shown in the bar graph, the total GSK-3β decrease after using β-actin standardized protein loading, (C) GSK-3β expression in N2a/AβPPswe treated with different drugs: Cells were plated in 6 well plate for overnight and then drugs were added into each designated wells in duplicate. Cells were lysed after 36 hours incubation. Proteins were loaded onto SDS-page gel and then blotted with each antibody after transfer onto PVDF membrane. Groups are: CTRL, Control; M1T2, 10^{-5} M Melatonin + 2.5 nM THC; M2T2, 10^{-6} M Melatonin + 2.5 nM THC; T1, THC 25 nM; T2, THC 2.5 nM; T3, THC 0.25 nM, (D) Expression of pGSK-3β following melatonin and THC treatment in N2a/AβPPswe cells. *The same batch protein samples were used in this test as in Fig. 7C. Bands were quantified. One-way ANOVA was applied to the data. $p < 0.05$ when compared with control group. $^{**}p < 0.01$ when compared with control group. Groups are: Ctrl, Control; M1T2, 10^{-5} M Melatonin + 2.5 nM THC; M2T2, 10^{-6} M Melatonin + 2.5 nM THC; T1, THC 25 nM; T2, THC 2.5 nM; T3, THC 0.25 nM.

feine. Further evidence shows that N2a/AβPPswe cells, treated twice with THC, show an even greater reduction in Aβ levels at slightly higher concentrations. Although it might have been predicted that caffeine and THC may function in a synergistic effect to reduce the Aβ load in N2a/AβPPswe cells, no synergy was observed.

The MTT assay confirmed that cells treated at efficacious concentration of THC showed no toxicity, suggesting such a treatment to be safe and effective for further experimentation in the AD animal model. However, valid arguments have transpired in recent times regarding the concern for acute and long-term memory impairment with the use of THC. It has been

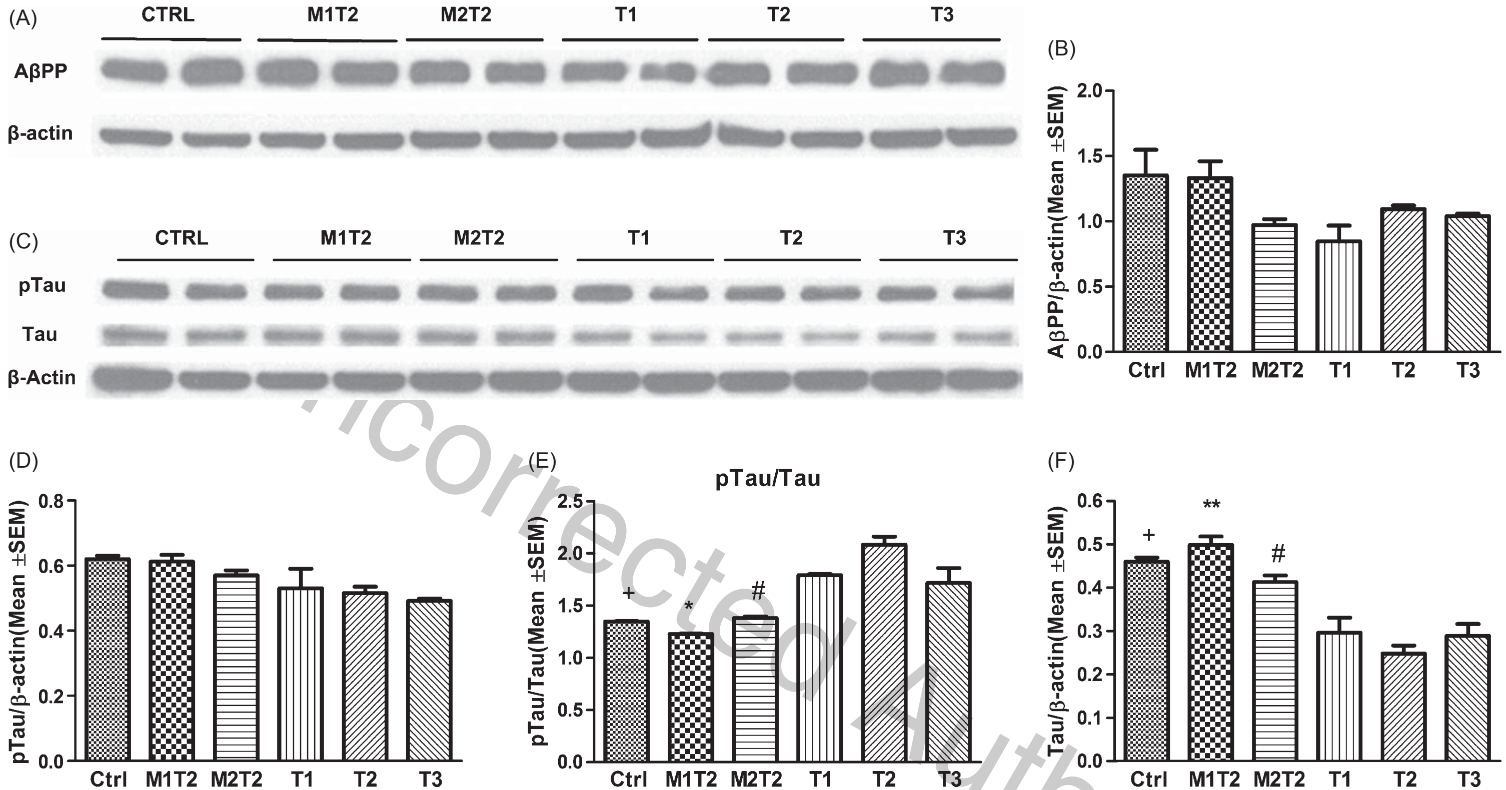

Fig. 8. (A) AβPP expression in N2a/AβPPswe treated with different drugs. The sample protein samples as in Fig. 7C were used for western blotting assay. 6E10 anti-Aβ antibody was used to detect AβPP and β-Actin was detected as protein loading control. Groups are: CTRL, Control; M1T2, 10^{-5} M Melatonin + 2.5 nM THC; M2T2, 10^{-6} M Melatonin + 2.5 nM THC; T1, THC 25 nM; T2, THC 2.5 nM; T3, THC 0.25 nM, (B) Quantification result of AβPP in western blotting: We used quantification method to further compare the differences among drug treatment to AβPP level. There are no statistical significant differences among all treatment ($p > 0.05$). This data indicates that THC did not change AβPP expression level. Groups are: Ctrl, Control; M1T2, 10^{-5} M Melatonin + 2.5 nM THC; M2T2, 10^{-6} M Melatonin + 2.5 nM THC; T1, THC 25 nM; T2, THC 2.5 nM; T3, THC 0.25 nM, (C) Tau expression in N2a/AβPPswe treated with different drugs. The sample protein samples as in Fig. 7C were used for western blotting assay. Anti-Tau and pTau antibodies were used to detect AβPP and β-Actin was detected as protein loading control. Groups are: CTRL, Control; M1T2, 10^{-5} M Melatonin + 2.5 nM THC; M2T2, 10^{-6} M Melatonin + 2.5 nM THC; T1, THC 25 nM; T2, THC 2.5 nM; T3, THC 0.25 nM, (D) No significant difference of pTau expression shown among six groups in N2a/AβPPswe cells. THC treatment has no function to pTau expression. Groups are: Ctrl, Control; M1T2, 10^{-5} M Melatonin + 2.5 nM THC; M2T2, 10^{-6} M Melatonin + 2.5 nM THC; T1, THC 25 nM; T2, THC 2.5 nM; T3, THC 0.25 nM, (E) Expression of pTau/Tau following melatonin and THC treatment in N2a/AβPPswe cells. $^{+}p < 0.05$ when compared with THC 25 nM and THC 0.25 nM groups. $^{*}p < 0.01$ when compared with THC 25 nM, THC 2.5 nM, and THC 0.25 nM groups. $^{\#}p < 0.05$ when compared with THC 25 nM and THC 2.5 nM groups. Groups are: Ctrl, Control; M1T2, 10^{-5} M Melatonin + 2.5 nM THC; M2T2, 10^{-6} M Melatonin + 2.5 nM THC; T1, THC 25 nM; T2, THC 2.5 nM; T3, THC 0.25 nM, (F) Expression of tau following melatonin and THC treatment in N2a/AβPPswe cells. $^{+}p < 0.05$ when compared with the THC 25 nM, THC 2.5 nM, and THC 0.25 nM groups. $^{**}p < 0.01$ when compared with the THC 25 nM, THC 2.5 nM, and THC 0.25 nM groups. $^{\#}p < 0.05$ when compared with THC 2.5 nM group. Groups are: Ctrl, Control; M1T2, 10^{-5} M Melatonin + 2.5 nM THC; M2T2, 10^{-6} M Melatonin + 2.5 nM THC; T1, THC 25 nM; T2, THC 2.5 nM; T3, THC 0.25 nM.

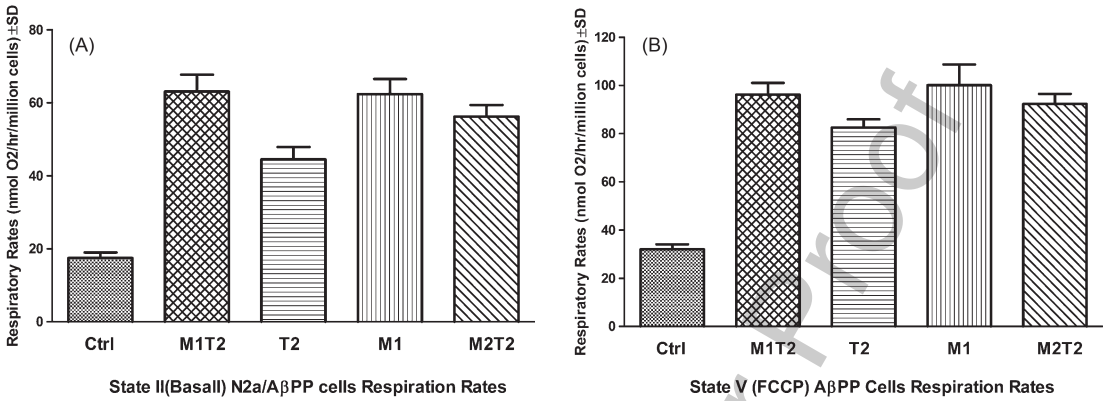

Fig. 9. (A) The enhancement of mitochondria function to cells treated with different: N2a/AβPPswe cells were cultured in 10 cm tissue culture plate and then treated with drugs for 36 hours and mitochondria were harvested and tested for their ability of using oxygen utilization. Ctrl, Control; M1T2, 10^{-5} M Melatonin + 2.5 nM THC; M2T2, 10^{-6} M Melatonin + 2.5 nM THC; T2, THC 2.5 nM; M1, 10^{-5} M Melatonin, (B) The enhancement of mitochondria function to cells treated with different: N2a/AβPPswe cells were cultured in 10 cm tissue culture plate and then treated with drugs for 36 hours and mitochondria were harvested and tested for their ability of using oxygen utilization. Ctrl, Control; M1T2, 10^{-5} M Melatonin + 2.5 nM THC; M2T2, 10^{-6} M Melatonin + 2.5 nM THC; T2, THC 2.5 nM; M1, 10^{-5} M Melatonin.

Table 1
Difference and percent decrease of $A\beta_{40}$ (pg/ml) in THC treated cells at 2.5 μg/ml compared with the control at different time points

Time Point	6 h	24 h	48 h
Control	1064.025	5303	5935.525
THC 2.5 μg/ml	965.827	3648.975	2894.175
Percentage of decreased $A\beta_{40}$	9.23%	31.19%	51.24%

shown that memory impairment was identified in rats treated with THC [42]. It should be clear, however, that the memory impairment observed occurred at concentrations more than a thousand times higher than what is presented here as a beneficial treatment in AD model N2a/AβPPswe cells. The concentrations used in the study are considered to be extremely low, as the concentrations that we focused on in the study were from 2.5 nM of THC down to 0.25 nM of THC. Although some studies with ultra-low doses of THC have indicated neurotoxic roles [42], newer research shows a neuroprotective role and actually promotes elevation of phosphorylated cAMP response element-binding protein (pCREB) by increasing the levels of brain-derived neurotrophic factor in the frontal cortex [43]. Furthermore, the dosing used in our study is a lower concentration than that in the aforementioned research. Therefore, we believe that THC has a therapeutic value, and that at low enough doses, the potential benefits strongly prevail over the risks associated with THC and memory impairment.

In addition to the Aβ concentration suppression, benefits of THC, analyzed with a western blot and ThT assay, confirmed anti-Aβ aggregate properties by a dose-dependent decrease in fluorescence uptake, and a decrease in intensity of aggregated Aβ in a dose-dependent manner. The positive results suggest possible intermolecular force interactions, preventing the molecular aggregation of Aβ peptides. The conducted ELISA, to ensure the intermolecular interaction of THC with Aβ did not block the major B-cell epitope, showed no interference with antibody binding, which indicated that regardless of the molecular interaction of THC with Aβ, an immune response should not be inhibited.

One pathway in which THC function was shown through the cannabinoid receptor inhibition with rimonabant. The dose- and time-dependent increase of Aβ with respect to CB_1 inhibition was noted. It is likely that the time deference was observed due to the slow interaction of rimonabant with the CB_1 receptor. However, the difference in Aβ concentration becomes more evident at the later time points. Lastly, we showed a dose-dependent decrease in GSK-3β expression influenced by THC.

To date, no Aβ specific therapeutic options for AD have been approved. While progression is being made in this field, rigorous efforts focus on developing compounds that can address or possess the inhibition of Aβ synthesis and anti-Aβ aggregation properties or characteristics that down regulate GSK-3β and pGSK-3β. Our results demonstrate that THC possesses all of the above mentioned properties. All of these areas address major etiological characteristics of AD. GSK-3β, pGSK-3β, and Aβ-plaque brain concentrations, the hallmark of AD, are major targets for current AD research. Furthermore, we have shown that THC functions are pathway dependent of the endogenous cannabinoid CB_1 receptor recently discovered to possibly function in AD disease modulation by suppressing microglial activation upon receptor interaction. Notwithstanding, it should also be noted that low doses of THC are used to address the above mentioned targets, thus avoiding risks induced by THC associated with memory impairment and risks associated with toxicity. In addition, we also discovered that low doses of THC can also enhance mitochondria function and has no negative drug interactions to melatonin, a potential therapeutic for AD.

Here we have presented a promising compound that addresses many major targets for AD therapeutics currently being research. We have shown THC, at an extremely low dose level (2.5 nM), has the proclivity to slow or halt AD progression by dampening the synthesis of the major pathological marker of AD, Aβ. Also, our lab has elucidated a potential mechanism responsible for the anti-pathological properties of THC with respect to AD. Furthermore, we have clearly exhibited lack of toxicity at low concentrations of both THC and caffeine individually. In conclusion, we believe the multifaceted functions of THC will ultimately decrease downstream tau hyperphosphorylation and neuronal death thereby halting or slowing the progression of this devastating disease.

DISCLOSURE STATEMENT

Authors' disclosures available online (http://www.j-alz.com/disclosures/view.php?id=2309).

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