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Antroquinonol administration in animal preclinical studies for Alzheimer's disease (AD): A new avenue for modifying progression of AD pathophysiology

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ABSTRACT

Despite the rise of Alzheimer's disease (AD) in an ageing population, no cure is currently available for this disorder. This study assessed the role of a natural compound, Antroquinonol, in modifying the progression of AD when administered at the start and/or before appearance of symptoms and when the disease was well established, in a transgenic animal model. Antroquinonol was administered daily for 8 weeks, in 11 week (early stage) and 9 month (late stage) male transgenic mice (3 times Transgenic mice $PS1_{M146V}$, APP_{Swe}, and tau_{P301L}, 3 Tg XAD) and their respective aged controls. Behavioural testing (including Elevated Plus Maze Watermaze, Recognition object testing and Y maze) was performed at the end of the drug administration. In addition AD biomarkers (Amyloid beta 42 (Aβ42), tau and phospho-tau levels), oxidative stress and inflammatory markers, were assessed in tested mice brains after their sacrifice at the end of the treatment. When administered before the start of symptoms at 11 weeks, Antroquinonol treatment at 34 mg/kg (D2) and more consistently at 75 mg/kg (D3), had a significant effect on reducing systemic inflammatory markers (Interleukin 1, IL-1β and TNF-α) and AD biomarker (Amyloid Beta 42, Aβ42 and tau) levels in the brain. The reduction of behavioural impairment reported for 3TgXAD mice was observed significantly for the D3 drug dose only and for all behavioural tests, when administered at 11 weeks.

Similarly, beneficial effects of Antroquinonol (at higher dose D3) were noted in the transgenic mice in terms of AD biomarkers (tau and phosphorylated-tau), systemic inflammatory (IL-1β), brain anti-inflammatory (Nrf2) and oxidative (3-Nitrotyrosine, 3NT) markers. Improvement of memory impairment was also reported when Antroquinonol (D3) was administered at late stage (9 months).

Since Antroquinonol has been used without adverse effects in previous successful clinical trials, this drug may offer a new avenue of treatment to modify AD development and progression.

1. Introduction

With a growing ageing population, Alzheimer's Disease (AD), the most common form of dementia, has been rising for the last few decades, reaching an overwhelming 50 million worldwide. AD is a progressive neurodegenerative disease clinically characterized by gradual cognitive decline including loss of memory, orientation and reasoning, along with behavioural disturbances (Davis et al., 1999). AD hallmarks in the brain include the aggregation of amyloid plaques and neurofibrillary tangles (NFTs) associated with an increase of oxidative stress and neuro-inflammation (Dickson, 1997). The sequential cleavage of the amyloid precursor protein (APP) by enzymes, β-secretase and γ-secretase beta, yields an excessive production of extracellular Aβ peptides, leading to the formation of amyloid plaques or senile plaques (Spires-Jones and Hyman, 2014). Along with these plaques, the hyperphosphorylation of tau provokes its detachment from the microtubules, and forms tangles

Abbreviations: **AMPK**, 5′ adenosine monophosphate-activated protein kinase; **APP**, Amyloid precursor protein; **APPβ**, secreted amino-terminals APPβ fragment; **cdck5**, cyclin dependant kinase 5; **CTFβ**, carboxyterminal fragment-β; **Aβ**, amyloid-β peptides; **AICD**, APP Intracellular Cytoplasmic/C-terminal Domain; **sAPPα**, secreted amino-terminals APPα fragment; **CTFα**, carboxyterminal fragment-α; **GSK3β**, Glycogen synthase kinase 3 beta; **IFNγ**, Interferon gamma; **IL-1**, Interleukin 1; **IL-6**, Interleukin 6; **MAPK**, Mitogen activated protein kinase; **NF-kB**, nuclear factor kappa-light-chain-enhancer of activated B cells; **NMDA Rc**, methyl-D-aspartate receptors receptor; **TNF-α**, Tumor Necrosis factor alpha; **ROS**, reactive oxygen species.

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inside neurons (Spires-Jones and Hyman, 2014). The presence of NFTs critically disturbs the intracellular transport leading, along with senile plaques, to neurodegeneration and consequently an imbalance of neurotransmitters (acetylcholine, serotonin, and glutamate) in the region (Spires-Jones and Hyman, 2014; Yiannopoulou and Papageorgiou, 2020). There are only two classes of drugs approved to treat AD which includes acetylcholinesterase (AChE) inhibitors (donepezil, galantamine, and rivastigmine) and N-methyl-D-aspartate receptor antagonists (NMDA; memantine). The inhibition of AchE for AD treatment is based on the cholinergic hypothesis suggesting a progressive degeneration of cholinergic neurons in the basal forebrain and temporal lobe, both critical regions for learning and memory (Yiannopoulou and Papageorgiou, 2020; Anand et al., 2017). For moderate to more severe forms of the disease, memantine, preventing the overactivation of NMDA receptors and consequently glutamatergic neurodegeneration, can also be administered in combination with donepezil (Cummings et al., 2019; van Bokhoven et al., 2021). Thousands of drugs are currently tested in preclinical studies using animal models, with many of these studies involving genetically modified mouse models for AD (Scearce-Levie et al., 2020). The recent production of genetically modified mice now also allows the exploration *of* genetic risk factors in the context of different biological challenges and drug discovery (Cacabelos et al., 2021). While hundreds of drugs have demonstrated preclinical efficacy for ameliorating cognitive impairment in animal models, none have yet confirmed efficacy in human clinical trials (Scearce-Levie et al., 2020). More recently, Aducanumab, an engineered monoclonal antibody able to traverse the brain blood barrier and bind directly to Aβ peptides, was approved through the Food and Drug Administration (FDA) accelerated program to be administered to patients before obtaining the results of the phase 4 clinical trial planned to be available in 2030 (Sevigny et al., 2016). However, its capacity to affect clinical symptoms such as reduction of cognitive decline is yet to be established (Sevigny et al., 2016).

Although there have been many preclinical studies and clinical trials for the prevention and/or treatment of AD, much of the current therapy aims at reducing Alzheimer's symptoms, which is mostly effective for mild to moderate forms of the disease (Tam et al., 2019). A focus on disease-modifying treatments or therapies have recently increased with research aimed at altering the progression of AD by working on several pathophysiological mechanisms-in contrast to traditional therapy which is focused in reducing AD symptoms (Breijyeh and Karaman, 2020).

Emphasis on emergent natural medicine for modifying AD progression has been rising over the last two decades, potentially due medicinal plants offering synergistic effects on AD pathophysiological pathways (Tam et al., 2019; Cassidy et al., 2020; Mancuso et al., 2012). Compounds extracted from leaves, flowers, roots and fungi have been tested for a variety of conditions for their anti-inflammatory, anti-oxidant and/or potential anti-amyloidogenic activity in sporadic AD, with these sometimes presenting fewer side effects compared to synthetic counterparts (Wei, 2016; Sun et al., 2013).

More than 160 compounds have been extracted from the rare Taiwenese fungus named *Antrodia Camphorata* (or *Antrodia cinnamomea* or *Taiwanofungus camphoratus*) and studied for their various potential biological activities including anti-cancer, anti-oxidant, anti-inflammatory, anti-diabetic, anti-microbial, anti-ageing, hepatoprotective, neuroprotective, anti-hypertensive, anti-hyperlipidemic, and immunomodulatory properties (Lu et al., 2013; Senthil Kumar et al., 2020).

Among the phytochemicals extracted, Antroquinonol (trade name Hocena), a tetrahydro-ubiquinone, constitutes the first drug granted orphan drug status by the FDA for the treatment of liver and pancreatic cancer, as well as acute myeloid leukemia (Angamuthu et al., 2019). It has also been approved for Phase 2 clinical trials in non-small cell lung cancer (NSCLC) and more recently in a Phase 2 clinical trial for treating pneumonia induced by Covid-19, due to its anti-viral, anti-inflammatory and anti-fibrotic actions reported in preclinical studies (Villaume et al., 2016).

A previous study has also shown an anti-amyloidogenic effect of Antroquinonol, along with an improvement of learning and memory ability in the Morris test in a transgenic animal model of AD (carrying mutations in *APP* gene) (Chang et al., 2015). Since the use of APP transgenic mice in preclinical studies explores only the effects of the drug in the accumulation of amyloid-β plaques in the brain, it is important to test Antroquinonol in an animal model of AD which also includes tau dysfunction in the context of drug discovery. Consequently, we have here explored the anti-inflammatory, anti-oxidant and anti-amyloidogenic properties of Antroquinonol in an animal model for AD presenting both APP and tau dysfunction. We have also evaluated the potential of Antroquinonol to reduce AD brain biomarkers, as well as learning and memory impairment, when administered at early (preventive effects) and late stage (therapeutic effects) AD pathology, using a well-established transgenic mouse model for this disease.

2. Materials and methods

2.1. Animals and ethical considerations

Since tau pathology is similar in both genders (Hirata-Fukae et al., 2008) and to avoid any confounding effect of estrogen and/or progesterone on tested markers and behavioural tests (Villa et al., 2015a; Frick et al., 2018; Tuscher et al., 2015; Morimoto et al., 2008), a male mouse 3 times Transgenic TG (Pre-Selenin $PS1_{M146V}$, APP_{Swe}, and tau_{P301L}) animal model of AD (3TgXAD) and related controls (Bl6-129SF2/J) were chosen for this study and obtained from the Jackson Lab, USA. Both estrogen and progesterone can have some positive effects on inflammation, oxidative stress and on working (Frick et al., 2018; Tuscher et al., 2015; Morimoto et al., 2008; Villa et al., 2015b). An *in vitro* study showed a decrease of Aβ peptide generation in cultured cells and primary neurons from estrogen-deprived mice, when estrogen was administered in comparison to no estrogen supplied cultures (Xu et al., 2006). In addition, when estrogen-deprived mice were treated with estrogen, this treatment reversed the elevated levels of brain Aβ peptides in mouse brains (Xu et al., 2006), reinforcing the choice of including males only in this study.

A breeding colony was rapidly established to provide animals at 11 weeks (for analysing the effects of Antroquinonol administration prior and/or at the start of AD pathology-preventive study) and at around 9 months of age (for analysing the effects of Antroquinonol administration when AD pathology is well-established- therapeutic study). The mice were housed (1–5 max per cage) in a pathogen-free laboratory with *ad libitum* access to food and water with a 12-h light-dark cycle at constant pressure, temperature and humidity at the Medical Engineering Research Facility, QUT, Brisbane, Queensland, Australia. Mice were provided with environmental enrichment including weekly rotated tissue paper and wood chunks, a nesting box and sunflower seeds for foraging.

All experimental procedures were approved by the Animal Ethics Committee, Queensland University of Technology, and conducted in accordance with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes* (2004) as well as with the principles of laboratory animal care (in: th (Ed.) and Guide for t, 2011).

2.2. Antroquinonol administration

Antroquinonol was isolated and characterized, as detailed in a previous study (Lee et al., 2007). Different drug concentrations of Antroquinonol were prepared freshly on a weekly basis, for daily administration for 8 weeks.

To assess the role of Antroquinonol administered at an early stage, 11 week old male 3TgXAD mice and their related controls were weighed and assigned into different groups (6 treatment groups per strain including VEH (vehicle for Antroquinonol which was corn oil), dose 1 of Antroquinonol at 7 mg/kg (D1); dose 2 of Antroquinonol at 34 mg/kg (D2) and dose 3 of Antroquinonol at 75 mg/kg (D3). In addition to these four groups per strain, a positive control group of mice received Allopregnanolone (ALLO) (Sigma Aldrich, Australia; 10 mg/kg, *s.c.* a week before behavioural testing) due to its ability to reverse the neurogenic and

cognitive deficits observed in the 3TgXAD mice, as shown in a previous study when administered 1 week before behavioural testing (Wang et al., 2010). Since the strain of mice and similar behavioural tests to assess memory deficits were used in this study, allopregnanolone was appropriate to use in our study with same dosage and protocol of administration as previously published (Wang et al., 2010). Non treated 3TgXAD and their related controls were also included in the study.

To evaluate the role of Antroquinonol administered at late stage of pathology, the same arrangement of mice groups occurred with mice aged around 8–9 months at the start of the drug administration (see Fig. 1).

For each respective study, a total of 12 groups of mice (n = 8 to 18 for each respective group) was tested. Animals were handled daily in order to get used to human contact before starting the administration of the drugs/vehicle by oral gavage (0.05 ml) with two lengths of stainless animal feeding needles utilized according to the age of the mice (Fisher Scientific, Australia).

Mice were sacrificed after the last session of behavioural testing through carbon dioxide, and brain tissue (separated randomly into half hemispheres) was rapidly collected and kept at -80 °C until further analysis. Blood was collected by cardiac puncture and plasma was prepared and stored for ELISA analysis at -20 °C.

2.3. Behavioural testing

Object recognition testing was performed after habituation to an open-field box and according to a protocol previously described (Fernandez et al., 2012; Jafari et al., 2013). An objection recognition (OR) test, corresponding to the ratio of the amount of time spent exploring the novel object over the total time spent exploring both objects, was used to assess learning and memory function.

The Elevated plus maze (EPM) testing was performed as previously described (Durand et al., 2000). All sessions were videotaped and then analysed through the Ethovision tracking system. Time spent in the closed and open arms were recorded as well as the total distance travelled in the EPM.

Performance in the spontaneous alternation task was also assessed using a Y-maze as previously described (Jafari et al., 2013). Testing was for 10 free-choice arm entries or 16 min, whichever occurred first. Mice placed in the start arm must enter (whole body, including tail) the left or right open goal arm and return to the choice area, and then enter the opposite arm for each trial. Time to accomplish the ten alternations was reported and compared between the tested groups.

The Watermaze test was performed during the last days of treatment, as previously described (Vorhees and Williams, 2006). Briefly, each mouse was allowed to swim for 60 s and the time spent to reach the platform was noted. Each mouse had 20 training trials divided equally during a 5-day period. In each trial, mice that successfully located the platform were allowed to stay on the platform for 10 s, whereas those that failed to locate the platform within the defined time frame, were placed on the platform for 10 s by the experimenter. A single probe trial was conducted by removing the platform (target-off test) on the 5th day (90 min after the last training session), in which all mice started from the farthest point opposite to the original target platform location and were allowed to swim for 60 s. All sessions were videotaped and time and distance swum within the quadrant were analysed through the Ethovision tracking system.

2.4. Enzyme-linked immunosorbent assay (ELISA)

Since AD related brain dysfunction affects all cortical lobes and subcortical structures (Pini et al., 2016), randomised half brain hemisphere brains were homogenized in 5 M guanidine/5 mM Tris buffer (pH 8.0) using a tissue homogeniser before being prepared and diluted according to ELISA kits manufacturer. ELISA assays were performed according to the manufacturer's protocol in brain tissue (Invitrogen Mouse Aβ42 ELISA (Davis et al., 1999)for AD markers and Abcam 3-NitroTyrosine (3NT) as oxidative marker (Bandookwala and Sengupta, 2020) ELISA Kit ab210603, Australia), and in plasma (R&D Systems IL-1β and TNF-α Quantikine ELISA, Australia).

2.5. Immunoblotting

Randomised half brain hemispheres were gently homogenized in lysis buffer (50 mM Tris pH 7.5, 50% glycerol), containing protease inhibitor (Sigma Aldrich, Australia) as previously described (Andrews et al., 2018). Sample proteins were separated via 7–20% pre-cast bis-Tris polyacrylamide gel (Biorad, Australia) electrophoresis, transferred to nitrocellulose membrane and probed with primary antibody for tau (ab64193), phosphorylated-tau or p-tau (ab4841) as AD biomarkers (Davis et al., 1999) and Nrf2 (ab62352) as an anti-inflammatory marker. Nrf2 was chosen as an anti-inflammatory marker due to its role in AD pathophysiology, as previously reported in mice (Chang et al., 2015; Fragoulis et al., 2017) and human studies (Serini and Calviello, 2016). In addition, Nrf2 was reported to be upregulated by Antroquinonol treatment in brains from another transgenic AD animal model (APP/PS1) (Chang et al., 2015).

Samples were run in duplicate across 6–7 gels per protein and were loaded in a randomized order with even numbers of 3TgXAD and control samples per group, per gel, to minimize the effects of gel-to-gel variability on results. A pooled sample, used as a positive control, was loaded onto each gel to account for any gel-to-gel variability. Membranes were washed and probed with horseradish peroxidase (HRP) conjugated affinity-purified secondary antibody goat anti-rabbit (anti-Rabbit IgG, #7074 from Cell Signaling). Protein signals were visualized using a chemiluminescent HRP substrate detection system (Clarity ECL, Biorad) and quantified by a luminescence imaging system Fusion FX Spectra chemiluminescence system (Vilber Lourmat, Fisher Biotec).

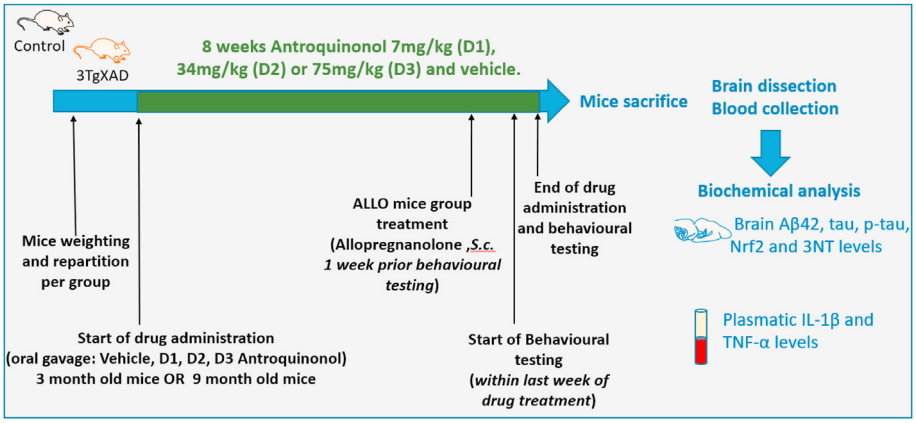

Fig. 1. Schematisation of Antroquinonol administration at early and late stage in two mice cohorts (3 and 9 month-old mice 3TgXAD and their related control).

2.6. Statistical analysis

Statistical analysis of data collected from both behavioural testing and biomarker assays, was analysed using Graph Pad Prism (Version 7; Graph Pad, La Jolla, USA) and SPSS (Version 25; IBM, New York, USA). To determine the number of mice per group that was required to achieve a significant result ($p < 0.05$ at 90% confidence), power calculations were conducted using JMP (Version 11, SAS Institute, North Carolina, USA). Based on our previous work with testing learning and memory in transgenic mice (Fernandez et al., 2012) and previously published work testing Antroquinonol (Chang et al., 2015), it was determined that 8–12 animals per group were required to ensure that the behavioural data was statistically significant. For all tests performed, the significance was set to $P < 0.05$ and outliers were removed from analysis. All data was run through a Kolmogorov-Smirnov test within SPSS to ensure normality and transformed if necessary. One Way ANOVA performed with SPSS analysed the effects of the treatment within the tested groups. Two Way ANOVA analysed the effects of both groups (3TgXAD and Controls) and treatment for each respective study. Following these two tests, a Tukey test was undertaken for *post hoc* analysis of all data.

3. Results

3.1. Behavioural testing-early stage antroquinonol administration

When Antroquinonol was administered at early stage of the pathology, anxiety related behaviour and cognitive abilities were improved significantly in 3TgXAD treated with 75 mg/kg (D3) and less consistently with 34 mg/kg (D2) of Antroquinonol compared to 3TgXAD vehicle treated mice (see Fig. 2A).

A significant anxiogenic effect of Antroquinonol assessed by time spent in closed arms of EPM was reported in 3TgXAD mice compared to controls mice ($F(5, 61) = 3.32$, $p = 0.011$) (Fig. 2A). When looking at the effects of Antroquinonol within the 3TgXAD mice group, mice treated with the highest dose of the drug (D3) spent significantly less time in the closed arms compared to the vehicle group ($p < 0.05$), suggesting a reduction of anxiety for mice treated with D3 Antroquinonol compared to mice treated with vehicle. Similarly, a significant difference was reported for the time spent in open arms for 3TgXAD mice group ($F(5, 61) = 2.56$, $p = 0.042$), with mice treated with higher dose of Antroquinonol (D3) spending more time in the open-arm compared to vehicle treated mice ($p < 0.05$). No significant difference was found for the control mice group regarding their time spent in in open and closed arms within ($p > 0.05$). With respect to distance travelled in EPM (see Figure 2A), 3TgXAD mice treated with D2 ($p < 0.01$) and D3 ($p < 0.001$) Antroquinonol treatment groups displayed significantly less anxious behaviour compared to vehicle treated mice. However, there was no significant differences reported within the control mice group for distance travelled in EPM ($p > 0.05$). Mice treated with allopregnanolone spent more time in open-arms and covered more distance than the non-treated mice ($p < 0.05$). There were significant differences between 3TgXAD and the controls for both times spent in open and closed arms as well as travelled distance ($p < 0.001$).

When looking at the effects of Antroquinonol administration on the cognitive performance, memory impairment reported in 3TgXAD vehicle treated mice was reversed in the object recognition test (Fig. 2B), Y maze

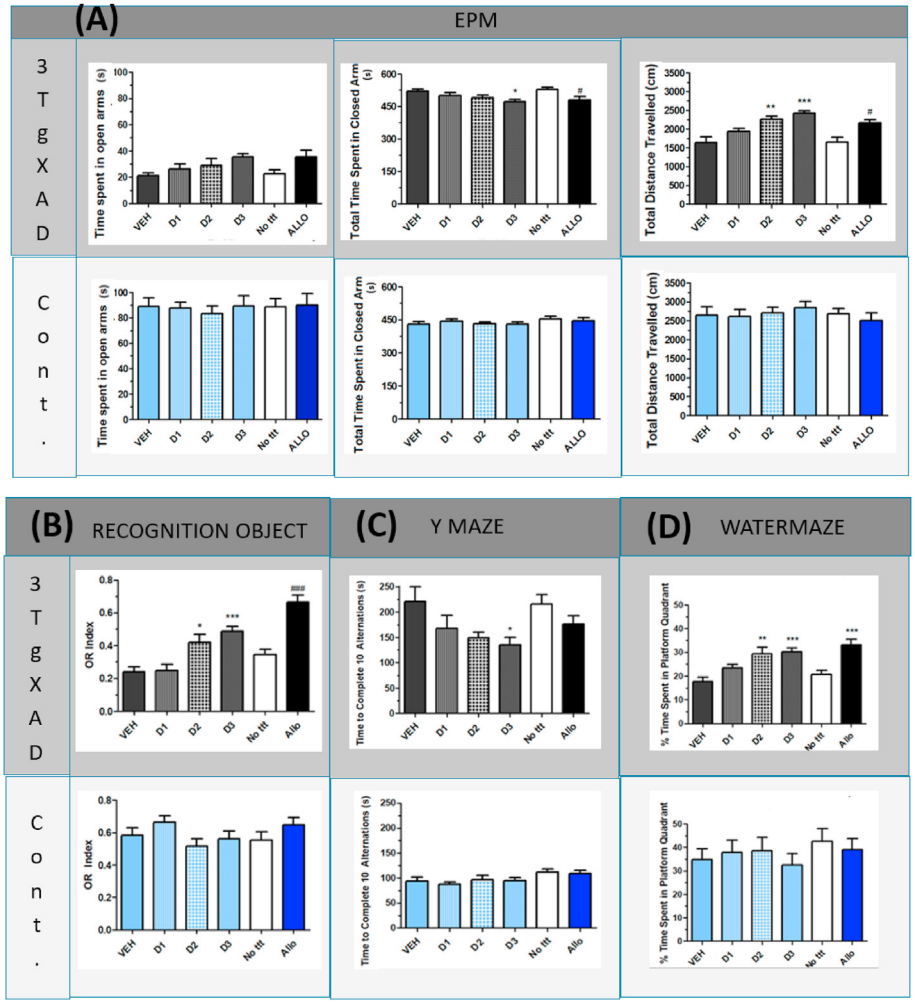

Fig. 2. Behavioural testing for 3TgXAD and control mice treated or not with Antroquinonol (D1 at 3 month old ($n = 7$ to 14, after removal of outliers). Graphs were plotted as mean values ± SEM.* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$(A). EPM results with time spent in open arms, time spent in closed arms and total distance travelled during the test.(B). Object recognition test. The analysis of Objection Recognition (OR) test revealed a significant difference between the tested groups in the 3TgXAD mice model ($p < 0.001$).(C). Y maze results. A significant difference in the mean time required to perform ten alternations between vehicle treated mice and D3 treated mice was found in 3TgXAD group ($p < 0.05$), but no significant difference was reported in the control group ($p > 0.05$).(D). Time spent in platform quadrant in Watermaze Morris test. In the Morris test, spatial memory was improved significantly within 3TgXAD treated mice, with D2 ($p < 0.01$) and with D3 ($p < 0.001$) spending around 40% more time in quadrant platform than the vehicle treated mice.

(Fig. 2C) and Morris tests (Fig. 2D) when the mice were administered with D3 Antroquinonol ($p < 0.05$), and as well as when 3TgXAD were administered with D2 Antroquinonol in object recognition and Morris tests ($p < 0.05$, see Fig. 2 B, C and D).

The analysis of the Objection Recognition test revealed a significant difference within the 3TgXAD mice group ($F(5, 57) = 18.94$, $p < 0.001$), with D2 treated mice and D3 treated mice showing an increase of time spent exploring the new object compared to transgenic vehicle treated mice (18% of increase for D2 treated mice ($p = 0.036$) and 28% for D3 treated mice ($p = 0.001$)) (Fig. 2B). The positive control group, ALLO 3TgXAD treated mice, spent significantly more time with the new object compared to all other 3TgXAD mice groups ($p < 0.001$). In the control mice group, no significant difference was reported regarding the time spent with the new object ($p < 0.05$). However, the control mice group spent significantly more time than the 3TgXAD mice group ($F(11,105) = 9.07$, $p < 0.01$), illustrating a better memory performance than the transgenic mice.

Looking at working memory performance in the Y maze, a significant difference in the time required to perform ten alternations was found within the 3TgXAD mice group with D3 treated mice being significantly faster to complete the task, than the vehicle treated mice ($p = 0.042$). There was no statistically significant differences within the control mice cohort and between the two strains of mice ($p > 0.05$).

In the Morris test, spatial memory was improved significantly within the 3TgXAD group, for D2 treated mice ($p < 0.01$) and for D3 treated mice ($p < 0.001$), compared to vehicle treated mice. The 3TgXAD D3 treated mice spent around 40% more time in the quadrant platform than the vehicle treated mice ($F(5, 63) = 7.54$, $p < 0.001$; Fig. 2D). Positive control group (ALLO) reversed spatial cognitive deficit, which was observed in both non-treated and vehicle treated 3TgXAD groups ($p < 0.001$), by spending over 35% time in the platform quadrant. No statistical differences were reported between any of the treatment groups in the control group of mice ($p > 0.05$). Control mice group spent significantly more time in the quadrant platform compared to the 3TgXAD mice cohort ($F(11, 121) = 4.81$, $p < 0.001$).

3.2. Biomarker analyses – early stage antroquinonol administration

When Antroquinonol was administered at an early stage (3 month old mice), Aβ42 brain levels were significantly higher in 3TgXAD mice compared to the control cohort ($p < 0.01$; Fig. 3A). However, D3 ($p = 0.023$), D2 ($p = 0.019$) and ALLO ($P < 0.001$) 3TgXAD treated mice showed significantly lower brain levels of Aβ42 compared to vehicle treated mice (Fig. 3A). Looking at tau and p-tau levels in the brain (Fig. 3B and C), no significant difference was indicated between 3TgXAD and control groups ($p < 0.05$). Within the 3TgXAD, only the D3 treated mice showed a significantly lower level of tau ($p = 0.015$) in the brain, compared to the vehicle treated mice (Fig. 3B).

As regards the inflammatory markers measured in the plasma, both IL-1β and TNF-α levels were significantly higher in 3TgXAD mice groups, compared to the control mice cohort (Fig. 3 D and E); $p < 0.05$). However, the levels of IL-1β in the 3TgXAD mice group were significantly lower in the D2, D3 and ALLO treated mice compared to the vehicle treated mice (Fig. 3 D, $p < 0.01$). For TNF-α levels, only D3 ($p = 0.031$) and ALLO ($p = 0.002$) 3TgXAD treated mice showed significantly lower levels of this inflammatory marker in plasma compared to 3TgXAD vehicle group (Fig. 3E). No significant difference was reported for the levels of anti-inflammatory marker Nrf2 measured in the brain of 3TgXAD mice and their respective controls (Fig. 3G; $p > 0.05$). Oxidation assessed by the measurement of 3NT levels in brain was also not significantly different in both 3TgXAD group and control group (Fig. 3F; $p > 0.05$).

3.3. Behavioural testing -late stage antroquinonol administration

When Antroquinonol was administered at a later stage (9-month-old mice), improvement of memory impairment was seen in 3TgXAD vehicle and was observed for EPM, the recognition object test, Ymaze and watermaze tests.

In the EPM test, 3TgXAD D3 mice spent significantly less time in closed arms compared to vehicle treated transgenic mice ($p = 0.013$;

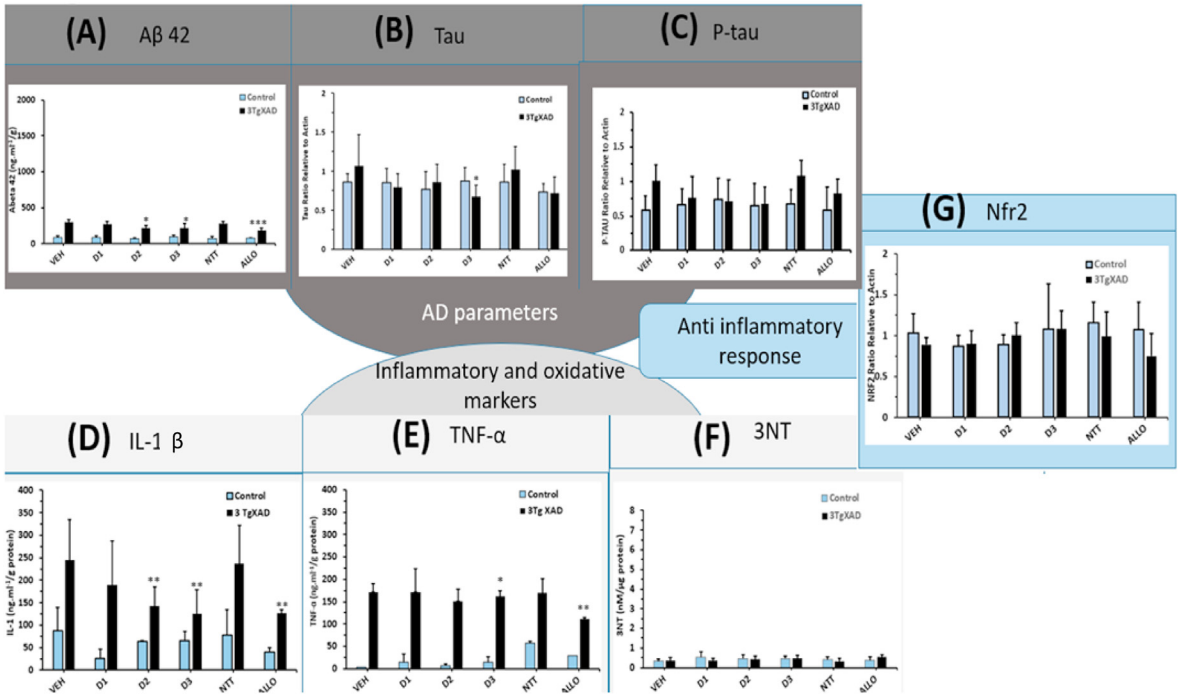

Fig. 3. Assessment of AD biomarkers, oxidative stress anti-inflammatory markers in brain tissue (half-hemisphere randomly assigned in groups) and plasmatic inflammatory markers from 3TgXAD and control mice administered or not with Antroquinonol or vehicle (n = 4 to 8 after removal of outliers) at 3-month-old. (A) Aβ42, (B) tau, (C) p-tau, (D) IL-1 β, (E) TNF-α, (F) Nrf2 and (G) 3NT levels. Graphs were plotted as mean values ± SEM.* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Fig. 2A), confirming the anxiolytic effect of Antroquinonol administration reported in previous study. There was no significant difference between the time spent in open and closed arms for the control group mice, as well as the distance travelled during the test for both 3TgXAD and control mice ($p > 0.05$).

The beneficial effect of Antroquinonol treatment was also reported in the recognition object test (Fig. 4B) and in Y maze tests (Fig. 4C) for D3 treated 3Tgx AD mice compared to their respective controls ($p < 0.05$). The 3TgXAD mice spent significantly less time with new object compared to the control mice ($F(1,166) = 42.7$, $p < 0.001$). A significant difference for the OR test was found within the 3TgXAD group ($F(5, 80) = 6.2$, $p < 0.001$) but not in the control group ($p > 0.05$), with the transgenic D3 treating mice spending significantly more time with the new object compared to the vehicle 3TgXAD treated mice ($p < 0.001$). A significant

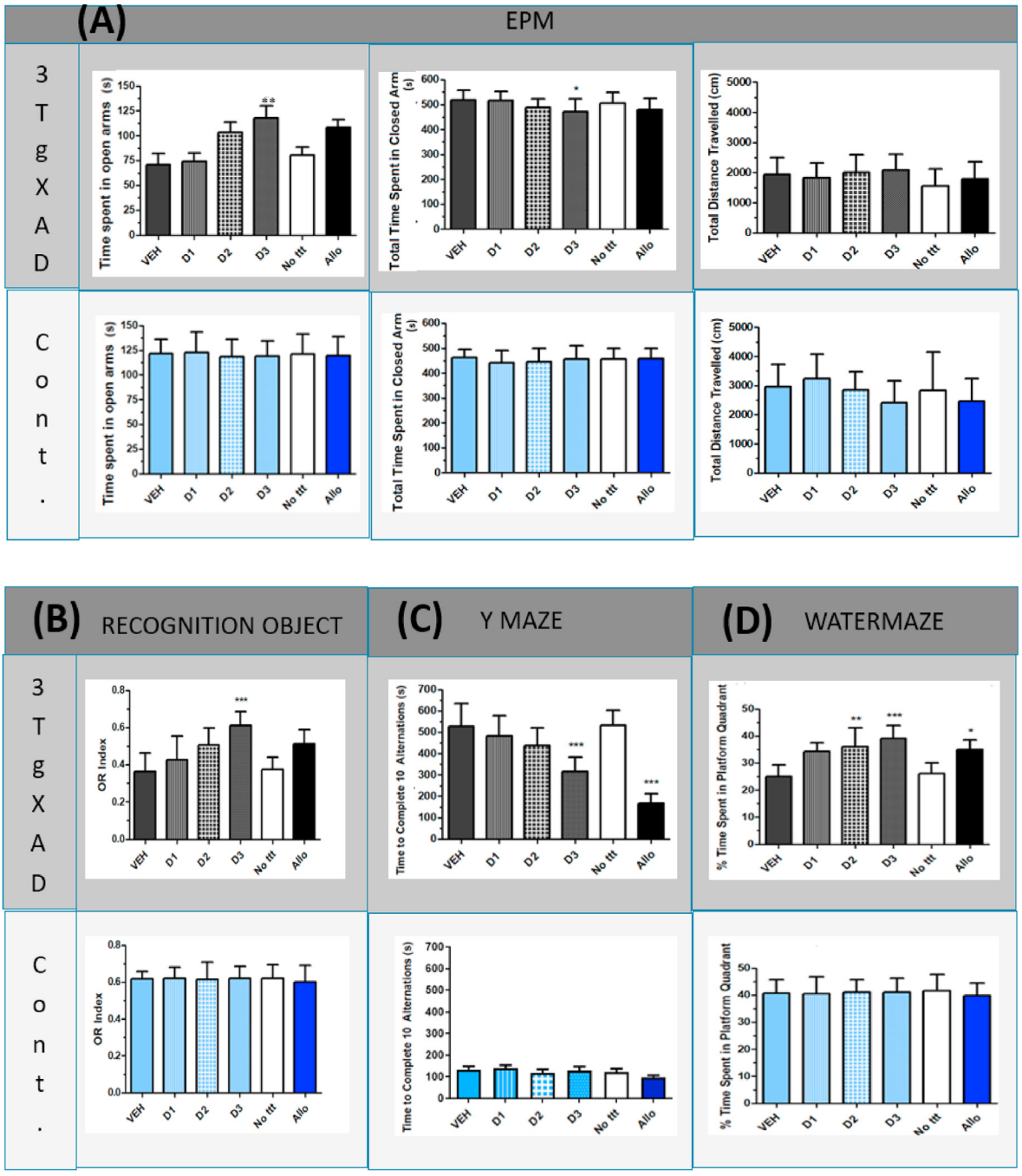

Fig. 4. Behavioural testing for 3TgXAD and control mice treated or not with Antroquinonol (D1 at 9 month old ($n = 10$ to 18, after removal of outliers). Graphs were plotted as mean values ± SEM.* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

(A). EPM results with time spent in open arms, time spent in closed arms and total distance travelled during the test. The 3TgXAD mice spent significantly less time in open and closed arms compared to their respective controls $p < =0.001$, Fig. 4A). Transgenic mice showed significant difference of time spent in open arms for the mice treated with D3 compared to vehicle treated mice ($p < 0.05$).(B) Object recognition test. The 3TgXAD mice spent significantly less time with new object compared to the control mice ($p < 0.001$) while the transgenic D3 treated mice spent significantly more time with the new object ($p < 0.001$) compared to 3TgXAD vehicle mice.(C) Y maze. Transgenic treated with D3 and ALLO treated mice were significant faster to complete the task in Y maze compared to 3TgXAD vehicle treated mice ($p < 0.001$).(D). Time spent in platform quadrant in Watermaze Morris test. Spatial memory tested in Watermaze was significantly lower in transgenic mice compared to control mice ($p < 0.001$). Transgenic treated mice with D2, D3 and ALLO spent more time in the platform quadrant of the Watermaze compare to the 3TgXAD vehicle treated mice.

difference between transgenic mice and their related controls was also found in Y maze tests, with transgenic mice spending more time to complete 16 alternations ($F(1,177) = 421.5$, $p < 0.001$, Fig. 4C). Transgenic D3 ($p = 0.001$) and ALLO treated mice ($p < 0.001$) were significantly faster to complete the task in Y maze compared to 3TgXAD vehicle treated mice ($F(5,76) = 16.51$, $p < 0.001$). There was no significant difference between the tested group within the control group of mice for both the Y maze and Watermaze performances ($p > 0.05$, Fig. 4C and D, respectively). However, spatial memory tested in Watermaze was significantly lower in the transgenic mice compared to control mice ($F(1, 191) = 34.56$, $P < 0.001$) as previously reported. Transgenic Antroquinonol D2 ($p = 0.007$), D3 ($p = 0.001$) and ALLO treated mice ($p = 0.031$) spent more time in the platform quadrant of the Watermaze compared to the 3TgXAD vehicle treated mice.

3.4. Biomarker analyses-late stage antroquinonol administration

Regarding the levels of AD biomarkers in the mice administered with Antroquinonol at 9 months old, 3TgXAD mice showed a significantly higher level of Aβ42 and p-tau in the brain compared to the control mice group ($F(1,71) = 169.1$, $p < 0.001$) and $F(1, 80) = 13.2$, $p = 0.001$ respectively, Fig. 5A and C). Interestingly, 3TgXAD D1 ($p = 0.017$) and D2 ($p < 0.001$) treated mice showed p-tau levels significantly lower in the brain compared to vehicle treated mice (Fig. 5C). Both tau and p-tau levels were found to have significantly lower levels in D3 3TgXAD treated mice compared to vehicle treated mice ($p = 0.023$ and $p < 0.001$, respectively; Fig. 5B and C).

Looking at the inflammatory markers in the plasma, both IL-1β and TNF-α levels were found to be higher in the transgenic mice group compared to the control mice group ($F(1, 64) = 6.325$, <0.001 and $F(1, 61) = 72.211$, $p < 0.001$ respectively. D3 treatment ($p = 0.001$) was also efficient at decreasing plasmatic IL-1 β levels in 3TgXAD mice, compared to vehicle treated mice. The control mice group did not show any significant difference for both measured inflammatory markers ($p > 0.05$).

Oxidative stress measured by 3NT levels in the brain was significantly higher in transgenic mice compared to control mice ($F(1, 61) = 278.121$, $p < 0.001$). Transgenic D3 treated mice showed a significant lower level of 3NT ($p = 0.008$) compared to 3TgXAD vehicle treated mice ($p = 0.003$).

Also as reported previously in the literature with a different animal model of AD (Chang et al., 2015), Nrf2 levels were significantly higher in brains from 3TgXAD D3 treated mice ($p = 0.004$) compared to 3TgXAD vehicle treated mice ($p < 0.001$).

4. Discussion

The role of Antroquinonol in the context of AD pathophysiology was assessed in this study using a well-established AD animal model by administering the drug at early and later stages of the development of the pathology. Our results showed a beneficial effect at the behavioural and neurochemical levels (for plasmatic inflammatory markers and both AD brain biomarkers, Aβ 42 and tau) when the tested drug was administered at early stage and at the highest dose (75 mg/kg). When AD symptoms were more advanced, the highest tested dose of Antroquinonol also improved behavioural impairment reported in 3TgXAD in the majority of the performed tests, but in addition was also able to reduce tau pathology (tau and p-tau levels) and oxidative markers (3NT levels) in the brain, as well as inflammatory markers (IL-1β and TNF-α levels) in the plasma.

Biomarkers for both amyloid (Aβ42) and tau pathologies (using antibodies targeting S262 for tau and p-threonine T205 for p-tau) were present in the brain of 3TgXAD (non-treated and/or receiving drug-vehicle) at a moderate level in mice sacrificed around 5 months and more abundantly in the mice sacrificed around 11 months (Wang et al., 2020), reflecting the progression of AD pathophysiology in the tested animal model. The level of inflammation assessed by IL-1β and TNF levels in plasma was also significantly higher in the plasma of 3TgXAD mice at 5 months, compared to 11 month in accordance with previous studies (Patel et al., 2005; Janelsins et al., 2005) and potentially reflecting neuro-inflammation and activation of microglia (Fig. 6 (Jones, 2001).).

Antroquinonol at higher dose (75 mg/kg) was able to significantly reduce, Aβ and tau levels in the brain at the earlier stages of AD pathology, as well as tau and p-tau brain levels at later stages of the pathology. These results are consistent with the literature reporting amyloid pathogenicity taking place before the formation of NFTs (Davis

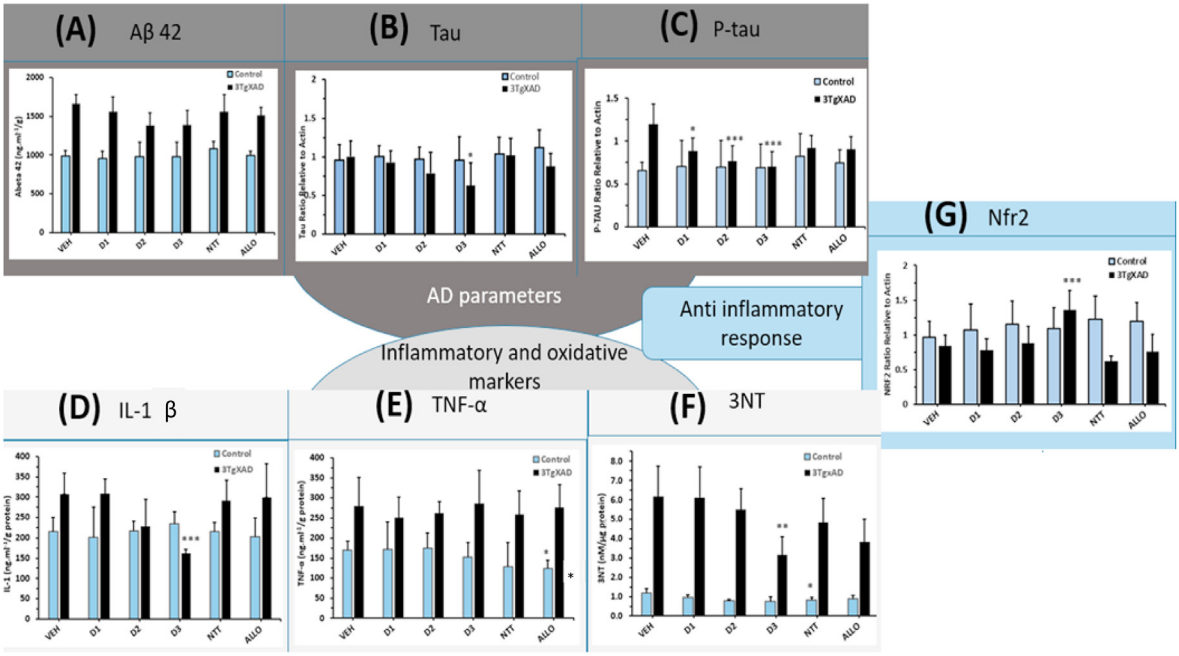

Fig. 5. Assessment of AD biomarkers, oxidative stress anti-inflammatory markers in brain tissue (half-hemisphere randomly assigned in groups) and plasmatic inflammatory markers from 3TgXAD and control mice administered or not with Antroquinonol or vehicle (n = 4 to 8 after removal of outliers) at 9-month-old. A) Aβ42, (B) tau, (C) p-tau, (D) IL-1β, (E) TNF-α, (F) Nrf2 and 3NT levels. Graphs were plotted as mean values ± SEM* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

et al., 1999; Dickson, 1997). NFTs are formed from the aggregation of tau oligomers and hyperphosphorylated tau proteins, which lead to microtubule dysfunction and neurotoxicity (Iqbal et al., 1998). Kinases such as Adenosine monophosphate protein kinase (AMPK), Mitogen activated protein kinase (MAPK) and Glycogen synthase kinase 3 beta (GSK-3β), phosphorylate tau at over 30 phosphorylation sites since tau protein exhibits a high number of serine and threonine in its sequence (Steinhilb et al., 2007). Interestingly, Fyn Kinase, which regulates the activity of these kinases, is also responsible for the phosphorylation of Nrf2 (Bahn et al., 2019). When non-phosphorylated, Nrf2 acts as a transcription factor and inhibits the expression of *BACE 1* coding for β-secretase enzyme (see Fig. 6). Since Antroquinonol acts on both NF-kB (Thiyagarajan et al., 2015) and Nrf2 (Chang et al., 2015; Kumar et al., 2011) expression, this drug could indirectly reduce the levels of Aβ in the brain. Additional studies will be necessary to determine the role of Fyn Kinase in the context of Antroquinonol treatment in 3TgXAD animal model.

Antroquinonol anti-inflammatory properties, particularly at higher dose of administration (75 mg/kg), was also illustrated by the significant reduction of plasmatic IL-1β and TNF-α levels in 5 month-old 3TgXAD mice and a decrease of plasmatic IL-1β levels in 11 month-old 3TgXAD mice, compared to their respective controls. Activated microglia (M1 phenotype, producing cytokines such as IL-1β and TNF-α in brain) contributes to AD neurodegeneration (including failing to clear pathological protein aggregation) (Block et al., 2007; Schwab et al., 2010; Streit et al., 2004). Interestingly, several studies have demonstrated the cytoprotective properties toward Aβ peptide-induced toxicity exerted by Nrf2 activation and related to its anti-inflammatory effects (Serini and Calviello, 2016; Cores et al., 2020). Nrf2 activation reduces the amounts of several inflammatory cytokines such as TNF-α, IL-1β, IL-6, due to a shifting of the microglia state from the M1 (pro-inflammatory) to a M2 (anti-inflammatory) phenotype (Song and Suk, 2017). When induced by expression of Interferon-γ (IFN-γ), M1 microglia activation was shown to lead to an increase in amyloid plaques in another AD transgenic mouse model (APP/PS1) (Weekman et al., 2014). Conversely, M2 phenotype activation (via stimulation of IL-4 expression or administration) decreased β-amyloid deposition in both *in vitro* and in an animal model (Latta et al., 2015).

Furthermore, the reduction of Aβ levels by high dose Antroquinonol treatment in 3TgXAD may also lead to the decrease of activation of Fyn kinase (Larson et al., 2012; Um et al., 2012) and consequently to a decline of phosphorylation of tau proteins (p-tau) (Fig. 6) as well as a reduction of NMDA receptor phosphorylation (inducing its internalisation). Since NMDA receptor underlies synaptic plasticity processes, its phosphorylation by Fyn Kinase may lead to the modification of the threshold for hippocampal synaptic plasticity and consequently to learning and memory impairment (Trepanier et al., 2012). Antroquinonol at higher dose (75 mg/kg) was able to reverse systematically the behavioural impairment observed in 3TgXAD (including anxiety and learning and memory issues) to levels found in the control mice in younger and older tested mice (Figs. 2 and 4) confirming the therapeutic potential of this drug in AD symptomology (Chang et al., 2015).

Both Fyn Kinase (Nygaard, 2018) and more recently Nrf2 (Osama et al., 2020) have attracted interest as new therapeutic targets for AD, with the ability to modify reactive oxygen species (ROS) (Abe et al., 2000) and expression of nitric oxide synthase (iNOS) (Ramsey et al., 2007) (Fig. 6). Oxidative stress, such as free radicals like ROS or nitric oxide synthesis, contributes significantly to AD development in many ways, including Aβ metal ion redox potential and mitochondria dysfunction (Fig. 6). In this study, the oxidative stress marker, 3NT, was significantly lower in 5-month-old 3TgXAD brain compared to 11 month-old transgenic mouse brains ($p < 0.001$) in accordance with progression of AD pathophysiology (Aluise et al., 2010). High levels of

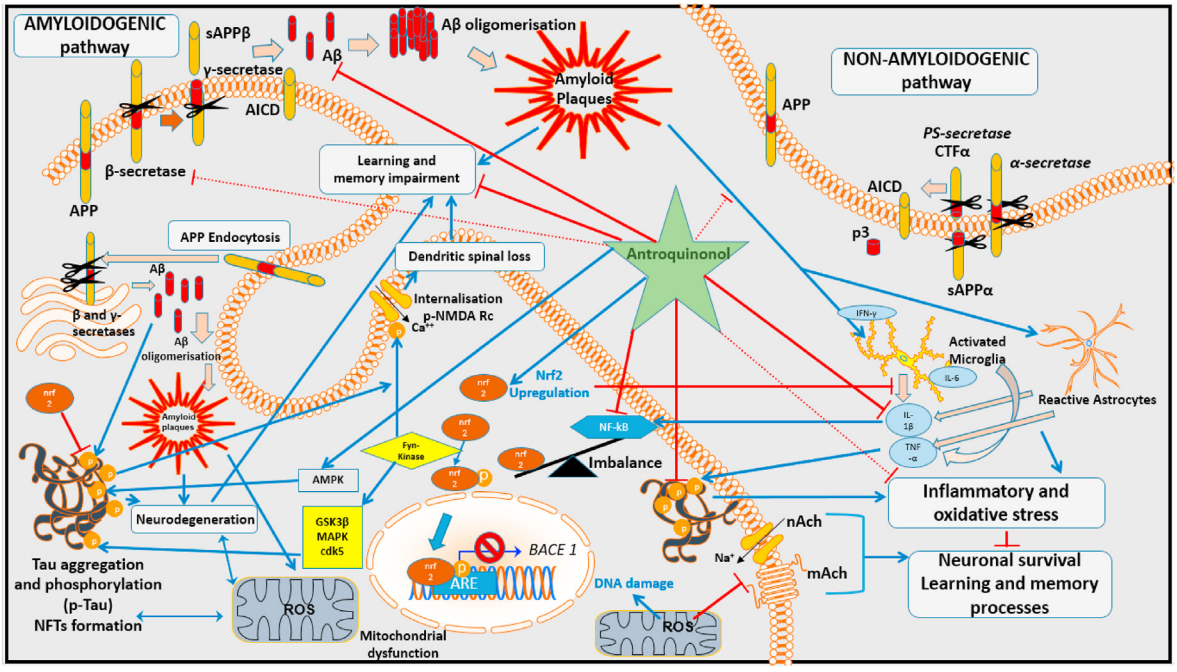

Fig. 6. Summary of the role of Antroquinonol in the context of AD pathophysiology.
Antroquinonol administration leads to a decrease of Aβ levels in the brain, which is forms through the cleavage of APP. Briefly, APP can be processed through non-amyloidogenic or amyloidogenic pathways, with the latter being more prominent in AD brains. Cleavage by β-secretase of APP produces sAPPβ and CTFβ (after APP endocytosis). CTFβ is then cut by γ-secretase into Aβ and AICD fragments. Exocytosis of Aβ and sAPPβ back into the extracellular space then occurs. Aβ accumulates, and its oligomerisation, leads to the formation of amyloid plaques in the brain. The non-amyloidogenic pathway consists of APP cleavage by α-secretase; which produces sAPPα, and CTFα, with CTFα being further cleaved by γ-secretase, leading to generation of p3 and AICD fragments. All products part of non-amyloidogenic pathway are non-pathogenic. Antroquinonol can also reduce inflammation in the neurons notably by its action on Nrf2 expression and reduction of NF-kB, TNF-α and IL-1β levels produced by reactive neuroglial cells. Actions of Antroquinonol can therefore lead to reduction of learning and memory impairment, neuronal loss and inflammatory and oxidative responses.

Antroquinonol significantly reduced the 3NT levels when AD like pathophysiology was at a more advanced stage in the transgenic mice ($p < 0.001$), confirming the efficiency of this drug to act on another dimension of AD pathology with an improvement of learning and memory performance.

Although Antroquinonol has been previously studied in the context of AD pathophysiology (Chang et al., 2015), the APP transgenic animal model used in the study was limited to amyloid pathology only and lacked investigation in relation to tau pathology. The choice of an animal model to mirror the main factors underlying pathology is one important aspect to success in the translation of animal preclinical studies to human clinical trials. Here, we have for the first time showed the efficiency of Antroquinonol to not only efficiently reduce early AD hallmarks and inflammatory markers but also to reduce an AD associated oxidative stress marker at a later stage of the disease in a well-established animal model of the disease. However, limitations of this research must be considered. Randomised half-brains have been employed in the tissue brain analysis for biomarkers. This could lead to a potential "dilution" of the reported effects of Antroquinonol compared to the use of brain specific structures for testing brain biomarkers (such as pre-frontal cortex and hippocampus) relevant to studying AD and memory processes (Preston and Eichenbaum, 2013). Additional *in vitro* investigations will be also necessary to characterise the role of Antroquinonol at the molecular level in the context of AD pathophysiology.

Considering both the high social and economic costs of AD in our growing elderly society, there is an urgency to find new strategies for the prevention and treatment of this pathology. Since Antroquinonol has been utilized without adverse effects in previous successful clinical trials and is currently used for other pathologies, its consideration as a preventive and/or therapeutic AD agent at early and/or later stage of AD pathophysiology now appears timely and topical.

Disclosure form

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References

Abe, J., Okuda, M., Huang, Q., Yoshizumi, M., Berk, B.C., 2000. Reactive oxygen species activate p90 ribosomal S6 kinase via Fyn and Ras. J. Biol. Chem. 275 (3), 1739–1748.

Aluise, C.D., Robinson, R.A., Beckett, T.L., Murphy, M.P., Cai, J., Pierce, W.M., Markesbery, W.R., Butterfield, D.A., 2010. Preclinical Alzheimer disease: brain oxidative stress, Abeta peptide and proteomics. Neurobiol. Dis. 39 (2), 221–228.

Anand, A., Patience, A.A., Sharma, N., Khurana, N., 2017. The present and future of pharmacotherapy of Alzheimer's disease: a comprehensive review. Eur. J. Pharmacol. 815, 364–375.

Andrews, J.L., Newell, K.A., Matosin, N., Huang, X.F., Fernandez, F., 2018. Perinatal administration of phencyclidine alters expression of Lingo-1 signaling pathway proteins in the prefrontal cortex of juvenile and adult rats. Neuronal. Signal 2 (3), NS20180059.

Angamuthu, V., Shanmugavadivu, M., Nagarajan, G., Velmurugan, B.K., 2019. Pharmacological activities of antroquinonol- Mini review. Chem. Biol. Interact. 297, 8–15.

Bahn, G., Park, J.S., Yun, U.J., Lee, Y.J., Choi, Y., Park, J.S., Baek, S.H., Choi, B.Y., Cho, Y.S., Kim, H.K., Han, J., Sul, J.H., Baik, S.H., Lim, J., Wakabayashi, N., Bae, S.H., Han, J.W., Arumugam, T.V., Mattson, M.P., Jo, D.G., 2019. NRF2/ARE pathway negatively regulates BACE1 expression and ameliorates cognitive deficits in mouse Alzheimer's models. Proc. Natl. Acad. Sci. U. S. A. 116 (25), 12516–12523.

Bandookwala, M., Sengupta, P., 2020. 3-Nitrotyrosine: a versatile oxidative stress biomarker for major neurodegenerative diseases. Int. J. Neurosci. 130 (10), 1047–1062.

Block, M.L., Zecca, L., Hong, J.S., 2007. Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. Nat. Rev. Neurosci. 8 (1), 57–69.

Breijyeh, Z., Karaman, R., 2020. Comprehensive review on alzheimer's disease: causes and treatment. Molecules 25 (24).

Cacabelos, R., Carrera, I., Martinez-Iglesias, O., Cacabelos, N., Naidoo, V., 2021. What is the gold standard model for Alzheimer's disease drug discovery and development? Expet Opin. Drug Discov. 16 (12), 1415–1440.

Cassidy, L., Fernandez, F., Johnson, J.B., Naiker, M., Owoola, A.G., Broszczak, D.A., 2020. Oxidative stress in alzheimer's disease: a review on emergent natural polyphenolic therapeutics. Compl. Ther. Med. 49, 102294.

Chang, W.H., Chen, M.C., Cheng, I.H., 2015. Antroquinonol lowers brain amyloid-beta levels and improves spatial learning and memory in a transgenic mouse model of alzheimer's disease. Sci. Rep. 5, 15067.

Cores, A., Piquero, M., Villacampa, M., Leon, R., Menendez, J.C., 2020. NRF2 regulation processes as a source of potential drug targets against neurodegenerative diseases. Biomolecules 10 (6).

Cummings, J.L., Tong, G., Ballard, C., 2019. Treatment combinations for alzheimer's disease: current and future pharmacotherapy options. J. Alzheimers Dis. 67 (3), 779–794.

Davis, D.G., Schmitt, F.A., Wekstein, D.R., Markesbery, W.R., 1999. Alzheimer neuropathologic alterations in aged cognitively normal subjects. J. Neuropathol. Exp. Neurol. 58 (4), 376–388.

Dickson, D.W., 1997. The pathogenesis of senile plaques. J. Neuropathol. Exp. Neurol. 56 (4), 321–339.

Durand, M., Aguerre, S., Fernandez, F., Edno, L., Combourieu, I., Mormede, P., Chaouloff, F., 2000. Strain-dependent neurochemical and neuroendocrine effects of desipramine, but not fluoxetine or imipramine, in spontaneously hypertensive and Wistar-Kyoto rats. Neuropharmacology 39 (12), 2464–2477.

Fernandez, F., Soon, I., Li, Z., Kuan, T.C., Min, D.H., Wong, E.S., Demidov, O.N., Paterson, M.C., Dawe, G., Bulavin, D.V., Xiao, Z.C., 2012. Wip1 phosphatase positively modulates dendritic spine morphology and memory processes through the p38MAPK signaling pathway. Cell Adhes. Migrat. 6 (4), 333–343.

Fragoulis, A., Siegl, S., Fendt, M., Jansen, S., Soppa, U., Brandenburg, L.O., Pufe, T., Weis, J., Wruck, C.J., 2017. Oral administration of methysticin improves cognitive deficits in a mouse model of Alzheimer's disease. Redox Biol. 12, 843–853.

Frick, K.M., Kim, J., Koss, W.A., 2018. Estradiol and hippocampal memory in female and male rodents. Curr. Opin. Behav. Sci. 23, 65–74.

Hirata-Fukae, C., Li, H.F., Hoe, H.S., Gray, A.J., Minami, S.S., Hamada, K., Niikura, T., Hua, F., Tsukagoshi-Nagai, H., Horikoshi-Sakuraba, Y., Mughal, M., Rebeck, G.W., LaFerla, F.M., Mattson, M.P., Iwata, N., Saido, T.C., Klein, W.L., Duff, K.E., Aisen, P.S., Matsuoka, Y., 2008. Females exhibit more extensive amyloid, but not tau, pathology in an Alzheimer transgenic model. Brain Res. 1216, 92–103.

in: th (Ed.), Guide for the Care and Use of Laboratory Animals, Washington (DC), 2011.

Iqbal, K., Alonso, A.C., Gong, C.X., Khatoon, S., Pei, J.J., Wang, J.Z., Grundke-Iqbal, I., 1998. Mechanisms of neurofibrillary degeneration and the formation of neurofibrillary tangles. J. Neural. Transm. Suppl. 53, 169–180.

Jafari, S., Huang, X.F., Andrews, J.L., Fernandez-Enright, F., 2013. In vivo pharmacological evaluations of novel olanzapine analogues in rats: a potential new avenue for the treatment of schizophrenia. PLoS One 8 (12), e80979.

Janelsins, M.C., Mastrangelo, M.A., Oddo, S., LaFerla, F.M., Federoff, H.J., Bowers, W.J., 2005. Early correlation of microglial activation with enhanced tumor necrosis factor-alpha and monocyte chemoattractant protein-1 expression specifically within the entorhinal cortex of triple transgenic Alzheimer's disease mice. J. Neuroinflammation 2, 23.

Jones, R.W., 2001. Inflammation and Alzheimer's disease. Lancet 358 (9280), 436–437.

Kumar, K.J., Chu, F.H., Hsieh, H.W., Liao, J.W., Li, W.H., Lin, J.C., Shaw, J.F., Wang, S.Y., 2011. Antroquinonol from ethanolic extract of mycelium of Antrodia cinnamomea protects hepatic cells from ethanol-induced oxidative stress through Nrf-2 activation. J. Ethnopharmacol. 136 (1), 168–177.

Larson, M., Sherman, M.A., Amar, F., Nuvolone, M., Schneider, J.A., Bennett, D.A., Aguzzi, A., Lesne, S.E., 2012. The complex PrP(c)-Fyn couples human oligomeric Abeta with pathological tau changes in Alzheimer's disease. J. Neurosci. 32 (47), 16857-71a.

Latta, C.H., Sudduth, T.L., Weekman, E.M., Brothers, H.M., Abner, E.L., Popa, G.J., Mendenhall, M.D., Gonzalez-Oregon, F., Braun, K., Wilcock, D.M., 2015. Determining the role of IL-4 induced neuroinflammation in microglial activity and amyloid-beta using BV2 microglial cells and APP/PS1 transgenic mice. J. Neuroinflammation 12, 41.

Lee, T.H., Lee, C.K., Tsou, W.L., Liu, S.Y., Kuo, M.T., Wen, W.C., 2007. A new cytotoxic agent from solid-state fermented mycelium of Antrodia camphorata. Planta Med. 73 (13), 1412–1415.

Lu, M.C., El-Shazly, M., Wu, T.Y., Du, Y.C., Chang, T.T., Chen, C.F., Hsu, Y.M., Lai, K.H., Chiu, C.P., Chang, F.R., Wu, Y.C., 2013. Recent research and development of Antrodia cinnamomea. Pharmacol. Ther. 139 (2), 124–156.

Mancuso, C., Siciliano, R., Barone, E., Preziosi, P., 2012. Natural substances and Alzheimer's disease: from preclinical studies to evidence based medicine. Biochim. Biophys. Acta 1822 (5), 616–624.

Morimoto, K., Uji, M., Ueyama, T., Kimura, H., Kohno, T., Takamata, A., Yano, S., Yoshida, K., 2008. Estrogen replacement suppresses pressor response and oxidative stress induced by cage-switch stress in ovariectomized rats. Ann. N. Y. Acad. Sci. 1148, 213–218.

Nygaard, H.B., 2018. Targeting Fyn kinase in alzheimer's disease. Biol. Psychiatr. 83 (4), 369–376.
Osama, A., Zhang, J., Yao, J., Yao, X., Fang, J., 2020. Nrf2: a dark horse in Alzheimer's disease treatment. Ageing Res. Rev. 64, 101206.
Patel, N.S., Paris, D., Mathura, V., Quadros, A.N., Crawford, F.C., Mullan, M.J., 2005. Inflammatory cytokine levels correlate with amyloid load in transgenic mouse models of Alzheimer's disease. J. Neuroinflammation 2 (1), 9.
Pini, L., Pievani, M., Bocchetta, M., Altomare, D., Bosco, P., Cavedo, E., Galluzzi, S., Marizzoni, M., Frisoni, G.B., 2016. Brain atrophy in Alzheimer's Disease and aging. Ageing Res. Rev. 30, 25–48.
Preston, A.R., Eichenbaum, H., 2013. Interplay of hippocampus and prefrontal cortex in memory. Curr. Biol. 23 (17), R764–R773.
Ramsey, C.P., Glass, C.A., Montgomery, M.B., Lindl, K.A., Ritson, G.P., Chia, L.A., Hamilton, R.L., Chu, C.T., Jordan-Sciutto, K.L., 2007. Expression of Nrf2 in neurodegenerative diseases. J. Neuropathol. Exp. Neurol. 66 (1), 75–85.
Scearce-Levie, K., Sanchez, P.E., Lewcock, J.W., 2020. Leveraging preclinical models for the development of Alzheimer disease therapeutics. Nat. Rev. Drug Discov. 19 (7), 447–462.
Schwab, C., Klegeris, A., McGeer, P.L., 2010. Inflammation in transgenic mouse models of neurodegenerative disorders. Biochim. Biophys. Acta 1802 (10), 889–902.
Senthil Kumar, K.J., Gokila Vani, M., Chen, C.Y., Hsiao, W.W., Li, J., Lin, Z.X., Chu, F.H., Yen, G.C., Wang, S.Y., 2020. A mechanistic and empirical review of antcins, a new class of phytosterols of formosan fungi origin. J. Food Drug Anal. 28 (1), 38–59.
Serini, S., Calviello, G., 2016. Reduction of oxidative/nitrosative stress in brain and its involvement in the neuroprotective effect of n-3 PUFA in alzheimer's disease. Curr. Alzheimer Res. 13 (2), 123–134.
Sevigny, J., Chiao, P., Bussière, T., Weinreb, P.H., Williams, L., Maier, M., Dunstan, R., Salloway, S., Chen, T., Ling, Y., O'Gorman, J., Qian, F., Arastu, M., Li, M., Chollate, S., Brennan, M.S., Quintero-Monzon, O., Scannevin, R.H., Arnold, H.M., Engber, T., Rhodes, K., Ferrero, J., Hang, Y., Mikulskis, A., Grimm, J., Hock, C., Nitsch, R.M., Sandrock, A., 2016. The antibody aducanumab reduces Aβ plaques in Alzheimer's disease. Nature 537 (7618), 50–56.
Song, G.J., Suk, K., 2017. Pharmacological modulation of functional phenotypes of microglia in neurodegenerative diseases. Front. Aging Neurosci. 9, 139.
Spires-Jones, T.L., Hyman, B.T., 2014. The intersection of amyloid beta and tau at synapses in Alzheimer's disease. Neuron 82 (4), 756–771.
Steinhilb, M.L., Dias-Santagata, D., Fulga, T.A., Felch, D.L., Feany, M.B., 2007. Tau phosphorylation sites work in concert to promote neurotoxicity in vivo. Mol. Biol. Cell 18 (12), 5060–5068.
Streit, W.J., Mrak, R.E., Griffin, W.S., 2004. Microglia and neuroinflammation: a pathological perspective. J. Neuroinflammation 1 (1), 14.
Sun, Z.K., Yang, H.Q., Chen, S.D., 2013. Traditional Chinese medicine: a promising candidate for the treatment of Alzheimer's disease. Transl. Neurodegener. 2 (1), 6.
Tam, C., Wong, J.H., Ng, T.B., Tsui, S.K.W., Zuo, T., 2019. Drugs for targeted therapies of alzheimer's disease. Curr. Med. Chem. 26 (2), 335–359.
Thiyagarajan, V., Tsai, M.J., Weng, C.F., 2015. Antroquinonol targets FAK-signaling pathway suppressed cell migration, invasion, and tumor growth of C6 glioma. PLoS One 10 (10), e0141285.
Trepanier, C.H., Jackson, M.F., MacDonald, J.F., 2012. Regulation of NMDA receptors by the tyrosine kinase Fyn. FEBS J. 279 (1), 12–19.
Tuscher, J.J., Fortress, A.M., Kim, J., Frick, K.M., 2015. Regulation of object recognition and object placement by ovarian sex steroid hormones. Behav. Brain Res. 285, 140–157.
Um, J.W., Nygaard, H.B., Heiss, J.K., Kostylev, M.A., Stagi, M., Vortmeyer, A., Wisniewski, T., Gunther, E.C., Strittmatter, S.M., 2012. Alzheimer amyloid-beta oligomer bound to postsynaptic prion protein activates Fyn to impair neurons. Nat. Neurosci. 15 (9), 1227–1235.
van Bokhoven, P., de Wilde, A., Vermunt, L., Leferink, P.S., Heetveld, S., Cummings, J., Scheltens, P., Vijverberg, E.G.B., 2021. The Alzheimer's disease drug development landscape. Alzheimer's Res. Ther. 13 (1), 186.
Villa, A., Rizzi, N., Vegeto, E., Ciana, P., Maggi, A., 2015a. Estrogen accelerates the resolution of inflammation in macrophagic cells. Sci. Rep. 5 (1), 15224.
Villa, A., Rizzi, N., Vegeto, E., Ciana, P., Maggi, A., 2015b. Estrogen accelerates the resolution of inflammation in macrophagic cells. Sci. Rep. 5, 15224.
Villaume, M.T., Sella, E., Saul, G., Borzilleri, R.M., Fargnoli, J., Johnston, K.A., Zhang, H., Fereshteh, M.P., Dhar, T.G., Baran, P.S., Antroquinonol, A., 2016. Scalable synthesis and preclinical biology of a phase 2 drug candidate. ACS Cent. Sci. 2 (1), 27–31.
Vorhees, C.V., Williams, M.T., 2006. Morris water maze: procedures for assessing spatial and related forms of learning and memory. Nat. Protoc. 1 (2), 848–858.
Wang, J.M., Singh, C., Liu, L., Irwin, R.W., Chen, S., Chung, E.J., Thompson, R.F., Brinton, R.D., 2010. Allopregnanolone reverses neurogenic and cognitive deficits in mouse model of Alzheimer's disease. Proc. Natl. Acad. Sci. U. S. A. 107 (14), 6498–6503.
Wang, L., Li, N., Shi, F.X., Xu, W.Q., Cao, Y., Lei, Y., Wang, J.Z., Tian, Q., Zhou, X.W., 2020. Upregulation of AMPK ameliorates alzheimer's disease-like tau pathology and memory impairment. Mol. Neurobiol. 57 (8), 3349–3361.
Weekman, E.M., Sudduth, T.L., Abner, E.L., Popa, G.J., Mendenhall, M.D., Brothers, H.M., Braun, K., Greenstein, A., Wilcock, D.M., 2014. Transition from an M1 to a mixed neuroinflammatory phenotype increases amyloid deposition in APP/PS1 transgenic mice. J. Neuroinflammation 11, 127.
Wei, S., 2016. Potential therapeutic action of natural products from traditional Chinese medicine on Alzheimer's disease animal models targeting neurotrophic factors. Fundam. Clin. Pharmacol. 30 (6), 490–501.
Xu, H., Wang, R., Zhang, Y.W., Zhang, X., 2006. Estrogen, beta-amyloid metabolism/trafficking, and Alzheimer's disease. Ann. N. Y. Acad. Sci. 1089, 324–342.
Yiannopoulou, K.G., Papageorgiou, S.G., 2020. Current and future treatments in alzheimer disease: an update. J. Cent. Nerv. Syst. Dis. 12, 1179573520907397.