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# Targeting cannabinoid receptor activation and BACE-1 activity counteracts TgAPP mice memory impairment and Alzheimer's disease lymphoblasts alterations

**Emilio Nuñez-Borque[1]*, Pedro González-Naranjo[2]*, Fernando Bartolomé[3,4], Carolina Alquézar[1†], Alejandro Reinares[1], Concepción Pérez[2], Maria L. Ceballos[4,5], Juan A. Páez[2], Nuria E. Campillo[1], and Ángeles Martín-Requero[1,4]**

[1]Centro de Investigaciones Biológicas (CSIC) Madrid, [2]Instituto de Química Médica (CSIC), [3]Neurodegenerative Disorders group, Instituto de Investigación Hospital 12 de Octubre. [4]Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Madrid, [5]Insituto Cajal (CSIC), Madrid.

*Equal contribution

†Present address: Department of Neurology, Memory and Aging Center, University of California, Box 1207, San Francisco, CA, 94158, USA.

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Address for correspondence:

Dr. Ángeles Martín-Requero, Dr. Nuria E. Campillo
Centro de Investigaciones Biológicas (CSIC)
Ramiro de Maeztu 9, 28040 Madrid, SPAIN
Phone: 34-91-837-3112
Email: amrequero@cib.csic.es, nuria.campillo@cscic.es
AM-R ORCID: 0000-0002-3416-9440
NEC ORCID: 0000-0002-9948-2665

**ABSTRACT**

Alzheimer's disease (AD), the leading cause of dementia in the elderly, is a neurodegenerative disorder marked by progressive impairment of cognitive ability. Patients with AD display neuropathological lesions including senile plaques, neurofibrillary tangles, and neuronal loss. There are no disease-modifying drugs currently available. With the number of affected individuals increasing dramatically throughout the world, there is obvious urgent need for effective treatment strategy for AD. The multifactorial nature of AD encouraged the development of multifunctional compounds, able to interact with several putative targets. Here, we have evaluated the effects of two in-house designed cannabinoid receptors (CB) agonists showing inhibitory actions on β-secretase-1 (BACE-1) (NP137) and BACE-1/butyrylcholinesterase (BuChE) (NP148), on cellular models of AD, including immortalized lymphocytes from late-onset AD patients. Furthermore, the performance of TgAPP mice in a spatial navigation task was investigated following chronic administration of NP137 and NP148. We report here that NP137 and NP148 showed neuroprotective effects in amyloid β-treated primary cortical neurons, and NP137 in particular rescued the cognitive deficit of TgAPP mice. The latter compound was able to blunt the abnormal cell response to serum addition or withdrawal of lymphoblasts derived from AD patients. It is suggested that NP137 could be a good drug candidate for future treatment of AD.

## Introduction

Alzheimer's disease (AD) is the most common form of dementia in the elderly affecting around 30 million people worldwide. Neuropathologically, AD is characterized by the extracellular accumulation of amyloid-β (Aβ) plaques and intracellular neurofibrillary tangles (NFTs) of hyperphosphorylated tau protein, associated with neuronal cell death. Nevertheless, the cause of neuronal death remains elusive. AD is a multifactorial disease involving different etiopathogenic mechanisms, including APP pathogenic cleavage, neurotransmitter dyshomeostasis, protein misfolding, mitochondrial dysfunction, oxidative stress, and neuronal abortive cell cycle re-entry among others [1,2].

Currently available treatments for AD are symptomatic and do not prevent or slow the progression of the disease [3]. Thus disease-modifying therapy is urgently needed to treat the growing number of affected individuals, and a major goal of research of AD therapeutics. Considering the multifactorial nature of AD, a great effort has been devoted to develop multitarget compounds able to interfere with several of the proposed pathogenic causes. Our group has been engaged during the last decade, in the development of multitarget drugs for AD treatment [4-6]. Recently, we have reported the synthesis of a family of new indazolylketones derivatives with a multitarget profile, showing activity at cannabinoid receptors (CBRs) as agonists, inhibitors of butyrylcholinesterase (BuChE) and β-secretase-1 (BACE-1) [5]. Further biological studies in cellular and *in vivo* models of AD with two of the most interesting multitarget compounds are described in this work. Specific, NP137 a mixed cannabinoid agonist and BACE-1 inhibitor and NP148 a cannabinoid agonist and BACE-1/BuChE inhibitor have been studied.

Cannabinoids exert a wide spectrum of central and peripheral effects by activating specific cannabinoid receptors CB1 and CB2. CB1 receptors are expressed mainly in

nervous system [7], while CB2 receptors are more abundant in cells and organs of the immune system [8], although some reports indicate that CB2 receptors are also expressed in brain, not only in glial cells but in neurons as well [9,10]. The interest in cannabinoids application in AD comes from the observation that the endocannabinoid system is dramatically altered in AD brain [11]. Indeed, a decrease in CB1 receptor protein has been reported along a reduction in cannabinoid agonist G-protein coupling, increased CB2 immunoreactivity in senile plaques, decreased anandamide levels and of the major endocannabinoid degrading enzymes [12-15]. More important is the fact that cannabinoids exert neuroprotective effects in different experimental conditions of acute brain injury, such as excitotoxicity, glucose deprivation and hypoxia, or cerebral ischemia [16,17]. Morever, cannabinoid agonists had been shown to reduce Aβ-induced memory impairment, while reducing the neuroinflammatory response and brain Aβ levels in AD animal models [18-20,12].

BACE-1 is a transmembrane aspartic protease implicated in the generation of Aβ peptides through the cleavage of APP. Moreover, BACE-1 is increased in the brain cortex of most patients with sporadic AD [21,22] and in AD transgenic mice [23,24]. These evidences make BACE-1 an attractive therapeutic target in AD.

BuChE together with AChE belong to cholinesterase (ChEs) family of enzymes and play a role in acetylcholine regulation and in the cholinergic signaling. BuChE, in a different way to AChE, is non-substrate specific and is distributed throughout the body [25]. Two decades ago, the relevance of BuChE in human brain and specifically in AD was underestimated owing to its low expression [26]. However, during the last years, several studies have shown the importance of BuChE within the nervous system to be pivotal in the late stages of AD [27]. AChE knockouts establish central cholinergic pathways and can use BuChE to hydrolyze acetylcholine [28,29].

Given the complex nature of the neuropathology of AD we considered that targeting several molecular pathways at the same time would be interesting. Therefore, in this work we have analyzed the effects of two multitarget compounds, CB agonists and inhibitor of BACE-1 (NP137) or BACE-1/BuChE (NP148), on neuronal cells *in vitro* and murine model of AD *in vivo*. Furthermore, the effects of NP137 have been evaluated in peripheral cells from late-onset AD patients.

We show here that both multitarget CB agonists NP137 and NP148 showed neuroprotective effects against Aβ-induced cell death in primary rat cortical neurons. Furthermore, it is reported that following chronic administration of NP137, but not NP148, improved the performance of TgAPP mice in a spatial navigation task, without altering motor activity. NP137 was able to normalized the response of lymphoblasts derived from AD patients to serum stimulation or withdrawal.

## Materials and Methods

All components for cell culture were obtained from Invitrogen (Carlsbad, CA, USA). PVDF (polyvinylidene difluoride) membranes for Western blots were purchased from Bio-Rad (Richmond, CA, USA). The enhanced chemiluminiscence (ECL) system was from Amersham (Uppsala, Sweden). All other reagents were of molecular grade. Antibodies against β-actin (sc-81178), pRb (sc-500), p27 (sc-528), were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, USA) and anti-Lamin B1 was purchased from Calbiochem (Billerica, MA, USA). Rabbit polyclonal antibodies (pAbs) against human phospho-Akt (Ser473), phospho-ERK1/2, anti-total Akt were obtained from Cell Signaling (Beverly, MA, USA).

### Compound preparation

NP137 (4-methoxybenzyl)(3-(3-(piperidinopropoxy)indazol-1-yl)ketone) and NP148 ((2-benzyloxyphenyl)(3-(3-piperidinopropoxy)indazol-1-yl)ketone) were synthesized as previously described [4,5]. Chemical details of NP137 and NP148 [5] together with effective permeability values that predict its ability to cross the blood brain barrier (BBB) are provided in Table 1.

The compounds were dissolved in 100% dimethyl sulfoxide (DMSO) as a 10 mM stock and diluted with phosphate-buffered saline (PBS; Invitrogen) to final concentrations. $A\beta_{42}$ powder (AnaSpec, Inc., San Jose, CA), was dissolved in acetic acid (0.1M) obtaining a 2μg/μL stock. Then $A\beta_{42}$ was oligomerized in no phenol red DMEM for 24h at 4 ºC. The final concentration in the rat primary cortical neuron cultures was 5 μM. All dilutions of stock were prepared fresh before addition to the culture medium.

### Primary cortical neuronal cultures

Pregnant Wistar rats (3-5 months) were obtained from the inbred colony of the Research

Institute, Hospital Doce de Octubre, Madrid, Spain. Primary cortical neuronal cultures were prepared from rat embryos (E15–16) according to methods described previously [30,31]. 3 x $10^4$ cells per well were plated in pre-treated poly-D-lysine-coated 96-well plates. Cells were maintained at 37°C in a 5% $CO_2$ humidified atmosphere in Neurobasal A medium (Gibco) containing 2 mM L-glutamine, and 10% B27 Supplement (Invitrogen). Nine days after cell culture preparation, cortical neurons were treated with 5μM $A\beta_{42}$ for 24 h and pre-treated or not for 1 h with increasing concentrations of NP137. After treatment, cultures were processed for cell viability assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as previously described [32]. All animals were handled and cared for Council Directive 2010/63/UE of September 22, 2010.

**Animals and treatments**

Male TgAPP (line 2576) harboring two Swedish mutations of 12 months of age at the start of the experiments were used. Controls consisted of littermate wild type mice (WT). Animals were group-housed (4-5 animals per cage) under standard conditions (12:12 h light/dark cycle) with food chow and water accessed *ad libitum*. All procedures were performed in agreement with the ethical regulations on the use and welfare of experimental animals of the EU and the Spanish Ministry of Agriculture, and the bioethical committee of the CSIC approved them.

Oral administration was selected since it is highly suitable for long-term treatment and its translation into the Clinic. The compounds under study were administered in the drinking water at a dose of 1 mg/kg/day, starting at 12 months of age, for 3.5 months. This dose was selected considering our previous studies with cannabinoid compounds [19,20] and their *in vitro* potency. Compounds were first dissolved in 1 ml of DMSO, and thereafter

diluted in water. The volume drank by the mice was monitored every other day and the compound concentration was adjusted to the weight of the animals (assessed weekly) and the fluid drank. All animals had a similar body weight both at the start and at the end of the experiments (not shown). The amount of water drank was similar across the different groups discarding any reinforcing effects of the drugs (not shown).

**Behavioral tests**

All the procedures were performed in isolated rooms at the same time of the day to avoid circadian effects (from 9.00 h to 14.00 h).

Motor activity was monitored in activity cages (Digiscan; AccuScan Instruments, Columbus, OH, USA) for 10 min. The apparatus consisted of several independent open fields of 20 cm x 20 cm, with photobeam sensors. Horizontal activity, measured as distance travelled (cm), and vertical activity (number of rears) over 10 min were recorded.

Spatial learning and memory were assessed by means of the water maze as described in reference [12]. In brief, the mice were trained to find a hidden platform in a pool of 100 cm diameter, over 5 days. Each day the animals were submitted to 4 trials of 60 sec duration, at least 30 min apart, and the latency to reach the platform was recorded. Mice failing to reach the platform were gently placed on it and removed after 15 sec. On the sixth day the platform was removed and the mice were allowed to swim for 90 sec. The time spent in each quadrant of the pool was recorded. The behavior of the animals was acquired with a video camera and analyzed (Noldus Technology, The Netherlands).

***Ex vivo* enzymatic assays**

Mice were killed by decapitation, the brain was dissected on a cold plate and samples stored at -80ºC until assayed. Cerebral cortical samples were homogenized in 20 volumes of Tris HCl 50 mM, pH 7.7, with a Polytron. Samples were centrifuged at

3,000 g and the supernatants were used to assess AChE and BuChE activities. In brief, 20 µl of supernatant was assayed in a final volume of 200 µl, in 100 mM phosphate buffer (pH 8) containing either acetylthiocholine or butirylthiocholine (0.4 mM) as substrates of the reaction, and 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB, Ellman's reagent, 0.2 mM), and the absorbance was read at 412 nm, after 30 min incubation at 30ºC. Samples were assayed in triplicate and the results are expressed as pmol/min x mg of tissue (wet weight). While the activity of AChE was detected in all the samples, BuChE activity was beyond the level of detection.

**Cell lines**

A summary of demographic characteristics of all subjects enrolled in this study is reported in Table 2. A total of 20 patients were diagnosed in the department of Neurology of the University Hospital Doce de Octubre (Madrid, Spain) of probable Alzheimer's disease according to NINCDS-ADRDA (National Institute of Neurological and Communicative Diseases and Stroke-Alzheimer's Disease and Related Disorders Association) criteria. Establishment of lymphoblastic cell lines and culture was performed in our laboratory as previously described [33]. All study protocols were approved by the Hospital Doce de Octubre and the Spanish Council of Higher Research Institutional Review Board and are in accordance with National and European Union Guidelines. In all cases, peripheral blood samples were taken after written informed consent of the patients or their relatives.

**Determination of cell proliferation, cell viability and cell cycle**

Cell proliferation was determined by total cell counting, using a TC10™ Automated Cell Counter, Bio-Rad Laboratories, S.A. (Madrid, Spain). EBV-immortalized lymphocytes from control and AD patients were seeded at an initial cell concentration of 1 x $10^6$ cells x $mL^{-1}$ and enumerated everyday thereafter. Cells failing to exclude Trypan blue were considered nonviable. Cell viability was determined by the MTT assay. Cell survival was

estimated as the percentage of the value of untreated controls. Morphological evaluation was performed using an inverted phase contrast Olympus microscope IX-50. Images were taken with a CCD camera Hamamatsu 9100-02. For cell cycle analysis, cells were fixed in 75% ethanol for 1 hour at room temperature. Subsequent centrifugation of the samples was followed by incubation of cells PBS containing 1 μg/mL of RNase at room temperature for 20 minutes and staining with propidium iodide (PI; 25 μg/mL). Cells were analyzed in an EPICS-XL cytofluorimeter (Coulter Científica, Móstoles, Spain). Estimates of cell cycle phase distributions were obtained by computer analysis of DNA content distributions.

**Immunoblotting analysis**

Cells were collected by centrifugation, washed with PBS and total protein extracts were obtained by lysing them as previously described [34]. To separate the cytosolic and nuclear fractions, cells were harvested, washed in PBS and then lysed in ice-cold hypotonic buffer as previously described [35]. After extraction on ice for 15 min, 0.5% Nonidet P-40 was added and the lysed cells were centrifuged at 4,000 rpm for 10 min. Supernatants containing cytosolic proteins were separated and pellets were resuspended in hypertonic buffer to lysate the nucleus. The protein content of the extracts was determined by the Pierce BCA Protein Assay kit (Thermo Scientific). Equal amounts of proteins were resolved by SDS–polyacrylamide gel electrophoresis. The proteins were then transferred to polyvinylidene fluoride (PVDF) membranes for immunodetection. Primary antibodies were used at the following concentrations: anti-pRb (1:1000); anti-p27 (1:500); anti-phospho-Akt (Ser473) (1:1000); anti-Akt (1:1000); anti-β-actin (1:500); anti-phospho-ERK1/2 (Thr286) (1:1000); anti-ERK1/2 (1:500). The relative band intensities were quantified using Image Studio Lite software (LI-COR Biotechnology).

**Statistical analysis**

Graphical representation and statistical analyses were performed with Graph Pad Prism 8 (La Jolla, CA, USA). All the statistical data are presented as mean ± standard error of the mean (SEM). Normality was checked with the Shapiro-Wilk test. Parametric tests were therefore used in the statistical analysis. Statistical significance was estimated by both one-way and two-way analysis of variance (ANOVA) followed by the Fisher's test for multiple comparisons. In the water maze experiments repeated measures ANOVA was used, followed by one-way ANOVA at each time point. A value of $p < 0.05$ was considered significant.

## Results

### Neuroprotective effects of NP137 and NP148 on β-amyloid-induced cell death in rat primary cortical neuronal cultures.

We first evaluated the potential neuroprotective effects of multitarget CB agonist and BACE-1 inhibitor, (NP137) and NP148, a CB agonist with a profile of BACE-1 and BuChE inhibitor in rat primary cortical neuronal cultures. For these experiments, rat primary cortical neurons were preincubated in the absence or in the presence of increasing concentrations of NP137 or NP148 and then exposed for 24 h to Aβ. As shown in Fig. 1, cortical neurons' viability was markedly decreased after the cell was exposed to Aβ as determined by MTT, while pretreated with NP137 or NP148 (2.5 and 5μM) the Aβ-induced cell death was significantly attenuated.

### NP137, but not NP148, counteracts cognitive deficits in TgAPP mice

We next seek to evaluate the effects of NP137 and NP148 in a murine model of AD. To this end, mice received prolonged oral administration of compounds (1 mg/kg/day po).

As shown in Fig. 2 (A-B) TgAPP mice showed learning deficits in the water maze as compared to WT animals receiving vehicle. Indeed, WT mice learned the task over the five training days, as judged by the decrease in latency time to reach the platform, while TgAPP mice needed more time. Prolonged oral administration of NP137 delayed the learning of WT animals, and there was a significant difference in the time to reach the platform at day 3 and day 4 compared to WT mice receiving vehicle, although at day 5 all WT mice, regardless their treatment, had the same latency (Fig. 2A). In contrast, long-term administration of NP137 to TgAPP mice completely restored their cognitive abilities, and their latencies to reach the platform were almost identical to WT vehicle treated mice. NP148 continuous administration neither altered the learning of WT nor

modified the learning deficits of TgAPP mice. In fact, the animals on NP148 treatment improved at day 2 in comparison to day 1 performance, but the latency was maintained until day 5. Next, we examined the time spent in each quadrant of the pool on day 6, following the training period. There was a significant decrease in the time TgAPP mice spent in the platform quadrant compared to WT vehicle treated animals. This decrease was fully reversed but continuous administration of NP137, but unaffected by NP148 treatment. In summary, NP137 was able to restore the learning and memory deficits shown by TgAPP mice.

Given that the water maze relies in locomotor activity it was necessary to assess any effects of the new compounds on that parameter. All the animal groups displayed a similar speed in the water maze, and the speed displayed on day 6 is shown in Fig. 2D. In agreement with these results no differences in the distance travelled by mice treated for 3.5 months were observed, and the number of rearings (vertical activity) were neither significantly different, although the compounds under study tended to increase the vertical activity, a measure of exploration in a novel environment. In summary, NP137 is able to counteract both the learning and memory deficits shown by TgAPP mice following prolonged administration.

As shown in Fig. 2G, AChE enzymatic activity was similar across all the experimental, in accordance with the absence of effect of the new compounds in in vitro experiments [5].

**Activation of CB receptors normalized the response of AD lymphoblasts to serum stimulation or withdrawal.**

Since NP137 improved learning memory deficit in a murine model of AD, we considered interesting to evaluate the effects of this compound on the survival/death fate of human

immortalized lymphocytes from AD patients in response to serum stimulation or deprivation.

Previous work from this laboratory demonstrated that AD lymphoblasts present neoplastic-like features, enhanced cell proliferation in the presence of serum, and increased resistance to cell death induce by serum deprivation. Moreover, it was shown that the cell responses to serum stimulation or withdrawal of AD lymphoblasts are not dependent on disease severity, as we did not observed differences between cells derived from mild, moderate or severe AD patients [36,37]. Therefore, experiments were performed with cell lines randomly selected from control individuals and from the three groups of AD patients. Data in Fig. 3 show, as expected, enhanced proliferation of AD lymphoblasts in the presence of serum, as well as increased resistance to serum deprivation-induced cell death in comparison with lymphoblasts derived from control, non-demented individuals [38]. The addition of NP137 (5μM) blunted the increased proliferative activity of AD cells, without affecting the rate of proliferation of control cells (Fig. 3). On the other hand, the presence of NP137 restored the normal cellular response to serum withdrawal, observing similar values of dying cells in both control and AD cultures 72 h after NP137 addition (Fig. 3). In agreement with the fact that NP137 did not affect the rate of proliferation of control cells, suggesting that NP137 does not have cytotoxic effects, we did not observe morphological changes in control or AD lymphoblasts after NP137 treatment (Fig. 4A), Moreover, we did not find significant changes in the proportion of sub-$G_0/G_1$ hypodiploid cells, characteristic of apoptosis/necrosis, in control and AD lymphoblasts in response to NP137 addition under proliferative conditions (Fig. 4B).

Overactivation of PI3K/Akt leading to decreased levels of the CDK inhibitor p27 is a distinct feature of immortalized lymphocytes from AD patients [39] accounting for the

increased proliferative activity. For this reason, we have analyzed the effects of NP137 on PI3K/Akt activity and in the cellular content of p27. Cells were preincubated for 30 min in the presence of NP137 before serum stimulation PI3K/AKT activity was determined by assessing the levels of Akt phosphorylation with a phospho-specific anti-Akt antibody, 24 h after serum addition. As shown in Fig. 4, NP137 prevents the increase in Akt phosphorylation in AD cells, without affecting the phosphorylation status of Akt in control cells. This effect of NP137 was accompanied by the restoration of the p27 levels in AD lymphoblasts to similar values observed in control cells (Fig. 2). As expected, reciprocal changes between levels of p27 and phosphorylation status of pRb protein were found (Fig. 5).

**NP137 restored normal ERK1/2 activity and p21 levels in AD cells under serum deprivation**

The higher resistance of AD lymphoblasts to death induced by serum deprivation was associated with a lower sustained stimulation of ERK1/2 activity and with elevated levels of the p21 protein [38,39]. Fig. 6 shows that indeed, ERK1/2 activity, as monitored by the enhanced phosphorylation of ERK1/2, is severely reduced in AD cells, and in parallel p21 levels are higher. The addition of NP137 normalized both the phosphorylation status of ERK1/2 and p27 content of AD lymphoblasts (Fig. 6).

**Effects of NP137 on subcellular localization of p21**

It was reported previously that the protein p21 exert different nuclear and cytosolic functions [40,41]. The antiapoptotic effect of increased levels of p21 in AD lymphoblasts was associated with a shift of p21 from nucleus to the cytosolic compartment [39]. On these grounds, we decided to explore the effect of NP137 in the subcellular localization of p21 in control and AD lymphoblasts by performing nuclear and cytoplasmic fractionation experiments. Fig. 7 shows that p21 is accumulated in the cytoplasm rather

than in nucleus in serum-deprived lymphoblasts. AD cells show a significant increase in p21 cytosolic levels, that was reduced by the presence of NP137.

**Discussion**

Multitarget cannabinoids had been considered a promising therapeutic strategy for AD treatment. Here we report that NP137, and NP148, two in-house designed multitarget CB agonists showing BACE-1 and/or BuChE activities, showed neuroprotective actions against Aβ-induced cell death in primary rat cortical neurons and in particular NP137 improved cognitive performance in TgAPP transgenic mice. In addition, NP137 normalized the response of human AD lynphoblasts to serum stimulation or deprivation.

Both NP137 and NP148 were able to partially prevent the cell death induced by Aβ in primary rat cortical neuronal cultures. These results are in consonance with previous work showing similar neuroprotective effect of other CB2 agonist [36].

These promising results encouraged performing studies in a murine model of AD with both compounds. The compounds under study differentially affected the cognitive impairment of TgAPP mice after their continuous administration for 3 and a half months. NP137, but not NP148, was able to prevent the learning deficit in a spatial navigation task. Indeed, the enhanced latency to find the platform of TgAPP mice (vehicle treated) was counteracted by prolonged oral administration of the drug. Moreover, there was a significant recovery of the AD mice memory by NP137, as judged by the increased time spent in the target quadrant compared to TgAPP vehicle treated. These results are in line with previous reports showing amelioration of the learning and memory deficits by chronic cannabinoid agonist administration in AD models [19,20,42]. However, in WT mice we noticed a delay in their learning abilities, although at the end of the training period all animals learned the task. Previously a deleterious effect of cannabinoids had been observed in young mice following continuous administration of a low dose of $\Delta^9$-tetra-hydro-cannabinol, while mature or aged animals showed beneficial effects [43]. Interestingly, hippocampal gene expression changes paralleled those effects.

Furthermore, we recently reported that fatty acid amide hydrolase activity was similarly reduced in aged rat synaptic endings and human AD brains, but CB1/CB2 agonists reduced availability of anandamide in AD while they increased it in aging [14].

It is well known that CB1 and CB1/CB2 mixed agonists acute administration reduces motor activity [44,45], both horizontal and vertical activity, and it is considered a detrimental effect. However, upon repeated administration of such cannabinoids, tolerance develops and the hypomotor effect disappears [46]. Tolerance likely accounts for the absence of a significant effect of prolonged administration of either NP137 or NP148 on motor activity.

Several works have reported that AChE and BuChE inhibitors are able to restore the cognitive deficits shown in different models relevant to AD [47-49]. Interestingly, the beneficial effects on cognition are highly dependent on the dose, detrimental effects being observed with higher doses [50,47]. This fact has been attributed to high levels of ACh, which might act on the autoreceptors inhibiting its synthesis and release in the presynaptic neurons, subsequently impeding the acquisition of information. Therefore, it could be argued that the inability of NP148 that shares a similar cannabinoid agonist profile with NP137 [5], in restoring the cognitive deficits of TgAPP is related to its *in vitro* potency in inhibiting BuChE activity. In any case, BACE-1 inhibition, by decreasing Aβ generation and neuroinflammation *in vivo*, could contribute to the beneficial effects of NP137 [51].

Immortalized lymphocytes from AD and other neurodegenerative disorders have proven to be a suitable human cell-based platform to evaluate at preclinical level the potential of drug candidates for the treatment of these disorders. We evaluated the effects of NP137 in the cellular response to serum. It is assumed that cell cycle alterations in non-neuronal cells from AD patients are systemic manifestation of the disease, as aberrant cell cycle

entry is observed in affected neurons in AD brains [52]. Our results indicate that NP137 was effective in preventing the enhanced serum stimulation of cell proliferation observed in lymphoblasts from AD patients. NP137 counteracted the overactivation of PI3K/Akt, resulting in down regulation of the CDK inhibitor p27 levels and subsequent recovering of normal levels of phosphorylated pRb protein. These findings are in consonance with previous work from our laboratory [36] in which the effects of a CB2 agonist/BuChE inhibitor was studied. Together these results support a predominant role of CB receptor activation, rather than BACE-1, or BuChE activities in peripheral cells.

It was previously reported that lymphoblasts from AD patients showed an increased resistance to serum deprivation-induced cell death [38,39]. Here we show that NP137 was able to blunt the activation of the signaling cascade leading to cell death in response to serum withdrawal, namely decreased ERK1/2 activation and enhanced levels of p21, together with cytosolic accumulation of p21 [39]. These features appear to confer AD cells a survival advantage similar to that described for cancer cells [53]. In agreement with previous work showing antitumoral effects of CB receptor activation in C6 glioma cells by up-regulation of ERK1/2 activity [54], our results indicate that NP137 addition restored ERK1/2 phosphorylation levels and activity, decreased up-regulated p21 levels, and normalize p21 subcellular localization, thus sensitizing AD cells to apoptosis.

Together, our results indicate that CB receptor activation modulates the functional relationship PI3K/Akt or ERKs activities in serum-induced signaling in immortalized lymphocytes, controlling cell fate (proliferation/death or survival) depending on growth factor availability. The proposed scenario is represented schematically in Fig. 8.

In summary, our work revealed that the dual CB agonist and potent BACE-1 inhibitor, NP137, has the capacity to restore abnormal features of AD lymphoblasts. Moreover, NP137 showed neuroprotective effects in primary rat neuronal cultures, and rescued the

cognitive deficit of TgAPP mice. We, therefore, believe that NP137 could be a promising candidate for AD treatment.

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**Authorship Contributions**

EN, PGN, FB, CA, AR, CP, MLC, JAP, NEC, AMR

Participated in research design: JAP, MLC, NEC and A M-R

Conducted experiments: EN-B, PGN, AR, CP, FB, CA, and MLC

Contributed new cannabinoids: JAP, PGN

Performed data analysis: All authors contributed to data analysis

Wrote or contributed to the writing of the manuscript: MLC, NEC, and AM-R.

**DISCLOSURE**

None of the authors has any conflict to disclose

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**Legend to the figures**

**Fig. 1**

**Neuroprotective effects of NP137 and NP148 on Aβ-induced death in rat primary cortical neurons**

Rat cortical primary neurons were exposed to 5 μM Aβ for 24 hours in the presence or in the absence of 2.5, 5 and 10 μM of NP137 (left panel) or NP148 (right panel). The number of viable cells was measured by MTT assay. Each data point represents the mean ± SEM of three replications in 5 different experiments. *p<0.05, ***p<0.001significantly different from untreated cells; and †p<0.05, ††p<0.01, and †††p<0.001 significantly different from Aβ-treated cells.

**Fig. 2.**

**NP137 counteracts cognitive deficits in TgAPP mice.** Following oral administration for 3.5 months starting at 12 months of age the effect of the compounds NP137 and NP148 on a spatial navigation task was assessed. Latency (sec) to reach the hidden platform in the water maze of vehicle treated (veh) or treated with NP137 and NP148 over the 5 training days. A) Effects on WT mice. NP137 delayed WT mice learning. B) Effects on TgAPP mice. NP137 counteracted TgAPP mice learning deficits. C) Time spent in the target quadrant (platform location). TgAPP (vehicle treated) mice spent less time in the target quadrant, while NP137 prevented that reduction. D) All mice showed similar speed while swimming in the water maze on the probe trial day ($6^{th}$ day). E) All animals displayed a similar locomotor activity (distance travelled in cm over 10 min) in the open field. F) Number of rears (vertical activity) were not significantly different across the different groups. G) AChE enzymatic activity in cerebral cortex samples were

measured in triplicates. Results are expressed in pmol/min x mg of tissue (wet weight). *p<0.05 vs WT mice, # p<0.05 vs Tg APP + veh mice.

**Fig. 3**

**NP137 normalized the cell response to serum stimulation or withdrawal**

Immortalized lymphocytes from control and late-onset AD patients were seeded at an initial density of 1 x $10^6$ x $ml^{-1}$ and incubated with or without 10% FBS in the absence or in presence of NP137 (5μM). Aliquots were taken for cell counting 72 hours after the drug administration. Data shown are the mean ± SEM for 10 observations carried out with cell lines from different individuals. *p<0.05, significantly different from control cells. †p<0.05, significantly different from untreated AD cells.

**Fig. 4**

**NP137 does not induce morphological alterations or apoptosis in control and AD lymphoblasts.**

Immortalized lymphocytes from control and late-onset AD patients were seeded at an initial density of 1 x $10^6$ x $ml^{-1}$ and incubated with 10% FBS in the absence or in the presence of NP137 (5μM). A) Morphological images were taken using an inverted phase contrast Olympus microscope IX-50 with x40 objective. Images were taken with a CCD camera Hamamatsu 9100-02. Scale bar: 50 μm. B) Cell cycle analysis of control and AD lymphoblasts after treatment with NP137. The percentage of cells in Sub $G_1$ is indicated for each condition.

**Fig. 5**

**NP137 restores the phosphorylation status of Akt and pRb proteins, and cellular content of p27 in AD lymphoblasts.**

Immortalized lymphocytes from control and AD patients, were seeded at an initial density of 1 x $10^6$ x $ml^{-1}$, and incubated in RPMI medium containing 10% FBS in the absence or in the presence of the CB agonist NP137 (5μM). Cell extracts were prepared 24 hours after drug administration to determine levels of phosphorylation of Akt, p27 and pRb by Western blot using phospho-specific antibodies. Representative immunoblots are shown. The densitometric data represent the mean ± SEM of 6-10 different experiments *$p<0.05$; **$p<0.01$ significantly different from control cells. †$p<0.05$ ††$p<0.01$ significantly different from untreated AD cells.

**Fig. 6**

**NP137 increases ERK1/2 activation and p21 levels in serum-deprived AD lymphoblasts**

Immortalized lymphocytes from control and AD patients, were seeded at an initial density of 1 x $10^6$ x $ml^{-1}$ and incubated in serum deprived-RPMI medium in the absence or in the presence of NP137 (5μM). Cell extracts were prepared 72 hours after drug administration to determine levels of phosphorylation of ERK1/2, and cellular levels of p21 by Western blot. Representative immunoblots are shown. The densitometric data represent the mean ± SEM of 5 different experiments, *$p<0.05$, significantly different from control cells. †$p<0.05$, significantly different from untreated AD cells.

**Fig. 7**

**NP137 counteracts the enhanced p21 nucleo-cytoplasmic shuttling in serum-deprived AD lymphoblasts**

Lymphoblasts from control and AD patients were seeded at an initial density of 1 x $10^6$ x $ml^{-1}$, incubated in the absence of serum for 72 hours, and then fractionated to determine by immunoblot analysis the subcellular localization of p21. Antibodies to α-tubulin and

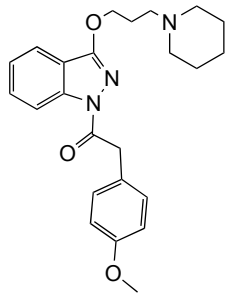
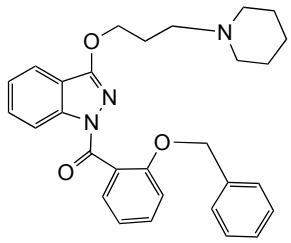
to lamin B1 were used as control of purity and loading of cytoplasmic and nuclear protein extracts, respectively. A representative immunoblot is shown, whereas the densitometric analysis is presented below. Data represent ± SEM of 5 different experiments. ***p<0.001, significantly different from control cells, †††p<0.001, significantly different from untreated AD cells.

**Fig. 8**

**Diagram summarizing the effects of targeting CB receptors on fate of AD lymphoblasts upon serum stimulation or withdrawal.**

In the presence of serum AD cells show enhanced activation of PI3K/Akt leading to decreased levels of p27, and activation of cyclin/CDK/pRb, therefore favoring the progression of cells through the cell cycle. In the absence of serum, ERK1/2 activity is downregulated in comparison with control cells, which in turn induces an increase on the cellular content of p21, as well as favors the cytosolic localization of p21, which then seems to protect AD lymphoblasts from the serum deprivation-induced apoptosis.

**Table 1. Activity profile of NP137 and NP148**

| Structure | Code | CBR[a] (Emax) | BACE-1[b] %Inhibition (10 μM) | BuChE[c] $IC_{50}$ | logBB [55] |
|---|---|---|---|---|---|
|  | NP137 | Agonist<br>CB1/CB2 | 60 ± 8 | >10(42%) | BBB+ |
|  | NP148 | Agonist<br>CB1/CB2 | 38.35 ±1.11 | 2.5 ± 1.2 nM | BBB+ |

[a]Receptor binding studies were performed using membrane fractions of human CB1 or CB2 receptor transfected cells (HEK293EBNA). [b]Percentage of inhibition of BACE-1 at 10 μM. [c]$IC_{50}$ half maximal inhibitory concentration values (mean ± standard error of the mean) were determined from three different experiments using 0.5 mM of BuChE as the substrate [48]. BBB prediction was estimated as previously reported [55].

**Table 2. Summary of the study population**

| | CONTROL | AD |
|---|---|---|
| Age | 68 ± 10 | 74 ± 6 |
| Age range | 50-83 | 56-82 |
| Gender | | |
| Male | 8 | 11 |
| Female | 12 | 9 |
| Total | 20 | 20 |

Control: individuals with no sign of neurological disease. AD: patients with a diagnosis of probable AD. Values are expressed as means ± SD

Figure 1

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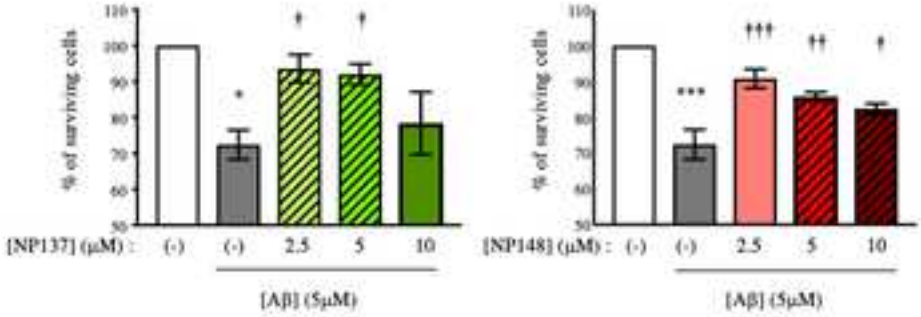


Figure 1

Figure 2

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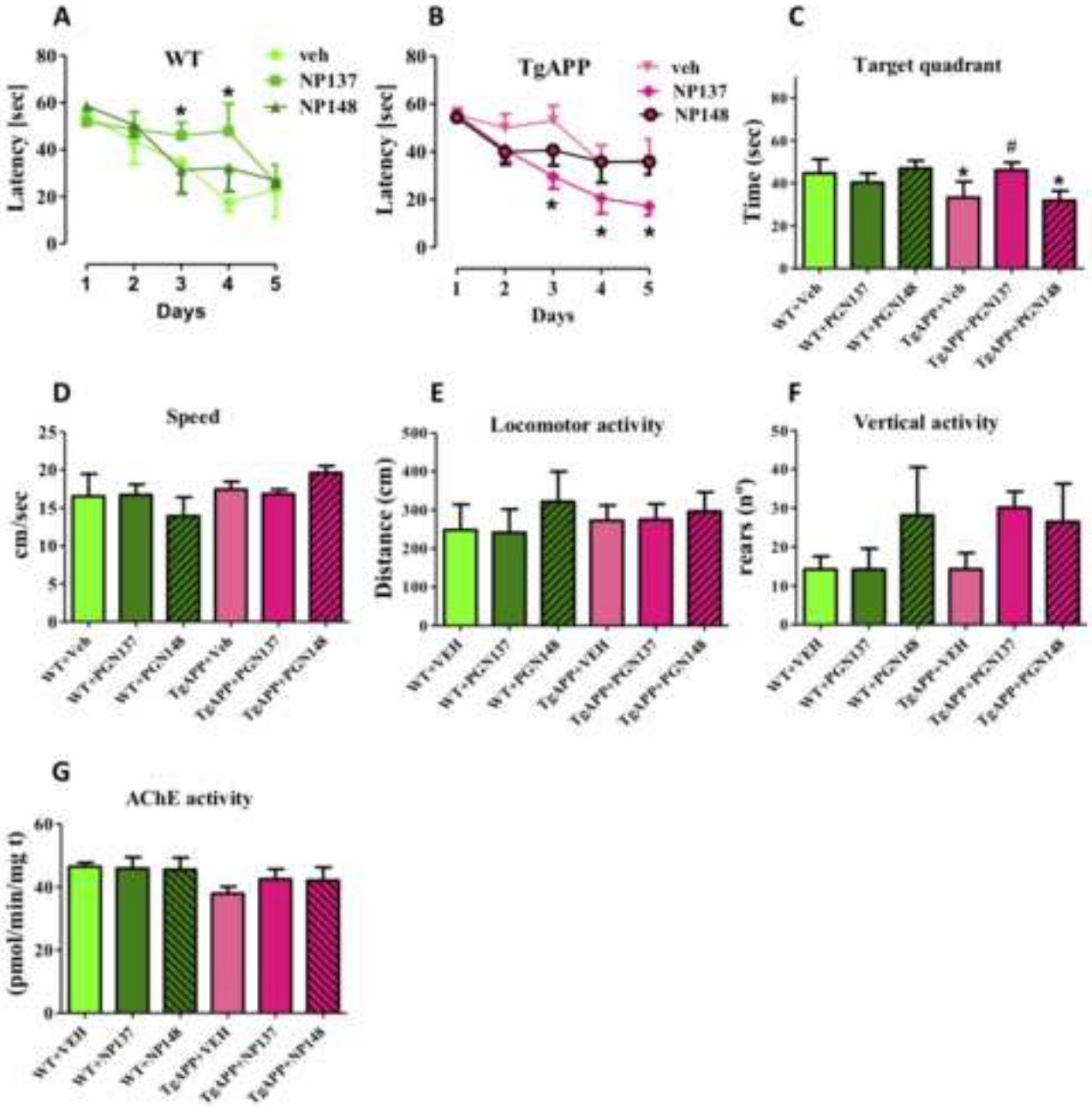


Figure.2

Figure 3

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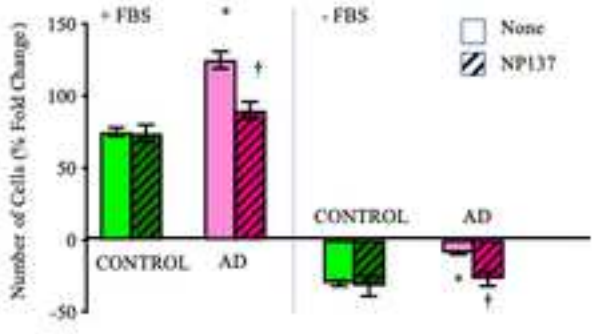


Figure 3

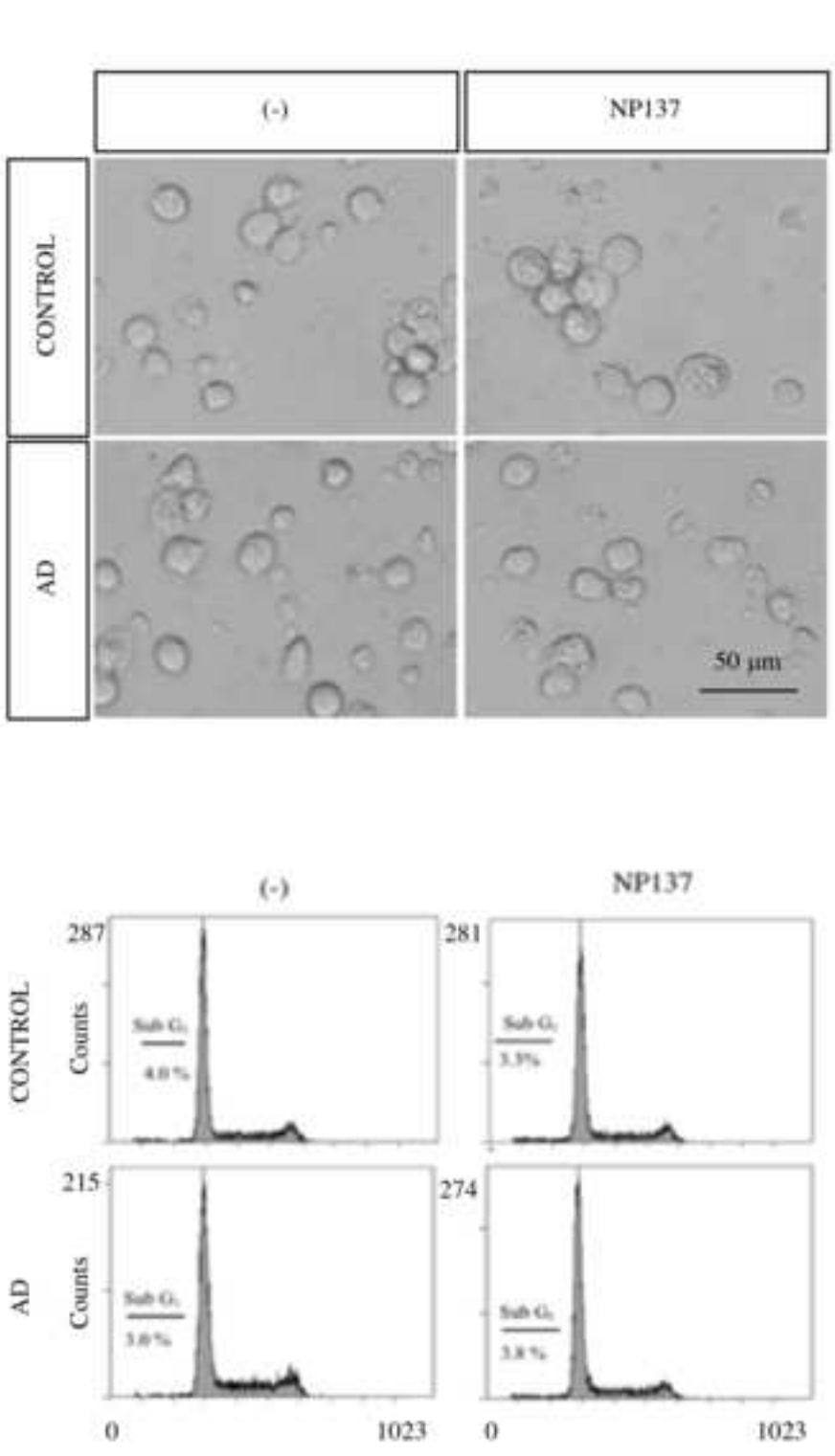


Figure 4

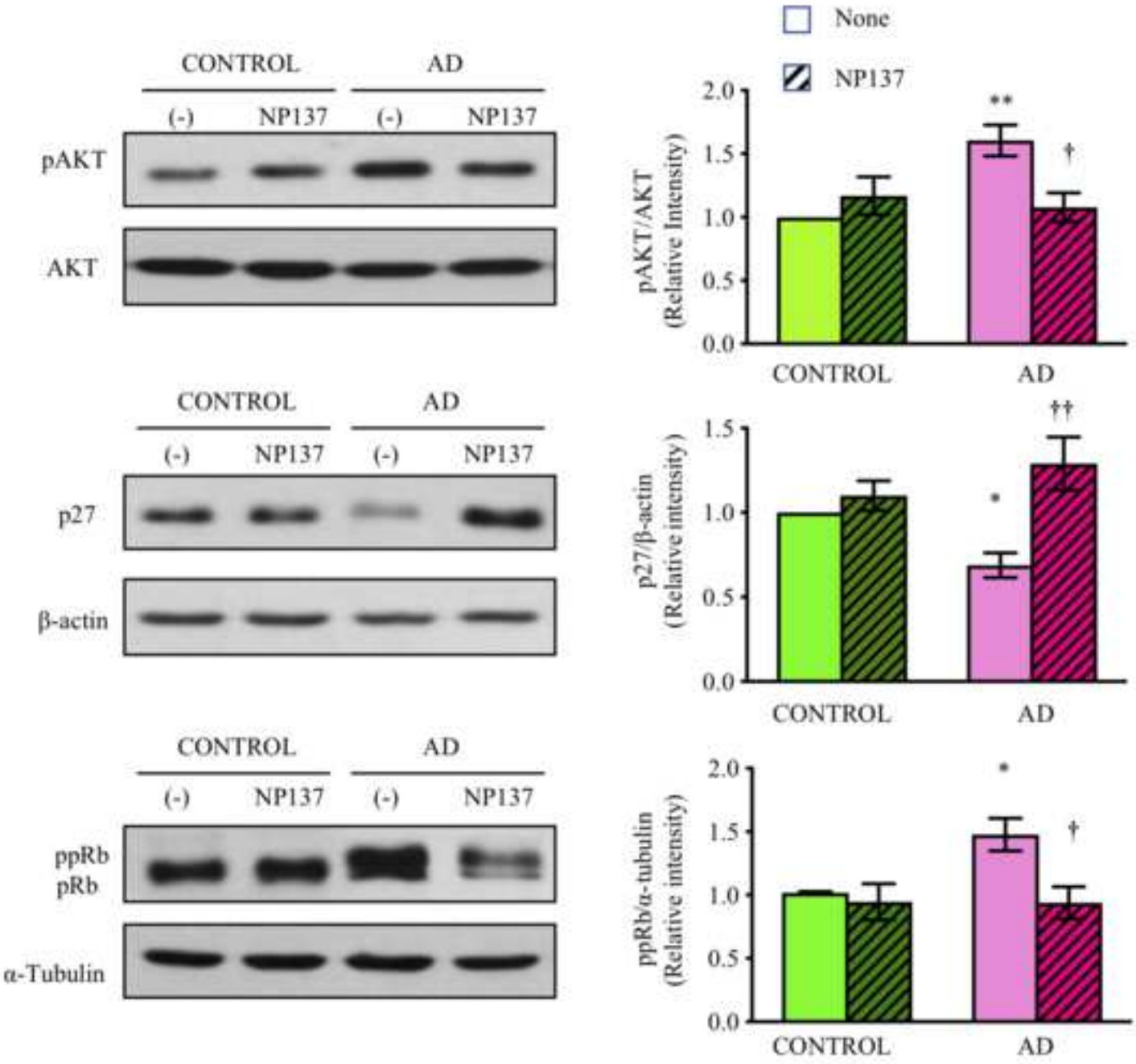


Figure 5

Figure 6

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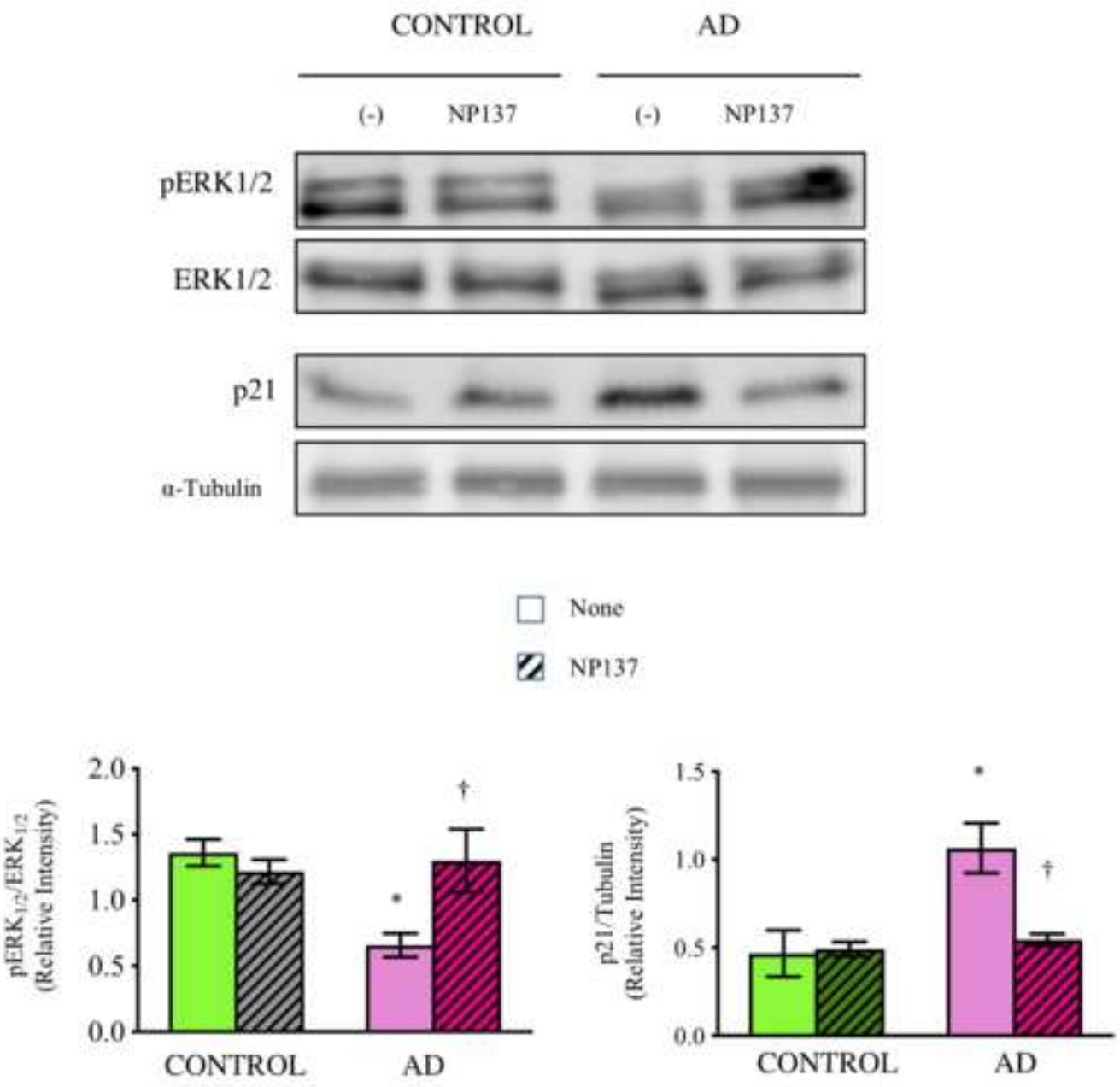
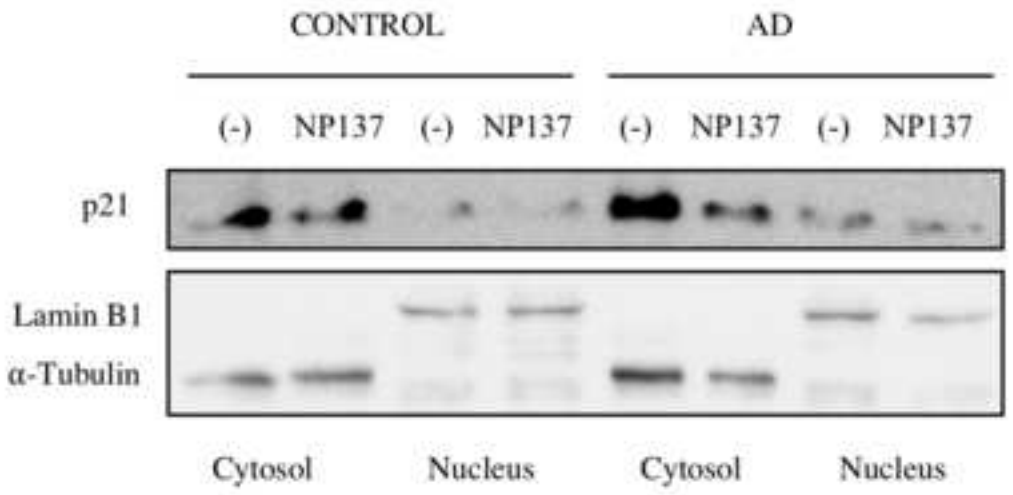



Figure 6



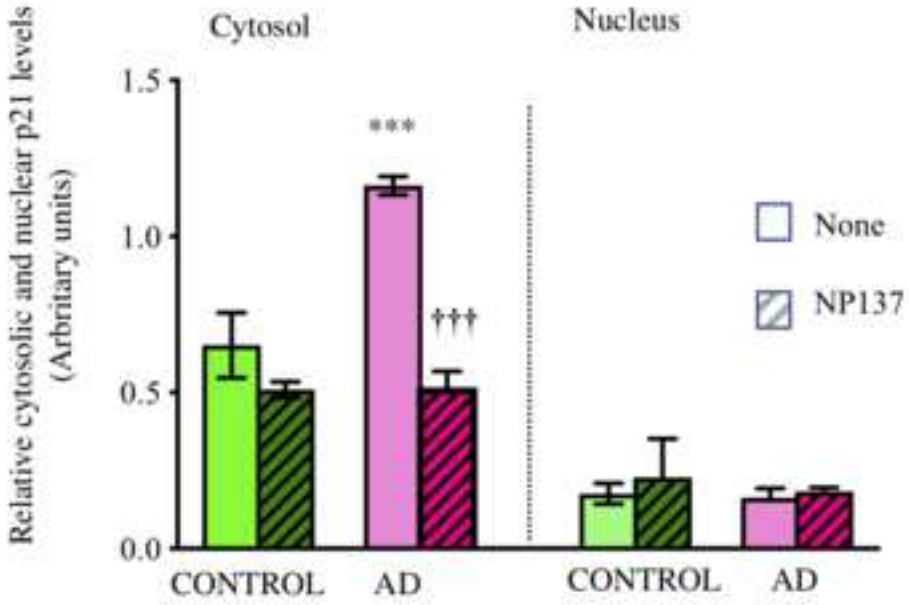




Figure 7

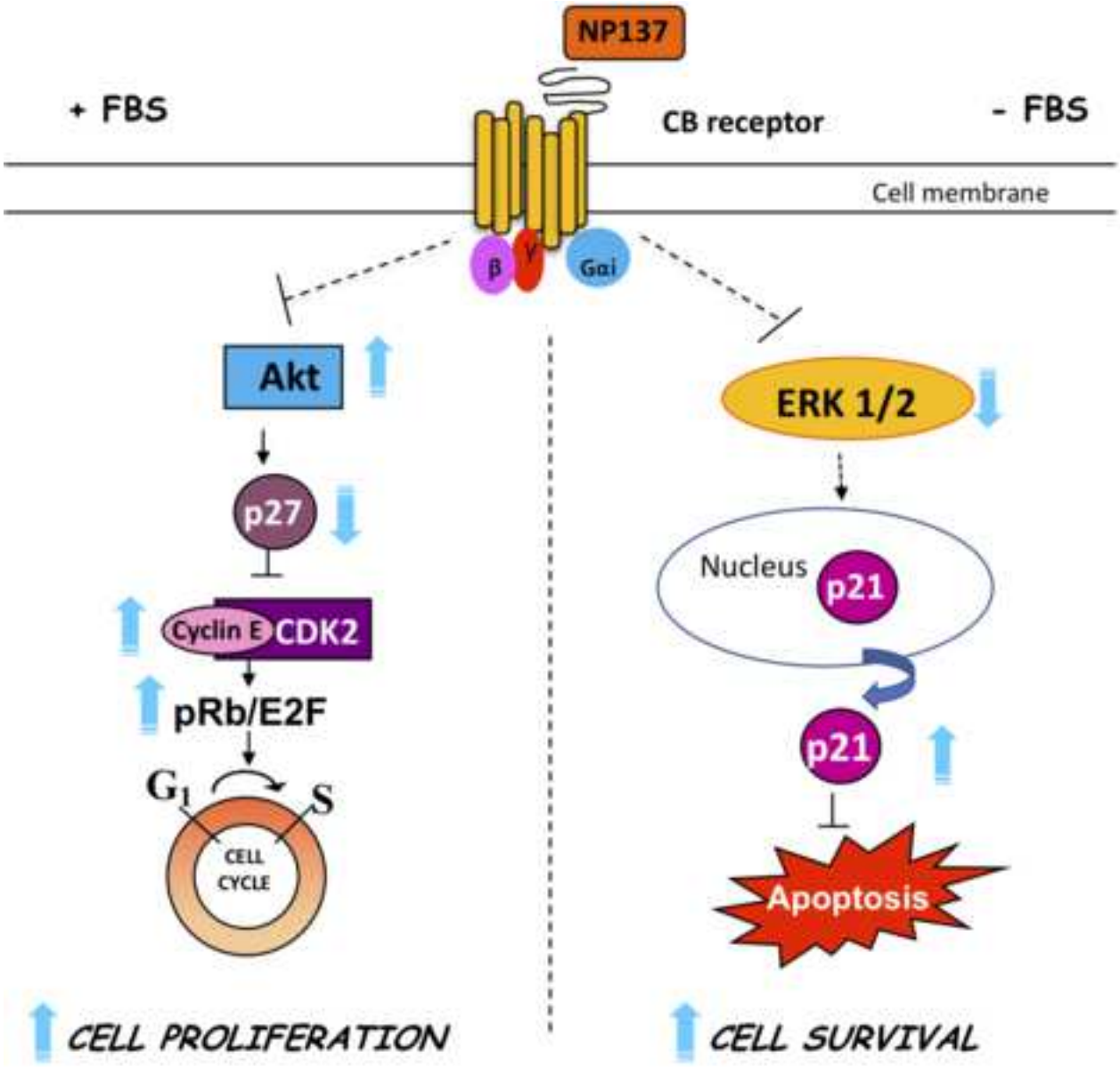



Figure 8