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**Activation of cannabinoid type 2 receptor by a novel indazole
derivative normalizes survival pattern of lymphoblasts from late-onset
Alzheimer's disease patients**

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ABSTRACT

Background: Alzheimer's disease (AD) is a multifactorial disorder for which there are no yet disease-modifying treatment. CB2 receptors have emerged as a promising therapeutic target for AD, because they are expressed in neuronal and glial cells and their activation has no psychoactive effects.

Objective: The aim of this work was to investigate whether activation of CB2 receptor would restore the aberrant enhanced proliferative activity characteristic of immortalized lymphocytes from late-onset AD patients. It is assumed that cell cycle dysfunction occurs in both peripheral cells and neurons in AD patients contributing to the instigation of the disease.

Methods: Lymphoblastoid cell lines from AD patients and age-matched control individuals were treated with a new in-house designed dual drug PGN33, that behaves as CB2 agonist and Butyryl Cholinesterase (BuChE) inhibitor. The effects of this compound on the rate of cell proliferation and levels of key regulatory proteins were analyzed. In addition, we investigated the potential neuroprotective action of PGN33 in β -amyloid ($A\beta$) treated neuronal cells.

Results: We report here that PGN33 normalized the increased proliferative activity of AD lymphoblasts. The compound blunted the calmodulin-dependent overactivation of the PI3K/Akt pathway, by restoring the cyclin dependent kinase (CDK) inhibitor p27 levels, which in turn reduced the activity of the CDK/pRb cascade. Moreover, this CB2 agonist prevented $A\beta$ -induced cell death in neuronal cells.

Conclusion: Our results suggest that the activation of CB2 receptors could be considered a useful therapeutic approach for AD.

KEY POINTS

- PGN33, an in-house designed CB2 agonist, is able to normalize enhanced proliferative activity of lymphoblasts from Alzheimer's disease patients by attenuating the calmodulin-dependent overactivation of PI3K/Akt pathway.
- PGN33 has neuroprotective effects on β -amyloid-induced cell death in primary rat cortical neuronal cultures
- PGN33 could be considered a promising candidate for future treatment of AD.

1. INTRODUCTION

Alzheimer's disease (AD), the most common cause of dementia in the elderly, is a neurodegenerative disorder marked by progressive impairment of cognitive ability. The neuropathological hallmarks for AD are β -amyloid ($A\beta$) plaques, neurofibrillary tangles, and regionalized neuronal loss. The causes of the disease are largely unknown but many factors are implicated, including toxic effects of aggregated amyloid and tau proteins [1, 2], inflammation [3] and oxidative damage [4], and cell cycle disturbances [5-7].

With increasing life expectancy across the world, dementia is a rapidly growing socioeconomic and medical problem. There are no effective disease-modifying drugs currently available. Drug development for AD has proven to be very difficult. Five drugs are approved for the treatment of AD including four cholinesterase inhibitors (tacrine, donepezil, rivastigmine, galantamine) and an N-methyl-D-aspartate (NMDA) receptor antagonist (memantine) [8, 9]. Moreover, in the last years all clinical trials with new drugs have failed. Therefore, it is a priority to explore new alternatives for the development of new drugs.

AD has multifactorial nature, where interrelated events result in plaque and neurofibrillary tangles formation, cholinergic deficit and neuronal loss, current therapeutic strategies are directed to address multiple etiological targets [10]. In this regard, we have designed and synthesized new indazole ethers that possess different activities as CB2 agonists and inhibitors of butyrylcholinesterase (BuChE) activity and β -secretase-1 (BACE-1) [11].

In the last few years, there has been a growing interest in the use of cannabinoids for AD patients, since the cannabinoid system is dramatically altered in AD brain [12]. Cannabinoids compounds could act on different pharmacological targets, besides interacting with the endocannabinoid system. CB2 receptors were first found in

peripheral organs as spleen or thymus, as well as in macrophages, B and NK cells [13], however increasing evidence during this last decade support the involvement of CB2 receptors and signaling in neurogenesis, axon guidance, and neuronal excitability [14-17] excitability. On the other hand, deletion of CB2 receptors in mouse hippocampus has been found to be associated with deficits in memory and synaptic plasticity [18]. Therefore, it is believed that non-psychoactive CB2 receptors could have potential as a therapeutic target for neurodegenerative disorders including AD (reviewed in [19]).

As it is well known, AD is characterized by marked cholinergic dysfunction [20]. Depletion of acetylcholine together with significant reduction of the acetylcholinesterase (AChE) activity and increased BuChE activity are observed in the cortex and hippocampus of AD patients [21]. These changes are thought to contribute to the loss of memory and cognition during disease progression. In addition, BuChE has been associated with A β plaques and neurofibrillary tangles [22, 23]. These features emphasize the potential of BuChE as a therapeutic target for AD [24].

The present work was undertaken to explore the potential of a new CB2 agonist with additional properties as inhibitor of BuChE, which could restore observed pathological features of peripheral cells from late onset AD patients.

Our previous work highlighted the role of Ca²⁺/Calmodulin-dependent activation of PI3K/Akt in controlling cell proliferation of immortalized lymphocytes from AD patients [25, 26]. It was demonstrated that the cell cycle regulatory failure observed in AD lymphoblasts resulted from enhanced degradation of the CDK inhibitor p27, with the concomitant stimulation of phosphorylation of the pRb family of proteins and thus favoring the progression of the cell cycle at the G1/S checkpoint [27]. It was suggested that these cell cycle-related alterations could have pathogenic significance in AD, as it is believed that unscheduled cell cycle entry underlies neuronal loss in neurodegenerative

disorders [7, 28-30]. The re-entry of quiescent neurons into the cell cycle may result in a mitotic failure and cell death [5, 6, 31]. Moreover, lymphoblastoid cell lines from AD patients have been previously used as a platform to test the therapeutic potential of certain drugs [32-34]. Here we report the ability of one in-house designed CB2 agonist with additional cholinergic activity in normalizing the aberrant proliferation and signaling of lymphoblasts from AD patients. In addition, this compound shows neuroprotective effects against A β -induced cell death in primary rat cortical neurons and human neuroblastoma SH-SY5Y cells.

2. METHODS

2.1. Materials.

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), α -tubulin (B-5-1-2) sc-23948, pRb (sc-500), p27 (sc-528), pCaMKII (Thr286) (sc-12886-R), Akt (C-20) (sc-1618), and CB2 (3C7) (sc-293188) were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Rabbit polyclonal antibody against human phospho-Akt (Ser473) #9271 was obtained from Cell Signaling (Beverly, MA, USA). Rabbit polyclonal antibody against human NeuN (ABN78) was obtained from Merk Millipore (Darmstadt, Germany). Rabbit polyclonal antibody against human glial fibrillary acidic protein (GFAP; Z0334) was obtained from Dako (Agilent; Cal, US).

2.2. Compound preparation

CB2 agonist, PGN33 (1-(2-diisopropilaminoethyl)-3-(4-methoxybenzyloxy) indazole was synthesized as previously described [11]. PGN154, a potent inhibitor of BuChE (IC₅₀ 0.15±0.02 μ M) was synthesized in our group (*PCT/ES2016/070906,2016*). Chemical details of PGN33 together with effective permeability values, that predict its ability to cross the blood brain barrier (BBB) [35] are provided in Table 1. The effect of PGN33 on BuChE activity was assessed using BuChE from human serum (3units/mg of protein) and 0.4 mM butyrylthiocholin as substrate in Elmans reagent (5,5-dithio-bis (2-nitrobenzoic acid) as described [11]. the values of IC₅₀ were calculated by UV spectroscopy from the absorbance changes at 412 nm. Experiments were performed in triplicate. The commercial CB2 agonist JWH133 was from Tocris (Cookson Ltd., UK)

and the CB2 antagonist AM630 was provided by Sigma (Alcobendas, Spain). Compounds were dissolved in 100% dimethyl sulfoxide (DMSO) as a 10 mM stock and diluted with phosphate-buffered saline (PBS; Invitrogen) to their final concentrations. A β ₄₂ powder (AnaSpec, Inc., San Jose, CA), was dissolved in acetic acid (0.1M) obtaining a 2 μ g/ μ L stock. Then A β ₄₂ was oligomerized in no phenol red DMEM for 24h at 4°C. The final concentration in the rat primary cortical neuron cultures was 5 μ M. All dilutions of stock were prepared fresh before addition to the culture medium. All vehicles were confirmed to have no biological effects.

2.3. Cell lines

A summary of demographic characteristics of all subjects enrolled in this study is reported in Table 2. A total of 30 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. 16 patients had mild AD (DSM-III-R, Mini Mental State Examination (MMSE) score between 18-24), 7 had moderate AD (MMSE: 10-18), and 7 had severe AD (MMSE: <10). A group of 29 age-matched individuals without history or clinical signs of neurological or psychiatric disease was used controls. Establishment of lymphoblastic cell lines and culture was performed in our laboratory as previously described by infecting peripheral blood lymphocytes with the Epstein-Barr virus [36]. All study protocols were approved by the Hospital Universitario 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.

Human neuroblastoma (SH-SY5Y) cells were purchased from the European Collection of Cell Cultures (Health Protection Agency, Salisbury, UK), and were propagated in Dulbecco's modified Eagle medium containing L-glutamine (2 mM), 1% nonessential amino acids, 1% penicillin/streptomycin, and 10% fetal bovine serum (FBS) under humidified 5% CO₂ and 95% air. On attaining semiconfluency, cells were pre-treated with PGN33 and/or AM630 during 1 h before the addition of A β . 4 h later, cultures were processed for cell viability assay or western blotting.

2. 4. Rat primary 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 [37, 38]. 3 x 10⁴ cells per well were plated in pre-treated poly-D-lysine-coated 96-well plates for cell viability assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) or BrdU (5-bromo-2'-deoxyuridine) incorporation into DNA as previously described [39], [40], respectively. For immunoblotting 4 x 10⁵ cells per well were plated in pre-treated poly-D-lysine-coated 6-well plates. Cells were maintained at 37°C in a 5% CO₂ humidified atmosphere in Neurobasal A medium (Gibco) containing 2 mM L-glutamine, and 10% B27 Supplement (Invitrogen). Cortical neurons were treated with 5 μ M A β ₄₂ for 24 h and pre-treated or not for 1 h with increasing concentrations of PGN33. The concentration of A β ₄₂ (5 μ M) was chosen on the basis of previous studies in our and other laboratories [41, 42]. After treatment, cultures were processed for cell viability assay, BrdU incorporation or immunoblotting. All animals were handled and cared for Council Directive 2010/63/UE of September 22, 2010.

2. 5. 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 1×10^6 cells $\times \text{mL}^{-1}$ and enumerated everyday thereafter for three days. Cells failing to exclude Trypan blue were considered nonviable. 5-bromo-2'-deoxyuridine (BrdU) incorporation was determined in rat primary neuronal cultures method using an enzyme-linked immunoassay kit procured from Roche (Madrid, Spain). Cell viability was determined by the MTT assay (3-[4,5-Dimethylthiazol-2-yl]-2,5-Diphenyltetrazolium Bromide), as previously described [39]. Cell survival was normalized to untreated controls and is presented as percentage. Cell cycle analysis was performed using a standard method [43]. Cells were fixed in 75% ethanol for 1 hour at room temperature. Subsequent centrifugation of the samples was followed by incubation of cells in phosphate-buffered saline (PBS) containing 1 $\mu\text{g/mL}$ of RNase at room temperature for 20 minutes and staining with propidium iodide (PI; 25 $\mu\text{g/mL}$). 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.

2. 6. Immunoblotting analysis

Cells were harvested, washed in PBS and then lysed in ice-cold lysis buffer as previously described [40]. Protein estimation was determined by the Pierce BCA Protein Assay kit (Thermo Scientific). 50-100 μg of protein were fractionated on a SDS polyacrylamide gel, and transferred to Poly (vinylidene) fluoride (PVDF) membranes (Millipore, Billerica, MA, USA) and immunodetected as described previously [40]. 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-CaMKII (Thr286) (1:1000); anti-CaMKII (1:500); anti CB2 (1:200) anti

1 NeuN (1:1000); anti GFAP (1:1000). The relative band intensities were quantified using
2 Image Studio Lite software (LI-COR Biotechnology, NE, USA).
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4 **2. 7. Statistical analysis**

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7 Statistical analyses were performed with Graph Pad Prism 6 (La Jolla, CA, USA). All the
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9 statistical data are presented as mean $\pm$ standard error of the mean (SEM). All the
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11 experiments were replicated at least 3 times. Normality was checked with the Shapiro-
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13 Wilk test. Parametric tests were therefore used in the statistical analysis. Statistical
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15 significance was estimated by both one-way and two-way analysis of variance (ANOVA)
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17 followed by the Bonferroni's test for multiple comparisons. A value of $p < 0.05$ was
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19 considered significant.
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3. RESULTS

3. 1. Proliferative activity of lymphoblasts from control and AD patients.

The effect on proliferation of the CB2 agonist PGN33 was determined using the colorimetric MTT assay. First, a dose-response curve was evaluated in lymphoblasts from late-onset AD patients compared to control cells for 72 hours. As shown in Fig. 1A, PGN33 reduced viability of AD lymphoblasts, in a dose-dependent manner, while viability of control cells was found affected only at the highest concentration of PGN33 (Fig. 1A). Then, the effect on proliferation was analyzed using the 5 μ M concentration in lymphoblasts from controls and AD patients by total cell counting excluding Trypan blue. Fig. 1B shows a time course analysis of the proliferative activity of control and AD lymphoblasts in the absence and presence of PGN33. In agreement with previous reports [26, 27], AD lymphoblasts showed higher proliferative activity than control cells. The CB2 agonist PGN33 selectively prevented the enhanced stimulation of proliferation of AD lymphoblasts, showing no significant effects on control cells (Fig. 1B). Fig 1C shows that proliferation of lymphoblasts from AD patients was not affected by the degree of disease progression. Cell lines from mild, moderate or severe AD patients showed similar rates of cell growth, with no significant differences in the efficacy of PGN33 in blocking cell proliferation (Fig. 1C). Fig. 1D shows that there were no significant changes in the proportion of sub-G₀/G₁ hypodiploid cells, characteristic of apoptosis/necrosis, in control and AD lymphoblasts after PGN33 treatment, thus indicating that the decreased cell number in AD lymphoblasts induced by PGN33 truly reflects a decrease in cell proliferation.

The effects of PGN33 inhibiting proliferation of AD lymphoblasts were comparable with that induced by the reference CB2 agonist JWH133 (Fig.2). On the other hand, the effect of PGN33 was prevented by the specific CB2 antagonist, AM630. The addition of the

1 potent BuChE inhibitor, PGN154 (IC_{50} 0.15 ± 0.02 μ M), which does not bind to CB2
2 receptors (*PCT/ES2016/070906*, 2016), had no appreciable effects on the proliferative
3 activity of AD lymphoblasts (Fig.2). The above observations suggest that the
4 antiproliferative effect of PGN33 requires CB2 receptor activation, but it is independent
5 of BuChE activity.
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11 **3.2. Effects of CB2 agonists on downstream signaling pathway**

12 We first analyzed the effects of perturbing the CB2 receptor signaling by using pertussis
13 toxin (PTX) to inhibit the coupling of the receptor with $G_{i/o}$ proteins, and the calmodulin
14 (CaM) antagonist, calmidazolium (CMZ) or the specific PI3K/Akt inhibitor Ly294002,
15 as the Ca^{2+} /CaM/PI3K/Akt was previously found to be increased un AD lymphoblasts
16 [27]. Table 3 shows the influence of these effectors on cell viability in comparison with
17 the CB2 agonist PGN33. The inhibition of cell survival by PTX or Ly294002 was
18 approximately 25% in control and AD cells. In contrast, and in agreement with previous
19 reports from this laboratory [26], the antagonist of CaM, CMZ, exclusively inhibited the
20 proliferation of lymphoblasts from AD patients. The CB2 agonist PGN33 mimicked the
21 effects of the CaM antagonist (Table 3) suggesting that PGN33 prevents the Ca^{2+} /CaM-
22 mediated stimulation of PI3K/Akt.
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42 The effects of PGN33 on CaM or PI3K/Akt signaling were assessed in control and AD
43 lymphoblasts by monitoring the phosphorylation status of CaMKII or Akt by using
44 phospho-specific anti CaMKII or anti Akt antibodies. Cells were preincubated with the
45 corresponding drug 30 minutes before serum stimulation, and the levels of the
46 phosphorylated proteins were determined 24 hours later. As expected, lymphoblasts from
47 AD individuals showed increased levels of p-CaMKII and p-Akt, thus indicating higher
48 activity of these two kinases in comparison with that of control cells. Incubation of cells
49 with PGN33 blunted the enhanced phosphorylated levels of Akt and CaMKII
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characteristic of AD lymphoblasts without affecting the phosphorylation of these proteins in control cells (Fig. 3A). These results suggest that PGN33 prevents rather than inhibits the activation of the PI3K/Akt pathway. We next sought to check whether PGN33 is effectively blocking the increase in the levels of phosphorylated pRb. Figure 4 shows how this treatment was effective in decreasing the phosphorylation of pRb in AD lymphoblasts up to levels similar to those of control cells. We did not detect any differences in either levels of these two proteins or in the effects of PGN33, depending on disease severity (not shown).

Increased pRb levels in AD lymphoblasts could be the consequence of reduced cellular content of the CDK inhibitor p27, which in turn allows cell cycle progression [27, 32]. Indeed, the PGN33-induced inhibition of cell proliferation and pRb phosphorylation in AD lymphoblasts were accompanied by increased levels of p27 protein (Fig. 4).

3.3. Neuroprotective effects of PGN33 on A β -induced death in AD neuronal models.

The above results obtained in lymphoblastoid cell lines were then validated in neuronal models of AD disease. We assessed the expression levels of CB2 receptors in SH-SY5Y and rat primary neuronal cultures by Western blotting (Fig. 5A) using human lymphoblasts lysates as control. A prominent band of approximately 45Kd was found in the three types of cells. The expression of CB2 receptors was found higher in lymphocytes than in neuronal cells (Fig.5A). Levels of neuronal and glial markers NeuN and GFAP indicated that primary culture contained mostly post-mitotic neurons (Fig. 5A). PGN33 effect on survival of A β -treated human neuroblastoma SH-SY5Y cells is shown in Fig. 5B. A β induced death of SH-SY5Y cells (Fig. 5B), and PGN33 reduced cell death significantly (Fig. 5A). To assess the involvement of CB2 receptors in this effect, cells were pre-treated with PGN33 in presence of increasing concentrations of selective CB2 antagonist AM630. The antagonist had no effect in control cells, but significantly

reduced, in a concentration-dependent manner, survival of neuroblastoma cells treated with A β and PGN33 (Fig. 5B), suggesting that neuroprotective effect of PGN33 on A β -induced cell death is mediated by CB2 receptors. Then, we evaluated the effects of PGN33 in post-mitotic neuronal cells. For these experiments rat primary neuronal cultures were pre-treated with escalating concentrations of PGN33 for 1 h before A β exposure, and cell viability was analyzed 24 h later by MTT assay. As shown in Fig. 5C PGN33 prevented, in a dose-dependent manner, the cell death induced by A β in these non-dividing neurons. Maximal effects were found at 5 μ M A β concentration. It was previously reported that A β treatment activates cell cycle in primary cultures of rat cortical neurons [44]. For this reason, we determined the effects of A β on the rate of BrdU incorporation into the DNA, in the absence or in the presence of PGN33. Figure 5D shows that indeed PGN33 was able to partially block the increase in DNA synthesis induced by A β . Additionally, the cellular levels of pRb and p27 proteins were determined. We found that PGN33 blocked the A β -induced increased phosphorylation of pRB by restoring the normal levels of p27 (Fig. 5E).

DISCUSSION

There is growing evidence of the potential of CB2 agonists in the treatment of neurodegenerative diseases including AD [19]. CB2 receptors seem to be increased in the brain of patients with neurodegeneration [45] and CB2 agonists have shown neuroprotective effects in several models of brain injury [46, 47]. Regarding AD, it was reported that selective CB2 agonists were able to prevent memory deficit in A β -injected rats and mice [48]. Moreover, a specific role for CB2 receptor in the modulation of tau phosphorylation was also suggested in the APP/PS1 mice. [49]. Recently, a novel cerebral role for CB2 receptors has been found demonstrating that the activation of CB2 receptors increased brain glucose uptake in a murine model of AD [50]. On the other hand, CB2 agonists are devoid of psychoactive adverse effects associated with CB1 receptor activation [51].

In this study, we assessed the effects of a novel dual CB2 agonist and BuChE inhibitor, PGN33 [11], on proliferation of lymphoblasts from late onset AD patients.

The existence of systemic manifestations in AD subjects is now widely accepted [52]. Abnormalities in metabolic and biochemical processes in AD brain are also present in peripheral cells, such as fibroblasts and lymphocytes, from patients, [53]. Thus, peripheral cells and particularly lymphocytes had been used often to study cellular or molecular alterations of pathological significance (see [54] for a review). Cell cycle-related events are now considered an important pathogenic mechanism for neurodegenerative disorders including AD [29], with alterations observed in both, neurons and peripheral cells [5, 55]. For these reasons, we found interesting to evaluate the potential of drugs able to interfere with cell cycle activation for AD treatment. We have evaluated the antiproliferative effects of a novel CB2 agonist, PGN33, on immortalized lymphocytes from AD patients. Additionally, we have investigated the

1 effects of PGN33 on A β -induced death of primary neuronal cultures, which is associated
2 with cell cycle reactivation in these post-mitotic neurons [44]. It is worth highlighting our
3 previous work showing similar cell cycle alterations in either Epstein–Barr virus-
4 immortalized lymphocytes or freshly isolated lymphocytes from AD patients [26, 27, 56],
5 indicating that cell cycle control failure is an essential feature of AD pathogenesis that is
6 not affected by the viral transformation. Therefore, lymphoblastoid cell lines from AD
7 patients could be useful as suitable platform for testing novel therapies.

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17 CB2 agonist PGN33 efficiently blocked the serum-mediated enhancement of
18 proliferation characteristic of AD lymphoblasts, without significantly affecting the rate
19 of proliferation of control cells. The concentration used in this work (5 μ M) is in
20 accordance with previous reports showing the effects of synthetic cannabinoid agonists
21 in immune cells [57]. The PGN33 effect was not due to increased cell death as this drug
22 did not change the percentage of cells showing hypodiploid nuclei when the cell cycle
23 status was analyzed. The effect of PGN33 was blocked by the CB2 receptor antagonist
24 AM630 indicating its dependency on receptor activation.

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36 Since we had previously reported a Ca²⁺/CaM-dependent overactivation of PI3K/Akt,
37 leading to increased phosphorylation of pRb protein [27], we sought to investigate the
38 effects of PGN33 on the phosphorylation status of CaMKII, Akt and pRb. Treatment of
39 AD cells with PGN33 prevented the previously reported upregulation of phosphorylated
40 CaMKII, Akt and pRb. The higher phosphorylation of pRb protein observed in AD
41 lymphoblasts resulted from diminished levels of the CDK inhibitor p27 [26, 27]. Here we
42 show that PGN33 was indeed capable of restoring p27 levels up to values found in control
43 cells. The proposed scenario is schematically represented in Figure 6. Activation of CB2
44 receptors prevented the Ca²⁺/CaM-induced overactivation of PI3K/Akt pathway, which
45 in turn restored the levels of p27, reducing CDK activity and cell cycle activation. The
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1 effect of PGN33 preventing the phosphorylation of Akt is in line with previous reports
2 indicating similar effects of CB2 synthetic agonists in blocking the EGF inducing
3 activation of Akt in non-small cell lung cancer [58]. The molecular mechanism involved
4 in the effect of PG33 preventing the overactivation of CaM-dependent activation of
5 PI3K/Akt cannot be ascertained with the present data. However this blockage may result
6 from changes in the activity of the Src family of tyrosin kinases induced by CB2 signaling
7 [59, 60]. Phosphorylation of CaM at Tyr99 by a Src kinase is required for CaM binding
8 to the p85 subunit and subsequent PI3K activation [61]. Changes in proliferative activity
9 and state of activation of PI3K/Akt and CaMKII in AD lymphoblasts did not correlate
10 with disease severity as PGN33 was equally effective in cell lines derived from early or
11 advanced disease patients. These observations may indicate the cell disturbances reported
12 here are early manifestations of the disease, in good agreement with the cell cycle theory,
13 which states that neurodegeneration, induced by cell cycle activation, is an early feature
14 in the instigation of the disease[62].
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34 BuChE activity was reported to be increased in AD brain associated with loss of episodic
35 memory [63] and A β burden [64]. However, the effect of PGN33 seems to be independent
36 of its ability to inhibit BuChE activity as PGN154, a BuChE specific inhibitor without
37 CB2 agonist capacity, had no effect on proliferation of lymphoblasts. Further work
38 analyzing the effects of this compound in animal models of AD will help to assess if its
39 inhibiting BuChE activity conveys any additional potential for AD management.
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50 Although alterations detected in peripheral cells from patients might not fully reflect those
51 occurring in the AD brain, it may be worthwhile mentioning that an increased
52 phosphorylation of Akt in AD temporal cortex neurons [65] was associated with increased
53 levels of Akt substrates and lower levels of p27 protein [66]. Similarly, impaired CaMKII
54 activation was also detected in AD brain, associated with increased phosphorylation of
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1 tau and neurofibrillary tangle formation [67]. Moreover, PGN33 was able to partially
2 prevent the cell cycle activation and cell death induced by A β in primary rat cortical
3 neuronal cultures. PGN33 treatment reduced the A β -induced increased levels of pRb
4 phosphorylation and normalized the cellular content of p27. These results add further
5 support to the hypothesis that cell cycle control failure underlies neuronal loss and
6 indicate the usefulness of lymphoblasts from AD patients to study molecular events
7 linked to AD etiopathogenesis.
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9 On the other hand, the activation of CB2 receptors in cells of the immune system might
10 also have beneficial effects on AD, as systemic inflammation is known to play an
11 important role in the instigation and progression of AD [52]. CB2 receptors are highly
12 expressed in lymphocytes [13], therefore their activation could contribute to ameliorate
13 the pro-inflammatory cytokine production [68]. Moreover, it has been reported that the
14 CB2 agonist JWH-015 was able to protect and rescue peripheral blood lymphocytes from
15 A β - and H₂O₂-induced apoptosis [69]. Altogether, these observations add further support
16 to the idea that CB2 agonists could have neuroprotective effects in AD.
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18 Our findings suggest that PGN33, acting through a CB2 receptor-mediated mechanism
19 deserves further studies to establish its potential in the therapy of AD.
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COMPLIANCE WITH ETHICAL STANDARDS

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Conflict of interest

None of the authors has any conflict of interest to report

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Legends to the Figures

Fig.1

Effects of CB2 agonist PGN33 on the proliferation of lymphoblasts from control and late-onset AD patients.

Immortalized lymphocytes from control and AD patients were seeded at an initial density of $1 \times 10^6 \times \text{ml}^{-1}$ in the absence or in presence of PGN33, for 72 hours. A) For the MTT assay a total of 100,000 cells per well were seeded in a 96-well plate in presence of increasing doses (0-10 μM) of this drug. Results represent the percentage of cell survival of treated cells referred to untreated ones. B) Effect of PGN33 (5 μM) on proliferation of control and AD lymphoblasts. Aliquots were taken for cell counting 72 hours after the drug administration. Data shown are the mean $\pm$ SEM for fifteen observations carried out with cell lines from different individuals. *** $p < 0.001$, significantly different from control cells. †† $p < 0.01$, significantly different from untreated AD cells. C) Lymphoblasts from mild, moderate or severe AD were cultured for three days as above. Data shown are the mean $\pm$ SEM. *** $p < 0.001$, significantly different from control cells. †† $p < 0.01$, significantly different from untreated AD cells. D) Cell cycle analysis of control and AD lymphoblasts after treatment with 5 μM PGN33 during 24 h. The mean $\pm$ SEM of the percentage of cells in Sub G1 of four independent experiments is indicated for each condition.

Fig. 2

The antiproliferative effects of CB2 Receptor activation were prevented by the specific CB2 antagonist AM630

Immortalized lymphocytes from control and AD patients were seeded at an initial density of $1 \times 10^6 \times \text{ml}^{-1}$ in the absence or in presence of the PGN33 (5 μM), with and without the

1 CB2 antagonist AM630 (5 μ M). In addition, the effects of equal concentration of the
2 commercial CB2 agonist JWH133 and the non-CB2 agonist, inhibitor of BuChE PGN154
3 were determined 72 hours after their addition. Box plot represents the proliferative
4 activity of control (white) and AD lymphoblasts (black). ***p<0.001 significantly
5 different from control cells. †p<0.05, ††p<0.01 and †††p<0.001, significantly different
6 from untreated AD cells, respectively.
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17 **Fig. 3**

18 **Effects of the CB2 agonist PGN33 on the activation of PI3K/Akt and CaMKII**

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Immortalized lymphocytes from control and AD patients, were seeded at an initial density
of 1 x 10⁶ x ml⁻¹, and were incubated in RPMI medium containing 10% FBS in the
absence or in the presence of the CB2 agonist PGN33 (5 μ M). Cell extracts were prepared
24 hours after drug administration to determine CaMKII and PI3K/Akt activation by
Western blot using phospho-specific antibodies anti CaMKII and Akt. Representative
immunoblots are shown. The densitometric data represent the mean $\pm$ SEM of four
different experiments. **p<0.01 and ***p<0.001, significantly different from control
cells. †p<0.05 and ††p<0.01, significantly different from untreated AD cells.

Fig. 4

Effects of the CB2 agonists PGN33 on the phosphorylation status of the pRb protein and the levels of p27

Immortalized lymphocytes from control and AD patients, were seeded in the same
experimental conditions that in the figure 3. Cell extracts were prepared 24 hours after
PGN33 treatment to analyze pRb and p27 levels. Representative immunoblots are shown.
The densitometric data represent the mean $\pm$ SEM of, at least four different experiments.

* $p < 0.05$ and ** $p < 0.01$, significantly different from control cells; † $p < 0.05$, significantly different from untreated AD cells. ppRb = hyperphosphorylated pRb.

Fig.5

Neuroprotective effects of PGN33 on β -amyloid-induced death in neuronal cells

A) Lysates from lymphoblasts, SH-SY5Y and rat primary neuronal cultures were used to determine the protein levels of CB2 receptors. NeuN and GFAP protein levels were used as neuronal and glial cells markers, respectively. B) Neuroblastoma SH-SY5Y cells were incubated in absence or presence of $5\mu\text{M}$ $\text{A}\beta$ for 24 hours with and without $10\mu\text{M}$ PGN33 alone or in combination of increasing concentrations of AM630 ($0.5\text{--}2\mu\text{M}$) added 1h prior the $\text{A}\beta$ incubation. Number of viable cells after drug treatments were measured by MTT assay. Each data point represents the mean $\pm$ SEM for four different experiments. (** $p < 0.001$ significantly different from SH-SY5Y untreated cells; †† $p < 0.01$ significantly different from $\text{A}\beta$ -treated cells). C) Rat cortical primary neurons were exposed to $5\mu\text{M}$ $\text{A}\beta$ for 24 hours in presence or absence of different concentrations of PGN33 ($0\text{--}10\mu\text{M}$). The number of viable cells was measured by MTT assay. Each data point represents the mean $\pm$ SEM of three replications in four different experiments. * $p < 0.05$ significantly different from untreated cells; and †† $p < 0.01$ significantly different from $\text{A}\beta$ -treated cells. D) Rat cortical primary neurons were incubated as above in 96-well plates; 24 hours later, cells were pulsed with $10\mu\text{M}$ 5-bromo-2'-deoxyuridine for 4 hours (BrdU). DNA synthesis was assessed by BrdU incorporation method according to the manufacturer's instructions. * $p < 0.05$ significantly different from untreated cells; and †† $p < 0.01$ significantly different from $\text{A}\beta$ -treated cells. E) Rat cortical primary neurons were incubated in the absence or in the presence of $5\mu\text{M}$ $\text{A}\beta$ for 24 h and then harvested to prepare cell lysates to determine pRb and p27 levels by Western blotting.

Representative immunoblots are presented. ppRb = hyperphosphorylated pRb.

Fig.6

Diagram summarizing the effects of targeting CB2 receptors on the proliferative activity of AD lymphoblasts

Lymphoblasts from AD patients show an overactivation of the PI3K/Akt compared with that of control cells. Treatment of AD cells with the CB2 agonist PGN33, prevented the Ca^{2+} /CaM-dependent activation of PI3K/Akt, normalized the cellular content of p27, and restored normal rates of pRb phosphorylation and cell cell cycle activation.

Table 1

PGN33 characteristics

Compound	Structure	Binding affinity CB1 ^a Ki (μM)	Binding affinity CB2 ^a Ki (μM)	IC ₅₀ BuChE ^b (μM)	BBB prediction
PGN33 (C ₂₅ H ₂₈ N ₄ O)		>40	7.7 ± 2	4.8 ± 0.31	+

^aReceptors binding studies were performed using membrane fractions of human CB1 or CB2 receptor transfected cells (HEK293EBNA)^b. IC₅₀ values (mean ± SEM) were determined from 3 different experiments using 0.5 mM butyrlthiochloride as substrate [11]. Blood Brain Barrier (BBB) prediction was estimated as previously reported [35]. ND: not determined.

Table 2
Summary of study population

	N	Age	Age range	Gender (Male/Female)
CONTROL	29	69±10	(43-83)	10/19
AD				
Total	30	75±7	(59-84)	12/18
Mild	16	76±2	(66-83)	7/9
Moderate	7	78±2	(59-84)	3/4
Severe	7	74±3	(60-81)	2/5

Control: healthy control individuals with no sign of neurological disease; AD: patients with a diagnosis of probable AD. Values are the mean ± SD.

Table 3

Effects of effectors of the CB2R signalling on cell viability

Cell treatment	Control	AD
	% of MTT Reduction	
PTX (2ng/ml)	73±2	72±3
Ly294002 (10µM)	69±3	68±4
CMZ (1µM)	89±2	72±3**
PGN33 (5µM)	86±1.5	67±5*

Lymphoblasts from control and AD patients were incubated in RPMI medium containing 10% FBS for 72 h with the indicated concentrations of the pertussis toxin (PTX), calmidazolium (CMZ), Ly294002 and the CB2 agonist. Cell viability was assessed by measuring the MTT reduction. Independent experiments with different cell lines were carried out in triplicate. Results are expressed as % of the viability of cells at day 0 and are the mean ± SEM of, at least, 3 different experiments. Statistical difference: *p<0.05, **p<0.01, from lymphoblasts from control individuals.

Fig. 1

Figure 2

[Click here to download Figure FIGURE 2.jpg](#)

Fig. 2

Fig. 3

Fig. 4

Fig. 6