Caniferolide A, a macrolide from *Streptomyces caniferus*, attenuates neuroinflammation, oxidative stress, amyloid-beta and tau pathology *in vitro*

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ABSTRACT. The macrolide caniferolide A was isolated from extracts of a culture of the marine-derived actinomycete *Streptomyces caniferus* and its ability to ameliorate Alzheimer's disease (AD) hallmarks was determined. The compound reduced neuroinflammatory markers in BV2 microglial cells activated with lipopolysaccharide (LPS), being able to block NFκB-p65 translocation to the nucleus and to activate Nrf2 pathway. It also produced a decrease in pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α), reactive oxygen species (ROS) and nitric oxide release, and inhibited iNOS, JNK and p38 activities. Moreover, the compound blocked BACE1 activity and attenuated Aβ-activation of microglia by drastically diminishing ROS levels. The phosphorylated state of tau protein was evaluated in SH-SY5Y tau441 cells. Caniferolide A reduced Thr212 and Ser214 phosphorylation by targeting p38 and JNK MAPK

kinases. On the other side, the antioxidant properties of the macrolide were determined in an oxidative stress model with SH-SY5Y cells treated with H_2O_2. The compound diminished ROS levels and increased cell viability and GSH content by activating the nuclear factor Nrf2. Finally, the neuroprotective properties of the compound were confirmed in two trans-well co-culture systems with activated BV2 cells (both with LPS and Aβ) and wild type and transfected SH-SY5Y cells. The addition of caniferolide A to microglial cells produced a significant increase in the survival of neuroblastoma in both cases. These results indicate that the compound is able to target many pathological markers of AD, suggesting that caniferolide A could be an interesting drug lead for a polypharmacological approach to the illness.

KEYWORDS: Streptomyces, p38, NFκB, Nrf2, Alzheimer`s disease.

1. Introduction

Streptomyces spp are Gram-positive bacteria included in the phylum Actinobacteria. Streptomycetes can live in many ecosystems that include extreme environments as the deep sea and arid habitats [1, 2], or associated to other organisms [3, 4]. *Streptomyces* members produce several secondary metabolites with ecological functions such as competition with other microorganisms or mediation in symbiotic relationships [5]. Many of these metabolites are bioactive compounds, most of them with antibiotic properties[6]. Macrolides isolated from *Streptomyces* spp. have displayed immunosuppressive [7], antibacterial [8] and neuroprotective activities [9, 10]. Particularly, the 36-membered macrolide GT-35 [11] and the structurally related metabolites PM100117 and PM100118[12], axenomycins [13-15] and deplelides [16] have shown antitumor, antibiotic and antifungal activities.

Amyloid-beta (Aβ) and tau aggregation are main hallmarks of Alzheimer's disease (AD). Accumulated Aβ and hyperphosphorylated tau contribute to other pathological processes of the

illness as oxidative stress, neuroinflammation, mitochondrial dysfunction or neurotransmitter deficits [17]. Aβ triggers an inflammatory response in microglia that promotes neurodegeneration. At the beginning of the disease, activated microglia phagocyte and remove Aβ, but the continued release of cytokines, chemokines and aggregated Aβ produces a chronic inflammatory response that can induce neuronal death. Neurotoxic microglia releases pro-inflammatory cytokines, nitric oxide (NO) and reactive oxygen species (ROS), creating a toxic environment that contributes to the progression of AD [18]. The transcription factor kappa-light-chain-enhancer of activated B cells (NFκB) is the main controller of inflammation. RelB, c-Rel, p50, p52 and p65 constitute the transcription factor. The five domains form homo- and heterodimers and are retained in the cytosol by IκB. The stimulation of IκB kinase complex produces the phosphorylation of IκB proteins, leading to the release of NFκB dimers that translocate to the nucleus. Particularly, the increase of p65: p50 dimers augments pro-inflammatory and pro-apoptotic genes expression [19].

Tau is a microtubule-associated protein that stabilizes the neurons cytoskeleton. In AD, tau is hyperphosphorylated, loses its function and accumulates, resulting in the formation of toxic neurofibrillary tangles. The abnormal phosphorylation of this protein is due to an imbalance between the activity of kinases and phosphatases [17]. Tau overexpression produces mitochondrial dysfunction by impairing oxidative phosphorylation and affecting to the mitochondrial pore opening[20]. Neurofibrillary tangles also contribute to neuroinflammation, misfolded tau is enough to activate NFκB and mitogen activated kinases (MAPK) pathways and increase the release of pro-inflammatory cytokines [21]. On the other side, high levels of cytokines and ROS increase the activity of kinases responsible of tau hyperphosphorylation [22]. These examples illustrate the complex connexions between the pathological processes in AD, which result in a feedback cycle that finally leads to the neuronal loss observed in the illness. Due to this complexity, the simultaneous modulation of the multiple mechanisms implicated in

the pathology has been proposed as a promising therapeutic approach. Current approved drugs for the treatment of AD are acetylcholinesterase inhibitors and N-methyl D-aspartate antagonists and only offer temporary benefits to patients. Therefore, a big effort has been made to find new compounds with the ability of targeting several hallmarks of the disease[17].

In this context, caniferolide A was isolated from the marine-derived actinomycete *Streptomyces caniferus* and its ability to modulate neuroinflammation, oxidative stress, tau phosphorylation and Aβ cleavage was tested *in vitro*.

2. Material and methods

2.1 Chemicals and solutions

5-(and-6)-carboxy-2′, 7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA), tetramethylrhodamine methyl ester (TMRM) and Thiol Tracker™Violet, were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Human beta-amyloid peptide (1-42) was purchased from Abcam (Cambridge, UK). Other chemical were reagent grade and purchased from Sigma-Aldrich (Madrid, Spain).

2.2 Isolation and structure of caniferolide A

Caniferolide A was isolated from extracts of a 1 L culture of *Streptomyces caniferus* CA-271066 in APM9-modifed medium (glucose 50 g/L, soluble starch 12 g/L, soy flour 30 g/L, $CoCl_2 \cdot 6H_2O$ 2 mg/L, sea salts 30 g/L, calcium carbonate 7 g/L, pH 7). The inoculated flasks (20×250 mL Erlenmeyer flasks each containing 50 mL of medium) were incubated in a rotary shaker at 28 °C, 70% relative humidity and 220 rpm for 6 days before harvesting.

Ethyl acetate extraction and chromatographic separation of the crude obtained using Sephadex LH-20 (MeOH/$CHCl_3$, 2:1) followed by semipreparative reversed-phase HPLC (Agilent Zorbax RX-C8, 9.4 × 250 mm, 7 µm; 3 mL/min, UV detection at 210 and 254 nm,

with a linear gradient of CH_3CN/H_2O from 40 to 50% CH_3CN in 40 min) yielded 13.0 mg of the compound. Its structural elucidation, including the determination of the absolute configuration, was accomplished by ESI-TOF mass spectrometry and 1D and 2D NMR spectroscopy, combined with analysis of the biosynthetic gene cluster. The compound turned out to be the 19-*O*-sulphate derivative of GT-35, an antifungal macrolide previously reported in a Japanese patent[11]. Full details on the isolation and structural elucidation of the compound will be published elsewhere[23].

2.3 Cell culture

SH-SY5Y human neuroblastoma cells were obtained from American Type Culture Collection (ATCC), number CRL2266. Dulbecco´s Modified Eagle´s medium: Nutrient Mix F-12 (DMEM/F-12) with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin and 1% glutamax was used as culture medium.

SH-SY5Y tau441 (SH-SY5Y-TMHT441) human neuroblastoma cell line was provided by QPS Austria GmbH. This cellular line is stably transfected with V337M and R406W human mutations. The transfection produces the phosphorylation of tau in residues as Thr231, Thr181 and Ser396 and increases the activity of enzymes as glycogen synthase kinase-3 beta, or c-Jun N-terminal kinase (JNK) [24]. Cells were maintained in DMEM/F-12 medium supplemented with 10% FBS, glutamax, non-essential aminoacids and 10 mg/mL gentamicin. 0.6% geneticin was added to the culture medium for cell line maintenance.

BV2 murine microglial cells were purchased from Interlab Cell Line Collection (ICLC), number ATL03001. RPMI 1640 medium with 10 % FBS, 100 U/mL penicillin and 100 µg/mL streptomycin was used to cell culture.

Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cell lines were dissociated using 0.05% trypsin/EDTA. All the reagents were provided by Thermo Fischer Scientific.

2.4 Protein extraction

BV2 and SH-SY5Y cells were cultured in 12-well plates at $1x10^6$ cells per well and allowed to settle down for 24 h. SH-SY5Y-TMHT441 cell line was seeded until reaching 60% confluence and differentiated with retinoic acid at 10 µM for 7 *div*. BV2 cells were treated with the compound (0.001-0.1 µM) for 1 h. Then, lipopolysaccharide (LPS) at 500 ng/mL was added for 24 h. SH-SY5Y and SH-SY5Y-TMHT441 cells were treated with the compound (0.001-0.1 µM) for 6 and 48 h, respectively. Cells were washed with PBS and a hypotonic solution was added (20 mM Tris-HCl pH 7.4, 10 mM NaCl and 3 mM $MgCl_2$, containing a phosphatase/protease inhibitors cocktail). Cells were incubated for 15 min on ice and centrifuged at 3000 rpm, 4 °C for 15 min. Cytosolic fraction was obtained from the supernatant and the protein concentration was quantified with Direct Detect instrument (Merck Millipore). The pellet of BV2 and SH-SY5Y cells was resuspended in a nuclear extraction buffer (100 mM Tris pH 7.4, 2 mM Na_3VO_4, 100 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 1 mM EGTA, 0.1% SDS, 1 mM NaF, 0.5% deoxycholate, and 20 mM $Na_4P_2O_7$, containing 1 mM PMSF and a protease inhibitor cocktail). Samples were incubated on ice for 30 min, vortexing in intervals of 10 min, and centrifuged at 14000 g, 4 °C for 30 min. The nuclear fraction was quantified with Bradford method.

2.5 Western blot assays

Electrophoresis was resolved in 4-20 % sodium dodecyl sulphate polyacrylamide gels (Biorad, Hercules, CA, USA), containing 20 µg of cytosolic protein or 10 µg of nuclear fraction. Proteins were transferred to PVDF membranes (Merck Millipore). Snap i.d. system (Merck

Millipore) was used to block membranes and antibody incubation. Supersignal West Pico Luminiscent Substrate and Supersignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and Diversity GeneSnap system and software (Syngene, Cambridge, U.K.) were used for protein bands detection. Primary antibodies are summarized in Table 1. The ratio between phosphorylated and total protein levels was calculated to determine the activation of kinases. Protein band intensity was corrected by lamin B1 and β-actin in nuclear and cytosolic fractions, respectively. Experiments were performed at least three times by duplicate.

2.6 Evaluation of reactive oxygen species levels

The release of ROS was quantified with the fluorescent dye carboxy-H_2DCFDA (5-(and-6)-carboxy-2′, 7′-dichlorodihydrofluorescein diacetate). Serum-free medium was used to wash cells and 20 μM carboxy-H_2DCFDA was added. After 1 h at 37 °C, PBS was added to each well during 30 min. Then, the fluorescence was read at 527 nm, with an excitation wavelength of 495 nm. Experiments were carried out three times by triplicate.

2.7 Determination of cytokines release

BV2 microglial cells were treated with the compound (0.001-0.1 μM) and 500 ng/mL LPS and the levels of IL-1β, IL-6, TNF-α and IL-10 were assessed. IL-1 β release was determined with a Mouse IL-1β ELISA kit (Thermo Fisher Scientific). IL-6, TNF-α and IL-10 were evaluated with a Mouse High Sensitivity T Cell Magnetic Bead Panel (Merck Millipore), following manufacturer's instructions. Luminex 200 ™ instrument and xPONENT® software (LuminexCorp, Austin, TX) were used to collect the data. All experiments were performed three independent times by duplicate.

2.8 Nitric oxide measurement

The levels of NO were determined after the pre-treatment of BV2 cells with the compound (0.001-0.1 μM) for 1 h and the addition of LPS during 23 h. Cells were cultured in 12-well plates in DMEM without phenol red. Griess Reagent Kit (Thermo Fisher Scientific) was used to evaluate the spontaneous oxidation of NO to nitrite. Briefly, 150 μL of medium were mixed with 20 μL of Griess reagent. After 30 min at room temperature, the absorbance was measured at 548 nm in a spectrophotometer plate reader. Experiments were performed three times.

2.9 β-secretase inhibition assay

A fluorescence resonance energy transfer (FRET) kit (Thermo Fisher Scientific) was used to determine the capacity of the macrolide to inhibit the activity of BACE1. The substrate of the enzyme has two fluorophores, a fluorescence donor and a quenching acceptor. When the protease is active, the fluorophore is separated and the fluorescence increases. Therefore, a reduction in the fluorescence indicates that the compound is able to inhibit the activity of the enzyme. The assay was performed as described in manufacturer's instructions. The assay was carried our three independent times.

2.10 Trans-well co-cultures

SH-SY5Y and SH-SY5Y-TMHT441 neuroblastoma cells were seeded in 24-well plates. SH-SY5Y-TMHT441 cells were differentiated with 10 μM retinoic acid for 7 *div*. BV2 microglial cells were seeded in culture inserts (0.4 μM pore size, Merck-Millipore) placed above neuroblastoma cells and allowed to grow for 24 h. After this time, microglial cells co-cultured with SH-SY5Y were treated with the compound for 1 h (0.001, 0.01 and 0.1 μM) and 500 ng/mL LPS for 23 h. On the other side, BV2 cells co-cultured with SH-SY5Y-TMHT441 cells were treated with the macrolide (0.001, 0.01 and 0.1 μM) and activated with 1μM Aβ. Aβ (1-42) was dissolved in DMSO at 500 μM and fibrils were incubated at 37 °C for 24 h in culture medium as previously described [25].

The effects of activated-microglia on the viability of neuroblastoma cells were determined with MTT (3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay. After treatments, SH-SY5Y cells were washed three times with saline solution. Then, MTT (500 µg/mL) was added to the cells for 1h at 37 °C and 300 rpm. Sodium dodecyl sulphate at 5% was incorporated to each well to disaggregate cells and the absorbance was measured at 595 nm with a plate reader. The experiments were carried out three times.

2.11 Mitochondrial membrane potential and cell viability assays

SH-SY5Y cells were co-treated with the compound at concentrations ranging from 0.001 to 0.1 µM and 150 µM H_2O_2 for 6 h. The ability of the *Streptomyces* secondary metabolite to repolarize the mitochondrial membrane was determined with TMRM (tetramethylrhodamine methyl ester) dye. Cells were rinsed twice with saline solution and the fluorescent dye at 1 µM was incorporated to the wells. After 30 min of incubation at 37 °C, cells were solubilized with H_2O and DMSO at 50 %. The fluorescence was read with a spectrophotometer plate reader (535 nm excitation and 590 nm emission).

The ability of the compound to protect SH-SY5Y cells from H_2O_2-induced injury was assessed with MTT assay as described above. All the assays were performed three times.

2.12 Evaluation of glutathione levels

SH-SY5Y neuroblastoma cells were treated as described before and glutathione (GSH) levels were determined with Thiol Tracker™ Violet dye, following manufacturer's instructions. Cells were washed twice with PBS and loaded with the fluorescence dye at 10 µM for 30 min at 37°C. The fluorescence was monitored in a spectrophotometer plate reader (404 nm excitation and emission at 526 nm). The assay was carried out three independent times.

2.13 Statistical analysis

Data are presented as mean ± SEM of at least three independent experiments. Statistical comparison was determined by one way ANOVA with Dunnett's post hoc analysis and statistical significance was considered at *$p < 0.05$, **$p<0.01$ and ***$p<0.001$.

3. Results

3.1 Caniferolide A modulates the phenotypic state of LPS-activated BV2 microglial cells

BV2 cells have been stablished as an appropriate model for evaluating neuroinflammation *in vitro* [26]. The activation of these cells with LPS, a constituent of the membrane of Gram-negative bacteria, has been widely used to induce an inflammatory response in these cells [27, 28].

Firstly, BV2 microglial cells were treated with the compound (Figure 1) at concentrations ranging from 0.001 to 1 μM for 1 h and 500 ng/mL LPS during 23 h and the cell viability was assessed by MTT test. The addition of caniferolide A at 1 μM reduced the survival of microglial cells (data not shown). Thus, the compound was used at lower concentrations in the following experiments.

The transcription factors nuclear factor E2-related factor 2 (Nrf2) and NFκB are key pathways in the modulation of the phenotypic state of microglia. In the nucleus, an increase in Nrf2 and a decrease in the domain p65 of NFκB have been related to a prevalence of the neuroprotective microglial phenotype [29]. In this context, we evaluated the expression of both transcription factors in nuclear and cytosolic fractions by western blot. As Figure 2a shows, p65 levels were significantly reduced when microglial cells were pre-treated with the macrolide at all the concentrations assayed. The addition of the compound also induced the translocation of Nrf2 to the nucleus, with the highest effect at 0.001 μM (Figure 2b).

In view of these results, we determined if caniferolide A was decreasing the release of pro-inflammatory factors in BV2 cells. The treatment with the compound at 0.001 μM significantly

reduced ROS levels (Figure 3a) to a percentage of 92.3± 0.6% (p <0.05) with respect to cells treated with LPS alone. Regarding IL-1β, the macrolide diminished the release of this cytokine at all the concentrations tested (Figure 3b), displaying the greater inhibition at 0.1 μM (46.9± 8.1%, p<0.01). As Figure 3c shows, the addition of 500 ng/mL LPS produced an increment in the levels of IL-6 of 77.0 ±5.7% (p <0.001) with respect to inactivated cells. The release of this cytokine decreased when microglia is treated with the macrolide at the highest concentrations, reaching a 43.0 ±1.4% (p<0.01) of LPS control cells at 0.1 μM. Finally, caniferolide A produced a great effect on the release of TNF-α, reducing its levels to percentages among 55.2- 70.9 % of LPS control cells (Figure 3d).

Figure 3e presents the results regarding the anti-inflammatory cytokine IL-10. Caniferolide A at 0.01 and 0.1 μM induced an increase in the levels of IL-10 up to 159.8 ±12.9% (p<0.05) and 156.0 ±12.4% (p<0.05), respectively.

High levels of NO and the activation of MAPK kinases are related to the pro-inflammatory response in microglial cells [30, 31]. The effects of the compound on these inflammatory markers were evaluated. The addition of caniferolide A to LPS-activated BV2 cells significantly reduced the release of NO to the medium at 0.001 (49.7±6.9%, p<0.01), 0.01 (53.3±6.9%, p<0.01) and 0.1 μM (62.2 ±2.3%, p<0.05) compared to cells treated with LPS alone (Figure 4a). We also determined the expression of iNOS, the enzyme that releases NO. As can be seen in Figure 4b, the compound decreased the expression levels of the protein at the three concentrations assayed, being statistically significant at 0.1 μM (34.9 ± 10.5%, p<0.05). Interestingly, the macrolide reduced the activation of p38 MAPK to levels around 55% of LPS control cells at all the concentrations tested (Figure 4c). On the other hand, the compound did not display any significant effect on the expression of extracellular signal-regulated kinase (ERK) (Figure 4d). With respect to JNK, the treatment with the compound produced a reduction in the

phosphorylation of this kinase, reaching an inhibition of 78. 1 % at 0.1 μM ($p< 0.01$) (Figure 4e).

In order to confirm the anti-inflammatory properties of caniferolide A, a trans-well co-culture was carried out with BV2 microglial cells and SH-SY5Y neuroblastoma cell line. Microglia was pre-treated with the compound (0.001-0.1 μM) and stimulated with LPS and the survival of SH-SY5Y cells was assessed (Figure 5). Microglial cells activated with 500 ng/mL LPS produced a decrease in neuroblastoma cell viability of 59.2±2.1% ($p<0.05$) with respect to SH-SY5Y cells co-cultured with inactivated BV2. When the macrolide was added to the inserts, a significant increase in neuroblastoma cell viability occurred at 0.001, 0.01 and 0.1 μM (122.9±15%, $p<0.01$; 97.4±9.2%, $p<0.05$; 117.4±18.4%, $p<0.01$, respectively).

3.2 Effects of caniferolide A on an in vitro model of tau hyperphosphorylation

The phosphorylated state of tau is regulated by the activity of several kinases as ERK, glycogen synthase-3β, cyclin-dependent kinase 5 or, as mentioned before, p38[32]. The SH-SY5Y-TMHT441 cells have been stablished as a useful tool for tau screening *in vitro* [24, 33, 34]. Neuroblastoma cells were differentiated with retinoic acid and treated with the compound during 48 h. The phosphorylated levels of tau were determined by western blot with three antibodies that identify some residues of tau characteristic of AD: Ser202 (AT8 antibody), Thr212 and Ser214 (AT100) and Thr181 (AT270). Tau-5 was used to detect total tau levels. Caniferolide A produced a reduction in AT8 levels at 0.1 μM, although the decrease is not statistically significant (Figure 6a). The addition of the compound at the highest concentration also diminished the phosphorylation at Thr212 and Ser214 (Figure 6b), whereas the macrolide did not affect to the expression levels of AT270 (Figure 6c). Due to the regulation of tau phosphorylation by MAPK kinases, the expression levels of the enzymes were determined. Caniferolide A was able to diminish the activation of p38, as it happened in microglial cells,

with the greater reduction at 0.1 μM (36.4 ±15.4 %, p<0.05) (Figure 6d). The expression of ERK was not affected by the treatment with the compound (Figure 6e), but the macrolide also produced a decrease in the phosphorylated state of JNK at the higher concentration (43.2±1.9%, p< 0.05) (Figure 6f).

3.3 Caniferolide A attenuates Aβ-activation of BV2 microglial cells

In AD, the activation of microglia is mainly produced by Aβ oligomers. At first, activated-microglial cells phagocyte the toxic proteins producing the clearance of Aβ plaques, but the sustained stimulation of the cells leads to the release of toxic factors and the subsequent neuronal death [18]. We tested the ability of caniferolide A to modulate the activation of BV2 microglial cells with Aβ. At first, a FRET assay was carried out to determine if the compound was able to inhibit BACE1, the key enzyme in the cleavage of Aβ (Figure 7a). The macrolide showed a dose-dependent response, being statistically significant at 0.1 μM (58.9±7.6%, p<0.01). In view of these results, BV2 cells were treated with caniferolide A for 1 h and activated with 1 μM Aβ during 23 h. After this time, the levels of ROS were evaluated. As Figure 7b shows, the addition of Aβ produced an increase in ROS release of 27.5±3.7% (p<0.05) compared to control cells. When microglial cells are pre-treated with the compound, ROS levels decreased until percentages around 50% of inactivated cells.

To determine if the modulation of the inflammatory response by caniferolide A in Aβ-activated BV2 cells could improve neuronal survival, microglia was co-cultured with SH-SY5Y-TMHT441 cells. Neuroblastoma cells were differentiated with 10 μM retinoic acid for 7 *div* and BV2 cells were seeded in culture inserts placed above the neuronal cell line. Microglia was treated with the compound and Aβ as described before and the cell viability of SH-SY5Y-TMHT441 cells was evaluated by MTT assay (Figure 7c). When caniferolide A was added to

activated-BV2 cells, the survival of neuroblastoma increased to levels of cells co-cultured with inactivated microglia, reaching a 118.8±9.2 % (p<0.05) at 0.01 μM.

3.4 Caniferolide A protects SH-SY5Y neuroblastoma cells from oxidative damage

In neurodegenerative diseases, neuronal electronic transport chain failures produce an increase in ROS release [35]. As mentioned before, chronic microglia activation also contributes to the increment in the levels of ROS and reactive nitrogen species. These events produce an oxidative stress environment that can promote neuronal death [36, 37]. Therefore, the effects of caniferolide A on a neuronal oxidative stress model were assessed. SH-SY5Y cells were treated with 150 μM H_2O_2 to induce oxidative damage and the compound at non-toxic concentrations (0.001-0.1μM) was added to determine its antioxidant properties. After 6 h of incubation, the levels of ROS were evaluated (Figure 8a). When neuroblastoma cells were treated with 150 μM H_2O_2 alone, ROS release increases until 130.0±10.4% (p<0.05) of untreated cells. Caniferolide A reduced ROS levels at all the concentrations tested, with levels between 78-87% (p <0.01) of control cells. The ability of the macrolide to protect SH-SY5Y cells from oxidative injury and increase cell survival was determined with MTT test (Figure 8b). The addition of 150 μM H_2O_2 reduced cell viability until 61.0±6.2% (p <0.01). Once again, the compound displayed beneficial effects at the three concentrations assayed, being 0.1 μM the most effective treatment with an increment in cell viability to 99.4±10.2% (p<0.01) with respect to control cells. The effects of caniferolide A on the mitochondrial membrane potential were also evaluated (Figure 8c), but none of the treatments showed a significant recovery of the membrane potential. The principal non-enzymatic antioxidant in cells is GSH. Therefore, its levels were determined after treatment with the compound (Figure 8d). When caniferolide A was added to SH-SY5Y cells, the GSH content significantly augmented at 0.01 (94.2 ±6.0%, p<0.05) and 0.1 μM (96.3± 3.3%, p<0.05) compared to cells treated only with H_2O_2.

The transcription factor Nrf2 induces the expression of antioxidant genes as those that encode the enzymes of GSH cycle (glutathione peroxidase, glutathione transferase) [37]. The ability of caniferolide A to activate the translocation to the nucleus of Nrf2 was evaluated by western blot. Cells were treated with the compound for 6 h and a significant increase in the expression of nuclear Nrf2 was observed at the highest concentrations (0.01 and 0.1 μM) (Figure 8e).

4. Discussion

Until now the research of compounds for the treatment of AD has shown disappointing results. Most of the drugs tested were focused on a single target of the pathology (inhibitors of BACE1, antioxidants, blockers of GSK3β, etc.). AD is a multifactorial illness with a complex network of altered metabolic routes and the modulation of various pathological processes by one drug has been proposed as a promising approach for the treatment of the disease [38]. The results obtained with the *Streptomyces caniferus* secondary metabolite caniferolide A in different cellular models make them a potential candidate compound for further investigations in AD models.

With respect to neuroinflammation, the macrolide was able to block the nuclear translocation of NFκB-p65 and to activate the transcription factor Nrf2. These pathways have been related to two opposite phenotypes of microglial cells. When microglia presents the pro-inflammatory or neurotoxic phenotype, NFκB pathway is activated and induces the expression on pro-inflammatory genes, whereas Nrf2 nuclear levels are low. On the other side, the neuroprotective or alternative phenotype is characterized by decreased NFκB activation and increased expression of antioxidant genes induced by the translocation of Nrf2 to the nucleus [29]. The results obtained with caniferolide A indicate that the compound is able to modulate the phenotypic state of microglia towards the neuroprotective phase. This was confirmed by the reduction observed in the release of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α.

Moreover, the macrolide increases IL-10, an anti-inflammatory cytokine related to the neuroprotective state of microglia [39].

When microglial cells are activated, the translocation of NFκB-p65 to the nucleus produces an increase in the expression of iNOS, the enzyme responsible of NO production. NO is particularly harmful in combination with increased levels of ROS. The increase in reactive oxygen and nitrogen species (RONS) generated by microglia plays a major role in the death of surrounding neurons. RONS activate neuronal apoptosis by stimulation of mitochondrial permeability transition pore, activation of p53 and p38 MAPK kinase pathways and induction of endoplasmic reticulum stress [40]. Caniferolide A induced a decrease in iNOS expression, NO and ROS release by blocking NFκB pathway, enhancing the immunomodulatory effects of the macrolide on activated-microglia.

MAPK kinases are activated in response to stress stimuli as inflammatory cytokines or oxidative stress. With respect to p38, the enzyme regulates the expression of many genes involved in neuroinflammation as those coding for TNF-α or IL-1β. In AD, the activation of p38 also produces mitochondrial stress that can lead to cell death, an increase in intracellular calcium and ROS production, and contributes to the hyperphosphorylated state of tau protein. On the other side, JNK activation has been detected in brains from AD patients and is known to directly induce tau phosphorylation[41]. This enzyme is also involved in the induction of pro-inflammatory enzymes and the release of pro-inflammatory mediators[42]. Thus, the inhibition of p38 and JNK has been proposed as a potential target for the treatment of the neurodegenerative pathology [41, 43]. Interestingly, the macrolide caniferolide A was able to reduce the activation of both MAPK kinases in microglial and neuronal cells. In microglia, the inhibition contributes to the decrease in pro-inflammatory factors induced by the compound. In neuroblastoma cells, a reduction in tau phosphorylation was detected in the presence of the macrolide, probably related to the blockage of p38 and JNK activity.

Caniferolide A also displayed anti-neuroinflammatory effects upon Aβ stimulation. The compound drastically reduced ROS levels and inhibited BACE1 activity. This enzyme catalyses the irreversible step of Aβ cleavage from the amyloid precursor protein. BACE1 is a key target in AD and several clinical trials have been carried out with inhibitors of its activity [44].

The anti-inflammatory properties of the *Streptomyces* secondary metabolite were supported by the results obtained when activated-microglial cells (both with LPS and Aβ) were co-cultured with neuroblastoma cells. Wild type and transfected- SH-SY5Y cell survival was increased in the presence of the compound, confirming the neuroprotective effects of the macrolide.

In AD, the accumulation of misfolded proteins (Aβ and tau) produces mitochondrial dysfunction and increased levels of ROS release in neurons [20, 44]. Also, as mentioned before, microglial activation contributes to the oxidant environment observed in this illness. In this context, the use of antioxidant molecules has been proposed for the treatment of neurodegeneration [45]. Our results show that caniferolide A has antioxidant properties in an *in vitro* model of oxidative stress. The compound increased cell survival, was able to reduce ROS levels and recovered GSH content. This effect is mediated by the ability of caniferolide A to translocate Nrf2 to the nucleus. In addition to its anti-inflammatory characteristics, Nrf2 is a key regulator of the expression of antioxidant genes. When it translocates to the nucleus, the transcription factor binds to the antioxidant response element and induces the expression of genes that encode for enzymes of GSH cycle, and important detoxifying molecules as superoxide dismutase or catalase. Nrf2 is also implicated in the availability of substrates to mitochondrial metabolism [46]. Compounds that activate Nrf2 are called indirect antioxidants because they induce the endogenous antioxidant machinery of the cell producing a longer effect than direct antioxidants, with shorter half-lives. These compounds can exert other beneficial

effects because Nrf2 is implicated in the modulation of the proteasome function, the lipid metabolism or neuroinflammation [47].

In summary, caniferolide A was able to modulate microglial cells state towards the neuroprotective phenotype by blocking NFκB, and p38 and JNK MAPK kinases. The compound also reduced tau phosphorylation by inhibiting p38 and JNK kinases and acts as an indirect antioxidant through the translocation of Nrf2 to the nucleus. It is noteworthy that the macrolide inhibits BACE1 activity, the key enzyme in the processing of Aβ. Taken together, our results indicate that caniferolide A could be a promising compound for AD treatment. This work opens the door to further studies to clarify its potential as a candidate drug for a polypharmacological approach against the neurodegenerative disease.

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Author Contributions

R. A. performed *in vitro* experiments. R.L., D.O., O.G and F.R. isolated and characterized the compound. E. A., A. A. and L.M. B. did critical discussion and work design. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript

Notes

The authors declare no competing financial interest

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Tables

Table 1. Antibodies used in western blot assays.

Antibody	Host	Dilution	Source
NfκB-p65	Rabbit	1:10000	Abcam
Nrf2	Rabbit	1:1000	Millipore
iNOS	Rabbit	1:1000	Abcam
Phospho p38	Rabbit	1:1000	Abcam
p38	Rabbit	1:1000	Abcam
Phospho ERK	Rabbit	1:1000	BD Biosciences
ERK	Mouse	1:1000	BD Biosciences
Phospho JNK	1:500	Mouse	BD Biosciences
JNK	1:1000	Mouse	BD Biosciences
AT8	Mouse	1:500	Thermo Fisher
AT100	Mouse	1:1000	Thermo Fisher
AT270	Mouse	1:1000	Thermo Fisher
Tau-5	Mouse	1:1000	Millipore
Lamin B1	Rabbit	1:1000	Abcam
Actin	Mouse	1:10000	Millipore

Figures

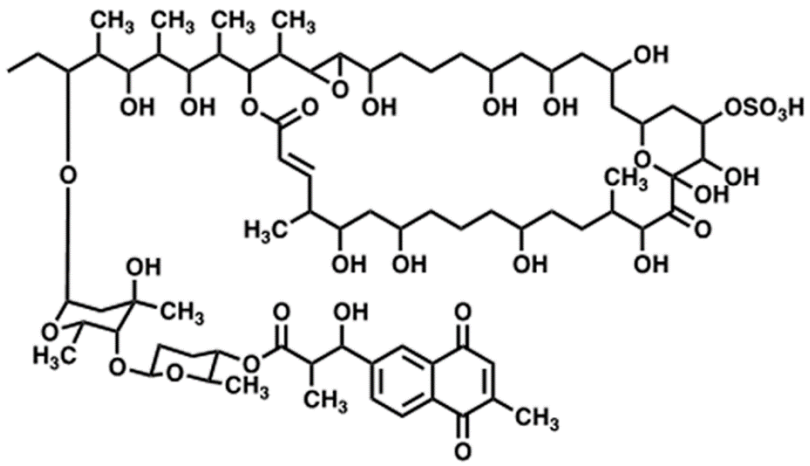

Figure 1. Chemical structure of the compound

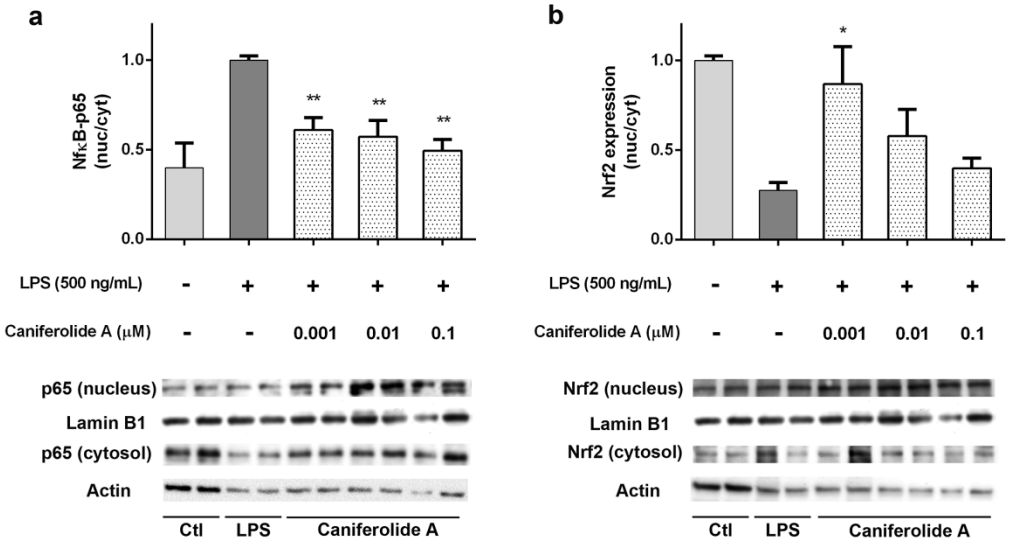

Figure 2. Caniferolide A modulates NFκB and Nrf2 pathways in microglia. BV2 cells were treated with the compound (0.001-0.1 μM) for 1 h and 500 ng/mL LPS was added during 23 h. The expression levels of (a) NFκB-p65 and (b) Nrf2 transcription factors were determined in nuclear and cytosolic fractions by western blot. Results are expressed as ratio of nuclear/cytosolic protein expression and corrected with lamin B1 and actin, respectively. Data are mean± SEM of three independent replicates performed by duplicate and compared to cells treated with LPS alone by one way ANOVA and post hoc Dunnett's tests.*p<0.05, **p<0.01

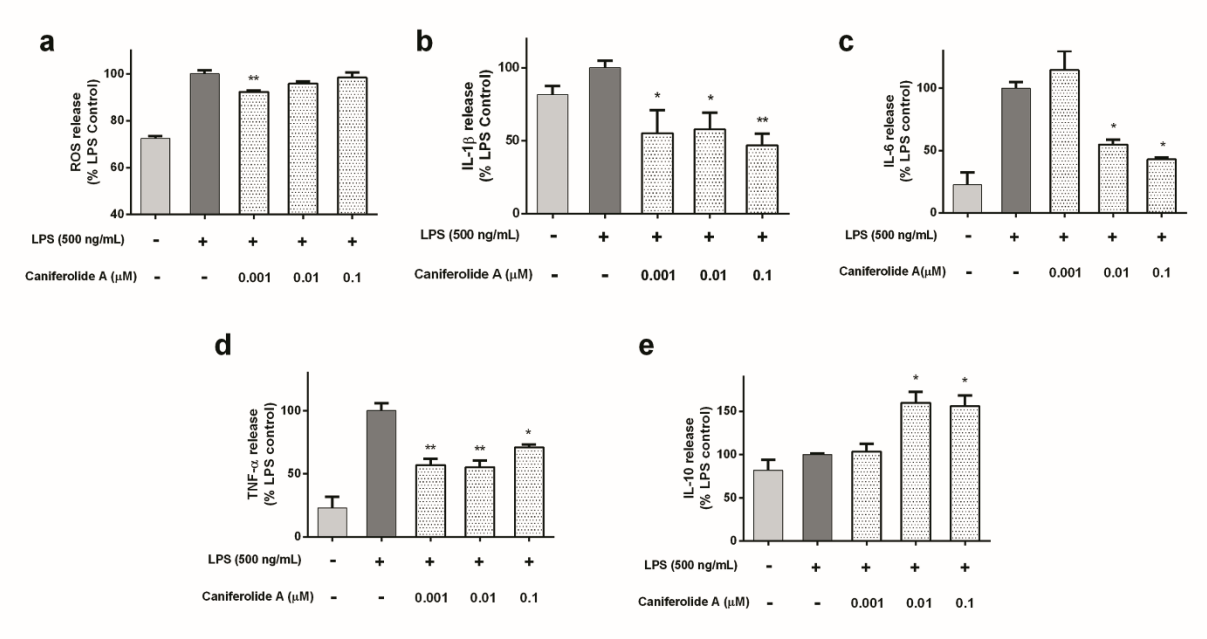

Figure 3. Effects of caniferolide A on ROS and cytokines release by microglia. The compound (0.001-0.1 μM) was added to BV2 cells for 1 h. Then, microglia was activated with 500 ng/mL LPS for 24 h. (a) Intracellular ROS levels were measured with carboxy-H_2DCFDA fluorescent dye. (b) IL-1β release was determined in cells supernatant with an ELISA assay kit. Levels of (c) IL-6, (d) TNF-α and (e) IL-10 were evaluated with Luminex. Data are mean± SEM of three independent experiments expressed in percentage of cells treated only with LPS. One way ANOVA and Dunnett's tests were used to determine statistical differences between treatments. *p<0.05, **p<0.01

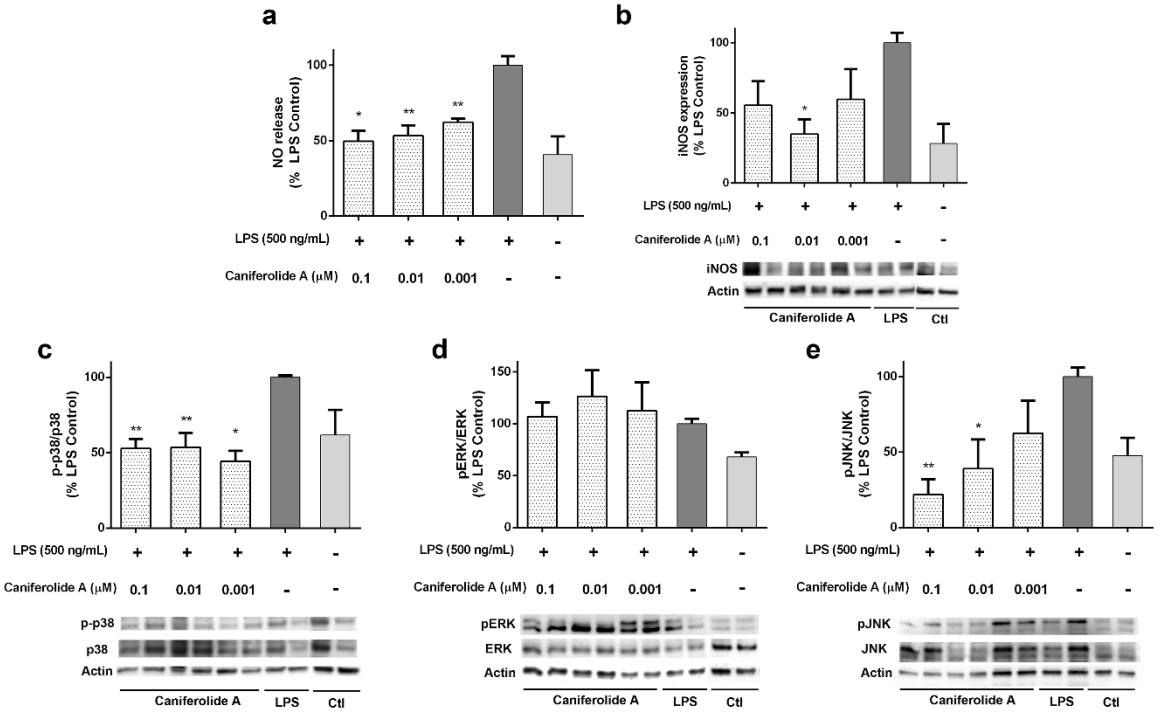

Figure 4. Effects of caniferolide A on NO release and MAPK kinases activation in BV2 cells. After treatment with *Streptomyces* secondary metabolite (0.001-0.1 μM) and LPS for 24 h, (a) the levels of NO release to the medium were evaluated with Griess reagent, and the expression of (b) iNOS, (c) p38, (d) ERK and (e) JNK were measured by western blot. The activation of kinases was assessed as the ratio between phosphorylated and total protein levels. Data are mean ±SEM of three independent experiments and expressed as percentage of LPS control cells. Bar graphs are inverted to match with blots order. *p<0.05, **p<0.01

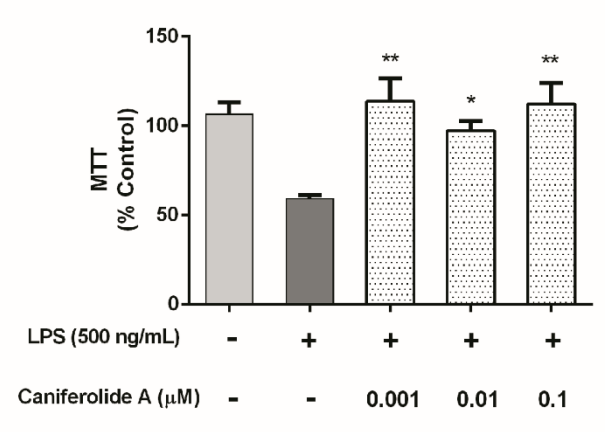

Figure 5. Caniferolide A increases SH-SY5Y survival in a co-culture with microglial cells. BV2 cells were seeded in trans-well inserts placed above neuroblastoma cells. Microglia was pre-treated with the macrolide (0.001-0.1 μM) and activated with 500 ng/mL LPS for 24 h. The effects on SH-SY5Y viability were determined with MTT assay. Results are expressed as mean ±SEM of three independent experiments performed by duplicate and compared to LPS control cells. Statistical differences were evaluated with one way ANOVA test followed by Dunnett's post hoc test. *p<0.05, **p<0.01

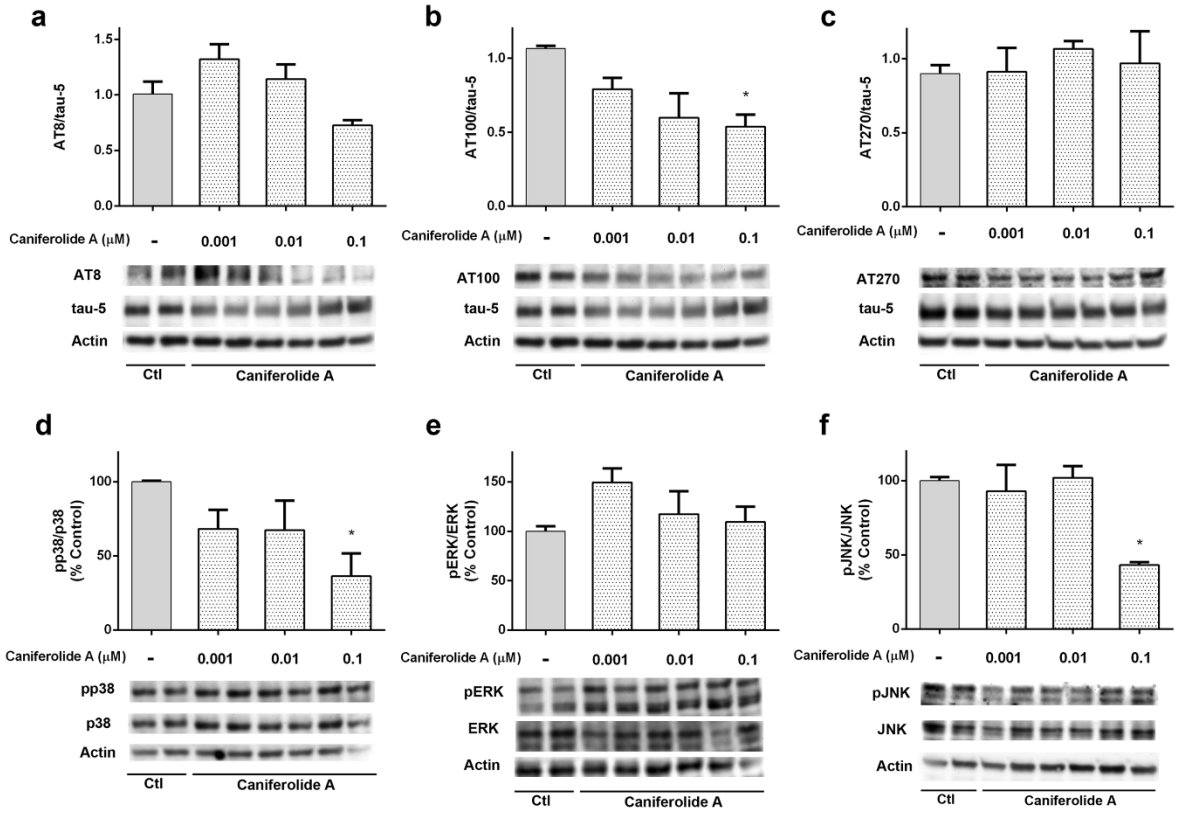

Figure 6. Effects of caniferolide A on tau phosphorylation. Differentiated SH-SY5Y-TMHT441 cells were treated with the compound (0.001-0.1 μM) for 48 h. Cells were lysed and the expression levels of (a) AT8, (b) AT100, (c) AT270, (d) p38, (e) ERK and (f) JNK were determined by western blot. Tau phosphorylation rate is expressed as levels of phosphorylated epitopes (AT8, AT100, and AT270) between total tau levels (tau-5). Activation of kinases is calculated as the ratio of phosphorylated/total protein levels. Values are mean ±SEM of three independent experiments performed by duplicate. Statistical differences were determined by one way ANOVA test followed by Dunnett's post hoc test. *p<0.05

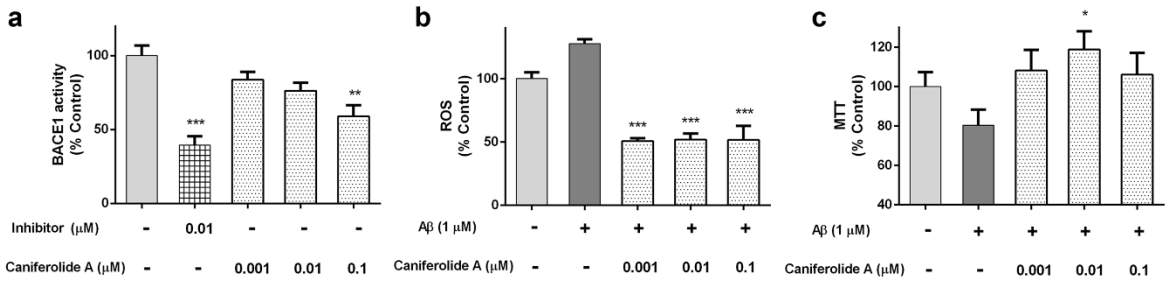

Figure 7. Caniferolide A inhibits BACE1 and reduces Aβ-induced activation of microglia. (a) BACE1 activity was determined with a FRET assay. A statine-derived inhibitor at 0.01μM was used as control. (b) ROS levels were measured in BV2 cells after pre-treatment with caniferolide A (0.001-1 μM) for 1 h and activation with 1 μM Aβ (1-42) during 23 h. (c) Viability of differentiated SH-SY5Y-TMHT441 cells after a trans-well co-culture with BV2 cells treated with the compound (0.1-0.001 μM) and 1 μM Aβ (1-42). Data are mean± SEM of three independent experiments. Statistical differences were determined with one way ANOVA and Dunnett's post hoc tests. *p<0.05, **p<0.01, ***p<0.001

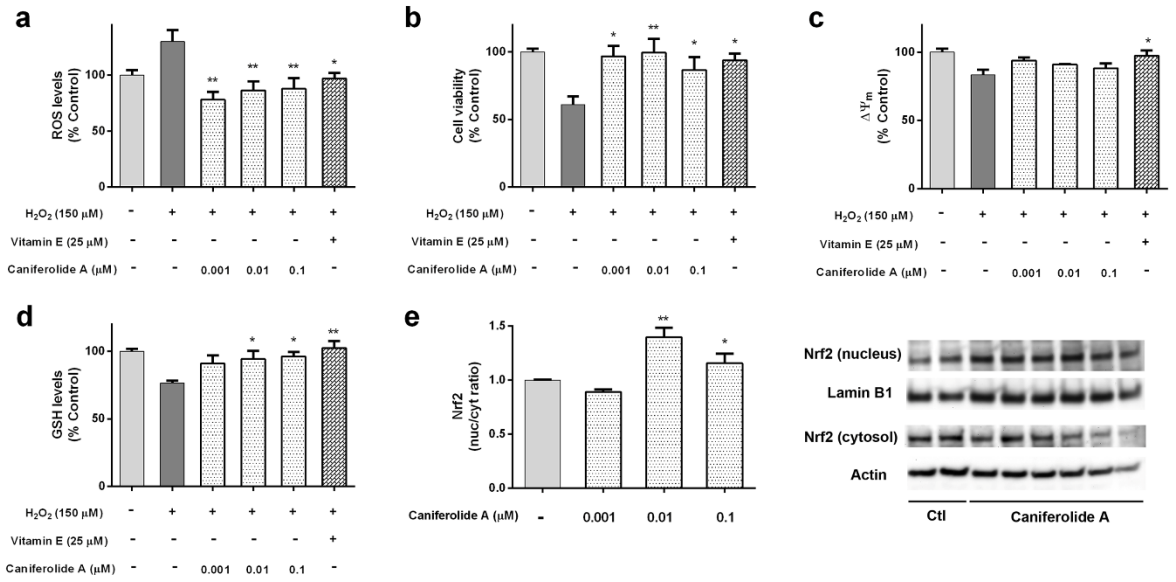

Figure 8. Antioxidant properties of caniferolide A. SH-SY5Y cells were treated with the *Streptomyces caniferus* secondary metabolite (0.001-0.1 μM) and 150 μM H_2O_2 for 6 h. Vitamin E at 25 μM was used as positive control. (a) Intracellular reactive oxygen species levels, (b) the effects on cell viability, (c) mitochondrial membrane potential and (d) glutathione levels were evaluated. Data are mean± SEM of three independent replicates. Values are expressed as percentage of untreated control cells and compared to cells treated with 150 μM H_2O_2 alone. (e) The ability of caniferolide A to translocate Nrf2 to the nucleus was determined after treatment with the compound (0.001-0.1 μM) for 6 h. Nrf2 translocation was calculated as the ratio between nuclear and cytosolic levels of the protein. Data are mean ± SEM of three independent experiments performed by duplicate. One way ANOVA and Dunnett's tests were used to determine the statistical significance between treatments. *p<0.05, **p<0.01

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