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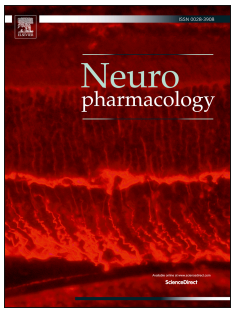

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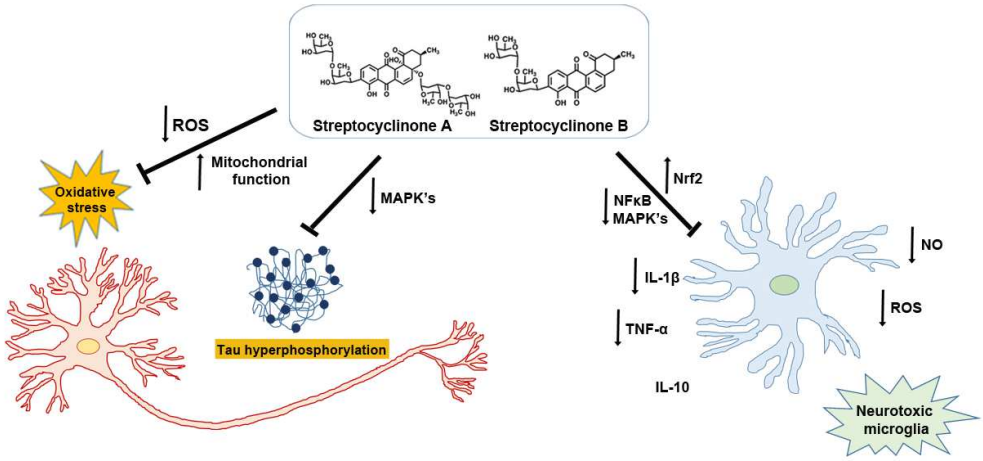

Streptocyclinones A and B ameliorate Alzheimer's disease pathological processes *in vitro*

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Abstract

Alzheimer's disease (AD) is a pathology characterized by the abnormal accumulation of amyloid-beta (Aβ) and hyperphosphorylated tau. Oxidative stress and neuroinflammation are also strongly related to this disease. The ability of two new glycosylated angucyclinones, streptocyclinones A and B (**1** and **2**), isolated from *Streptomyces* sp to improve AD hallmarks was evaluated. Compounds were able to protect SH-SY5Y neuroblastoma cells from H_2O_2-induced oxidative injury by activating the nuclear factor E2-related factor (Nrf2). Their capacity to modulate neuroinflammation was tested in lipopolysaccharide-activated BV2 microglial cells. Compounds reduced the release of pro-inflammatory factors, inhibited the activation of NFκB and mitogen activated kinases (MAPK), and induced the translocation of Nrf2 to the nucleus of microglial cells. A trans-well co-culture was established to determine the effect of microglia treated with streptocyclinones on the survival of SH-SY5Y cells. The cell viability of neuroblastoma cells increased when the compounds were added to BV2 cells. SH-SY5Y-TMHT441 cells were used to determine the effect of compounds on tau phosphorylation. Both compounds reduced tau hyperphophorylation by targeting MAPK kinases. Moreover, streptocyclinone B (**2**) was able to inhibit the activity of β-secretase 1 and decrease the release of reactive oxygen species in BV2 cells stimulated with Aβ. With the same co-culture trans-well

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid-beta; Nrf2, nuclear factor E2-related factor; NFκB, nuclear factor kappa-light enhancer of activated B cells; MAPK, mitogen activated kinase; BACE1, β-secretase 1; ROS, reactive oxygen species; IL-6, interleukin 6; IL-1β, interleukin 1-beta; TNF-α, tumor necrosis factor-α ; NO, nitric oxide; IL-10, interleukin 10; GSKβ3, glycogen synthase kinase-β3; APP, amyloid precursor protein; JNK, c-Jun N-terminal kinase; GSH, glutathione; LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase; ERK 1/2, extracellular signal-regulated kinases

system, the treatment of Aβ-stimulated microglia with compound **2** augmented the viability of SH-SY5Y-TMHT441 cells. The results presented in this work provide evidences of the multitarget activities displayed by these new *Streptomyces* compounds, making them good candidates for further studies in the treatment of AD.

Keywords: *Streptomyces*, Alzheimer disease, neuroinflammation, oxidative stress, Nrf2, p38

1. Introduction

Alzheimer's disease (AD) is a complex disorder characterized by its multifactorial nature. There are several processes involved in this pathology as tau hyperphosphorylation, disruption of calcium homeostasis, inflammation, amyloid-β (Aβ) aggregation, mitochondrial dysfunction and oxidative stress (Kumar et al., 2018b; Tong et al., 2018). These pathological hallmarks are closely interconnected and produce a vicious cycle that leads to neurodegeneration. In this sense, the treatments focused in one aspect of the illness have shown disappointing results and the polypharmacological approach has emerged as a promising strategy to face the complex etiology of AD (Rosini et al., 2016).

Neurons are extremely vulnerable to the accumulation of reactive oxygen species (ROS), which can even produce the death of these cells. Mitochondria of aging neurons are responsible of the augmented levels of radical species observed in neurodegenerative diseases. Failures in the electronic transport chain lead to the release of ROS that, together with a reduction in the efficacy of the antioxidant systems, increase the oxidative stress in the brain (Cheignon et al., 2018). The nuclear factor E2-related factor 2 (Nrf2) is the most important pathway implicated in the regulation of antioxidant enzymes. This transcription factor has been also related to the availability of substrates for mitochondrial metabolism. So, the activation of this pathway can produce a reduction in oxidative stress by enhancing the antioxidant systems and improving mitochondrial function (Esteras et al., 2016).

Neuroinflammation is a pathological hallmark in AD, mainly driven by microglial cells. Microglia are the immune cells of the central nervous system and represent the first line of host

defence against brain damage. These cells have two opposite phenotypes with neurotoxic or neuroprotective effects, which represent extremes of a broad range of phenotypic states. The pro-inflammatory or neurodegenerative phenotype (M1) is characterized by the release of toxic factors as interleukin-6 (IL-6), interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), ROS or nitric oxide (NO) and the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), the master regulator of the inflammatory response. The neuroprotective phenotype (M2) is characterized by augmented levels of anti-inflammatory molecules as interleukin-4 and interleukin-10 (IL-10), increased phagocytic capacity, a reduction in NO levels and the activation of Nrf2 translocation to the nucleus (Nakagawa and Chiba, 2015; Tang and Le, 2016). In neurodegenerative diseases there is an imbalance towards M1 phenotype, so the modulation of this disequilibrium to shift phenotype M1 to M2 has been suggested as a therapeutic target in the treatment of these disorders (Pena-Altamira et al., 2016).

AD is characterized by the abnormal aggregation of two proteins: tau and Aβ. Tau is associated with microtubules and plays a key role in the stabilization of neuronal cytoskeleton. In this pathology, tau is hyperphosphorylated and aggregated into neurofibrillary tangles, producing a destabilization of microtubules and impairing neuron's transport system (Panza et al., 2016). Tau phosphorylation is controlled by kinases and phosphatases and the dysregulation of this balance produces the hyperphosphorylation of the protein. Several kinases as cyclin-dependent kinase-5, mitogen-activated protein kinases (MAPK) or glycogen synthase kinase-3β (GSK3β) regulate the phosphorylation state of tau (Martin et al., 2013).

Moreover, the irregular processing of the amyloid precursor protein (APP) by β-secretase 1 (BACE1) and γ-secretase leads to the accumulation of Aβ. The protein is secreted to the extracellular space where it aggregates and forms toxic oligomers and fibrils. Aggregated Aβ species have a central role in AD by generating synaptic toxicity, mitochondrial dysfunction, oxidative stress and activation of immune cells (Polanco et al., 2018).

Streptomyces genus is a group of Gram-positive bacteria that belongs to the phylum Actinobacteria. Secondary metabolites isolated from members of this genus have displayed beneficial effects against oxidative stress in neuronal models (Eftekharzadeh et al., 2010), inhibition of neuroinflammation (Jang et al., 2006; Kim et al., 2002) and beneficial effects on AD hallmarks (Leiros et al., 2015a).

In this study, we evaluated the potential of two new amphiphilic glycosylated angucyclinones, streptocyclinones A and B (**1** and **2**), isolated from *Streptomyces* sp to ameliorate AD hallmarks. Their antioxidant properties and their ability to modulate the inflammatory response were tested in SH-SY5Y human neuroblastoma cells and BV2 murine microglial cells, respectively. SH-SY5Y cells have been widely used in oxidative stress models (Sun et al., 2016) and BV2 cells have been characterized as an appropriate model for the study of neuroinflammation (Henn et al., 2009). Finally, the effects of compounds on tau phosphorylation were determined in SH-SY5Y tau441 cells (SH-SY5Y-TMHT441). This cell line has been established as an *in vitro* model for AD screening since it is stably transfected with the human V337M and R406W mutations, resulting in tau phosphorylation in residues related to this illness (eg., Thr231, Thr181) and increased activity of kinases as c-Jun N-terminal kinases (JNK) or GSK3β (Leiros et al., 2015a; Leiros et al., 2015b; Loffler et al., 2012).

2. Materials and Methods

2.1 Chemicals and solutions

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

2.2 Isolation

Compounds were isolated from extracts of a culture of *Streptomyces* sp. CA-237351 using a two steps cultivation methodology. At first, a seed culture of the strain was obtained by inoculating three 50 mL tubes each containing 14 mL of ATCC-2-M medium (soluble starch 20 g/L, glucose 10 g/L, NZ Amine Type E 5 g/L, meat extract 3 g/L, peptone 5 g/L, yeast extract 5 g/L, sea salts 30 g/L, calcium carbonate 1 g/L, pH 7) with 0.7 mL of a freshly thawed inoculum stock of the producing strain. Tubes were incubated in a rotary shaker at 28 °C, 70% relative humidity and 220 rpm for about 96 h.

The fresh inoculum was mixed and employed to inoculate twenty 250 mL flasks, each containing 50 mL of FR23 medium (2.5% v/v) (sugar cane honey 20 g/L, glucose 5 g/L, soluble starch (potato) 30 g/L, cotton seed flour 20 g/L, pH adjusted to 7.0). The inoculated flasks were incubated in a rotary shaker at 28 °C, 70% relative humidity, and 220 rpm for six days before harvesting. 10.4 mg of streptocyclinone A (**1**) and 6.8 mg of streptocyclinone B (**2**) were obtained after extraction and bioassay-guided chromatographic separation using Sephadex LH-20 (MeOH/$CHCl_3$ 3:1) and semipreparative HPLC (gradient H_2O/CH_3CN from 25 to 40 % CH_3CN in 45 min) of the culture broth.

Structures of compounds **1** and **2** were established by HRMS (Bruker maXis ESI-TOF spectrometer) and 1D and 2D NMR (Bruker Avance III 500 MHz), and comparison with data published for other structurally related molecules (Ueda et al., 2011) (see supplementary information for structure elucidation details).

2.3 Cell culture

Human neuroblastoma SH-SY5Y cell line was purchased from American Type Culture Collection (ATCC), number CRL2266. The cells were maintained in Dulbecco´s Modified Eagle´s medium: Nutrient Mix F-12 (DMEM/F-12) supplemented with 10% fetal bovine serum (FBS), glutamax, 100 U/mL penicillin and 100 µg/mL streptomycin.

The human neuroblastoma cell line SH-SY5Y tau441 (SH-SY5Y-TMHT441) was kindly supplied by QPS Austria GmbH (Loffler et al., 2012). Cells were cultured in DMEM/F-12

medium supplemented with 10% FBS, 10 mg/mL gentamicin, glutamax and non-essential aminoacids. For the cell line maintenance, 0.6% geneticin was added to the culture medium.

BV2 cell line was purchased from Interlab Cell Line Collection (ICLC), number ATL03001. Cells were maintained in RPMI 1640 medium with 10 % FBS, 100 U/mL penicillin and 100 µg/mL streptomycin.

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

2.4 Oxidative stress assays

All the assays were performed in 96-well plates. SH-SY5Y cells were seeded at a density of $5x10^4$ cells per well and allowed to grow during 24 h. Cells were treated with compounds at various concentrations (0.001-1 µM) and 150 µM H_2O_2 for 6 h. Four parameters were examined: cell viability, ROS levels with the fluorescence dye carboxy-H_2DCFDA, mitochondrial membrane potential ($\Delta\Psi_m$) and glutathione (GSH) levels. The endogenous antioxidant vitamin E at 25 µM was used to validate the *in vitro* model in all the assays. All experiments were performed at least three times.

2.4.1 *Cell viability and mitochondrial membrane potential assays*

The neuroprotective effects of the compounds on the viability of SH-SY5Y cells in the presence of 150 µM H_2O_2 were determined with MTT (3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay. Cells were washed three times with saline solution and 200 µL of MTT (500 µg/mL) dissolved in saline buffer were added to each well. Following 1 h of incubation at 37ºC, cells were disaggregated with 5% sodium dodecyl sulphate. Absorbance of formazan crystals was measured at 595 nm with a spectrophotometer plate reader. Saponin was used as death control and its absorbance was subtracted from the other data.

The changes in mitochondrial membrane potential ($\Delta\Psi_m$) were monitored with TMRM dye. Cells were washed twice with saline solution and 1 µM TMRM was added to each well for 30 min at 37ºC. After the incubation, cells were solubilized with DMSO and H_2O at 50% and the fluorescence was monitored with a spectrophotometer plate reader (535 nm excitation and 590 nm emission).

2.4.2 *Evaluation of intracellular reactive oxygen species release and glutathione levels*

Intracellular levels of ROS were determined with carboxy-H_2DCFDA (5-(and-6)-carboxy-2′, 7′-dichlorodihydrofluorescein diacetate). After the treatment with compounds and H_2O_2, SH-SY5Y cells were washed twice with serum-free medium. Then, 20 µM carboxy-H_2DCFDA dissolved in serum-free medium was added to the cells. After 1 h at 37ºC, the medium containing the fluorescence dye was replaced with PBS. The plate was incubated for 30 min at 37ºC and fluorescence was read at 527 nm, with an excitation wavelength of 495 nm.

GSH levels were determined using Thiol Tracker™ Violet dye following manufacturer´s instructions. Briefly, cells were washed twice with PBS and loaded with 100 µL of pre-warmed Thiol Tracker™ Violet dye (10 µM) for 30 min at 37ºC. The fluorescence was measured at 404 nm excitation and emission at 526 nm.

2.5 Neuroinflammation assays

2.5.1 Fibrillar Aβ preparation

Aβ fibrils were prepared as previously described (An et al., 2017). Aβ (1-42) was dissolved at 500 µM in DMSO and fibrils were incubated in culture medium for 24 h at 37ºC.

2.5.2 Determination of nitric oxide and reactive oxygen species levels

For determining NO levels, BV2 cells were cultured in 12-well plates at 1×10^6 cells per well in DMEM without phenol red. Microglial cells were pre-treated with the compounds (0.001-1 µM) for 1 h. Then, LPS at 1 µg/mL was added to each well during 23 h to activate the cells. NO

levels were monitored using the Griess Reagent Kit (Thermo Fisher Scientific) following manufacturer's instructions.

In order to evaluate ROS release, BV2 cells were seeded in 96-well plates at 4×10^4 cells per well. Cells were treated with compounds as described before and 500 ng/mL LPS or 1 µM Aβ were added for 23 h. After this time, the levels of ROS were analysed following the protocol described for SH-SY5Y cells. All the assays were performed at least three times.

2.5.3 Measurement of cytokines release

BV2 murine microglial cells were pre-treated with the compounds at various concentrations (0.001, 0.01, 0.1, and 1 µM) for 1 h. After this time, LPS at 500 ng/mL was added to each well for 23 h. The release of IL-1β and IL-6 to the medium were measured with a Mouse IL-1β ELISA Kit and a Mouse IL-6 ELISA kit (Thermo Fisher Scientific), following manufacturer's instructions.

IL-10 and TNF-α levels were determined with a Mouse High Sensitivity T Cell Magnetic Bead Panel (Merck Millipore, Darmstadt, Germany), following manufacturer's instructions. The levels of cytokines release to the medium were measured in Luminex 200 ™ instrument (LuminexCorp, Austin, TX) and adquired with xPONENT® software.

2.6 Western blotting

For western blot analysis, BV2 and SH-SY5Y cells were seeded in 12-well plates at a density of 1×10^6 cells per well. SH-SY5Y-TMHT441 cells were seeded at a 60% confluence and differentiated with 10 µM retinoic acid for 7 *div*. After treatment with compounds, cells were washed twice with ice-cold PBS. Then, an ice-cold hypotonic solution buffer (20 mM Tris-HCl pH 7.4, 10 mM NaCl and 3 mM $MgCl_2$, containing a complete phosphatase/protease inhibitors cocktail) was added. Cells were incubated on ice for 15 min and centrifuged at 3000 rpm, 4 ºC during 10 min. The supernatant was collected as the cytosolic fraction. The pellet of SH-SY5Y and BV2 cells was resuspended in an ice-cold 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 for 30 min, vortexing in 10 min intervals, and centrifuged at 14000 g and 4°C for 30 min. The supernatant was saved for nuclear protein fraction. This fraction was quantified by Bradford method, whereas cytosolic protein fraction was quantified using Direct Detect system (Merck Millipore). Samples containing 20 µg (cytosolic fraction) or 10 µg (nuclear fraction) were used for electrophoresis, which was resolved in a 4-10% sodium dodecyl sulphate polyacrylamide gel (Biorad, Hercules, CA, USA) and transferred onto PVDF membranes (Merck Millipore). Snap i.d protein detection system was used for membrane blocking and antibody incubation. Protein bands were detected using 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.). Primary antibodies are summarized in Table 1. Protein signal was corrected by β-actin in cytosolic fraction and lamin B1 in nuclear fraction. The ratio of phosphorylated *versus* total protein levels was determined to verify the activation of kinases.

2.7 β- secretase inhibition assay

The ability of the compounds to inhibit BACE1 activity was determined with a fluorescence resonance energy transfer kit (FRET) (Thermo Fisher Scientific), following manufacturer's instructions.

2.8 Trans-well co-cultures

SH-SY5Y cells were seeded in 24-well plates at a density of 2.5×10^5 cells per well. BV2 microglial cells were seeded in cell culture inserts (0.4 µM pore size, Merck-Millipore) at a density of 2.5×10^5 cells per insert. After 24 h, BV2 cells were pre-treated for 1 h with compounds (0.001-1 µM) and 500 ng/mL LPS was added to each insert during 23 h.

SH-SY5Y-TMHT441 cells were seeded at 2×10^5 cells per well and differentiated with 10 µM retinoic acid for 7 *div*. Then, BV2 cells were seeded in cell culture inserts as described above.

Cells were pre-treated with compound 2 (0.001-1 µM) for 1 h and 1 µM Aβ was added to the inserts for 23 h to activate microglial cells.

MTT assay was carried out as described before, in order to determine the survival of SH-SY5Y and SH-SY5Y-TMHT441 cells in the presence of BV2 microglial cells.

2.9 Statistical Analyses

Data are presented as mean ± SEM. Differences were evaluated by one way ANOVA with Dunnett's post hoc test and statistical significance was considered at $p < 0.05$.

3. Results

3.1 Streptocyclinones A and B display neuroprotective effects against oxidative stress in SH-SY5Y neuroblastoma cells.

At first, SH-SY5Y cells were treated with compounds **1** and **2** (Figure 1) at various concentrations (0.001-1 µM) for 24 h and the cytotoxicity was evaluated with MTT assay. None of the compounds displayed cytoxicity against this cell line at the concentrations tested (data not shown).

In order to determine the antioxidant properties of the compounds, oxidative stress was induced with the well-known oxidant H_2O_2 at 150 µM. Neuroblastoma cells were co-treated with the compounds and H_2O_2 for 6 h and the protective effects of the compounds were tested. The endogenous antioxidant vitamin E at 25 µM was used as positive control in all the assays.

The effect of the compounds on cell viability in the presence of H_2O_2 was determined by MTT test. One way ANOVA test revealed significant differences between treatments ($F_{10, 33} = 4.48$, p=0.0009). Particularly, the addition of 150 µM H_2O_2 produced a reduction of 39.0 ±13.8% (p<0.01) in cell viability (Figure 2a) *vs.* control cells. Treatment with compound **1** (0.1 and 1 µM) and compound **2** (0.01, 0.1 and 1 µM) significantly protected SH-SY5Y cells with an increase in viability until percentages between 95-105% of control cells. Regarding $\Delta\Psi_m$

(Figure 2b), the differences between groups were significant ($F_{10, 33}$ = 7.91, p<0.0001) and the addition of compound **1** repolarized the mitochondrial membrane at 0.001, 0.01 and 0.1 µM (106.7 ±4.1%, p<0.001; 100.6 ±2.2 %, p<0.001; 94.0± 2.4%, p<0.05). Compound **2** also resulted in a significant recovery of $\Delta\Psi_m$ at the lowest concentrations (0.001, 0.01 and 0.1 µM), with levels of 101.2 ±0.5% (p<0.001), 102.8 ±4.9% (p<0.01) and 95.6 ±0.8% (p<0.01), respectively.

Treatment with the two compounds significantly reduced the increase in ROS levels produced by 150 µM H_2O_2 (135.4 ±12.6%, p<0.05). One way ANOVA test showed a significant effect between treatments ($F_{10, 22}$ = 2.67, p=0.0336) (Figure 2c). Compound **1** showed antioxidant properties at all the concentrations assayed, decreasing ROS release to 74.9±7.8 % (p<0.01) at 0.01 µM. Treatment with **2** significantly reduced ROS at 0.01, 0.1, and 1 µM to levels lower than control cells, reaching a 71.7 ±15.7%, (p<0.05) at 0.1 µM. The levels of GSH, the main non-enzymatic antioxidant in cells, were also evaluated (Figure 2d). Statistical analysis with one way ANOVA revealed significant differences among groups ($F_{10, 22}$ = 3.75, p=0.0035). H_2O_2 produced a drop in GSH levels to 80.5 ±3.2% (p<0.05), but the addition of compounds **1** and **2** at 1 µM significantly increased the levels of the antioxidant molecule to 101.0 ±2.6% (p<0.05), 104.8 ±1.2% (p<0.05), respectively.

Nrf2 activation is related with the improvement of mitochondrial function and the enhancement of antioxidant functions (Esteras et al., 2016). In view of our results, the ability of compounds to translocate the nuclear factor Nrf2 was determined. SH-SY5Y cells were treated with compounds (0.001-1 µM) for 6 h and the expression levels of the transcription factor were evaluated by western blot. Both compounds produced an increase in Nrf2 expression in the nucleus (Figure 2e) compared to control cells ($F_{8, 18}$ = 10.08, p=0.0001). Compound **1** activated the translocation of the nuclear factor at 0.01, 0.1 and 1 µM, with levels around a 160 % of control cells. Compound **2** increased Nrf2 levels at all the concentrations assayed, in a dose-dependent manner, reaching a 194.4 ±7.9% (p<0.001) at 1 µM. The expression of the transcription factor in the cytosolic fraction did not show significant differences ($F_{8, 18}$ = 0.79,

p=0.617) (Figure 2f). Our results confirm that the antioxidant effect produced by *Streptomyces* compounds on SH-SY5Y cells is mediated by the translocation of Nrf2 to the nucleus.

3.2 Streptomyces secondary metabolites modulate neuroinflammation in LPS-activated BV2 microglial cells

BV2 murine microglial cell line has been established as a suitable model for studying neuroinflammation *in vitro* and assessing the potential of anti-inflammatory drugs (Gresa-Arribas et al., 2012; Henn et al., 2009).

Firstly, the cytotoxicity of *Streptomyces* secondary metabolites was determined with MTT test. BV2 cells were treated with compounds (0.001-1 µM) for 24 h. None of the treatments reduced cell viability (data not shown). Therefore, the anti-inflammatory effects of the compounds were evaluated by pre-treating BV2 cells with streptocyclinones for 1 h, followed by activation with 500 ng/mL LPS during 23 h. LPS is the major component of the outer membrane of Gram-negative bacteria, and is a well-known inducer of inflammatory response in microglial cells (Han et al., 2017). The stimulation with 500 ng/mL LPS significantly increased ROS levels a 38.2 ±1.9% (p<0.001) *vs* control cells (Figure 3a). Also, the differences among treatments were significant ($F_{9, 20}$ =13.80, p<0.0001). The addition of compound **1** decreased the ROS release at 1 µM (88.5±2.2%, p<0.05) compared to LPS control cells. Treatment with compound **2** reduced ROS levels at 0.001, 0.1 and 1 µM, with this concentration producing the highest decline, until 81.6±1.5% (p<0.001).

An increase in the release of NO by microglial cells is also associated with neurodegeneration, since this molecule facilitates neuronal death (Yuste et al., 2015). The levels of NO in BV2 supernatant were assessed with Griess reagent, which allows to detect the spontaneous oxidation of NO to nitrite under physiological conditions(Green et al., 1982). One way ANOVA analysis showed significant differences between treatments ($F_{9, 30}$ = 5.81, p=0.0002). The addition of LPS induced a rise in NO levels of 59.3 ±6.0% (p<0.001) (Figure 3b). Pre-treatment with compound **1** diminished the release of the pro-inflammatory molecule at all the concentrations tested until

percentages around 50% of LPS control cells. Compound **2** also produced a decrease in NO levels at 0.1 (64.1±14.1%, $p<0.01$) and 1 µM (65.7± 4.1 %, $p<0.01$). In order to confirm the results obtained with Griess reagent, the expression of inducible nitric oxide synthase (iNOS), the enzyme responsible of NO release, was measured by western blot (Figure 3c). The statistical analysis with one way ANOVA test presented significant differences ($F_{9, 30} = 2.57$, $p=0.0313$). The activation of BV2 cells with 500 ng/mL LPS produced a high increase in the expression of the enzyme compared to inactivated cells (95.8 ±7.1%, $p<0.001$). Both streptocyclinones significantly reduced the expression levels of iNOS, compound **1** at 0.01 and 0.1 µM (63.2± 9.4%, $p<0.05$ and 60.3 ±7.1%, $p<0.05$, respectively) and compound **2** at 0.1 and 1 µM (54.1± 16.5%, $p<0.05$ and 53.8 ±1.8%, $p<0.05$, respectively).

The release of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α to the medium was also measured. The statistical analysis of IL-1β release presented a significant effect among treatments ($F_{9, 20} = 3.13$, $p=0.0307$). The addition of 500 ng/mL LPS induced an increase in the levels of IL-1β of 29.3±1.6% ($p<0.05$) compared to untreated cells. Compounds **1** and **2** significantly diminished the release of this cytokine at all the concentrations tested, with percentages between 69 -75% of LPS control cells (Figure 4a).

None of the compounds was able to decrease the levels of IL-6, but the addition of LPS produced a significant increase compared to inactivated cells ($F_{9, 20} = 6.39$, $p=0.0003$) (Figure 4b). However, both compounds reduced TNF-α release ($F_{9, 20} = 6.00$, $p=0.0008$) (Figure 4c). The increase in TNF-α levels produced by the addition of 500 ng/mL LPS (77.0± 5.7%, $p<0.001$) was significantly diminished by compound **1** at 0.1 and 1 µM until a percentage of 45.4% ($p<0.01$). The treatment with compound **2** at 0.001, 0.01 and 1 µM also induced a decline in TNF-α, reducing the pro-inflammatory molecule to levels between 46.1 and 54.2 %.

In regard to the anti-inflammatory cytokine IL-10, treatment with the two streptocyclinones significantly augmented its levels ($F_{9, 20} = 5.88$, $p=0.0009$) (Figure 4d). Interestingly, compound **1** at 1 µM generated an increase in IL-10 levels of 141.8 ±14.5 % ($p<0.05$), and compound **2** at

0.001 and 0.1 µM induced the release of this cytokine until 140.6 ±3.1 % (p<0.05) and 138.2± 4.6 (p<0.05), respectively.

The transcription factors NFκB and Nrf2 are main regulators of pro- and anti-inflammatory phenotypes of microglia (Rojo et al., 2014). NFκB is a complex constituted by p-65, RelB, c-Rel, p-50 and p-52. The five proteins form homo- and heterodimers that regulate NFκB activity. An increase in p65/p50 dimers in the nucleus induces the expression of pro-inflammatory genes (Srinivasan and Lahiri, 2015). The expression levels of Nrf2 and NFκB-p65 in cytosolic and nuclear fractions were assessed by western blot (Figure 5a-d). BV2 cells were treated with streptocyclinones (0.001-1 µM) for 1 h and 500 ng/mL LPS was added during 23 h. As Figure 5a shows, compounds **1** and **2** were able to significantly increase Nrf2 levels in the nucleus. The statistical differences between treatments were significant ($F_{9, 30}$ = 2.84, p=0.0152). When 500 ng/mL LPS was added to BV2 cells, Nrf2 levels in the nucleus decreased a 53.3 ±15.1% (p<0.01) *vs* control cells. Pre-treatment with compound **1** at 0.01 and 0.1 µM increased Nrf2 expression until percentages of 135.9±18.0% (p<0.05) and 138.5 ±36.4% (p<0.05), respectively. Compound **2** presented a dose-dependent response, with a significant effect at 0.1 and 1 µM, reaching a percentage of 111.8±10.5% (p<0.01) at the highest concentration. The levels of Nrf2 expression in cytosolic fraction were not affected by the addition of *Streptomyces* secondary metabolites ($F_{9, 30}$ = 2.14, p=0.0629) (Figure 5b).

The nuclear expression of NFκB-p65 presented a significant effect among treatments ($F_{9, 30}$ = 2.92, p=0.0194). The increase in BV2 cells treated with LPS (57.5±7.0%, p<0.01) was diminished by both compounds (Figure 5c). Compound **1** showed inhibitory activity at the highest concentrations, 0.1 (57.1±11.7%, p<0.05) and 1 µM (57.4±6.4%, p<0.01) and compound **2** reduced the expression of p65 at 0.01, 0.1 and 1 µM, although it was only statistically significant at 0.01 (80.5±7.3%, p<0.05) and 1 µM (76.3±14.9%, p<0.05). Once again, the expression of the transcription factor in the cytosol did not present significant differences between treatments ($F_{9, 30}$ = 0.53, p=0.8418) (Figure 5d).

Extracellular signal–regulated (ERK 1/2) and p38 kinases are activated in response of stress signals as oxidative damage or inflammation and their inhibition is related with the neuroprotective phenotype of microglia (Kim and Choi, 2015). In this context, we analysed the effect of *Streptomyces* compounds on the phosphorylation of these enzymes (Figure 5e-f). As Figure 5e shows, pre-treatment with streptocyclinones reduced p38 activation ($F_{9, 20}$ = 5.76, p=0.0006). Particularly, compound **1** diminished p38 phosphorylation at all the concentrations, but it was statistically significant at 0.001, 0.1 and 1 µM, displaying the biggest inhibitory effect at 0.1 µM (48.0±17.6%, p<0.01). Compound **2** also diminished p38 activation at all the concentrations assayed, highlighting the effect at 0.001 µM (32.5 ±8.4%, p<0.001). On the other hand, ERK 1/2 phosphorylation was inhibited only by compound **1** (Figure 5f) at 0.001 (62.7 ±10.4%, p<0.01) and 0.1 µM (71.1± 6.6%, p<0.05). One way ANOVA test of ERK 1/2 expression showed significant differences ($F_{9, 20}$ = 6.45, p=0.0005).

In view of these results a trans-well co-culture system was established with BV2 and SH-SY5Y cells with the objective of determine the effect of LPS-activated microglial cells treated with compounds on the survival of neuroblastoma cells. Firstly, we assessed the cytotoxicity of the treatments in SH-SY5Y cells, without BV2 cells. None of the treatments affected to the viability of neuroblastoma cells (data not shown). On the other hand, in the trans-well system the differences among treatments presented significant differences ($F_{9, 19}$ = 5.56, p=0.0008). The activation of microglia with LPS produced a significant reduction in the viability of SH-SY5Y cells, with a decrease of 40.8 ±2.1% (p<0.01) compared to neuroblastoma cells co-cultured with inactivated BV2 cells (Figure 6). Pre-treatment with compound **1** protected SH-SY5Y cells at all the concentrations, reaching percentages between 96.9 and 111.11% of control cells. Addition of compound **2** to LPS-activated BV2 cells resulted in a protective effect on SH-SY5Y viability at 0.1 (91.5 ±4.7 %, p<0.05) and 1 µM (92.7±10.2%, p<0.05).

3.3 Streptocyclinones reduce tau hyperphosphorylation by inhibiting MAPK kinases

In order to evaluate the effects of *Streptomyces* compounds on the phosphorylated state of tau, we used the SH-SY5Y-TMHT441 cell line, a well-established *in vitro* model for tau screening (Leiros et al., 2015a; Leiros et al., 2015b; Loffler et al., 2012). Cells were differentiated for 7 *div* and compounds at concentrations ranging from 0.001 to 1 µM were added for 48 h. Tau expression levels were evaluated by western blot with three specific antibodies that recognize different phosphorylated residues typical of AD: AT8 (pSer202), AT270 (pThr181) and AT100 (pThr212 and pSer214). Total tau levels were determined with Tau-5 antibody. As Figure 7a shows, AT8 expression was reduced by the two compounds ($F_{8, 18} = 3.67$, p=0.0186), compound **1** was effective at 0.01 and 0.1 µM, displaying the biggest reduction at 0.1 µM (60.4±11.8%, p<0.05). Compound **2** decreased pSer202 at 0.01, 0.1 and 1 µM, the most potent treatment, with a reduction of 57.9±7.7% (p<0.05). Phosphorylation at Thr181, recognized by AT270 antibody, presented a significant effect among treatments ($F_{8, 18} = 4.12$, p=0.0119). Compound **1** diminished AT270 expression at the lowest concentration (0.001 µM) until 64.5±6.2% (p<0.05). Compound **2** produced a decrease at 0.01 (70.9±1.1%, p<0.05) and 0.1 µM (72.2±5.3%, p<0.05) (Figure 7b). With regard to AT100 expression, one way ANOVA test showed significant differences between treatments ($F_{8, 18} = 3.89$, p=0.0204). Compound **1** induced a dose-dependent response, being statistically significant at 1 µM, with a percentage of 63.1 ±13.0% (p<0.05) (Figure 7c). Figure 7d shows total tau levels, which did not present significant differences ($F_{8, 18} = 0.79$, p=0.6222).

The tau residues analysed can be phosphorylated by members of MAPK kinase family (ERK 1/2, JNK and p38) and other protein kinases as GSK3β (Martin et al., 2013). The activation state of kinases was also studied by western blot (Figure 8). Analysis with one way ANOVA of p38 phosphorylation revealed statistical differences ($F_{8, 18} = 3.27$, p=0.0285). Compound **2** reduced the activation of p38 at all the concentrations assayed, presenting significant differences at 0.01 and 1 µM (69.8± 8.6%, p<0.05 and 59.0±5.1%, p<0.01, respectively) (Figure 8a). In regard to ERK 1/2, the addition of **1** reduced the activation of this enzyme at the lowest concentrations, being 0.001 µM the most effective treatment, with a percentage of 59.7 ±10.0%

(p<0.05) (Figure 8b). One way ANOVA test between all the treatments showed significant differences ($F_{8, 18}$ = 2.94, p=0.0453). Figure 8c presents the results corresponding to JNK kinase ($F_{8, 18}$ = 4.94, p=0.0069). Both *Streptomyces* compounds produced a dose-dependent inhibition of JNK phosphorylation, reaching a 49.7±8.9 % (p<0.05) when 1 µM of compound **1** is added and a 35.9±8.1% (p<0.01) when cells are treated with **2** at the same concentration. Regarding GSK3β, the phosphorylated levels were determined with an antibody that recognizes pSer9, that is, the inactivated form of the kinase. Therefore, an increase in phosphorylated levels indicated an inhibition of this enzyme. None of the compounds displayed significant effects on the activity of GSK3β ($F_{8, 18}$ = 0.72, p=0.6717) (Figure 8d).

3.4 Streptocyclinone B inhibits β-secretase activity and attenuates amyloid beta-activation of BV2 microglial cells

BACE1 is the enzyme that initiates the production of Aβ. Therefore, it has been proposed as a good target for the reduction of Aβ (Kumar et al., 2018a). The ability of compounds to inhibit the activity of BACE1 was tested with a FRET assay kit. A statine-derived inhibitor was used as positive control in order to check the validity of the assay. Statistical analysis with one way ANOVA test revealed significant differences ($F_{9, 20}$ = 8.160, p=0.0002) (Figure 9a). Specifically, compound **1** did not inhibit BACE1 activity at any of the concentrations tested, whereas compound **2** presented a dose-dependent effect, with the highest inhibition at 1 µM (51.2±6.1%, p<0.01).

In view of the results obtained with **2** in LPS-activated BV2 cells and in BACE1 assay, this compound was chosen to evaluate its effects on BV2 microglial cells activated with Aβ. Treatment with Aβ and the compound produced significant differences ($F_{5, 12}$ = 39.54, p<0.0001) (Figure 9b). The addition of 1 µM Aβ produced a significant increase in the release of ROS by microglia (27.5±3.7%, p<0.05). Compound **2** reduced the levels of ROS at all the concentrations tested (0.001-1 µM) to percentages varying between 79.3% and 41.5%, suggesting that this *Streptomyces* secondary metabolite is able to modulate the activation of

microglia with Aβ. In order to confirm these results, Aβ-activated BV2 cells were co-cultured with SH-SY5Y-TMHT441. Prior to co-culture experiments, the cytotoxicity of the co-treatment with **2** and 1 µM Aβ in neuroblastoma cells was assessed. The treatments did not affect to the survival of the cells (data not shown). In the co-culture, one way ANOVA test showed a significant effect among groups ($F_{5, 12}$ = 9.34, p=0.0085). The addition of compound **2** to microglial cells activated with Aβ resulted in an increase in the survival of neuroblastoma cells at 0.1 and 1 µM until 110.5±11.1% (p<0.05) and 118.9±2.7% (p<0.01), respectively (Figure 9c).

4. Discussion

Almost a half of known natural products are produced by members of the phylum Actinobacteria (Lucas et al., 2013). Among actinomycetes, *Streptomyces* genus is the most prolific source of pharmacologically active compounds. Several bioactive secondary metabolites from different *Streptomyces* spp. with antimicrobial, anticancer, anti-inflammatory or antiviral activities have been described (Manivasagan et al., 2014). In this work we evaluated the ability to ameliorate AD hallmarks of two new glycosylated angucyclinones isolated from a *Streptomyces* sp, streptocyclinones A and B.

Oxidative stress is an early event in AD. Increasing age produces a rise in ROS levels and a reduction in the ability of antioxidant systems to neutralize these molecules. Mitochondria are the main producers of ROS and are particularly vulnerable to oxidative damage (Cheignon et al., 2018). Augmented ROS levels induce the opening of the mitochondrial permeability transition pore, a non-selective channel that dissipates the membrane potential and can lead to cell death (Kalani et al., 2018). The results obtained indicate that both streptocyclinones are able to reduce H_2O_2-induced injury in an *in vitro* model of oxidative stress. *Streptomyces* secondary metabolites also diminish ROS release and are able to protect mitochondrial function by recovering $\Delta\Psi_m$ and restoring GSH levels. These neuroprotective effects seem to be mediated by the ability of the compounds to activate the translocation of the transcription factor Nrf2 to

the nucleus. Targeting this pathway has been proposed as a therapeutic option to ameliorate neurodegeneration because of its essential role in mitochondrial metabolism and in the regulation of antioxidant and detoxifying enzymes (Esteras et al., 2016).

Nrf2 is also implicated in the modulation of neuroinflammation. High levels of ROS activate microglial NFκB, increasing the release of pro-inflammatory cytokines, which induce an increment in ROS levels, creating a vicious cycle (von Bernhardi et al., 2015). This cycle can be deactivated by the translocation to the nucleus of Nrf2. The expression of a battery of antioxidant genes produced by Nrf2 activation lead to a decrease in ROS levels and a subsequent reduction in the release of pro-inflammatory cytokines (Rojo et al., 2014). There are evidences of a functional cross-talk between Nrf2 and NFκB pathways. It has been recently discovered that Nrf2 can repress the transcription of genes encoding pro-inflammatory cytokines in microglial cells (Quinti et al., 2017). On the other side, the p65 subunit of NFκB can exert a negative effect on the expression of antioxidant genes induced by Nrf2 (Wardyn et al., 2015).

Augmented levels of Aβ, ROS, NO and cytokines induce the phosphorylation of MAPK kinases. In microglia, activation of p38 and ERK 1/2 cascades is characteristic of M1 neurotoxic phenotype and contribute to iNOS, TNF-α and IL-1β increase. Thus, the inhibition of these pathways is a promising approach for the treatment of neurodegeneration (Munoz and Ammit, 2010; Sun and Nan, 2017). Both compounds reduce p38 phosphorylation and streptocyclinone B also blocks ERK 1/2 cascade. These inhibition of MAPK kinases, the reduction of pro-inflammatory markers (NO, iNOS, ROS, IL-1β and TNF-α), the increase in the anti-inflammatory cytokine IL-10, and the regulation of Nrf2 and NFκB-p65 expression suggest that these *Streptomyces* secondary metabolites are able to modulate microglial state towards M2 phenotype. These findings are confirmed by the protective effect produced by the compounds on SH-SY5Y cells co-cultured with LPS-stimulated microglial cells.

Release of microglial cytokines as IL-1β, IL-6 or TNF-α can promote tau phosphorylation by increasing the activity of tau kinases as GSK3β, p38 and JNK (Domingues et al., 2017). Abnormal tau phosphorylation plays a critical role in AD pathogenesis. Hyperphosphorylated tau promotes neurotoxicity, increases intracellular calcium and causes synaptic impairment (Kumar et al., 2018b). Our results reveal that both *Streptomyces* compounds reduce tau hyperphosphorylation by reducing the activity of MAPK kinases. Streptocyclinone A inhibits ERK 1/2 and JNK, whereas streptocyclinone B diminishes the phosphorylated state of tau by targeting p38 and JNK. It is noteworthy that streptocyclinone B reduces p38 activation both in neuronal and microglial cells. In addition to its contribution to microglial toxicity and tau phosphorylation, p38 MAPK is involved in the activation of astrocytes, which contribute to brain inflammation, and affects synaptic plasticity by the inhibition of the long-term potentiation(Munoz and Ammit, 2010). High levels of p38 MAPK have been found at early stages in AD brains (Sun et al., 2003) and the blockage of this pathway suppresses the release of pro-inflammatory cytokines and attenuates synaptic dysfunction in an AD mouse model (Munoz et al., 2007). In this context, the modulation of p38 MAPK has been proposed as a therapeutic approach for the treatment of AD due to its central role in this pathology (Lee and Kim, 2017).

Finally, Aβ aggregates trigger an inflammatory response in microglial cells that contributes to neurodegeneration in AD. At first, the activation of microglia produces the clearance of aggregated proteins by phagocytosis, but the sustained exposure to cytokines, chemokines and Aβ results in a chronic inflammatory response that can produce neuronal loss(von Bernhardi et al., 2015). Aβ also increases mitochondrial dysfunction by altering the respiratory chain and produces damage to the organelles through lipid peroxidation (Cheignon et al., 2018). Streptocyclinone B inhibits the activity of BACE1 and is able to reduce the activation of microglia when Aβ is added, producing a protective effect on neuronal cells. So, it would be interesting to carry out further investigations with this compound in order to clarify its effects on microglial and neuronal cells in the presence of Aβ aggregates.

5. Conclusion

In this work we showed the ability of two new compounds isolated from *Streptomyces* sp. to target AD hallmarks. Streptocyclinones A and B presented neuroprotective and antioxidant properties and were able to modulate the inflammatory state of microglia. The multitarget approaches to re-establish the normal regulation of microglia and reduce ROS release have emerged as promising tools to the treatment of neurodegeneration (von Bernhardi et al., 2015). Moreover, these *Streptomyces* compounds reduced more specific AD targets. Both molecules diminished tau hyperphosphorylation by inhibiting MAPK kinases. Streptocyclinone B also decreased BACE1 activity and reduced the activation of microglia with Aβ, increasing the survival of neuronal cells. These findings make the two new glycosylated angucyclinones drug candidates for multitargeted studies against AD.

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Declarations of interest

None

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Figure captions

Table 1. Primary antibodies used in western blot assays.

Figure 1. Chemical structures of streptocyclinones A (**1**) and B (**2**)

Figure 2. Antioxidant effects of streptocyclinones on SH-SY5Y cells. Compounds (0.001-1 µM) and 150 µM H_2O_2 were added to SH-SY5Y cells for 6 h. Their protective effects on cell viability (a) and their capacity to recover the mitochondrial membrane potential (b) were evaluated. Also, the ability of compounds to decrease ROS release (c) and increase GSH levels (d) was measured. Vitamin E (VitE) at 25 µM was used as positive control in all the assays. Data are mean± SEM of three independent experiments performed in triplicate and compared to cells treated with H_2O_2 alone by one way ANOVA and Dunnett's post- hoc test. *p<0.05, **p<0.01 and ***p<0.001. Nrf2 expression in the nuclear (e) and cytosolic fraction (f) of SH-SY5Y cells was assessed by western blot after treatment with compounds (0.001-1 µM) for 6 h. Results are mean ± SEM of three independent replicates performed by duplicate and compared to untreated control cells. *p<0.05, **p<0.01 and ***p<0.001.

Figure 3. Compounds reduce ROS and NO release in BV2 microglial cells. (a) ROS levels were evaluated after treatment with streptocyclinones (0.001-1 µM) for 1 h and 500 ng/mL LPS for 23 h. (b) BV2 cells were co-treated with compounds and 1 µg/mL LPS and NO release was measured with Griess reagent. (c) Expression of iNOS determined with western blot. Values are presented as percentage of cells treated with LPS alone. Data are mean± SEM of four independent replicates and compared to LPS control cells with one way ANOVA and Dunnett's tests. *p<0.05, **p<0.01 and ***p<0.001.

Figure 4. Effect of *Streptomyces* compounds on cytokines release by BV2 microglial cells. Compounds at concentrations ranging from 0.001 to 1 µM were added to microglial cells for 1 h. After this time, cells were activated with 500 ng/mL. IL-1β (a) and IL-6 (b) levels were measured by ELISA. TNF-α (c) and IL-10 (d) release were assessed with a Magnetic Bead Panel and Luminex instrument. Data are mean± SEM of three independent experiments

expressed in percentage of LPS control cells. Treatments are compared to cells stimulated with LPS by one way ANOVA and Dunnett's post-hoc tests. *p<0.05 and **p<0.01.

Figure 5. Streptocyclinones modulate the phenotypic state of BV2 microglial cells. *Streptomyces* secondary metabolites (0.001-1 µM) were added to BV2 cells 1 h before the stimulation with 500 ng/mL LPS. Nrf2 expression levels were assessed in nuclear (a) and cytosolic (b) fractions. The effect of compounds on the domain p65 of NFκB were also determined in the nucleus (c) and the cytosol (d) of microglial cells. The expression levels of p38 (e) and ERK 1/2 (f) kinases were analysed as the ratio between active/total protein levels. Data are mean± SEM of four independent experiments performed by duplicate. Statistical differences were analysed with one way ANOVA and Dunnett's post-hoc test. *p<0.05, **p<0.01 and ***p<0.001.

Figure 6. Trans-well co-culture with BV2 and SH-SY5Y cells. Microglial cells were seeded in culture inserts placed above neuroblastoma cells. BV2 cells were treated with compounds (0.001-1 µM) and 500 ng/mL LPS and the cell viability of SH-SY5Y cells was determined with MTT assay. Data are mean± SEM of three independent experiments performed in triplicate. Results are expressed in percentage of untreated control cells and compared to LPS control cells with one way ANOVA and Dunnnett's tests. *p<0.05 and **p<0.01.

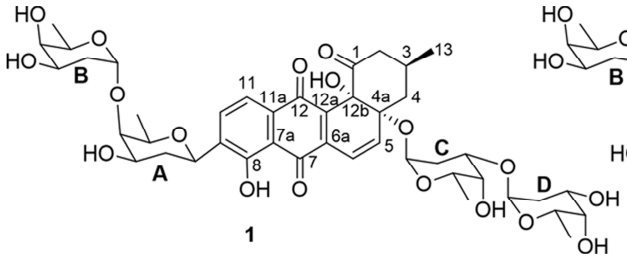
Figure 7. *Streptomyces* compounds reduce tau phosphorylation. SH-SY5Y-TMHT441 cells were differentiated for 7 *div* and treated with compounds (0.001-1 µM) during 48 h. Phosphorylated tau was analysed in cell lysates by western blot with AT8 (a), AT270 (b) and AT100 (c) antibodies. (d) Total tau levels were determined with tau-5 antibody. Values are expressed in percentage of control cells. Data are mean ± SEM of three experiments performed in duplicate and compared to control cells with one way ANOVA test followed by Dunnett's post-hoc test. *p<0.05 and **p<0.01.

Figure 8. Streptocyclinones inhibit MAPK kinases in SH-SY5Y-TMHT441 cells. Compounds (0.001- 1 µM) were added for 48 h to differentiated neuroblastoma cells. The expression levels

of p38 (a), ERK 1/2 (b), JNK (c) and GSK3β (d) were determined by western blot. Results were analysed as ratio between active p38/total levels, active ERK 1/2 /total levels, active JNK/ total levels and inactive GSK3β/ total levels. Values are expressed in percentage of control cells. Data are mean ±SEM of three independent replicates performed in duplicate. One way ANOVA and Dunnett's tests were used to analyse the statistical differences between treatments with compounds and control cells. *p<0.05 and **p<0.01.

Figure 9. Effects of *Streptomyces* compounds on Aβ. (a) The ability of compounds to inhibit BACE1 activity was determined with a FRET assay. A statine-derived inhibitor (Inh) was used to test the validity of the assay. BV2 cells were pre-treated with compound **2** (0.001-1 µM) for 1 h and stimulated with 1 µM Aβ (1-42) during 23 h. After treatments, the levels of ROS were determined (b) and the effect of Aβ-activated microglial cells on SH-SY5Y-TMHT441 cells survival in a trans-well co-culture system was evaluated with MTT test (c). Data are mean ± SEM of three independent replicates expressed in percentage of control cells and compared to microglia treated with Aβ alone by one way ANOVA and Dunnett's tests. *p<0.05, **p<0.01 and ***p<0.001.

Antibody	Dilution	Host	Brand
iNOS	1:1000	Rabbit	Abcam
Nrf2	1:1000	Rabbit	Millipore
NfκB-p65	1:10000	Rabbit	Abcam
Phospho p38	1:1000	Rabbit	Abcam
p38	1:1000	Rabbit	Abcam
Phospho ERK 1/2	1:1000	Rabbit	BD Biosciences
ERK 1/2	1:1000	Mouse	BD Biosciences
AT8	1:500	Mouse	Thermo Fisher
AT100	1:1000	Mouse	Thermo Fisher
AT270	1:1000	Mouse	Thermo Fisher
Tau-5	1:1000	Mouse	Millipore
Phopho JNK	1:500	Mouse	BD Biosciences
JNK	1:1000	Mouse	BD Biosciences
Phospho GSK3β	1:1000	Mouse	Millipore
GSK3β	1:1000	Mouse	Millipore
Lamin B1	1:1000	Rabbit	Abcam
β-actin	1:10000	Mouse	Millipore



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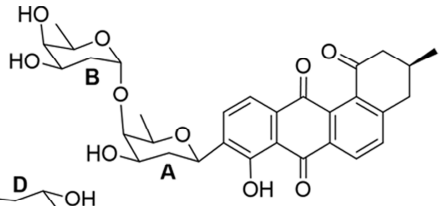

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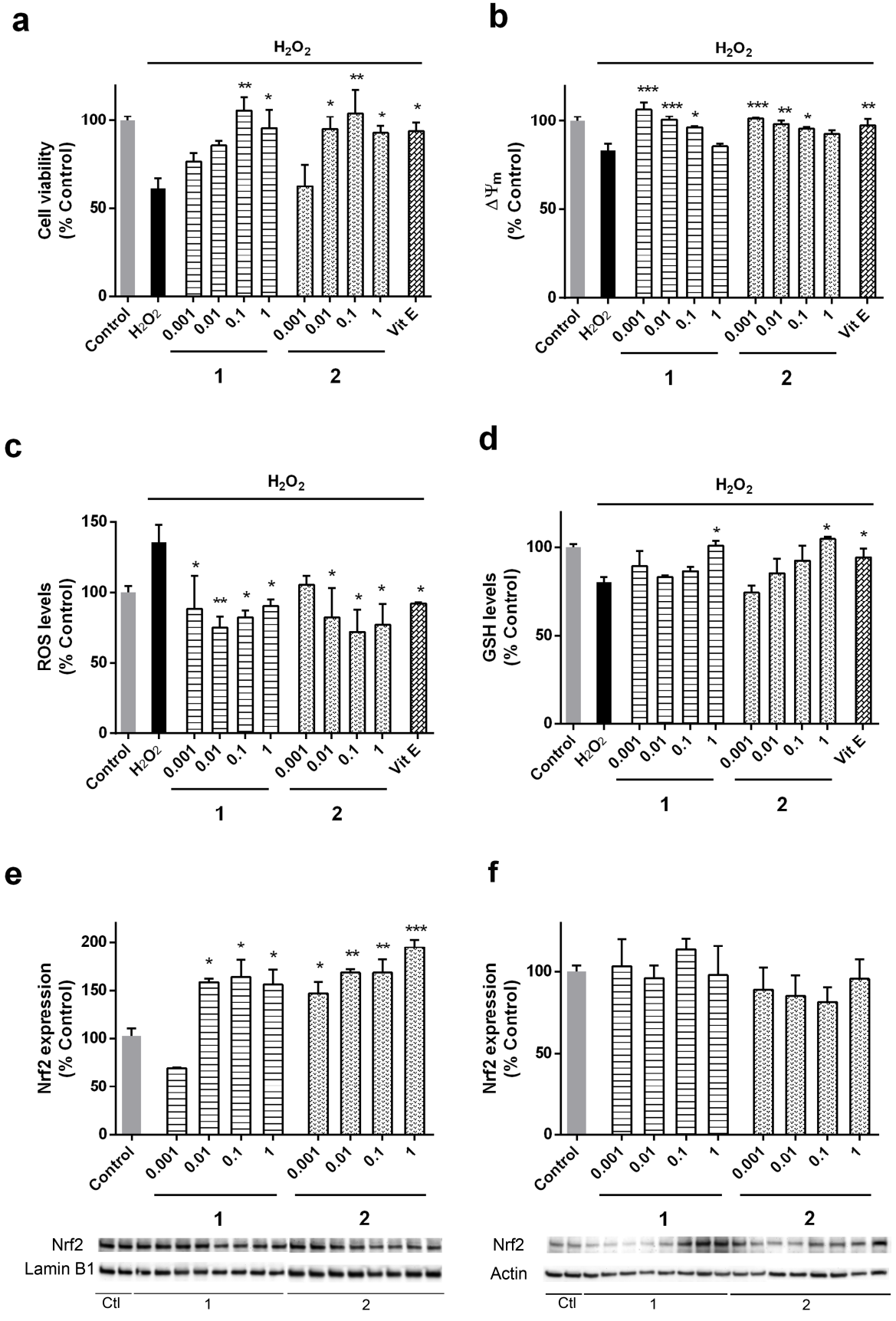
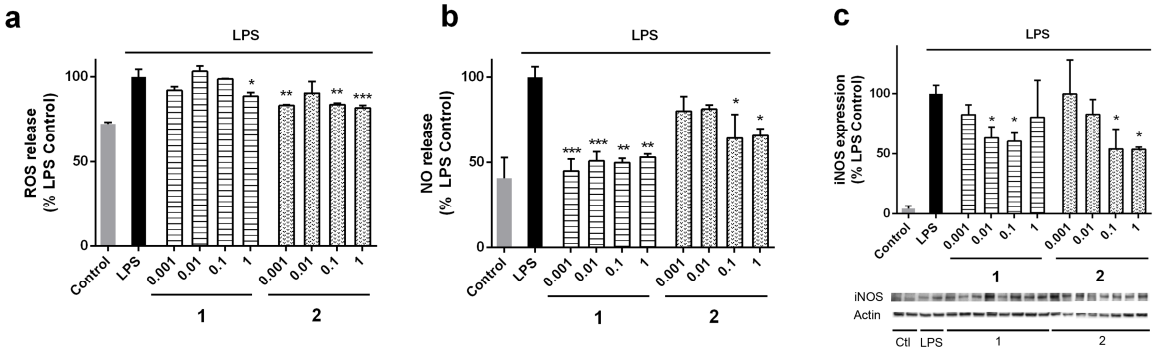
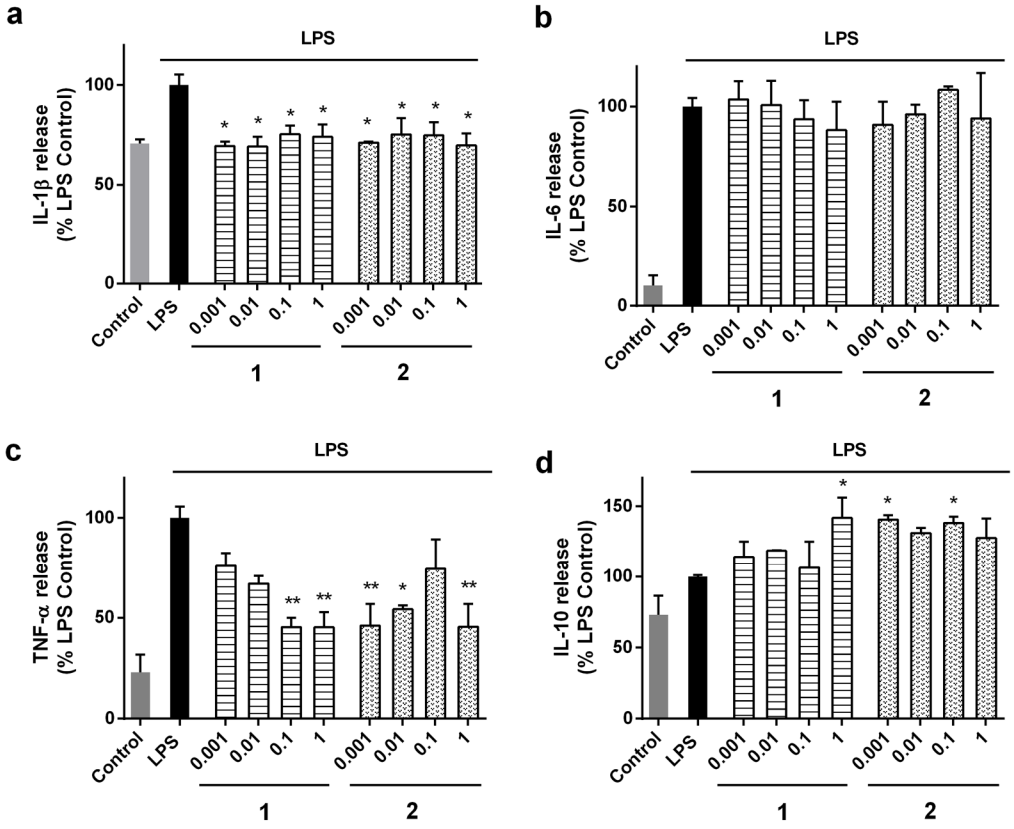
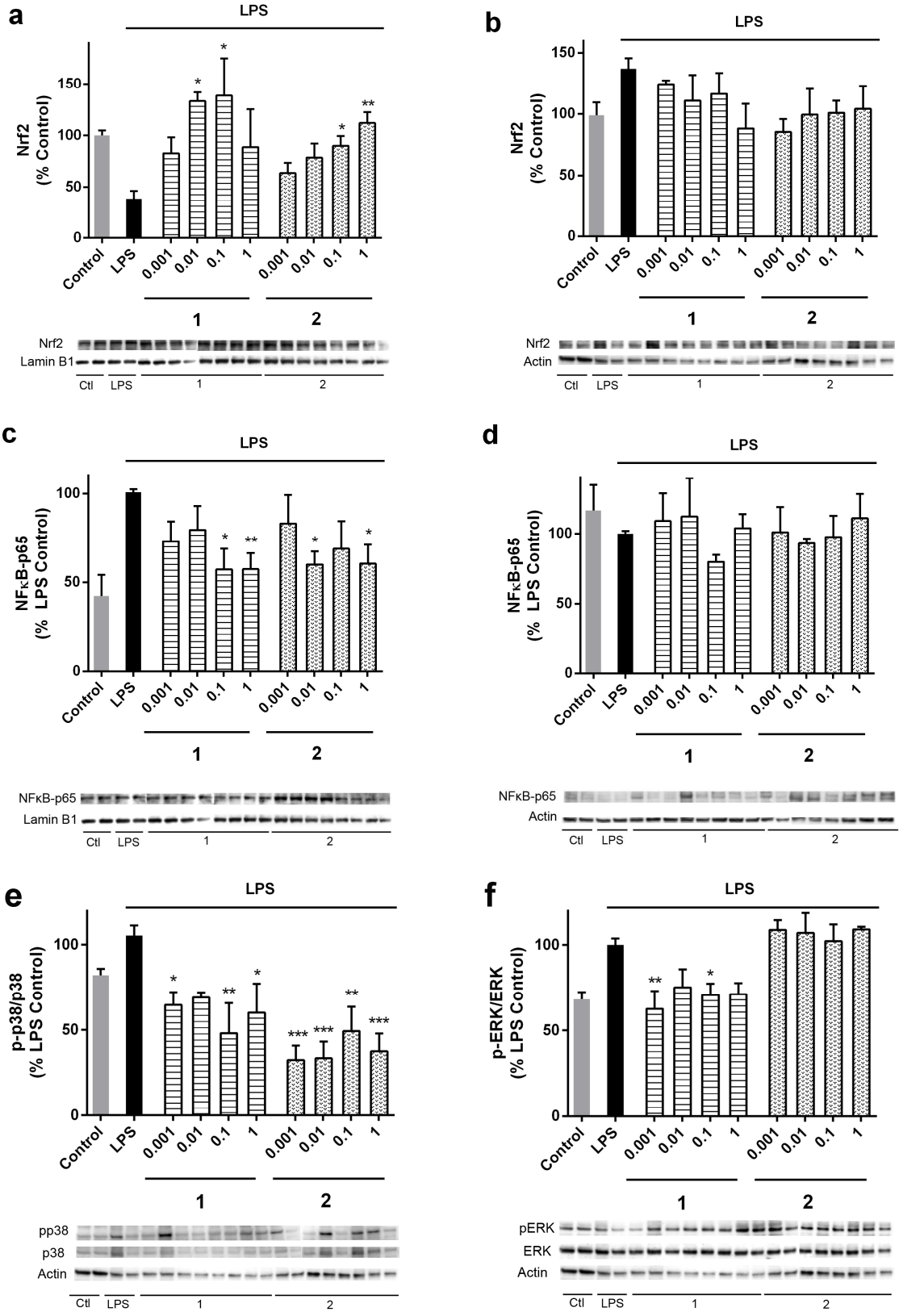
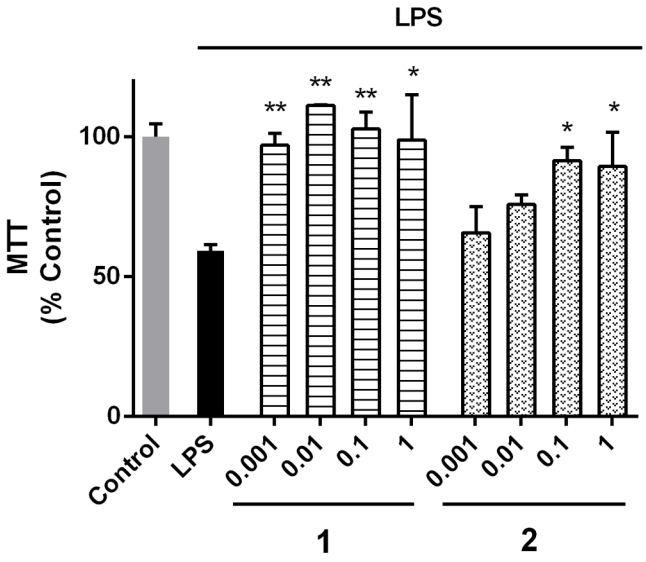
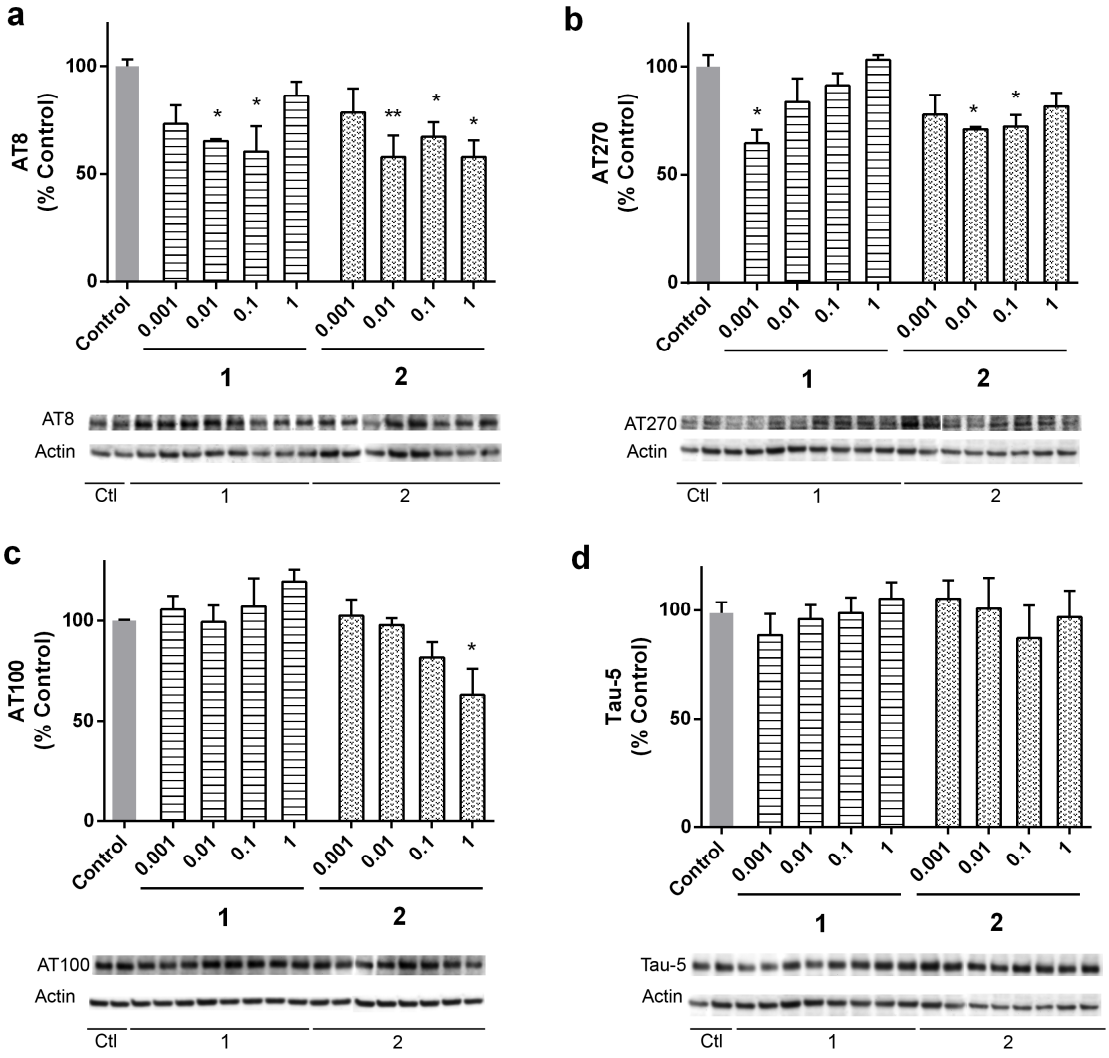

a
b
c
d
e
f
H_2O_2
Cell viability (% Control)
$\Delta\Psi_m$ (% Control)
ROS levels (% Control)
GSH levels (% Control)
Nrf2 expression (% Control)
Control
H_2O_2
0.001
0.01
0.1
1
Vit E
1
2
Nrf2
Lamin B1
Actin
Ctl

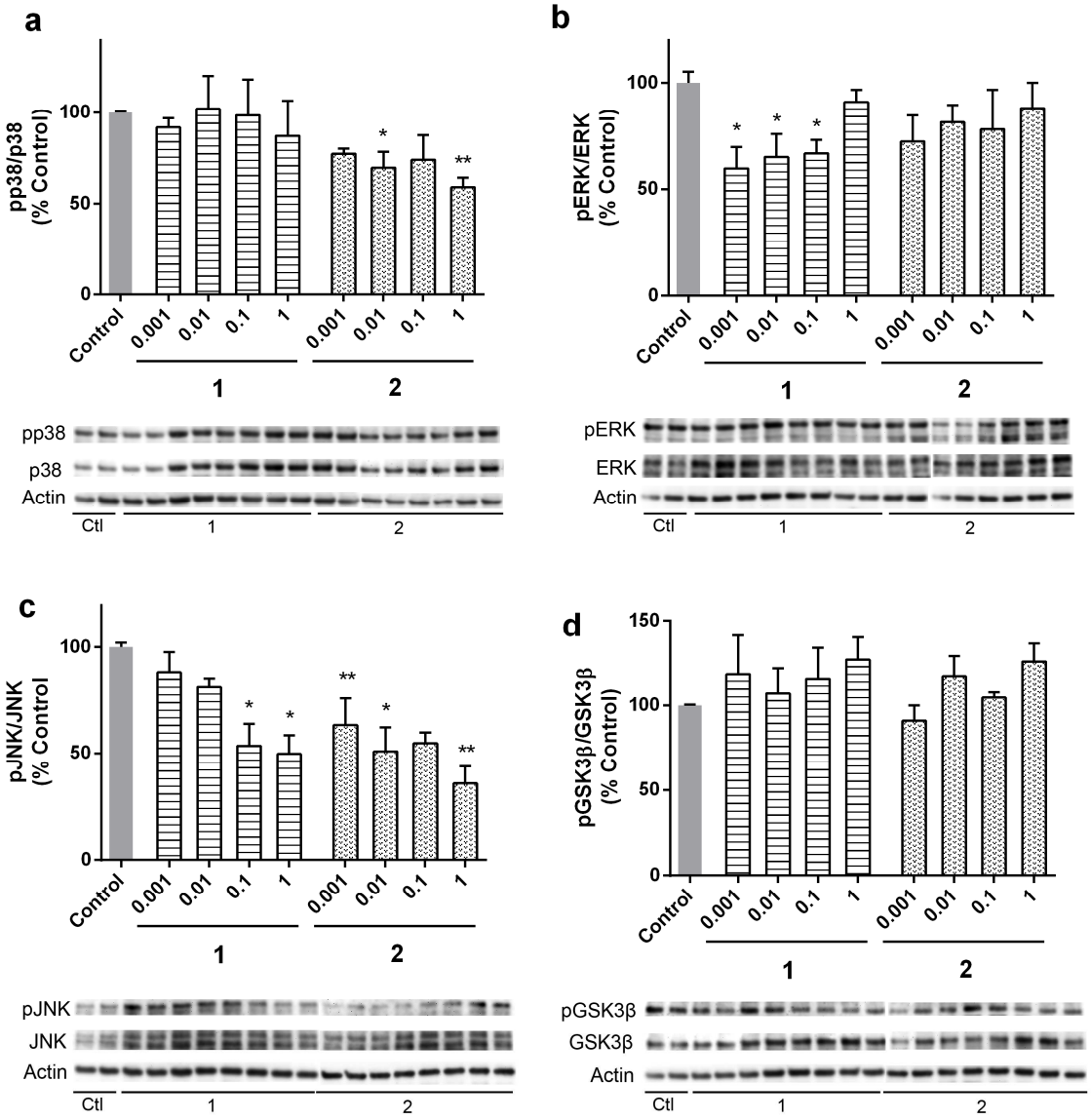





a
LPS
Nrf2 (% Control)
150
100
50
0
Control
LPS
0.001
0.01
0.1
1
0.001
0.01
0.1
1
1
2
Nrf2
Lamin B1
Ctl
LPS
1
2
b
LPS
Nrf2 (% Control)
150
100
50
0
Control
LPS
0.001
0.01
0.1
1
0.001
0.01
0.1
1
1
2
Nrf2
Actin
Ctl
LPS
1
2
c
LPS
NFκB-p65 (% LPS Control)
100
50
0
Control
LPS
0.001
0.01
0.1
1
0.001
0.01
0.1
1
1
2
NFκB-p65
Lamin B1
Ctl
LPS
1
2
d
LPS
NFκB-p65 (% LPS Control)
100
50
0
Control
LPS
0.001
0.01
0.1
1
0.001
0.01
0.1
1
1
2
NFκB-p65
Actin
Ctl
LPS
1
2
e
LPS
p-p38/p38 (% LPS Control)
100
50
0
Control
LPS
0.001
0.01
0.1
1
0.001
0.01
0.1
1
1
2
pp38
p38
Actin
Ctl
LPS
1
2
f
LPS
p-ERK/ERK (% LPS Control)
100
50
0
Control
LPS
0.001
0.01
0.1
1
0.001
0.01
0.1
1
1
2
pERK
ERK
Actin
Ctl
LPS
1
2



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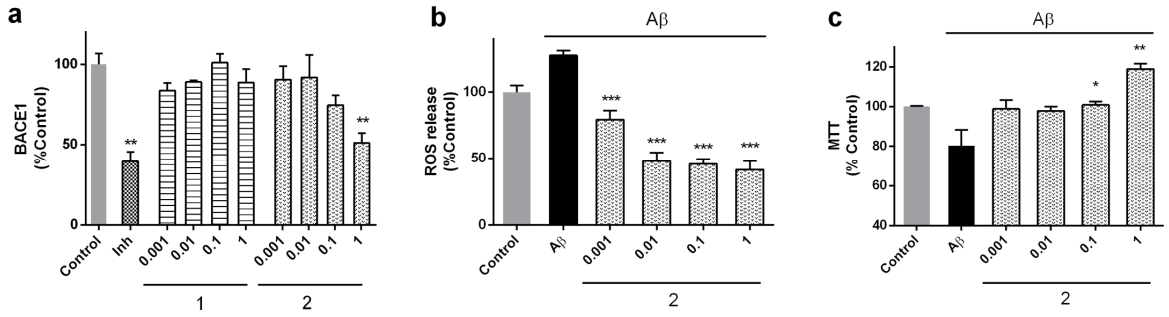
pp38/p38 (% Control)
100
50
0
Control
0.001
0.01
0.1
1
0.001
0.01
0.1
1
1
2
*
**
pp38
p38
Actin
Ctl
1
2
b
pERK/ERK (% Control)
100
50
0
*
*
*
Control
0.001
0.01
0.1
1
0.001
0.01
0.1
1
1
2
pERK
ERK
Actin
Ctl
1
2
c
pJNK/JNK (% Control
100
50
0
*
*
**
*
**
Control
0.001
0.01
0.1
1
0.001
0.01
0.1
1
1
2
pJNK
JNK
Actin
Ctl
1
2
d
pGSK3β/GSK3β (% Control)
150
100
50
0
Control
0.001
0.01
0.1
1
0.001
0.01
0.1
1
1
2
pGSK3β
GSK3β
Actin
Ctl
1
2



Highlights

-Streptocyclinones protect SH-SY5Y cells from oxidative stress by activating Nrf2

-Compounds modulate neuroinflammation by NFκB and MAPKs inhibition and Nrf2 activation

-Tau hyperphosphorylation is reduced by compounds through inhibition of MAPK kinases

-Streptocyclinone B inhibits BACE1 and decreases ROS release in Aβ-activated BV2 cells

-Neuronal cells are protected by compounds in trans-well co-cultures with microglia