

Supporting Information

Gracilin A derivatives target early events in Alzheimer's disease: *in vitro* effects on neuroinflammation and oxidative stress

Rebeca Alvariño, Eva Alonso, Mikail E. Abbasov, Christian M. Chaheine, Michael L. Conner, Daniel Romo, Amparo Alfonso, Luis M. Botana

Figures

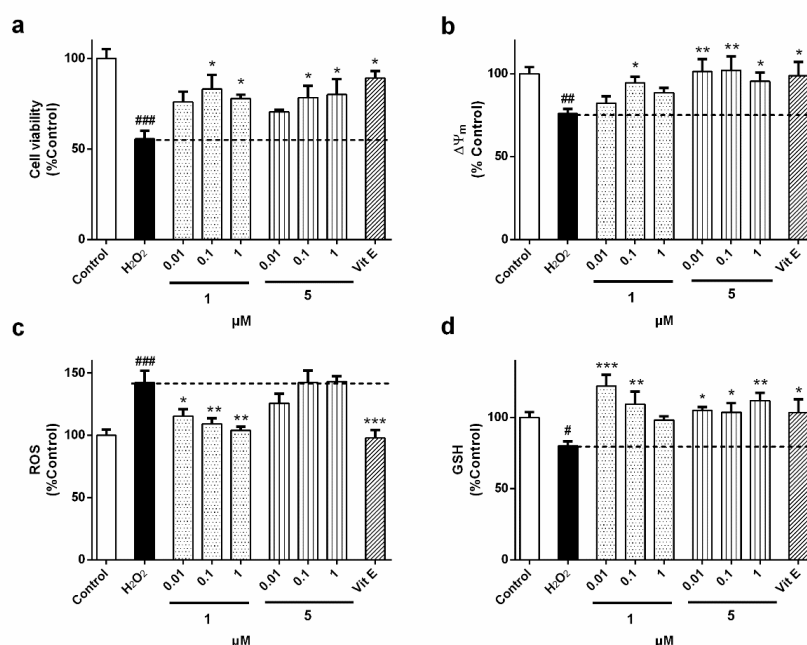


Figure S1. Neuroprotective effects of derivatives **1** and **5** in an oxidative stress model. SH-SY5Y cells were treated with the compounds and 150 μM H₂O₂ for 6 h. The dotted line indicates the levels of cells treated with H₂O₂ alone. Vitamin E (Vit E) was used as positive control (a) Effect of compounds on cell viability. (b) Recovery of mitochondrial membrane potential (ΔΨ_m) by derivatives **1** and **5**. (c) ROS levels after treatment with H₂O₂ and compounds. (d) Evaluation of GSH content in neuroblastoma cells. Data are mean± SEM of four independent replicates and expressed as percentage of control cells. Treatments with compounds are compared to H₂O₂ control cells by one-way ANOVA test and post hoc Dunnett's test. **p*<0.05, ***p*<0.01, ****p*<0.001. Cells treated with H₂O₂ alone compared to untreated control cells. #*p*<0.05, ##*p*<0.01, ###*p*<0.001

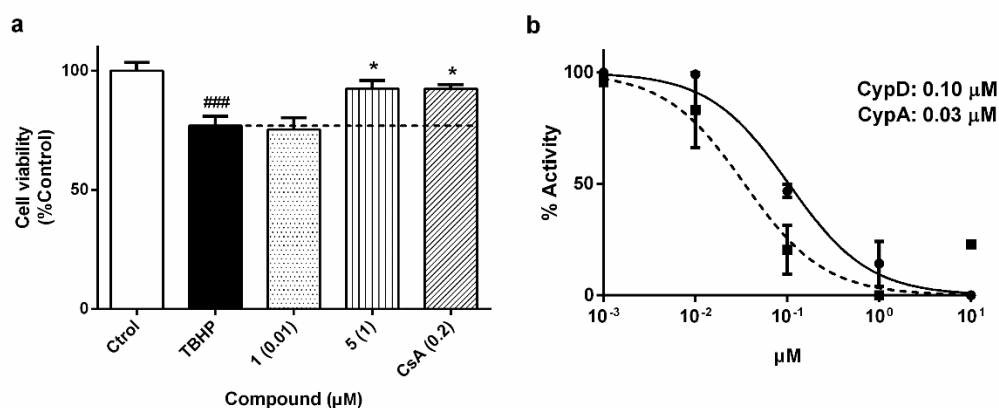


Figure S2. Effect of derivatives **1** and **5** on the mitochondrial permeability transition pore (mPTP). (a) mPTP opening in SH-SY5Y cells was determined by flow cytometry. Cells were treated with compounds and 1mM *tert*-Butyl hydroperoxide (TBHP). Values are expressed as percentage of control cells. Mean \pm SEM of four independent experiments. The dotted line indicates the level of cells treated with TBHP alone. Statistical significance was determined with one-way ANOVA and Dunnett's test. * $p < 0.05$, compared to TBHP control cells. ### $p < 0.001$ compared to control cells. Cyclosporine A (CsA) was used as positive control (b) Inhibition of cyclophilins (Cyp's) enzymatic activity by derivative **5**. Solid line represents CypD inhibition and dotted line shows CypA activity. Half-maximal inhibitory concentrations (IC_{50}) are presented in the graph. Data are presented as percentage of the maximal activity. Values are mean \pm SEM of four independent replicates

Materials and Methods

All the experiments were carried out as previously described¹.

Oxidative stress assays

SH-SY5Y human neuroblastoma cells were seeded in 96-well plates at 5×10^4 cells per wells and allow to grow for 24 h. Then, cells were treated with compounds **1** and **5** at non-toxic concentrations (0.01-1 μ M) and 150 μ M H_2O_2 for 6 h. After this time, cell survival, mitochondrial membrane potential ($\Delta\Psi_m$), reactive oxygen species (ROS) and glutathione (GSH) levels were evaluated. All the assays were performed four independent times and the antioxidant vitamin E at 25 μ M was used as positive control to validate the *in vitro* model.

The neuroprotective effects on the viability of neuroblastoma cells were determined with MTT test following the method described for the trans-well assay in the manuscript. $\Delta\Psi_m$ was assayed with the fluorescence dye tetramethylrhodamine methyl ester (TMRM) (Thermo Fisher Scientific). SH-SY5Y cells were washed twice with saline buffer and 1 μ M TMRM was added for 30 min, 300 rpm and 37°C. Then, cells were solubilized with DMSO and H_2O_2 at 50% and the fluorescence was read at 535nm excitation and 590 nm emission.

ROS levels were determined with the protocol described in the manuscript for BV2 microglial cells. Finally, GSH was evaluated using Thiol Tracker™ Violet dye (Thermo Fisher Scientific), following manufacturer's instructions. Briefly, neuroblastoma cells were rinsed twice with PBS and 10 μ M Thiol Tracker™ Violet was added to each well for 30 min, 300 rpm at 37°C. Then, the fluorescence was measured in a plate reader (404 nm excitation and emission at 526 nm).

Mitochondrial permeability transition pore measurement

The ability of compounds to block the opening of the mitochondrial permeability transition pore (mPTP) was evaluated with the MitoProbe™ Transition Pore Assay Kit (Thermo Fisher Scientific). Neuroblastoma cells were resuspended in PBS/ Ca^{+2} at 1×10^6 cells/mL and loaded with the fluorescent dye Calcein-AM for 15 min at 37°C. After this time, 0.4 mM CoCl_2 and derivatives

1 and **5** at 0.01 and 1 μ M were added. SH-SY5Y cells were incubated for 15 min at 37°C, centrifuged and resuspended in PBS. Cyclosporine A (CsA) at 0.2 μ M was used as positive control. The opening of the pore was induced with *tert*-Butyl hydroperoxide (TBHP) at 1 mM just before the analysis. Fluorescence (488 nm excitation and 517 nm emission) was evaluated by flow cytometry with the ImageStream MKII (Amnis Corporation, Merck-Millipore) instrument. IDEAS Application vs 6.0 software (Amnis Corporation, Merck-Millipore) was used to analyse the fluorescence of 10000 events.

Cyclophilins D and A enzymatic inhibition assay

The inhibition of the the peptidyl-prolyl *cis-trans* isomerase (PPIase) activity of cyclophilin D (CypD) and cyclophilin A (CypA) by **5** was determined by monitoring the rate of hydrolysis of *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide by chymotrypsin. The assay buffer (20 mM Tris-HCl, 50 mM NaCl, pH 7.8) with CypD or CypA (1 nM), and the derivative at different concentrations (0.0001-10 μ M) were pre-cooled for 1 h at 4°C. Then, 0.4 mg/mL chymotrypsin dissolved in 1 mM HCl was incorporated to each well. The addition of the substrate at 0.1 mg/mL in 500 mM LiCl in tetrahydrofuran started the reaction. A spectrophotometer plate reader was used for the measurement of the absorbance at 380 nm. The blank rates of hydrolysis (in absence of Cyp's) were subtracted from the rates in the presence of the enzyme. The half-maximal inhibitory concentration (IC₅₀) was calculated with GraphPad Prism 6.0 software using a log (inhibitor) vs. response model.

References

1. Abbasov, M. E.; Alvarino, R.; Chaheine, C. M.; Alonso, E.; Sanchez, J. A.; Conner, M. L.; Alfonso, A.; Jaspars, M.; Botana, L. M.; Romo, D., Simplified immunosuppressive and neuroprotective agents based on gracilin A. *Nat Chem* **2019**, *11* (4), 342-350.