**STUDY No. HCS1708019**

# ASSESSMENT OF NEUROPROCTECTION EFFECTS OF THE DIETARY SUPPLEMENT “ADN TÉLOMÉRACTIVES” ON ALZHEIMER AND PARKINSON MODELS

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# 1 - AIM OF THE STUDY

The aim of this study was to assess the *in vitro* neuroprotection effects the dietary supplement "ADN Téloméractives" toward Parkinson's cell models used to study neurodegenerative diseases. Neurodegenerative diseases models can be set up in laboratory using differentiated neuron cell lines. With chemicals inducers, Parkinson diseases models are obtained from the human neuroblastoma SH-SY5Y cell line. The neuroprotective properties of the two candidates are assessed following a neurotoxicity assay, by counting neurons and measuring neurites length.

# 2 - MATERIALS

## *2.1 Cell culture*

**Table 1:** Cell culture materials

| Material | Supplier | Reference | Batch |
|---|---|---|---|
| **SH-SY5Y** | **ATCC** | **CRL-2266** | **13C014** |
| **Trypsin / EDTA** | Promocell | C41000 | 417M214 |
| **HAM F12** | **EUROBIO** | **CM1H120001** | **690434** |
| **Fetal Calf Serum** | EUROBIO | S1810-500 | - |
| **Greiner advanced 96 wells plates** | **Dutscher** | **655986** | **E160433M** |

## *2.2 Chemicals*

**Table 2:** Chemicals

| Compounds | Supplier | Reference |
|---|---|---|
| **Rotenone** | TCI | R0090-25G |
| **ADN Téloméractives comprimés** | HBN | 002 07/2018 |

## *2.3 Immunostaining*

**Table 3:** Immunostaining materials

| Materials | Supplier | Reference |
|---|---|---|
| **PBS 1X** | Eurobio | CS1PBS01 |
| **Acetone** | Sigma | 24201-25-R |
| **Methanol** | Sigma | 32213-25L |
| **Hoecsht 33258** | Sigma | B2883 |

| | | |
|---|---|---|
| **Rabbit polyclonal antibody to Tubulin 3** | Abcam | Ab18207 |
| **Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488** | Molecular Probes | A11034 |

# 3 - METHODS

### *3.1 Cell culture and differentiation*

SH-SY5Y were grown and subcultured according to supplier's recommendations.

All assays have been done in technical triplicate using 96 wells microplates. SH-SY5Y cells were differentiated by exposure to retinoic acid (10µM) for 4 days.

### *3.2 Chemicals*

The extract was prepared following our customer recommendation. The working solutions were freshly prepared with a 12 final concentrations range from 0.00125 – 0.0025 – 0.0050 – 0.0125 – 0.025 – 0.05 -0.125 – 0.25 – 0.5 – 1.25 mg/mL.

### *3.3 Cell fixation and staining*

The cells were then fixed using a mix of Ethanol and acetone at room temperature for 5 minutes, prior to immunostaining of nuclei with Hoechst and B3 tubulin with a primary antibody anti-B3 tubulin and a secondary antibody conjugated to an Alexa 488 fluorochrome.

### *3.4 Neuroprotection assay*

Cytotoxicity was assessed using cell nucleus staining and cell counting.

The neuroprotective properties of the two candidates were assessed following a neurotoxicity assay based on neurons counting and neurites morphological assessment. Then, cells were exposed to aqueous extracts of the dietary supplement "ADN Téloméractives", rotenone (Parkinson's disease inducer) for 24 hours. After fixation and labelling, neurites outgrowth and cell count were assessed using MetaXpress (Molecular Devices).

### *3.5 Cell Imaging and images analysis*

The images were acquired on an automated ImageXpress® Micro XLS microscope (Molecular Devices), with a 20X objective. 2 dichroic filters were used simultaneously to select specifically the detection of the different probes used. 4 fields per well have been acquired and analyzed individually.

The images were analyzed using MetaXpress (Molecular Devices) software.

### *3.6 Statistical Analysis*

Statistical comparison of results versus control conditions were performed using a ANOVA followed by Dunett test post hoc. Significance was expressed with the symbol " * " when $p<0.05$.

## 4 - RESULTS & DISCUSSION

### *4.1 Cytotoxicity*

Cytotoxicity was assessed on differentiated SH-SY5Y after 24 hours of exposure. Product was tested co incubate with fixed concentration of Rotenone. As Rotenone is also cytotoxic, cytoxic effect of the product in combination with rotenone is non-interpretable, although IC50 was evaluated at 0.47 mg/mL.

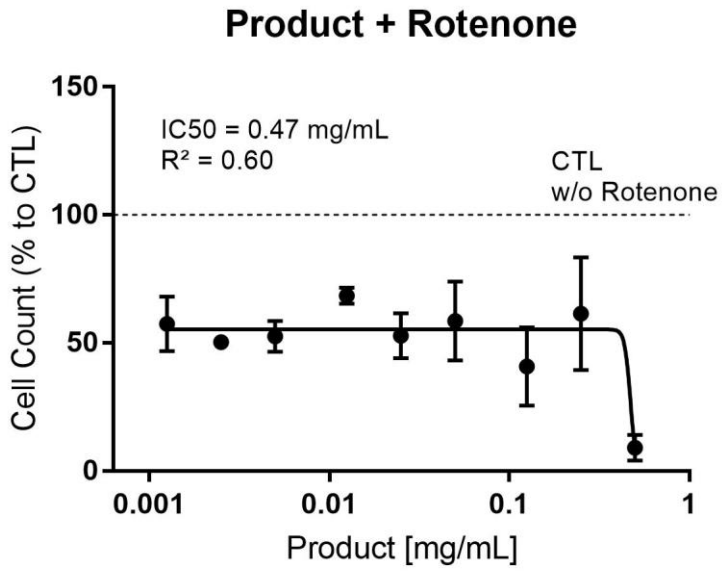


**Figure 1:** Cytotoxicity of the aqueous extract or "ADN Téloméractives", after 24 hours of exposure. Product was tested alone or co incubated with fixed concentrations of Rotenone. 100% = cell count of the cells receiving medium alone. Results are expressed as a mean +/- SD of 3 replicates.

The highest concentration of aqueous extract tested (1.25 mg/mL) was not presented for IC50 calculation due to high toxicity.

### *4.2 Neuroprotective effect of product on neurites lengths on Parkinson model*

The aqueous extract at 0.00125 mg/mL significantly protected the neurites length against induction of Parkinson phenotypes after 24 hours of exposure to 0.00125 mg/mL.

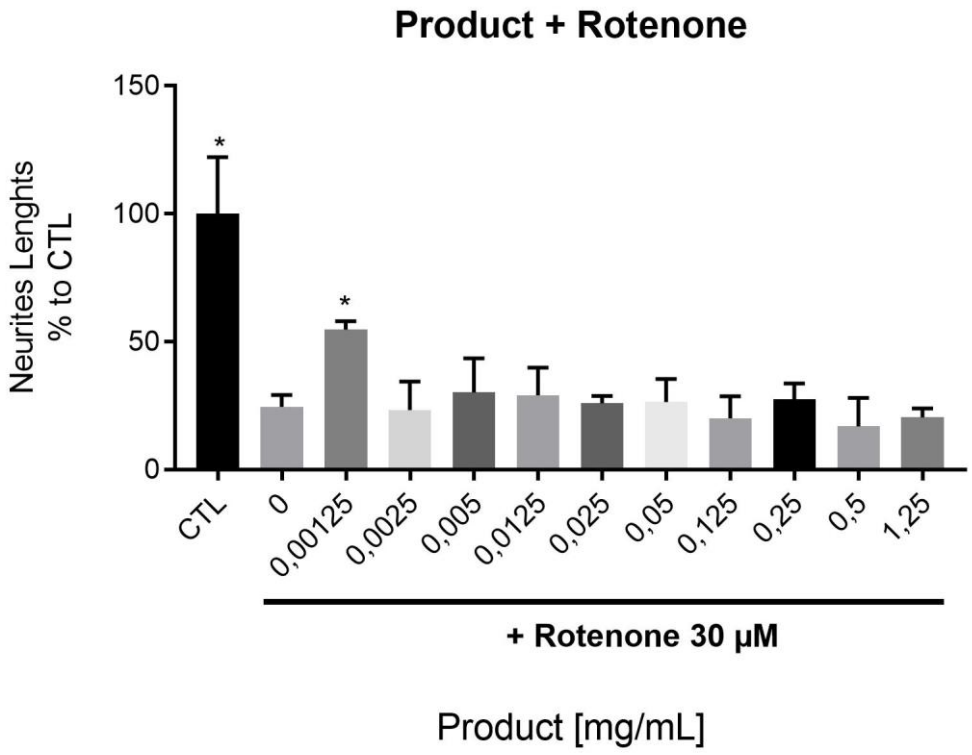


**Figure 6:** Neuroprotective effect of product on neurites lengths after 24 hours in Parkinson model. Results are expressed as a mean +/- SD of 3 replicates.

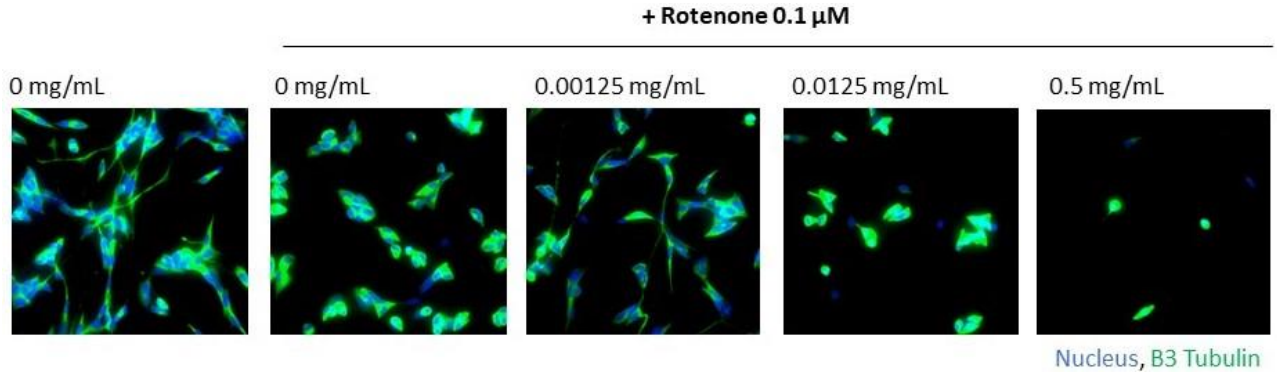


**Figure 7:** Illustrations of product dose effect on differentiated SH-SY5Y exposed to rotenone. 20X magnification, nuclei in blue, B3 tubulin in green.

Differentiated SH-SY5Y cells, when exposed to rotenone, generate a Parkinson phenotype through the inhibition of the mitochondrial complex 1 protein. This mitochondrial toxicity and the subsequent oxidative stress induce a neurites loss, especially in dopaminergic neurons, which characterize Parkinson pathology. The aqueous extract exposed at 0.00125 mg/mL significantly protect the differentiated SH-SY5Y cells versus deleterious effect of rotenone.

## 5 - DATA

Raw data are stored in excel files and can be provided on demand, or as an attached annex to the final report. Images in high quality can also be provided on demand through hard drive transfer.

# SIGNATURE PAGE

| | Signature: | Date: |
|---|---|---|
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