

Neuroprotective Properties of Eudesmin on a Cellular Model of Amyloid- β Peptide Toxicity

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Abstract.

Background: Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive cognitive impairment and memory loss. One of the hallmarks in AD is amyloid- β peptide (A β) accumulation, where the soluble oligomers of A β (A β O) are the most toxic species, deteriorating the synaptic function, membrane integrity, and neuronal structures, which ultimately lead to apoptosis. Currently, there are no drugs to arrest AD progression, and current scientific efforts are focused on searching for novel leads to control this disease. Lignans are compounds extracted from conifers and have several medicinal properties. Eudesmin (Eu) is an extractable lignan from the wood of *Araucaria araucana*, a native tree from Chile. This metabolite has shown a range of biological properties, including the ability to control inflammation and antibacterial effects.

Objective: In this study, the neuroprotective abilities of Eu on synaptic failure induced by A β O were analyzed.

Methods: Using neuronal models, PC12 cells, and *in silico* simulations we evaluated the neuroprotective effect of Eu (30 nM) against the toxicity induced by A β O.

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Results: In primary cultures from mouse hippocampus, Eu preserved the synaptic structure against A β O toxicity, maintaining stable levels of the presynaptic protein SV2 at the same concentration. Eu also averted synapsis failure from the A β O toxicity by sustaining the frequencies of cytosolic Ca²⁺ transients. Finally, we found that Eu (30 nM) interacts with the A β aggregation process inducing a decrease in A β O toxicity, suggesting an alternative mechanism to explain the neuroprotective activity of Eu.

Conclusion: We believe that Eu represents a novel lead that reduces the A β toxicity, opening new research venues for lignans as neuroprotective agents.

Keywords: A β interaction, Alzheimer's disease, amyloid- β peptide, eudesmin, neuroprotection

INTRODUCTION

Alzheimer's disease (AD) is the most prevalent cause of dementia and neurodegenerative disorders, which are clinically characterized by memory loss and cognitive impairment [1–3]. The histopathological hallmarks of AD are related to protein accumulation of intracellular hyperphosphorylated tau protein and extracellular amyloid- β peptide (A β) deposits [4, 5]. The aggregation of A β peptide into senile plaques has been considered a major route for the development of AD. The A β peptide is produced by the cleavage of amyloid- β protein precursor (A β PP) by β -secretase and γ -secretase [6]. These cleavage products are imprecise, resulting in different A β species that can self-aggregate leading to several structures such as protofibrils, fibrils, or plaques. The aggregation process consists of an initial nucleation phase where the monomers undergo a structural change from alpha-helix to beta-sheet, which induces aggregation. This second stage is the elongation phase, where trimers, oligomers, protofibrils, fibrils, and finally, plaques of fibrillar aggregates or senile plaques are formed [7–9]. The γ -secretase product at position 40 (A β ₁₋₄₀) and 42 (A β ₁₋₄₂) has hydrophobic and fibrillogenic properties and are the most abundant species [9]. The senile plaques are mainly composed of A β ₁₋₄₀ [10, 11], which are very toxic species and able to form pores in the cellular membrane, allowing a disruption in ionic homeostasis [12, 13]. The membrane perforation induced by A β is associated with an increase in intracellular calcium levels [14], and as demonstrated more recently, with increased passage of ions and molecules as large as glucose and ATP through cell membranes [8, 11, 15, 16]. The increase in extracellular ATP can lead to the activation of P2X purinergic receptors in neurons and glia [17]. These receptors are permeable to Ca²⁺ and can induce the activation of microglia and mitochondrial dysfunction. These

events are known to induce synaptic failure and neuronal death, facilitating neurodegeneration [15, 18–20]. During this process, several neurodegenerative events occur, including neuronal dysfunction, microglial response, astrogliosis, and apoptosis [18, 21, 22].

Several hypotheses have been proposed to understand the origin of AD, including malfunction of the cholinergic system [23], extracellular depositions of A β peptide [24], hyperphosphorylation of tau protein in the intracellular space [25], and an increase in oxidative stress [26]. However, most treatments to control AD are focused on cholinergic therapies (i.e., acetylcholinesterase inhibitors) [27]. Galantamine is an alkaloid obtained from *Galanthus nivalis* showing activity as an acetylcholinesterase inhibitor and nicotinic positive allosteric modulator. This alkaloid has been used to treat cognitive impairment since the cholinergic theory for AD was established [28, 29]. Moreover, other plant metabolites have also been reported as attractive neuroactive compounds with anti-inflammatory, analgesic, or antioxidant properties [8, 30].

Natural products, including polyphenols and alkaloids, have several effects that can potentially benefit central nervous system (CNS) function. For instance, some natural products exhibit antioxidant properties and anti-inflammatory activity, contributing to their neuroprotective effects [17, 30, 31]. Benzofurans are biomedically relevant scaffolds and have been reported as inhibitors of the A β peptide fibrillation process, suggesting these chemical cores could help control neurodegenerative disorders such as AD [31–35]. Recently, our team has demonstrated the biological and neuroprotective activity of fomanoxin, the most prevalent natural benzofuran isolated from the Russulale fungus, *Aleurodiscus vitellinus*, an edible mushroom native to Andean-Patagonian forests. This benzofuran can preserve synaptic structure and function, illustrated by experiments where

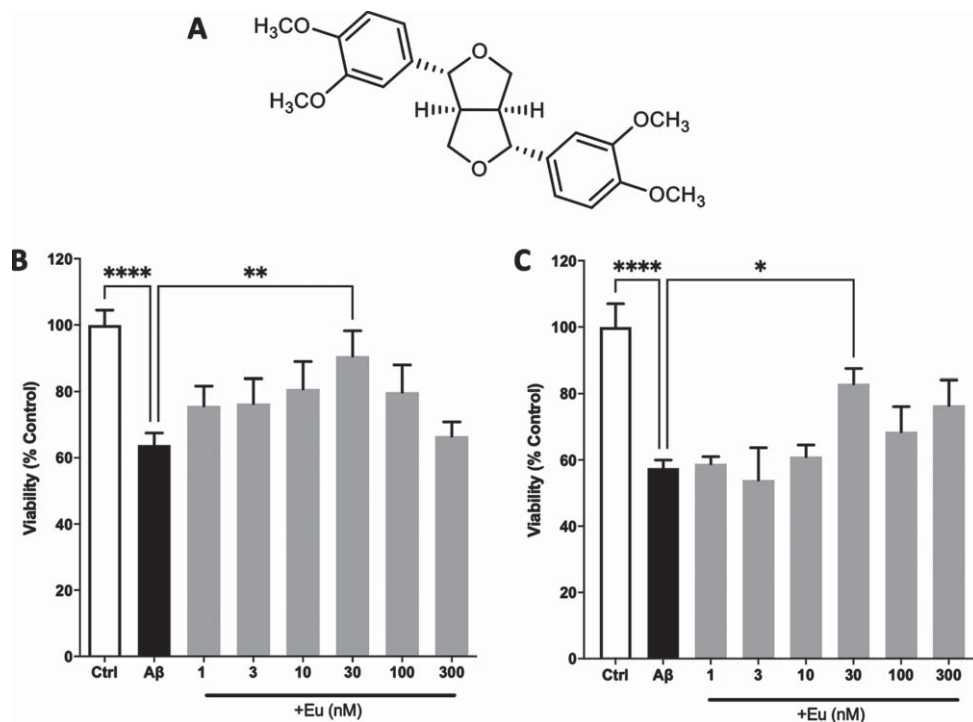


Fig. 1. Neuroprotective effect of Eudesmin against A β O s . Chemical structure of Eudesmin (A). PC12 cells (B) and cortical neurons (C) were co-incubated for 24 h with A β O s (0.5 μ M) and increasing concentrations of Eudesmin (Eu 1–300 nM). Cellular viability was measured by MTT assay. Values are expressed as the percentage of the control group (without A β O s treatment). * p < 0.05, ** p < 0.01, **** p < 0.0001. n = 3.

10⁻⁶M of fomannoxin can induce a reversion or protection against A β soluble oligomers and disrupts their aggregation [36]. Lignans isolated from conifers species have been recently studied and shown to exhibit similar anti-inflammatory, antihypertensive, and antioxidant properties [37–40]. Additionally, these privileged natural scaffolds have been studied as potential phytohormones for, among others, the treatment or prevention of cancer and neuroprotective properties [38]. Different type of lignans have been isolated from almost all vascular plants, but their specific role is not entirely understood [41]. It has been suggested that they are part of the plants' defense system against pathogens [2, 3].

Eudesmin (Eu) (Fig. 1A) is a tetrahydrofurofuranoid lignan that has been isolated from numerous medicinal plants and shown several biological activities including cytotoxic, antibacterial, antifungal, anti-inflammatory, immunostimulant, and inhibitory effects on tumor necrosis factor-alpha (TNF- α) production as well as improving the neurite outgrowth [11, 39, 40, 42, 43]. Moreover, Eu has been reported mainly from the genus *Artemisia*, exhibiting nitric oxide (NO) inhibitory effects and cytotoxic activities

[39, 40]. In the present study, we use Eu isolated from the wood of *Araucaria araucana*, a Chilean native species to evaluate its neuroprotective abilities using an *in vitro* model of AD with A β soluble oligomers treatments [41]. We investigated the cells viability response, presynaptic preservation, and established a direct relationship with calcium transient recordings and presynaptic markers. This experimental evidence suggests an alternative mechanism to explain the neuroprotective activity of Eu, evaluating the effect of this lignan on the interaction with A β during the aggregation process considering *in vitro* assays and *in silico* models. Computational studies then indicated the specific residues involved in the interaction between Eu and A β .

MATERIALS AND METHODS

PC12 cells culture

PC12 cells were cultured in DMEM 1X (Corning, USA) with 4.5 g/L glucose, L-glutamine, and sodium pyruvate supplemented with 5% fetal bovine serum (Gibco, USA), 5% horse serum (HyClone, USA), and

1% penicillin/streptomycin (Gibco, USA). The cells (passage 20) were incubated under standard conditions (37°C, 5% CO₂), plated at a concentration of 50,000 cells/well for experiments and used 24 h after.

Primary cultures of mouse hippocampal neurons

Hippocampal neurons were obtained from 18-day pregnant mouse embryos C57BL/6J following ethical regulations established by NIH and University of Concepción. The cells were plated at density of 320,000 cells/ml. The cells were cultured in DMEM supplemented with 10% horse serum (HyClone, USA), 4 µg/ml DNAase (HyClone, USA), and L-glutamine (HyClone, USA). Plating media was replaced after 24 h by DMEM supplemented by 5% horse serum (HyClone, USA), 5% fetal bovine serum (Gibco, USA), and 0.5 % N3 (a mixture of defined nutrients). Hippocampal neurons were maintained under standard conditions (37°C, 5% CO₂) in a thermo-regulated incubator. All experiments were performed at 9-10 days *in vitro* (DIV).

Eudesmin obtention and isolation

Eu extract were obtained from knots of *Araucaria araucana* (Molina) K. Koch collected in May 2018 on Nahuelbuta range (Biobío Region, 37°41'43.56"S73°07'49.34"W). Samples were identified by the botanist Dr. Roberto Rodríguez from the University of Concepcion. 100 g of *A. araucana* wood were chopped, dried at 40°C for 8 h, milled to 1–3 mm. Soxhlet extraction was made with 2–5 g of sample placed in a cellulose thimble with acetone (250 mL) and refluxed for 12 h. The organic layer was filtered through a GV Durapore filter (0.22 µm pore size, 13 mm diameter, Millipore, Bedford, MA, USA) and evaporated under reduced pressure, and dried in vacuum to give the extract. Subsequently, isolation of Eu (yield 1% w/w) was obtained after dissolve the extract in hot ethanol (70°C) and kept in cold in 4°C for 8 h. Eu was frozen at –20°C until use with a 96–98% purity as previously described [41].

Preparation of Eudesmin stock

10 mg of Eu extract were dissolved in 2587.69 µL DMSO and stored in aliquots at 10 mM. The aliquots were then diluted in DPBS 1x (Gibco, USA) leaving a stock of 1 mM. Four serial dilutions were made in DPBS 1X (Gibco, USA) to achieve the experimental

conditions. All the experiments with Eu were at a concentration of 1, 3, 10, 30, 100, 300, and 1000 nM.

Peptide Aβ₁₋₄₀ preparation

Human synthetic Aβ₁₋₄₀ peptide (GenicBio, Shanghai, China) was reconstituted in DMSO to a concentration of 2.3 mM and stored at –20°C. Subsequently, the peptide was aggregated in sterile 1X Phosphate-Buffered Saline (Corning, USA) at 80 mM. For the formation of oligomers, Aβ₁₋₄₀ was stirred vertically at 500 rpm to 37°C for 4 h in a Thermomixer Compact (Eppendorf AG, Hamburg, Germany). All treatments with Aβ₁₋₄₀ oligomers (AβOs) were made at a final concentration of 0.5 mM.

Cellular viability

Cellular viability changes were evaluated by the *in vitro* MTT assay kit (Sigma-Aldrich, USA) that measures the ability of mitochondria to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT salt) to formazan, which has a purple color. The cells were subjected to 1, 3, 10, 30, 100, 300, and 1000 nM of Eu, and then incubated for 2 h in MTT (1 mg/ml). The insoluble formazan was solubilized in 100 µL of isopropanol, and the absorbance was read in a NOVostar multiplate reader at 570 nm (MG Labtech, Germany).

Immunofluorescence

Control and treated hippocampal neurons were fixed with 4% paraformaldehyde for 15 min at 4°C. Afterward, the cells were permeabilized and blocked with 10 % horse serum (HyClone, USA) plus 0.1 % Triton X-100 for 15 min at room temperature. The neurons were subsequently incubated with monoclonal anti-SV2 antibody (1:200, Hybridoma Bank) and polyclonal anti MAP2 (1:200, Synaptic Systems) for 1 h at room temperature (RT) followed by incubation with the corresponding second antibody conjugated to AlexaFluor 488 and Cy3 (Jackson ImmunoResearch, USA) for 1 h at RT. Finally, the samples were mounted in Fluorescent Mounting Media (Dako, Agilent, USA) and the images were obtained using a laser scanning microscope LSM780 (63X oil immersion objective, Zeiss, Germany). Image processing was made with Image J (NIH, Bethesda, MD, USA).

Western blot

Equal amounts of protein obtained from hippocampal neurons treated with A β Os (0.5 μ M), Eu (30 nM), and A β Os plus Eu (A β Os+ Eu) for 24 h, were separated on 10% SDS-polyacrylamide gels. Protein bands were transferred into PVDF membranes (250 mA, 120 min), blocked with 5% nonfat milk, and incubated with primary antibodies using the following concentrations: 1:1000 anti-SV2 (mouse; Hybridoma Bank; USA) and 1:1000 β -actin (mouse; Santa Cruz Biotechnology, USA). A secondary HRP anti-mouse antibody was used at 1:5000 (Santa Cruz Biotechnology, USA). Immunoreactive bands were revealed using Clarity TM Western ECL substrate (Bio-Rad Laboratories, USA) and visualized with an Odyssey FC detection system (Li-Cor, Lincoln, NE, USA). Quantification of the signals was made with the Image Studio program (Image Studio Inc, USA) and β -actin were used as an internal control for the experiments and quantification.

Calcium imaging

Neurons were treated with A β Os (0.5 μ M), Eu (30 nM), and A β Os + Eu for 24 h and were loaded with 1 μ M Fluo-4AM; (Invitrogen, Carlsbad, CA, USA) for 20 min at 37°C, washed 2 times with normal external solution and the mounted on an inverted fluorescent microscope Nikon TE-2000-U (Nikon, Japan). The cells were briefly illuminated (200 ms) and the regions of interest (ROI) were selected on neuronal soma emitting Fluo-4 fluorescence (ex 480 nm, em 510 nm). Images were collected at 1 s intervals during a continuous 200-s exposure using an EMCCD iXon- 16-bit camera (Andor, Northern Ireland). The data were analyzed with the GraphPad Prism program (GraphPad, San Diego, CA, USA).

Thioflavin T fluorescence assay

A β ₁₋₄₀ aggregation assay was performed on a 96-microwell plates in the presence of Eu (30 and 300 nM) with 20 μ M Thioflavin T (ThT, ex: 440 nm, em: 485 nm, Sigma-Aldrich). The ThT fluorescence intensity of the aggregation process of A β was measured every 3 min for 4 h using a NOVostar multiplate reader (MG Labtech, Germany). The plate was kept at 37°C with an orbital agitation of 500 rpm.

In silico prediction (molecular docking simulations)

Protein-ligand docking was performed using the structure of the A β ₁₋₄₀ monomer from the Protein DataBank (PDB ID: 1AML) obtained by NMR. The A β ₁₋₄₀ structure was prepared to incorporate hydrogens, assign bond orders, fill in missing side chains, and generate protonation states at pH 7 \pm 0.2 using Maestro (Schrödinger, LLC, New York, NY, USA). The structure of Eu was obtained in the PubChem database (CID: 234823) and prepared using LigPrep (Schrödinger, LLC) before docking protein-ligand [44]. Initially, a docking protein-ligand was performed using Autodock Vina [45] in which the monomer A β ₁₋₄₀ was used as the protein target. Based on these preliminary results, a site-directed docking was performed with Glide (Schrödinger, LLC, New York, NY, 2018) using a receptor grid of 20Å centered on the N-terminal amino acids, with an extra precision (XP) configuration. Analysis of the interface A β -Eu included structural and energetic parameters performed by the same software. Additionally, a theoretical MM-GBSA Δ G bind was calculated using Prime (Schrödinger, LLC). All images were created using PyMol (version 1.5, DeLano Scientific LLC).

Data analysis

Statistical analyses were performed using GraphPad Prism (GraphPad, San Diego, CA, USA). The values are expressed as mean \pm S.E.M. for 3 or more independent experiments and as a percentage of the control (without treatment). Statistical differences were determined using T Student's test or One-way ANOVA with Tukey or Kruskal Wallis post-test. * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001 was considered statistically significant.

RESULTS

Eudesmin has neuroprotective effects against A β oligomers on neuronal models

To determinate the neuroprotective effects Eu against A β Os, PC12 cells and hippocampal primary neurons were co-incubated with A β Os, during 24 h (0.5 μ M) in the presence of Eu (1, 3, 10, 30, 100, and 300 nM) (Fig. 1A). The cellular viability was measured using MTT assay. Figure 1B and 1C show that A β Os significantly reduced cellular viability in PC12

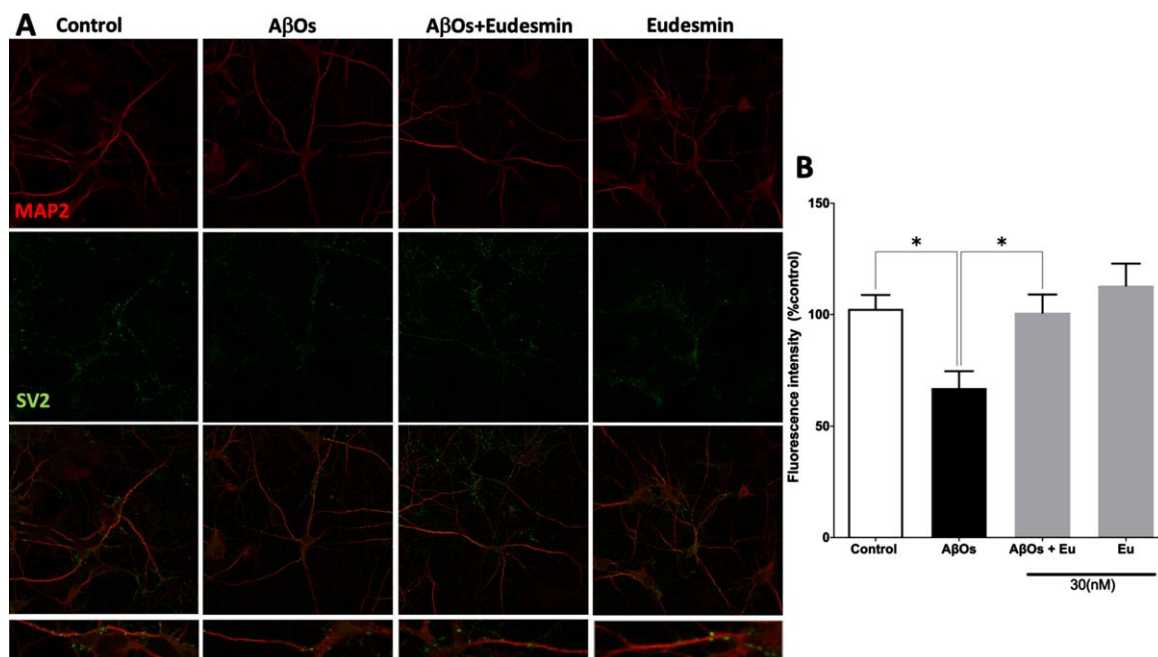


Fig. 2. Neuroprotective effect of Eudesmin on synaptic markers. Immunofluorescence confocal images of hippocampal neurons co-incubated for 24 h with AβOs (0.5 μM) and Eudesmin (Eu 30 nM) to analyze MAP2 (red) and SV2 (green), a zoom of each treatment was represented (A). Quantification of total fluorescence SV2 intensity was measured using Image J software (B). Values are expressed as the percentage of the control group (without AβOs treatment). Values are mean ± SEM, $n = 3$ using one-way ANOVA and Bonferroni test. $*p < 0.05$.

cells (36%) and cortical neurons (42%), respectively. Eu (30 nM) exerted a significant neuroprotective effect on PC12 cells, increasing the viability by 25.4% over control experiments (Fig. 1B). Equally, cortical neurons co-treated with Eu (30 nM) underwent an increase in their viability by 26.7% (Fig. 1C). Furthermore, we also observed that Eu concentrations have no intrinsic toxic properties on our cellular and neuronal models (Supplementary Figure 1).

Eu preserve the synaptic structure by conservation of exocytotic SV2 protein

In order to establish a correlation between neuronal viability and synaptic function, we initially analyzed the SV2 immunoreactivity using Eu (30 nM) and AβOs treatments (Fig. 2A) by immunofluorescence experimental approaches, using confocal microscopy techniques. The total fluorescence intensity of SV2 was quantified. The SV2 immunoreactivity showed a significant decrease of 34.5%, in AβOs-treated neurons (Aβ: 65.5 ± 7.30% of the control) (Fig. 2B). These results correlate with our observations in viability studies and suggest that part of this neuroprotective effects may be associated to synaptic function preservation. Eu treatments showed flu-

orescence intensity similar to controls (Fig. 2B). Moreover, western blot analysis of SV2 signal (Fig. 3A) rendered similar results. AβOs treatments showed a significant decline in SV2 band intensity, 53.5% (Aβ: 46.5 ± 7.05% of the control). Eu co-treatment prevents this decrease (Fig. 3B).

Eudesmin's synaptic preservation against AβOs is related to Ca²⁺ signals conservation

To reinforce our previous results and establish a deeper understanding of the observed neuroprotective properties of Eu, we decided to study the synaptic function preservation by means of intracellular spontaneous Ca²⁺ signals, using hippocampal neurons in the presence of AβOs and co-treated with Eu (30 nM) (Fig. 4A). After 24 h, we evaluated calcium transients and quantified the frequency of Ca²⁺ spikes for 200 s (Fig. 4B). AβOs treatments reduced the spikes frequency approximately 30% (Aβ: 71.8 ± 4.54% of the control), in accordance with our previous work [19, 46, 47]. Co-incubation with Eu leads to the preservation of calcium transient's frequencies on neurons. This result suggests that the synaptic structural network conservation is associated with the preservation of functional synapses.

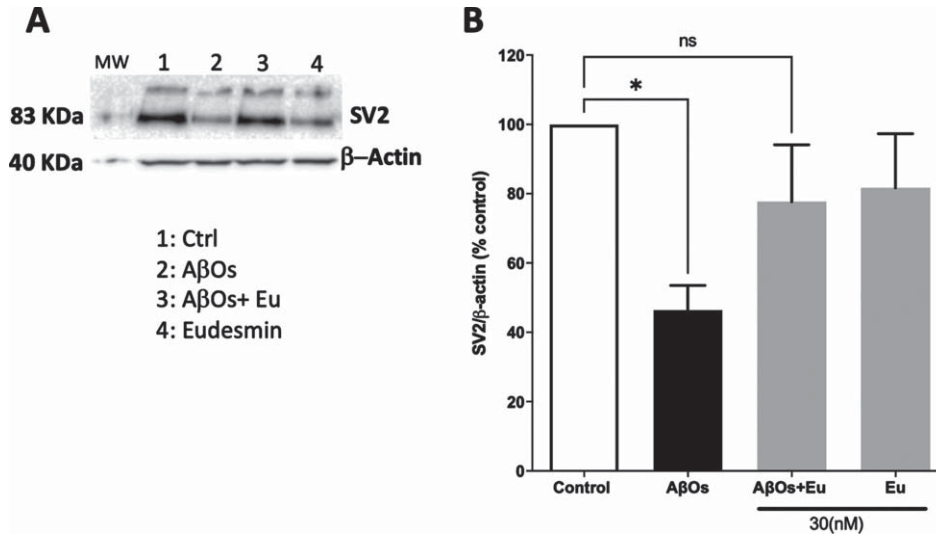


Fig. 3. Neuroprotective effect of Eudesmin on synaptic markers: SV2 detection. Western blot images of neuronal cultures co-incubated for 24 h with A β O s (0.5 μ M) and Eudesmin (Eu 30 nM) SV2 total expression (A). Total expression intensity was measured using the Odyssey FC detection system. β -actin detection was used as reference. Values are expressed as the percentage of the control group (without A β O s treatment) (B). Values are mean \pm SEM, $n = 3$ using one-way ANOVA and Bonferroni test. * $p < 0.05$ versus control group.

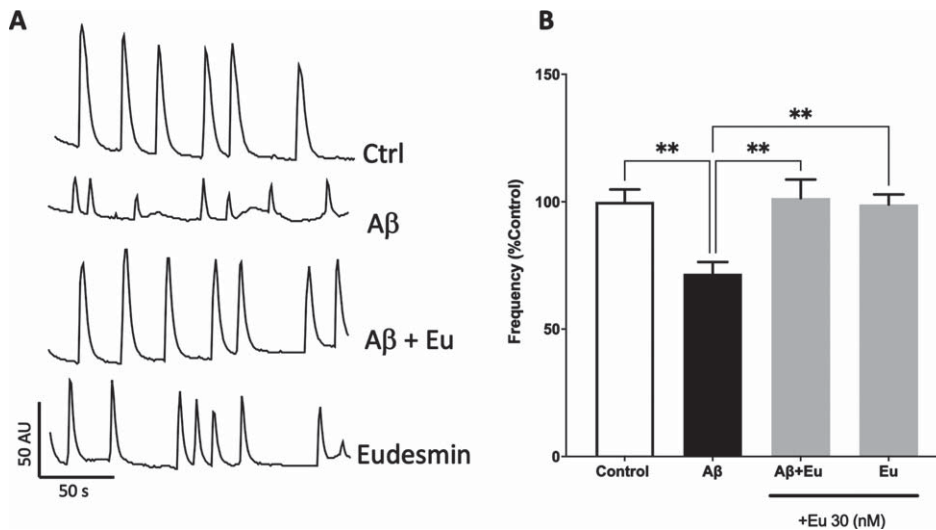


Fig. 4. Eudesmin restored calcium transients in neurons treated with A β O s . Hippocampal neurons were co-incubated for 24 h with A β O s (0.5 μ M) and Eudesmin (30 nM). Calcium transients were measured using Fluo-4AM for 200 s. Representative images of calcium transients in cells treated to the different indicated treatments (A). Quantification of calcium transient's frequency. Percentage values are expressed as the percentage of the control group (without A β O s treatment) (B). Values are mean \pm SEM, $n = 3$ using Kruskal-Wallis test, with Dunn's post-test correction. ** $p < 0.01$.

Moreover, Eu has no toxic effects on synaptic communication after treatments and was able to recover the reduction induced by A β O s respect to control condition (A β +Eu: $101 \pm 7.28\%$; Eu: $98.9 \pm 3.97\%$) (Fig. 4B).

Eudesmin induces alterations on A β O s aggregation pattern and reduce their toxicity

Considering the Eu neuroprotective abilities and synaptic function preservation, we sought to stab-

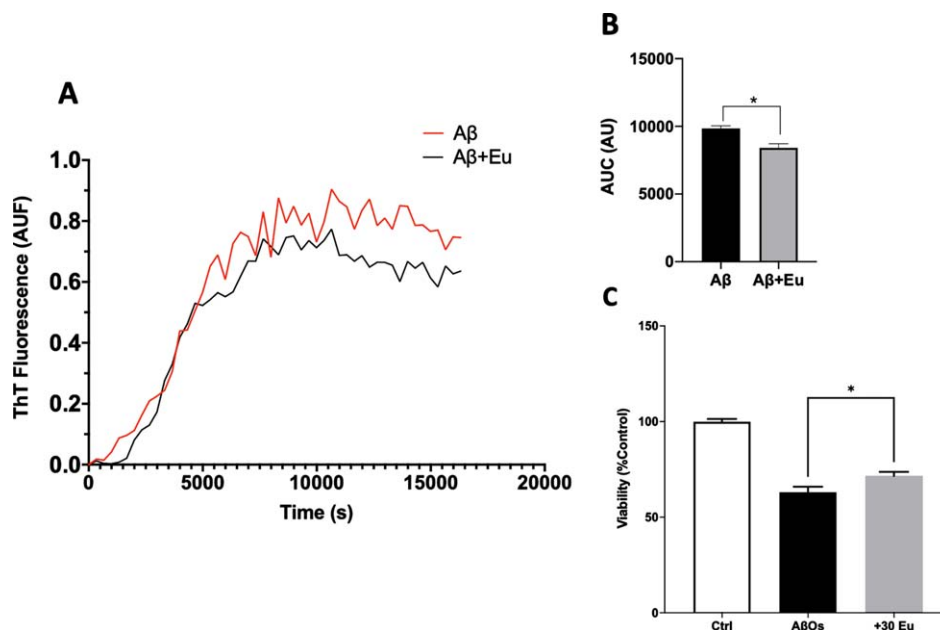


Fig. 5. Eudesmin effects on A β aggregation. A β ₁₋₄₀ (80 μ M) aggregation was measured using Thioflavin T (ThT 20 μ M) evaluating fluorescence every 3 min for 350 min at 37°C, in absence of Eudesmin 30 nM (red) or its presence (black) (A). Area under curve quantification (arbitrary units) shown the effect of Eudesmin over the background fluorescence of ThT (B). Viability quantification evaluated on PC12 cells using MTT to compare response against A β O_s or A β O_s+Eu (30 nM) (C). Values are mean \pm SEM, $n=3$ using one-way ANOVA and Tukey or Welch's test. * $p < 0.05$.

lish whether Eu influences the aggregation kinetics and presence of A β ₁₋₄₀ toxic species [36, 48]. An aggregation assay using Thioflavin T (ThT) was performed. We monitored the fluorescence of ThT in A β and A β plus 30 or 300 nM of Eu and observed that Eu (30 nM) decreases the maximum fluorescence of A β aggregation pattern (Fig. 5A, black line). Also, it was observed that the area under the curve (AUC, Fig. 5B; A β : 9860 \pm 325.2 AU; A β +Eu: 8428 \pm 498.6 AU) is correlated with the PC12 viability assay using A β aggregated in the presence of Eu (30 nM, Fig. 5C). This shows a correlation between the capacity of Eu to interfere with the aggregation of A β and the ability of this lignan to protect PC12 cells from the toxic effects of A β . The effect was more pronounced when a higher concentration of Eu (300 nM) was used (Fig. 6A, B), suggesting a concentration-dependent effect (AUC, Fig. 6B; A β : 10313 \pm 274.7 AU; A β +Eu: 6000 \pm 297.0 AU). The A β O_s generated in the presence of Eu 300 nM showed to be less toxic than those produced in their absence (Fig. 6C). These results showed that Eu has an additional mechanism of action, which promotes neuronal protection and is complementary with the

previously observed synaptic effects (structural and functional).

Eudesmin induces a A β -Eu stable complex that could be responsible for reduced A β O_s toxicity, in silico evaluation

We sought to evaluate the interaction of Eu with A β ₁₋₄₀ monomers using in silico techniques. The NMR structure of the A β monomer (PDB ID: 1AML) was used for prediction corresponding to A β ₁₋₄₀. First, a complex using docking protein-ligand was created (Fig. 7A) in order to evaluate the ability of Eu interaction with A β monomer. Eu was able to interact favorably in structural and energetic terms showing a docking score of -2.91 calculated by Glide and a ΔG bind of -48.44 kcal/mol obtained using an MM-GBSA approach calculated with Prime. A more detailed analysis of the A β -Eu interaction site showed a region that includes the amino acids ¹DAEFRHDS⁸, 15Q, 18V, and 22E, detecting the formation of 3 hydrogen bonds with the 5R, 6H, and Q15, in a primarily polar region (Fig. 7B). Thus, these results predict a stable and favorable interac-

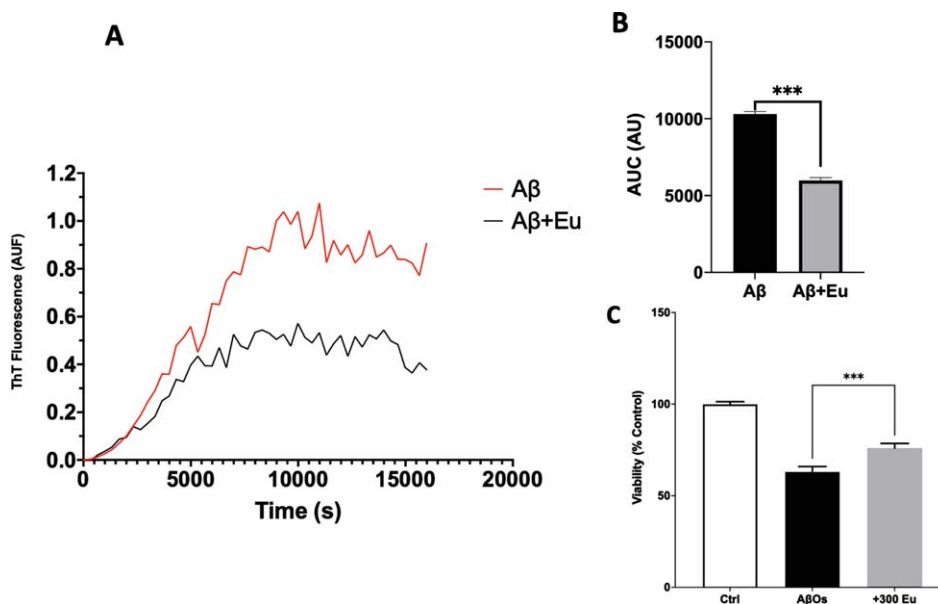


Fig. 6. Eudesmin effects on A β aggregation depends on concentration. A β ₁₋₄₀ (80 μ M) aggregation was measured using Thioflavin T (ThT 20 μ M) evaluating fluorescence every 3 min for 350 min at 37°C, in absence of Eudesmin 300 nM (red) or in presence (black) (A). Area under curve quantification (arbitrary units) shown the effect of Eudesmin over the background fluorescence of ThT (B). Viability quantification evaluated on PC12 cells using MTT to compare response against A β Os or A β Os+Eu (300 nM) (C). Values are mean \pm SEM, $n = 3$ using one-way ANOVA and Tukey or Welch's test. *** $p < 0.001$.

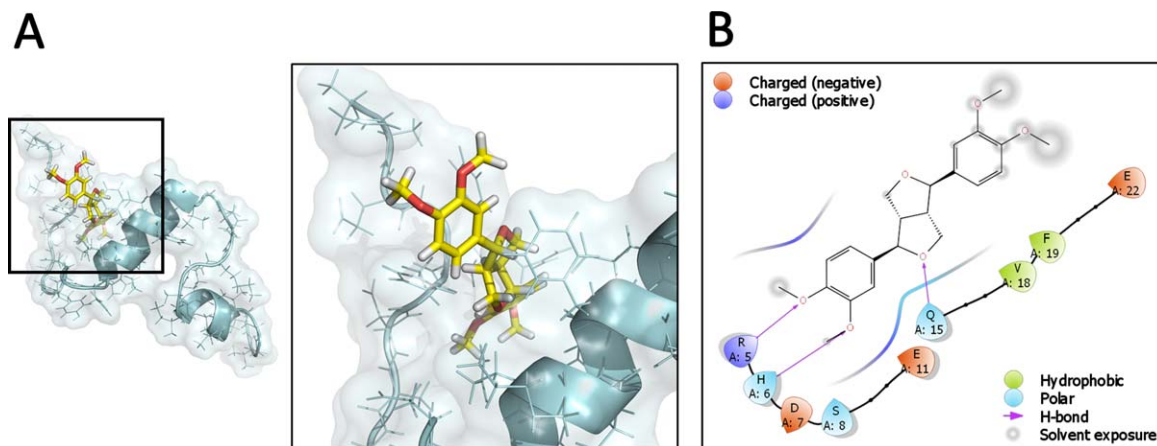


Fig. 7. Predicted interaction of the A β ₁₋₄₀ monomer with Eudesmin. Complex between A β and Eudesmin generated by docking protein-ligand. Cartoon representation and surface of A β ₁₋₄₀ monomer has been colored cyan, while the carbon backbone and oxygen atoms of Eudesmin are shown yellow and red, respectively (A). Schematic representation of the interactions presents at the A β -Eu interface in a region defined with a cutoff of 4Å (B).

tion between Eu and the A β monomer at the initial state prior to aggregation.

DISCUSSION

The discovery of natural products displaying neuroprotective and synaptic preservation allows a new strategy for treating or preventing the development

of diseases related to cognitive impairment, such as AD [30, 36, 49]. In this work, we uncover the previously unknown neuroprotective properties of Eu, a lignan containing a tetrahydrofuran core. It has been reported that other lignan similar to Eu, Sesamin, has showed neuroprotective effects involving anti-inflammatory paths [50, 51]. We isolated Eu from *Araucaria araucana*, a native conifer from

Chile that indigenous people have used as food and traditional medicine [41]. Eu did not show toxicity in the concentration range investigated (1–1000 nM), using a cellular viability assay (Supplementary Figure 1). However, Eu showed neuroprotective effects *in vitro*, in PC12 cell lines and neuronal primary cultures assays [31, 36, 46, 48]. Remarkably, Eu proved to be rather potent, showing neuroprotective effects at 30 nM (in PC12 cells and neuronal cultures) instead of 1 μ M, as our previously reports for a natural product such as fomannoxin [36]. Eu also showed beneficial effects on synaptic preservation. The expression of SV2 protein as presynaptic marker was evaluated by immunofluorescence and western blot at the same neuroprotective concentration, and we found that Eu (30 nM) preserves the SV2 protein expression during chronic A β O treatments (24 h). Similar results have been reported in other *in vitro* models for AD where SV2 expression was diminished by pore formation induced by A β oligomers and characterized by calcium entry inducing an ionic dys-homeostasis [12, 35, 47]. Also, a reduced detection of SV2 protein was observed on AD patients [12]. In our study, we observed a strong neuroprotective effect of Eu by conserving the synaptic structure as well as maintaining synaptic communication. To analyze neuronal transmission, we evaluate the effect of Eu co-treatments on calcium transient's frequency as an indicator of synaptic function, at the same concentration previously described as neuroprotective (30 nM). Eu does not affect the calcium transient's frequencies observed under control conditions and prevents the detrimental effect of A β O on Ca⁺² homeostasis. Considering that neurotransmitter release is correlated to intracellular calcium, we expected that Eu preserves the presynaptic proteins and the intracellular calcium transients, similarly to other natural products with neuroprotective activity [52]. Thus, we propose an intracellular mechanism that explains the neuroprotective activity of Eu by presynaptic structure preservation, including SV2 and calcium transients. These data are related to similar compounds previously described by our group, including benzofurans that have been reported with the capacity to interfere with the A β aggregation process [31, 35, 36]. Using ThT assays, we evaluated the effects of Eu on aggregation process, and we quantify the area under the curve. Then, the viability response was analyzed using cellular model experiments in the presence of A β O and co-treated with Eu. At low concentrations of Eu (30 nM), a slight effect on A β aggregation was observed, and

therefore a minor neuroprotective effect on viability response was detected. However, at high concentrations of Eu (300 nM), an increase in both aggregation and viability response effects was detected. These concentration-related effects of Eu on the A β O aggregation led us to investigate the A β -Eu complex, using *in silico* techniques. These results indicate the stable formation of complexes where key hydrophobic residues such as phenylalanine and valine are required for the Eu effect on the aggregation of A β . Also, we observe three specific hydrogens bonds that can stabilize the A β -Eu complex formation. The stable A β -Eu complex could be responsible for the reduced toxicity observed on viability assays.

Finally, we believe that Eu represents a novel lead that reduces the A β toxicity, opening new research venues for lignans as neuroprotective agents. Future efforts will focus on *in vivo* experimental approaches in order to gain a deep understanding of the neuroprotective mechanism of Eu.

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SUPPLEMENTARY MATERIAL

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