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The standardized *Centella asiatica* extract ECa 233 regulates the catalytic activities of β APP-cleaving secretases in human cell lines

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ABSTRACT: Alzheimer's disease is a neurodegenerative disorder characterized by the accumulation of amyloid peptides in the brain. While the production of A β is dependent on the cleavage of the β -amyloid precursor protein by the β -secretase BACE1, the α -secretase activity, mainly supported by ADAM10, counterbalances this pathway by both preventing A β production and triggering the release of the neuroprotective soluble APP alpha (sAPP α) metabolite. For this reason, strategies aimed at promoting α -secretase and/or blocking β -secretase seem to be indicated for the purpose of containing the disease. Here we investigated the effects of ECa 233, a standardized extract of the plant *Centella asiatica*, on β APP levels and sAPP α secretion as well as on the expression and catalytic activity of the α -secretases ADAM10 and ADAM17 and the β -secretase BACE1 in human cells. Our results interestingly demonstrate that the ECa 233 extract is able to significantly stimulate α -secretase activity and to inhibit β -secretase activity in a dose-dependent manner in the human SH-SY5Y neuroblastoma cell line. In conclusion, these results reveal an original doubly beneficial effect of ECa 233, which is both capable of promoting the non-amyloidogenic α -secretase activity and interfering with the amyloidogenic pathway and thereby stands as a promising candidate for the future development of mild, safe and preventive therapeutic treatment of Alzheimer's disease.

KEYWORDS: Alzheimer's disease, soluble APP alpha, *Centella asiatica*, alpha-secretase, beta-secretase

INTRODUCTION

Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder and is by far the leading cause of dementia in the elderly, thus representing a major global public health problem. The disease first manifests itself in the appearance of short-term memory impairment followed by a more general alteration in cognitive functions leading irremediably to death. One of the main hallmark of AD is the abnormal load of extracellular β -amyloid peptide (A β) leading to the occurrence of senile plaques that first form in the entorhinal cortex and then in the hippocampus before spreading into the cerebral cortex. In addition, the exacerbation of several deleterious pathogenic processes accompanies the development of the disease among which we can cite oxidative stress, neuroinflammation, mitochondrial dysfunction, altered calcium homeostasis and apoptosis.

The production of A β peptides is initiated by cleav-

age of the β -amyloid precursor protein (β APP) by the β -secretase BACE1 and ends with the hydrolysis of the C99 fragment by the heterotetrameric γ -secretase complex [1]. Concomitantly with this amyloidogenic pathway, β APP is also, and predominantly, the target of the α -secretase activity, which fulfills a doubly beneficial action by interfering with A β production (since it operates in the middle of the A β sequence) and giving rise to the secretion of the neuroprotective, neurotrophic and memory-enhancing sAPP α metabolite, thus establishing α -secretase activation as a promising anti-AD therapeutic strategy [2]. However, this approach faces a major problem which is the large number of proteins other than β APP fulfilling vital physiological functions and which are the target of the two major enzymes responsible for this activity, ADAM10 and ADAM17 [3]. In this context, the use of plant extracts and plant-derived active compounds is increasingly gaining popularity and has been considered in recent years as a credible alternative to anti-AD

pharmacotherapies, principally because the side effects associated with their use are very moderate or even nonexistent [4].

Centella asiatica (Linn.) is a plant of the Apiaceae family that grows in swamps of temperate and tropical areas and holds an important place in the international market of medicinal plants. Chemically, it is mainly composed of the pentacyclic triterpenoids asiaticoside and madecassoside and their respective metabolites asiatic acid and madecassic acid [5]. During the past twenty years, a number of beneficial effects have been attributed to extracts of this plant or to its active ingredients against various pathological conditions [6]. Hence, methanol and water extracts of *C. asiatica* were reported to display anti-hyperlipidemic properties in murine [7]. It has also been shown that *C. asiatica* hydrogel accelerates wound healing in rabbits [8], a property that can reasonably be attributed to asiaticoside as evidenced in rats [9]. In addition, *C. asiatica* water extract was shown to alleviate epilepsy-provoked cognitive impairments in a mouse model of the disease [10] and to reduce adriamycin-induced myocardial failure in rats [11]. Finally, asiatic acid was reported to provide neuroprotection in a mouse model of focal cerebral ischemia [12]. The neuroprotective tonus of *C. asiatica* was further confirmed by several studies. Firstly, a chloroform:methanolic extract of the plant was shown to be neuroprotective in mono sodium glutamate-treated rats [13]. Secondly, asiaticoside, asiatic acid and medecassoside were established as the probable responsible active components mediating neuroprotection as documented *in vivo* following D-galactose-induced cognitive impairments in mice [14].

Further evidence of the beneficial effects of *C. asiatica* on brain function was brought by the demonstration that basal learning and memory performance can be improved following ingestion of aqueous extract of whole plant (200 mg/kg) for 14 days in rats [15], a phenomenon also observed following intraperitoneal injection of asiatic acid (30 mg/kg), suggesting that this triterpenoid group is, at least partly, supporting the memory-enhancing capability of the extracts [16]. Moreover, oral administration of an ethanol extract of the plant (300–1500 mg/kg) was shown to ameliorate learning and memory impairment induced by transient bilateral common carotid artery occlusion in mice [17].

Because learning and memory are severely impaired in AD, the hypothesis that *C. asiatica* could behave as an ameliorating factor with respect to the disease logically appeared and several studies have evidenced some positive effects of aqueous extract of the plant on AD pathology. Thus, it ameliorates behavior in a streptozotocin-injected rat model of AD [18], reduces A β -induced neurodegeneration in hippocampal neurons [19], alleviates behavioral deficits in the Tg2576 transgenic mouse model of the disease and conveys neuroprotection in 5 \times FAD transgenic mice [20].

Although the anti-oxidant and anti-inflammatory properties of *C. asiatica* are likely to contribute to its anti-AD effects [6], whether it can also interfere with β APP processing via the regulation of β APP-cleaving secretases remained unexplored to this day. Here we show that the non-toxic [21] and well-characterized [22] standardized *C. asiatica* extract ECa 233 stimulates α -secretase and inhibits β -secretase catalytic activities without modifying the expression of the involved proteins. These results thus established ECa 233 as a beneficial regulator of both the non-amyloidogenic and the amyloidogenic processing of β APP, altogether making it a possible anti-AD preventive compound.

MATERIALS AND METHODS

Materials

DMEM, fetal bovine serum (FBS) and penicillin-streptomycin mix (Pen/Strep) were from Invitrogen (Carlsbad, CA, USA). Tris buffer and glycine were from VWR Amresco lifesciences (Solon, CA, USA). Polyclonal anti- β APP antibody (A8717), monoclonal anti- β -actin (A2228), dimethyl sulfoxide (DMSO), SDS and sodium bicarbonate were from Sigma (St. Louis, MO, USA). Polyclonal anti-ADAM10 (AB19026) and polyclonal anti-ADAM17 (AB19027) were from Millipore (Bedford, MA, USA). Monoclonal anti-BACE1 (ab108394) was from Abcam (Cambridge, UK). Skim milk powder was from Bio Basic (Singapore). Monoclonal anti- β -amyloid antibody (2B3), which was used to specifically detect sAPP α was from IBL (Minneapolis, MN, USA). ECL reagent and ammonium persulphate were from GE Health care (Pisataway, NJ, USA). O-Phenanthroline was from Calbiochem (San Diego, CA, USA). Goat anti-mouse (polyclonal 7076) and goat anti-rabbit (polyclonal 7074) peroxidase-conjugated secondary antibodies were from Cell Signaling (Beverly, MA, USA).

Cell lines and treatments

Human HEK293 cells were cultured at 37°C, 5% CO₂ in DMEM supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (50 mg/ml). Human SH-SY5Y neuroblastoma cells were grown at 37°C, 5% CO₂ in high glucose-DMEM supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (50 mg/ml). The standardized ECa 233 extract was obtained as previously described by a patent-pending method [23] and contains 53.1% madecassoside and 32.3% asiaticoside as determined by quantification by HPLC and liquid chromatography-tandem mass spectrometry (LC-MS/MS) as previously reported [24]. A 100 mg/ml stock solution (in 100% DMSO) was first prepared from which serial dilutions were performed in order to get four 100 \times intermediate solutions (10 mg/ml, 1 mg/ml, 100 μ g/ml and 10 μ g/ml) in 10% DMSO. Cells (80% confluence) were treated for

24 h with 10 μ l of 100 \times concentrations in a total volume of 1 ml of complete media (final concentrations: 0.1 μ g/ml, 1 μ g/ml, 10 μ g/ml, 100 μ g/ml; 0.1% DMSO) before being processed for sAPP α secretion and western blot analysis. For non-treated controls, DMSO was also adjusted to 0.1%.

sAPP α secretion and detection

Secretion and detection of sAPP α in HEK293 and SH-SY5Y cells with the human-specific monoclonal anti-sAPP α antibody (2B3). Briefly, following treatments in complete media, media was removed and cells were incubated with fresh DMEM (1 ml) and allowed to secrete for 5 h. Then, 10% TCA precipitation of the whole medium was performed and the precipitate was subjected to electrophoresis through 10% SDS-PAGE gels, transferred onto nitrocellulose membranes (100 min, 90 volts), incubated in 5% non-fat milk blocking solution for 30 min and incubated overnight at 4 $^{\circ}$ C with 2B3 (1 μ g/ml). After three washes with PBST (PBS containing 0.05% Tween 20), membranes were then incubated with a HRP-conjugated anti-mouse IgG antibody (dilution 1/3000), rinsed three times with PBST incubated with ECL reagent and signals were detected using an Azure c400 (Azure Biosystems, Dublin, CA, USA). Band densities were measured with the Image J software (<http://imagej.nih.gov/ij>).

Western blot analyses

Cells were collected with phosphate-buffered saline (PBS)-EDTA and resuspended in 70 to 100 μ l of lysis buffer (10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.5% triton X-100, 0.5% deoxycholate, 5 mM EDTA). Protein concentrations were determined by the Bradford method and 20–40 μ g proteins were loaded onto 10% of SDS-PAGE gels, which were run at 100 volts for 2–2.5 h. Proteins were then transferred onto nitrocellulose membranes for 60–120 min at 90 V. Protein transfer was verified by Ponceau red staining, and nitrocellulose membranes were subsequently incubated in 5% non-fat milk blocking solution for 45 min. Membranes were then incubated with primary antibodies directed against β APP (dilution 1/2000), ADAM10 (dilution 1/500), BACE1 (dilution 1/1000) or β -actin (dilution 1/5000) on a platform shaker overnight at 4 $^{\circ}$ C. Bound antibodies were detected using goat anti-mouse (dilution 1/3000, polyclonal 7076, Cell Signaling) or goat anti-rabbit peroxidase-conjugated antibody (dilution 1/3000, polyclonal 7074, Cell Signaling). After 3 washes with PBST, membranes were incubated with a HRP-conjugated anti-rabbit (ADAM10, β APP and BACE1) or anti-mouse (β -actin) secondary antibody (1/3000) for 2 h, rinsed 3 times with PBST and processed as described above. All protein levels were normalized using β -actin as an internal standard.

α -secretase fluorimetric assay on intact cells

The α -secretase catalytic activity was measured following a procedure previously described [25]. Briefly, SH-SY5Y cells were treated in duplicate without (control) or with ECa 233 for 24 h at 37 $^{\circ}$ C in 1 ml of DMEM containing 1% FBS. Cells were then incubated for 30 min at 37 $^{\circ}$ C in the absence or in the presence of the general metalloprotease inhibitor o-phenanthroline (100 μ M) in 1.5 ml of PBS. Then, the α -secretase-specific JMV2770 substrate (10 μ M) was directly added into the media and cells were kept at 37 $^{\circ}$ C. Every 15 min, 100 μ l of media were collected and the α -secretase-specific activity corresponding to the o-phenanthroline-sensitive fluorescence was recorded in black 96-well plates at 320 nm and 420 nm excitation and emission wavelengths respectively.

β -secretase fluorimetric assay on cell homogenates

SH-SY5Y cells were cultured in 35 mm-dishes until they reach 80% confluence, treated without (control) or with ECa 233 for 24 h at 37 $^{\circ}$ C in DMEM containing 1% FBS and assayed for their β -secretase activity. Briefly, cells were collected, lysed with Tris 10 mM pH 7.5, homogenized and kept on ice. Samples were assayed for their protein contents with the Bradford method and adjusted to a 3 μ g/ μ l concentration. Thirty μ g of each samples (10 μ l) diluted in 10 mM sodium acetate buffer pH 4.5 were incubated for 30 min at 37 $^{\circ}$ C in black 96-well plates (in a final volume of 100 μ l) in the absence (triplicate) or in the presence (triplicate) of the β -secretase specific inhibitor JMV1197. Then, the β -secretase-specific JMV2236 substrate (10 μ M) was added to all samples and plates were maintained at 37 $^{\circ}$ C. Every 15 min, the β -secretase-specific activity corresponding to the JMV1197-sensitive fluorescence was recorded at 320 nm and 420 nm excitation and emission wavelengths respectively.

Statistical analysis

Statistical analyses were performed with the Prism software (GraphPad, San Diego, USA) using an unpaired *t*-test for pairwise comparisons. All results were expressed as means \pm SEM and the *p* values equal to or less than 0.05 were considered significant.

RESULTS

Effects of ECa 233 on sAPP α production and β APP, ADAM10, ADAM17 and BACE1 protein levels in cultured human SH-SY5Y cells

We first examined the effect of 24 h-treatments with four different doses of ECa 233 (0.1, 1, 10 and 100 μ g/ml) for their ability to promote the secretion of the β APP-derived sAPP α metabolite produced by α -secretase in cultured SH-SY5Y human neuroblastoma cells. The results showed no significant differences whatever the dose considered when compared

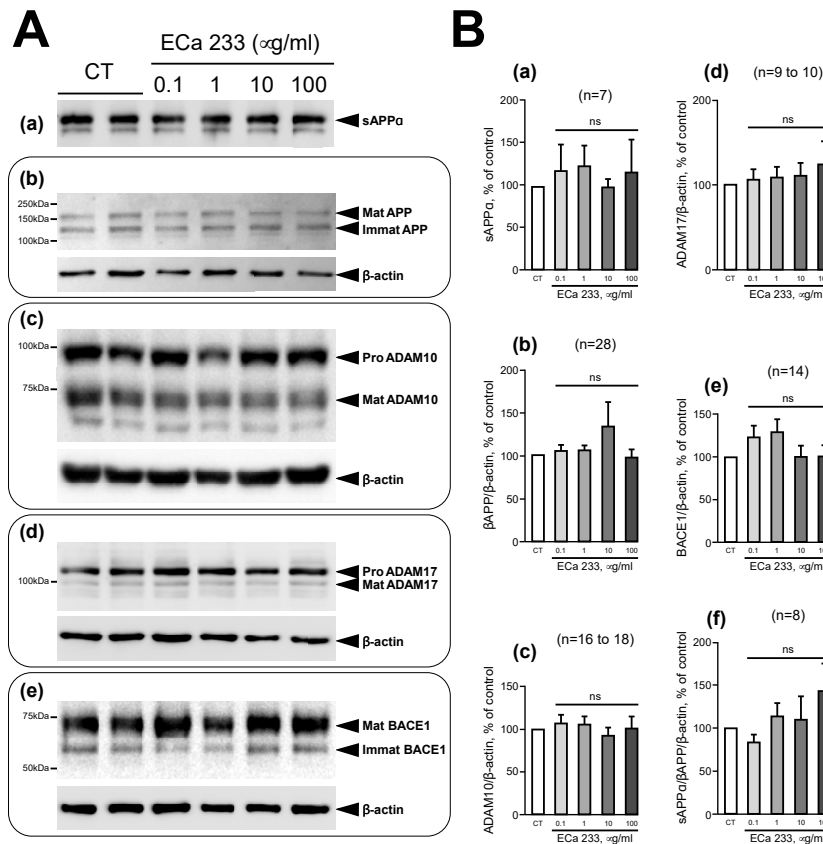


Fig. 1 Effect of ECa 233 on sAPP α secretion, β APP, ADAM10, ADAM17, BACE1 protein levels and the ratio sAPP α / β APP in human SH-SY5Y cells. (A) Representative gels of western blot analysis of sAPP α production in media (a) as well as β APP (b), ADAM10 (c), ADAM17 (d) and BACE1 (e) and their respective β -actin in lysates following treatment of cultured SH-SY5Y cells without (CT) or with the indicated concentrations of ECa 233 for 24 h. (B) Statistical analysis of the data for sAPP α (a), β APP (b), ADAM10 (c), ADAM17 (d), BACE1 (e) and the ratio sAPP α / β APP (f). Bars correspond to the densitometric analyses (β APP, the ratio sAPP α / β APP, ADAM10, ADAM17 and BACE1 being normalized with β -actin), are expressed as a percent of control taken as 100, and are the means \pm SE of the indicated number of independent determinations (n); ns, no statistical difference.

to controls (Fig. 1A(a) and B(a)) although a slight upward trend was observed (Fig. 1B(a)). We then wanted to determine whether ECa 233 could modify the protein levels of β APP, the two α -secretases ADAM10 and ADAM17 and of the β -secretase BACE1. No significant variation in the immunoreactivities of these proteins could be demonstrated by western blot analysis (Fig. 1A(b-e) and B(b-e)), thus showing that there is no impact of ECa 233 on the expression of key proteins of β APP metabolism. Because the secretion of sAPP α is dependent both on the hydrolysis of β APP by α -secretase activity and on the expression of β APP itself, and in order to increase the stringency of our measurements, we also measured the effect of ECa 233 on the sAPP α / β APP ratio under the same conditions. Despite the fact that it seems that ECa 233 increases this ratio in a dose-dependent manner, the statistical analysis of the results did not allow us to highlight any significant differences (Fig. 1B(f)).

Effects of ECa 233 on sAPP α production and β APP, ADAM10, ADAM17 and BACE1 protein levels in cultured human HEK293 cells

In order to test the same parameters in a different cell line, we conducted the same experimental protocols with human HEK293 cells. The results show, as observed in SH-SY5Y cells, that ECa 233 did not modify the protein levels of β APP, ADAM10, ADAM17 and BACE1 (Fig. 2A(b-e) and B(b-e)) and appeared to increase, although not significantly, the production of sAPP α (Fig. 2A(a) and B(a)). Nevertheless, we could establish that treatment with ECa 233 at 100 μ g/ml significantly increases the sAPP α / β APP ratio (Fig. 2B(f)).

Based on these results, we then wanted to determine whether ECa 233 was able to modulate the catalytic activity of α secretase and β -secretase, which compete for β APP processing and thus control the

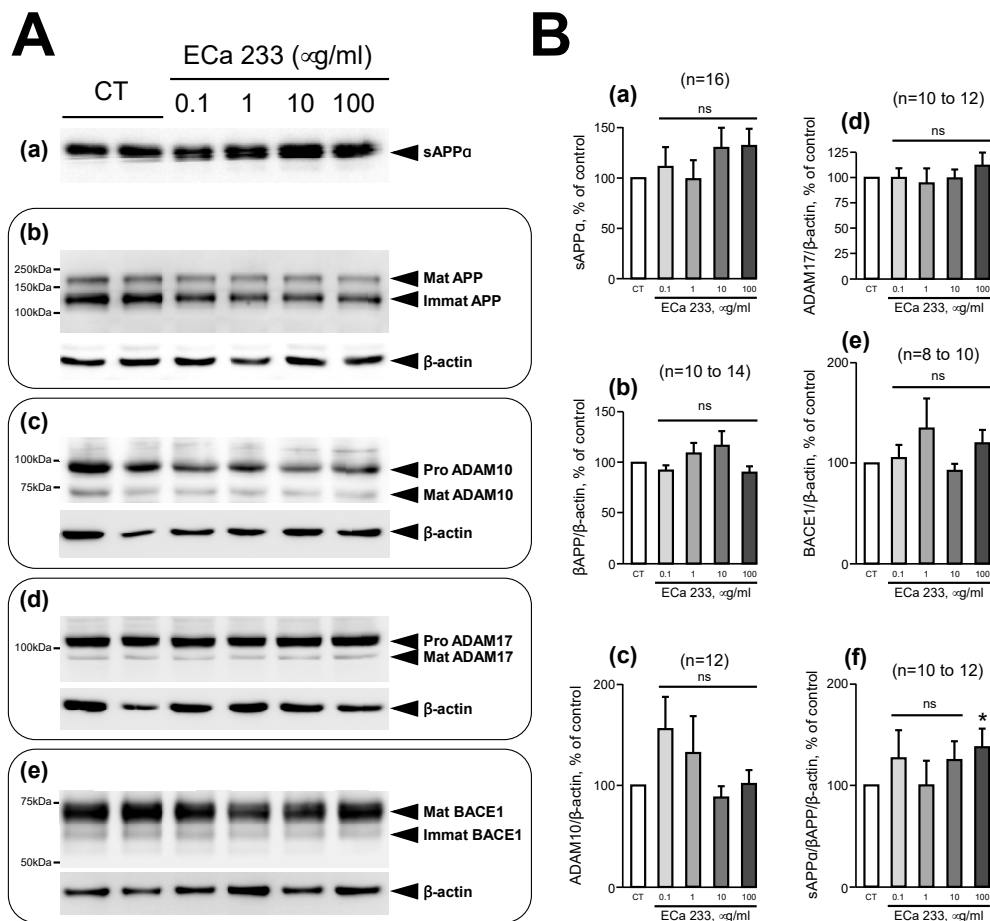


Fig. 2 Effect of ECa 233 on sAPP α secretion, β APP, ADAM10, ADAM17, BACE1 protein levels and the ratio sAPP α / β APP in human HEK293 cells. (A) Representative gels of western blot analysis of sAPP α production in media (a) as well as β APP (b), ADAM10 (c), ADAM17 (d) and BACE1 (e) and their respective β -actin in lysates following treatment of cultured HEK293 cells without (CT) or with the indicated concentrations of ECa 233 for 24 h. (B) Statistical analysis of the data for sAPP α (a), β APP (b), ADAM10 (c), ADAM17 (d), BACE1 (e) and the ratio sAPP α / β APP (f). Bars correspond to the densitometric analyses (β APP the ratio sAPP α / β APP, ADAM10, ADAM17 and BACE1 being normalized with β -actin), are expressed as a percent of control taken as 100, and are the means \pm SE of the indicated number of independent determinations (n); * $p < 0.05$; ns, no statistical difference.

balancing between the amyloidogenic and the non-amyloidogenic pathways.

Effects of ECa 233 on the α -secretase catalytic activity in cultured human SH-SY5Y neuroblastoma cells

In a first set of experiments, we examined the impact of increasing concentrations (0.1 up to 100 μ g/ml) of ECa 233 on the α -secretase activity by measuring the phenanthroline-sensitive hydrolysis of the fluorimetric JMV2770 substrate by cultured SH-SY5Y cells. Despite an increasing trend, no significant difference was noted at 0.1 μ g/ml concentration (Fig. 3A(a)). However, our results indicated that ECa 233 dose-dependently enhances the JMV2770-hydrolyzing activity as shown by an increased significance (p value) from 1 to

100 μ g/ml concentrations (Fig. 3A(b-d) and B).

Effects of ECa 233 on the β -secretase catalytic activity in cultured human SH-SY5Y neuroblastoma cells

Finally, we undertook to determine if ECa 233 could behave as an inhibitor of the amyloidogenic β -secretase catalytic activity. Taking advantage of a well-characterized BACE1-selective fluorimetric assay, we have measured the impact of the treatment of SH-SY5Y cells with the extract for 24 h at the four previously used concentrations. Indeed, the JMV1197-sensitive hydrolysis of the fluorimetric JMV2236 substrate measured in SH-SY5Y cell extracts at acidic pH was gradually reduced as the concentrations of ECa 233 increased (Fig. 4A and B), thereby evidencing

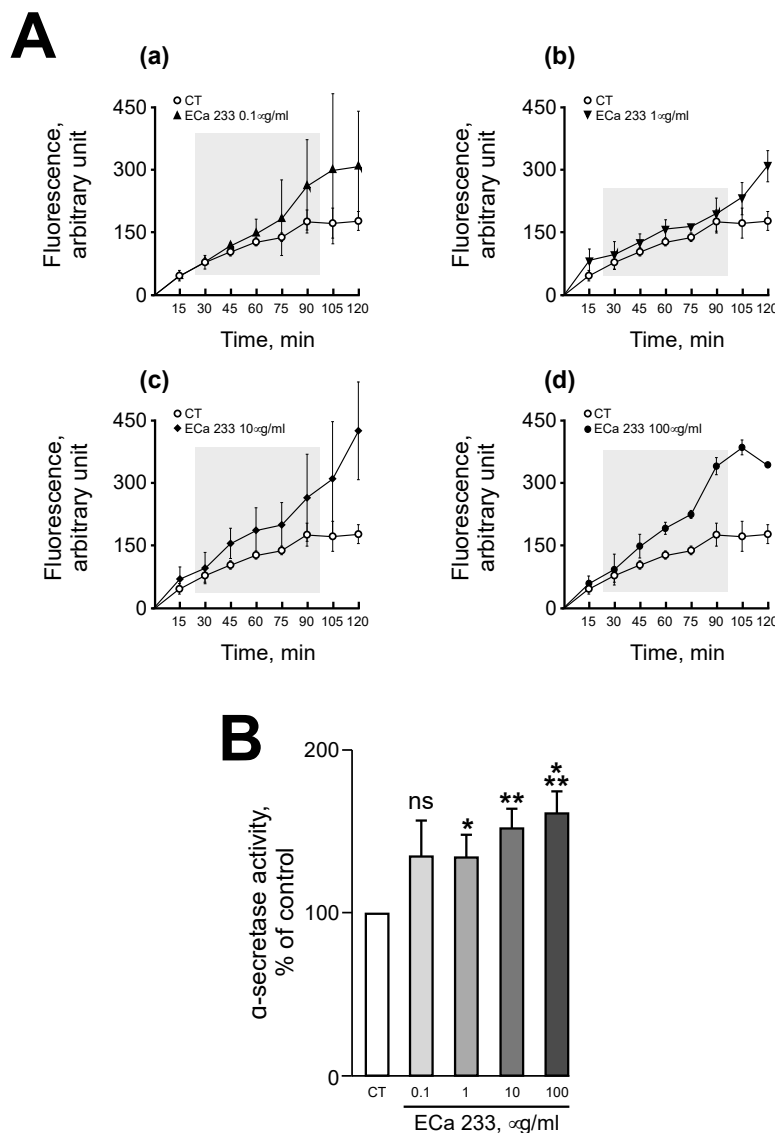


Fig. 3 Effects of ECa 233 on the α -secretase catalytic activity in SH-SY5Y intact cells. (A) The α -secretase catalytic activity (phenanthroline-sensitive hydrolysis of the fluorimetric substrate JMV2770) was measured on cultured SH-SY5Y cells incubated in the absence (control, white circle) or in the presence of 0.1 $\mu\text{g/ml}$ (a), 1 $\mu\text{g/ml}$ (b), 10 $\mu\text{g/ml}$ (c) and 100 $\mu\text{g/ml}$ (d) of ECa 233 for 24 h. The curves represent the mean specific fluorescence \pm SE from 2 independent experiments including two controls each. (B) Statistical analysis of the data. Bars are expressed as a percentage of control (white bars, non-treated cells) calculated from the linear parts of the curves (initial velocity, light grey area) and are the means \pm SE of 11 independent determinations; * $p < 0.02$; ** $p < 0.0003$; *** $p < 0.0002$; ns, no statistical difference.

a dose-dependent effect of the extract on the limiting factor of the amyloidogenic processing of β APP with more than 50% inhibition observed at 100 $\mu\text{g/ml}$ when compared to control untreated cells (Fig. 4A(d) and B).

DISCUSSION

AD is a yet incurable neurodegenerative disorder characterized by a progressive severe loss of memory and cognitive functions. The reason why available medical treatments are still incapable to cure AD symp-

oms efficiently mostly resides in the fact that AD is a complex and multifactorial disease with multiple pathological processes. Over the past decades, a huge effort, although in vain, has been made to develop novel synthetic drugs with disease-modifying properties and few side effects [26]. As a consequence, compounds extracted from natural sources logically gained popularity and the notion of a preventive rather than a curative approach aimed at combating AD is increasingly taken into account.

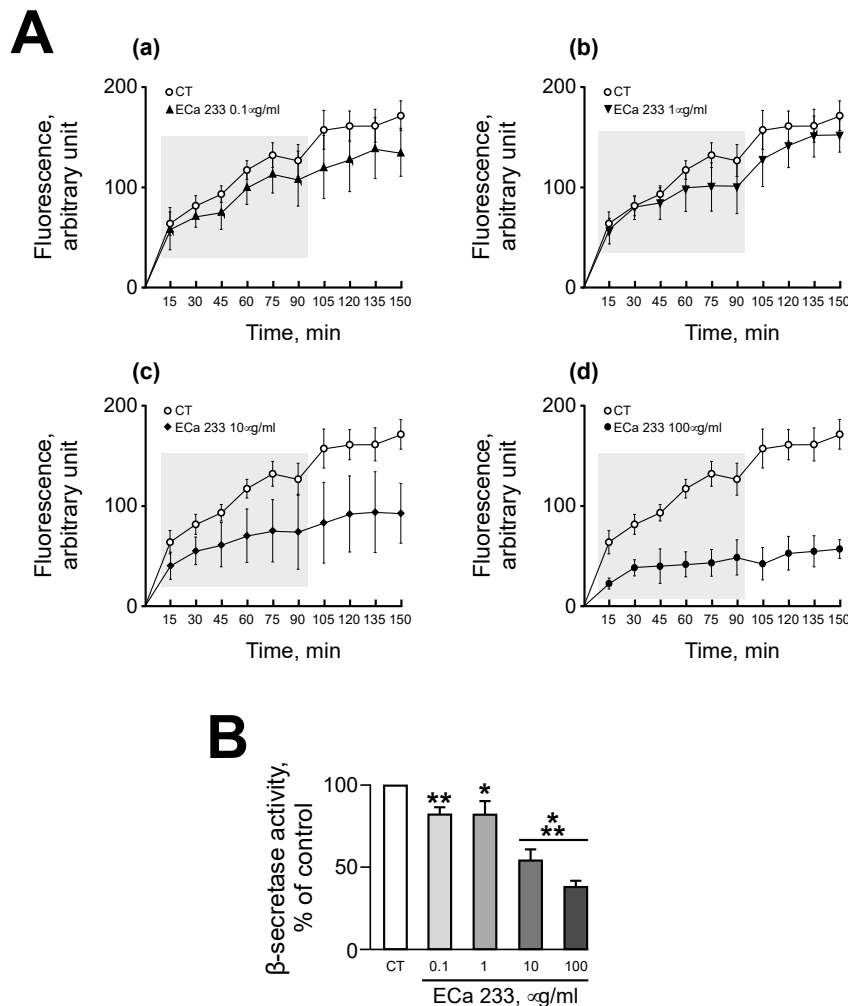


Fig. 4 Effects of ECa 233 on the β -secretase catalytic activity in homogenates of SH-SY5Y cells. (A) The β -secretase catalytic activity (JMV1197-sensitive hydrolysis of the fluorimetric substrate JMV2236) was measured in the homogenates of SH-SY5Y cells beforehand incubated in the absence (control, white circle) or in the presence of 0.1 $\mu\text{g/ml}$ (a), 1 $\mu\text{g/ml}$ (b), 10 $\mu\text{g/ml}$ (c) and 100 $\mu\text{g/ml}$ (d) of ECa 233 for 24 h. The curves represent the mean specific fluorescence \pm SE from 5 independent experiments including two controls each. (B) Statistical analysis of the data. Bars are expressed as a percentage of control (white bars, non-treated cells) calculated from the linear parts of the curves (initial velocity, light grey area) and are the means \pm SE of 24 independent determinations; * $p < 0.05$; ** $p < 0.0002$; *** $p < 0.0001$.

The possible use of *C. asiatica* extracts to counteract the development of AD is based on the observation of its beneficial effects on certain pathogenic processes associated with the disease. Firstly, they have been largely reported as antioxidant factors *in vitro* [27] as well as *in vivo* [7, 15] with asiaticoside most likely supporting this function as evidenced by its ability to increase enzymatic and non-enzymatic antioxidants [9]. Secondly, *C. asiatica* extracts were shown to convey anti-inflammatory effects *in vitro* in macrophages [28] and *in vivo* in rats [29], a property shared by the main components asiatic acid, asiaticoside, madecassic acid and madecassoside as evidenced *in vivo* [28]. Thirdly, its widely described neuroprotective properties are

arguably based on its ability to promote dendritic arborization of hippocampal neurons [30] and to stimulate nerve regeneration [31]. Finally, some possible underlying molecular mechanisms explaining its positive impact on AD, were evidenced such as an increased phosphorylation of CREB in neuroblastoma cells expressing $A\beta_{42}$ [32] and the anti-acetylcholinesterase activity of asiatic acid [33]. In the end, all of these data strongly argue in favor of a protective action of *C. asiatica* against AD pathogenesis.

Because there exists a fluctuation in the amounts of the biological active constituents in differently prepared crude extracts of *C. asiatica*, well-controlled methods were established to prepare ECa 233 as a

standardized extract of the plant that consistently contains at least 80% of the two triterpenoid glycosides madecassoside and asiaticoside with a ratio between them maintained at $1.5 \pm 0.5:1$ [23]. Importantly, ECa 233 has been previously shown to trigger a certain number of beneficial effects, both at the periphery on osteoarthritis [34] and in the central nervous system as illustrated by an anxiolytic activity in stressed mice [24], most likely via a fine tuning of neuronal activity [35], as well as a propensity to reduce ischemia-induced neuronal damage and cognitive dysfunction in rodents [36,37]. Beyond these protective effects under pathological conditions, it is interesting to note that ECa 233 also shows some memory-enhancing properties under physiological conditions in rats that is accompanied by an increase in synaptic plasticity, thereby suggesting that the extract provides memory tonic [38], a function which may be based on the fact that ECa 233 is able to stimulate neurites outgrowth as demonstrated in human neuroblastoma cells [39].

Last but not least, ECa 233 was reported to convey neuroprotection under conditions closely associated with AD pathogenesis. Hence, the extract alleviates neuroinflammation processes in LPS-treated macrophages [40] and reduces memory and learning deficits as well as hippocampal cell loss provoked by intracerebroventricular injection of A β in mice [41]. However, whether it could control the metabolism of β APP via the modulation of β APP-cleaving secretases remained until this day without answer. The present demonstration that ECa 233 can influence the metabolism of β APP in two ways, by activating its non-amyloidogenic cleavage by α -secretase and at the same time inhibiting its hydrolysis by the amyloidogenic β -secretase activity, unveil new mechanisms through which the extract can operate and adds additional weight as to its neuroprotective properties with respect to AD.

The fact that we did not evidence some statistically significant differences in sAPP α production under ECa 233 treatment, despite an observed upward trend, is most likely due to a certain lack of sensitivity of the western blot technique that is reflecting sAPP α steady state. Nevertheless, the more stringent α - and β -secretase fluorimetric assays, which specifically measure one single parameter without interfering with off-side biological processes, clearly evidenced some positive effects of the extract as shown by our findings that it displays both pro- α -secretase and anti- β -secretase properties.

It is important to underline here that ECa 233 in the concentrations that modulate secretase activities were previously shown to have no effect on cell viability in human neuroblastoma cells following 24 h treatment [39], thereby indicating harmlessness of this extract under our experimental conditions. Finally, *C. asiatica* is largely consumed as food while ECa 233

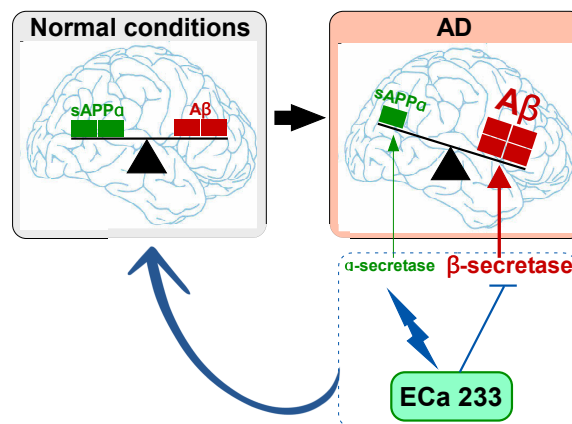


Fig. 5 Schematic outline of the doubly beneficial action of ECa 233 regarding β APP processing. Alzheimer's disease is characterized by an imbalance of β APP metabolism in favor of the amyloidogenic pathway leading to an increased A β production and a decrease in sAPP α levels. Our results suggest that ECa 233 extract-based treatment could restore this balance by doubly modulating, in an opposite way, the α -secretase activity (activation) and the β -secretase activity (inhibition).

capsules does not trigger adverse effects in humans after single and multiple ingestion of 250 or 500 mg of the extract [21]. As a whole and in light of our results, the very favorable safety profile of ECa 233 opens the way to the possible use of this extract in order to prevent the development of AD by intervening on the early mechanisms of the pathology.

CONCLUSION

Overall, our work described original findings demonstrated that the standardized *C. asiatica* extract ECa 233 conveys both pro- α -secretase and anti- β -secretase effects (Fig. 5). This indeed paves the way for the possible development, so far sparingly explored, of compounds of natural origin able to control in a doubly beneficial manner the metabolism of β APP by both decreasing the production of A β and increasing the production of the neurotrophic, neuroprotective and neurogenic sAPP α metabolite. Thus, ECa 233 is representing a new class of factors to be developed as natural therapeutic tool aimed at preventing the development of Alzheimer's disease.

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