Gamma-Secretase Inhibitor Activity of a Pterocarpus erinaceus Extract

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Abstract

Background: Accumulation of β-amloid peptides (Aβ) and its progressive deposition into amyloid plaques are key events in the aetiology of Alzheimer’s disease (AD). To date, AD treatment is symptomatic and consists of drugs treating the cognitive decline. Objective: Identifying molecules specifically targeting Aβ production or aggregation represents a huge challenge in the development of specific AD treatments. Several molecules reported as γ-secretase inhibitors or modulators have been evaluated, but so far none of them have proven to be selective or fully efficient. We have previously investigated the potential interest of plant extracts and we reported that \textit{Pterocarpus erinaceus} stem-bark extract was active on Aβ release. Our aim here was to characterize the mechanisms by which this extract reduces Aβ levels. Methods: We tested \textit{P. erinaceus} extract at non-toxic concentrations on cells expressing the human amyloid precursor protein (APP695) or its amyloidogenic β-cleaved C-terminal fragment (C99), as well as on neuronal cell lines. \textit{P. erinaceus} extract was found to inhibit Aβ release. We further showed that this extract inhibited γ-secretase activity in cell-free and in vitro assays, strongly suggesting that \textit{P. erinaceus} extract is a natural γ-secretase inhibitor. Importantly, this extract did not inhibit γ-secretase-dependent Notch intracellular domain release. Conclusion: \textit{P. erinaceus} extract appears as a new potent γ-secretase inhibitor selective towards APP processing.

Introduction

The β-amyloid peptide (Aβ) accumulation in the brain is the key event in Alzheimer’s disease (AD) amyloid cascade [1]. This can be due to Aβ peptide excessive production, its reduced clearance and/or impaired transport through the blood brain barrier. Aβ can trigger neuronal damage in different ways, e.g. by aggregating into senile plaques causing oxidative stress in the surrounding neurons and synapse disorders, by forming toxic soluble oligomers or by intraneuronal accumulation leading to neuronal apoptosis [2–4].

The metabolic pathways involved in Aβ production have been extensively studied in the last two decades [5, 6], leading to the identification of the amyloidogenic secretases, namely β- and γ-secretases. β-Secretase cleaves...
amyloid precursor protein (APP) ectodomain, generating the soluble β-APP and a membrane-anchored β-cleaved C-terminal fragment (β-CTF or C99), which is further processed by γ-secretase to release Aβ. APP can alternatively be cleaved by α-secretase into α-CTF or C83, which is also a substrate of the γ-secretase. Aβ production is precluded in this non-amyloidogenic pathway. In both pathways, γ-secretase proteolysis of α- and β-CTFs generates the soluble APP intracellular domain (AICD), acting as nuclear signalling peptide involved in the transcriptional regulation of APP target genes [7, 8].

The development of disease-modifying drugs is a major challenge and would be a sizeable asset for future AD treatments. Plants are a well-known source of new bioactive compounds with novel structures that have served throughout history and continue to serve as models enriching our therapeutic armamentarium. Many natural molecules have been isolated, acting on several targets in AD [9–11]. Phytochemicals can be sorted into different structural classes and by their mechanism of action, according to the known AD pathways. Different classes of molecules (e.g. alkaloids, terpenoids, polyketides) have been reported to inhibit β-secretase activity, but no direct inhibitor of γ-secretase has been found from natural sources so far [9], although few natural compounds may act indirectly on this enzymatic complex such as beauveriolide III acting through a decrease of acylated cholesterol [12] or luteolin through GSK-3 inhibition [13].

We previously reported [14] that the Soxhlet aqueous extract of the stem-bark of *Pterocarpus erinaceus* Poir. (Fabaceae family), used by Beninese traditional practitioners for blood-related diseases and learning problems, lowered Aβ production in cell models. In order to characterize the mechanism of the active extract and to be able to evaluate its therapeutic interest, we decided to further investigate how the extract precisely regulates Aβ production by addressing the following questions: (1) does the extract inhibit the amyloidogenic processing, in particular γ-secretase activity, and (2) is the inhibition by *P. erinaceus* extract specific towards APP processing?

We report here that *P. erinaceus* extract reduces Aβ production by directly inhibiting γ-secretase activity. Interestingly, the effect observed can be compared to that of DAPT, a well-known γ-secretase inhibitor [15] in both treated cells and cell-free assays. Furthermore, the treatment of cells with *P. erinaceus* extract does not functionally inhibit the γ-secretase-dependent cleavage of Notch, as evidenced by Notch intracellular domain (NICD) transactivation assay and NICD release in the cell. Together, these results strongly support the idea that *P. erinaceus* aqueous extract acts as an APP-specific γ-secretase modulator.

### Materials and Methods

**Chemicals and Reagents**

Analytical-grade and UPLC-grade solvents were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany), epicatechin and catechin standards from Extrasynthese (Genay, France), CHAPS from Merck KGaA (Darmstadt, Germany), culture media and reagents, Fungizone (ampoterin B), Lipopectamine and NuPAGE 4–12% bis-tris gels from Invitrogen (Carlsbad, Calif., USA), and penicillin-streptomycin solution and G418 from BioWhittaker (Walkersville, Md., USA). Reagents for luciferase assay (Dual-Glo luciferase assay kit) were purchased from Promega (Madison, Wisc., USA), DAPT from Calbiochem (Carmarillo, Calif., USA), pCS2 Notch1 ΔEMV-6MT plasmid [16] from Aldgene (Cambridge, Mass., USA), and Complete™ protease inhibitor cocktail from Roche (Basel, Switzerland). Human-specific anti-APP WO-2 antibody was obtained from Millipore (Billerica, Mass., USA), anti-APP C-terminal antibody from Sigma-Aldrich (St. Louis, Mo., USA), anti-myc antibody from Cell Signalling (Danvers, Mass., USA), their secondary antibodies from Amersham Bioscience (Uppsal, Sweden), ECL reagents from Perkin Elmer Inc. (Waltham, Mass., USA), mouse-specific anti-βIIITub antibody from Chemicon International (Temecula, Calif., USA), anti-MAP2 antibody and fluorescent nucleic acid stain DAPI from Sigma-Aldrich, and Alexa-labelled secondary antibodies from Molecular Probes, Invitrogen. For tannin quantification, swine hide powder was obtained from SCR European Pharmacopoeia; phospho-molybdo-tungstic reagent was prepared according to the European Pharmacopoeia (2012); the necessary salts for this preparation, as well as Na2CO3, were purchased from Acros (Geel, Belgium).

**Plant Material and Extract Preparation**

Stem-bark of *P. erinaceus* Poir. was collected and vouchered in Benin and dried, powdered and extracted on a Soxhlet apparatus as previously described [14]. The yield of the water extract, calculated according to the weight of dry plant powder, was 23.55%, being very close to the yield obtained previously. For the assays, aqueous extract was dissolved at 20 mg/ml in water-ethanol 2:1 and used at concentrations that were previously shown to be non-toxic [14].

**HPLC-PDA-ESI-HRMS and Extract Fingerprint**

HPLC analysis was performed on an Accela HPLC system hyphenated to a PDA and an LTQ-Orbitrap XL mass spectrometry system, as already described [17], with the following modifications. HPLC separation was performed on a reverse-phase C18 column (LiChrospher-LiChroCART RP18e, 250 mm length, 4.6 mm width, 5 μm of particle diameter) equipped with a C18 pre-column (5 mm length), both from Merck Millipore (Darmstadt, Germany). Migration was done at 800 μl/min with two eluents: (1) H2O-MeOH-HCOOH 90:10:0.1 and (2) MeOH with HCOOH 0.1%, and with the following gradient: 0–40 min, B 0–50%; 40–60 min, B 50–100%; 60–65 min, B 100%; 65–66 min, B 100–0%; 66–76 min, B 0%. The conditions of the ESI in-
terface (used in positive ionization mode) were: ion spray voltage +5 kV, capillary temperature and voltage 275°C and +5 V and tube lens voltage +45 V. Prior to injection to HPLC, P. erinaceus extract sample was dissolved in MeOH (4 mg/ml). Reference compounds were used for comparisons of retention times and mass spectrometry.

**Cell Culture, Transfection and Treatment**

The Chinese hamster ovary (CHO) cells stably expressing human APP695 were cultivated as previously described [14]. C99 (corresponding to β-CTF) was transiently expressed by transfection of CHO cells with pSVK3-C99 plasmid [18]. Mouse embryonic fibroblast (MEF) cell lines were cultured in DMEM/Ham’s F12 supplemented with 10% FBS and antibiotics. NG108-15 cells were plated in monolayers on culture dishes pretreated with 10 μg/ml poly-L-lysine in phosphate-buffered saline (PBS), were cultured in DMEM with high concentrations of glucose and supplemented with 10 mM hypoxanthine, 0.1 mM aminopterin, 1.6 mM thymidine and 10% FBS and were maintained at 37°C in a 5% CO₂ atmosphere; neuronal differentiation was induced by reducing FBS concentration to 1%. CHO and NG108-15 cells were treated by P. erinaceus extract or DAPT at indicated concentrations 1 day after transfection or after differentiation (7 days of culture), respectively. Culture media were harvested 16 h after treatments and recovered by quick centrifugation (28,000 g).

**Immunofluorescence**

Differentiated NG108-15 cells were fixed with 4% v/v formaldehyde in PBS at room temperature for 15 min and then permeabilized with 0.25% Triton X100 (v/v) in PBS for 30 min. Non-specific binding was prevented by 30 min of preincubation in 5% goat serum/Triton 0.1% in PBS, followed by overnight incubation with primary antibodies βIIITub (1:6,000) and MAP2 (1:500). After 3 washes with PBS, samples were incubated for 1 h with Alexa-labelled secondary antibody (1:500) and DAPI (1:2,000). After 3 additional washes, preparations were mounted in Fluoprep (BioMérieux) and examined with an EVOS fluorescence microscope (AMG, Mill Creek, Wash., USA).

**Western Blotting**

Western blotting was performed on 4–12% NuPAGE bis-tris gels [14]. Membranes were incubated overnight (4°C) with the primary antibody (0.5 μg/ml for the human APP-specific WO-2 antibody, 1/5,000 for the anti-APP C-terminal antibody and 1/1,000 for the anti-nect antibody), then washed and incubated for 1 h with horseradish-peroxidase-conjugated secondary antibody (1/10,000 of the anti-mouse sheep IgG, 1/15,000 of the anti-rabbit donkey IgG) and revealed by electrochemiluminescence. Signals were quantified using the Quantity One® software coupled to the Gel Doc 2000 device (Bio-Rad, Hercules, Calif., USA).

**Aβ Quantification**

Aβ₃₈, Aβ₄₀, and Aβ₄₂ were quantified in culture media by multiplex Electro-Chemiluminescence Immuno-Assay (ECLIA), using 6E10 (human Aβ triplex kit) and 4G8 (rodent Aβ triplex kit) antibodies and MSD SECTOR®Imager 2400 (Meso Scale Discovery, Gaithersburg, Md., USA) following a procedure already described [14]. Preliminary experiments and plate design in triplex assays allowed ensuring that there were no significant cross-reactions between Aβ₃₈, Aβ₄₀ and Aβ₄₂ quantifications.

**Cell-Free APP Cleavage Assay**

Measurements of γ-secretase activity in cell-free assays were adapted from previous procedures [19, 20]. All steps were carried out at 4°C unless otherwise indicated. Confluent CHO-APP cells were washed with PBS, scraped in 0.5 ml hypotonic MOPS buffer (1 mM MOPS, 1 mM KCl, pH 7), incubated for 10 min on ice and homogenized in a tight-fitting homogenizing syringe. Homogenates were cleared by centrifugation at 1,000 g for 15 min to remove cell debris and nuclei. The post-nuclear supernatants were centrifuged (40 min at 16,000 g), and the pellets containing crude membranes were recovered in an assay buffer (150 mM sodium citrate, pH 6.4) with either DAPT (250 nM or 10 μM) or P. erinaceus extract (200 μg/ml). Membranes were incubated at 37°C for 2 h. Samples were put on ice at the end of the incubation, sonicated in Laemmli buffer and loaded for Western blotting to monitor AICD release. Preliminary experiments were carried out to define optimal incubation time (2 h) for AICD detection.

**In vitro Fluorogenic γ-Secretase Assays**

γ-Secretase assays with small fluorogenic peptides were adapted from Wang et al. [21]. Mouse embryonic fibroblast (MEF) knock-out cells for PS1 and PS2 (PS−) were used as negative controls [22]. All steps were carried out at 4°C unless otherwise indicated. CHO and MEF cells were recovered by centrifugation at 500 g for 5 min in PBS. Pellets were homogenized in lysis buffer (5 mM Tris-HCl, 5 mM EDTA, 5 mM EGTA, pH 7.4, containing Complete protease inhibitor cocktail). The homogenates were centrifuged at 800 g for 10 min to remove nuclei and large debris, which were homogenized and centrifuged again. The pooled supernatants were centrifuged at 25,000 g for 1 h and the membrane pellets were resuspended in a reaction buffer (50 mM Tris-HCl, 2 mM EDTA, 150 mM KCl, pH 6.8, containing Complete protease inhibitor cocktail). Membranes were homogenized by gentle agitation for 1 h and adjusted to 1 mg/ml of protein in a solubilizing buffer (50 mM Tris-HCl, 2 mM EDTA, 150 mM KCl, pH 6.8, 0.25% CHAPS). Membrane protein (30 μg) samples were prepared in a final volume of 100 μl in reaction buffer containing (or not) the molecule/extract to be tested. Negative controls (without membranes or fluorogenic probe) were performed in the same conditions. Samples were incubated for 3 min at 37°C prior to adding fluorprobe (6 μM), and further incubated for 5 h at 37°C. Reaction was stopped on ice, and samples were centrifuged for 15 min at 16,100 g. Fluorescence was monitored on supernatants (excitation/emission 335/440 nm) using a Victor X3 Multilabel Plate Reader (PerkinElmer, Waltham, Mass., USA). Two fluoroprobes were used: the first one (Nma-G-G-V-Y-L-A-T-V-K(Dnp)R-R-R-NH₂; Merck) mimics the γ-cleavage site of APP, and the second one (Nma-G-C-G-V-L-L-K(Dnp)R-R-NH₂; Sigma-Aldrich) mimics the GVI sequence of the S3 Notch γ-cleavage site. In preliminary experiments, optimal incubation time was found to be 5 h for protein membrane concentrations above 150 μg/ml.

**Reporter Gene Assay (Dual Luciferase Assay)**

CHO cells were seeded in 12-well plates and co-transfected 24 h later with the HES1-luciferase reporter gene [22] and the pRL-TK Renilla luciferase control vector (Promega) at a 4:1 ratio, to correct for transfection efficiency; 1 day after transfection, the cells were treated for 16 h prior to measurement of luciferase activities, following the manufacturer’s instructions (Dual-Glo luciferase assay; Promega). MEF cells were seeded in 24-well plates and co...

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transfected 24 h later with the pCS2 Notch1 ΔEMV-6MT plasmid [16] and the two reporter luciferase constructs described above; 1 day after transfection the cells were treated for 16 h, washed with PBS and recovered either for luciferase activity or for Western blotting using the anti-myc antibody to monitor NICD production.

Statistical Analysis
The number of samples under each experimental condition is indicated in the figure legends. Statistical analyses were done by unpaired t test or ANOVA followed by Tukey’s multiple comparison post hoc test (Prism 4.0 and 6.0; GraphPad Software Inc., San Diego, Calif., USA).

Results

P. erinaceus Extract HPLC-MS Fingerprint
P. erinaceus extract was submitted to an HPLC-PDA-MS analysis. The fingerprint chromatogram is given in figure 1. The peak at 27.15 min corresponds to epicatechin ([M + H]+ = 291.0967 amu); some (epi)catechin dimers and trimers, respectively. Tan-nins were also detected, with a foaming index of about 400, according to the Pharmacopée Française (2010).

P. erinaceus Extract Displays γ-Secretase Inhibitor-Like Effects on Aβ Production and APP Processing
P. erinaceus stem-bark water extract was used at concentrations that we previously showed to be non-toxic [14]. Its effects on Aβ production and APP processing were tested on CHO cells expressing either human APP695 or C99; the latter corresponds to the cleaved β-CTF fused to the signal peptide of APP (fig. 2a), a construct that has been widely used to focus on γ-secretase cleavage in Aβ production [18]. The effects of P. erinaceus extract were compared to those of DAPT, a reference γ-secretase inhibitor [15]. After 16 h of treatment the CTF/APP ratio was measured in cell lysates by Western blotting, and the medium was collected for Aβ quantification by ECLIA. Accumulation of CTFs was observed upon treatment of CHO-APP by DAPT or by P. erinaceus extract (fig. 2b, c). The same treatment did not increase soluble APP (α and β) release from APP-expressing cells as measured by

Fig. 1. HPLC-ESI-HR-MS of P. erinaceus extract. Chromatograms in ESI-MS positive ion mode. a Total ion current. b Selected ions (289–292, and 576–580, and 864–867 amu) corresponding to epicatechin (peak indicated by an arrow) and (epi)catechin dimers and trimers, respectively.
ECLIA (data not shown); the accumulation of CTFs can therefore be considered as the hallmark of a decreased γ-cleavage. Using ECLIA, we measured Aβ concentration in the extracellular medium of CHO-APP cells treated with P. erinaceus extract or DAPT. A significant decrease in Aβ38, Aβ40 and Aβ42 was observed after treatment by P. erinaceus extract at both 200 and 100 μg/ml (fig. 2d, e).

It is important to note here that, although the effects of P. erinaceus extract and DAPT were similar on CTF accumulation, DAPT at 250 nM showed more potent inhibitory effects on Aβ38 and Aβ40 production than P. erinaceus at the highest non-toxic concentration (200 μg/ml).

We carried out similar experiments on cells expressing C99, corresponding to the β-CTF fused to the signal peptide of APP. This construct bypasses the first amyloidogenic cleavage (β-) and thus allows focus on γ-cleavage in Aβ production. An accumulation of CTFs is also observed upon treatment by DAPT or P. erinaceus extract, but it is of note that the accumulation is mainly an accumulation of α-CTF (fig. 3a). Indeed, it has been previously shown that β-CTF or C99, which are the amyloidogenic substrates of γ-secretase, are efficiently converted to α-CTF by the cells prior to γ-cleavage. Inhibition of γ-secretase therefore results in the accumulation of α-CTFs [18]. Furthermore, treatment by P. erinaceus extract at 200 μg/ml leads to a significant decrease in Aβ38, Aβ40 and Aβ42 (fig. 3b, c).

Together, our results indicated that P. erinaceus extract was likely to decrease Aβ production by inhibiting the γ-cleavage of APP. We further studied in cell-free and in vitro assays whether this extract could directly inhibit γ-secretase activity.

**P. erinaceus Extract Inhibits γ-Secretase Activity in Cell-Free and in vitro Assays**

Membranes isolated from CHO cells expressing human APP were treated for 2 h by DAPT or P. erinaceus extract.
extract at several concentrations. The cleavage of APP by γ-secretase was monitored by the accumulation of AICD in the assay medium [20]. We normalized AICD levels to CTFs present in the membrane assays. Both DAPT and *P. erinaceus* extract significantly decreased AICD release (fig. 4 a, b). The effect of *P. erinaceus* extract was concentration-dependant but less potent than the one observed for DAPT, which is consistent with the data obtained on Aβ production (fig. 2 c, e). It is also interesting to note that the maximal inhibitory effects of DAPT were reached in cell-free assays at concentrations at least 10-fold higher than those used on living cells to block Aβ production (fig. 2 e, 3 c, 4 b).

We next sought to measure the direct inhibition of γ-secretase by *P. erinaceus* extract by an in vitro assay using cell membrane preparations and a γ-secretase fluorogenic substrate mimicking the APP γ-site. To validate this assay, we first carried out the experiments on PS+ (expressing) or PS– (double knock-out for presenilins 1 and 2) MEF cells. γ-Secretase activity is abolished by the absence of PS1 and PS2 [22]. The fluorescence signal measured with membranes from PS– cells was about 53% of the signal measured in PS+ cells (fig. 5 a). This remaining signal is due to contaminating enzymatic activities which are not related to genuine γ-secretase, as shown by the lack of effect of DAPT and *P. erinaceus* extract in PS– cells (fig. 5 a). DAPT and *P. erinaceus* extract were further tested at different concentrations on γ-secretase activity from CHO membrane preparations – the cell type used to anal-

### Fig. 3. Effects of *P. erinaceus* extract on Aβ production in CHO cells expressing C99. Ctl = Control; Pteroc. = *P. erinaceus*. a Western-blot analysis of cell lysates from CHO-C99 cells treated with DAPT or *P. erinaceus* aqueous extract. Cells were treated for 16 h with *P. erinaceus* extract at 200 μg/ml or with 250 nM DAPT. Western blots were revealed with the WO-2 (upper panel) or the C-ter antibody (lower panel). The expected positions of α-CTF and C99 are indicated. NT = Non-transfected cells. b Measurement of Aβ concentration in extracellular media from CHO-C99 cells. Cells were treated for 16 h as indicated; human Aβ isoforms (38, 40, 42) were quantified by multiplex ECLIA. Results (means ± SEM) are given as pg/ml. c Aβ measurements were normalized and results are shown as percentage non-treated cells (Ctl). * p < 0.05; ** p < 0.01 (n = 6).

### Table 1. Effect of *P. erinaceus* extract on Aβ production in CHO cells expressing C99.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Aβ38 (pg/ml)</th>
<th>Aβ40 (pg/ml)</th>
<th>Aβ42 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctl</td>
<td>224.0 ± 9.5</td>
<td>1494.9 ± 130</td>
<td>128.3 ± 74.4</td>
</tr>
<tr>
<td>DAPT</td>
<td>9.5 ± 0.0</td>
<td>130.2 ± 4.3</td>
<td>6.4 ± 0.0</td>
</tr>
<tr>
<td><em>P. erinaceus</em> 200</td>
<td>178.2 ± 8.8</td>
<td>816.2 ± 57.8</td>
<td>74.4 ± 7.5</td>
</tr>
</tbody>
</table>

### Fig. 4. Cell-free assays. Membranes were isolated from CHO cells expressing human APP695 and incubated in vitro. Ctl = Control; Pteroc. = *P. erinaceus*. a Western blotting of the treated membranes analysed with C-ter antibody; the expected positions of α-CTF, β-CTF and AICD are indicated. b Quantification of AICD/CTF ratio. Results (means ± SEM) were normalized to control (non-treated, 2 h of incubation). * p < 0.05; ** p < 0.01 (n = 4).
yse APP processing and Aβ production (fig. 2, 3). Both P. erinaceus extract and DAPT displayed dose-dependent inhibition of substrate cleavage, the maximal effects being reached at 250 μg/ml for P. erinaceus and 10 μM for DAPT (fig. 5b), which is in line with the results obtained in cell-free assays. Note that the remaining fluorescence measured at these concentrations was similar to that measured in MEF PS– and PS+ cells after similar treatments (fig. 5a).

**Notch Cleavage by γ-Secretase Is Not Inhibited by P. erinaceus Extract**

We repeated the previous membrane assay on CHO membranes using fluorogenic peptides mimicking either the APP or Notch γ-cleavage sites. CHO membranes were treated with DAPT and P. erinaceus extract in order to compare the cleavage of APP and Notch fluoroprobes (fig. 6a). Both treatments displayed an inhibition of substrate cleavage, but P. erinaceus bark extract at 200 μg/ml was significantly more active against the APP-mimicking substrate than against the Notch-mimicking substrate (p = 0.02). The remaining fluorescence signals measured with DAPT or extract were similar to the signal measured in PS– cell membrane preparations (fig. 5a).

We further assessed the effect of P. erinaceus extract on Notch γ-cleavage in cells by a transactivation assay [22]. Notch γ-cleavage releases the NICD that activates transcription of Notch target genes [23], among which HES1 (hairy and enhancer of split 1). Transcriptional activity of an HES1-luciferase reporter gene provides a good functional measurement of NICD production. To evaluate NICD production by both Western blotting and transactivation assay, MEF cells were co-transfected with myc-tagged ΔE-Notch and dual reporter genes (fig. 6b, c). In PS– cells, the transcriptional activity of the HES1 promoter is reduced by 50% compared to control PS+ cells (fig. 6c). DAPT treatment (at 1 μM and above) significantly inhibited NICD-dependent transactivation. Under the same conditions, NICD production was abolished as shown by Western blotting (fig. 6b).

Strikingly, P. erinaceus extract, even at the highest concentration tested (200 μg/ml), did not decrease HES1 reporter gene transactivation activity, and did not affect NICD release from ΔE-Notch construct (fig. 6b, c). Inhibitory effects of DAPT and P. erinaceus have finally been tested on endogenously expressed Notch in CHO cells. In line with the results in MEF cells, DAPT (at 1 μM and above) significantly reduced luciferase activity whereas no inhibitory effects were detected even at the highest concentration (200 μg/ml) of P. erinaceus extract (fig. 6d).

Together, these data indicated that P. erinaceus extract selectively inhibited the γ-cleavage of APP but had no significant effect on Notch processing.

**P. erinaceus Extract Decreases Aβ in Cultured Neuronal Lines**

We finally evaluated the effects of P. erinaceus extract on neuronal cells. Differentiated NG108-15 cell lines exhibited specific neuronal proteins and morphological
characteristics as shown by immunocytochemistry. We observed a positive staining for protein markers of neuronal differentiation: βIIIITub and MAP2, recognising microtubules of neuronal-specific origin and dendrites, respectively (fig. 7a, b). APP processing and Aβ production were tested on differentiated neuronal cells treated with P. erinaceus extract and DAPT. After 16 h of treatment, CTF accumulation was measured in cell lysates by Western blotting (fig. 7c) and the medium was collected for Aβ quantification by ECLIA (fig. 7d).

**Fig. 6.** Effect of P. erinaceus extract on NICD release; 3 experiments were done to assess the selectivity of the extract for APP vs. Notch processing. Ctl = Control; Pteroc. = P. erinaceus. a The in vitro γ-secretase assay was carried out on membranes isolated from CHO cells and incubated for 5 h with DAPT and P. erinaceus extract, in the presence of 1 of 2 fluoroprobes (mimicking either APP γ-site or Notch S3-site) as indicated. Results (means ± SEM) are given as normalized relative fluorescence values. * p < 0.05; ** p < 0.01 (n = 3 at least) compared to control (non-treated). b, c MEF cells were co-transfected with a HES1-luciferase reporter gene, the pRL-TK Renilla construct and myc-tagged ΔE-Notch plasmid, and treated with P. erinaceus extract or DAPT at the indicated concentrations. NICD release was measured by Western blotting, using an anti-myc antibody (b) and a transactivation assay (c). d CHO cells were transfected with a HES1-luciferase reporter gene and the pRL-TK Renilla construct and treated with DAPT or with P. erinaceus extract at the indicated concentrations. Endogenous NICD release was measured by a functional transactivation assay. Luminescence values (c, d) were normalized by calculating firefly luciferase/Renilla luciferase ratio. Results (means ± SEM) are given as percentage of activity measured in control cells (non-treated PS+ MEF or CHO, respectively). * p < 0.05; ** p < 0.01 (n = 6) compared to control.
Accumulation of CTFs was observed upon treatment by *P. erinaceus* extract at the highest non-toxic concentration of 100 μg/ml or by 5 μM DAPT, the reference γ-secretase inhibitor, demonstrating a decreased γ-cleavage. In the extracellular medium of neuronal cells, Aβ40 and Aβ42 levels were significantly decreased after treatment by *P. erinaceus* extract in a concentration-dependent manner at 75 and 100 μg/ml (fig. 7d).

**Discussion**

Up to now, several studies have reported a potential therapeutic interest for plant extracts, especially those used in traditional medicine, in the treatment of AD [24]; one of them is the standardized extract of *Ginkgo biloba* (EGb761), which is an officially registered drug against AD in Germany and in Belgium, and which works through polyvalent activities, including inhibition of Aβ oligomerization and neurotoxicity [25, 26]. Currently, the five other drugs that are approved for AD are either acetylcholinesterase inhibitors or modulators of the glutamatergic transmission. Among the former class, galantamine is an alkaloid occurring in several Amaryllidaceae, and rivastigmine is a derivate of the natural alkaloid eserine. Numerous other natural compounds were found to act on several AD-related mechanisms [9], mostly by inhibiting cholinesterase and/or Aβ oligomerization (e.g. rosmarinic acid), by inhibiting β-secretase and/or tau hyperphosphorylation (e.g. luteolin), or by promoting synaptic plasticity and nerve growth (e.g. catalpol) [9, 27, 28].

**Fig. 7.** Effect of *P. erinaceus* extract on neuronal cells. Ctl = Control; Pteroc. = *P. erinaceus*. Differentiated NG108-15 cells were stained for the neuronal proteins βIIIITub (a) and MAP2 (b). Scale bar, 200 μm. c Western blot analysis of cell lysates from differentiated NG108-15 cells treated with DAPT or *P. erinaceus* aqueous extract. Cells were treated for 16 h with *P. erinaceus* extract at 50, 75 and 100 μg/ml or with 250 nM and 5 μM DAPT. Western blots were revealed with the C-ter antibody. d Measurement of Aβ concentration in extracellular media from differentiated NG108-15 cells. Cells were treated for 16 h as indicated; rodent Aβ isoforms (38, 40, 42) were quantified by multiplex ECLIA (4G8 antibody). Aβ measurements were normalized and results (means ± SEM) are shown as percentage of non-treated cells (Ctl). * p < 0.05; ** p < 0.001 (n = 3).
The current registered treatments were developed as symptomatic treatments and unfortunately do not block the progression of the disease. It is therefore of prime importance to discover new molecules or approaches with disease-modifying properties. In our study, we report the γ-secretase modulating properties of a medicinal plant extract (P. erinaceus) that presents an interesting therapeutic potential.

In a previous study [14], we evaluated the modulatory effects of a wide range of plant natural extracts on Aβ production at non-toxic concentrations. P. erinaceus extract was shown to decrease Aβ levels in the culture medium from CHO cells. Here, we report that this extract significantly decreases extracellular levels of Aβ from CHO cells expressing either human APP695 or C99, which corresponds to the amyloidogenic CTF of APP and neuronal cells. The effects of the plant extract were systematically compared throughout the study to those of DAPT, a reference γ-secretase inhibitor. The inhibitory effects of P. erinaceus extract on Aβ production measured in CHO cells were very significant but still less important than those of DAPT at sub-maximal concentration (250 nM; see fig. 2, 3), particularly in cells expressing C99 (fig. 3). Although C99 corresponds to the β-CTF of APP, one must keep in mind that, when directly expressed, it is present in compartments where β-CTF is not necessarily located, and might thus be less accessible to some classes of inhibitors. We also observed that DAPT inhibited more potently the production of the short Aβ isoforms (Aβ38 compared to Aβ42), whereas P. erinaceus extract at 200 μg/ml displayed similar effects to those observed for DAPT at 250 nM on Aβ42 released from CHO cells expressing human APP695. In neuronal cells, P. erinaceus extract significantly inhibited Aβ release, but its effects were weaker than in transfected CHO cells, in particular on Aβ38 isoform. This could result from the APP isoforms studied (human C99 vs. endogenous rodent APP) and underlines the importance of evaluating active compounds in different cell lines, including neurons. However, it is particularly interesting to note that P. erinaceus extract also potently inhibits Aβ42 in neuronal models, since Aβ42 is the isoform reported to be the most toxic when it accumulates in the brain or inside the neurons [3, 29].

γ-Secretase Inhibition by P. erinaceus Extract

A decrease in Aβ levels in cell media could result mainly from decreased production, from inhibition of secretion or from accelerated degradation. The fact that inhibitory effects were measured on C99 directly expressed in the cells argues in favour of an inhibition of γ-secretase. Other interpretations could still be proposed in addition to an effect of γ-cleavage. For instance, P. erinaceus extract could also act on APP/C99 trafficking which is a key mechanism for these substrates to meet the secretases [30, 31]. Two in vitro assays were carried out to measure the direct effects of the plant extract on γ-secretase activity – measurement of APP γ-cleavage on crude membrane preparations and in vitro measurement of γ-secretase activity with a fluorogenic substrate. Assays on crude membrane preparations were adapted from previously described procedures [19, 20] and an in vitro enzymatic assay using a fluorogenic substrate [21] was newly established. These assays were crucial to show a direct effect of the molecules on γ-secretase activity, avoiding the possible interferences due to side effects on cell metabolism or cell trafficking. In appropriate experimental conditions, the use of fluorogenic substrates allows a quantitative measurement of γ-secretase catalytic activity. However, experiments carried out in PS+ and PS− MEF cells revealed that a sizeable background was due to PS-independent enzymatic activities (fig. 5a). This is probably resulting from the ability of a γ-secretase synthetic substrate to interact in these assays with enzymes that the endogenous substrates will not meet in a compartmentalized living cell. Nevertheless, we observed that pharmacological inhibition of γ-secretase and the absence of presenilins led to identical and sizeable inhibition in enzymatic assays, showing that genuine γ-secretase activity was measured. For instance, none of the compounds tested had inhibitory effects in PS− cell extracts (fig. 5a). P. erinaceus extract and DAPT inhibited γ-secretase activity in a dose-dependent manner (fig. 5b). Complementary assays also showed that P. erinaceus extract and DAPT directly inhibited γ-secretase activity. In the crude membrane extract assay (fig. 4a), AICD production was concomitant to a decrease in β-CTF but not α-CTF, which is also in line with the recent hypothesis that AICD is preferentially produced from APP in a β-secretase dependent manner [32]. It is of note that inhibition of DAPT in in vitro assays was observed at concentrations higher than those used to achieve inhibition of Aβ production in cultured cells (10 μM vs. 250 nM). On the contrary, P. erinaceus extract displayed similar effects on Aβ release and γ-secretase activity at the concentration of 200 μg/ml. Taken together, our data indicate that P. erinaceus extract directly inhibits γ-secretase activity. We cannot firmly rule out at this stage the hypothesis that cellular processes like endocytosis, which are known to be essential in Aβ production [31], could also be targeted in the effects of P. erinaceus extract observed in treated cells.

Hage/Marinangeli/Stanga/Octave/Quetin-Leclercq/Kienlen-Campard

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Evaluation of *P. erinaceus* effects on cell biology and cell trafficking await further experiments which are beyond the scope of this study.

**Selectivity of *P. erinaceus* Extracts towards APP Cleavage**

Another issue that needs to be addressed for γ-secretase inhibitors/modulators, from a therapeutic perspective, is their selectivity. The different γ-secretases are intricate and unusual proteolytic complexes [33] with an increasing number of substrates [34]. Some of them such as the Notch proteins are critically involved in cell functions; in fact, the PS1 KO lethal phenotype is linked to the loss of Notch function during development [35]. It is therefore of prime importance to isolate molecules able to modulate APP processing without displaying detrimental Notch-dependent side effects [23, 36].

Indeed, much concern has arisen about γ-secretase inhibitors in vivo; the few compounds that reached clinical assays were stopped either because of lack of cognitive improvement (tarenflurbil) or because of heavy side effects, mainly Notch-related, including carcinoma (semagacestat, avagacestat) [37]. APP-selective γ-secretase inhibitors are still needed as an alternative to the current symptomatic treatments because, on one hand, even a partial inhibition of this enzyme would decrease Aβ production enough to prevent oligomerization and also reverse the cognitive decline in Tg2576 mice [38], and on the other hand, selectivity should prevent the side effects observed up to now.

We used a transactivation assay and Western blotting to measure Notch cleavage and further NICD release in whole cells. To that end, we used a previously described set-up [16] in MEF cells. Cells were transfected by a ΔE-Notch construct allowing us to monitor the myc-tagged NICD release and to measure NICD activity by the transactivation of a HES1-luciferase reporter gene. In contrast to DAPT, the *P. erinaceus* extract did not inhibit NICD-dependent transactivation of the HES1 promoter and did not block the release of NICD from ΔE-Notch (fig. 6b, c). Comparable results were obtained for endogenous NICD release in CHO cells (fig. 6d). Transcriptional activity observed in PS– cells or upon γ-secretase inhibition reflects basal regulation by other transcription factors. Taken together, our results strongly support the hypothesis that *P. erinaceus* extract inhibits the γ-cleavage of APP without affecting Notch. This is a key observation for the potential therapeutic interest of this plant extract.

The selectivity towards APP has already been described for some other rare chemicals [39], but it relies on an unknown molecular basis. This could be due to the particular structural features of the APP transmembrane domain compared to other γ-secretase substrates [18, 34], and in particular to the structure of the transmembrane helices that define the cleavage properties of APP [40]. Another explanation for the variation of activities of the γ-secretase (especially for APP γ-cleavage vs. Notch S3/APP ε-cleavage) could be found in the manifold interactions with other membrane entities [41, 42].

**Conclusion**

*P. erinaceus* bark aqueous extract, used in Beninese traditional medicine to treat cognitive deficits, appears promising in treating amyloidosis in AD. In cellular models, including neuronal cell lines, it strongly decreases production of Aβ by inhibiting γ-secretase-mediated cleavage of APP. The direct inhibition of γ-secretase was also assessed in enzymatic assays in isolated membranes. No molecule directly inhibiting γ-secretase has been isolated from natural sources until now [9]. Furthermore, the extract was shown in cellular models not to inhibit Notch S3 cleavage, which is a major concern for the therapeutic use of γ-secretase inhibitors because of the strong side effects linked to Notch cascade inhibition.

The next step should be the isolation and identification by bio-guided fractionation of the constituents responsible for the observed in vitro activities of the plant extract. *P. erinaceus* is known to contain catechic polyphenols, which seem to be at least partly responsible for the observed activities. Although the activity was lowered after tannin removal, a sizeable inhibitory effect remained, indicating that other classes of molecules are also active [14]. Catechin tannins comprise small but also high-molecular-weight molecules which are broken down in the intestine to smaller polyphenols that are absorbed and cross the blood-brain barrier in vivo [43], several of them having shown a specificity for AD-related events in vivo, e.g. protection against neurodegeneration, decrease of amyloid deposition in the brain and inhibition of tau aggregation [9, 44].

Further studies are needed in animals to test the efficiency of *P. erinaceus* extracts in vivo (and on a longer term than can be done in vitro) to assess their safety and bioavailability, which is especially critical for potential drugs intended to act in the central nervous system [36]. This will hopefully fully confirm the therapeutic interest of *P. erinaceus* extracts for AD.
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