

Effects of γ -Secretase Inhibition on the Amyloid β Isoform Pattern in a Mouse Model of Alzheimer's Disease

Erik Portelius^a Bin Zhang^b Mikael K. Gustavsson^a Gunnar Brinkmalm^a
Ann Westman-Brinkmalm^a Henrik Zetterberg^a Virginia M.-Y. Lee^b
John Q. Trojanowski^b Kaj Blennow^a

^aInstitute of Neuroscience and Physiology, Department of Psychiatry and Neurochemistry, Sahlgrenska Academy, University of Gothenburg, Mölndal, Sweden; ^bCenter for Neurodegenerative Disease Research, Department of Pathology and Laboratory Medicine, and Institute on Aging, University of Pennsylvania School of Medicine, Philadelphia, Pa., USA

Key Words

Alzheimer's · Amyloid β · Mass spectrometry · γ -Secretase · Immunoprecipitation · Transgenic mouse

Abstract

Background: Accumulation of amyloid β (A β) in the brain is believed to represent one of the earliest events in the Alzheimer disease process. A β is generated from amyloid precursor protein after sequential cleavage by β - and γ -secretase. Alternatively, α -secretase cleaves within the A β sequence, thus, precluding the formation of A β . A lot of research has focused on A β production, while less is known about the non-amyloidogenic pathway. We have previously shown that A β is present in human cerebrospinal fluid (CSF) as several shorter C-terminal truncated isoforms (e.g. A β 1–15 and A β 1–16), and that the levels of these shorter isoforms are elevated in media from cells that have been treated with γ -secretase inhibitors. **Objective:** To explore the effect of N-[N-(3,5-difluorophenacetyl-L-alanyl)]-5-phenylglycine t-butyl ester (DAPT), a γ -secretase-inhibitor, treatment on the A β isoform pattern in brain tissue and CSF from Tg2576 mice. **Methods:** Immunoprecipitation using the anti-A β antibodies 6E10 and 4G8 was combined with either matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

try or nanoflow liquid chromatography and tandem mass spectrometry. **Results:** All fragments longer than and including A β 1–17 displayed a tendency towards decreased levels upon γ -secretase inhibition, whereas A β 1–15 and A β 1–16 indicated slightly elevated levels during treatment. **Conclusion:** These data suggest that A β 1–15 and A β 1–16 may be generated through a third metabolic pathway independent of γ -secretase, and that these A β isoforms may serve as biomarkers for secretase inhibitor treatment.

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Introduction

Alzheimer's disease (AD) is the most common form of dementia, with characteristic pathological hallmarks that include intracellular neurofibrillary tangles and extracellular senile plaques [1]. The plaques consist mainly of β -amyloid (A β) [2–4], which is generated by cleavage of amyloid precursor protein (APP) by 2 different enzymes, β - and γ -secretase known as the amyloidogenic pathway [5]. In the alternative 'non-amyloidogenic pathway', α -secretase cleaves within the A β sequence precluding the formation of A β [6–8]. The β -site APP-cleaving enzyme 1 gene (*BACE1*) encodes the β -secretase activity [9–12], while γ -

secretase is a protease complex consisting of at least 4 essential components: the homologous presenilins 1 and 2 (PS1 and 2), nicastrin, Aph-1 and Pen-2 [13]. The α -secretase candidates so far identified all belong to the 'A Disintegrin and Metalloprotease' (ADAM) family (ADAM9, ADAM10 and ADAM17) [14].

We and others have previously shown that the combination of immunoprecipitation (IP) and mass spectrometry (MS) is a useful analytical method in targeted $A\beta$ proteomics for simultaneous identification and quantification of $A\beta$ isoforms with high mass accuracy in a single analysis [15]. Using IP-MS, it has been shown that $A\beta$ is manifested in several isoforms in the human brain and cerebrospinal fluid (CSF), having both C- and N-terminal truncations [16, 17]. Further, IP-MS has also been used for analyzing the $A\beta$ isoform pattern in the brains of transgenic (Tg) mice and $A\beta$ isoforms in cell media [18, 19].

We have recently shown that the levels of shorter $A\beta$ isoforms ($A\beta$ 1–14, $A\beta$ 1–15 and $A\beta$ 1–16) are elevated in media from cells which have been treated with γ -secretase inhibitors [20]. Here, we analyze the effects of γ -secretase inhibition on the $A\beta$ isoform pattern in CSF and the brain in a Tg mouse model of AD, Tg2576 [21]. We show that all $A\beta$ isoforms longer than and including $A\beta$ 1–17 are γ -secretase dependent, whereas the shorter isoforms $A\beta$ 1–15 and $A\beta$ 1–16 are processed through a novel APP metabolic pathway.

Material and Methods

Experimental Animals

In this study, we used Tg2576 mice overexpressing APP with Swedish mutations (hAPP695.SWE) on a C57B6/SJL background [21]. Male Tg2576 mice were mated with female C57B6/SJL wild-type mice. Offspring were genotyped as described previously [22], and were used as hemizygotes (+/-) of APP and wild-type mice.

Treatment Protocol

The protocols for mouse treatment and sample collection were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania. Six-month-old Tg2576 mice were subcutaneously injected with either N-[N-(3,5-difluorophenacetyl-L-alanyl)]-S-phenylglycine t-butyl ester (DAPT, 100 mg/kg, Calbiochem, Calif., USA), a γ -secretase inhibitor, or corn oil in 5% ethanol as control (n = 5/group, all females). Mouse CSF and brain tissues were collected 6 h after treatment.

CSF Collections

To collect CSF, mice were anesthetized by an intraperitoneal injection of ketamine hydrochloride (100 mg/kg) and xylazine (10 mg/kg). The skin and muscle above the cisterna magna were exposed and the meninges were punctured using a 30-gauge needle.

CSF samples were aspirated from just below the surface of the punctured meninges using a P20 micropipette. The samples were stored at -80°C as described earlier [23, 24].

Mouse Brain Tissue Collection

After CSF collection, the mice were decapitated after lethal anesthesia. Different regions of the exposed brains were dissected, i.e. the cortex, hippocampus, cerebellum and remaining regions. All brain samples were stored at -80°C until further study, as described earlier [25].

$A\beta$ Extraction

The brain samples (30–120 mg) were homogenized (Pellet Pestle®, Sigma-Aldrich, St. Louis, Mo., USA) on ice in Tris-buffered saline (20 mM Tris, 137 mM NaCl, pH 7.6) with complete protease inhibitor tablets (Roche, Basel, Switzerland). The extraction ratio (brain tissue:Tris-buffered saline) was 1:5 (w/v). Formic acid (FA) was added to the sample (final concentration 70%) followed by sonication (power: 15 amplitude microns; tune: 'middle') and centrifugation at 30,000 g for 1 h at 4°C . The FA-soluble $A\beta$ extract was dried and dissolved in FA, and finally neutralized using 0.5 M Tris.

Immunoprecipitation and Mass Spectrometry

IP using the KingFisher magnetic particle processor (Thermo Scientific, Waltman, Mass., USA) and mass spectrometric analysis using MALDI-TOF MS were performed as described earlier [17]. Briefly, an aliquot (8 μg) of the anti- $A\beta$ antibody 6E10 (Signet Laboratories, Dedham, Mass., USA), which is reactive to amino acids 1–17, was added to 50 μl magnetic Dynabeads M-280 Sheep Anti-Mouse IgG (Invitrogen, Carlsbad, Calif., USA). The antibody-coated beads were added to 5–10 μl CSF, and diluted to 1 ml in 0.025% Tween 20 in phosphate-buffered saline (pH 7.4). After washing, using the KingFisher magnetic particle processor, the $A\beta$ isoforms were eluted using 100 μl 0.5% FA. The neutralized $A\beta$ extract from brain tissue was immunoprecipitated by adding 50 μl 6E10-coated beads (8 μg) and 50 μl 4G8-coated beads (8 μg , Signet, reactive to amino acid 17–24).

MALDI-TOF MS measurements were performed using an Autoflex instrument (Bruker Daltonics, Bremen, Germany) operating in linear mode at 19 kV acceleration voltage. Each spectrum represents an average of 1,500 shots acquired 75 at a time. The MALDI samples were prepared with the seed layer method using α -cyano-4-hydroxycinnamic acid as the matrix.

LC-MS/MS was conducted by nanoflow liquid chromatography coupled to electrospray ionization Fourier transform ion cyclotron resonance tandem mass spectrometry (LC-ESI-FTICR-MS/MS) with an Ettan MDLC (GE Healthcare, Uppsala, Sweden) coupled to an LTQ-FT (ThermoFisher Scientific, Bremen, Germany), a hybrid linear quadrupole ion trap-Fourier transform ion cyclotron resonance mass spectrometer equipped with a 7-T magnet, as described previously [26].

Results

To test whether the IP-MS method using MALDI-TOFMS was sufficiently sensitive for analyzing such small volumes as 5–10 μl CSF, a pilot study was conducted using untreated Tg2576 mice ranging in age from 3–4

months up to older than 15 months. We analyzed 4 age groups with 3 mice in each group. Using IP-MS, 6 different peaks at different mass-to-charge ratios (m/z) were reproducibly detected, which by mass were assigned to be 6 isoforms of A β (A β 1-15, A β 1-16, A β 1-17, A β 1-19, A β 1-38 and A β 1-40). The data revealed an age-dependent shift in the A β pattern; the older Tg2576 mice produced more A β 1-40 and less A β 1-17 than the younger animals (fig. 1). Mice aged 6–7 months were used for the γ -secretase-inhibitor treatment study.

Using IP-MS, a study including 5 mice treated with γ -secretase inhibitor (DAPT) and 5 treated with corn oil (vehicle) was performed. All 6 isoforms were reproducibly detected in the CSF of all mice. As seen in figure 2a,b, treatment with DAPT greatly increased the mass spectrometric signal for A β 1-16 ($p < 0.01$, Mann-Whitney U exact test). The signal corresponding to A β 1-15 showed a tendency towards increased levels, while the signals for the longer isoforms, including A β 1-17, indicated slightly decreased levels (fig. 2c).

From the same mice included in the CSF study, the hippocampus, cortex and cerebellum were analyzed using IP and MALDI-TOFMS to study changes in the A β isoform pattern in response to γ -secretase inhibition. The detected A β isoforms were also confirmed using LC-MS/MS (data not shown). Figure 3a–b displays MALDI-TOF mass spectra from FA-extracted A β isoforms from the hippocampus, where A β 1-15, A β 1-17, A β 1-19, A β 1-38, A β 1-40 and A β 1-42 were reproducibly detected in all mice. A β 1-16 was not detected in all samples, and was omitted from the evaluation. The same trend as for CSF was noted; the short A β isoforms indicated slightly increased levels, while isoforms longer than and including A β 1-17 showed a tendency towards decreased levels (fig. 3c). Similar data were obtained in the cerebellum (fig. 3d) and cortex (fig. 3e), although the number of reproducibly detected A β isoforms differed between the different brain regions.

Discussion

Using MALDI-TOFMS measurement on 6E10 immunoprecipitated A β from CSF, a distinct pattern of 6 different peaks at different m/z was detected in both treated and untreated mice. They were, by mass, assigned to be 6 isoforms of A β (A β 1-16, A β 1-17, A β 1-18, A β 1-19, A β 1-38 and A β 1-40). In human CSF, more than 20 different A β isoforms have been detected [15], while in this study on mouse CSF, 6 isoforms were reproducibly detected.

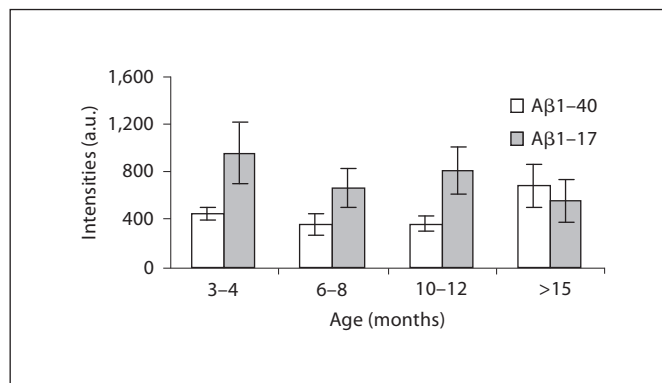


Fig. 1. MALDI-TOFMS intensities for A β 1-17 and A β 1-40. The mass spectrometric signals for A β 1-17 were divided by 3.

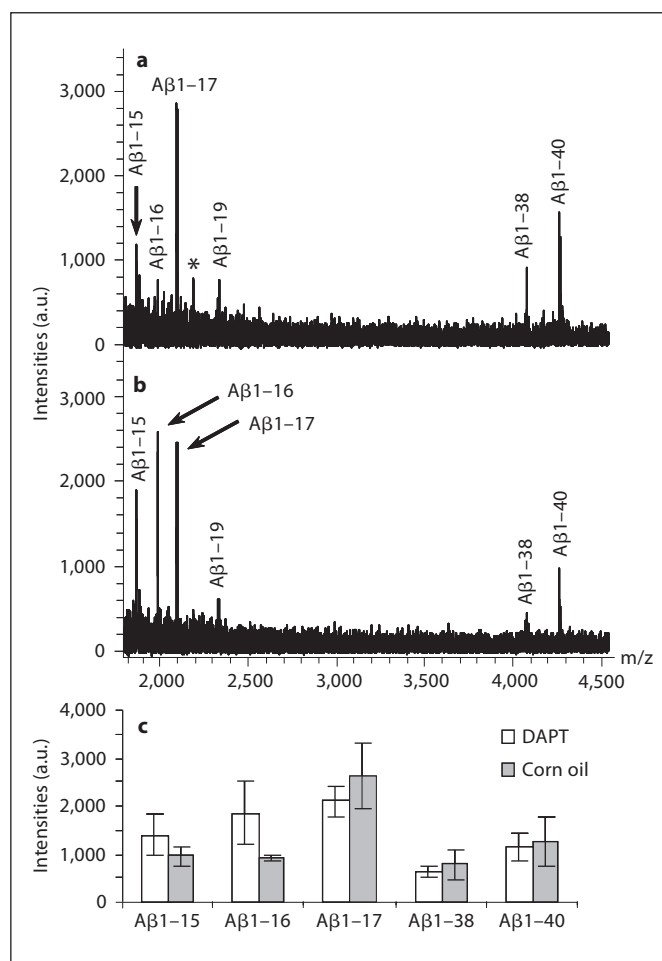


Fig. 2. MALDI-TOF mass spectra showing the A β isoform pattern in CSF from vehicle (a) and γ -secretase-inhibitor treated (DAPT; b) mice. * Unidentified peak. The average MALDI-TOF mass spectrometric signals for the detected A β isoforms are displayed from the 2 different treatment groups (c).

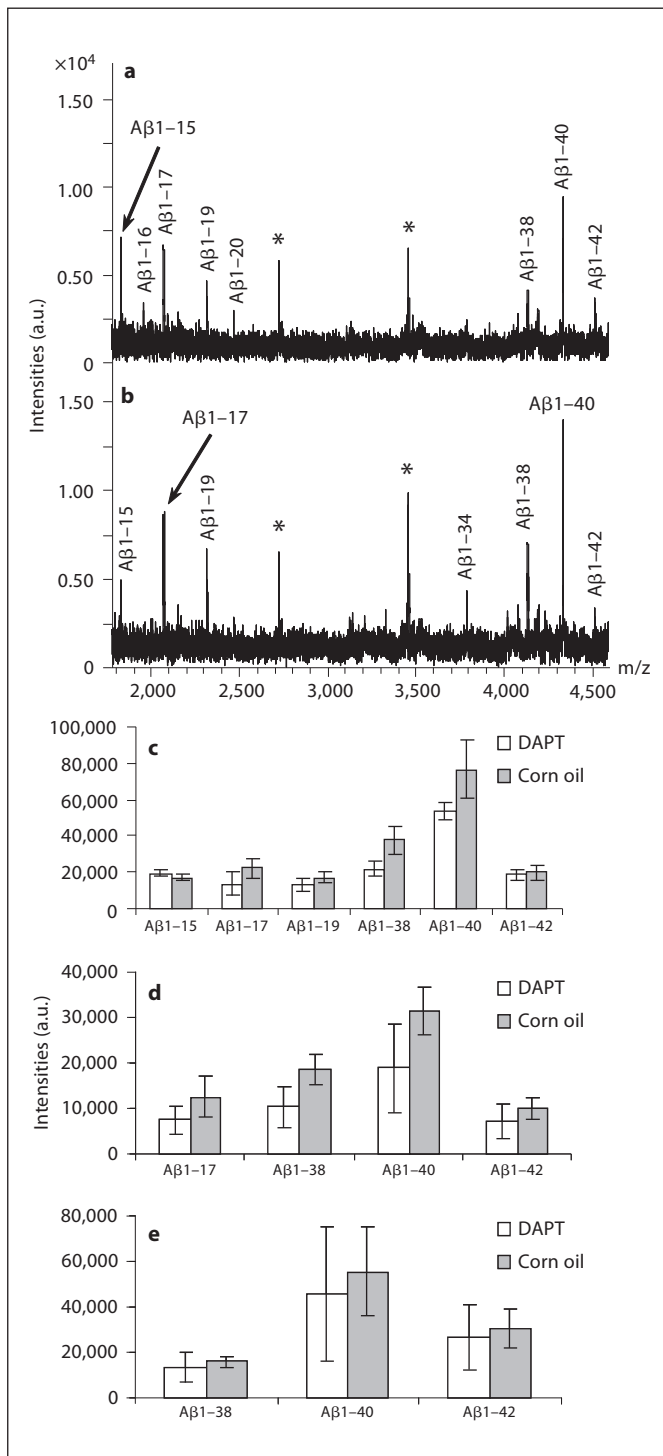


Fig. 3. MALDI-TOF mass spectra showing the A β isoform pattern in the hippocampus from γ -secretase inhibitor- (DAPT; **a**) and vehicle- (**b**) treated mice. * Unidentified peaks. The average MALDI-TOF mass spectrometric signals for the detected A β isoforms are displayed from the 2 different treatment groups in the hippocampus (**c**), cerebellum (**d**) and cortex (**e**).

The fewer A β isoforms detected in mouse CSF probably reflects the small volume of CSF available for analysis (5–10 μ l, compared with 1 ml in human CSF studies).

Analysis of the A β isoform pattern in CSF from mice of varying ages indicated an age-dependent increase in the longer and more aggregation-prone A β peptides. If verified in human studies, this may provide an explanation for the strong age-dependency of amyloid pathology in the brain.

The isoform patterns varied in different brain regions. In the IP extract from approximately 30 mg hippocampus, 6 different isoforms were detected compared with only 3 in the cortex (93–115 mg). This may be due to either the method used not being sufficiently sensitive for detecting low abundant isoforms or that the shorter isoforms (A β 1–15, A β 1–17 and A β 1–19) are absent in the cortex. Further, by normalizing the peaks to the sum of the intensities of the 3 most abundantly detected isoforms in all brain regions (A β 1–38, A β 1–40 and A β 1–42), the cortex displayed an approximate 2-fold increase in the A β 1–40/A β 1–42 ratio (in both DAPT and untreated). A β 1–42 also had the highest mass spectrometric signal in the cortex. It should be noted that the ratio between the A β 1–42 and A β 1–40 peaks in the mass spectrum cannot be interpreted as a direct reflection of their relative abundance since the ionization efficiency might be different for the 2 peptides and that A β 1–42 is more hydrophobic and less soluble than A β 1–40. However, these data suggest that A β 1–42 is present at higher levels in the cortex relative to the other brain areas studied.

In the two APP processing pathways described in the literature, the ‘amyloidogenic’ and ‘non-amyloidogenic’, either β - or α -secretase cleaves APP followed by γ -secretase-mediated cleavage of the remaining C-terminal APP fragment [27]. We show here that CSF A β 1–15 and A β 1–16 indicated slightly increased levels, whereas the levels of all fragments longer than and including A β 1–17 were slightly decreased in response to γ -secretase inhibitor treatment. These data are in agreement with previous results on γ -secretase-inhibitor-treated cells [20]. Similar results were obtained using IP-MS on formic-acid-extracted A β isoforms from the hippocampus, cerebellum and cortex. This suggests that all fragments longer than and including A β 1–17 depend on γ -secretase, directly or indirectly, while A β 1–15 and A β 1–16 do not. These shorter A β isoforms may be processed along a third APP-processing pathway involving concerted β - and α -secretase-mediated cleavages of APP. Additional knowledge on this putative third APP processing pathway may be gained from ongoing secretase inhibitor studies in humans.

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