Pharmacological Modulation of GSAP Reduces Amyloid-β Levels and Tau Phosphorylation in a Mouse Model of Alzheimer’s Disease with Plaques and Tangles

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Handling Associate Editor: Patrizia Mecocci

Accepted 11 February 2014

Abstract. Accumulation of neurotoxic amyloid-β (Aβ) is a major hallmark of Alzheimer’s disease (AD) pathology and an important player in its clinical manifestations. Formation of Aβ is controlled by the availability of an enzyme called γ-secretase. Despite its blockers being attractive therapeutic tools for lowering Aβ, this approach has failed because of their serious toxic side-effects. The discovery of the γ-secretase activating protein (GSAP), a co-factor for this protease which facilitates Aβ production without affecting other pathways responsible for the toxicity, is giving us the opportunity to develop a safer anti-Aβ therapy. In this study we have characterized the effect of Imatinib, an inhibitor of GSAP, in the 3 × Tg mice, a mouse model of AD with plaques and tangles. Compared with controls, mice receiving the drug had a significant reduction in brain Aβ levels and deposition, but no changes in the steady state levels of AβPP, BACE-1, ADAM-10, or the four components of the γ-secretase complex. By contrast, Imatinib-treated animals had a significant increase in CTF-Aβ and a significant reduction in GSAP expression levels. Additionally, we observed that tau phosphorylation was reduced at specific epitopes together with its insoluble fraction.

In vitro studies confirmed that Imatinib prevents Aβ formation by modulating γ-secretase activity and GSAP levels. Our findings represent the first in vivo demonstration of the biological role that GSAP plays in the development of the AD-like neuropathologies. They establish this protein as a viable target for a safer anti-Aβ therapeutic approach in AD.

Keywords: Amyloid-β, γ-secretase, imatinib, tau protein, transgenic mice

INTRODUCTION

Alzheimer’s disease (AD) is characterized by elevated levels of amyloid-β (Aβ)-peptides that are produced by β- and γ-secretases [1]. As a corollary, any approach aimed at blocking the production of Aβ by interfering with these enzymes could be seen as a valid therapeutic strategy. However, for instance, full blockade of γ-secretase has deleterious effects because this enzyme is also an active player in the proteolytic processing of other substrates besides the amyloid-β protein precursor (AβPP) such as Notch-1 and cadherins, both of which are important in transducing biologically relevant signals within the cells.
ARTICLE

METHODS

Mice and treatment

All animal procedures were approved by the Institutional Animal Care and Usage Committee, and in accordance with the National Institute of Health guidelines.

The 3×Tg mice harboring a mutant AβPP (KM670/671NL), a human mutant PS1 (M146V) knockin, and tau (P301L) transgenes were used in this study. They were kept in a pathogen-free environment, on a 12·h light/dark cycle and had access to food and water ad libitum. A total of twelve mice were available for this study, with 3 female and 3 male mice per group. 3×Tg mice (age: 10 month, n = 6 mice per group; 3 male and 3 female mice) were treated with artificial cerebrospinal fluid (1.3 CaCl2, 1.2 MgSO4, 3KCl, 0.4 KH2PO4, 25 NaHCO3, and 122 NaCl, pH 7.35) or Imatinib (STI571, Gleevec®) (Selleckchem), a tyrosine-kinase inhibitor which previously used in the treatment of multiple cancers, at 40 mg/Kg/day dose (a concentration of 80 g/μl entered), as previously described[9]. Briefly, mice were anesthetized by intraperitoneal injection of ketamine hydrochloride (1 mg/10 g) and xylazine (0.1 mg/10 g). After being shaved on the head and back, a cannula was inserted into the right lateral ventricle using stereotaxic coordinates (frontal pole (F-P), 0.5 mm; mediolateral (M-L), 1.0 mm; dorsoventral (D-V), 2.7–3.3 mm). Instant dental cement was used to secure the cannula to the skull, and an osmotic pump (Model 1007D, Alzet, Cupertino, CA, USA) was placed subcutaneously on the back. Alzet 1007D osmotic pumps have an infusion rate of 0.5 μl/h and a 7-day pumping capacity; thus the pumps subcutaneously delivered 40 μg/h of Imatinib for the duration of the experiments. After surgery mice were housed individually. After one week treatment, mice were sacrificed. After killing, mice were perfused with ice-cold 0.9% phosphate-buffered saline containing EDTA (2 mmol/l pH 7.4). Brain was removed, gently rinsed in cold 0.9% phosphate-buffered saline and immediately dissected in two halves. One half was immediately stored at −80 °C for biochemistry, the other half was fixed in 4% paraformaldehyde in phosphate-buffered saline, pH 7.4 for immunohistochemistry studies.

Western blot analysis

Proteins were extracted in EIA buffer containing 250 mM Tris base, 750 mM NaCl, 5% NP-40, 25 mM EDTA, 2.5% sodium deoxycholate, 0.5% SDS and an EDTA-free protease inhibitor cocktail tablet (Roche Applied Science), sonicated, centrifuged at 13,000 rpm for 45 min at 4 °C, and supernatants used for immunoblot analysis, as previously described[10, 11]. Total protein concentration was determined by using BCA Protein Assay Kit (Pierce, Rockford, IL). Samples were electrophoretically separated using 10% Bis-Tris gels or 3–8% Tris-acetate gel (for GSAP full length) and Tris-tricine gels (16.5% of acrylamide for GSAP-16kDa) (Bio-Rad, Richmond, CA), according to the molecular weight of the target molecule, and then transferred onto nitrocellulose membranes (Bio-Rad). They were blocked with Odyssey blocking buffer for 1 h, and then incubated with primary antibodies described in Table 1 overnight at 4 °C. After three washing cycles with T-TBS, membranes were incubated
Table 1

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Immunogen</th>
<th>Host</th>
<th>Application</th>
<th>Source</th>
</tr>
</thead>
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<tr>
<td>PION/GSAP</td>
<td>19 amino acid peptide near the carboxy terminus of human PION: as 41–105 mapping near the N-terminus of TMP21 of human origin</td>
<td>Rabbit</td>
<td>WB</td>
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<td>aa 732–748 of human ADAM 10</td>
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<td>Millipore</td>
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<td>PS-1</td>
<td>aa around valine 293 of human presenilin 1</td>
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<td>Cell Signaling</td>
</tr>
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<td>sAβP1</td>
<td>Synthetic peptide of the C-terminal part of human sAβP1</td>
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<td>CTFs</td>
<td>a synthetic peptide [C(CKMKQQNGYENPPKFFEQQM)] corresponding to amino acids 731–770 of human precursor protein (AβPP), conjugated to KLH</td>
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<td>Dr. P Davies</td>
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WB, western blot; IHC, immunohistochemistry.

with IRDye 800CW or IRDye 680CW-labeled secondary antibodies (LI-COR Bioscience, NE) at 22°C for 1 h. Signals were developed with Odyssey Infrared Imaging Systems (LI-COR Bioscience). Actin was always used as an internal loading control.

Sarkosyl insolubility assay

The assay for insoluble tau was performed as previously described [12]. Briefly, ultraconcentration and sarkosyl extraction (30 min in 1% sarkosyl) was used to obtain soluble and insoluble fractions of tau. Insoluble fractions were washed one time with 1% sarkosyl, then immunoblotted with HT-7 antibody.

Quantitative analysis of Aβ peptides

Mouse brain homogenates were sequentially extracted in RIPA for the Aβ1-40 and Aβ1-42 soluble fractions, and then in formic acid (FA) for the Aβ1-40 and Aβ1-42 insoluble fractions, and then assayed by a sensitive sandwich ELISA kits (WAKO Chem., Richmond, VA) as previously described [10, 11].

Immunohistochemistry

Immunostaining was performed as reported previously by our group [10–12]. Briefly, serial coronal sections were mounted on 3-aminopropyl triethoxysilane (APES)-coated slides. Every eighth section from the habenular to the posterior commissure (8–10 sections per animal) was examined using unbiased stereological principles. Sections were deparaffinized, hydrated, and pretreated with FA (88%) and subsequently with 3% H2O2 in methanol. Sections were then blocked in 2% fetal bovine serum before incubation with primary antibody overnight at 4°C (Wako Chemicals, Richmond, VA). After washing, sections were incubated with biotinylated anti-mouse IgG (Vector Lab) and then developed by using the avidin–biotin complex method (Vector Lab) with 3,3′-diaminobenzidine (DAB) as a chromogen. Light
microscopic images were used to calculate the area occupied by Aβ-immunoreactivity by using the software Image-ProPlus for Windows version 5.0 (Media Cybernetics). The threshold optical density that discriminated staining from background was determined and kept constant for all quantifications. The area occupied by Aβ-immunoreactivity was measured by the software and divided by the total area of interest to obtain the percentage area of Aβ-immunoreactivity.

Cell culture

The N2A (neuro-2A neuroblastoma) neuronal cells stably expressing human AβPP carrying the K670 N, M671 L Swedish mutation (AβPPsw) were grown in DMEM-Dulbecco’s modified Eagle medium (11965-092, Gibco) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 400 μg/ml G418 (Gibco), at 37 °C in the presence of 5% CO2, as previously described [13]. Cells were grown to 70% confluence and then treated with Imatinib. Compared with controls, we observed that supernatants form N2A-AβPPSwe neuronal cells) and incubated them with Imatinib. By contrast, compared with controls treated mice had a significant increase in the levels of the active GSAP (i.e., GSAP 16 kDa) (Fig. 2C). Next, we explored the effect of GSAP pharmacological blockade on tau phosphorylation. As shown in Fig. 3, although we did not observe any changes in the levels of soluble tau between the two groups, compared with vehicle, the Imatinib-treated mice had a significant decrease in its phosphorylated form at epitopes S396/S404, as recognized by the antibody PHF-13, and at S202/T205 as recognized by the antibody AT8. By contrast, no changes were observed for tau phosphorylated at T181, and T231/S235 as recognized by the antibodies AT270 and AT180, respectively. Addi-

Results

In vivo study

Imatinib reduces Aβ levels and deposition

Ten month old 3×Tg mice were randomized to receive Imatinib or vehicle intrathecally over 7 days by means of implanted osmotic minipumps. At the end of this period mice were sacrificed, brains harvested and assessed for levels and deposition of Aβ. As shown in Fig. 1A–D, we observed that mice receiving Imatinib had a significant decrease in the amount of RIPA-soluble and FA-soluble Aβ1-40 and Aβ1-42. Confirming the ELISA data, we found that the same condition led to a significant reduction in Aβ deposition in their brains (Fig. 1E, F). To investigate the mechanism responsible for this change, we assessed the steady state levels of the amyloid-β protein precursor (AβPP) along with its cleaving enzymes and products. While no differences were observed for total AβPP, BACE-1, ADAM-10, sAβPPsw, sAβPPa, or the four components of the γ-secretase pathway (Supplementary Figure 1), a significant increase in the levels of CTF-β, but not CTF-α, was observed in the brains of mice receiving the active drug (Fig. 2A, B). In addi-

In vitro study

Imatinib reduces Aβ formation

To support our in vivo findings, we conducted a series of in vitro experiments using neuronal cells that express the same AβPP Swedish mutation as the 3×Tg mice (i.e., N2A-AβPPsw neuronal cells) and incubated them with Imatinib. Compared with controls, we observed that supernatants form N2A-AβPPsw cells treated with Imatinib (10 μM) produced significant less Aβ1-40 (Fig. 4A). This change was not associated with any significant differences between the two groups in terms of AβPP, BACE-1, ADAM-10, sAβPPsw, sAβPPa, PS1, Nicastrin, Pen-2, and APH-1, the four components of the γ-secretase complex.
Fig. 1. Imatinib reduces Aβ levels and deposition in the brains of 3×Tg mice. A,C) Radioimmunoprecipitation assay (RIPA)-soluble extractable Aβ1-40 and Aβ1-42 in brain homogenates of 3×Tg mice receiving Imatinib or vehicle. B,D) FA extractable Aβ1-40 and Aβ1-42 in brain homogenates of 3×Tg mice receiving Imatinib or vehicle (*p ≤ 0.04). E) Representative images of brain sections from 3×Tg mice treated with Imatinib or vehicle immunostained with the pan-Aβ antibody 4G8. F) Quantification of the area occupied with Aβ immunoreactivity in the brains of the same groups of mice (*p = 0.004).

(Fig. 4B and Supplementary Figure 2). By contrast, we observed that cells incubate with Imatinib had a significant increase in the levels of CTF-β but no changes in the CTF-α were detected (Fig. 4B, C).

Finally, we found that lysates form cells incubated with Imatinib had a significant reduction in steady state levels of GSAP 16kDa, but no changes in its precursor (i.e., GSAP-FL, 98kDa), nor in two distinct γ-secretase modulatory proteins, TMP21 and CD147 (Fig. 4D, E).

**DISCUSSION**

Despite some criticism, the anti-Aβ therapy still holds a strong rationale as a therapeutic strategy for AD. Thus, the modulation of γ-secretase activity to selectively decrease Aβ levels without inhibiting cleavage of other biologically relevant substrates (i.e., Notch) is a very attractive therapeutic approach for AD, which with the identification of the novel protein GSAP is now closer than ever to become a therapeutic reality for the disease. However, despite we know that GSAP derives from a larger precursor protein, and that its immunoreactivity is increased in AD brains where it associated with Aβ-containing amyloid plaques, whether the protein is a suitable target in vivo for a viable therapeutic approach in AD remains to be investigated. Conflicting reports have been published on in vivo studies which implemented a pharmacological approach to block the availability of GSAP for the processing of AβPP and therefore to lower Aβ formation. Thus, it was reported that peripheral administration of Imatinib in mice decreased Aβ levels in plasma and that this was sufficient to reduce Aβ in the brains [14]. Contrary to these findings, Hussain et al. found that peripheral administration of the same drug to rats had no effect on Aβ [7]. Since it is known that Imatinib does not cross the blood-brain barrier, in the present study we delivered the drug directly to the...
brain (i.e., intrathecal delivery) by means of implanted osmotic minipumps. In addition, because no data are available on the effect that this therapeutic regimen may have in a transgenic mouse model of AD, the drug was administered to the 3×Tg, which develop amyloid plaques and neurofibrillary tangles [8].

At the end of the treatment period we observed that compared with controls, mice receiving Imatinib had a significant reduction in their brain levels of both soluble and insoluble Aβ1-40 and Aβ1-42. This finding was confirmed by the assessment of the amyloid burden, which measure the Aβ peptide immunoreactive areas deposited in the brains. Despite the fact that the 3×Tg mice manifest sex difference in Aβ accumulation [15], in our study we did not observe it. Because we found that Imatinib treatment resulted in much less amount of Aβ peptides, next we investigated the possible mechanism involved in this effect. First, we observed that there were no differences between the mice receiving the drug and control regarding the steady state levels of AβPP, BACE-1, ADAM-10, and the four components of the γ-secretase complex. By contrast, confirming the functional blockade of the complex we observed a significant increase in the levels of the CTF-β, which we interpret as an accumulation of the substrate that cannot be further processed. In addition, we observed that the drug significantly reduced the levels of GSAP 16 kDa, but had no effect on its precursor, GSAP-FL, and on two distinct γ-secretase modulatory proteins, suggesting specificity in the action of Imatinib. Importantly, we confirmed these data also by using an in vitro approach in which neuronal cells were incubated with Imatinib. Because of the significant reduction in Aβ immunoreactivity in the brains of the treated mice, and the relatively short treatment period we assayed also some of the enzymes involved in Aβ clearance from the central nervous system, but no changes were observed at least for neprilysin and IDE. However, because we did not measure in our mice plasma levels of Aβ, we cannot rule out that the stark reduction in brain Aβ levels could also be secondary to a peripheral effect of the drug.
Fig. 3. Imatinib reduces tau phosphorylation in the brains of 3 × Tg mice. A) Representative western blot analyses for soluble and insoluble (sarkosyl soluble) total tau (HT-7), and phosphorylated at residues S396/S404, recognized by the antibody PHF1, at S396, recognized by the antibody PHF13, at S202/T205 recognized by the antibody AT8, at T181, recognized by the antibody AT 270, and at T231/S235 as recognized by the antibodies AT180 in brain homogenates of 3 × Tg mice treated with Imatinib or vehicle. B) Densitometric analyses of the immunoreactivities to the antibodies presented in the previous panel (∗p < 0.05). C) Representative images of brain sections from 3 × Tg mice treated with Imatinib or control immunostained with anti-tau antibody HT-7. D) Densitometric analysis of the immunoreactivity to the antibody presented in the previous panel.

Besides the Aβ pathology, AD is also characterized by the presence of abundant intracellular neurofibrillary tangles which are formed by mainly the hyperphosphorylated microtubule-associated protein tau [16, 17]. However, since some data suggest that Aβ can modulate cellular events leading to phosphorylation specific changes in tau [18], we were curious to see if this was the case in our experimental setting. To this end, the implementation of the 3 × Tg mouse model offered a unique opportunity to address this very important question. In the current study, we showed for the first time that Imatinib also influences tau metabolism by showing that its administration results in a significant reduction in tau phosphorylation. Interestingly, this effect was selective, because we found a decrease in phosphorylated tau at S396/S404 (detected by the antibody PHF-1), S396 (detected by the antibody PHF-13) and S202/T205 (detected by the antibody AT8). By contrast, we did not find any difference when other phosphoepitopes such as AT180 and AT270 were assayed. Confirming the effect on these specific tau epitopes, which are considered mid to late stage tau pathology, we also observed an effect on the insoluble fraction of tau which was significantly reduced in the brains of mice receiving Imatinib.

In conclusion, our study establish for the first time to our knowledge that Imatinib is a valid therapeutic tool against AD neuropathologies by targeting GSAP, which becomes now a viable AD therapeutic target. Because we have no data on the effect that Imatinib may have on the behavioral phenotype of the mouse model implemented in the current study, a note of caution is also necessary. Additionally, since our study is a short term intervention, it also would be very informative and interesting to investigate the long-term effect of such a treatment.

However, the established successful application of Imatinib in the cancer field, together with its inability to affect Notch signaling make this drug as a potentially attractive ad safe Aβ lowering compound for
the treatment of AD. Development of Imatinib-like drugs which when given orally can easily accumulate in the brain and reach their target would be necessary to facilitate and accelerate their clinical application and achieve their potential therapeutic benefits.

ACKNOWLEDGMENTS

This work was supported in part by a grant from the Alzheimer Art Quilt Initiative. J.C. and D.P. designed the study, developed the experimental design, performed data analysis, and wrote the article. E.L. and C.P.G. participate in the animal studies and performed some of the minipumps implantation. All authors discussed the results and commented on the manuscript. Authors’ disclosures available online (http://www.j-alz.com/disclosures/view.php?id=2157).

SUPPLEMENTARY MATERIAL

Supplementary figures are available in the electronic version of this article: http://dx.doi.org/10.3233/JAD-140105.

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