The novel tetrahydrofuran derivative ANAVEX2-73 attenuated GSK-3β activation and Tau hyperphosphorylation in a nontransgenic Alzheimer's disease model in mice

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INTRODUCTION

- Alzheimer’s disease (AD) is the most prevalent of the neurodegenerative dementia. The two physiopathological hallmarks of the disease are extracellular deposition of amyloid-β (Aβ) proteins and intracellular neurofibrillary tangles (NFT). NFT are mainly constituted of hyperphosphorylated forms of the microtubule-associated Tau protein. Indeed, in AD, Tau protein is hyperphosphorylated on several threonine and serine residues by different kinases, including the glycogen synthase kinase-3β (GSK3β). GSK3β is notably activated when phosphorylated on the T^{216} and is inactivated when phosphorylated on S^{9}. The kinase Akt, that could be activated when phosphorylated on S^{473}, is responsible for the phosphorylation of GSK3β on S^{9} (see Fig. 3a). Accumulation of Aβ proteins and resulting neurodegenerative processes in the brain of AD patients is concomittent and putatively synergistically enhanced by Tau hyperphosphorylation and aggregation. We described a non-transgenic model of AD in mice [1] and rats [2] induced by intracerebroventricular injection of the Aβ fragment Aβ_{25-35} in oligomeric form that activates GSK3β and provokes the hyper- and abnormal phosphorylation of Tau protein, in the rodent hippocampus and cortex. We use this model to evaluate the neuroprotective of drugs acting selectively or non selectively as activators of the sigma-1 (σ₁) chaperone protein [3-7].

- In particular, tetrahydro-N, N-dimethyl-2, 2-diphenyl-3-furanmethanamine (ANAVEX2-73) is a novel compound binding to muscarinic acetylcholine and σ₁ receptors with affinities in the low micromolar range [5]. We previously reported that the drug showed anti-amnesic and neuroprotective potential against Aβ_{25-35} toxicity in mice [5]. ANAVEX2-73 attenuated the oxidative stress, induction of caspases, cellular loss and memory deficits observed within weeks after Aβ_{25-35} injection [5].

- We here analyzed the time-course of the activation of Akt and GSK3β kinases at day 1, 3, 5 and 7 after the intracerebroventricular Aβ_{25-35} injection in mice and the resulting induction of Tau hyper- and abnormal phosphorylation. The neuroprotective effects of ANAVEX2-73, the selective σ₁ protein agonist PRE-084 and the muscarinic receptor agonist xanomeline were first checked using a novel object recognition test and then analyzed on the kinases activities and Tau phosphorylation. We report that the compounds are able to attenuate Aβ-induced Tau phosphorylation through Akt activation and GSK3β inactivation.
MATERIAL AND METHODS

Animals

Male Swiss mice (Deppe), aged 7-9 weeks and weighing 32 ± 2 g were housed in plastic cages in groups with free access to food and water, except during behavioral experiments. They were kept in a regulated environment (23 ± 1°C, 40-60% humidity) under a 12 h light/dark cycle (light on at 6:00 A.M.). All animal procedures were conducted in strict adherence to European Union Directive of 24 November 1986 (86/609).

Drugs and administration procedures

The amyloid-β(25-35) (Aβ25-35) and scrambled Aβ25-35 (Sc Aβ) peptides were from Genepep. They were solubilized in sterile distilled water at a concentration of 3 mg/ml and stored at -20°C until use. Before injection, peptides were aggregated by incubation at 37°C for 4 days. They were administered intracerebroventricularly (i.c.v.) in a final volume of 3 µl per mouse. Tetrahydro-3,N-dimethyl-2,2-diphenyl-3H-tetramethanamine hydrochloride (ANAVEX-2-73) was synthesized in the laboratory (Anavex Life Science). PRE-084 was a gift from Dr. Tsung-Ping Su. Xenodermolone was purchased from Eli Lilly. Drugs were solubilized in physiological saline at the concentration of 5 mg/ml. They were then brought up to each dose by dilution and injected in a volume of 100 µl/20 g body weight. Animals were used between Days 1 to 9 days after i.c.v. injections for behavioral testing or sacrifice before biochemical measures.

Western blotting

Mice were decapitated at indicated days after Aβ25-35 peptide injection. The hippocampus were removed on an ice-cold Peristalt and stored at -80°C. Tissues were homogenized by sonication in lysis buffer (Tris-HCl 125 mM pH 6.8; sodium deoxycholate, SDS, 4%; glycerol 20%) including protease and phosphatase inhibitors cocktails (Roche Diagnostics), heated to 70°C for 10 min and prepared for centrifugation. Protein concentration was determined using the Pierce BCA assay (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer’s instructions.

Proteins, 50 µg per lane, were resolved on a 10% SDS-polyacrylamid gel. Proteins were then transferred to a PVDF membrane. After 1 h blocking in 5% nonfat dry milk in Tris-buffered saline 20 mM, pH = 7.5, containing 0.1% Tween20 (TEV2-T), membranes were incubated overnight at 4°C with the primary antibodies: rabbit anti-phospho-Ser423-Akt antibody (52K71 from Cell Signaling Technology), mouse anti-phospho-Tyr216-GSK-3β (BD Biosciences), rabbit anti-phospho-Ser42-GSK3β (9336 from Cell Signaling Technology), mouse anti-human PHF-Tau AT8 clone (MN1020, Pierce Biotechnology) and mouse anti-human PHF-Tau AT100 clone (MN1060, Pierce Biotechnology). After brief washes, membranes were incubated for 1 h at room temperature with corresponding secondary antibody, goat anti-rabbit IgG peroxidase conjugate or goat anti-mouse IgG peroxidase conjugate (respectively A6154 and A4410 from Sigma-Aldrich). The immunoreactive bands were visualized with the Enhanced ChemiLuminescence (ECL) reagent (Pierce Biotechnology) using a Lum-Imager F1 Workstation. Then, membranes were stripped using the Restore™ Western Blot Stripping Buffer (Pierce Biotechnology) and reprobed with rabbit anti-GSK-3β polyclonal antibody (sc-9166, Santa Cruz Biotechnology), mouse anti-Tau monoclonal antibody (MA1-38710, Pierce Biotechnology), rabbit anti-Akt antibody (89727, Cell Signaling Technology). The intensity of peroxidase activity was quantified using the ImageJ® software (NIH).

Novel object recognition test

Six days after peptide and drug injections, mice were placed individually in a squared open-field (50 cm x 50 cm x 50 cm high) made in white Plexiglas with a floor equipped with infrared light emitting diodes. During a 10-min duration session, the locomotor activity of the animals was captured through an IR-sensitive camera and analyzed using the Videotrack® software (Viewpoint). The activity was analyzed as total distance traveled (m), locomotor speed (cm/s) and percentage of presence in the 25 x 25 cm central area. On day 7 after injections, two identical objects (50 ml plastic vials with caps) were placed at defined positions at two opposite edges of the central area. Each mouse was placed in the open-field and the exploratory activity was recorded during a 10-min duration session. The activity was analyzed using the nosotrack® protocol (Viewpoint), in terms of number of contact with objects and duration of contacts. On day 8 after the injections, the object in position #2 was replaced by a novel one (a soft plastic chair feet protection) differing in color shape and texture from the familiar object. Each mouse was placed again in the open-field and the exploratory activity recorded during a 10-min duration session. The activity was analyzed similarly. The preferential exploration index was calculated as the ratio of the number (or duration) of contacts with the object in position #2 over the total number (or duration) of contacts with the two objects. As the analyses in terms of number of contacts or duration of contacts led to strictly similar results, only the number of contacts is presented. Animals showing less that 10 contacts with objects during the day 7 or day 8 session were discarded from the study.
None of the treatment affected open field responses prior to the novel object recognition test.

(a, c, e) Total locomotor activity (m), (b, d, f) presence in the center of the open field, in percentage of the total time. Animals were treated i.p. with ANA/EX2-73 (0.1, 0.3, 1 mg/kg), PRE-084 (0.5, 1 mg/kg), xenonamine (0.5, 1 mg/kg) or saline solution (V). 20 min before the ANA/EX2-73 or Sc.A6 (8 nmol) i.c.v. injection. Six days after injection, they were habituated for 10 min to the open field arena. n = 13-15 per group, F_{(4,87)} = 0.38, p > 0.05 in (e); F_{(4,49)} = 0.61, p > 0.05 in (b); n = 13-15, F_{(3,32)} = 1.33, p > 0.05 in (c); F_{(4,49)} = 0.14, p > 0.05 in (d); n = 13-16, F_{(4,54)} = 0.42, p > 0.05 in (e); F_{(4,54)} = 0.22, p > 0.05 in (f)
ANAEX2-73, PRE-084 and xanomeline prevented the Aβ25-35-induced novel object recognition deficits

(a, c, e) Day 1 session, (b, d, f) day 2 session. Animals were treated i.p. with ANAEX2-73 (0.1, 0.3, 1 mg/kg), PRE-084 (0.5, 1 mg/kg), xanomeline (0.5, 1 mg/kg) or saline solution (V), 20 min before the Aβ25-35 or ScAβ (9 nmol) i.c.v. injection. Data showed the preferential exploration index calculated as the ratio of number of contact with the object in position 2 over the total number of contacts with the two objects, expressed as percentage. n = 13-15 per group. F(4,56) = 6.99, p < 0.05 in (a), F(4,56) = 3.45, p < 0.05 in (b), F(4,56) = 0.83, p > 0.05 in (c), F(4,56) = 3.01, p < 0.05 in (d), n = 13-15, F(4,56) = 0.67, p < 0.05 in (e), F(4,56) = 3.03, p < 0.05 in (f), **p < 0.01 vs. the (ScAβ) V-treated group, Dunnett's test.
Aβ<sub>25-35</sub> time-dependently affected Akt and GSK3β phosphorylation in the mouse hippocampus

(a) Signaling pathways involved, (b) P(ΔG<sup>25-35</sup>)Akt/total Akt ratio, (c) P(ΔG<sup>25-35</sup>)GSK3β/total GSK3β ratio, and (d) P(Y<sup>9</sup>)GSK3β/total GSK3β ratio. Abbreviations in (a): PDK1, phosphoinositide 3-kinase; PKA, protein kinase A; PYK2, proline-rich tyrosine kinase 2; Akt, serine/threonine protein kinase; GSK3β, glycogen synthase kinase-3β. Typical blots are shown above the graphs. Lanes were from the same blot but placed in the same treatment order as shown for the graph. Mice were administered i.c.v. with Sc.Aβ or Aβ<sub>25-35</sub> peptide (9 nmoI) and sacrificed 1, 3, 5, 7 days after injection. ANOVA: n = 9-11 per group, F(4,49) = 3.40, p < 0.05 in (b); n = 7-12, F(4,42) = 2.71, p < 0.05 in (c); n = 5-6, F(4,37) = 3.23, p < 0.05 in (d); n = 6, F(4,37) = 3.23, p < 0.05 in (e) n = 7, F(4,37) = 3.23, p < 0.05 in (f). *p < 0.05, **p < 0.01, ***p < 0.001 vs. the Sc.Aβ-treated group; Dunnett’s test.
ANAVEX2-73, PRE-084 and xanomeline prevented the Aβ25-35-induced decrease in Akt phosphorylation 1 and 7 days after injection

\[(a, b, c) P(S)^{Thr}_{Akt\text{total}}\] Akt ratio analyzed 1 day after Aβ25-35 injection. 
\[(d, e, f) P(S)^{Thr}_{Akt\text{total}}\] Akt ratio analyzed 7 days after Aβ25-35 injection. Mice were administered with ANAVEX2-73 (0.1-1 mg/kg i.p.), PRE-084 (0.5, 1 mg/kg i.p.), xanomeline (0.5, 1 mg/kg i.p.) or saline 20 min before the i.c.v. injection of Aβ25-35 peptide (9 nmoi i.c.v.) or ScAβ, 1 or 7 days before sacrifice. n = 4-6, F(4,39) = 4.23, p < 0.05 in (a); n = 4-6, F(3,23) = 1.63, p > 0.05 in (b); n = 4-6, F(1,17) = 2.14, p > 0.05 in (c); n = 10-13, F(4,45) = 3.32, p < 0.05 in (d); n = 5-13, F(3,39) = 3.40, p < 0.05 in (e); n = 5-10, F(1,29) = 4.73, p < 0.01 in (f). * p < 0.05; ** p < 0.01 vs. the (Sc.Aβ+V)-treated group. # p < 0.05, ## p = 0.01 vs. the (Aβ25-35+V)-treated group. Dunnett's test.
ANAVEX2-73, PRE-084 and xanomeline prevented the Aβ25-35-induced activation of GSK3β in the mouse hippocampus 7 days after injection.

Mice were administered with ANAVEX2-73 (0.1-1 mg/kg i.p.), PRE-084 (0.5, 1 mg/kg i.p.), xanomeline (0.5, 1 mg/kg i.p.) or saline 20 min before the i.c.v. injection of Aβ25-35 peptide (9 nmol i.c.v.) or Sc.Aβ, 7 days before sacrifice. n = 7-9; F(4,28) = 4.62, p < 0.05 in (a); n = 7-12; F(4,28) = 2.81, p < 0.05 in (a); n = 5-15; F(4,28) = 2.91, p < 0.05 in (c); n = 5-14; F(4,28) = 4.28, p < 0.05 in (b); n = 5-6; F(4,28) = 9.51, p < 0.001 in (d); n = 5-12; F(4,28) = 5.09, p < 0.01 in (f); *p < 0.05, **p < 0.01, ***p < 0.001 vs. the (Sc.Aβ+V)-treated group; #p < 0.05, ##p < 0.01 vs. the (Aβ25-35+V)-treated group. Dunnett’s test.
ANAVEX2-73, PRE-084 and xanomeline prevented the Aβ_{25-35}-induced hyperphosphorylation of Tau in the mouse hippocampus 7 days after injection.

(a, c, e) $\frac{P(\text{capped})}{\text{total}}$ Tau/total Tau ratio and (b, d, f) $P(\text{Thr}^392)$ Tau/total Tau ratio. Mice were administered with ANAVEX2-73 (0.1-1 mg/kg ip.), PRE-084 (0.5, 1 mg/kg ip.), xanomeline (0.5, 1 mg/kg ip.) or saline 29 min before the i.c.v. injection of Aβ_{25-35} peptide 8 nmol i.c.v. or Sc. Aβ, 7 days before sacrifice. n = 7-14, $F_{(2,18)} = 24.53$ p < 0.01 in (a); n = 7, $F_{(1,18)} = 10.5$, p < 0.001 in (b); n = 5-7, $F_{(2,18)} = 3.89$, p < 0.05 in (c); n = 5-11, $F_{(2,18)} = 4.10$, p < 0.05 in (d); n = 5-11, $F_{(3,18)} = 4.10$, p < 0.05 in (e); n = 4-14, $F_{(2,18)} = 3.98$, p = 0.05 in (f). * p < 0.05, ** p < 0.01, *** p < 0.001 vs. the (Sc.Aβ+V)-treated group; # p < 0.05, ## p < 0.01 vs. the (Aβ_{25-35}+V)-treated group; Dunnet’s test.
DISCUSSION

- We here first confirmed that ANAXEX2-73 dose-dependently prevented the $\alpha_\beta_{25-35}$-induced memory deficits in the novel object recognition test, assessing long-term recognition memory (Figs. 1,2). The active dose-range of ANAVEX2-73 was 0.3-1 mg/kg, as already described to led to anti-amnesic and neuroprotective effects [5]. The $\sigma_1$ receptor agonist, PRE-084 (1 mg/kg) and the muscarinic receptor agonist xanomeline (0.5 mg/kg), were also able to prevent the recognition memory deficits. These observations confirmed that activation of the $\sigma_1$ receptor or muscarinic (and particularly M1) receptor efficiently activated neuroprotective pathways in $\alpha_\beta_{25-35}$-treated mice.

- We characterized the time-course impact on kinases activation in the hippocampus of mice of the $\alpha_\beta_{25-35}$ peptide intracerebroventricular injection (Fig. 3). Using an ex vivo Western blot approach, we showed that P(S\textsuperscript{473})Akt level is rapidly decreased at days 1 and 3 after injection, suggesting a transient decrease in Akt activity at day 1 to 3. Akt is a serine-threonine kinase which plays a role in multiple cellular pathways, and it particularly inactivates GSK3$\beta$ by phosphorylating the kinase on S\textsuperscript{9}. The level of P(S\textsuperscript{9})GSK3$\beta$ was significantly decreased 7 days after the ICV injection. In parallel, the level of P(Y\textsuperscript{216})GSK3b, promoting activation of the kinase, was significantly increased at all time tested. This decrease in P(S\textsuperscript{9})GSK3$\beta$ level and concomittent increase in P(Y\textsuperscript{216})GSK3$\beta$ level could explain the $\alpha_\beta_{25-35}$-induced hyperphosphorylation of Tau protein, which has already been described [8,9].

- The ANAVEX2-73 treatment prevented the P(S\textsuperscript{473})AKT decreases at day 1 and 7 in the dose-range tested. This effect was shared by PRE-084 and xanomeline (Fig. 4). These observations showed that $\sigma_1$ protein or muscarinic receptor activation maintained Akt activity in $\alpha_\beta_{25-35}$-treated mice.
ANAVEX2-73 attenuated and xanomeline prevented the \( \text{A}^{25-35} \)-induced decrease in \( P(S^{9})\text{GSK3}\beta \) level. This effect was not shared by PRE-084. But all drugs significantly prevented the increase in \( P(Y^{210})\text{GSK3}\beta \) level in the dose-ranges tested (Fig. 5). These observations showed that \( \sigma_{1} \) protein or muscarinic receptor activation decreased GSK3\( \beta \) activity in \( \text{A}^{25-35} \)-treated mice.

Increased phosphorylation of Tau protein on S\( ^{202} \) and T\( ^{205} \) (AT8 clone) and on T\( ^{212} \) and S\( ^{214} \) (AT100 clone) are commonly find in AD and potentially responsible for the formation of NFT. We observed here that Tau appeared hyperphosphorylated in all these epitopes 7 days after \( \text{A}^{25-35} \) injection in mice (Fig. 6). Moreover, ANAVEX2-73, PRE-084 and xanomeline treatments led to a blockade of \( \text{A}^{25-35} \)-induced increases in Tau phosphorylation in the dose-ranges tested and using both antibody clones. The drugs prevented the Tauopathy in the \( \text{A}^{25-35} \) nontransgenic model of AD in mice.

The present study confirmed that ANAVEX2-73, a mixed muscarinic and \( \sigma_{1} \) receptors agonist, showed marked neurrrprotective activity, particularly by preventing Tau hyperphosphorylation due to Akt inactivation and GSK3\( \beta \) activation. The mixed pharmacological activity of the compound explain its relatively low active dose in vivo as compared with its in vitro affinities for the muscarinic and \( \sigma_{1} \) targets.

REFERENCES
