AZP2006 a first in class new neuroprotective compound for the treatment of Alzheimer's disease and related neurodegenerative diseases.

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ABSTRACT

Alzheimer disease (AD) affects mainly people over the age of 65 years, suffering from different clinical symptoms such as progressive decline in memory, thinking, language, and learning capacity. AD is pathologically characterized by extracellular senile plaques composed of amyloid-β (Aβ) peptides (Aβ plaques) and intracellular neurofibrillary tangles composed of hyperphosphorylated tau. Synapse loss is widespread and pronounced. In AD, the chronic Aβ accumulation causes cerebral neuroinflammation by activating microglia.

AZP2006 is currently in clinical development phase 2a in Progressive Supranuclear Palsy (PSP) patients. We previously showed that AZP2006 was protective in different vivo and vitro models of PSP reducing the hyperphosphorylation of Tau protein, the deposits in animals and the cognitive deficits. We showed that AZP2006 was able to reduce the neuroinflammation and could protect neurons via the release of a growth factor: the progranulin (PRGN). Here, we investigated the neuroprotective effect in in vitro /vivo models of AD. In addition, mechanistic studies were conducted to deeply investigate its mode of action. Using primary culture of cortical/hippocampal neurons injured with in Aβ1-42 oligomers in presence and absence of microglial cells we showed that AZP2006 rescued neurons from the injury reducing the hyperphosphorylation of Tau, protecting synapses and neurite connections.

Using in vivo models, we showed that AZP2006 was able to protect but also to restore cognitive impairment, to increase the synapse number, to reduce the microglial inflammation and the hyperphosphorylated Tau protein accumulation.

In addition, mode of action studies showed that AZP2006 displayed a high affinity for the lysosomes and was able to increase the PRGN/Prosaposin complex stability and finally the release of PRGN involved in the neuron survival and neurite outgrowth. We showed that the inhibition of these factors fully abolished the neuroprotective effect and the synaptogenesis induced by AZP2006. In addition, AZP2006 was able to antagonize Toll-like receptor 9 (TLR9) highly involved in the inflammatory process. This dual effect makes AZP2006 a serious candidate for the treatment of neurodegenerative disorders such as AD.

MATERIALS AND METHODS

***In vitro* investigations** : Primary rat cortical neurons (E15) were cultured as described by Callizot *et al.*, 2013 with modifications. The cells were seeded at a density of 30,000 per well in 96-well plates (for immunostaining).

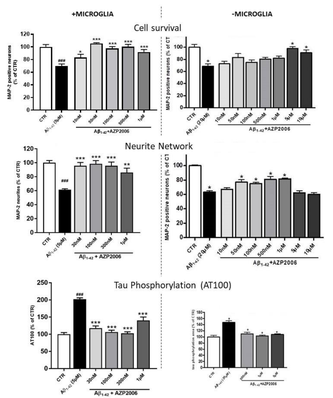
Pharmacological treatments: Aβ1-42 (20 µM ~2 µM of AβO for 24 h or 5 µM~0,5 of AβO for 72 h) was applied on day 11. Untreated cultures served as controls. AZP2006 (at different concentrations) was applied immediately after the neurotoxic agent. 72 hours after introduction, the cell culture supernatant was collected for PRGN quantification (Elisa assay Kit). Anti-PRGN Ab was added in the culture medium 1h before AZP2006 and Aβ.

Staining of cortical neurons and microglia and automatic microscopic analysis: After intoxication, neurons were fixed. The cells were incubated with : a) chicken polyclonal antibody anti microtubule-associated-protein 2 (MAP-2), and/or b) mouse monoclonal antibody anti OX-41 (microglia) and/or c) mouse monoclonal antibody anti phospho tau (AT-100) to evaluate hyperpho Tau protein aggregation into neurons. The immuno-labelled cultures were automatically analysed with MetaXpress (Molecular Devices, USA) at X20 magnification. For the gene expression silencing, siRNA targeting the expression of GRN and PSAP (Sigma-Aldrich) were transfected on day before the Aβ injury.

***In vivo* investigations** : The potential protective effect of the long-term administration of AZP2006 was tested in the mouse model of accelerated ageing: the Senescence-Accelerated Mouse-Prone 8 (SAMP-8). This model displays hyperphosphorylation of Tau, abnormal Aβ accumulation, increased oxidative stress and gliosis. SAMP-8 mice also show impaired immune response and deficits of learning and memory. SAMP-8 mice were treated p.o. (drinking water) with vehicle or 3mg/kg/day AZP2006 from the age of 1, 2, 4 or 6 months and evaluated each 2 months until the age of 10 months.

Figure 1

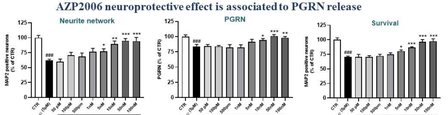
AZP2006 exhibits neuroprotective effect on primary cortical neurons in presence of microglia



Effect of AZP2006 on neuron survival, neurite network and tau phosphorylation (AT-100) levels on primary rat. Effect was observed on cortical neurons injured with Aβ1-42 peptides (72h (+**microglia**) or 24h (-**microglia**)). Data expressed as percentage of control show the mean ± SEM (100% = no Aβ, no compound). One-way ANOVA followed by Dunnett's test, n=4-6. *p< 0.05 was considered significant.

Figure 2

AZP2006 neuroprotective effect is associated to PGRN release



AZP2006 neuroprotective effect associated to PGRN release. Effect was observed on cortical neurons injured with Aβ1-42 peptides (72h (+**microglia**)) . Data expressed as percentage of control show the mean ± SEM (100% = no $Aβ_{1-42}$, no compound). One-way ANOVA followed by Fisher's test, n=5-6. *, ** and *** or ### indicate p<0.05, p<0.01 and p<0.001 respectively vs $Aβ_{1-42}$ condition (*) or vs CTR condition (#).

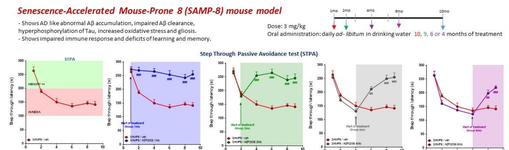
AZP2006 reduces neuroinflammation induced by Aβ1-42 injury in presence of microglia

Figure 3

Effect of AZP2006 on microglia activation (OX-41), IL-6, IL-1β and TNFα release in primary rat culture of cortical neurons cultured with microglia and injured with Aβ1-42 peptides for 72h (5 µM corresponding to 0.5 µM of AβO). Data exp expressed as percentage of control show the mean ± SEM (100% = no β amyloid, no compound). One-way ANOVA followed by Fisher's test, n=4-6. # or *, ## or ** and ### or *** indicate p<0.05, p<0.01 and p<0.001 respectively vs CTR condition (#) or vs Aβ1-42 condition (*).

Figure 4

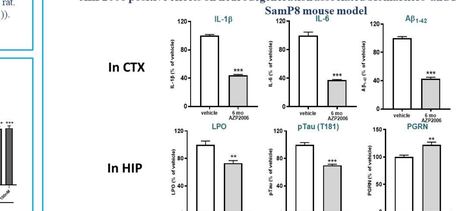
AZP2006 prevents and restores cognition deficit in SamP8 mouse model



Neuroprotective effects of AZP2006 in the SAMP-8 mouse model. AZP2006 protected mice from memory deficits evaluated in the STPA (A and B) tests (n=7-12). Mean ± SEM. One-way ANOVA, significant differences when compared with vehicle treated animals. #, ## and ### indicate p<0.05, p<0.01 and p<0.001 respectively.

Figure 5

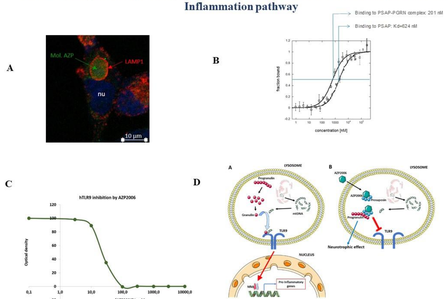
AZP2006 positive effects on neurodegeneration associated biomarkers and increase of PGRN in SamP8 mouse model



Neuroprotective effects of AZP2006 in the SAMP-8 mouse model. Mice were treated from the age of 6 to 10 month of age. AZP2006 decreased the levels of IL-1β,IL-6 in cortex and Aβ1-42 , AZP2006, also decreased LPO, pTau (T181) and increased (n=4-7). Mean ± SEM t-test. Significant differences when compared with vehicle treated animals ** and *** indicate p<0.01 and p<0.001 respectively.

Figure 6

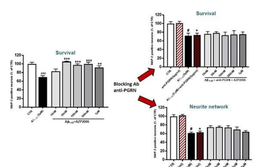
AZP2006 targets the lysosome, associate with PSAP-PGRN complex and prevents TLR9 Inflammation pathway



(A) Immunostaining on SY5Y APPwt cells. A dansyl-AZP2006 analog is localized inside a LAMP1 positive vesicle.
(B) AZP2006 binds to PSAP with a better affinity when complexed to PGRN (Microscale thermophoresis).
(C) In recombinant HEK-293 cells functionally over expressing a human or mouse TLR9 protein, AZP2006 inhibited both human and mouse TLR9 activation with an IC50=35 nM affinity
(D) Postulated AZP2006 molecular mode of action

Figure 7

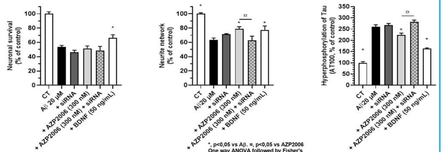
AZP2006 promotes neuronal survival and neurite network via PGRN



AZP2006 promotes survival and neurite network via PGRN. Data expressed as percentage of control show the mean ± SEM (100% = no $Aβ_{1-42}$, no compound). One-way ANOVA followed by Dunnett's test, n=6. # or *, ** and *** or ### indicate p<0.05, p<0.01 and p<0.001 respectively vs CTR condition (#) or vs $Aβ_{1-42}$ condition (*).

Figure 8

AZP2006 neuroprotective effects require both PSAP and PGRN



(A) Neuronal survival and (B) length of the neurite network of primary cortical neurons after an Aβ1-42 injury. Expressions of PSAP and PGRN were silenced by siRNA. (C) Hyperphosphorylation of Tau (AT100) in primary cortical neurons neurons after an Aβ1-42 injury. AZP2006 neuroprotective effect is lost in absence of PSAP and PGRN expression.

CONCLUSIONS

AZP2006 is a first in class new chemical entity that:

- **Exhibits strong neuroprotective effects both in vitro and in vivo in presence of microglia**
- **Prevents cell death, increases neurite network and reduces Tau hyperphosphorylation**
- **Prevents neuroinflammation**
- **Increases Progranulin levels both in vitro and in vivo.**
- **Requires Progranulin and Prosaposin to prevent cell death and increase neurite network**

AZP2006 effects requires Progranulin (PGRN) in association with Prosaposin (PSAP). We hypothetize that AZP2006 stabilize the PGRN-PSAP complex and therefore:

- **Prevents PGRN processing into pro-inflammatory factor granulin (thus reducing Neuroinflammation)**
- **Allows increased PGRN secretion by microglia (thus inducing Neuronal growth end regeneration)**

This dual effect makes AZP2006 a serious candidate for the treatment of neurodegenerative disorders such as AD.

References

Callizot et al., (2013) J Neurosci Res. 2013;91(5):706-16 / Laurent et al., (2017) Brain. 2017 Jan;14(1):184-200 / Meunier et al., (2013) Eur J Pharmacol. 2013 Jan 5;698(1-3):193-9

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