SETTING A RATIONAL SCREENING PROGRAMME FOR TRANSTHYRETIN-Aβ BINDING STABILIZING COMPOUNDS THAT MAY LEAD TO POTENTIAL ALZHEIMER'S DISEASE MODULATING DRUGS

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1. Summary

A number of physiological studies indicate that transthyretin (TTR) plays a role in Alzheimer's disease (AD) pathogenesis. Reduced levels of TTR in human cerebrospinal fluid (CSF) are found in AD patients, making TTR a promising candidate biomarker of AD disease progression. TTR naturally occurs as a tetramer forming two binding pockets occupied by thyroid hormones, endogenous ligands, while transported in plasma and CSF. TTR is the main Aβ-binding-protein in human CSF. In vivo studies using TTR antibodies and deletion of TTR gene suggest that TTR is neuroprotective in animal models of AD. TTR binding also neutralizes Aβ oligomers cytotoxicity in vitro. Binding of TTR to Aβ oligomers can be enhanced in vitro by compounds that, in turn, bind and stabilize the TTR tetramer. Preliminary in vivo studies have shown that when one of these compounds, iododiflunisal, is administered to an AD mouse model it decreases brain Aβ levels and deposition and improves cognitive functions in these mice.

The main aims of this project were to investigate the molecular mechanisms involving TTR in AD and to settle a drug discovery program for the discovery of small-molecule chaperones that enhance the TTR/Aβ interaction.

Thanks to a multidisciplinary consortium integrated by five research teams the project achieved new knowledge at molecular level and discovered a set of small-molecule compounds, among them some drugs, that are enhancers of the TTR/Aβ interaction and potential candidates for AD therapeutics.

2. Results

We would like to summarize here the main achievements of the project in the context of the overall goals of the project, as well as in the process of drug discovery and development of new compounds for the possible treatment of Alzheimer's disease. These achievements are the result of a strong coordinated effort between five participating interdisciplinary research teams. Accordingly, the results have emerged from the following activities:

a) Computational studies
b) Biological screening
c) Structural studies
d) High-throughput screening assays
e) Biophysical studies
f) In vivo studies

COMPUTATIONAL SCREENING

a) Build a model of TTR / Aβ interaction

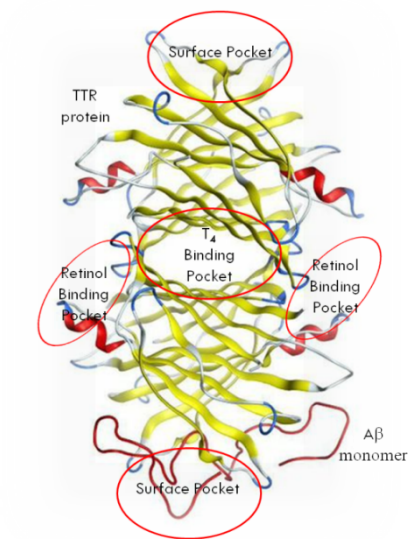

Figure 1.
A model of the TTR/Aβ monomer complex, highlighting the interactions in the surface pockets.

We obtained a refined computer model of the TTR/Aβ monomer interaction, using protein-protein computational docking techniques (Figure 1). This model was further validated by NMR studies.

b) Virtual screening to obtain compounds that interact with the TTR/Aβ complex

We selected compounds using computational techniques (virtual screening / docking), starting from databases of compounds that either are known TTR tetramer stabilizers, or compounds in the market (registered drugs) or in clinical phases, in a drug discovery process named repurposing or repositioning of known compounds for the treatment of new diseases. Our refined TTR/Aβ model was also used to select additional compounds that were advanced in clinical trials or had been approved as registered drugs for AD or age-related disorders, or compounds that had been shown to interact with TTR in the literature, or else that were similar to some of the compounds we had selected, in order to expand the list of compounds to be assayed in experimental screening assays and in structural studies.

We have built a computational model of the TTR/Aβ interaction, which we have used to select around 100 compounds (virtual hits) from around 10,000 by virtual screening.

Nearly 90 compounds were acquired or obtained and sent to the experimental screening team partner for their biological screening for TTR affinity and for TTR selectivity and stability.

BIOLOGICAL SCREENING

a) Validate virtual screening hits in experimental assays for TTR affinity and for TTR selectivity and stability

We designed and performed a battery of in vitro tests to screen for TTR stabilizers prior to the test of the interaction between the TTR tetramer and Aβ peptide in the presence or absence of compounds.

We analysed the effect of 90 compounds in TTR stability using different assays:

a. Qualitative analysis: Thyroxine (T4) competition displacement assay using a gel electrophoresis approach, using either the recombinant wtTTR and/or using human plasma.

b. T4 competition quantitative assay, using recombinant wtTTR. This approach is applied to compounds selected in qualitative analysis.

c. Stability assay, using a semi-denaturing gel electrophoresis approach. Compounds that produce a tetramer/monomer (i.e. folded/ monomeric TTR) ratio higher than the one found in control (absence of compound), are considered TTR stabilizers.

b) Selection of compounds for ternary assays with selected compounds.

A cyclic methodology was used (computational workflows, chemical knowledge, and biochemical experimental screening) that resulted in more than 50 compounds showing good TTR interactions.

STRUCTURAL STUDIES

a) Validate TTR / Aβ model through NMR

We carried out a computational prediction of the putative aggregating regions in the TTR and Aβ sequences, using the TANGO software, which provided information on the contribution of each key residue to the amyloidogenicity capacity of the whole molecules. A preferred interaction was suggested between residues 17-21 at the central hydrophobic core of Aβ with the hydrophobic patch formed by residues 93-97 in the TTR sequence (VVFTA), located at the surface pocket of the protein. Fittingly, the

described peptide sequence contains the major NMR-based epitope motif described above for the interaction of Aβ(12-28) with TTR.

We have employed NMR spectroscopy methods assisted by computational protocols to analyse the molecular mechanisms underpinning these molecular recognition processes. We have used STD-NMR to examine the interaction between TTR and different Aβ peptide sequences. The NMR results have revealed the specific interaction between TTR and the main recognition element of Aβ, the Aβ(12-28) peptide. These NMR-based findings have also been supported by a preliminary computational model of the TTR-Aβ complex.

The NMR protocol has also been applied to study the molecular recognition process in the presence of T4 ligands, particularly of our lead compound, iododiflunisal (IDIF), allowing us to propose a molecular model for the TTR-IDIF-Aβ interaction.

This model provides a structural view of the involvement of TTR and T4 channel-binder molecules in AD, potential key information for designing compounds useful in the AD arena. The key structural features of the interaction between TTR and the Aβ(12-28) peptide, the essential recognition element of Aβ, have been unravelled by STD-NMR spectroscopy methods in solution. Molecular aspects related to the role of the TTR stabilizer IDIF on the TTR-Aβ complex have also been examined.

b) Structural analysis of SMCs/TTR/Aβ interactions through NMR

We applied STD-NMR techniques to the system TTR-Aβ(12-28), trying to provide a structural basis for the positive effects exerted by IDIF in the AD features observed in animal experiments. The obtained NMR data strongly suggested that both molecules, IDIF and Aβ(12-28), are able to simultaneously bind to TTR, although at different binding sites. Thus, the homotetrameric TTR stabilized by T4-ligands is still able to properly interact with the peptide, indirectly supporting the demonstrated ability of T4-ligands to improve the TTR-Aβ interaction.

To obtain site specific information about the TTR epitopes involved in the interaction with Aβ monomers or small size oligomers we employed protein-based NMR methods. In particular, experiments based on monitoring changes on 15N-labelled protein signals were used to identify the amino acids of the TTR that are key for the interaction. Due

to the difficulties in dealing with the full Aβ peptide we analysed the interaction between TTR and the peptide fragment Aβ(12-28). The comparison of 1H-15N TROSY spectra of the protein in the absence and in the presence of three equivalents of Aβ(12-28) showed significant differences. Analysis of the total shift perturbation per residue allowed us to detect the most affected amino acids by the binding of Aβ(12-28) peptide to TTR, which were mapped on the TTR structure.

c) CRYSTALLIZATION STUDIES

In the crystallization trials of binary complexes, we described two new structures: two new binary complexes (TTR/stabilizer) were obtained. Also, the structural features that lead to the stabilizing effect of the compounds were identified.

To obtain the structure of ternary complexes [TTR/stabilizer/Aβ sequence] a myriad crystallization trials were performed. Several Aβ fragments were tested, namely fragments (1-16), (20-29), (29-40), (31-35), (1-40) and (1-42). TTR crystals were obtained, and X-ray diffraction data were collected using synchrotron radiation (ESRF, Grenoble, France and ALBA, Barcelona, Spain). The structures were solved. Unfortunately, in all these trials the Aβ fragments were not observed in the electron density maps.

HIGH THROUGHPUT SCREENING and BIOPHYSICAL STUDIES

a) Validate small-molecule chaperones (SMCs) selected for enhancing the TTR / Aβ interaction through a HTS Ternary Assay

The development and validation of a high throughput screening (HTS) assay was a key issue in the drug discovery project. We set up a turbidimetry-based screening assay, using our recombinant TTR protein and a short version of Aβ, [Aβ(12-28)], the key sequence of interaction discovered in our NMR studies. The assay has been validated and optimized for a HTS screening assay. Optimization of experimental parameters for Aβ(12-28) aggregation was conducted using a 2^3 factorial design. The assay was checked for reproducibility by statistical analysis and validated against control compounds. With our validated HTS assay we performed the screening assay in each of the more than 50 small compounds that were selected in the biological assays.

b) Selection of candidate SMCs for in vivo assays

ISOTHERMAL TITRATION CALORIMETRY

ITC studies were performed using a MicroCal VP-ITC calorimeter equipment (www.Malvern.com). This biophysical technique allowed us to obtain the full thermodynamic characterization of the binary interaction [TTR + full Aβ (1-42)], and of the ternary biomolecular interaction, [TTR+ IDIF + full Aβ (1-42)]. ITC results confirmed the chaperoning effect of our small-molecule IDIF at enhancing the TTR/Aβ interaction. ITC studies were performed in the prioritized list of small compounds selected in our HTS screening assay.

A set of ten small-molecule chaperones have been discovered, some of which are registered drugs.

c) COMPLEMENTARY ASSAYS: TEM and TOXICITY studies

TRANSMISSION ELECTRON MICROSCOPY (TEM)

TEM is a high-resolution imaging technique that enables identification and classification of aggregates (protofibrils, amyloid fibrils containing varying numbers of strands, and amorphous aggregates). Nevertheless, TEM imaging provides no quantitative information about aggregation kinetics or about the concentrations of the observed fibrils. With TEM analysis, we showed that selected SMCs in complex with TTR prevent the aggregation and fibrils formation by Aβ peptide.

EFFECT OF SMCs ON Aβ PEPTIDE TOXICITY

We evaluated apoptosis, through measurement of caspase 3 activation, induced by Aβ peptide incubated with TTR or with TTR pre-incubated with a small set of small molecule chaperones (SMCs), showing that the presence of the SMC further enhanced the ability of TTR to prevent Aβ toxicity.

IN VIVO STUDIES

a) Distribution of TTR and of TTR-compounds complexes in vivo through radiolabelling and animal imaging assays;

[^{131}I]-labelled stabilizer IDIF was synthesized for the first time. After intravenous administration in wild type mice the in vivo biodistribution of the following compounds, [^{131}I]IDIF, [^{131}I]TTR and the binary complexes [^{131}I]TTR-IDIF (labelled in TTR) and TTR- [^{131}I]IDIF (labelled in IDIF) were investigated.

Results confirm the capacity of TTR to cross the blood-brain barrier (BBB) and suggest that the formation of TTR-IDIF complexes enhances BBB permeability of both IDIF and TTR.

b) Longitudinal studies in animal models of Alzheimer's disease

We tackled the therapeutic efficacy of two compounds (IDIF and tolcapone) *in vivo* using our AD/TTR Tg mice transgenic model of AD (model AβPPswe/PS1A246E/TTR).

Iododiflunisal was synthesized and scaled up in our labs and tolcapone was isolated from the drug TASMAR acquired at a pharmacy. For in vivo treatments the corresponding water soluble salts were prepared following described protocols.

Longitudinal PET imaging studies were performed with the β-amyloid tracer [^{18}F]-florbetaben. Hippocampus-to-cerebellum uptake ratios were determined to assess the presence of plaques as a surrogate of treatment efficacy.

We have tested two compounds in longitudinal PET imaging studies in animal models of Alzheimer's disease. These in vivo experiments are still ongoing and final conclusions cannot be derived yet. Ex vivo studies to confirm in vivo findings are also in progress.

3. Possible clinical implications derived from results

In terms of drug discovery and development, our consortium has achieved in this project the two aims proposed: understanding the TTR /Aβ interaction and discovering a set of small compounds that enhance the interaction of TTR and Aβ, thus opening a new avenue for the treatment of Alzheimer's disease.

Estimated figures of victims affected by AD are at a staggering 100 million worldwide by 2050. The scale of the problem is in marked contrast to the lack of solutions available to efficiently tackle this major threat to individuals and society. Current pharmacotherapy for Alzheimer's disease may help lessen cognitive and functional decline, but it is not disease-modifying or curative. There is an urgent need to develop new treatments for AD. Understanding how the disease operates and identifying novel molecules are two crucial aspects in deciphering AD.

Our consortium has worked during the last few years in this framework integrating knowledge and efforts from five multidisciplinary teams. The discovery and selection of a group of small-molecule chaperones (SMCs), compounds that increase the interaction between transthyretin and the Aβ peptide, opens a new strategy for a therapeutic treatment of Alzheimer's disease. Some of the selected compounds are repurposed drugs, and thus once the effect on the reduction of amyloid burden in AD transgenic mice is confirmed, they could eventually move into clinical trials without additional toxicological analyses, since the compounds are already approved as treatments for other diseases. For the selected compounds which are not drugs, animal assays should be run first Then, if the positive effect on the reduction of amyloid fibrils is confirmed, a preclinical safety / clinical development process should be established and carried out before the compound can reach the late stage clinical trials.

4. Publications

PATENTABILITY STUDY *(in progress)*

Articles

L. M. Santos, D. Rodrigues, M. Alemi S.C. Silva, C. A. Ribeiro, I. Cardoso. Resveratrol administration increases Transthyretin protein levels ameliorating AD features-importance of transthyretin tetrameric stability. *Mol. Med.* **2016**, 22, 597-607.

M. Alemi, S. C. Silva, I. Santana, I. Cardoso. Transthyretin stability is critical in assisting beta amyloid clearance - Relevance of Transthyretin stabilization in Alzheimer's Disease. *CNS Neurosci. Ther.* **2017**, *23*, 605-619.

C.S. Silva, J. Eira, C. A. Ribeiro, Â. Oliveira, M. M. Sousa, I. Cardoso, M. A. Liz. Transthyretin neuroprotection in Alzheimer's disease is dependent on proteolysis. *Neurobiol. Aging.* **2017**, *59*, 10-14.

A. Gimeno, L. M. Santos, M. Alemi, J. Rivas, D. Blasi, E.Y. Cotrina, J. Llop, G. Valencia, I. Cardoso, J. Quintana, G. Arsequell, J. Jiménez-Barbero. Insights on the Interaction between Transthyretin and Aβ in Solution. A Saturation Transfer Difference (STD) NMR Analysis of the Role of Iododiflunisal. *J. Med. Chem.* **2017**, *60*, 5749-5758.

E. Y. Cotrina, A. Gimeno, J. Llop, J. Jiménez-Barbero, J. Quintana, G. Valencia, I. Cardoso, G. Arsequell. A Simple Assay for Screening Chaperones of the Interaction Between Transthyretin- and beta-Amyloid Peptides to Identify Potential Alzheimer's Disease Therapeutics (*submitted*).

TTRAD-consortium

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