Targeting transthyretin in Alzheimer's disease: drug discovery of small-molecule chaperones as disease-modifying drug candidates for Alzheimer's disease

*Ellen Y. Cotrina,[#] Luis Miguel Santos,^{l,⊥} Josep Rivas,[†] Daniel Blasi,[†] José Pedro
Leite,^{l,⊥,‡} Márcia A. Liz,^{l,⊥} Maria Antònia Busquets,[±] Antoni Planas,[~] Rafel Prohens,[°]
Ana Gimeno,[≠] Jesús Jiménez-Barbero^{≠,l}, Luis Gales,^{l,⊥,‡} Jordi Llop,[‡] Jordi Quintana,[‡]
^{*} Isabel Cardoso,^{l,⊥,*,‡} and Gemma Arsequell,^{#,*,‡}*

[#]Institut de Química Avançada de Catalunya (I.Q.A.C.-C.S.I.C.), 08034 Barcelona,
Spain.

^lIBMC - Instituto de Biologia Celular e Molecular, 4200-135 Porto, Portugal.

[⊥]i3S – Instituto de Investigação e Inovação em Saúde, Universidade do Porto, 4200-135
Porto, Portugal.

[†]Plataforma Drug Discovery, Parc Científic de Barcelona (PCB), 08028 Barcelona,
Spain.

[‡]Instituto de Ciências Biomédicas Abel Salazar (ICBAS), 4050-013 Porto, Portugal.

[±] Facultat de Farmàcia i Ciències de l'Alimentació. University of Barcelona. 08028

Barcelona

[~] Institut Químic de Sarrià, Universitat Ramon Llull, 08017 Barcelona, Spain

[°] Centres Científics i Tecnològics, Universitat de Barcelona, 08028 Barcelona, Spain

[≡] CIC bioGUNE, Basque Research and Technology Alliance (BRTA), Bizkaia

Technology Park, Building 800, 48160 Derio, Spain.

^l Ikerbasque, Basque Foundation for Science, 48009 Bilbao, Spain.

^l CIC biomaGUNE, Basque Research and Technology Alliance (BRTA), 20014 San

Sebastian, Spain

[‡] Equally contributing corresponding authors

* To whom correspondence should be addressed

KEYWORDS. Targeting transthyretin, small molecule chaperones (SMCs), AD drug discovery, transthyretin tetramer stability, transthyretin / A β interaction, HTS screening, protein-protein interactions, repurposing, multi-target screening, computational screening, Alzheimer's disease, AD disease-modifying drugs

ABSTRACT

Transthyretin (TTR) has a well-established role in neuroprotection, evidenced in Alzheimer's Disease (AD). By targeting TTR we have setup a drug discovery program of small-molecule compounds that act as chaperones, enhancing TTR/ amyloid- β peptide (A β) interactions. In a first stage, we carried out two computational drug repurposing approaches. In a second stage, the computationally selected compounds were assessed for their ability to bind and stabilize the TTR tetramer, using thyroxine displacement tests, and by assessing the level of monomers, respectively. In a third stage, the selected 53 best performing molecules were run through our in-house validated high-throughput screening ternary test. By targeting TTR in our AD drug discovery program, small-molecule chaperones (SMCs) have been discovered, providing the basis for a novel target for Alzheimer's disease (AD) based on their enhancement of the TTR/A β interaction. Among the SMCs, we have found our lead small-molecule compound Iododiflunisal (IDIF), a molecule in the discovery phase, one investigational drug (luteolin), and 3 marketed drugs (sulindac, olsalazine and flufenamic), which could be directly repurposed or repositioned for clinical use. Importantly, we found that not all TTR tetramer stabilizers are good SMCs *in vitro*, emphasizing the importance of our discovery program. A small set of these SMCs will be prioritized to enter preclinical safety studies, to validate TTR as a target *in vivo*, and to select one repurposed drug as a candidate to enter clinical trials for AD. We envisage that this new target will feed the currently exhausted pipeline of drugs in phase I for AD with the goal of increasing AD disease-modifying therapies.

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disease and is the leading cause of dementia. AD is characterized by accumulation of amyloid- β (A β) aggregates in various conformations, filamentous intraneuronal inclusions mainly constituted by hyperphosphorylated Tau protein (p-Tau), and synaptic dysfunction and neuronal loss.¹

One of the most difficult challenges in AD research is to find a disease modifying therapy (DMT),² this is, a therapy with an agent that produces an enduring (permanent) change in the course of AD. Up to now, AD has no currently approved DMTs. The five drugs available in the market are involved in only improving symptoms and are highly patient dependent, and no putative disease modifying drugs or new symptomatic treatments have achieved regulatory approval since 2003.³ There are 121 agents in the current AD treatment pipeline, 36 of them in phase III, 65 in phase II and 27 in phase I clinical trials. Out of these, 97 agents (80%) are in disease modification trials.⁴

AD drug development is not delivering new drugs in the last decades. Among the many explanations for such failure, one is the lack of new chemical entities from drug discovery approaches that reach the clinical Phase I level.⁵⁻⁸ There is an urgent need to find a treatment that prevents, delays the onset or slows the progression of this devastating disease.

With the aim of contributing with new candidate drugs that, after preclinical tests, may feed the currently exhausted pipeline of drugs in phase I for AD, we have settled a drug discovery program targeting transthyretin (TTR) and searching for small-molecule chaperones that may improve the neuroprotective function of TTR in AD.

Transthyretin (TTR), a homotetrameric protein mainly synthesized by the liver and the choroid plexus (CP), and secreted into the blood and the cerebrospinal fluid (CSF), respectively, has been specially recognised for its functions as a transporter protein of thyroxine (T₄) and retinol.⁹ The importance of TTR has also been robustly established in AD pathogenesis.¹⁰ It is most remarkable that TTR is the main A β binding protein in the CSF.¹¹⁻¹³ This binding is believed to naturally prevent A β aggregation and toxicity in this media. Comparative analysis of TTR evidenced decreased levels of this protein, not only in the CSF^{14,15} but also in plasma¹⁶⁻¹⁸ of AD patients, compared to controls. AD TTR-hemizygous mice showed increased A β production and deposition, compared to AD TTR WT littermates,¹⁹ whereas overexpressing human TTR WT in an AD mouse model decreased neuropathology and A β deposition.²⁰ Regarding the mechanisms proposed as underlying TTR neuroprotection in AD, *ex vivo* and *in vitro* studies, established that TTR has the ability to bind to A β , avoiding its aggregation and toxicity.²¹⁻²⁶ Recent reports suggest that TTR binds to A β oligomers and inhibits primary and secondary nucleation processes.^{25,26} TTR was also shown to assist A β brain efflux through the blood-brain barrier, and to promote its degradation in the liver. Results also revealed that TTR regulates the expression of LRP1, by increasing its levels, both in the brain and in the liver.²⁷ Recent data implicated TTR reduction in the thickening of the basement membrane of brain microvessels in AD mice,²⁸ an alteration that also occurs in the AD brain.²⁹

TTR tetrameric stability appears as a key factor in its interaction with A β peptide. Interestingly, TTR stability is also a key factor in Familial Amyloid Polyneuropathy (FAP)^{30,31} a systemic amyloidosis with a special involvement of the peripheral nerve system, which results from the aggregation, deposition and toxicity of mutated TTR. Since it is believed that tetramer dissociation into monomers is the first step leading to

TTR amyloid formation,³² TTR tetrameric stabilization has been defined as the basis for one of the possible therapeutic strategies in FAP.³³⁻³⁵ Such stabilization can be achieved through the use of small compounds sharing molecular structural similarities with thyroxine (T₄) which bind in the T₄ central binding channel.^{36,37} The search for TTR stabilizing ligands using virtual screening and computational procedures has been carried out previously³⁸⁻⁴⁰ and there is a large number of TTR and TTR-ligand three dimensional structures reported at the PDB.⁴¹ These computational and structural studies have been instrumental in the design, discovery and development of TTR stabilizing compounds that have either reached the market, like Tafamidis,^{42, 43} or that have reached clinical phases, like Tolcapone, a repurposed drug approved for the treatment of Parkinson's disease (Figure 1).⁴⁴

Figure 1. Chemical structures of TTR tetramer stabilizers: the orphan drug Tafamidis, the Non-steroidal anti-inflammatory drug (NSAID) diflunisal (DIF), the repurposed drug tolcapone for Familial amyloid polyneuropathy (FAP) and our lead compound iododiflunisal (IDIF).

We have demonstrated by early *in vitro* studies that TTR and A β bind together and that this interaction can be enhanced by a small set of TTR tetramer-stabilizing compounds,⁴⁵ one of them iododiflunisal (IDIF), a iodinated analog of the NSAID diflunisal (DIF) (Figure 1).⁴⁶⁻⁴⁸ More importantly, *in vivo* administration of IDIF to a mouse model of AD, resulted in decreased brain A β levels and deposition,^{49,50} and improving the cognitive functions that are impaired in this AD-like neuropathology.⁴⁹

Drug repurposing of already known drugs for new indications is a drug discovery approach increasingly being used to search for new therapies for diseases with unmet clinical needs. Both *in silico*^{51,52} and experimental⁵³ approaches on drug repurposing have been recently reviewed, as well as the application of this approach to drug discovery for AD.⁵⁴⁻⁵⁶ Furthermore, another interesting strategy in drug discovery is the multi-target approach, which aims to have a synergistic effect thanks to the interaction of a given ligand with different targets that are involved in a disease's pathway. Therefore, these synergies through target promiscuity could be translated into lower doses and reduced side effects.^{57,58}

In this paper, we report the discovery of TTR stabilizers through two complementary *in silico* drug repurposing approaches, as well as the systematic biological evaluation of the selected compounds on TTR stability, through competition with T₄ for TTR binding, and by measuring the amount of monomers. This process has led to the prioritization of a set of 53 TTR tetramer stabilizing compounds and to an analysis of their ability to enhance the TTR/A β interaction. Using our high throughput screening (HTS) proprietary ternary assay,²⁴ we have evaluated these 53 small-molecule compounds and have obtained a subset of small molecule chaperones (SMCs), among them our lead compound IDIF, one clinical phase compound (luteolin), and three approved drugs for other diseases (sulindac, olsalazine and flufenamic acid), that could enter clinical trials for AD as repurposed drugs.

RESULTS AND DISCUSSION

Computational studies

In order to find small molecule chaperones that enhance the TTR / A β interaction, two computational pipelines have been established, addressing drug repurposing and polypharmacology, as shown in Figure 2. The first approach started with the construction of a TTR/A β model as described in the Materials and Methods section in the Supporting Information. Then, compounds from the Integrity database (<https://integrity.thomson-pharma.com/integrity/xmlxsl/>) and from the literature were selected, so as they were either (i) known TTR binders, (ii) amyloid peptide binding ligands, or (iii) both, or (iv) compounds under study in clinical phases for the treatment of Alzheimer's disease. The use of the CARLSBAD software and database⁵⁹ allowed us to find privileged scaffolds with reported biological activity against both TTR and A β systems. This scaffold hunting methodology allowed us to find a group of compounds containing the stilbene substructure and able to interact with both entities (TTR and A β). Then, this stilbene scaffold was used to select molecules by substructure search in the Integrity-derived and literature-derived databases already mentioned, and the selected compounds were then prioritized by docking into our TTR/A β model. In total, 48 compounds with docking scores similar to IDIF (used as the cross-reference compound in the docking experiments) were prioritized, and 20 of them, that were commercially available, were selected and acquired to carry out the experimental assays.

Figure 2. Computational workflows for the selection of modulators of TTR/A β interaction (A) Based on drug repurposing and multi-target approach; (B) Based on multi-target computational docking and searches on common pharmacophores for several targets.

A second computational approach was carried out in order to exploit the drug-promiscuity and polypharmacology in a multitarget-based pipeline. This methodology allowed us to identify molecules able to bind TTR that also bind additional targets that have been associated with the treatment of AD and that are relevant in its pathological pathways. As described in the Materials and Methods section in the Supporting Information, this strategy includes multi-target molecular docking and molecular searches through common pharmacophore models to a set of targets (Figure 2B). Starting from the

Thomson Reuters Integrity database (<https://integrity.thomson-pharma.com/integrity/xmlxsl/>), which contains small molecules and drugs in the market or advanced clinical phases, we selected nearly 1400 compounds that were reported in preclinical, clinical or marketed phase and that had their protein target reported as one or more of the following, which have been associated to AD: APP (amyloid precursor protein); AchE (acetylcholinesterase); IL-10 (interleukin-10); α -, β - and γ -secretases; Tau protein; GABA and glutamate receptors. All these compounds were then docked into the TTR model, prioritizing a pool of 134 molecules that would theoretically bind the TTR T₄-channel with an affinity similar to IDIF. These molecules were then analyzed theoretically through cross-docking into the following protein structures: IL-10 (Interleukin-10) (PDB code 1ILK); inhibitor domain of APP (amyloid precursor protein; β -secretase (memapsin 2) complexed with inhibitor OM99-2 (PDB code 1FKN); human acetylcholinesterase (AChE) complexed with fasciculin-II (PDB code 1B41); metabotropic glutamate receptor 1 with LY341495 antagonist (PDB code 3KS9); metabotropic glutamate receptor 5 with glutamate (PDB code 3LMK). All these docking experiments lead to a prioritized list of 85 molecules, which were filtered to 27 molecules that would theoretically bind to TTR/A β complex (by docking experiments) and 4 additional AD related targets (multi-target docking approach for ligand selection). In addition, the 134 molecules that were selected before through docking with TTR were further analyzed computationally through the software LigandScout.⁶⁰ A common pharmacophore was built starting from the binding sites of TTR, β -secretase, metabotropic glutamate receptor 1 and metabotropic glutamate receptor 5, and then the 134 molecules were screened against this pharmacophore; 13 of them coincided with the list of 27 molecules derived from the multi-target docking, but we were able to select 4 additional molecules that matched with the common pharmacophore. The Table S1 in the

supplementary material contains all the chemical structures of the compounds that were selected through these computational procedures, as well as other molecules (that had been reported as TTR ligands, in the literature, and from previous studies in our laboratory), which were also selected after docking them into our TTR-A β model, and the results of the associated experimental assays for all the selected molecules, which are described hereunder.

Thyroxine binding assays and TTR stability assays

To confirm the ability of the proposed compounds to bind TTR we designed a flow chart through which all the selected compounds were experimentally assayed (Figure 3).

The first assay, to which all compounds were submitted, was a T₄ displacement assay.^{44,46} In brief, radiolabeled [¹²⁵I]-T₄ was incubated alone or in the presence of each of the compounds with TTR, and then samples were run in a native PAGE gel. Compounds that are able to bind to TTR in the T₄ central binding channel, and thus to displace [¹²⁵I]-T₄, result in decreased intensity of the [¹²⁵I]-T₄/TTR band. When using plasma as a source of TTR, which is the case of the present work, this approach also enables the visualization of the bands corresponding to the other T₄ transporter proteins, such as thyroxine-binding globulin (TBG) and albumin. IDIF was used as a reference compound (see representative example in Figure 3A). This assay, although not quantitative, is of utmost importance, in particular when using human plasma, since it also reveals the effect of the compounds in the other referred T₄ transporter proteins, and therefore, on their specificity for TTR binding. Using this approach, we characterized the behavior of the compounds regarding their ability to displace T₄ and their specificity for TTR binding (see Table S1 in the Supporting Information for a complete view of the behavior of all compounds in the qualitative T₄ displacement assay).

Figure 3. Flowchart to which compounds were subjected to determine experimentally which compounds bind to and stabilize TTR, and representative images of the results obtained in each assay. (A) The T₄ binding gel electrophoresis assay, using human plasma incubated with [¹²⁵I]-T₄ and with various compounds as competitors. The migration of the different plasma T₄ binding proteins is indicated. (B) The displacement of [¹²⁵I]-T₄ from WT TTR by competition with the compounds. The curves were obtained using various compounds indicated as competitive inhibitors. (C) The TTR stability assay, in which samples were analysed by Western Blot after incubation of the compounds with WT-TTR, under semi-denaturing conditions; the quantification plot presents the intensity

of monomer band, relative to the control condition which was considered one. (See compounds in Table S1 at the SI).

In addition to high affinity for TTR, compounds should also be specific for this protein, since lack of specificity, together with the high abundance of other plasma proteins, such as albumin, for instance, could lead to failure in therapeutics. However, at this stage of our selection, we mainly aimed at identifying strong TTR stabilizers which will be further investigated for their capacity to enhance the TTR/A β interaction. Thus, at this point all compounds that completely or significantly displaced T₄ from TTR, were selected for the quantitative assay regardless of their specificity (Table S1). Selected compounds were then evaluated in a quantitative T₄ assay to determine their EC₅₀ and values were compared to the EC₅₀ of T₄ (a representative example is given in Figure 3B, results in Table S2). Analysis of results enabled us to conclude that only compounds that showed complete T₄ displacement in the qualitative assay, produced EC₅₀ T₄/ EC₅₀ compound ratios higher than 1, *i.e.*, with affinity to TTR higher than the one of T₄. Nevertheless, a ratio of EC₅₀ T₄/ EC₅₀ compound of 0.5 or superior was used to identify a pool of compounds able to bind TTR efficiently and therefore those that should be tested in our proprietary HTS ternary assay.²⁴ In some instances, compounds revealed a non-standard or ambiguous behavior, hindering the statistical analysis performed for the rest of the compounds.

Nonetheless, and in order to avoid elimination of compounds that could stabilize TTR by pathways other than binding to the T₄ binding site, all of the non-selected compounds were assessed in a TTR stability assay.⁶¹ This approach was employed to test and to evaluate the ability of the non-selected compounds to stabilize TTR, by electrophoretic means, under semi-denaturing conditions, as described in the Material

and Methods section at the Supporting Information. As readout, we assessed the levels of monomeric TTR upon incubation with each compound and values were compared to those obtained in the absence of compound.

Figure 3C displays representative results obtained in this stability assay showing different levels of the TTR monomer. The graphic represents the intensity of the monomer band, considering the value of 1 for the control. Compounds that reduced the intensity of the monomer band to at least half of the intensity in the control, were considered for the final list of the compounds to enter a future ternary assay (Table S1).

Altogether, compounds selected from the T₄ quantitative and the TTR stability assays constitute a subset of 53 molecules amenable to be screened using our proprietary HTS ternary assay (See Table S4 in Supporting Information).

Crystal structures of TTR:ligand complexes

From this final list of compounds to enter a future ternary assay, two compounds, **35** and **73**, were selected for the structure elucidation of the TTR complexes by X-ray diffraction, because their TTR-ligand structures have not been previously analyzed (See Table S3 in Supporting Information). Both compounds bind in the so-called thyroxine binding sites (Figure 4). Compound **35** (Figure 4, left panel) binds very deeply with the iodinated ring located at the outmost part of the T₄ channel. This binding induces the rotation of Ser117 side chains of the four monomers creating new strong intermonomer hydrogen bonds between these residues. The Lys15 residues, located at the entrance of the binding sites, establish interactions with the carboxylate substituents of the ligand. Compound **73** does not bind so deeply in the channel as **35** (Figure 4, right panel) and, consequently, it does not induce the formation of such strong interactions between the Ser117 residues of the TTR tetramer. The Lys15 residues, in the TTR: **73** complex, are

not pointing to the center of the channel and are not involved in interactions with the compound.

Figure 4. Close view of one binding site of the crystal structures of TTR:**35** (left, PDB ID 6EP1) and TTR:**73** (right, PDB ID 6EOY). The two-symmetry related positions of each compound are shown with carbon atoms in grey and orange. The 2Fo-Fc electron density maps at 1 σ are drawn as a blue mesh around compounds **35** and **73** and residues Lys15 and Ser117 highlighted in stick representation.

Selection of small-molecule chaperones (SMCs): HTS ternary assay, ITC studies and Tht assays

To efficiently screen for potential small-molecule chaperones of the TTR/A β interaction, we have used our recently in house developed and validated high-throughput assay that relies on the ability of the test compounds to prevent A β aggregation in solutions of preformed TTR/A β complexes.²⁴ The assay makes use of A β (12-28) which has analogous properties as full length A β peptides but is a less expensive and more stable peptide. Thus, the recombinant wtTTR is incubated with the test compound (ratio

TTR/compound 1:2) during 1h and then, A β (12-28) is added to the complex and UV monitoring of turbidity for 6 h allows to determine its potency. A complete high-throughput screening in this ternary assay was carried out for all the 53 compounds previously selected from computational and biological studies (see Table S5 and Figures S1 to S10 in the Supporting Information).

After classifying the compounds according to their potency at reducing A β aggregation a prioritized list was obtained (See Fig 5). In this list we found our lead compound IDIF, the natural product luteolin (LUT), a compound in clinical phases, and three registered drugs, olsalazine (OLS), sulindac (SUL) and flufenamic acid (FLU), among others.

Figure 5. HTS assays. Kinetics of aggregation with best chaperones: (A) binary [A β (12-28) + TTR] and ternary interactions [A β (12-28) + (TTR+IDIF)]; (B) binary [A β (12-28) + TTR] and ternary interactions [A β (12-28) + (TTR+SMC)] with SMCs: LUT, SUL, OLS, and FLU. Ternary interactions [A β (12-28) + (TTR+DIF)] are added for comparison purposes.

Furthermore, Isothermal Titration Calorimetry (ITC) technique has been used to corroborate the chaperoning effect of five of the best small-molecule compounds as described previously.²³ Thus, we have compared the binding in the A β (12-28)/TTR/SMC ternary complexes with the binary interaction A β (12-28)/TTR (Supporting Information,

Figures S11 and S12). In addition, a full thermodynamic characterization of the ternary complexes with A β (1–40) and TTR and each of the five best selected SMCs was obtained (Figure 6). The binary TTR/A β (1–40) (1:1) complex formation shows a dissociation constant of $K_d = 6.49 \mu\text{M}$, However, IDIF enhances this interaction between TTR and A β (1–40), since the K_d is reduced to $3.34 \mu\text{M}$ when TTR in the presence of IDIF is titrated with A β (1–40). Similar improvements were observed for the ternary complexes with the best SMCs (*i.e.* $K_d = 1.52 \mu\text{M}$ (LUT); $3.42 \mu\text{M}$ (SUL); $0.81 \mu\text{M}$ (OLS) and $2.34 \mu\text{M}$ (FLU)).

Figure 6: ITC studies of the: (A) binary A β (1–40)/TTR complex; and (B) of the ternary complexes A β (1–40)/(TTR/SMC) (SMC: IDIF, LUT, SUL, OLS AND FLU).

To complement the results gathered so far, we performed the aggregation kinetics of A β (1–42) in the presence of the five selected SMCs using by ThT assays, obtaining coherent results with the turbidity measurements observed with A β (12–28) (Figure 7). As shown in Figure 7A the aggregation kinetics of A β (1–42) in the presence of TTR complexed with IDIF are concentration dependent of this small-compound.

Figure 7: Aggregation kinetics of Aβ(1-42) monitored by ThT fluorescence assays:

(A) Dose/response studies: kinetics of aggregation of Aβ(1-42) in the presence of (TTR+IDIF), fixed ratio Aβ(1-42) /TTR to (4:1) and increasing the ratio of TTR /IDIF from (1:0) to (1:2); and (B) binary [Aβ(1-42) + TTR] at a ratio (2:1) and ternary interactions [Aβ(1-42) + (TTR+SMC)] at a ratio (2:1:2) (SMCs: LUT, SUL, OLS, and FLU).

CONCLUSIONS

In this paper, we have first taken a combined computational / experimental approach to select a pool of chemically diverse compounds, that may enhance the ability of TTR to sequester the Aβ peptide. In order to carry out this comprehensive study, two complementary computational procedures were used: a) drug repurposing through virtual screening and pharmacophore searches, starting from a large set of compounds that have either reached the market or are in clinical phases, and docking them into our built TTR/Aβ molecular model; and b) a multi-target approach, where we selected molecules that were theoretically interacting with our built TTR/Aβ molecular model, but also

would theoretically interact with a group of four protein targets that have been associated with AD.

All of the selected compounds that underwent these computational procedures, and other known TTR ligands, were sequentially assayed in a screening cascade covering TTR binding through both a T₄ displacement assay (qualitative) and a TTR – T₄ competition assay (quantitative), followed by a TTR stabilizing assay.

The combined virtual and experimental screening approach has led to 53 highly diverse compounds that strongly stabilize TTR. Among these compounds, compound **35** and the drug olsalazine had not been previously described as TTR stabilizers and may be relevant for TTR-related therapies.

Of note, these 53 compounds were good candidates for the analysis of the modulation of the TTR/A β interactions, and they were analyzed through our robust HTS ternary assay. The results of this ternary assay show a prioritized list of small molecule chaperones (SMCs) of the TTR/A β interaction providing the basis for a novel therapeutic target for Alzheimer's disease (AD). Among the SMCs, we have found small-molecules as our lead small-molecule compound IDIF which is one of the best SMCs, and also three marketed drugs (olsalazine, sulindac, and flufenamic acid) and one investigational drug (luteolin), which could be directly repurposed or repositioned for clinical use. The orphan drug Tafamidis and the repurposed Parkinson's drug Tolcapone have been reported to be excellent TTR tetramer stabilisers, but these drugs have no chaperoning effect in our *in vitro* assays, showing that not all good TTR tetramer kinetic stabilisers are good SMCs.

Our drug discovery program offers the possibility to explore the chemical space by HTS of commercially available libraries of drugs/compounds to find new SMCs. Further work will be carried out to explore the activity of some chemical scaffolds such as the biphenyl one present in IDIF, so as to build structure-activity relationships from the HTS

ternary assay results. In particular, the SMC IDIF will be optimized for ADME properties and to improve blood brain barrier (BBB) passage.

A small set of these SMCs will be prioritized to enter preclinical safety studies, analyzing their *in vivo* behavior, in order to select one repurposed drug as a candidate to enter clinical trials for AD.

These selected small-molecule chaperones provide the basis for a novel target for Alzheimer's disease, based on targeting transthyretin. We envisage that this new target will feed the currently exhausted pipeline of drugs in phase I for AD with the goal of increasing AD disease-modifying therapies. In addition, the three marketed drugs reported in this work as SMCs of the TTR / Abeta interaction (sulindac, olsalazine and flufenamic acid) could directly enter a clinical phase program as candidate AD therapies, as an example of drug repurposing.

METHODS AND MATERIALS

General procedure for molecular models preparation

A molecular model of TTR was generated using the three-dimensional coordinates of the TTR protein structure (PDB code 1DVQ), available at the Protein Data Bank (PDB) (www.rcsb.org). For hydrogen atoms refinement and energy minimization, the Protonate 3D package implemented in MOE 2015.10 was employed to add hydrogen atoms to TTR which were further submitted to an energy-minimization process.⁶² Partial charges were obtained by computing the electrostatic potentials in the optimized structures. The energy minimization step was carried out using a distance-dependent dielectric constant and a cut-off distance of 10 Å for the van der Waals interactions. In the final step, the refinement was accomplished using 1000 cycles of steepest descents followed by conjugate gradients until the maximum gradient of the energy was smaller than 0.05 kcal/mol Å².

The same procedure and parameters were used to compute the following proteins used in this work: IL-10 (interleukin-10) (PDB code 1ILK); inhibitor domain of APP (amyloid precursor protein (Aβ) (PDB code 1AAP); β secretase with inhibitor OM99-2 (PDB code 1FKN); human acetylcholinesterase with Fasciculin-II (PDB code 1B41); metabotropic glutamate receptor 1 with LY341495 antagonist (PDB code 3KS9); metabotropic glutamate receptor 5 with glutamate (PDB code 3LMK).

Docking experiments (TTR/Aβ computational model) procedure

The MOE 2015.10 package⁶² was used to perform the docking studies between the refined TTR X-Ray crystallographic structure (as template) and Aβ peptide (as ligand). Alpha Triangle was used as placement method; Alpha HB as score function, and AMBER10 as forcefield in the first refinement step of the docking solutions. A rescoring

step was also implemented in this computational pipeline, using London DG as function score, and again AMBER10 as forcefield for the last refinement step of this docking study.

Docking experiments (virtual screening) general procedure

The MOE 2015.10 package was used to perform the docking studies between the refined TTR protein: A β peptide system. Molecules computed as ligand for the docking experiments were pretreated using the LygX application from the software package MOE. After that the experiment were performed as follows: Alpha Triangle was used as placement method; Alpha HB as score function, and MMFF94x as forcefield in the first refinement step of the docking solutions. A rescoring step was also implemented in this computational pipeline, using London DG as function score, and again MMFF94x as forcefield for the last refinement step.

Pharmacophore set-up

The Ligand Scout 3.0 software package⁶⁰ was used to build-up a pharmacophore starting from the binding sites of TTR protein (T₄ binding pocket), β -secretase, metabotropic glutamate receptor 1 and metabotropic glutamate receptor 5. The pharmacophore resulting from this pocket alignment process was used to screen a set of molecules coming from the second pipeline explained in the next section.

Selection pipeline of ligands

Two complementary approaches were carried out to select the ligands to be docked in the TTR molecular model:

Pipeline A: starting from the Thomson Reuters Integrity database (<https://integrity.thomson-pharma.com/integrity/xmlxsl/>), which contains small molecules and drugs in the market or advanced clinical phases, nearly 3200 compounds were selected and sorted according to which protein or disease was their main target: Amyloid peptide, transthyretin, both or Alzheimer's disease in general. In addition, nearly 500 compounds were also added to this list that have been gathered from the literature or that had been previously explored in our laboratories, as potential TTR stabilizers.

Then the CARLSBAD database and software,⁵⁹ which contains integrated data on compounds and their target binding affinities, were used to select compounds that had experimentally shown affinity vs. both TTR and the amyloid peptide. This led to a common stilbene substructure scaffold that was then used to carry out a substructure search among the original selection of nearly 3700 compounds. The resulting 48 compounds with the stilbene substructure were then docked into our TTR:A β computational model.⁶³

Pipeline B: starting from the Thomson Reuters Integrity database (<https://integrity.thomson-pharma.com/integrity/xmlxsl/>), which contains small molecules and drugs in the market or in advanced clinical phases, we selected nearly 1400 compounds that were reported in preclinical, clinical or marketed phase and that had their protein target reported as one or more of the following, which have been associated to AD: APP (amyloid precursor protein); AchE (acetylcholinesterase); IL-10 (interleukin-10); α -, β - and γ -secretases; tau protein; GABA and glutamate receptors. All these compounds were then docked into the TTR model, prioritizing a pool of 134 molecules that would theoretically bind the TTR T₄ channel with an affinity similar to IDIF. These molecules were then virtually analyzed through docking with software MOE 2015.10 into the following protein structures: IL-10 (interleukin-10); inhibitor domain of APP

(amyloid precursor protein (A β); β -secretase with inhibitor OM99-2; acetylcholinesterase (AChE) with Fasciculin-II; metabotropic glutamate receptor 1 with LY341495 antagonist; metabotropic glutamate receptor 5 with glutamate. All these docking processes lead to a prioritized list of 85 molecules, which were then docked into our TTR:A β computational model, leading to 27 molecules that would theoretically bind to the TTR:A β complex and to 4 additional AD related targets (A β , IL-10, β -secretase and acetylcholinesterase). In addition, the initial set of 134 molecules that had been selected through docking with the TTR:A β model, were further analyzed computationally through the software Ligand Scout; 13 of them coincided with the list of 27 molecules derived from the multi-target docking, but we were able to select 4 additional molecules that matched with the common pharmacophore.

The compounds prioritized through these two computational (*in silico*) complementary approaches that were commercially available (Molport, <http://www.molport.com>), were acquired, in order to carry out the experimental TTR binding, competition, and stability assays (See Table S1 in Supporting Information). Just only in few cases, if a selected compound was not commercially available but another compound with a very similar structure was available, then that similar compound was processed through the computational procedures described, and acquired if it would match with either the multi-target docking or the common pharmacophore filters. Finally, some compounds in the market and/or in clinical phases, that are reported in the literature as good TTR stabilizers and/or as AD therapies under clinical studies, were also analyzed through the computational filters described above, and included in the final list of compounds to be experimentally assayed.

Recombinant wild-type human (wt rhTTR) production and purification

Human wild type rhTTR gene was cloned into a pET expression system and transformed into *E. coli* BL21(DE3) Star.⁶⁴ The phTTRwt-I/pET-38b(+) plasmid was provided by Prof. Antoni Planas (IQS, URL).⁶⁵ The production of recombinant protein was performed at Erlenmeyer scale, protein production and purification were done as described previously following an optimized version of our protocol.⁶⁶ wt rhTTR was produced using a pET expression System. The expressed protein only contains an additional methionine on the *N*-terminus if compared to the mature natural human protein sequence. wt rhTTR protein was expressed in *E. coli* BL21-(DE3) cells harboring the corresponding plasmid. Expression cultures in 2xYT rich medium containing 100 µg/mL kanamycin were grown at 37 °C to an optical density (at 600 nm) of 4 (OD₆₀₀≈4), then induced by addition of IPTG (1 mM final concentration), grown at 37 °C for 20 h, and harvested by centrifugation at 4 °C, 10000 rpm for 10 min and resuspended in cell lysis buffer (0,5 M Tris-HCl, pH 7.6). Cell disruption and lysis were performed by French press followed by a sonication step at 4 °C. Cell debris were discarded after centrifugation at 4 °C, 11000 rpm for 30 min. Intracellular proteins were fractionated by ammonium sulfate precipitation in three steps. Each precipitation was followed by centrifugation at 12 °C, 12500 rpm for 30 min. The pellets were analyzed by SDS-PAGE (14% acrylamide). The TTR-containing fractions were resuspended in 20 mM Tris-HCl, 0.1 M NaCl, pH 7.6 (buffer A) and dialyzed against the same buffer. It was purified by Ion exchange chromatography using a Q-Sepharose High Performance (Amersham Biosciences) anion exchange column and eluting with a NaCl linear gradient using 0.1 M NaCl in 20 mM Tris-HCl pH 7.6 buffer A to 0.5 M NaCl 20 mM Tris-HCl pH 7.6 (buffer B). All TTR-enriched fractions were dialyzed against deionized water in three steps and were lyophilized. The protein was further purified by gel filtration chromatography using a Superdex 75 prep grade resin (GE Healthcare Bio-Sciences AB) and eluting with 20

mM Tris pH 7.6, 0.1 M NaCl. Purest fractions were combined and dialyzed against deionized water and lyophilized. The purity of protein preparations was >95% as judged by SDS-PAGE. Average production yields were 150-200 mg of purified protein per liter of culture. Protein concentration was determined spectrophotometrically at 280 nm using calculated extinction coefficient value of $17780 \text{ M}^{-1}\cdot\text{cm}^{-1}$ for wtTTR. The protein was stored at -20 °C.

Thyroxine binding assays

Qualitative studies of the displacement of T₄ from WT TTR were carried out by incubation of 5 µL of human plasma, with ¹²⁵I-T₄ (specific radioactivity $\approx 1200 \mu\text{Ci}/\mu\text{g}$; Perkin Elmer) in the presence of the different compounds (final concentration of 666 µM).^{44,46} Protein separation was carried out in a native PAGE system using glycine/acetate buffer. The gel was dried and revealed using an X-ray film.

For the quantitative analysis, T₄ binding competition assays based on a gel filtration procedure was used, as previously described.⁶⁷ Briefly, 50 µl of a diluted sample (120 nM human recombinant TTR) was incubated with 50 µl of either cold T₄ or compound solutions of variable concentrations ranging from 0 to 1000 nM and with a constant amount of labeled ¹²⁵I-T₄ (~50,000 cpm). This solution was counted in a gamma spectrometer and incubated at 4 °C overnight. Protein bound ¹²⁵I-T₄ and free ¹²⁵I-T₄ were separated by gel filtration through a 1 ml BioGel P6DG (Bio-Rad) column. The bound fraction was eluted while free T₄ was retained on the BioGel matrix. The eluate containing the bound T₄ was collected and counted. Bound T₄ was expressed as percentage of total T₄ added. Each assay was performed in duplicate. Analysis of the binding data was performed with the GraphPad Prism program (version 5.0, San Diego, CA) and data was expressed as the EC50 ratio (EC50 T₄/EC50 compound).

TTR stability assay⁶¹

Recombinant wt-TTR (333 μ M) was incubated alone or in the presence of different compounds for 1h at 37 °C. Then, urea 8 M (used as denaturing agent) and sample buffer without SDS were added. Samples were then run in a 15% acrylamide gel, prepared without SDS, and transferred onto a nitrocellulose membrane (AmershamTM GE Healthcare – Protan 0.2 μ m), using a wet system (Bio-Rad Criterion Blotter). The membranes were blocked 1 hour at RT with 5% non-fat dry milk (DM) in PBS containing 0,05% Tween-20 (PBS-T) and then incubated with primary antibody anti-human TTR (Dako; 1:3000) in 3% DM/PBS-T. Then, washed membranes were incubated for 1 hour at RT with sheep anti-rabbit immunoglobulins conjugated with horseradish peroxidase (The binding Site; 1:5000) in 3% DM/PBS-T. The blots were developed using ClarityTM Western ECL substrate (Bio-Rad) and levels of monomeric TTR were detected and visualized using a chemiluminescence detection system (ChemiDoc, Bio-Rad).

Crystal structures of TTR:35 and TTR:73 complexes

Co-crystallization.

TTR (9.9 mg.ml⁻¹) was incubated with each compound (molar ratios **35**/TTR = 20 and **73**/TTR = 50) at 4 °C on, in HEPES buffer 10mM, pH = 7.5. Crystals suitable for X-ray diffraction were obtained by hanging-drop vapour-diffusion techniques at 20 °C. Crystals were grown within 1 week by mixing 2 μ l of the protein:compound solutions with 2 μ l of reservoir solution. The reservoir solutions used in the crystallization trials contained acetate buffer 0.2 M pH 4.8-5.4, ammonium sulfate 1.8-2.2 M, 7% glycerol. Crystals were

transferred to reservoir solutions containing increasing concentrations of glycerol (10–25%) and flash frozen in liquid nitrogen.

Data collection, processing and refinement.

X-ray diffraction data sets were collected using synchrotron radiation at the XALOC beamline at the ALBA synchrotron center (Barcelona), ID30B beam line at the ESRF (European Synchrotron Radiation Facility, Grenoble Cedex, France) and Proxima 2 beam line at the SOLEIL synchrotron (Paris).

Diffraction images were processed with the XDS Program Package⁶⁸ and the diffraction intensities converted to structure factors in the CCP4 format.⁶⁹ A random 5% sample of the reflection data was flagged for R-free calculations⁷⁰ during model building and refinement. A summary of the data collection and refinement statistics is presented in Table 2. Initial molecular replacement phases were generated with PhaserMR,⁷¹ using as initial model one monomer of the complex TTR:IDIF (PDB ID 1Y1D).⁴⁸ The final models were obtained after further cycles of refinement, carried out with Coot⁷² and PHENIX.⁷³

HTS ternary assay

In this assay²⁴ the following stock solutions were used: Buffer A: 25 mM HEPES buffer, 10 mM glycine, pH 7.4 was prepared in the absence of salt. Protein (TTR) stock: 9,5 mg/mL (170 μ M) in 25 mM HEPES buffer, 10 mM glycine, pH 7.4 and 5% DMSO (final concentration) was prepared in the absence of salt (buffer A). For the *A β* peptide stock: 0,4 mg/mL (200 μ M) in 25 mM HEPES buffer, 10 mM glycine, pH 7.4 and 5% DMSO (final concentration). For the small-molecule compound IDIF, a first solution of

3,76 mg/mL (10 mM) in DMSO was prepared. The final stock of the small-molecule IDIF was prepared by mixing 50 μ L of the previous DMSO solution with 950 μ L of buffer A (the final concentration of 5% DMSO).

First, the small-molecule compound and TTR complex was formed. To this end, 60 μ L of TTR stock was dispensed into the wells of a 96-well microplate. 40 μ L of small-molecule stock was added to give final concentrations of 100 μ M. The plate was introduced in the microplate reader (SpectraMax M5 Multi-Mode Microplate Readers, Molecular Devices Corporation, California, USA) and incubated for 1h at 37 °C with orbital shaking 15 s every 30 min. Then, 100 μ L of A β solution was added to the well to give a final concentration of 100 μ M.

Other wells of the 96-well microplate are filled with: a) Buffer alone: 200 μ L of buffer A solution was added to the well; b) Negative control of A β aggregation: 200 μ L of A β (1-11) stock solution in buffer A was dispensed into the wells; c) Testing TTR aggregation: 60 μ L of TTR stock were dispensed into the wells of a 96-well microplate and 140 μ L of buffer A were added; d) For the A β (12-28) aggregation: 100 μ L of A β (12-28) stock solution is dispensed into the wells and 100 μ L of buffer A were added. In order to see if the small-molecule compounds affect the A β aggregation, we run control experiments to test the aggregation of A β in the presence of each of the compounds. None of them affected the A β aggregation.

The plate was incubated at 37 °C in a thermostated microplate reader with orbital shaking 15 s every minute for 30 min. The absorbance at 340 nm was monitored for 6 h at 30 min intervals. Data were collected and analyzed using Microsoft Excel software. All assays were done in duplicate.

$$RA (\%) = \left[1 - \left(\frac{Abs_c}{Abs_{A\beta} + Abs_c} \right) \right] * 100 \quad (1)$$

The parameter monitored in this assay was used to calculate the percent reduction of formation of aggregates (RA %) according to equation 1, where $Abs_{A\beta}$ and Abs_c are the final absorbance of the samples, in the absence or in the presence of the small-molecule compound/TTR complex; respectively.

Thioflavin-T (ThT) fluorescence assays

The robustness of our HTS turbidimetry-based method was further validated on the basis of comparative by Thioflavin-T (ThT) fluorescence assays on the same system. The ThT fluorescence was monitored at 37 °C using Gemini XPS plate reader (Molecular Devices) at an excitation wavelength of 440 nm and an emission wavelength of 490 nm. Thioflavin-T (ThT) was dissolved in 25 mM HEPES buffer, 10 mM glycine, pH 7.4 and 5 % DMSO to a final concentration of 25 μ M. Aggregation of A β (12-28) 50 μ M was performed in the presence of 25 μ M ThT. All solutions were dissolved in the same buffer. TTR was added to a final concentration of 25 μ M. IDIF was added to a final concentration of 50 μ M. Aggregation of A β (1-42) 20 μ M was performed with 10 μ M TTR and 20 μ M of ligand. For the ternary complex, TTR was incubated first with IDIF for 1h, then A β (12-28) was added. The final volume was 200 μ L for all samples. Fluorescence intensity at 490 nm of each sample was monitored after each 2h for 8h, and then at 21h. Measurements were performed as independent triplicates. Recorded values were averaged and background measurements (buffer containing 25 μ M ThT) were subtracted. Measurements were performed as independent triplicates. Recorded values were averaged and background measurements (buffer containing 25 μ M ThT) were subtracted.

Isothermal Titration Calorimetry (ITC) studies

Experiments were carried out in a VP-ITC (MicroCal, LLC, Northampton, Ma, USA). In a titration experiment,²³ the ligand in the syringe is added in small aliquots to the macromolecule in our case TTR protein in the calorimeter cell, which is filled with an effective volume that is sensed calorimetrically. The TTR solution of 20 μM , and $\text{A}\beta$ ($\text{A}\beta(12-28)$ or $\text{A}\beta(1-40)$), or ligand solutions of 200 μM were prepared in the same buffer. The titrant was injected over 20 or 30 times at a constant interval of 300 s with a 450 rpm rotating stirrer syringe into the sample cell containing its binding partner. All solutions were prepared with 25 mM HEPES buffer, 10 mM glycine, pH 7.4 and 5% DMSO (final concentration) and it was corroborated that at these conditions TTR and $\text{A}\beta(1-40)$ are stable. $\text{A}\beta(1-40)$ working solution was prepared at 200 μM and used immediately, to avoid premature aggregate formation. TTR stock solution was prepared at 40 μM . Ligand stock solution was prepared at 10 mM in DMSO. All solutions were prepared in the same buffer and filtered prior to use. In the control experiments, the titrant (ligand or $\text{A}\beta$) was injected into the buffer in the sample cell to measure the heat of dilution. This value of the heat of dilution was subtracted from the titration data. The experiments were performed at 25 °C. Titration data were analyzed by the evaluation software, MicroCal Origin, Version 7.0. The binding curves were fitted by a non-linear regression method to a one set of sites binding model. This leads to the calculation of K , n , ΔH , ΔS , and ΔG . Each experiment was conducted three times, and the mean value with standard deviations is provided.

ASSOCIATED CONTENT

Supporting Information.

Compounds assayed in different assays; selected compounds after computational screening; T₄ displacement assays; TTR stability assays; selected compounds for ternary assays; results from the HTS assays; kinetics of aggregation of A β (12-28) peptide with TTR or TTR complexed with representative compounds of the 53 selected molecules; ITC studies of the binary interaction A β (12-28) + TTR and the ternary interactions [A β (12-28) + [(TTR+SMC)]]; ITC studies of the binary interactions (TTR+SMC).

AUTHOR INFORMATION

Corresponding Author

*E-mail: jordiramon.quintana@upf.edu.

*E-mail: icardoso@ibmc.up.pt

*E-mail: gemma.arsequell@iqac.csic.es

ORCID ID Jordi Quintana: orcid.org/0000-0002-7059-7418.

ORCID ID Isabel Cardoso: orcid.org/0000-0003-2472-7055.

ORCID ID Gemma Arsequell: orcid.org/0000-0002-7009-3738.

Present Address

† Dr. Jordi Quintana, Research Programme on Biomedical Informatics, Universitat Pompeu Fabra (UPF), Barcelona, Spain.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. ‡These authors contributed equally to this work.

Funding Sources

The work was supported by a grant from the Fundació Marató de TV3 (neurodegenerative diseases call, project reference: 20140330-31-32-33-34, <http://www.ccma.cat/tv3/marato/en/projectes-financats/2013/212/>). The group at IBMC-i3S also acknowledges for funding through grant Norte-01-0145-FEDER-000008 -Porto Neurosciences and Neurologic Disease Research Initiative at I3S, supported by Norte Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (FEDER).

Notes

Dedicated to Dr. Gregorio Valencia

ACKNOWLEDGMENT

I. Cardoso works under the Investigator FCT Program which is financed by national funds through FCT and co-financed by ESF through HPOP, type 4.2 - Promotion of Scientific Employment. M. Alemi was a recipient of a Research Fellowship (BIM) funded by the project of Fundació La Marató de TV3, Spain, and L. M. Santos was a recipient of a fellowship from Norte 2020. J.P. Leite acknowledges the FCT fellowship SFRH/BD/129921/2017 (Portugal). IQAC-CSIC acknowledges a contract to Ellen Y. Cotrina funded by the project of Fundació Marató de TV3, Spain and a contract from Ford España - Fundación Apadrina la Ciencia. The group at CIC bioGUNE also acknowledges MINECO (Spain) for funding through grant CTQ2015-64597-C2-1-P and a Juan de la Cierva contract to A. Gimeno. We thank access to ALBA (XALOC), ESRF (ID30B) and Soleil (PROXIMA 1 and 2a) synchrotrons.

ABBREVIATIONS: AChE, Acetylcholinesterase; AD, Alzheimer Disease; TTR, transthyretin; APP, amyloid protein precursor; CSF, cerebrospinal fluid; IDIF, iododiflunisal; on, overnight.

We have settled a drug discovery approach targeting transthyretin (TTR) with the aim to increase the AD drug discovery pipeline in early stages. First, a combination of computational studies, drug repurposing and multitargeting together with experimental screening has afforded a set of highly diverse TTR stabilizers. Second, these candidates have been screened using our in-house designed and validated turbidimetry-based HTS ternary assay. A selection of new small molecule chaperones (SMCs) have been discovered, among them some registered drugs that can be repurposed. Targeting TTR could enlarge current early stage clinical drug development towards the goal of finding disease-modifying drugs for Alzheimer's disease.

References:

- (1) 2020 Alzheimer's disease facts and figures. *Alzheimers Dement*. 2020 Mar 10. doi: 10.1002/alz.12068.
- (2) Cummings, J., Fox, N. Defining Disease Modifying Therapy for Alzheimer's Disease. *J. Prev. Alzheimers Dis*. **2017**, 4, :109-115. doi: 10.14283/jpad.2017.12.
- (3) Cummings, J. Drug Development for Psychotropic, Cognitive-Enhancing, and Disease-Modifying Treatments for Alzheimer's Disease. *J. Neuropsychiatry Clin. Neurosci*. **2021**, 33, 3-13.
- (4) Cummings J, Lee G, Ritter A, Sabbagh M, Zhong K. Alzheimer's disease drug development pipeline: 2020. *Alzheimers Dement (N Y)*. **2020**, 6:e12050 . DOI: 10.1002/trc2.12050
- (5) Cummings, J. Lessons Learned from Alzheimer Disease: Clinical Trials with Negative Outcomes. *Clin. Transl. Sci*. **2018**, 11, 147-152. doi: 10.1111/cts.12491.
- (6) Molinuevo, J. L.; Minguillon, C.; Rami, L., Gispert, J. D. The Rationale Behind the New Alzheimer's Disease Conceptualization: Lessons Learned During the Last Decades. *J. Alzheimers Dis*. **2018**, 62, 1067-1077.
- (7) Friedman, L. G.; McKeahan, N.; Hara, Y.; Cummings, J. L.; Matthews, D. C.; Zhu J, Mohs R. C.; Wang, D.; Hendrix, S. B.; Quintana, M.; Schneider, L. S.; Grundman, M.; Dickson, S. P.; Feldman, H. H.; Jaeger, J.; Finger, E. C.; Michael Ryan, J.; Niehoff, D.; Dickinson, S. L.; Markowitz, J. T.; Owen, M.; Travaglia, A.; Fillit, H. M. Value-Generating Exploratory Trials in Neurodegenerative Dementias. *Neurology* **2021** Mar 5:10.1212/WNL.0000000000011774.

- (8) Cummings, J.; Ritter, A.; Zhong, K. Clinical Trials for Disease-Modifying Therapies in Alzheimer's Disease: A Primer, Lessons Learned, and a Blueprint for the Future. *J. Alzheimers Dis.* **2018**, *64*, S3-S22. doi: 10.3233/JAD-179901.
- (9) Blake, C. C.; Geisow, M. J.; Oatley, S. J.; Rerat, B.; Rerat, C. Structure of prealbumin: secondary, tertiary and quaternary interactions determined by Fourier refinement at 1.8 Å. *J. Mol. Biol.* **1978**, *121*, 339-356.
- (10) Gião, T.; Saavedra, J.; Cotrina, E.; Quintana, J.; Llop, J.; Arsequell, G.; Cardoso, I. Undiscovered Roles for Transthyretin: From a Transporter Protein to a New Therapeutic Target for Alzheimer's Disease. *Int J Mol Sci.* **2020**, *21*:2075. doi: 10.3390/ijms21062075.
- (11) Schwarzman, A. L.; Gregori, L.; Vitek, M. P.; Lyubski, S.; Strittmatter, W. J.; Enghilde, J. J.; Bhasin, R.; Silverman, J.; Weisgraber, K. H.; Coyle, P. K.; Zagorki, M. G.; Talafous, J.; Eisenberg, M.; Saunders, A. M.; Roses, A. D.; Goldgaber, D. Transthyretin sequesters amyloid beta protein and prevents amyloid formation. *Proc. Nat. Acad. Sci. U. S. A.* **1994**, *91*, 8368-8372.
- (12) Pate, K. M.; Murphy, R. M. Cerebrospinal Fluid Proteins as Regulators of Beta-amyloid Aggregation and Toxicity. *Isr. J. Chem.* **2017**, *57*, 602-612.
- (13) Davidsson, P.; Westman-Brinkmalm, A.; Nilsson, C. L.; Lindbjör, M.; Paulson, L.; Andreasen, N.; Sjögren, M.; Blennow, K. Proteome analysis of cerebrospinal fluid proteins in Alzheimer patients. *Neuroreport* **2002**, *13*, 611-615.
- (14) Serot, J. M.; Christmann, D.; Dubost, T.; Couturier, M. Cerebrospinal Fluid Transthyretin: Aging and Late Onset Alzheimer's Disease. *J. Neurol. Neurosurg. Psychiatry* **1997**, *63*, 506-550.

- (15) Gloeckner, S.F.; Meyne, F.; Wagner, F.; Heinemann, U.; Krasnianski, A.; Meissner, B.; Zerr, I. Quantitative analysis of transthyretin, tau and amyloid-beta in patients with dementia. *J Alzheimers Dis.* **2008**, *14*, 17-25.
- (16) Han, S. H.; Jung, E. S.; Sohn, J. H.; Hong, H. J.; Hong, H. S.; Kim, J. W.; Na, D. L.; Kim, M.; Kim, H.; Ha, H. J.; Kim, Y. H.; Huh, N.; Jung, M. W.; Mook-Jung, I. Human Serum Transthyretin Levels Correlate Inversely with Alzheimer's Disease. *J. Alzheimers Dis.* **2011**, *25*, 77–84. Doi: 10.3233/JAD-2011-102145.
- (17) Velayudhan, L.; Killick, R.; Hye, A.; Kinsey, A.; Güntert, A.; Lynham, S.; Ward, M.; Leung, R.; Lourdasamy, A.; To, A. W.; Powell, J.; Lovestone, S. Plasma Transthyretin as a Candidate Marker for Alzheimer's Disease. *J. Alzheimers Dis.* **2012**, *28*, 369–375.
- (18) Ribeiro, C. A.; Santana, I.; Oliveira, C.; Baldeiras, I.; Moreira, J.; Saraiva, M. J.; Cardoso, I. Transthyretin decrease in plasma of MCI and AD patients: investigation of mechanisms for disease modulation. *Curr. Alzheimer Res.* **2012**, *9*, 881-889.
- (19) Oliveira, S. M.; Ribeiro, C.A.; Cardoso, I.; Saraiva, M. J. Gender-dependent transthyretin modulation of brain amyloid- β levels: evidence from a mouse model of Alzheimer's disease. *J. Alzheimers Dis.* **2011**, *27*, 429-39.
- (20) Buxbaum, J. N.; Ye, Z.; Reixach, N.; Friske, L.; Levy, C.; Das, P.; Golde, T.; Masliah, E.; Roberts, A.R. ; Bartfai, T. Transthyretin protects Alzheimer's mice from the behavioral and biochemical effects of A β toxicity. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 2681-2686.

- (21) Costa, R.; Gonçalves, A.; Saraiva, M. J.; Cardoso, I.; Transthyretin binding to A-Beta peptide - Impact on A-Beta fibrillogenesis and toxicity. *FEBS Lett* **2008**, 582, 936-942.
- (22) Li, X.; Zhang, X.; Ladiwala, A. R. A.; Du, D.; Yadav, J. K.; Tessier, P. M.; Wright, P. E.; Kelly, J. W.; Buxbaum, J. N. Mechanisms of Transthyretin Inhibition of β -Amyloid Aggregation In Vitro. *J. Neurosci.* **2013**, 33, 19423-19433.
- (23) Cotrina, E. Y.; Gimeno, A.; Llop, J.; Jiménez-Barbero, J.; Quintana, J.; Valencia, G.; Cardoso, I.; Prohens, R.; Arsequell, G. Calorimetric Studies of Binary and Ternary Molecular Interactions between TTR, A β Peptides, and Small-Molecule Chaperones toward an Alternative Strategy for AD Drug Discovery. *J. Med. Chem.* **2020**, 63, 3205–3214.
- (24) Cotrina E.; Gimeno A; Llop J.; Jiménez-Barbero J.; Quintana J.; Prohens R.; Cardoso I; Arsequell G. An assay for screening potential drug candidates for Alzheimer's disease that act as chaperones of the Transthyretin and Amyloid- β peptides interaction. *Chemistry* **2020**, 26, 17462-17469.
- (25) Nilsson, L.; Pamrén, A.; Islam, T.; Brännström, K.; Golchin, S. A.; Pettersson, N.; Iakovleva, I.; Sandblad, L.; Gharibyan, A. L.; Olofsson, A. Transthyretin Interferes with A β Amyloid Formation by Redirecting Oligomeric Nuclei into Non-Amyloid Aggregates. *J. Mol. Biol.* **2018**, 430, 2722-2733.
- (26) Ghadami, S. A.; Chia, S.; Ruggeri, F. S.; Meisl, G.; Bemporad, F.; Habchi, J.; Cascella, R.; Dobson, C. M.; Vendruscolo, M.; Knowles, T. P. J.; Chiti, F. Transthyretin Inhibits Primary and Secondary Nucleations of Amyloid- β Peptide Aggregation and Reduces the Toxicity of Its Oligomers. *Biomacromolecules* **2020**, 21, 1112-1125.

- (27) Alemi, M.; Gaiteiro, C.; Ribeiro, C. A.; Santos, L. M.; Gomes, J. R.; Oliveira, S. M.; Couraud, P. O.; Weksler, B.; Romero, I.; Saraiva, M. J.; Cardoso, I. Transthyretin participates in beta-amyloid transport from the brain to the liver--involvement of the low-density lipoprotein receptor-related protein 1? *Sci Rep.* **2016** Feb 3;6:20164.
- (28) Ricardo Vieira, J.; Patrícia Moreira, A.; Oliveira, Â.; Alemi, M.; Cardoso, I. Collagen type IV in brain vessels of an AD mouse model: modulation by transthyretin? *Amyloid* **2019**, 26(sup1):138-139.
- (29) Howe, M. D.; McCullough, L. D.; Urayama, A. The Role of Basement Membranes in Cerebral Amyloid Angiopathy. *Front Physiol.* **2020** Nov 25;11:601320. doi: 10.3389/fphys.2020.601320.
- (30) Kerschen, P.; Planté-Bordeneuve, V. Current and Future Treatment Approaches in Transthyretin Familial Amyloid Polyneuropathy. *Curr. Treat. Options Neurol.* **2016**, 18(12):53.
- (31) Adams, D.; Cauquil, C.; Labeyrie, C. Familial amyloid polyneuropathy. *Curr. Opin. Neurol.* **2017**, 30, 481-489.
- (32) Cardoso, I; Goldsbury; C. S.; Müller, S. A.; Olivieri, V.; Wirtz, S.; Damas, A. M.; Aepli, U.; Saraiva, M. J. Transthyretin fibrillogenesis entails the assembly of monomers: a molecular model for in vitro assembled transthyretin amyloid-like fibrils. *J. Mol. Biol.* **2002**, 317, 683-695.
- (33) Almeida, M. R.; Gales, L.; Damas, A. M.; Cardoso, I; Saraiva, M. J. Small Transthyretin (TTR) Ligands as Possible Therapeutic Agents in TTR Amyloidoses. *Curr. Drug Targets CNS Neurol. Disord.* **2005**, 4, 587–596.

- (34) Adams, D.; Cauquil, C.; Labeyrie, C.; Beaudonnet, G.; Algalarrondo, V.; Théaudin, M. TTR kinetic stabilizers and TTR gene silencing: a new era in therapy for familial amyloidotic polyneuropathies. *Expert Opin. Pharmacother.* **2016**, *17*, 791-802.
- (35) Johnson, S. M.; Wiseman, R. L.; Sekijima, Y.; Green, N. S.; Adamski-Werner, S. L.; Kelly, J. W. Native state kinetic stabilization as a strategy to ameliorate protein misfolding diseases: a focus on the transthyretin amyloidosis. *Acc. Chem. Res.* **2005**, *38*, 911-921.
- (36) Yokoyama, T.; Mizuguchi, M. Transthyretin Amyloidogenesis Inhibitors: From Discovery to Current Developments. *J. Med. Chem.* **2020**, *3*, 14228–14242.
- (37) Nencetti, S.; Orlandini, E. TTR fibril formation inhibitors: is there a SAR?. *Curr. Med. Chem.* **2012**, *19*, 2356-2379.
- (38) Ortore, G.; Martinelli, A. Identification of novel Transthyretin Fibril Formation Inhibitors using structure-based Virtual Screening. *ChemMedChem.* **2017**, *12*, 1327-1334.
- (39) Simões, C. J.; Mukherjee, T.; Brito, R. M.; Jackson, R. M. Toward the discovery of functional transthyretin amyloid inhibitors: application of virtual screening methods. *J. Chem. Inf. Model.* **2010**, *50*, 1806-1820.
- (40) Connelly S.; Mortenson, D. E.; Choi, S.; Wilson, I. A.; Powers, E. T.; Kelly, J. W.; Johnson, S. M. Semi-quantitative models for identifying potent and selective transthyretin amyloidogenesis inhibitors. *Bioorg. Med. Chem. Lett.* **2017**, *27*, 3441-3449.

(41) Palaninathan, S. K. Nearly 200 X-Ray crystal structures of transthyretin: What do they tell us about this protein and the design of drugs for TTR amyloidoses?. *Curr. Med. Chem.* **2012**, *19*, 2324-2342.

(42) Coelho, T.; Merlini, G.; Bulawa, C. E.; Fleming, J. A.; Judge, D. P.; Kelly, J. W.; Maurer, M. S.; Planté-Bordeneuve, V.; Labaudinière, R.; Mundayat, R.; Riley, S.; Lombardo, I.; Huertas, P. Mechanism of Action and Clinical Application of Tafamidis in Hereditary Transthyretin Amyloidosis. *Neurol. Ther.* **2016**, *5*, 1-25.

(43) Nencetti, S.; Rossello, A.; Orlandini, E. Tafamidis (Vyndaqel): A Light for FAP Patients. *ChemMedChem* **2013**, *8*, 1617–1619.

(44) Sant'Anna, R.; Gallego, P.; Robinson, L. Z.; Pereira-Henriques, A.; Ferreira, N.; Pinheiro, F.; Esperante, S.; Pallares, I.; Huertas, O.; Almeida, M. R.; Reixach, N.; Insa, R.; Velazquez-Campoy, A.; Reverter, D.; Reig, N.; Ventura, S. Repositioning tolcapone as a potent inhibitor of transthyretin amyloidogenesis and associated cellular toxicity. *Nat. Commun.* **2016** Feb 23;7:10787.

(45) Ribeiro, C. A.; Saraiva, M.J.; Cardoso, I. Stability of the Transthyretin Molecule as a Key Factor in the Interaction with A-Beta Peptide – Relevance in Alzheimer's Disease. *PloS ONE* **7** **2012** (9):e45368. Doi: 10.1371/journal.pone.0045368.

(46) Almeida, M. R.; Macedo, B.; Cardoso, I.; Alves, I.; Valencia, G.; Arsequell, G.; Planas, A.; Saraiva, M. J. Selective binding to transthyretin and tetramer stabilization in serum from patients with familial amyloidotic polyneuropathy by an iodinated diflunisal derivative. *Biochem. J.* **2004**, *381*, 351-356.

(47) Mairal, T.; Nieto, J.; Pinto, M.; Almeida, M. R.; Gales, L.; Ballesteros, A.; Barluenga, J.; Pérez, J. J.; Vázquez, J. T.; Centeno, N. B.; Saraiva, M. J.; Damas, A. M.;

Planas, A.; Arsequell, G.; Valencia, G. Iodine atoms: a new molecular feature for the design of potent transthyretin fibrillogenesis inhibitors. *PLoS One* **2009**, *4*, e4124. Doi: 10.1371/journal.pone.0004124.

(48) Gales, L.; Macedo-Ribeiro, S.; Arsequell, G.; Valencia, G.; Saraiva, M. J.; Damas, A. M. Human transthyretin in complex with iododiflunisal: structural features associated with a potent amyloid inhibitor. *Biochem. J.* **2005**, *388*, 615-662.

(49) Ribeiro, C. A.; Oliveira, S. M.; Guido, L. F.; Magalhaes, A., Valencia, G.; Arsequell, G.; Saraiva, M. J.; Cardoso, I. Transthyretin stabilization by iododiflunisal promotes amyloid- β peptide clearance, decreases its deposition, and ameliorates cognitive deficits in an Alzheimer's disease mouse model. *J. Alzheimers Dis.* **2014**, *39*, 357-370. Doi: 10.3233/JAD-131355.

(50) Rej, L; Gómez-Vallejo, V.; Rios, X.; Cossio, U.; Baz, Z.; Mujica, E.; Gião, T.; Cotrina, E.Y.; Jiménez-Barbero, J.; Quintana, J; Arsequell, G.; Cardoso, I.; Llop, J. Oral treatment with iododiflunisal delays beta amyloid plaque formation in a transgenic mouse model of Alzheimer Disease: a Longitudinal in vivo molecular imaging study. *J. Alzheimer's Dis.* **2020** (Pub Date : 2020-08-10). DoI: 10.21203/rs.2.23011/v1.

(51) Martorana, A.; Perricone, U.; Lauria, A. The Repurposing of Old Drugs or Unsuccessful Lead Compounds by in Silico Approaches: New Advances and Perspectives". *Curr. Top. Med. Chem.* **2016**, *16*, 2088-2106.

(52) Oprea, T. I.; Overington, J. P.; Computational and Practical Aspects of Drug Repositioning. *Assay Drug Dev. Technol.* **2015**, *13*, 299-306.

(53) Parvathaneni, V.; Kulkarni, N. S.; Muth, A.; Gupta, V. Drug repurposing: a promising tool to accelerate the drug discovery process. *Drug Discov Today*. **2019**, *24*, 2076-2085.

(54) Corbett, A.; Williams, G.; Ballard, C. Drug repositioning in Alzheimer's disease. *Front. Biosci. (Schol Ed)*. **2015**, *7*, 184-8.

(55) Kim, T. W. Drug repositioning approaches for the discovery of new therapeutics for Alzheimer's disease. *Neurotherapeutics* **2015**, *12*, 132-142.

(56) Rodriguez, S.; Hug, C.; Todorov, P.; Moret, N.; Boswell, S. A.; Evans, K.; Zhou, G.; Johnson, N. T.; Hyman, B. T.; Sorger, P. K.; Albers, M. W.; Sokolov, A. Machine learning identifies candidates for drug repurposing in Alzheimer's disease. *Nat. Commun.* **2021** Feb 15;12(1):1033. doi: 10.1038/s41467-021-21330-0.

(57) Anighoro, A.; Bajorath, J.; Rastelli, G. Polypharmacology: challenges and opportunities in drug discovery, *J. Med. Chem.* **2014**, *57*, 7874-7887.

(58) Calzà, L.; Baldassarro, V. A.; Giuliani, A.; Lorenzini, L.; Fernandez, M.; Mangano, C.; Sivilia, S.; Alessandri, M.; Gusciglio, M.; Torricella, R.; Giardino, L. From the multifactorial nature of Alzheimer's disease to multitarget therapy: the contribution of the translational approach. *Curr. Top. Med. Chem.* **2012**, *13*, 1843-1852.

(59) Mathias, S. L.; Hines-Kay, J.; Yang, J. J.; Zahoransky-Kohalmi, G.; Bologa, C. G.; Ursu, O.; Oprea, T. I. The CARLSBAD database: a confederated database of chemical bioactivities. Database (Oxford). 2013 Jun 21; 2013:bat044. <http://datascience.unm.edu/carlsbad-platform/carlsbad/>.

- (60) Wolber, G; Langer, T. LigandScout: 3-D pharmacophores derived from protein-bound ligands and their use as virtual screening filters. *J. Chem. Inf. Model.* **2005**, *45*, 160–169.
- (61) Santos, L. M.; Rodrigues, D.; Alemi, M.; Silva, S. C.; Ribeiro, C. A., Cardoso, I. Resveratrol administration increases Transthyretin protein levels ameliorating AD features- importance of transthyretin tetrameric stability. *Mol. Med.* **2016**, *22*, 597-607.
- (62) Molecular Operating Environment (MOE), 2013.08; Chemical Computing Group ULC, 1010 Sherbooke St. West, Suite #910, Montreal, QC, Canada, H3A 2R7, 2017. URL [http:// chemcomp.com/](http://chemcomp.com/).
- (63) Gimeno, A.; Santos, L. M.; Alemi, M.; Rivas, J.; Blasi, D.; Cotrina, Y.; Llop, J.; Valencia, G.; Cardoso, I.; Quintana, J.; Arsequell, G.; Jiménez-Barbero, J. Insights on the Interaction between Transthyretin and A β in Solution. A Saturation Transfer Difference (STD) NMR Analysis of the Role of Iododiflunisal. *J. Med. Chem.* **2017**, *60*, 5749-5758.
- (64) Furuya, H.; Saraiva, M. J.; Gawinowicz, M. A.; Alves, I. L.; Costa, P. P.; Sasaki, H.; Goto, I.; Sakaki, Y. Production of Recombinant Human Transthyretin with Biological Activities toward the Understanding of the Molecular Basis of Familial Amyloidotic Polyneuropathy (FAP). *Biochemistry* **1991**, *30*, 2415–2421.
- (65) Dolado, I.; Nieto, J.; Saraiva, M. J. M.; Arsequell, G.; Valencia, G.; Planas, A. Kinetic assay for high-throughput screening of in vitro transthyretin amyloid fibrillogenesis inhibitors. *J. Comb. Chem.* **2005**, *7*, 246–252.
- (66) Cotrina, E. Y.; Vilà, M.; Nieto, J.; Arsequell, G.; Planas, A. Preparative Scale Production of Recombinant Human Transthyretin for Biophysical Studies of Protein-Ligand and Protein-Protein Interactions. *Int. J. Mol. Sci.* **2020**, *21*(24):9640.

- (67) Almeida, M.R., I.L. Alves, H. Terazaki, Y. Ando, and M.J. Saraiva. Comparative Studies of Two Transthyretin Variants with Protective Effects on Familial Amyloidotic Polyneuropathy: TTR R104H and TTR T119M. *Biochem. Biophys. Res. Commun.* **2000**, 270, 1024–1028.
- (68) Kabsch, W. Automatic Processing of Rotation Diffraction Data from Crystals of Initially Unknown Symmetry and Cell Constants. *J. Appl. Crystallogr.* **1993**, 26, 795–800.
- (69) Collaborative Computational Project, Number 4. The CCP4 suite: programs for protein crystallography. *Acta Crystallogr. D Biol. Crystallogr.* **1994**, 50, 760-763.
- (70) Brünger, A.T. Free R Value: A Novel Statistical Quantity for Assessing the Accuracy of Crystal Structures. *Nature* **1992**, 355, 472–475.
- (71) McCoy, A. J.; Grosse-Kunstleve, R. W.; Adams, P. D.; Winn, M. D.; Storoni, L. C.; Read, R. J. Phaser crystallographic software. *J. Appl Crystallogr.* **2007**, 40, 658-674.
- (72) Emsley, P.; Lohkamp, B.; Scott, W. G.; Cowtan, K. Features and development of Coot. *Acta Crystallogr. D Biol. Crystallogr.* **2010**, 66, 486-501.
- (73) Adams, P. D.; Afonine, P. V.; Bunkóczi, G.; Chen, V. B.; Davis, I. W.; Echols, N.; Headd, J. J.; Hung, L. W.; Kapral, G. J.; Grosse-Kunstleve, R. W.; McCoy, A. J.; Moriarty, N. W.; Oeffner, R.; Read, R. J.; Richardson, D. C.; Richardson, J. S.; Terwilliger, T. C.; Zwart, P. H. PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr. D Biol. Crystallogr.* **2010**, 66, 213-221.

Supporting Information

Targeting transthyretin in Alzheimer's disease: drug discovery of small-molecule chaperones as disease-modifying drug candidates for Alzheimer's disease

Ellen Y. Cotrina,[#] Luis Miguel Santos,^{1,1,1} Josep Rivas,[†] Daniel Blasi,[†] José Pedro Leite,^{1,1,1} [†] Márcia A. Liz,^{1,1} Maria Antònia Busquets,[±] Antoni Planas,[~] Rafel Prohens,[°] Ana Gimeno,[≠] Jesús Jiménez-Barbero[≠] ¹, Luis Gales,^{1,1,1} [†] Jordi Llop,[‡] Jordi Quintana,^{‡, *} Isabel Cardoso,^{1,1,1} ^{*, ‡}, and Gemma Arsequell,^{#, *, ‡}

[#] Institut de Química Avançada de Catalunya (I.Q.A.C.-C.S.I.C.), 08034 Barcelona, Spain.

¹ IBMC - Instituto de Biologia Celular e Molecular, 4200-135 Porto, Portugal.

¹ i3S – Instituto de Investigação e Inovação em Saúde, Universidade do Porto, 4200-135 Porto, Portugal.

[†] Plataforma Drug Discovery, Parc Científic de Barcelona (PCB), 08028 Barcelona, Spain.

[†] Instituto de Ciências Biomédicas Abel Salazar (ICBAS), 4050-013 Porto, Portugal.

[±] Facultat de Farmàcia i Ciències de l'Alimentació. University of Barcelona. 08028 Barcelona

[~] Institut Químic de Sarrià, Universitat Ramon Llull, 08017 Barcelona, Spain

[°] Centres Científics i Tecnològics, Universitat de Barcelona, 08028 Barcelona, Spain

[≠] CIC bioGUNE, Basque Research and Technology Alliance (BRTA), Bizkaia Technology Park, Building 800, 48160 Derio, Spain.

¹ Ikerbasque, Basque Foundation for Science, 48009 Bilbao, Spain.

[‡] CIC biomaGUNE, Basque Research and Technology Alliance (BRTA), 20014 San Sebastian, Spain

[‡] Equally contributing corresponding authors

* To whom correspondence should be addressed

* Correspondence to:

Dr. Jordi Quintana, e-mail: jordiramon.quintana@upf.edu;

Dr. Isabel Cardoso, e-mail: icardoso@ibmc.up.pt;

Dr. Gemma Arsequell, e-mail: gemma.arsequell@iqac.csic.es

Dr. Jordi Quintana. Research Programme on Biomedical Informatics, Universitat Pompeu Fabra (UPF-IMIM), 08003 Barcelona, Spain

Table of contents

	Pages
Table of compounds assayed in different assays (Table S1)	S3
Table of selected compounds (Table S2)	S19
Crystal structures of TTR:35 and TTR:73 complexes Data collection and refinement statistics (Table S3)	S20
Selected compounds for ternary assay (Abeta/TTR/compound/). Compounds renamed from 1B to 53B. (Table S4)	S21
Results from the HTS ternary assay: representative compounds	S26
Results of HTS screening of compounds 1B to 53B. Table S5.	S27
Isothermal Titration Calorimetry (ITC) studies with A β (12-28)	S28
ITC binary complexes (TTR + SMCs)	S29
References	S30

Table S1: Compounds assayed in different assays

# Our reference	STRUCTURE		Complete displacement of T4 from TTR	Significant displacement of T4 from TTR	No displacement or non-significant displacement of T4 from TTR	Specific for TTR binding	Increased T4 binding to TTR	Selected for T4 quantitative assay	EC50 ratio (T4/Compound)	Selected to the ternary assay from the T4 quantitative assay (EC50 ratio ≥ 0.5)	Selected to the ternary assay from the stability assay	SELECTED COMPOUNDS IN ORANGE	# DRUGS (In green)
1 MAR0002		TOLCAPONE	✓			✓		✓	1,06	✓		YES	1B
2 MAR0003			✓			✓		✓	2,05	✓		YES	2B
3 MAR0004		IDIF-β-Ala-β-Ala In house (synthesis)	✓			✓		✓	0,85	✓		YES	3B
4 MAR0005		In house library		✓		✓		✓	0,27				

5 MAR0006		DIFLUNISAL			✓	✓					✓	YES	4B
6 MAR0007		TAFAMIDIS	✓			✓		✓	0,45		✓	YES	5B
7 MAR0008		LUMIRACOXIB			✓						✓	YES	6B
8 MAR0009		MEFENAMIC ACID			✓						✓	YES	7B
9 MAR0010		MECLOFENAMIC ACID					✓				✓	YES	8B

10 MAR0011		FLUFENAMIC ACID		✓				✓	0,36		✓	YES	9B
11 MAR0012		TRIIODOPHENOL	✓			✓		✓	4,29	✓		YES	10B
12 MAR0013			✓					✓	3,64	✓		YES	11B
13 MAR0014		INDIGO			✓								
14 MAR0016		RESVERATROL		✓		✓					✓	YES	12B
15 MAR0017		CARBAMAZEPIN E <i>Isolated from Tegretol</i>					✓						
16 MAR0019		CYCLOBENZAPR INE <i>Isolated from Yurelax</i>					✓						

17 MAR0022		CLOZAPINE Isolated					✓				✓	YES	13B
18 MAR0024		OLSALAZINE O0146000 FLUKA		✓				✓	Not converged		✓	YES	14B
19 MAR0025		SULINDAC ALDRICH S-8139			✓						✓	YES	15B
20 MAR0030		SULFASALAZINE ALDRICH 4935			✓						✓	YES	16B
21 MAR0031		BALSALAZIDE DISODIUM			✓						✓	YES	17B

22 MAR0032		CLOMIPHENE CITRATE SALT <i>isolated</i>			✓								
23 MAR0036		LOXAPINE Succinate salt SIGMA L106			✓						✓	YES	18B
24 MAR0037		QUETIAPINE FUMARATE <i>isolated</i>					✓						
25 MAR0038		LUMEFANTRINE SIGMA L5420	✓			✓		✓	Not conve rged		✓	YES	19B
26 MAR0039		TAMOXIFEN CITRATE <i>isolated</i>			✓								
27 MAR0043		In house Compound (synthesis)			✓								

28 MAR0044		In house compound			✓							✓	YES	20B
29 MAR0045		MORDANT YELLOW 12 ALDRICH 19,511-1			✓							✓	YES	21B
30 MAR0048		DIETHYLSTILBESTROL (DES)	✓							0,47		✓	YES	22B
31 MAR0050		ALIZARIN YELLOW R			✓							✓	YES	23B
32 MAR0052		ALIZARIN YELLOW GG	✓			✓			✓	0,28		✓	YES	24B

33 MAR0054		In house Compound ¹			✓						✓	YES	25B
34 MAR0056		In house compound	✓			✓		✓	Not converged		✓	YES	26B
35 MAR0057		In house compound			✓						✓	YES	27B
36 MAR0060		CHRYSAMINE G Disodium salt			✓						✓	YES	28B
37 MAR0061		CONGO RED Disodium salt			✓								
38 MAR0064		CANERTINIB chlorhydrate SIGMA C7249			✓								

39 MAR0082		TRIFLUOPERAZINE Dihydrochloride SIGMA T8516					✓						
40 MAR0083		FLUPHENAZINE Dihydrochloride SIGMA F4765					✓						
41 MAR0084		GUANFACINE HYDROCHLORIDE SIGMA G1043			✓						✓	YES	29B
42 MAR0085		RILUZOLE SIGMA R116			✓						✓	YES	30B
43 MAR0086		LOFEXIDINE HYDROCHLORIDE SIGMA SML1019			✓								
44 MAR0089		MELPERONE HYDROCHLORIDE TOCRIS 2495			✓								

45 MAR0090		CLIOQUINOL	✓			✓		✓	0,84	✓		YES	31B
46 MAR0092		ARTICAINE HYDROCHLORID E FLUKA Y0000079			✓								
47 MAR0093		RALTEGRAVIR POTASSIUM ISENTRESS			✓								
48 MAR0094		IODODIFLUNISAL (IDIF)	✓					✓	0,86	✓		YES	32B
49 MAR0098		TAFAMIDIS ² Meglumine salt (water soluble)	✓			✓		✓	0,50	✓		YES	33B
50 MAR0099		HBTI <i>In house compound (by synthesis)</i> ³		✓		✓		✓	0,06		✓	YES	34B
51 MAR0101		HBXI <i>In house compound (by synthesis)</i>		✓		✓		✓	0,03

52 MAR0103		HBT 2-(2-hydroxyphenyl) benzothiazole			✓							✓	YES 35B
53 MAR0106		In house compound ¹			✓							✓	YES 36B
54 MAR0107		In house compound ¹				✓	✓					✓	YES 37B
55 MAR0108		HBX 2-(2-hydroxyphenyl) benzoxazole ALDRICH H50802			✓							✓	YES 38B
56 MAR0109		TOLFENAMIC ACID		✓					✓	0,38			
57 MAR0110		IODODIFLUNISAL Meglumine salt (water soluble) ⁴	✓						✓	0,85	✓		YES 39B

58 MAR0111		TOLCAPONE (TASMAR)	✓					✓	0,90	✓		YES	40B
59 MAR0112		DIFLUNISAL Meglumine salt (water soluble)		✓		✓		✓	0,08		✓	YES	41B
60 MAR0113		In house compound (library) ⁵	✓					✓	1,71	✓		YES	42B
61 MAR0114		In house compound (library)	✓			✓		✓	4,12	✓		YES	43B
62 MAR0115		In house compound (library)	✓			✓		✓	2,57	✓		YES	44B
63 MAR0116		In house compound (library)	✓			✓		✓	0,59	✓		YES	45B
64 MAR0117		ERB-041 PRINABEREL ALDRICH PZ0183	✓			✓		✓	0,35		✓	YES	46B
65 MAR0118		5F-203 ALDRICH SML0983			✓						✓	YES	47B

66 MAR0119		Lamotrigine (Bexal)			✓								
67 MAR0121		BZT ALDRICH 737593			✓						✓	YES	48B
68 MAR0122		ACECLOFENAC SIGMA A8861					✓						
69 MAR0123		PIOGLITAZONE SIGMA E6910	✓			✓		✓	Ambiguous		✓	YES	49B
70 MAR0124		GEMFIBROZIL ⁶ SIGMA 91823			✓						✓	YES	50B
71 MAR0125		LUTEOLIN ^{7,8} Extrasynthese 1125 S	✓			✓		✓	0,15		✓	YES	51B

72 MAR0126		ENTACAPONE SIGMA SML0654		✓		✓		✓	Not converged		✓	YES	52B
73 MAR0127		178804-04-1 MolPort-000-810-817 Vitas-M Laboratory STK027735			✓						✓	YES	53B
74 MAR0201		EGCG SIGMA E4143 INTEGRITY #183411 (5 TARGETS)			✓								
75 MAR0202		BMS 299897 TOCRIS 2870 502 (2) INTEGRITY # (5 TARGETS)			✓								
76 MAR0203		CITICOLINE SODIUM SALT HYDRATE SIGMA C0256 (6 TARGETS) AChE, β -secretase, mGluR5,			✓								

		mGluR1, APP, IL-10											
77 MAR0204		ENAMINE Ltd. COMPOUND 2 Z44862351 EN300-246102 (2 TARGETS) mGluR5, IL-10			✓								
78 MAR0205		(S)-3,4-DCPG TOCRIS 1302 (6 TARGETS) AChE, β-secretase, mGluR5, mGluR1, APP, IL-10			✓								
79 MAR0206		MAP4 TOCRIS 0711 (6 TARGETS) AChE, β-secretase, mGluR5, mGluR1, APP, IL-10			✓								
80 MAR0207	Similar to:	GGsTop GGT inhibitor TOCRIS 4452 SIMILAR to 1241 (6 TARGETS) AChE, β-secretase, mGluR5, mGluR1, APP, IL-10			✓								

81 MAR0208		L-AP4 TOCRIS 0103 (5 TARGETS) AChE, β -secretase, mGluR5, mGluR1, APP			✓							
82 MAR0209		AMN 082 dihydrochloride TOCRIS 2385 (4 TARGETS) AChE, β -secretase, APP, IL-10			✓							
83 MAR0210		COMPOUND 1 ENAMINE ENAMINE Ltd. EN300-04239 (3 TARGETS) AChE, β -secretase, APP					✓					
84 MAR0211		LY 487379 hydrochloride TOCRIS 3283 1134 (1) (1 TARGETS) β -secretase	✓			✓		✓	Ambiguous			

85 MAR0212		Salvianolic acid B SIGMA-ALDRICH Y0001559 (6 TARGETS) AChE, β -secretase, mGluR5, mGluR1, APP, IL-10			✓							
86 MAR0213		LPYFD-NH ₂ TOCRIS 4084 (4 TARGETS) AChE, β -secretase, mGluR1, APP			✓							
87 MAR0214		15-deoxy- Δ -12,14-7Prostaglandin J2 TOCRIS 2306 (2 TARGETS) mGluT5, IL-10			✓							

Table S2. EC50 ratios obtained in the T₄ quantitative assay.

EC50 ratio threshold equal to or higher than 0.5 was used to select compounds for the ternary assay.

Compound	EC50 ratio (T ₄ /compound)	Selected for ternary assay	Compound	EC50 ratio (T ₄ /compound)	Selected for ternary assay
IDIF	0,90		50	0,06	
1	1,06	✓	51	0,03	
2	2,05	✓	56	0,38	
3	0,85	✓	57	0,85	✓
4	0,27		58	0,90	✓
6	0,45		59	0,08	
10	0,36		60	1,71	✓
11	4,29	✓	61	4,12	✓
12	3,64	✓	62	2,57	✓
18	Not converged		63	0,59	✓
25	Not converged		64	0,35	
30	0,47		69	Ambiguous	
32	0,28		71	0,15	
34	Not converged		72	Not converged	
45	0,84	✓	84	Ambiguous	
48	0,86	✓			
49	0,50	✓			
Total: 14 compounds					

Crystal structures of TTR:35 and TTR:73 complexes

Data collection and refinement statistics

Table S3 Data collection and refinement statistics

Crystal data and data-collection statistics	TTR:35 PDB ID 6EP1	TTR:73 PDB ID 6EOY

Wavelength (Å)	1.033	0.973
Space Group	P 21212	P 21212
Unit-cell parameters (Å)		
a	42.96	42.97
b	84.87	85.64
c	64.75	64.24
Resolution range (Å)	84.866 - 1.299 (1.303 - 1.299)	35.72 - 1.38 (1.43 - 1.38)
Observed reflections	292370 (2710)	196098 (18525)
No. of unique reflections		
Completeness (%)	99.9 (61.5)	98.4 (95.7)
Multiplicity	5.0 (4.8)	4.0 (4.0)
$\langle I/\sigma(I) \rangle$	17.8 (1.5)	11.2 (1.1)
Rmerge(%)#	3.8 (85.1)	4.2 (125.7)
Refinement		
cryst(%)	16.3	18.6
Rfree(%)	18.3	19.8
RMSD for bonds (Å)	0.006	0.006
RMSD for angles (°)	0.870	0.959
Average main chain B-factor (Å ²)	20.27	23.82
Average side chain B-factor (Å ²)	25.16	26.89
Average drug B-factor (Å ²)	34.33	42.94
Average water B-factor (Å ²)	38.30	39.34
Average drug B-factor (Å ²)		
Average water B-factor (Å ²)	99.10	96.36
Ramachandran plot statistics (%)	0.90	3.64
Favoured regions		
Allowed regions		

$^{\#}R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the i^{th} observation of reflection hkl and $\langle I(hkl) \rangle$ is the weighted average intensity for all observations of reflection hkl .

Table S4: Selected compounds for ternary assay (TTR/compound/Abeta): compounds from 1B to 53B (in green those who are registered drugs).

#	STRUCTURE		# <i>DRUGS</i> (In green)
1 MAR0002		TOLCAPONE	1B
2 MAR0003			2B
3 MAR0004		IDIF- β -Ala- β -Ala	3B
5 MAR0006		DIFLUNISAL	4B
6 MAR0007		TAFAMIDIS	5B
7 MAR0008		LUMIRACOXIB	6B
8 MAR0009		MEFENAMIC ACID	7B
9 MAR0010		MECLOFENAMIC ACID	8B
10 MAR0011		FLUFENAMIC ACID	9B
11 MAR0012		TRIIDOPHENOL	10B
12 MAR0013			11B
14 MAR0016		RESVERATROL	12B

17 MAR0022		CLOZAPINE	13B
18 MAR0024		OLSALAZINE O0146000 FLUKA	14B
19 MAR0025		SULINDAC ALDRICH S-8139	15B
20 MAR0030		SULFASALAZINE ALDRICH 4935	16B
21 MAR0031		BALSALAZIDE DISODIUM	17B
23 MAR0036		LOXAPINE Succinate salt SIGMA L106	18B
25 MAR0038		LUMEFANTRINE SIGMA L5420	19B
28 MAR0044		<i>In house compound</i>	20B
29 MAR0045		MORDANT YELLOW 12 ALDRICH 19,511-1	21B
30 MAR0048		DIETHYLSTILBESTROL (DES)	22B

31 MAR0050		ALIZARIN YELLOW R	23B
32 MAR0052		ALIZARIN YELLOW GG	24B
33 MAR0054		<i>In house compound</i>	25B
34 MAR0056		<i>In house compound</i>	26B
35 MAR0057 (X-ray structure)		<i>In house compound</i>	27B
36 MAR0060		CHRYSAMINE G Disodium salt	28B
41 MAR0084		GUANFACINE HYDROCHLORIDE SIGMA G1043	29B
42 MAR0085		RILUZOLE SIGMA R116	30B
45 MAR0090		CLIOQUINOL	31B
48 MAR0094		IODODIFLUNISAL (IDIF)	32B
49 MAR0098		TAFAMIDIS Meglumine salt (water soluble)	33B

50 MAR0099		HBTI In house compound	34B
52 MAR0103		HBT 2-(2-hydroxyphenyl) benzothiazole	35B
53 MAR0106		In house compound	36B
54 MAR0107		In house compound	37B
55 MAR0108		HBX 2-(2-hydroxyphenyl) benzoxazole ALDRICH H50802	38B
57 MAR0110		IODODIFLUNISAL Meglumine salt (water soluble)	39B
58 MAR0111		TOLCAPONE (TASMAR)	40B
59 MAR0112		DIFLUNISAL Meglumine salt (water soluble)	41B
60 MAR0113		In house compound (library)	42B
61 MAR0114		In house compound (library)	43B
62 MAR0115		In house compound (library)	44B
63 MAR0116		In house compound (library)	45B
64 MAR0117		ERB-041 PRINABEREL ALDRICH PZ0183	46B
65 MAR0118		5F-203 ALDRICH SML0983	47B

67 MAR0121		BZT ALDRICH 737593	48B
69 MAR0123		PIOGLITAZONE SIGMA E6910	49B
70 MAR0124		GEMFIBROZIL SIGMA 91823	50B
71 MAR0125		LUTEOLIN Extrasynthese 1125 S	51B
72 MAR0126		ENTACAPONE SIGMA SML0654	52B
73 MAR0127 (X-ray structure)		178804-04-1 MolPort-000-810-817 Vitas-M Laboratory STK027735	53B

Kinetics of aggregation of A β (12-28) monitored by turbidimetry-based assay

- alone,
- in the presence of TTR or
- in the presence of TTR in complex with each SMC.

HTS screening of small-molecule compounds: Turbidimetry-based ternary aggregation assay in the presence of small-molecule compounds

Representative examples

Figure S1. HTS screening of representative examples: compounds 48B, 30B, 22B, 34B, 16B, 7B, 38B, 13B, 9B, 32B). The curve represents 13 readings every 30 minutes and the number of replicas is n=8 for the controls and n=3 for the screening of compounds. The statistical analysis has been carried out based on the confidence interval 95% (95%CI).

HTS TERNARY ASSAY

**Table S5: Results of HTS screening of compounds 1B to 53B.
(TTR/compound/Abeta)**

#	Experiment	Abs (final)	RA (%)
	Aβ(12-28)	0,124	-
	TTR + Aβ(12-28)	0,044	74
1B	TTR + 1B + Aβ(12-28)	0,032	79
2B	TTR + 2B + Aβ(12-28)	0,007	95
3B	TTR + 3B + Aβ(12-28)	0,030	81
4B	TTR + 4B + Aβ(12-28)	0,034	78
5B	TTR + 5B + Aβ(12-28)	0,043	74
6B	TTR + 6B + Aβ(12-28)	0,037	77
7B	TTR + 7B + Aβ(12-28)	0,037	77
8B	TTR + 8B + Aβ(12-28)	0,032	79
9B	TTR + 9B + Aβ(12-28)	0,014	90
10B	TTR + 10B + Aβ(12-28)	0,021	86
11B	TTR + 11B + Aβ(12-28)	0,018	88
12B	TTR + 12B + Aβ(12-28)	0,053	70
13B	TTR + 13B + Aβ(12-28)	0,014	90
14B	TTR + 14B + Aβ(12-28)	0,012	91
15B	TTR + 15B + Aβ(12-28)	0,009	93
16B	TTR + 16B + Aβ(12-28)	0,044	74
17B	TTR + 17B + Aβ(12-28)	0,054	70
18B	TTR + 18B + Aβ(12-28)	0,033	79
19B	TTR + 19B + Aβ(12-28)	0,008	94
20B	TTR + 20B + Aβ(12-28)	0,042	75
21B	TTR + 21B + Aβ(12-28)	--*	--
22B	TTR + 22B + Aβ(12-28)	0,049	72
23B	TTR + 23B + Aβ(12-28)	0,057	69
24B	TTR + 24B + Aβ(12-28)	0,047	73
25B	TTR + 25B + Aβ(12-28)	0,031	80
26B	TTR + 26B + Aβ(12-28)	0,041	75

27B	TTR + 27B + A β (12-28)	0,044	74
28B	TTR + 28B + A β (12-28)	0,043	74
29B	TTR + 29B + A β (12-28)	0,057	69
30B	TTR + 30B + A β (12-28)	0,055	69
31B	TTR + 31B + A β (12-28)	0,058	68
32B	TTR + 32B + A β (12-28)	0,001	99
33B	TTR + 33B + A β (12-28)	0,042	75
34B	TTR + 34B + A β (12-28)	0,034	78
35B	TTR + 35B + A β (12-28)	0,047	73
36B	TTR + 36B + A β (12-28)	--	--
37B	TTR + 37B + A β (12-28)	--	--
38B	TTR + 38B + A β (12-28)	0,028	82
39B	TTR + 39B + A β (12-28)	0,006	95
41B	TTR + 41B + A β (12-28)	0,037	77
42B	TTR + 42B + A β (12-28)	0,004	97
43B	TTR + 43B + A β (12-28)	0,027	82
44B	TTR + 44B + A β (12-28)	0,042	75
45B	TTR + 45B + A β (12-28)	0,007	95
46B	TTR + 46B + A β (12-28)	0,051	71
47B	TTR + 47B + A β (12-28)	0,058	68
48B	TTR + 48B + A β (12-28)	0,126	0
49B	TTR + 49B + A β (12-28)	0,097	56
50B	TTR + 50B + A β (12-28)	0,079	61
51B	TTR + 51B + A β (12-28)	0,003	98
52B	TTR + 52B + A β (12-28)	0,119	0
53B	TTR + 53B + A β (12-28)	0,061	67

* Note: (--) Those compounds gave some problems of solubility

Figure S2: ITC of the binary complex Aβ(12-28) + TTR; and ITC's of the ternary complexes [Aβ(12-28) + (TTR + SMC)], being SMC: IDIF, LUT, SUL, OLS, and FLU.

Isothermal titration calorimetry. Binary complexes, TTR + SMCs (IDIF, LUT, SUL, OLS, FLU)

(TAFAMIDIS AND TOLCAPONE added for comparison purposes)

Figure S3: ITC of the binary complexes TTR+SMCs; being SMC: IDIF, LUT, SUL, OLS, and FLU. TOLCA AND TAF A added for comparison purposes.

REFERENCES:

1. González, A.; Quirante, J.; Nieto, J.; Almeida, M. R.; Saraiva, M. J.; Planas, A.; Arsequell, G.; Valencia, G. Isatin derivatives, a novel class of transthyretin fibrillogenesis inhibitors. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 5270-5273.
2. Razavi, H.; Palaninathan, S. K.; Powers, E. T.; Wiseman, R. L.; Purkey, H. E.; Mohamedmohaideen, N. N.; Deechongkit, S.; Chiang, K. P.; Dendle, M. T.; Sacchettini, J. C.; Kelly, J. W. Benzoxazoles as transthyretin amyloid fibril inhibitors: Synthesis, evaluation, and mechanism of action. *Angew. Chem. Int. Ed.* **2003**, *42*, 2758–2761.
3. Rodríguez-Rodríguez, C.; Sánchez de Groot, N.; Rimola, A.; Alvarez-Larena, A.; Lloveras, V.; Vidal-Gancedo, J.; Ventura, S.; Vendrell, J.; Sodupe, M.; González-Duarte, P. Design, selection, and characterization of thioflavin-based intercalation compounds with metal chelating properties for application in Alzheimer's disease. *J. Am. Chem. Soc.* **2009**, *131*, 1436-1451.
4. Water soluble salts of an NSAID with meglumine/glucamine, P.A. Veronesi, Therpicon s.r.l., US Patent US-4,748,174 (May 31, 1988).
5. Vilaró, M.; Nieto, J.; La Parra, J. R.; Almeida, M. R.; Ballesteros, A.; Planas, A.; Arsequell, G.; Valencia, G. Tuning transthyretin amyloidosis inhibition properties of iododiflunisal by combinatorial engineering of the nonsalicylic ring substitutions. *ACS Comb Sci.* **2015**, *17*, 32-38.
6. Iakovleva, I.; Brännström, K.; Nilsson, L.; Gharibyan, A. L.; Begum, A.; Anan, I.; Walfridsson, M.; Sauer-Eriksson, A. E.; Olofsson, A. Enthalpic Forces Correlate with the Selectivity of Transthyretin-Stabilizing Ligands in Human Plasma. *J. Med. Chem.* **2015**, *58*, 6507-6515.
7. Nilsson, L.; Larsson, A.; Begum, A.; Iakovleva, I.; Carlsson, M.; Brännström, K.; Sauer-Eriksson, A. E.; Olofsson, A. Modifications of the 7-Hydroxyl Group of the Transthyretin Ligand Luteolin Provide Mechanistic Insights into Its Binding Properties and High Plasma Specificity. *PLoS One.* **2016** Apr 6;11(4):e0153112.
8. Iakovleva, I.; Begum, A.; Pokrzywa, M.; Walfridsson, M.; Sauer-Eriksson, A. E.; Olofsson, A. The flavonoid luteolin, but not luteolin-7-O-glucoside, prevents a transthyretin mediated toxic response. *PLoS One.* **2015** May 28;10(5):e0128222.