Design and redesign journey of a drug for transthyretin amyloidosis

Francisca Pinheiro, Salvador Ventura*

The misfolding and subsequent aggregation of proteins into amyloid fibrils underlie the onset of a variety of human disorders collectively known as amyloidosis. Transthyretin (TTR) is one of the > 30 amyloidogenic proteins identified to date and is associated with a group of highly debilitating and life-threatening disorders called TTR amyloidosis (ATTR). ATTR comprises senile systemic amyloidosis, which is linked to wild-type (WT) TTR aggregation, and hereditary ATTR, a dominantly inherited disorder caused by the deposition of one of over 130 TTR genetic variants. Senile systemic amyloidosis is a prevalent age-related amyloidosis, affecting up to 25% of the population over 80 years of age, and is characterized by the build-up of TTR fibrils in the myocardium. Regarding hereditary ATTR, the clinical presentation is highly heterogeneous, primarily affecting the peripheral nervous system (familial amyloid polyneuropathy – FAP) or the heart (familial amyloid cardiomyopathy). In rare cases, aggregation develops in the central nervous system, giving rise to a phenotype known as familial leptomeningeal amyloidosis (Carroll et al., 2022).

TTR is a 55 kDa homotetrameric protein primarily synthesized by the liver and the choroid plexus, being secreted into the blood and cerebrospinal fluid, respectively. In plasma, TTR transports the retinol-binding protein-retinol complex and acts as a secondary carrier for thyroxine (T_4), whereas in the cerebrospinal fluid, it is the main T_4 transporter (Sanguinetti et al., 2022). The native state of TTR features two identical T_4-binding sites defined by the interface between the dimers AB and CD. Tetramer dissociation at this interface generates dimers that rapidly dissociate into aggregation-prone monomers, constituting the rate-limiting step in TTR misfolding and amyloidogenesis (Foss et al., 2005; **Figure 1**). Disease-associated mutations enhance TTR amyloidogenesis by lowering its thermodynamic stability and/or the kinetic barrier for tetramer dissociation (Hammarstrom et al., 2002).

Historically, liver transplantation has been the only treatment option for ATTR, aiming to suppress the main source of mutant TTR. However, WT-TTR produced by the donor's liver can still accumulate in the heart and the peripheral nerves after transplantation. In recent years, TTR kinetic stabilizers and gene-silencing agents (e.g., patisiran and inotersen) have emerged as less invasive therapeutic options. TTR kinetic stabilizers are small molecules that bind to one or both T_4-binding sites, stabilizing the TTR native conformation, thereby preventing tetramer dissociation and, consequently, aggregation. While T_4 itself acts as a TTR kinetic stabilizer, less than 1% of TTR is bound to T_4 in blood (Yokoyama and Mizuguchi, 2020). This observation triggered an intensive search for molecules that could bind with high affinity and selectivity to TTR, but, to date, only one stabilizer, tafamidis, has reached the market for ATTR (Bulawa et al., 2012). Tafamidis was initially approved in Europe and Japan for early-stage FAP and was recently granted approval by the United States Food and Drug Administration for the treatment of ATTR-related cardiomyopathy (familial amyloid cardiomyopathy and senile systemic amyloidosis). Of note, the United States Food and Drug Administration has not approved the use of tafamidis in patients with early-stage FAP. Additionally, the observation that 30% of patients do not respond to tafamidis therapy (Monteiro et al., 2019) and its high cost, which poses a significant barrier to access for many patients, has fuelled the development of alternative TTR kinetic stabilizers.

In 2016, our group described tolcapone, a United States Food and Drug Administration-approved molecule for Parkinson's disease, as a new drug for ATTR. Tolcapone exhibits a high affinity and specificity for TTR, effectively stabilizing the tetramer and thus preventing amyloid formation. Importantly, tolcapone demonstrated superior efficacy compared to tafamidis in preclinical studies and induced a robust stabilization of TTR in patients with FAP. The higher potency of tolcapone relative to tafamidis was attributed to its more favorable enthalpic binding to TTR and, especially, to the absence of negative cooperativity among binding sites, which, in the case of tafamidis, substantially reduces its affinity for the second site. Tolcapone was identified through a drug repositioning strategy. However, this does not guarantee that the contacts it establishes with the protein are optimal, as tolcapone was not specifically designed for binding TTR. This is evident from the high-resolution crystal structures of TTR:tolcapone complexes, which revealed that, unlike tafamidis, tolcapone does not establish specific interactions with the inner binding pocket of TTR. Moreover, although tolcapone binds to the second binding site with a significantly higher affinity than tafamidis (K_d for tolcapone = 34 nM; K_{d2} for tafamidis = 260 nM), the binding of tafamidis to the first site is stronger, with a K_{d1} of 9.9 nM (Sant'Anna et al., 2016).

These observations laid the foundation for a multidisciplinary drug design program aimed at developing tolcapone derivatives with improved biological activity. However, X-ray crystal structures provide static snapshots, making it difficult to evaluate the dynamic impact of kinetic stabilizers on TTR stability and to evolve stronger TTR binders. Therefore, we decided to implement a novel approach in which we used molecular dynamics (MD) simulations to predict TTR-ligand interactions as well as the binding energetics, ranking molecules for experimental characterization according to these predictions (Pinheiro et al., 2022). This strategy led to the discovery of M-23, a molecule that binds to WT-TTR with an affinity higher than both tolcapone and tafamidis, positioning it as one of the strongest TTR binders reported to date. The crystal structure of M-23 bound to WT-TTR at 1.2 Å resolution revealed that, as intended, it keeps the interactions established by tolcapone in the outer and central parts of the binding pocket, while forming new and strong contacts with the residues lying on the inner part of the pocket (**Figure 2**). MD simulations predicted these interactions, which resulted in a significantly higher TTR stabilization both *in vitro* and in human plasma relative to tolcapone. These results highlighted the potential of MD simulations to guide the design of potent TTR kinetic stabilizers and positioned M-23 as a candidate for further preclinical and clinical development.

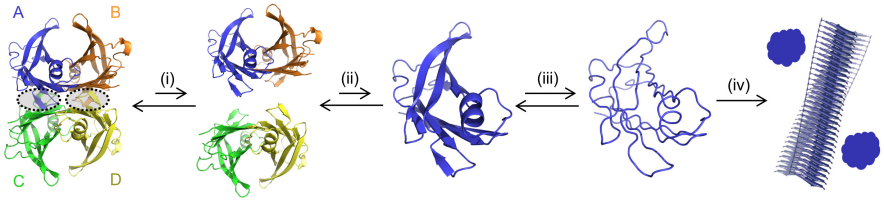

Figure 1 | TTR aggregation mechanism.
The most accepted theory for TTR aggregation defends that the tetramer first dissociates into dimers (i) that readily convert into monomers (ii), which partially unfold, forming an amyloidogenic intermediate (iii). This intermediate ultimately leads to the formation of amyloid fibrils and amorphous aggregates (iv). The letters A–D indicate the TTR subunits while the dashed circles denote the T_4-binding sites. Figure prepared using the PDB structure 1F41 and created with PyMOL (The PyMOL Molecular Graphics System, Version 2.5.7, Schrödinger, LLC) and Microsoft® PowerPoint® for Microsoft 365 MSO (Version 2403). TTR: Transthyretin.

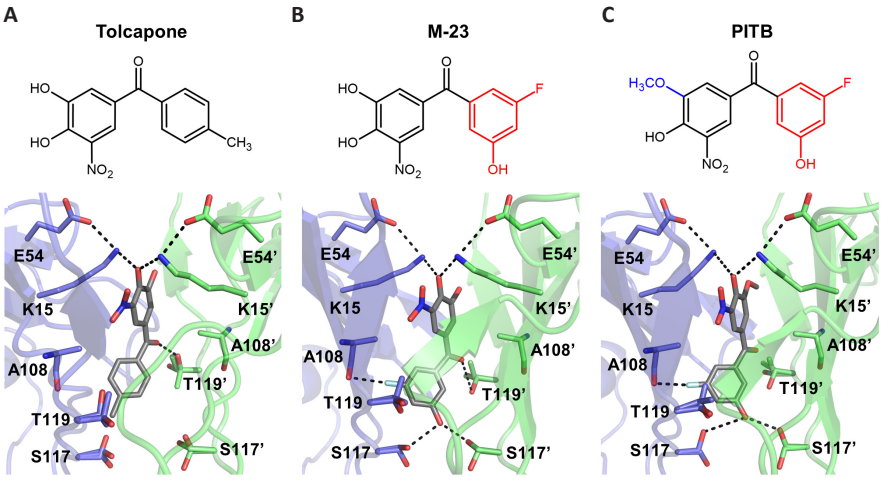

Figure 2 | Crystal structure of WT-TTR bound to tolcapone, M-23, and PITB.
Close-up view of one of the WT-TTR binding sites for tolcapone (A), M-23 (B) and PITB (C). The compounds and the most important TTR interacting residues are depicted as sticks. The dashed lines represent key contacts between each ligand and TTR. The chemical structures of the molecules are shown in the upper part of each panel, with the red and blue colors evidencing the differences between them. Figure prepared from PDB structures 4D7B (A), 7QC5 (B) and 8PM9 (C), and generated with PyMOL (The PyMOL Molecular Graphics System, Version 2.5.7, Schrödinger, LLC) and Microsoft® PowerPoint® for Microsoft 365 MSO (Version 2403). PITB: Pharmacokinetically Improved Transthyretin Binder; TTR: transthyretin; WT: wild-type.

Despite the excellent performance of M-23, a pharmacokinetic (PK) study in mice revealed that it exhibits a suboptimal PK profile, with low bioavailability (5.5%) and a short plasma half-life (0.9 hours), both disfavoring its use. Hence, we embarked on the design of a novel compound to circumvent the PK limitations of M-23 (Pinheiro et al., 2023). Importantly, we aimed to improve the PK properties of M-23 while preserving the main features that make it a stronger TTR stabilizer than tafamidis and tolcapone. In this pursuit, we retained the lower phenyl ring of M-23, which is thought to underlie its increased binding affinity and stabilizing activity, and redesigned the 3,4-dihydroxy-5-nitrophenyl moiety. Therefore, we took advantage of the structure of 3-O-methyltolcapone, a minor metabolite of tolcapone that presents a higher plasma half-life and keeps TTR stabilizing activity (Jorga et al., 1999; Loconte et al., 2020). Given the structural similarity between tolcapone and M-23, we hypothesized that the methylation of M-23 could also improve its PK properties, while maintaining the contacts established with TTR. Consistent with our prediction, we observed that the 3-O-methylation of M-23 occurs after its administration to mice and that the resulting minor derivative has a 3-fold longer half-life in plasma than M-23. Therefore, we chemically synthesized this derivative, which we named Pharmacokinetically Improved Transthyretin Binder (PITB), and investigated its activity as a TTR kinetic stabilizer (Pinheiro et al., 2023). Our data indicated that PITB binds with high affinity and no cooperativity to WT-TTR (K_d = 16 nM) and the two most prevalent disease-associated TTR variants V30M (K_d = 36 nM) and V122I (K_d = 14 nM). Additionally, PITB exhibits a higher affinity than tolcapone for these proteins, especially for the FAP-related V30M-TTR, to which it binds with an affinity > 12-fold higher. Consequently, PITB effectively stabilizes all three proteins, inhibiting their aggregation. Noteworthy, PITB exerts a higher tetramer stabilizing effect in plasma from both control individuals (WT-TTR) and TTR V30M carriers compared to tolcapone. The TTR/PITB crystal structures confirmed that, like M-23, PITB establishes additional interactions with the protein, likely explaining its higher potency in plasma relative to tolcapone (**Figure 2**). Furthermore, in contrast to tolcapone, PITB exhibited a strong safety profile, being devoid of toxicity against cultured human cells, even at high doses. Finally, PK studies conducted in mice demonstrated that, as intended, PITB presents optimal PK properties, with a half-life of 10.1 hours and an oral bioavailability of 85.1%. Interestingly, PITB exhibited even better PK parameters than tolcapone. In particular, PITB presented a 7-fold longer half-life than tolcapone, leading to a 2-fold higher exposure to this compound relative to tolcapone. All in all, these findings support the potential of PITB to become a potent therapeutic agent for ATTR, particularly for FAP caused by the V30M mutation, which is the most common mutation associated with hereditary ATTR cases.

Discussion and perspective: Tafamidis binds to the first T_4-binding cavity with a high affinity. However, as most TTR kinetic stabilizers described so far, it binds with negative cooperativity, decreasing its therapeutic potency. Remarkably, tolcapone binds to TTR with no cooperativity, enabling it to occupy both binding sites at lower concentrations, explaining its better performance *in vitro* and *in vivo*. This results from computational simulations in which the first TTR binding site was already occupied by tafamidis, leaving the second cavity available for docking of the repurposed compounds. However, the crystal structures of TTR:tolcapone and TTR:tafamidis complexes revealed that while the methyl-phenyl ring of tolcapone is better suited for the outer binding pocket, the dichlorophenyl ring of tafamidis docks better to the inner pocket. This immediately suggested that tolcapone could be redesigned to introduce new contacts in the inner binding pocket, thus improving its binding affinity and potency. Of note, contrary to most TTR kinetic stabilizers, including tafamidis, tolcapone does not possess halogen atoms and, thus, cannot form halogen bonds. Since halogen binding seems important for binding to TTR, we envisioned that substituting the 4-methyl-phenyl ring of tolcapone with different halogen moieties would increase its affinity for the protein.

The use of MD simulations played a key role in this task as it has allowed us to predict the binding energy of a large series of rationally designed tolcapone derivatives, but also to anticipate which protein-compound interactions are more relevant for binding, further guiding the design. This approach allowed us to narrow down the search for promising candidates without the need for extensive synthesis and experimental screening.

The biophysical characterization of the top-scoring compounds revealed that M-23 was a promising hit. As intended, M-23 establishes additional interactions in the inner part of the TTR binding pocket, which underlies its exceptional affinity for both binding sites and very high binding enthalpy. These results prompted us to further develop M-23 into a drug for ATTR and, in this context, our next step was to assess its PK properties in mice. This study showed that M-23 has suboptimal PK parameters, reinforcing the delicate balance that exists between target binding/efficacy and PK, as subtle changes in a compound chemical structure can significantly affect its pharmacological properties.

We conducted a structural optimization of M-23 to generate PITB, in an effort to improve the PK properties. The structural and biophysical characterization of PITB interaction with TTR showed that it binds with high affinity to the WT protein and especially to the prevalent V30M variant causing polyneuropathy, relative to any previous kinetic stabilizer. This remarkable selectivity, also evident in human plasma, was accompanied by significantly improved PK parameters. Importantly, PITB did not exhibit associated toxicity, making it a promising candidate for further preclinical and clinical development.

Overall, these studies underscore the benefits of a multidisciplinary approach that integrates structural analysis with biophysical and pharmacological characterization to identify optimal drug candidates. Moreover, we strongly advocate for the utilization of MD simulations for developing more potent and selective TTR binders. A continuous feedback loop between *in vitro*, *in vivo*, and computational analyses is expected to create a virtuous cycle that increases the likelihood of success in ATTR drug development.

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Authors have submitted a patent application to protect the use of PITB as a therapy for TTR amyloidosis. Title: Compound for the treatment of transthyretin amyloidosis. Property: Universitat Autònoma de Barcelona. Request number: EP2023/025333.

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OPEN PEER REVIEW REPORT1

Name of journal: Neural Regeneration Research
Manuscript NO: NRR-D-24-00056
Title: Design and Redesign Journey of a Drug for Transthyretin Amyloidosis
Reviewer's Name: Anna-Marie Babey
Reviewer's country: Australia

COMMENTS TO AUTHORS

Thank you for the opportunity to review this article, which addresses recent advances in transthyretin amyloidosis (ATTR) drug design and discovery. Given the recent improvements in the use of non-invasive investigations to diagnose ATTR, this is a timely topic. The strength of this paper lies in the step-by-step discussion of the discovery of M- 23 and its pharmacokinetic optimisation to create PITB. Unfortunately, the writing style of this narrative is casual and colloquial, which detracts from the value of this manuscript, making it seem more suited to a lay publication. Consequently, I am recommending minor revisions to be addressed prior to publication. Specific comments are provided below.

Content

. p.1, line 39 – a bit of detail would be useful to address the AB/CD dimer-dimer interface, as the interested but uninitiated will require additional information, and this is not directly identified in Figure 1

. p.3, line 57 – regarding the lack of toxicity against hepatic cells, were these experiments performed on cultured hepatocytes or in animals. There is confusion here because the next sentence specifically mentioned studies in mice, which implies that the studies on hepatic cells were not. This needs to be clarified.

. p.4 line 1 – it is unclear what is meant by *"an accumulation 2-fold higher"* – this could be taken to mean that the plasma concentration with repeated dosing is higher than that of tolcapone, but this would be expected when the oral bioavailability is higher, and the half-life is longer; clarification is required here

Writing Style

. use of words such as *"remarkably"* (p.2 line 18; p.3 line 48), *"impressive"* (p.3, line 61) and "exceptionally good" (p.4, lines 49-51) in reference to one's own work comes across as self-congratulatory; the narrative is sufficiently compelling that this is unnecessary and therefore, should be removed

. the phrase "meaning that it was the best compound from our candidates set" (p.2, lines 27-28) goes without saying and shouldn't need to be mentioned; the fact that it is mentioned makes the reader wonder what point you are trying to make and worse, could be construed as some type of excuse

. editorialising should be removed (*"Still, we remained steadfast in our commitment to develop a drug for ATTR"* on p.3, lines 11-13; *"Still, there was room for improvement"* on p.4, line 22; *"hopefully"* on p.4, line 31; *"seemed a good idea"* on p.4 lines 36-38) – the reader will understand the depth of your commitment, as well as the nature of this challenge without the need to make a statements such as these

. there are instances in which the word choice and/or phrasing is awkward, and should be revised; some examples include

- p.1, line 12 – disorders are *called* something, they are not *named* something
- p.2, line 50 – "*but also"* should be replaced with something like "*as well as"*
- p.3, line 22 – *"To this purpose"* should be replaced with something like *"Consequently"*

- p.3, line 43 – *"As a matter of fact"* should be replaced with something like *"Additionally"*
- p.3, lines 56-57 – *"exhibited a safety profile"* should either be modified by the inclusion of an adjective to describe the safety profile (e.g. "a strong safety profile") or should be rephrased
- p.4, line 11 – *"suffers from"* should be replaced with something like *"causes"*

. furthermore, by definition, negative cooperativity means that binding to the second docking site is weaker and therefore, the phrase *"and the binding to the second cavity is substantially weaker"* is redundant and appears to be overstating the obvious

- p.4, line 31 – it is *"contrary to"* not *"contrarily to"*
- p.4, line 58 – *"evidencing"* should be replaced with something like *"reinforcing"*
- p.5, line 13 – *"to identify"* or *"to create"* would be preferable to *"for finding"*

. there are some minor typographical errors that should be corrected

Referencing

. the reader should not be expected to assume that the entirety of the first paragraph was sourced from the review article by Sekijima, and therefore, if this is the source of all information in this paragraph, this in-text reference should appear at the end of the paragraph as well

- similarly, the second paragraph on p.2 (lines 13-40) the reference to the work published in the paper by Sant'Anna and colleagues should appear at the end of the paragraph to reinforce that the contents of the entire paragraph relate to this paper

. when it is mentioned that *"we decided to implement"*, the in-text reference (Pinheiro *et al.*, 2022) should appear with this sentence, not the subsequent sentence

. while I appreciate that the journal will format the references, it is still best to have a reference list that is complete and in a consistent format

- the Monteiro reference has missing capitalisation for the journal title and is missing the article number (i.e. e126526)
- the article title for the Pinheiro reference should be in sentence case