Inhibition of the mechano-enzymatic amyloidogenesis of transthyretin: role of ligand affinity, binding cooperativity and occupancy of the inner channel

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Dissociation of the native transthyretin (TTR) tetramer is widely accepted as the critical step in TTR amyloid fibrillogenesis. It is modelled by exposure of the protein to non-physiological low pH in vitro and is inhibited by small molecule compounds, such as the drug tafamidis. We have recently identified a new mechano-enzymatic pathway of TTR fibrillogenesis in vitro, catalysed by selective proteolytic cleavage, which produces a high yield of genuine amyloid fibrils. This pathway is efficiently inhibited only by ligands that occupy both binding sites in TTR. Tolcapone, which is bound with similar high affinity in both TTR binding sites without the usual negative cooperativity, is therefore of interest. Here we show that TTR fibrillogenesis by the mechano-enzymatic pathway is indeed more potently inhibited by tolcapone than by tafamidis but neither, even in large molar excess, completely prevents amyloid fibril formation. In contrast, mds84, the prototype of our previously reported bivalent ligand TTR ‘superstabiliser’ family, is notably more potent than the monovalent ligands and we show here that this apparently reflects the critical additional interactions of its linker within the TTR central channel. Our findings have major implications for therapeutic approaches in TTR amyloidosis.
sites in each native TTR tetramer. This is most efficiently achieved by bivalent ligands, exemplified by the palindromic compound mds84, that is spontaneously bound simultaneously and pseudo-irreversibly in both sites. Sant’Anna and colleagues have recently reported that tolcapone is a potent inhibitor of TTR dissociation and aggregation under denaturing conditions. They attribute its efficacy to occupation of both binding pockets with similar high affinity in contrast to the notable negative cooperativity observed with thyroxine itself and other monovalent ligands, including the TTR stabiliser drugs, tafamidis and diflunisal, which are now in clinical use.

This important observation prompted us to test the capacity of tolcapone to inhibit mechano-enzymatic amyloidogenesis of TTR using the V122I variant which is the most prevalent cause of hereditary cardiac amyloidosis.

Results and Discussion

We compared tolcapone with tafamidis and the experimental bifunctional compound, mds84 (Fig. 1A). When stirred with V122I TTR at 37 °C in PBS in the presence and absence of trypsin for 96 h, all three ligands inhibited proteolytic cleavage of the protein in a dose-dependent manner (Fig. 1B and Supplementary Fig. 1). At molar...
Consistent with their inhibition of proteolytic TTR cleavage, the three compounds also inhibited fibril formation (Fig. 1C). At a ligand:TTR tetramer molar ratio of 0.5:1, mds84 and tolcapone reduced fibril formation by approximately 90% and 60% respectively, and tafamidis only by 40%. At molar equivalence, inhibition increased at 60% with tafamidis, ~80% with tolcapone and remained at ~90% with mds84. At twofold and greater molar excess of ligand, both monovalent ligands, tafamidis and tolcapone inhibited TTR fibrillogenesis by the same amount (~90%) as mds84.

Even when we compared the effect of the three compounds on the inhibition of TTR acidic-mediated aggregation13, mds84 at equimolar concentration with TTR was the only ligand able to completely inhibit the process (Supplementary Information and Supplementary Fig. S2).

The inhibition of the mechano-enzymatic pathway of TTR amyloidogenesis, which we believe to be the most likely pathophysiological mechanism in vivo, depends on the occupation of both thyroxine binding sites. The superior potency of tolcapone among monovalent TTR ligands is consistent with its unique property of not inducing negative cooperativity5.

The much more effective inhibition of proteolysis-mediated TTR fibrillogenesis by mds84 results from the simultaneous occupation of both thyroxine binding sites, and the internal channel between them, by this panidromic molecule5. It occurs rapidly and completely at equimolar concentrations of TTR and ligand. Binding of mds84 by native TTR is pseudo irreversible under physiological conditions8 and generates a stable complex. This in contrast to the reversible monovalent ligands for which a higher molar excess is required to saturate both binding sites in vivo. These limitations on efficacy against mechano-enzymatic mediated amyloidogenesis may explain the modest therapeutic benefit of monovalent ligands in the clinical studies reported so far12.

To understand the superior inhibitory effect of bivalent ligands, we analysed the deposited X-ray structures of TTR complexed with monovalent ligands, tolcapone5 and tafamidis6, (Fig. 2) in comparison with bivalent ligands, mds84 and compounds 20 and 22 of Green et al.13 (Supplementary Fig. S3). For a general description of the crystal structures we refer to a very exhaustive review by Palaninathan14. There is no PDB structure of the mds84-V122I TTR complex so the analysis is restricted to wild type TTR. This choice is justified by the almost perfect superposition of the wild type and V122I TTR structures in the absence of ligands (Supplementary Fig. S4A,B) with minor deviations when bound to tolcapone (Supplementary Fig. S4C,D). The average root mean square deviations (rmsd) for the TTR backbone residues are 0.45 and 0.9 Å for the two proteins in their free and bound form, respectively. Moreover, Val122 and Ile122 side chains point towards the external part of the pocket cavity and residue 122 is not present in the halogen binding pockets (HBPs; see Methods). Importantly, neither monovalent nor bivalent molecules induce major rearrangements of the HBPs residues with rmsd values calculated for the backbone atoms of HBP1, HBP2 and HBP3 ranging from 0.2 to 0.3 Å (Table 1). The superposition of the binding pockets of TTR with and without ligands is shown in Supplementary Fig. S5. Also there is no correlation between the number of ligand-protein hydrogen bonds, (Table 1) and inhibitory efficacy. A full list of hydrogen bonds between the ligand and the HBPs is reported in the Supplementary Information. However, in contrast with the monovalent ligands which interact only with the ligand pocket itself, the methylene linker of the bivalent compounds occupies the narrow TTR inner channel with an interaction surface increased by 48 Å², for mds84 compared with tolcapone. Binding of mds84, compound 20 and compound 2213 by TTR creates, respectively, up to 9, 5 and 4 hydrophobic interactions, mainly with S117 Cδ1.0 kcal/mol for the three ligands respectively.

Occupancy of the central channel of TTR, estimated for mds84 to be around 80% of the void volume, may thus have a crucial role in preventing subunit sliding and also provide long distance stabilisation affecting the dynamics of CD loop, the selective cleavage of which potently primes amyloid fibrillogenesis. The pioneering study of Green et al.13 identified the enhanced stabilisation of TTR by bivalent ligands with a central linker but their compounds did not interact with native TTR. They were bound only by renaturation of dissociated denatured TTR around the ligand.13 In sharp contrast, both the mds84 prototype and its analogues are rapidly bound by the native TTR tetramer, traversing the inner channel to occupy it with the linker.13 The unique total binding site in TTR, comprising the two halogen binding pockets and the inner channel, is entered by these ligands with a decreased entropic cost compared to all monovalent ligands5. The key factors responsible for the ‘superstabiliser’ property of mds84 and its analogues are thus likely to be the combination of entropic gain, increased hydrophobic contributions of the linker and a reduced void volume in the interface cavity.

The mechanism of binding of TTR by bivalent compounds cannot be fully clarified on the basis of the available crystallographic structures. These data reveal that the TTR inner cavity is very narrow and therefore apparently inaccessible to the bulky groups of the ligands. We hypothesize that the tetramer dynamics may be larger than expected and current studies in our laboratory using NMR might shed light on this important issue.

Finally, even at large molar excess of tafamidis or tolcapone that inhibited obvious in vitro aggregation, ultracentrifugation of the reaction mixture yielded a small amount of insoluble material which stained with Congo red to give typical green birefringence (Fig. 3A and Supplementary Fig. S6) and was fibrillar in the electron microscope (Fig. 3B). In the presence of mds84 such insoluble material was minimal but extensive searching by electron microscopy revealed some fibrils (Fig. 3B). Evidently, the mechano-enzymatic mechanism enables slow formation of some fibrils even when both binding sites are fully occupied in most TTR molecules. Effective targeting of all TTR molecules with stabilizer drugs is much more challenging in vivo than in vitro and it is therefore crucial to consider the significant pathogenic impact of even minimal amyloid fibril formation in vivo. There are potentially major implications, that should not be ignored, both for interpretation of current clinical studies in TTR amyloidosis and for the design of future therapeutic approaches such as the exploitation of non-natural peptides inhibitors of TTR aggregation16 or antibodies recognizing cryptic epitope exposed only on the surface of aggregated TTR and not in the native tetrameric state17.
**Figure 2.** TTR binding sites in the presence of tolcapone and mds84. Wild type TTR binding sites occupied by tolcapone (A) and mds84 (B) with ligands shown as solvent accessible surfaces. For clarity, H₂O oxygens are shown as spheres with 50% of the van der Waals radius. (C) Wild type TTR-mds84 complex with highlighted distances of the principal hydrophobic contacts between the ligand methylene linker and TTR atoms.

**Table 1.** Structural comparisons of TTR halogen binding pockets with and without ligands. *The root mean square deviation was calculated for the backbone atoms of all the three HBPs for the wild type TTR complexes compared to the same protein without ligand (PDB 1dvq). **The hydrogen bonds considered are those between the ligand and the HBPs atoms including crystallographic water molecules.
Methods

Expression of recombinant TTR. V122I TTR was expressed using a pET3a vector containing the full-length cDNA for human V122I TTR in E. coli BL21 super competent cells. Expression colonies were grown to an optical density at 600 nm of 1.0 in Luria-Bertani broth containing 100 µg ml⁻¹ ampicillin at 37 °C; protein synthesis was induced with 1 mM isopropyl-β-D-thiogalactoside at 18 °C overnight. The following day cells were harvested by centrifugation at 2,150 g for 30 min, the pellet was suspended in buffer containing 25 mM Tris-HCl, 2 mM EDTA, 0.1% Triton, pH 7.4 and sonicated at 12 µm amplitude for 10 cycles (1 min on/1 min off). The intracellular proteins were fractionated by 2 cycles of ammonium sulphate precipitation. TTR, which precipitated between 30 and 60% ammonium sulphate, was dissolved in 25 mM Tris-HCl, 0.1 M NaCl, pH 8.0 and fractionated on a Superdex 75 Hi Load 26/60 gel filtration column (GE Healthcare Life Science) equilibrated and eluted in the same buffer. TTR enriched fractions were dialyzed overnight against 25 mM Tris-HCl, pH 8.0, reduced with dithiothreitol and then applied to a Q-Sepharose anion exchange column equilibrated in 25 mM Tris-HCl pH 8.0 and eluted with a linear 0–1 M NaCl gradient in the same solvent. TTR enriched fractions were pooled, concentrated and further purified on the Superdex 75 Hi Load 26/60 column. Fractions containing TTR were dialyzed against water at 4 °C for at least 3 days and then lyophilized. Purity was confirmed by SDS-PAGE and electrospray ionisation mass spectrometry.

Fibrillogenesis of V122I TTR in the presence of ligands. Fibrillogenesis experiments were performed in standard glass vials stirred at 1,500 r.p.m. (IKA magnetic stirrer) at 37 °C using 1 mg ml⁻¹ of V122I TTR (18 µM tetramer) in PBS at pH 7.4 in the presence and in the absence (control) of 5 ng µl⁻¹ of trypsin. Tolcapone, tafenamid and mds84 were dissolved individually at 10 mM in DMSO, followed by serial dilutions which, when added in appropriate volume to TTR, provided ligand:TTR tetramer molar ratios of 0.5:1, 1:1, 2:1 and 4:1 respectively. Turbidity at 400 nm was used to monitor fibril formation over time until it reached a plateau at 96 h. The thioflavin T assay could not be used because tolcapone and mds84 interfere with the fluorescence measurement. At the end of the aggregation, susceptibility to trypsin was monitored using SDS-homogeneous 15% PAGE (GE Healthcare) under reducing conditions. Two-way Anova was performed using GraphPad Prism 5 for pairwise multiple comparison among tafenamid, tolcapone and mds84.

Figure 3. Residual amyloid aggregates in the presence of excess of ligands. (A) Congo-red stained specimens viewed under intense cross polarized light in the absence of any ligands and in the presence of fourfold molar excess of mds84, tolcapone and tafenamid (Supplementary Fig. S6). Some fragments of amyloid are present with maximally inhibitory ligand concentrations (Fig. 1), although least with mds84. Scale bar, 100 µM. (B) Typical fibrillar structures detected by exhaustive analysis of negatively stained electron microscopy images of the same TTR-ligand preparations. Scale bar, 100 nm.
The pellet was harvested from each protein sample in the absence and in the presence of ligands by ultracentrifugation in a Beckman Optima TL ultracentrifuge at 135,000 g, 20 min. After resuspension of the pellet with a minimal volume of PBS, samples were stained with alkaline alcoholic Congo red and examined by high intensity cross polarized light microscopy. Amyloid load was scored in the Congo red stained pellet of TTR in the presence of fourfold molar excess of each ligand (Supplementary Fig. S6). A blind quantification was carried out by an expert operator on six slides per each group of treatment using the following grading score: 0 (no spot detected), 1 (occasional spots), 2 (green birefringent spots clearly visible and corresponding to the stained material in the bright field), 3 (surface homogeneously covered by green birefringent material). The non-parametric Kruskall–Wallis test for mds84 vs tafamidis (or tolcapone) was applied using GraphPad Prism 5.

Samples were also examined by negative staining transmission electron microscopy. Briefly, a drop of each sample was allowed to dry on formvar coated copper EM grids for 2 min before blotting with filter paper to remove excess solvent and staining with 2% w/v uranyl acetate for 1 min. After further blotting and drying in air, transmission electron microscope (Jeol 200EX) images were obtained at 80 kV.

**Structural analysis.** X-ray structures of wild type TTR alone (PDB codes 1DVQ and 5CN3) and complexed with tolcapone, tafamidis, mds84, compound 20 and 22 (PDB codes 4D7B, 3TCT, 3IPE, 2FBR and 2FLM, respectively) were examined using VMD, SPDBV20,21 and Pymol (PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC). The energetic contribution of the linker to the binding was estimated by the SeeSAR programme (https://www.biosolveit.de/SeeSAR). The halogen binding pockets considered in the analysis are formed by Met13, Lys15, Leu17, Lys18, Val121 (HBP1); Lys17, Ala108, Ala109, Leu110 (HBP2); Ala108, Ser117, Leu110, Thr119 (HBP3).

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Author Contributions
The study was conceived, designed and supervised by V.B. G.V., P.P.M., S.R., S.G., R.P performed research. A.C., J.D.G., P.N.H., M.B.P., G.W.T. contributed to experimental design and discussion. All the authors analysed and interpreted the data. The paper was written by V.B., P.P.M., G.W.T. and M.B.P. and reviewed and approved by all co-authors.

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