Kinetic Stabilization of Transthyretin and its Role as an Inhibitor of Aβ Amyloid Formation

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Success is not final, failure is not fatal: it is the courage to continue that counts.

– W. Churchill
# Table of Contents

Table of Contents .......................... i
List of papers ............................. iii
Abstract .................................. v
Abbreviations ............................. vi
1 Introduction to the field ............... 1
2 Protein folding and misfolding ......... 2
3 Amyloid ................................ 3
   3.1 Amyloid structure .................. 3
   3.2 Mechanisms of amyloid formation ... 4
4 Transthyretin ........................... 5
   4.1 Structure .......................... 5
   4.2 Expression and metabolism ....... 5
   4.3 Functions .......................... 7
   4.3.1 Transporter of T<sub>4</sub> ....... 7
   4.3.2 Transporter of retinol-binding protein .......................... 7
   4.3.3 Other functions .................. 8
   4.4 Mechanism of transthyretin amyloid formation .... 8
   4.5 Transthyretin amyloidosis ......... 9
      4.5.1 Non-hereditary TTR amyloidosis ........ 9
      4.5.2 Hereditary TTR amyloidosis .......... 9
         4.5.2.1 Familial amyloid polyneuropathy .... 10
         4.5.2.2 Familial amyloid cardiomyopathy .... 10
         4.5.2.3 Familial leptomeningeal amyloidosis .... 10
   4.6 Transthyretin amyloidosis therapies ...... 10
      4.6.1 Liver transplantation ............ 10
      4.6.2 Stabilizing drugs ............... 11
         4.6.2.1 Diflunisal .................. 11
         4.6.2.2 Tafamidis .................. 11
5 Amyloid-β and Alzheimer’s disease .... 13
   5.1 Amyloid-β .......................... 13
   5.2 Interaction between amyloid β and TTR .... 14
6 Aim of this thesis ...................... 16
7 Methods ................................ 17
   7.1 Urea denaturation assay ............ 17
   7.2 Human liver microsomes ............ 17
   7.3 Thioflavin-T fluorescence assay .... 18
   7.4 Acid-denaturation assay .......... 18
   7.5 Cytotoxicity assay ............... 18
   7.6 High-performance liquid chromatography .... 19
   7.7 X-ray crystallography ............ 19
List of papers

This thesis is based on the following papers, which are referred to in the text by the corresponding Roman numerals I–IV.


   Manuscript.

†These authors contributed equally to this work.
Papers by the author not included in the thesis:

  Small pH and salt variations radically alter the thermal stability of metal-binding domains in the copper transporter, Wilson disease protein. 

  The N-terminal region of amyloid β controls the aggregation rate and fibril stability at low pH through a gain of function mechanism. 
Abstract

Amyloid formation occurs when normally soluble proteins and peptides misfold and aggregate into intractable threadlike structures called fibrils. There are currently more than 30 proteins associated with this aberrant structure, including the Aβ peptide in Alzheimer’s disease (AD) and transthyretin (TTR) in TTR amyloidosis. TTR is a homotetrameric transporter protein present in both cerebrospinal fluid and plasma. Dissociation of its tetrameric structure is required for the formation of amyloid fibrils. Small molecule ligands able to bind and stabilize the tetrameric structure of TTR thus represent a potential therapeutic intervention. Interestingly, apart from TTR’s role as a toxic agent in TTR amyloidosis, it also has a role as an inhibitor of the Aβ toxicity associated with AD. The work presented in this thesis focused on small molecules that have the potential ability to prevent TTR amyloidosis. We also sought to gain a greater understanding of the interaction between TTR and the Aβ peptide with respect to Aβ fibril formation.

The ability of a drug to stabilize TTR is directly correlated to its binding affinity. However, since TTR is a plasma protein, it is of great importance that the drug binds selectively to TTR. In paper I, we used a newly developed urea denaturation assay, in combination with isothermal titration calorimetry, to show that, in a complex environment such as plasma, the enthalpy of binding correlates better with a drug’s ability to stabilize TTR than the binding affinity. In paper II, we modified the highly selective but rapidly degraded TTR ligand luteolin in order to increase its resistance against biotransformation. Using a liver-based microsome assay, in combination with HPLC, we show how the luteolin analogues have gained increased stability. However, using the urea assay, we also show that the analogues have lost much of luteolin’s selectivity. In paper III, we show that tetrabromobisphenol A is a highly selective binder of TTR in plasma and is able to rescue cells from TTR-induced toxicity. In paper IV, we studied the interaction of TTR with Aβ and its effect on Aβ fibril formation. We used a ThT fluorescence-based assay and dot blotting to show that TTR inhibits Aβ amyloid formation and promotes the formation of high molecular weight assemblies with an open N-terminus. Using surface plasmon resonance, we further show how TTR is unable to inhibit fibril elongation and instead targets the nucleation processes, both primary and fibril-catalyzed secondary nucleation. To conclude, we present new molecules with the ability to selectively stabilize TTR that can serve as scaffolds in drug design. We also elucidate TTR’s inhibiting effects on toxic Aβ amyloid formation.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Aa</td>
<td>Amino acid</td>
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<tr>
<td>Aβ</td>
<td>Amyloid beta</td>
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<td>AD</td>
<td>Alzheimer’s disease</td>
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<td>APP</td>
<td>Amyloid precursor protein</td>
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<tr>
<td>ATTRm</td>
<td>Mutant transthyretin amyloidosis</td>
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<tr>
<td>ATTRwt</td>
<td>Wild-type transthyretin amyloidosis</td>
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<tr>
<td>7-Cl-Lut</td>
<td>Luteolin-Cl</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>Half maximal inhibitory concentration</td>
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<tr>
<td>ITC</td>
<td>Isothermal titration calorimetry</td>
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<tr>
<td>FAC</td>
<td>Familial amyloid cardiomyopathy</td>
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<td>FAP</td>
<td>Familial amyloid polyneuropathy</td>
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<tr>
<td>FCSN</td>
<td>Fibril-catalyzed secondary nucleation</td>
</tr>
<tr>
<td>GEM</td>
<td>Gemfibrozil</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>HBP</td>
<td>Halogen binding pocket</td>
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<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>Kd</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>LUT</td>
<td>Luteolin</td>
</tr>
<tr>
<td>7-MeO-Lut</td>
<td>Luteolin-MeO</td>
</tr>
<tr>
<td>MST</td>
<td>Microscale thermophoresis</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>RP</td>
<td>Reversed phase</td>
</tr>
<tr>
<td>RBP</td>
<td>Retinol-binding protein</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>SSA</td>
<td>Senile systemic amyloidosis</td>
</tr>
<tr>
<td>TBBPA</td>
<td>Tetrabromobisphenol A</td>
</tr>
<tr>
<td>TBG</td>
<td>Thyroxine-binding globulin</td>
</tr>
<tr>
<td>TBP</td>
<td>Thyroxine binding pocket</td>
</tr>
<tr>
<td>ThT</td>
<td>Thioflavin-T</td>
</tr>
<tr>
<td>T₄</td>
<td>Thyroxine</td>
</tr>
<tr>
<td>T₃</td>
<td>Triiodothyronine</td>
</tr>
<tr>
<td>TTR</td>
<td>Transthyretin</td>
</tr>
<tr>
<td>UGT</td>
<td>UDP-glucuronosyltransferase</td>
</tr>
<tr>
<td>Wt</td>
<td>Wild-type</td>
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1 Introduction to the field

The term *amyloid* was first introduced in 1854 by Rudolph Virchow to denote a macroscopic tissue abnormality from the cerebral corpora amylacea that exhibited a positive iodine staining reaction. Concluding that the substance was cellulose, he named it amyloid, derived from the Latin *Amylum*, meaning starch-like [1].

Today we know that the amyloid structures that Virchow found were made of proteins and not cellulose, but the name remains. Deposits of amyloid occur when normally soluble peptides and proteins misfold and aggregates into intractable thread-like structures called fibrils. The formation of amyloid fibrils is associated with both a loss of function of the proteins involved and with the generation of often toxic intermediates in the process of the aggregation.

There are now approximately 50 known disorders that are associated with the misfolding of proteins into amyloid fibrils, including the Aβ peptide in Alzheimer’s disease, α-synuclein in Parkinson’s disease and transthyretin (TTR) in familial amyloid polyneuropathy (FAP) [2].
2 Protein folding and misfolding

Proteins are large biomolecules that exist in every living organism. They are composed of amino acids linked together into long chains, and to be able to function most proteins need to fold into a specific three-dimensional conformation [3].

The driving force for protein folding is the search for a conformation with the lowest free energy. Small alterations in the amino acid chain can create a new local free-energy minimum or even the formation of a new global free-energy minimum resulting in a different stable structure of the protein that might be prone to aggregation [4].

To avoid aggregation and amyloid formation, there has been a negative selection during evolution against specific patterns of residues, such as alternating hydrophobic and hydrophilic stretches [5]. Another factor that controls aggregation is charge and a high net charge either globally or locally might hinder the self-association [6].

Other protective mechanisms against amyloid formation include the cellular environment such as the presence of molecular chaperones and degradation processes that function to prevent the formation and accumulation of misfolded proteins [2].
3 Amyloid

Today more than 30 different proteins and peptides are associated with diseases in which a normally soluble protein misfolds and aggregates into amyloid fibrils [7,8]. The diseases are clinically heterogeneous and one or multiple organs can be affected. They can either be localized, meaning that the amyloid deposits form in the site of precursor protein expression or systemic, meaning that the precursor protein is expressed and secreted at one site distinct from the major sites of deposition [9].

3.1 Amyloid structure

Although the proteins forming the amyloid fibrils have vastly different amino acid sequences and native structures, they end up in a remarkably similar structure in the mature fibril. They form an unbranched filamentous structure with only a few nanometers in diameter but often several micrometers in length (Figure 1A). The fibril is built up by multiple protofilaments that twist around each other, and each protofilament adopts a cross-β structure in which β-strands run perpendicular along the fibril axis (Figure 1B) [10].

Figure 1. Fibril structure. A) Electron micrograph of amyloid fibrils formed by the Aβ_{40} peptide. B) Structure of an 11-residue peptide of TTR (aa 105–115) showing the cross-β structure in which the β-strands run perpendicular to the fibril axis (PDB ID: 2M5N) [11].

Mature amyloid fibrils were long considered to be the toxic species and the cause of cell death in amyloid diseases. Over the last few years, however, the field has shifted and now the pre-fibrillar species, the oligomers, are considered to be the most damaging species to the cell [12]. The oligomers are thought to exert their toxic function by displaying surfaces that normally are hidden in the natively folded
protein, such as hydrophobic patches [13]. These surfaces can interact inappropriately with other proteins and cellular membranes [14,15].

Oligomers at the beginning of the aggregation pathway are small and contain only a few molecules, whereas oligomers occurring later in the pathway are larger and are referred to as protofibrils. The protofibrils form spherical beads of 2–5 nm in diameter that can assemble into beaded chains or annular structures, each containing extensive β-sheet structure [16].

3.2 Mechanisms of amyloid formation

Several mechanisms of aggregation into an amyloid fold have been proposed. In nucleation-dependent polymerization the initiating step of aggregation is the formation of a nucleus with a different conformation than the precursor protein. This is termed primary nucleation. The nucleus is a sparsely populated species typically rich in β-sheet structure. The steps preceding the formation of a nucleus are energetically unfavorable, but once the nucleus is formed monomers can rapidly add to the nucleus in a template-dependent manner ultimately forming a fibril structure [17]. This mechanism has been proposed to be the aggregation pathway for the Aβ peptide. However, recent studies have shown the importance of secondary nucleation in the aggregation pathway of Aβ. This secondary nucleation process requires the presence of fibrils and is termed fibril-catalyzed secondary nucleation (FCSN). When a critical concentration of amyloid fibrils is reached, the FCSN will overtake the primary nucleation as the major source of oligomers where the nucleation is catalyzed by the fibril surface [18].

In the downhill polymerization mechanism, the rate-limiting step is the formation of a misfolded aggregation-prone monomer. Subsequent addition of misfolded monomers to the growing polymer is energetically favorable. This mechanism describes the aggregation pathway for the protein transthyretin (TTR).

The addition of exogenous nuclei or seeds can bypass the slow nucleation step and speed up the aggregation process in nucleation-dependent polymerization, but not in the case of downhill polymerization, at least not in the case of TTR amyloid formation [19].
4 Transthyretin

Transthyretin (TTR) is a highly conserved protein found in vertebrate species [20]. It is a 55 kDa homotetramer present in both plasma and cerebrospinal fluid (CSF). The name is derived from its well-described function as a transporter of thyroxine (T4) and holotransretin-binding protein (RBP). More recent studies have suggested that it also has other important roles, e.g., by functioning as a neuroprotective factor in Alzheimer’s disease [21-23]. Misfolding and aggregation of TTR into amyloid fibrils is linked to systemic organ dysfunction, peripheral neuropathy, cardiomyopathy, ophthalmopathy and central nervous system (CNS) amyloidosis [24]. Both wild-type and mutated TTR misfolds into amyloid fibrils, and to date more than 130 mutations in TTR have been described [25].

4.1 Structure

The first X-ray structure of TTR was determined in 1971 [26]. Each monomer in the homotetramer has a weight of 14 kDa and is composed of 127 amino acids [27] (Figure 2A). The monomers each consists of eight antiparallel $\beta$-strands, denoted A–H, that are organized in two four-stranded $\beta$-sheets (Figure 2B). A short $\alpha$-helix is located at the end of $\beta$-strand E. All $\beta$-strands are antiparallel except between strands A and G. The TTR dimer is formed by extensive contacts between strands F and H of both monomers, and the association between the monomers is strengthened by numerous hydrogen bonds. The contact region between the dimers, which forms the functional tetramer, is small and consists of hydrophobic and hydrophilic interactions between the loops that join strands G to H and A to B. A hydrophobic channel runs through the center of the tetramer forming the thyroxine binding pocket (TBP). The TBP is characterized by hydrophobic depressions referred to as halogen binding pockets (HBPs), and these are where the iodine atoms of T4 reside (Figure 2C). HBP 1 is the outer binding subsite and it is made up of the side chains of Ala108/108’, Thr106/106’, Met13/13’ and Lys15/15’. HBP 2 is made up of the side chains of Leu110/110’, Ala109/109’, Lys15/15’ and Leu17/17’ whereas HBP 3 consists of Ser117/117’, Thr119/119’, Ala109/109’ and Leu110/110’ (Figure 2C) [28].

4.2 Expression and metabolism

The liver is the main producer of TTR in the plasma, and it secretes 90% of the plasma TTR in humans [30]. The concentration of TTR in plasma ranges from 3.6–7.2 µM and varies with age; it is lower in healthy newborns compared to adults [31] and starts to decline after about 50 years of age [32]. The main source of TTR in the
CSF is produced by the choroid plexus [33]. TTR constitute approximately 25% of the CSF proteins, and the concentration ranges from 0.9–3.6 µM [34]. TTR is also synthesized in the retinal pigment epithelium of the eye [35], the pancreatic islet of Langerhans [36] and to a small extent in the heart, skeletal muscle, spleen [37] and placenta [38]. TTR serum concentration is a good marker of energy malnutrition due to its decreased levels during protein and calorie deprivation [39].

The in vivo half-life of TTR is approximately 2-3 days in humans. Based on studies with rats, the major sites of degradation are the liver, muscle and skin with the liver degrading 36–38%, the muscle 12–15% and the skin 8–10%. The kidneys, adipose tissue, testes and gastrointestinal tract (GI) degraded 1–8% of the TTR. The

Figure 2. TTR structure. A) The TTR tetramer (PDB ID: 5EN3 [29]). The red rectangles indicate the TBPs. B) The TTR dimer with the β-strands denoted A-H. The contact region between the dimers is indicated in magenta (PDB ID: 5EN3 [29]). C) A close-up view of the TBP. The pocket is shown together with the natural ligand T4 (PDB ID: 2ROX [28]).
catabolism of TTR is far from fully understood and no evidence for degradation in the tissues of the nervous system has been found [40].

4.3 Functions

4.3.1 Transporter of T4

Thyroid hormones are important for the regulation of metabolism, protein synthesis and cell differentiation [41]. The thyroid gland synthesizes the prohormone thyroxine (T4) and to a smaller extent the more active triiodothyronine (T3). Thyroxine is bound to three different thyroid hormone-binding proteins when circulating in the plasma; thyroxine-binding globulin (TBG), TTR and albumin. About 65% of thyroxine is transported by TBG, 20% by albumin and 15% by TTR [20]. In the CSF, TTR is the main carrier of T4 transporting 80% of the T4 [42]. Each TTR tetramer has two binding sites for thyroid hormones, but only one is significantly occupied in vivo because they exhibit negative cooperativity [43]. The dissociation constant (Kd) for the binding of the first T4 molecule is 13.6 nM [44] and the Kd for the second is around 1 µM [45]. The mechanism by which T4 is delivered into the cells is not fully understood. Some studies suggest that T4 enters the cell via passive diffusion after first dissociating from the carrier protein [46], while other studies claim that the uptake occurs while T4 is still bound to the carrier protein [47]. TTR seems to have a redundant role as the transporter of T4 because mice carrying a null mutation appeared normal, viable and fertile even though the total T4 levels in their plasma were reduced to 35% of the controls and the T3 levels were reduced to 65% of the controls [48].

4.3.2 Transporter of retinol-binding protein

TTR plays a role in vitamin A metabolism when it forms a complex with RBP. Vitamin A is important for several physiological processes, including growth and development, vision and reproduction [46]. RBP is mainly synthesized in the liver and is secreted into the plasma after it has bound to retinol and formed of a complex with TTR [49]. The binding of RBP to TTR is important to prevent RBP from being eliminated by glomerular filtration in the kidney [50]. A crystal structure of the TTR/RBP complex determined at 3.2 Å resolution showed that TTR was bound to two RBP molecules and that the two RBPs mostly interacted with opposite dimers of the TTR tetramer [51]. The concentration of RBP in plasma is about 2 µM [52] and TTR binds only one RBP in vivo. Both holo- and apo-RBP can bind TTR but the former binds with significantly higher affinity [53]. The binding of holo-RBP to TTR exhibits negative cooperativity, and the Kd for the first bound RBP is 150 nM and for the second it is 35 µM [54]. Mice with a null mutation in the TTR gene are
phenotypically normal although their plasma levels of retinol are below the level of
detection (<6% of the normal value) and the levels of RBP are 3% of the normal value [48].

4.3.3 Other functions

TTR is able to bind a wide variety of small molecules in its TBP [55-57]. This and the
fact that only a small fraction of TTR's TBP is occupied by T4 in plasma [20] suggests
that TTR might function as a detoxifier. By binding ligands present in the plasma as a
result of dietary intake or other environmental exposures, TTR can transport them to
the liver or kidneys for elimination [58].

TTR has also been shown to serve as a neuroprotector in Alzheimer's disease via its
interaction with the Aβ peptide and this is described more in section 5.2.

4.4 Mechanism of transthyretin amyloid formation

In order for TTR to aggregate into amyloid, the tetramer first needs to dissociate into
monomers, and then the natively folded monomer has to misfold (Figure 3) [59]. The
rate-limiting step is the tetramer dissociation, and after monomer misfolding, the
aggregation continues very efficiently since every formed growing specie is more
stable than the previous, i.e. the misfolded dimer is more stable than the misfolded
monomer and the misfolded trimer is more stable than the misfolded dimer. As
mentioned in section 3.2 the mechanism of TTR amyloid formation is termed
downhill polymerization and it does not require nucleus formation and it is not
amenable to seeding [19].

Both mutated TTR and wild-type (wt) can dissociate, misfold and form amyloid
fibrils. The structures of several TTR variants have been solved, and most of them do
not show significant changes in structure compared to the wt [60-62]. There are also
eamples of mutations that stabilize TTR and make the tetramer less prone to
dissociation than the wt. The T119M mutation can suppress the tetramer
destabilization of other mutants. Individuals heterozygous for T119M and a
pathogenic variant exhibit a more benign form of the disease than individuals
heterozygous for wt TTR and the same pathogenic variant [63].
Figure 3. Mechanism of TTR amyloid formation. The amyloid cascade starts with the rate-limiting tetramer dissociation. After monomer misfolding the aggregation into larger species (e.g. oligomers and fibrils) continues efficiently.

4.5 Transthyretin amyloidosis

TTR amyloidosis can be divided into two forms – non-hereditary and hereditary. The non-hereditary form is linked to aggregation of wt TTR and is termed ATTRwt or senile systemic amyloidosis (SSA). The hereditary forms, ATTRm, are caused by mutations in the TTR gene and results in autosomal dominant disease.

4.5.1 Non-hereditary TTR amyloidosis

SSA is an age-related systemic amyloidosis where the wt TTR aggregates. It mainly affects the heart, but it can also cause carpal tunnel syndrome [64], bladder and GI symptoms [65,66]. Postmortem studies have demonstrated that 12–25% of people over 80 years of age have wt TTR amyloid deposits in the heart [67,68]. In addition to full-length TTR, the fibrils in SSA patients also consist of C-terminal fragments of the protein starting at positions 46–52 [67].

4.5.2 Hereditary TTR amyloidosis

Hereditary TTR amyloidosis, ATTRm, is linked to more than 130 mutations in the TTR gene and is divided into three phenotypes – familial amyloid polyneuropathy (FAP), familial amyloid cardiomyopathy (FAC) and familial leptomeningeal amyloidosis [69].
4.5.2.1 Familial amyloid polyneuropathy

FAP is a group of multisystem diseases in which nerve lesions are caused by mutated TTR. The first identified and the most studied FAP mutation is V30M. It is endemic in northern Sweden (Skelleftesjukan), Portugal and Japan. The age of onset and penetrance varies between countries, with carriers in Sweden having a lower penetrance and later onset than carriers in Japan and Portugal, but the reason for the discrepancies between endemic areas is not known [70]. The earlier-onset patients develop the disease before 50 years of age and have a more severe clinical course [71]. The neuropathic symptoms usually start in the lower limbs with sensory loss and pain and progress upwards. By the time the sensory neuropathy has reached the knees, the hands usually become affected. Autonomic neuropathy includes orthostatic hypotension, constipation alternating with diarrhea, nausea and impotence. Amyloid buildup in the heart and kidney is also common [72]. Untreated FAP on average leads to death within 10 years after onset [73].

4.5.2.2 Familial amyloid cardiomyopathy

FAC is characterized by progressive cardiomyopathy. The V122I mutation is the most common variant world-wide, and it is estimated that ~4% of the African Americans (1.3 million people) are heterozygous for this mutation [74]. The age of onset is similar to SSA, >60 years of age, but FAC patients are much more likely to suffer from cardiac failure [75].

4.5.2.3 Familial leptomeningeal amyloidosis

Leptomeningeal amyloidosis in combination with cerebral amyloid angiopathy is associated with ten rare mutations in TTR e.g. D18G and A25T [76,77]. Patients with leptomeningeal amyloidosis show various CNS dysfunctions such as dementia, visual impairment, seizures, ataxia and intracranial hemorrhage [69].

4.6 Transthyretin amyloidosis therapies

4.6.1 Liver transplantation

Liver transplantation as a treatment for FAP started in the early 1990’s. The rationale was that since the liver is the main source of TTR in the plasma, replacing the liver producing the mutated TTR with one producing wt TTR would reduce the plasma concentration of the mutated protein. This was proven to be true, and liver transplantation reduced the variant protein by 95% [78]. The progression of
autonomic and peripheral neuropathy halted or even slightly improved in most patients [79]. However, for some patients deposition of amyloid in the myocardium continued with eventual heart failure as a result. It was shown that the wt TTR, from the donor liver, had continued to deposit in the heart [80]. Genotype is an important factor determining survival rate, with 74% of V30M patients surviving 10 years after liver transplantation compared to only 44% of the patients in the non-V30M group [81].

4.6.2 Stabilizing drugs

As tetramer dissociation is necessary for TTR amyloid fibril formation a therapeutic option is to stabilize the tetramer. Binding of several small molecules to the TBP of TTR has been shown to prevent the dissociation of the tetramer [55,82]. These small molecules are frequently denoted kinetic stabilizers and they stabilize the tetramer over the dissociative transition state by selectively bind to the tetrameric state. This binding increases the free energy of activation for dissociation and dramatically slows down the tetramer dissociation, which is rate limiting for TTR fibril formation [83].

The binding of a drug to TBP will not significantly disturb the transport of T₄ since only ~1% of TTR is involved in T₄-binding [20]. Two small molecules – diflunisal and tafamidis – have been shown to slow the progression of FAP in randomized clinical trials.

4.6.2.1 Diflunisal

Diflunisal is a well-known non-steroidal anti-inflammatory drug (NSAID) developed more than 40 years ago [84]. It binds TTR with negative cooperativity with a Kd₁ = 75 nM and Kd₂ = 1.1 µM [85]. Although having a modest selectivity for TTR in plasma, its high oral bioavailability allows it to reach substantial concentrations in vivo [86,87]. A statistically significant reduction in the rate of neurological progression and preserved quality of life among diflunisal-treated patients has been shown in a two-year randomized study [88]. However, due to the adverse effect of COX-1 inhibitory activity caution should be taken when treating patients with impaired renal function and prior GI dysfunctions [89,90].

4.6.2.2 Tafamidis

Tafamidis is a newly developed drug and is the first to be approved for treatment of FAP. It binds to TBP with a Kd₁ of 2 nM and a Kd₂ of 154 nM [91]. In an 18-month clinical study, tafamidis-treated patients showed fewer cases of disease progression compared with placebo-treated patients [92]. Additional studies have reported that it
is appears to be generally safe and can improve both cardiac and neurological outcomes [93,94]. However, it was not able to stop the disease progression in patients with more advanced stages of disease [95]. Tafamidis is currently being investigated in a clinical trial to evaluate its efficacy and safety for both SSA and FAC patients and this trial is expected to be completed in August 2018.
5 Amyloid β and Alzheimer’s disease

Alzheimer’s disease (AD) is the most common neurodegenerative disorder, accounting for around 70% of the late-onset dementia cases, with a current estimation of 46 million affected people worldwide [96]. Advanced age is the single most common major risk factor for AD, and most cases occur relatively late in life, >65 years of age, although ~5% occur earlier [97]. The disease is fatal and characterized by a progressive memory loss, particular short-term memory loss, and cognitive impairment with death occurring 7–10 years after the initial diagnosis [98]. The characteristic histopathological hallmarks of AD are extracellular neuritic plaques and intracellular neurofibrillary tangles located in the hippocampus, amygdala and association neocortex [99].

5.1 Amyloid β

The ~4 kDa natively unfolded amyloid β (Aβ) peptide is the main component of the neuritic plaques [100]. It is generated by sequential proteolytic processing of the amyloid precursor protein (APP). APP is a single-pass transmembrane protein with a large extracellular domain. APP can be cleaved through two different pathways – the amyloidogenic pathway and the non-amyloidogenic pathway (Figure 4). In the amyloidogenic pathway, APP is first cleaved by β-secretase, generating soluble sAPPβ and a C-terminal membrane-tethered fragment called C99. C99 is further cleaved by γ-secretase to generate Aβ peptides and an intracellular fragment called AICD. In the non-amyloidogenic pathway, α-secretase cleaves APP between amino acids 16 and 17 in the Aβ sequence, hindering the formation of Aβ. This releases sAPPα and leaves an 83 aa membrane-bound fragment called C83. The subsequent cleavage of C83 by γ-secretase produces the short p3 peptide and AICD [101].

Due to the fact that the cleavage by γ-secretase is imprecise, the C-terminus of the produced Aβ is heterogeneous. The most abundant Aβ peptide is the one ending at position 40 (Aβ40) (80-90%) followed by 42 (Aβ42) (5-10%). The slightly longer forms, especially Aβ42, are more hydrophobic and more toxic. Aβ42 is also the principal peptide found in neuritic plaques [102]. The total concentration of Aβ in the CSF is 1–2 nM [103] and the concentration of Aβ42 is around 160 pM [104]. The concentration of Aβ40 in AD patients is not significantly different from healthy controls, whereas the concentration of Aβ42 is lower in AD patients [105]. The production of Aβ40 and Aβ42 does not appear to be different in sporadic AD cases compared to controls, but the clearance is reduced in the AD patients [106].
APP is processed through two pathways – the non-amyloidogenic and the amyloidogenic pathways. The $A\beta$ peptide is formed in the amyloidogenic pathway by sequential cleavage by $\beta$-secretase and $\gamma$-secretase (with permission from Pamrén 2012).

Most cases of AD are sporadic and late onset, whereas the rare cases of autosomal-dominant AD are predominantly early onset. Mutations in three genes – $APP$, $PSEN1$ and $PSEN2$ (where $PSEN$ is coding for the presenilin protein, a subunit of $\gamma$-secretase) – are known to cause autosomal-dominant AD. Many mutations in these genes affect the metabolism of $A\beta$ by increasing the ratio of $A\beta_{42}/A\beta_{40}$ or by increasing the total production of $A\beta$ [107].

Although often considered to be a harmful byproduct from APP processing, $A\beta$ has a neurotrophic effect in differentiating neurons [108] and it plays a role in synaptic plasticity [109]. It has also been suggested to function as an antimicrobial agent in the brain [110].

5.2 Interaction between amyloid $\beta$ and TTR

The first report of an interaction between $A\beta$ and TTR came when it was discovered that CSF inhibited $A\beta$ fibril formation in vitro and that the protein responsible for the inhibition was TTR. It was proposed that $A\beta$ was sequestered by extracellular proteins and that the failure of sequestration might lead to the amyloid fibril formation seen in AD [21]. Supporting a role of TTR in AD is the decreased TTR levels in CSF from AD patients that negatively correlate with plaque burden [111,112]. This decrease is not seen in other types of dementia such as dementia with Lewy bodies or frontotemporal dementia [23].
Transgenic (tg) mouse models overexpressing APP with the Swedish mutation, APPswe, support the connection of the two proteins. There is an upregulation of TTR in these mice, and infusion of an antibody directed towards TTR shows a significant increase in Aβ deposition in hippocampal neurons compared to mice infused with a control antibody [113]. Another study with mice overexpressing APPswe and a mutation in PSEN1 with a deletion of one allele of the TTR gene showed elevated levels of Aβ deposition compared to mice without the deletion [114]. A further study with mice overexpressing the Swedish mutation showed that simultaneous overexpression of TTR was ameliorative with the mice demonstrating improved cognitive function and reduced Aβ deposition [115].

Also supporting a beneficial role of TTR is that the intracellular domain (AICD), generated by cleavage of APP, has been shown to bind to the TTR promoter and upregulate the production of TTR in the same manner that AICD upregulates the Aβ-degrading enzyme neprilysin [116].

The interaction of TTR with Aβ is beneficial for cultured cells, and several studies have shown that preincubation of Aβ with TTR neutralizes Aβ-induced cell toxicity [117,118].

The in vitro studies of the direct interaction between Aβ and TTR show a preferential binding of TTR to aggregates of Aβ but TTR binds monomers as well [115,119]. It has been suggested that TTR monomers bind Aβ better than TTR tetramers [120]. The Kd of the interaction between tetrameric TTR and monomeric Aβ has been determined with isothermal titration calorimetry (ITC) to be 24 µM [121].

The interaction site on TTR has been examined with crosslinking and peptide scanning using series of overlapping peptides derived from TTR, and strands A and G in the TBP and the EF helix were identified as potential binders, although only very minor binding was seen in the EF region in the peptide scanning [120,122]. An NMR study further confirmed the TBP and amino acids in close proximity to it as a potential Aβ binding site. The same group also suggested that the interaction site on Aβ is amino acids 18–21 by using dot blotting, due to the fact that this epitope on Aβ was blocked after incubation with TTR [121].

It has been suggested that TTR cleaves Aβ and disaggregates fibrils [123] but these results are not supported by other laboratories [121].
6 Aim of this thesis

It has been known for over 20 years that binding of a small molecule to the TBP of TTR inhibits the tetramer dissociation and the subsequent amyloid fibril formation of TTR. To date, only one small molecule inhibitor, tafamidis, has been approved for the treatment of TTR amyloidosis. It slows down the progression of FAP; however, it does not work in the later stages of the disease. Thus, there is a need for more efficient drugs.

TTR also has a role as an inhibitor of the Aβ toxicity associated with AD, but due to the transient nature and complexity of the Aβ species, the details of this interaction are still elusive.

The general aims of this thesis are therefore, to 1) find new candidates for treatment of TTR amyloidosis and 2) to gain a greater understanding of the interaction between TTR and the Aβ peptide.

Specific aims

Paper I: To elucidate the determinants of ligand binding specificity for TTR in plasma.

Paper II: To increase the metabolic stability of luteolin by synthesizing analogues while maintaining its high specificity in plasma.

Paper IV: To investigate the potential of tetrabromobisphenol A (TBBPA) as a drug for treating TTR amyloidosis.

Paper IV: To characterize the interaction between TTR and Aβ with respect to Aβ fibril formation.
7 Methods

7.1 Urea denaturation assay

The in vivo efficacy of a drug is dependent on several factors and among these is the selectivity of a ligand to its target over other components. The urea denaturation assay is used to study the relative stability of TTR as a function of drug concentration, and when used with plasma it also probes the drug selectivity in a complex environment similar to the in vivo milieu. The plasma, containing ~5 µM TTR, is first preincubated with various concentrations of a drug. Depending on how selective the drug is for TTR, it can either bind to TTR or to other components of the plasma. Urea is then added to the samples, and this will dissociate all apo-TTR tetramers into monomers whereas the drug-bound TTR remains stable. After the incubation with urea the plasma samples are separated according to size with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The TTR with drug bound, being a very stable protein, will remain tetrameric in the gel. The gel electrophoresis is followed by western blotting using a polyclonal TTR antibody followed by a horseradish peroxidase-labeled antibody. Bound antibodies are visualized on a photographic film using enhanced chemiluminescence. The level of dissociation, i.e. the monomeric band, is monitored instead of the tetrameric band due to cross-reactivity of the polyclonal antibody with the highly abundant plasma protein albumin in the same size range as tetrameric TTR. With no drug added, tetrameric TTR will be fully dissociated into monomers by the urea. If the drug binds TTR, and stabilizes the tetramer, the monomeric band will become fainter with increasing amount of drug, and this gives a measure of its ability to selectively stabilize TTR in plasma.

7.2 Human liver microsomes

In vitro screening assays with liver-derived experimental systems represent an effective approach for estimating the metabolic fates of a drug in vivo [124]. Microsomes are endoplasmic reticulum membrane vesicles prepared by homogenization of the liver followed by subsequent centrifugation steps. The first centrifugation step is a low-speed centrifugation that removes larger components such as the plasma membrane and the mitochondria. The second centrifugation step is performed at a higher speed resulting in the endoplasmic reticulum, which contains the phase-II conjugating enzymes UDP-glucuronosyltransferases (UGTs), ending up in the pellet. The membrane-bound UGTs catalyze the addition of glucuronic acid moieties to xenobiotics. The glucuronidation of a drug is studied by incubating it with the microsomes, and the reaction is initiated by adding UDP-glucuronic acid [125].
7.3  Thioflavin-T fluorescence assay

The fluorescent benzothiazole dye thioflavin-T (ThT) is an efficient and convenient tool for identifying amyloid fibrils due to its large enhancement of fluorescence emission upon fibril-binding. Measuring ThT fluorescence is the most commonly used technique to monitor fibrillation kinetics in real time. When binding to the fibrils, ThT displays a dramatic shift in excitation maximum, from 385 nm to 450 nm, and emission maximum, from 445 nm to 482 nm [126]. The large enhancement of fluorescence emission upon fibril binding is suggested to be due to the fact that the free rotation of the benzothiazole and aniline ring in solution quenches the excited states generated by photon excitation. The dye becomes locked upon fibril binding, and this preserves the excited state resulting in a high quantum yield of fluorescence [127]. It is generally accepted that ThT binds parallel to the fibril axis in the channel-like motifs formed by repeating side-chain interactions running across the β-strands [128].

7.4  Acid-denaturation assay

Amyloid formation of TTR can be induced by lowering the pH to 4.5 since this leads to disturbances of the tetrameric fold [129], dissociation, monomer misfolding and subsequent aggregation [130]. The formation of aggregates can be detected by measuring turbidity at 400 nm. This is a convenient assay to use when screening for ligands that bind to TTR since ligand binding will stabilize the tetrameric TTR and prevent the formation of aggregates and the resulting turbidity. TTR, incubated at low pH, in the absence of ligand is assigned to have 100% fibril conversion [131].

7.5  Cytotoxicity assay

Preservation of the native tetrameric structure impairs TTR’s ability to exert a cytotoxic effect [132]. This is utilized in a cell-viability assay using the human neuroblastoma cell line, SH-SY5Y. Cells are incubated with TTR with or without ligand before addition of the blue and nonfluorescent resazurin. The cytotoxic effect of TTR is monitored by a decrease in the reduction of resazurin to the pink and highly fluorescent resorufin. This reduction is suggested to be due to mitochondrial enzyme activity [133].
7.6 High-performance liquid chromatography

Chromatography is the separation of a complex mixture into its individual components via the partitioning between a mobile phase and a stationary phase. In high-performance liquid chromatography (HPLC), the compounds flow under high pressure via the mobile phase through a column containing the stationary phase. In the column the mixture is resolved into its components and the resolution is dependent on the interaction of the solute compounds with the stationary phase and liquid phase. A common separation mode is reversed phase (RP) where the stationary phase is non-polar and the mobile phase is polar. Mobile phases in RP typically consist of a gradient of water and an organic solvent, starting with the more polar water will elute the hydrophilic compounds and with the increase of the less polar organic solvent more hydrophobic compounds are eluted [134].

7.7 X-ray crystallography

X-ray crystallography is a technique to determine the three-dimensional structure of proteins at atomic resolution. The first and often rate-limiting step is the growth of a protein crystal with sufficiently high quality for structure determination. When a narrow parallel beam of x-rays is directed at the crystal, a small fraction of the x-rays are scattered by the sample. The scattered waves reinforce one another at certain points and appear as diffraction points, reflections, on a detector [135]. Analysis of the resulting diffraction pattern can be used to build an electron density map, which is a 3D image of the electron clouds. The calculation of the electron density map requires the wavelength of the incident light, the amplitude of the scattered x-rays and the phase of diffraction, but the phase of the diffraction cannot be determined from the pattern of reflections. One way to solve this issue is to use the phases from related solved structures, and this is called molecular replacement. The structural model of the protein is built within the electron density map, and this requires knowledge of the amino acid sequence of the protein [136].

7.8 Microscale thermophoresis

Microscale thermophoresis (MST) is a relatively new technique for analyzing the interaction between biomolecules. It measures thermophoresis; the directed movement of molecules in a temperature gradient [137]. Thermophoresis is sensitive to binding-induced changes of molecular properties such as changes in size, hydration shell or charge [138]. One of the interaction partners usually has to be labeled with a fluorescent probe, either on primary amines or on sulfhydryl groups. The thermophoresis is induced in small glass capillaries, containing the sample, by an
infrared laser (IR) that produces a microscopic temperature gradient. Simultaneously the fluorophores are excited and their emission is measured. This allows following of the depletion or accumulation of fluorophores within the temperature gradient produced by the laser. When deriving binding constants, multiple capillaries with constant concentrations of fluorescently labeled molecules and increasing concentrations of ligand are used [139].

7.9 Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) is a widely used technique to study protein-ligand interactions [140], and a single experiment allows one to determine the thermodynamic parameters of a binding interaction [141]. An ITC instrument has two cells; a sample cell containing the macromolecule and a reference cell containing buffer. The instrument uses a power compensation design that maintains the same temperature in both cells. The ligand is titrated into the sample cell during the experiment, and this causes heat to be either released or absorbed. Depending on if it is an exothermic or endothermic reaction the feedback power to the sample cell heater will decrease or increase respectively. The measurements consist of the time-dependent input of power required to maintain equal temperatures between the two cells [142]. After several injections, when the limiting reactant becomes saturated, the heat signal will approach zero. Fitting of the resulting binding curve gives the binding enthalpy (ΔH), the Kd and the stoichiometry. From these data Gibb’s free energy (ΔG) and the entropy (ΔS) are calculated via [143]:

\[
\Delta G = -RT \ln K_a \\
\Delta G = \Delta H - T \Delta S
\]

where R is the universal gas constant and T is the temperature in degrees Kelvin.

7.10 Surface plasmon resonance

Surface plasmon resonance (SPR) is an optical-based technique to measure real-time protein-ligand binding affinities and kinetics. It measures the refractive index changes in the vicinity of a thin metal layer in response to biomolecular interactions. The metal is usually a thin film of gold on a glass support forming the floor of a small-volume flow cell. One of the investigated molecules is immobilized on the sensor surface while the other molecule, the analyte, is injected in an aqueous solution through the flow cell [144]. SPR is an optical effect that occurs when monochromatic
polarized light is reflected from a thin metal film. Part of the incident light interacts with the delocalized electrons in the metal (the plasmon) and this results in a slight reduction in the intensity of the reflected light. The angle of incidence at which the SPR occurs is determined by the material absorbed onto the metal film. There is a direct relationship between the mass of the immobilized molecules and the change in resonance energy at the metal surface that can be used to study interactions in real time [136].
8 Results and discussion

The discoveries that the dissociation of TTR into monomers is required for the formation of amyloid fibrils and that binding of a small molecule into the TBP of TTR increases the activation energy required for tetramer dissociation led to a new therapeutic intervention for TTR amyloidosis [130,145]. Two small molecule stabilizers, tafamidis and the NSAID diflunisal, have been shown to slow down the rate of the progression of FAP in randomized clinical trials [88,146]. Tafamidis has been proven to have a beneficial effect on TTR amyloidosis, but it does not work in later stages of the disease [95]. Diflunisal is an NSAID, and with that comes adverse effects on the GI tract, and this adds an extra burden to FAP patients who already suffer from GI dysfunctions [147]. Thus, there is a need for new and improved small molecule stabilizers that work efficiently for all stages of the disease and have low incidences of adverse effects.

Papers I-III involved the search for a more efficient TTR tetramer-stabilizing drug. The last paper in this thesis, paper IV, was a characterization of the interaction of TTR with the amyloidogenic peptide Aβ that is associated with AD.

8.1 Paper I – Enthalpic Forces Correlate with the Selectivity of Transthyretin-Stabilizing Ligands in Human Plasma.

A clinical trial takes approximately 8 years to complete [148], and with hereditary TTR amyloidosis being an orphan disease [149] relatively little money is currently being invested in the development of new drugs to treat it. Since the TBP of TTR is able to accommodate a plethora of different structures, one way to bypass a long and costly clinical trial is to screen a library of drugs that have already been approved for other uses. We screened a chemical library (Prestwick Chemical Library) with the aim of finding new TTR tetramer-stabilizing ligands. From this screen, nine different ligands able to bind and stabilize TTR were identified (Figure 5). Six were already known to bind TTR, including diflunisal, diclofenac, niflumic acid, luteolin, apigenin and meclofenamic acid and three were unknown, including aceclofenac, gemfibrozil and tolfenamic acid [131,150,151].
Plasma can strongly compromise a ligand’s ability to stabilize TTR

TTR is present at ~5 µM in plasma, and this implies that at least 5 µM ligand is required for the full stabilization of the TTR tetramer, provided that the Kd is sufficiently low. With all of the other components that are present in plasma, it is crucial that the ligand binds selectively to TTR in order to avoid adverse effects and to keep the therapeutic dose low. To test the selectivity of the different ligands, we performed the urea denaturation assay described in section 7.1. Using this assay the half maximal inhibitory concentration (IC_{50}) was determined, i.e. the ligand concentration where 50% of TTR is dissociated into monomers (Figure 6). There was a large variation in the ligand concentrations required for stabilization, and although LUT and GEM were the two ligands with the highest binding affinities, 70 nM and 100 nM respectively, they had vastly different selectivities. LUT is a very selective binder of TTR with an IC_{50} of 7 µM, while GEM has an IC_{50} of 180 µM, nearly a 40-fold excess of the ligand relative to the TTR concentration in plasma.
Figure 6. Selective stabilization of TTR in plasma. Dissociation of the TTR-tetramer, in human plasma, is observed as presence of a monomeric TTR band on a Western blot. The inhibition (%) of tetramer dissociation is plotted versus drug concentration. A) Luteolin. B) Gemfibrozil.

Enthalpy is a better predictor of selectivity than binding affinity

Since tetramer stabilization is directly proportional to the affinity of a bound ligand in a pure system, it is of interest to investigate the correlation between $K_d$ and $IC_{50}$ in plasma. We determined the $K_d$ for the interaction between the ligands and TTR with ITC (Table 1).

Table 1. The properties of the ligands\(^*\).

<table>
<thead>
<tr>
<th>Binding affinity to TTR ($K_d$, nM)</th>
<th>LUT</th>
<th>API</th>
<th>DIF</th>
<th>TOA</th>
<th>MEA</th>
<th>NIF</th>
<th>DIC</th>
<th>ACL</th>
<th>GEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>70</td>
<td>250</td>
<td>580</td>
<td>150</td>
<td>480</td>
<td>260</td>
<td>1000</td>
<td>1200</td>
<td>100</td>
</tr>
<tr>
<td>$K_d$, plasma, pH</td>
<td>7.3</td>
<td>18.3</td>
<td>25.2</td>
<td>15.1</td>
<td>40.4</td>
<td>50.4</td>
<td>130.4</td>
<td>136.4</td>
<td>140.3</td>
</tr>
<tr>
<td>Enthalpy ((\Delta H)), kcal</td>
<td>-11.2</td>
<td>-9.6</td>
<td>-7.8</td>
<td>-9.4</td>
<td>-8.6</td>
<td>-7.0</td>
<td>-3.8</td>
<td>-6.6</td>
<td>-1.0</td>
</tr>
<tr>
<td>Entropy ((\Delta S)), kcal/298°C</td>
<td>-1.4</td>
<td>-0.6</td>
<td>0.7</td>
<td>0.1</td>
<td>1.8</td>
<td>2.2</td>
<td>4.3</td>
<td>1.6</td>
<td>8.6</td>
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<tr>
<td>Molecular weight, g/mol</td>
<td>286</td>
<td>270</td>
<td>250</td>
<td>241</td>
<td>295</td>
<td>250</td>
<td>206</td>
<td>304</td>
<td>250</td>
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<tr>
<td>$\log P$</td>
<td>1.4</td>
<td>1.7</td>
<td>1.4</td>
<td>5.2</td>
<td>5.2</td>
<td>3.7</td>
<td>n.a.</td>
<td>4.3</td>
<td>3.8</td>
</tr>
<tr>
<td>Hydrogen bond donors</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Hydrogen bond acceptors</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>5</td>
<td>3</td>
<td></td>
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<tr>
<td>Rotatable bonds</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>7</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Surface area, $\text{Å}^2$</td>
<td>107</td>
<td>87</td>
<td>57.5</td>
<td>49.3</td>
<td>49.3</td>
<td>62.2</td>
<td>49.3</td>
<td>75.6</td>
<td>66.5</td>
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<td>Charge</td>
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<td>0</td>
<td>-1</td>
<td>-1</td>
<td>-1</td>
<td>-1</td>
<td>-1</td>
<td>-1</td>
<td></td>
</tr>
</tbody>
</table>

\(^*\)The table lists the $\log P$, $\Delta G$, $\Delta S$, and the $K_d$. Also listed is the $IC_{50}$ in plasma for each compound, molecular weight, number of hydrogen bond donors/acceptors, and the specific change in water at pH 7. Ligands are luteolin (LUT), difluoromethyl (DIF), tolan (TOA), mephenytoin (MEA), nefluramine (NIF), diclofenac (DIC), aceclofenac (ACL), gemfibrozil (GEM) and tolfenamic acid (TOA).

Plotting the $K_d$ value of the ligands in a pure system versus the $IC_{50}$ in plasma showed some correlation between $K_d$ and $IC_{50}$ in plasma, although some ligands deviated strongly from linearity (Figure 7).
Figure 7. Kd vs. IC50. A plot of the Kd in PBS determined by ITC and the IC50 in plasma. The IC50 is reported as the mean ± SD of three independent experiments.

A thermodynamic characterization of the interaction is obtained when determining Kd with ITC, and plotting the enthalpic component versus the IC50 shows a better correlation than Kd versus IC50 (Figure 8A). By plotting the ratio of ΔH to ΔG, and thus omitting the factor of affinity, versus IC50 also shows correlation (Figure 8B) where a high contribution of ΔH is associated with high selectivity in plasma.

Figure 8. Enthalpy of binding vs. IC50 and ΔH/ΔG vs. IC50. A) A plot of the enthalpy of binding and the IC50 in plasma. B) A plot of the relative contribution of the enthalpy of binding and the Gibbs free energy of binding versus the IC50 in plasma. The IC50 is reported as the mean ± SD of three independent experiments.

We performed a structural analysis to determine if the structural properties of the ligands could give insight into their selectivity (Table 1). From this analysis, we concluded that the number of possible hydrogen bonds does not correlate with selectivity and this is probably due to the fact that it does not equal the amount of actual formed bonds in the interaction between TTR and the ligand. The most selective ligands, LUT, API and DIF, had the lowest numbers of rotatable bonds, and...
it is reasonable to assume that ligands with a more flexible structure are able to adapt to a larger number of different targets, hence losing selectivity.

To conclude, we have shown that the selectivity of TTR-stabilizing ligands can be significantly compromised by the presence of plasma and that the enthalpy of binding correlates better with selectivity than Kd.
8.2 **Paper II** - Modifications of the 7-Hydroxyl Group of the Transthyretin Ligand Luteolin Provide Mechanistic Insights into Its Binding Properties and High Plasma Specificity

We have previously shown that the plant-derived flavonoid luteolin displays a very high selectivity for TTR in plasma [152]. With its low incidence of adverse effects, it would be an interesting candidate for stabilizing the tetrameric structure of TTR if it were not for its rather low metabolic stability *in vivo* [153]. It is biotransformed in the liver by glucuronidation, and the preferred site for glucuronidation is the 7-OH position [154] (Figure 9). Glucuronidation at this position abrogates the molecule's ability to stabilize TTR [152]. In order to prevent this, we modified luteolin by replacing the 7-OH with a chloro (7-Cl-Lut) or a methoxy group (7-MeO-Lut) (Figure 9).

![Figure 9. Chemical structures of luteolin, luteolin-7-O-glucuronide and the modified variants.](image)

The 7-OH is replaced in the analogues with a chloro group (7-Cl-Lut) or a methoxy group (7-MeO-Lut).

**Substitution of the 7-OH of luteolin increases its metabolic stability**

To investigate the metabolic stability of luteolin and its analogues against glucuronidation, we used a liver microsome-based assay. UHPLC was used to follow the degradation of the drug into its metabolites over time (Figure 10). The chromatograms show, as expected, that the chloro and the methoxy groups are inert to glucuronidation.

The half-life was calculated by comparing the area of the peaks corresponding to the unmodified drugs with the initial area. Both substitutions improved the half-life of the drugs with 7-MeO-Lut having a half-life of 36 ± 8 min, about twice as long as luteolin's 16 ± 1 min. 7-Cl-Lut had a half-life of 52 ± 7 min, around a 3-fold increase compared to luteolin (Figure 11). The decrease in the concentration of the analogues over time is due to glucuronidation at the 3’ and 4’-OH positions (Figure 10).

Figure 11. Depletion profile of luteolin and the analogues. Luteolin (triangles), 7-Cl-Lut (squares) and 7-MeO-Lut (diamonds). Each timepoint represent the average of three independent samples (mean ± SD).
The crystal structures reveal an opposite binding mode

7-Cl-Lut binds to the TBP in the same manner as luteolin, but as a result of exchanging the 7-OH group with a chlorine the hydrogen bonds to the hydroxyl groups of Ser117 and Thr119 are lost. Interestingly, the TTR-7-MeO-Lut complex shows that 7-MeO-Lut is oriented in the binding site in the opposite direction compared to luteolin. This is due to the bulkier methoxy group that cannot be accommodated within the same space as the chlorine or the hydroxyl group of luteolin (Figure 12).

![Figure 12. Comparison between the binding sites. A) 7-Cl-Lut. B) 7-MeO-Lut. C) Luteolin.](image)

The plasma selectivity of the two luteolin analogues is decreased compared to luteolin

Binding affinity was measured MST. Both 7-Cl-Lut and 7-MeO-Lut lost some binding affinity with Kd values of 620 ± 150 nM and 390 ± 40 nM, respectively, compared to luteolin’s Kd value of 150 ± 70 nM, but they still represent high-affinity binders. The selectivities of luteolin and the analogues for TTR in plasma were measured with the urea denaturation assay, which provided IC₅₀ values. Luteolin, being a highly selective ligand, has an IC₅₀ of 5 µM, whereas both 7-Cl-Lut and 7-MeO-Lut lost a lot of luteolin’s selectivity and had IC₅₀ values of 65 µM and 105 µM, respectively (Figure 13).
Figure 13. Selective stabilization of TTR in plasma. A) Luteolin B) 7-Cl-Lut C) 7-MeO-Lut.

In summary, we show that it is possible to increase the half-life of luteolin by substituting the 7-OH with a methoxy group or a chlorine. The analogues bind with high affinity to TTR but unfortunately the substitutions have a large effect on their selectivity for TTR in plasma.
8.3 Paper III – Tetrabromobisphenol A is an Efficient Stabilizer of the Transthyretin Tetramer

TTR is able to bind a number of organohalogen compounds, including brominated flame retardants [155-157]. Here we characterized the interaction between TTR and one of these; 2,2′,6,6′-tetrabromo-4,4′-isopropylidenediphenol (tetrabromobisphenol A, TBBPA).

**TBBPA is a high-affinity binder to TTR and prevents TTR-induced cell toxicity**

The Kd of the interaction between TBBPA and TTR was determined with ITC to be 20 nM, and thus it represents a high-affinity binder. We used a cell viability assay based on human SH-SY5Y neuroblastoma cells to determine TBBPA’s ability to suppress TTR-induced toxicity by preserving the integrity of the TTR tetramer. We included diflunisal and tafamidis, two ligands that previously have been studied for their ability to stabilize the tetramer, for comparison purposes. Incubation with only TTR led to a 65% reduction in cell survival, whereas addition of both TBBPA and diflunisal increased the cell survival to essentially that of the untreated cells. The addition of tafamidis increased cell survival but to a lower extent than TBBPA and diflunisal. (Figure 14).

![Figure 14. The inhibitory effect of the compounds on TTR-mediated cytotoxicity.](image)

TTR only or TTR with inhibitor was added to SH-SY5Y cells. Data represent the mean ± SD (n =3, ** p <0.01; *** p <0.001).
TBBPA is highly selective in human plasma

The ability to bind selectively to TTR in human plasma was probed with the urea denaturation assay. TBBPA is a highly selective drug with an IC$_{50}$ of 3.3 ± 0.5 µM. Assuming that the plasma concentration of TTR is 5 µM, this suggests that TBBPA stabilizes TTR in a nearly stoichiometric ratio (Figure 15). The highly selective drug tafamidis was included for comparison purposes and has an IC$_{50}$ of 4.8 ± 0.6 µM.

![Figure 15. Selective stabilization of TTR in plasma. A) TBBPA. B) Tafamidis.](image.png)

Having a selectivity in plasma in the range of tafamidis, the only approved drug for treatment of FAP, it is interesting to discuss some of the studies investigating the potential harmful effects of TBBPA. The European food and safety authority (EFSA) concluded that there were no indications of carcinogenicity and that the current dietary exposure was of no concern [158]. However, effects on liver, kidney and spleen weight have been observed in mice administered 500 and 1000 mg·kg$^{-1}$·day$^{-1}$ over the course of 90 days [159]. TBBPA also has a carcinogenic effect in rodents when administered 250, 500 or 1000 mg·kg$^{-1}$·day$^{-1}$ 5 days/week for 2 years [160]. Apart from its acute toxicity and having a potential carcinogenic effect at very high doses, it also has a low bioavailability, being effectively metabolized by glucuronyl- and sulfotransferases [161].

Taken together, we show that TBBPA is a highly selective ligand for TTR in human plasma, but due to its toxicity at high doses and low bioavailability it is not a suitable candidate for the treatment of FAP. However it does represent an interesting scaffold in the search for new and better drugs targeting TTR amyloidosis.
8.4 Paper IV – Transthyretin Impairs Nucleation in the Path of Amyloid β Fibril Formation

Anomalous aggregation of the Aβ peptide is strongly correlated to AD. A delicate balance controls the assembly and clearance of Aβ and determines whether aggregation will occur or not [162]. The homotetrameric transporter protein TTR has been found to interact with Aβ and prevent the association into amyloid fibrils [21]. It is however not clear how TTR interferes with the toxic process of amyloid fibril formation and here we aim to elucidate this mechanism further.

**TTR prolongs the lag phase and lowers the ThT signal at steady state of Aβ40 fibril formation**

The aggregation of Aβ follows a nucleation-dependent mechanism and this implies that the rate limiting step is the formation of a small assembly denoted nucleus [17]. The nucleus is formed during the initial regime, the lag phase, and this process is termed primary nucleation. When fibrils have formed, fibril-catalyzed secondary nucleation (FCSN), where nuclei catalyzed laterally along the fibril surfaces start to dominate the production of nuclei [18,163]. By using a ThT-based setup, we followed the conversion of Aβ40 into amyloid fibrils as a function of TTR (Figure 16).

![Figure 16. Spontaneous fibril formation of Aβ40 in the presence of TTR.](image)

The result shows that TTR both prolongs the lag phase and lowers the plateau at steady state. The increase in lag phase indicates that TTR is affecting the primary nucleation and the lowering of the plateau shows that TTR is able to interfere with Aβ’s assembly into an amyloid fibril. Also the rate of fibril formation is slightly lowered during the exponential phase as a function of TTR.
TTR does not interfere with fibril elongation

The change in slope during Aβ40 fibril formation as a function of increasing TTR concentration can have at least two different explanations; either TTR affects fibril elongation or FCSN. We investigated TTR’s effect on fibril elongation by using SPR. Aβ40 fibrils where immobilized on the chip and probed with either a flow of monomeric Aβ40 or a mixture of Aβ40 monomers and TTR. In the absence of TTR, the injection of monomers leads to a build-up of a fibril (Figure 17). Injection of monomers in the presence of five times molar excess of TTR does not reduce the response, indicating that TTR’s inhibiting mechanism on Aβ fibril formation during the exponential phase not is mediated through an interference with elongation but rather trough FCSN (Figure 17). The result also suggests that TTR’s affinity to the monomer is negligible low. This is supported by a recent study which determined a Kd of 24 µM for the interaction [121].

![Figure 17. Aβ40 elongation is not affected by TTR. Immobilized Aβ40 fibrils probed with either monomeric Aβ40 or monomeric Aβ40 in the presence of five times molar excess of TTR.](image)

TTR cannot be efficiently precipitated using mature Aβ40 fibrils

FCSN implies that the formation of Aβ nuclei is catalyzed laterally on the fibril surface. To test whether TTR inhibits the FCSN via direct binding to the surfaces of the fibril, and thereby blocking FCSN, we incubated TTR with an increasing concentration of mature Aβ fibrils for 2 h. This was followed by a centrifugation step to remove aggregated material and potentially bound TTR. Figure 18 shows the result, indicating no significant reduction in the concentration of TTR as a function of mature Aβ fibrils.
TTR converts $\alpha\beta_{40}$ into an alternative fold

In order to further probe the ability of TTR to modulate $\alpha\beta_{40}$ fibril formation, we investigated the ability of TTR to interfere with seeded reactions. The result in Figure 19A shows how TTR both increases the lag phase and lowers the plateau at steady state. The lowering of the plateau at steady state indicates that TTR either keeps $\alpha\beta$ soluble or has converted it into a non-amyloid form, unable to induce ThT fluorescence. To evaluate the aggregation status, we ultra-centrifuged the sample, separating monomeric and low molecular weight from larger aggregates. Figure 19B shows an SDS-PAGE analysis of the supernatant corresponding to the samples in Figure 19A and displays that TTR, in a concentration dependent manner, prevents $\alpha\beta$ assembly. At a concentration of 2 $\mu$M TTR, the $\alpha\beta$ monomeric fraction is almost undetectable although the ThT signal for the same sample (Figure 19A) at steady state corresponds to $\sim$10% of the control sample without TTR. This strongly suggests that the major part of the sample has acquired another form of large assembly, distinct from amyloid fibrils and unable to bind ThT. To investigate this possibility we employed a dot blot analysis with an oligomer specific antibody, mAB-M [164]. MAB-M recognizes the epitope Glu3-Ser8 on $\alpha\beta$ and, being oligomer specific, it is unable to bind the fibrillar forms. Figure 19C displays the result where both total (non-centrifuged samples) and supernatants are analyzed. The result clearly shows that the aggregates formed in the presence of TTR acquire an alternative form that readily reacts with the antibody.

Figure 18. Analysis of the supernatant after incubating 100 nM TTR with various concentrations of mature $\alpha\beta_{40}$ fibrils.
Figure 19. TTR impairs the assembly into an amyloid fold. 5 µM of monomeric Aβ forty seeded with 1% of fibrils in the presence of various concentrations of TTR. A) ThT analysis of the reaction. B) SDS-PAGE and silver staining analysis of the supernatant. C) Dot-blot analysis using mAB-M on the total sample or the supernatant.

To conclude, we show that TTR cannot interfere with Aβ fibril elongation and as a result is not efficiently binding to either the fibril ends or the monomers. Neither can we detect any binding to the lateral surfaces of the fibril. The mechanism of inhibition is instead mediated through an interference with the process of nucleation, including primary nucleation and FCSN. We further show that TTR converts Aβ to a high weight non-amyloidogenic form with a more open N-terminus.
9 Concluding remarks and outlook

TTR amyloidosis is a progressive, and in many cases a fatal disorder characterized by the extracellular deposition of TTR in various tissues. The current standard first-line treatment is liver transplantation, but this procedure is only viable for a small subset of the hereditary TTR amyloidosis patients. Small molecule stabilizers that bind and stabilize the tetrameric structure of TTR have shown promising results in clinical trials, and one drug is currently approved for the treatment of FAP. However, there is still a need for more effective drugs.

One important factor to consider when designing a drug is its selectivity, the drug should bind only to its target and nothing else. We show that the selectivities of small molecule TTR stabilizers in plasma correlates better with the binding enthalpy than with the binding strength. Our results points to the importance of a high enthalpic contribution to the Gibbs free energy of binding when designing a selective inhibitor of TTR amyloid fibril formation, and that the enthalpy is a better predictor of selectivity than binding affinity.

Another important factor to consider in drug design is the resistance of a drug against biotransformation, the drug needs to be stable enough to exert its effect. We previously showed that the flavonoid luteolin is a highly selective binder of TTR in human plasma. It is, however, not a suitable candidate as a TTR stabilizer due to its rapid biotransformation in vivo by UGT enzymes. In an attempt to prolong its half-life we synthesized two analogues in which a hydroxyl group of luteolin was substituted by a chlorine or a methoxy group. The analogues had an improved resistance against modification by the UGT enzymes. Unfortunately they had also lost a substantial amount of luteolin’s selectivity for TTR in plasma. The analogue in which the hydroxyl group was replaced by a methoxy group bound within the TBP in the opposite direction as luteolin, showing the remarkable ability of TTR’s TBP to accommodate different ligands.

Another potent TTR amyloid inhibitor is tetrabromobisphenol A (TBBPA), a flame retardant that only shows toxicity at very high doses. We show that it has high selectivity for TTR in plasma, even higher than tafamidis which is currently the only approved drug for TTR amyloidosis. Its low bioavailability however impairs its usage as a therapeutic drug for TTR amyloidosis treatment.

For a long time the main functions of TTR was considered to already be discovered. Its role as a transporter protein for T₄ and holo-RBP has been well described and even its name is derived from these two abilities. However, the discovery of TTR’s ability to inhibit Aβ amyloid fibril formation in CSF has revealed a new important role for TTR. With our study of the interaction between TTR and Aβ, we elucidated
the mechanisms by which TTR perturb the Aβ fibril formation process. We showed that TTR cannot interfere with the process of fibril elongation and as a result cannot efficiently bind the monomers or fibrils of Aβ. Instead it targets nucleation, both primary and FCSN. We also revealed how TTR converts Aβ into high weight aggregates with an open N-terminus. In the light of an in vivo perspective, it makes sense that TTR targets the most toxic species; the low molecular weight aggregates, and not the monomer or the fibrils.

With the relatively recent discovery of the involvement of TTR in the inhibition of Aβ amyloid formation, it is clear that the functions of TTR need to be examined more closely in the future. One question to be resolved is why we have so much of the protein in plasma that is unoccupied by both T4 and holo-RBP. In fact, the majority of TTR is in its apo form. Why would it be favorable for the body to produce so much extra protein without any obvious task?

Another intriguing question is the large variation in penetrance of the V30M FAP mutation between countries, with Sweden having the lowest penetrance with only 11% of the carriers developing the disease by the age of 50 compared to Portuguese carriers where 80% develop the disease by the age 50 [165]. This indicates that something other than TTR is affecting the disease course, and the mutation might not be the sole cause of the disease, although it is a requirement. It is clear that there is a need for more research in order to understand TTR amyloidosis as a disease – perhaps not all of the blame can be put on TTR.
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