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Characterizing the effect of transthyretin amyloid on the heart

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Boston University
CHARACTERIZING THE EFFECT OF TRANSTHYRETIN AMYLOID ON THE HEART

by

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DEDICATION

I would like to dedicate this work to my parents Justus and Yuko, and my grandparents Yoshiko, Yozo, Otto, Julia, Lothar, and Martha. Thank you for all the support and inspiration.
ACKNOWLEDGMENTS

I would like to acknowledge everyone who has helped me these past six years. First and foremost I would like to thank Lawreen Connors for her mentorship, advice, and guidance throughout my time here as a PhD student. Evan Chiswick, Elena Klimtchuk, Amy Leung, and Hai-li Cui contributed their time and expertise in the Immuno-assay development (Evan) Circular dichroism (Elena) iPS cell (Amy) and immunohistochemistry (Hai-li) experiments. I also am extremely grateful for all the advice and input I received from everyone in the Amyloidosis Center. The Pathology Department has been an incredible support system as well, and I would particularly like to thank Debbie Kiley. I would also like to thank the members of my committee, Daniel Remick (chair), Lawreen Connors (first reader), George Murphy (second reader), John Berk, and Carl O’Hara, for their support and insightful discussion of my work throughout all these years. In addition to the many people who supported me at BU, I also must thank the rest of my support system both stateside, as well as back home in Amsterdam. Last, but not least, I would like to thank my family and in particular my parents, Yuko and Justus, for always supporting me.
CHARACTERIZING THE EFFECT OF TRANSTHYRETIN AMYLOID ON THE HEART

CLARISSA M. KOCH

Boston University School of Medicine 2015

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ABSTRACT

Transthyretin (TTR)-associated amyloidoses are diseases wherein wild-type or mutant TTR forms amyloid fibrils that infiltrate multiple organs. Wild-type TTR amyloidosis, ATTRwt, is a sporadic disease characterized by deposits that occur mainly in the heart. Alternatively, >100 TTR mutants cause inherited forms, ATTRm, frequently featuring cardiac amyloid deposits.

The goals of this research were to create a cell-based model of ATTR amyloidosis, to define the mechanism of cardiac TTR-associated amyloid at the cellular level, and to study several agents that could interrupt the amyloid process. We hypothesized that TTR oligomers were cardiotoxic and played a role in the mechanism of ATTR amyloidosis, and that cytotoxicity could be inhibited by diflunisal, doxycycline, and Kiacta®. Focusing on TTR proteins associated with cardiac amyloidosis (wild-type, L55P, V30A, and V122), we developed a thermal denaturation method for creating TTR oligomers that allowed us to study the direct effect of oligomers on cells. Congo red and thioflavin T analyses confirmed that the oligomers were on pathway to amyloid fibril formation. We tested the effect of TTR oligomers on rat and human cardiac cells by measuring cell viability and stress response (through live protease activity and qPCR).
TTR-L55P oligomers elicited a cytotoxic effect; fluorescent microscopy indicated cellular uptake of the oligomers and continued intra-cellular aggregation. Cytotoxicity was blocked when TTR was heated in the presence of doxycycline; the drug appeared to dissociate TTR aggregates or stabilize the monomeric forms. We also investigated retinol-binding protein (RBP), a natural binding partner of TTR. By immuno-histochemistry, RBP was demonstrated in ATTRwt and ATTRm ‘non-amyloid’ transplant heart tissues, localized to areas containing amyloid or in the case of the transplant tissue, regions that appeared to display ischemic damage. Serum RBP levels were significantly different in ATTR vs. age-matched controls (p = 0.03), and in ATTRwt vs. ATTRm (p <0.0001) by ELISA.

These data provide evidence that TTR oligomers are cardiotoxic, possibly due to cellular internalization and progressive intracellular aggregation. Furthermore, our results support the use of doxycycline as a therapeutic in ATTR to target these amyloidogenic oligomers, and suggest that RBP may have potential as a disease biomarker.
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<tr>
<td>°C</td>
<td>Degrees Centigrade</td>
</tr>
<tr>
<td>µM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>A</td>
<td>Alanine</td>
</tr>
<tr>
<td>AA</td>
<td>Amyloid A or secondary amyloidosis</td>
</tr>
<tr>
<td>ActA</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>AL</td>
<td>Immunoglobulin light chain or primary amyloidosis</td>
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<tr>
<td>ApoE</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>ATTR</td>
<td>TTR-associated amyloidosis</td>
</tr>
<tr>
<td>ATTRm</td>
<td>TTR-associated amyloidosis due to mutant protein</td>
</tr>
<tr>
<td>ATTRwt</td>
<td>TTR-associated amyloidosis due to wild-type protein</td>
</tr>
<tr>
<td>Aβ</td>
<td>Amyloid beta protein</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>BMP4</td>
<td>Bone morphogenetic protein 4</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
</tr>
<tr>
<td>cEB</td>
<td>Cardiac embroind body</td>
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<tr>
<td>CMP</td>
<td>Cardiomyopathy</td>
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<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
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<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CTS</td>
<td>Carpal tunnel syndrome</td>
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<td>Da</td>
<td>Dalton</td>
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</table>
dH₂O ................................................................. Distilled water
DMEM .............................................................. Dulbecco’s modified Eagle’s medium
E.coli ................................................................. Escherichia coli
ELISA ............................................................... Enzyme-linked immunosorbent assay
ER ................................................................. Endoplasmatic reticulum
ESC ................................................................. Embryonic stem cell
FAC ................................................................. Familial amyloid cardiomyopathy
FAP ................................................................. Familial amyloid polyneuropathy
FBS ................................................................. Fetal bovine serum
FITC ............................................................... Fluorescein isothiocyanate
GAG ................................................................. Glycosaminoglycan
GF-AFC ........................................................... Glycyl-phenylalanyl-amino-fluorocoumerin
H ................................................................. Histidin
H₂O ................................................................. Water
HCl ................................................................. Hydrogen chloride
HRP ............................................................... Horseradish peroxidase
I ................................................................. Isoleucine
IEF ............................................................... Isoelecctric focusing
IMAC ............................................................ Immobilized metal ion affinity chromatography
iPSC ............................................................. Induced pluripotent stem cell
IPTG ............................................................... Isopropyl β-D-1-thiogalactopyranoside
kD ................................................................. Kilodalton
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>L</td>
<td>Leucine</td>
</tr>
<tr>
<td>L</td>
<td>Liter</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny broth</td>
</tr>
<tr>
<td>M</td>
<td>Methionine</td>
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<tr>
<td>M</td>
<td>Molar</td>
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<tr>
<td>mg</td>
<td>Milligram</td>
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<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular weight cut-off</td>
</tr>
<tr>
<td>NaBH</td>
<td>Sodium borohydride</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NEAA</td>
<td>Non-essential amino acids</td>
</tr>
<tr>
<td>ng</td>
<td>Nanogram</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drug</td>
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<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>P</td>
<td>Proline</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
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<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>RBP</td>
<td>Retinol-binding protein</td>
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<tr>
<td>RhoK</td>
<td>Rho-associated kinase</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>rTTR</td>
<td>Recombinant transthyretin</td>
</tr>
<tr>
<td>SCA</td>
<td>Senile cardiac amyloidosis</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<tr>
<td>SSA</td>
<td>Senile systemic amyloidosis</td>
</tr>
<tr>
<td>ThT</td>
<td>Thioflavin T</td>
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<tr>
<td>TTR</td>
<td>Transthyretin</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
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<tr>
<td>V</td>
<td>Valine</td>
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CHAPTER 1. INTRODUCTION

1.1 Protein misfolding and amyloid formation

Proteins are key in the proper functioning of every system in our body; correct tertiary and quaternary folding of these biomolecules is therefore of vital importance. Many normal mechanisms and checkpoints are in place to monitor and ensure that proteins in the human body are present in native form and thus, properly functional. The correct conformational state of a protein is achieved through a multistep folding mechanism that is mediated by molecular chaperones. Chaperones facilitate and maintain protein native structure and are usually able to reverse incorrect or non-native folding.

When a protein adopts an irreversible non-native conformation, it is removed by the cell through autophagy or proteosomal degradation[1], [2]. Aberrantly folded proteins can accumulate in the cellular environment when degradative pathways are impaired or overwhelmed. Failure to remove misfolded proteins can have devastating consequences at the cellular and eventually tissue/organ levels; increased levels of non-native proteins can lead to self-association of the misfolded subunits, subsequent development of soluble oligomers, generation of large molecular aggregates, and ultimately the formation and deposition of insoluble amyloid fibrils (Figure 1).

Amyloid fibrils are non-branching, of indeterminate length, and rich in beta-sheet structure. More than 25 proteins can form the backbone of amyloid fibrils[3]. Despite the sequence heterogeneity of amyloid proteins and amorphous appearance of the deposits by light microscopy, all amyloid fibrils exhibit a highly organized ultrastructure.
It is this ordered characteristic of the fibrils that imparts the unique, but consistent, dye-binding properties used in the detection and identification of amyloid in tissue. One such special stain is Congo red[4]; amyloid fibrils bind to Congo red and display a salmon color when observed by standard light microscopy, and a unique apple green birefringence when viewed under polarized light[5] as demonstrated in Figure 2. This histopathologic testing with Congo red is considered to be the gold standard for diagnosing all forms of amyloid disease[6]. In addition to Congo red, Thioflavin-T has also become a common tool to detect amyloid used more widely in laboratory settings[7], [8].
Figure 1. Protein misfolding, degradation, and aggregation schema.

The transition from the native to misfolded state of a protein can be reversed by chaperones. When the native state cannot be preserved, misfolded proteins can be degraded through autophagy or proteasomal degradation. If the degradation pathway fails, misfolded proteins will accumulate, self-associate, and progress to form larger oligomers that ultimately deposit as amyloid fibrils in tissue. Reproduced with permission from Nature Publishing Group (Irwin et al.[2]).
1.2 Amyloidosis

Amyloid fibrils can deposit in many different organs and tissue types; depending on the amyloid protein and target tissue, the deposits will result in a variety of pathologies collectively known as the amyloid diseases or amyloidoses. Currently, approximately 30 amyloid proteins[9] have been identified, all capable of forming amyloid fibrils through a protein misfolding mechanism, but leading to very distinct pathophysiologies[10]. Some of the most well-known amyloidoses are Alzheimer’s disease (AD), Parkinson’s disease (PD), and Huntington’s disease (HD)[11], [12](Table 1). These diseases may be considered localized forms of amyloidosis characterized by amyloid deposition exclusively in one organ. In Alzheimer’s disease, for instance, Aβ protein plaques in the brain are the pathological hallmark associated with dementia and neurodegeneration in patients[13].

In addition to the localized forms, there are the systemic amyloidoses in which multiple organs can be affected. The most common systemic amyloidosis is immunoglobulin light chain or primary amyloidosis (AL), in which the amyloid is derived from the immunoglobulin light chain. In AL amyloidosis, the most frequently affected organs are the heart, kidney, liver, and peripheral and autonomic nervous systems. This multiorgan involvement results in a vast array of clinical features including cardiomyopathy, proteinuria, neuropathy, and hepatomegaly[14]. It is estimated that AL affects one in every 100,000 individuals in Western countries[15]. Other types of systemic amyloidosis include both acquired and inherited forms usually associated with one of the plasma proteins. We are particularly interested in the group of
systemic amyloid diseases linked to the plasma protein, transthyretin (TTR). The TTR-associated systemic amyloidoses are the focus of this dissertation and a further description will follow. While these diverse pathologies with unique clinical profiles are caused by proteins with varying structures and functions, they are all characterized by an identical process that involves protein aggregation and amyloid fibrillogensis. The formation of amyloid can occur, regardless of the source protein native structure, and interestingly even the soluble oligomers display the same distinct amyloid structural conformations[16] composed of beta-pleated sheet and anti-parallel fibrillar structures.

Though the presence of fibrillar amyloid deposits is well-established as a hallmark feature of all amyloid diseases, the extent to which these deposits cause such diverse clinical characteristics in these pathologies remains unclear and controversial. Though it was initially thought that the deposits were the main cause of disease, it is now believed that soluble amyloid protein oligomers[10], [12], [17] and pre-fibrillar species may be toxic entities that lead to disease presentation and progression in amyloidosis[18]. In 2005, Cleary et al. were the first to show that soluble oligomers of Aβ disrupted cognitive function in a rat model[19] and in 2008, Sörgjerd et al. demonstrated in cell-based studies that pre-fibrillar transthyretin (TTR) oligomers were toxic to neuroblastoma cells (SH-SY5Y), whereas mature amyloid fibrils were not[20].
Table 1. Proteins involved in systemic and localized amyloid diseases.


<table>
<thead>
<tr>
<th>Protein name</th>
<th>Abbreviation</th>
<th>Related pathology</th>
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<tr>
<td>Amyloid β peptide</td>
<td>Aβ</td>
<td>Alzheimer Disease; cerebral amyloid angiopathy</td>
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<tr>
<td></td>
<td></td>
<td>APP-related</td>
</tr>
<tr>
<td>Tau protein</td>
<td>MAPT</td>
<td>Alzheimer Disease; frontotemporal dementia; pick disease of the brain; progressive</td>
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<td>Dialysis-related amyloidosis</td>
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<td>AR/NR3C4</td>
<td>Spinal and bulbar muscular atrophy/Kennedy's disease</td>
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Figure 2. Amyloid deposits demonstrated by Congo red histology.

Light microscopic images demonstrating the presence of amyloid in heart tissue. (a) Congo red binds to the amyloid deposits as shown by the red staining, with the corresponding signature (b) apple green birefringence observed under polarized light. Images were taken at 10x magnification; scale bar represents 100 µm.
1.3 Transthyretin

One protein that can undergo protein misfolding and transition to amyloid fibril formation is transthyretin (TTR). Previously known as prealbumin, TTR is a 55 kD, homotetrameric protein mainly synthesized in the liver and choroid plexus; each of the four TTR subunits spans 127 amino acids in length[22], [23]. The protein is extremely stable structurally, a characteristic attributed to its rich beta-sheet content[24], and a sequence that has been highly conserved across vertebrate species[25]. In humans, TTR is mainly found in plasma and cerebrospinal fluid (CSF) at concentrations of 200-400 µg/mL and 10-40 µg/mL, respectively[26], [27]. TTR functions as a transport protein for thyroxine (T4), a thyroid hormone, and retinol (Vitamin A) which is carried as a ternary complex with retinol-binding protein (RBP)[23]. T4 is carried in one of two binding pockets formed at the dimer-dimer interface of tetrameric TTR. The T4 binding pockets have been shown to also have binding affinities for other small molecules such as diflunisal; diflunisal is a repurposed drug recently studied in an international clinical trial of patients with inherited forms of TTR-associated amyloidosis[28]. In addition, there are two sites on TTR for association with retinol-binding protein (RBP). Figure 7 shows the TTR-RBP₂ complex, also known as the transthyretin-retinol-binding protein complex. TTR binding sites for T4 and retinol-binding protein are separate and non-overlapping (Figure 3)[29].

More than 100 point mutations in coding regions of the TTR gene have been reported and the majority are associated with inherited forms of amyloidosis[23]. Previously known as familial amyloid polyneuropathy (FAP) or familial amyloid...
cardiomyopathy (FAC) depending on the clinical phenotype, these diseases are now collectively referred to as mutant transthyretin amyloidosis (ATTRm)[30]. In addition to variant forms, wild-type TTR can also be amyloidogenic, i.e. form amyloid deposits; the amyloid disease associated with wild-type transthyretin amyloidosis (ATTRwt) has previously been referred to as age-related, senile cardiac (SCA) and senile systemic amyloidosis (SSA)[31], [32].
Figure 3. X-ray crystal structure of the transthyretin tetramer.

Ribbon diagram of the transthyretin (TTR) tetramer. The tetramer is composed of four identical monomers, each 127 amino acids in length. There are two thyroxine (T4) binding pockets, one between each dimer-dimer interface and indicated by the arrows in the figure. Source [http://www.ott.csic.es/rdcsic/rdcsicsp/rdqu42esp.htm](http://www.ott.csic.es/rdcsic/rdcsicsp/rdqu42esp.htm).
1.4 Transthyretin-associated amyloidosis

The transthyretin-associated amyloidoses are systemic diseases in which wild-type or a variant form of TTR becomes destabilized and forms amyloid fibrils that can infiltrate multiple organs. Wild-type TTR amyloidosis, ATTRwt, causes a sporadic pathology characterized by deposits that occur mainly in the extracellular compartment of the heart. In addition, over 100 point mutations in the TTR gene are responsible for a vast array of familial pathologies, ATTRm, that affect many different tissue systems[33], [34]. It is generally believed that all ATTR diseases share a common mechanism of amyloidogenesis. Research studies suggest that the pathway begins with dissociation of the normally stable TTR tetramer into monomeric subunits; tetramer dissociation is thought to be the rate-limiting step for TTR amyloid fibril formation. Following tetramer dissociation, it is believed that the individual TTR monomers become misfolded, i.e. the subunits adopt a non-native conformation, and this leads to subunit association, formation of oligomeric and pre-fibrillar aggregates, and eventually insoluble amyloid fibrils[8].

ATTRm amyloidosis

There currently are over one hundred point mutations that have been reported in the TTR gene, most of which are amyloidogenic and lead to symptomatic amyloid disease[23]; these pathologies are designated as mutant TTR-associated (ATTRm) amyloidosis. Clinical presentation and age of onset in ATTRm amyloidosis varies based on the underlying TTR mutation. Certain amyloidogenic TTR variants commonly feature neuropathic involvement while others are linked to cardiomyopathy as the main clinical presentation (Figure 4). In the past, these ATTRm diseases were termed familial...
amyloid polyneuropathy (FAP) and familial amyloid cardiomyopathy (FAC), based on clinical phenotype[35]–[37]; this nomenclature is still used occasionally in the literature[38].

Kinetic and biophysical studies have shown that severity of disease and earlier age of onset correlate with the instability of the pathologic TTR variant protein[39], [40]. ATTRm amyloidosis featuring presentation of symptoms at a younger age and a more aggressive progression of disease appear to be caused by TTR variants that, in vitro, are biochemically unstable and show an increased propensity to form amyloid fibrils[23], [41]–[45]. Examples demonstrating this phenomenon are TTR-L55P, -V30A, and -V122I (Table 2). L55P, considered one of the most amyloidogenic of all TTR variants, and V30A are both associated with early disease onsets that occur in the early 20s and 30s, respectively. V122I features symptom development around age 60 or beyond[40], [46]. These three TTR variants, detailed further in the paragraphs below, were used in many of the studies presented in this thesis.

The L55P point mutation results in the replacement of a leucine (L) residue by proline (P) at N-terminal amino acid residue 55 of the protein; this mutation is considered to be one of the most lethal, giving rise to a highly destabilized TTR protein[47]. Patients with the L55P TTR variant develop symptoms much earlier than most other forms of ATTRm and disease progression is more aggressive. Clinical features include both cardiomyopathy and neuropathy (Table 2). It has been demonstrated that this particular amino acid substitution leads to a significant decrease in tetramer stability and an increased tendency of L55P to form amyloidogenic species[45]. Moreover, Yang et al.
showed that the L55P monomer is significantly destabilized in comparison to wild-type monomers[46].

The substitution of an alanine (A) for a valine (V) residue at position 30 in the TTR protein was first described in 1992[34]. In patients with this amyloidogenic mutation, there is extensive amyloid deposition in various organs including the liver, peripheral nerves, and pancreas; a relatively young age of onset is also featured in ATTR-V30A (Table 1)[33]. Similar to the TTR-L55P variant previously described, the TTR-V30A mutant has been shown to be kinetically destabilized and this results in a TTR tetramer that can dissociate to form amyloid fibrils more easily compared to the wild-type protein[48].

First reported by Gorevic et al. in 1989[31], V122I results from a point mutation in exon 4 of the TTR gene and involves a substitution of isoleucine (I) for valine (V) at N-terminal residue 122 [23] of the protein. This amino acid replacement has been shown to decrease the energy of activation in the rate-liming step of tetramer dissociation, leading to a higher amyloid fibril formation rate in vitro[49]. In 2006, it was reported that about 3% of the African American population carry the V122I mutation in the TTR gene[50]. In addition, Jacobson et al.[40] found that, in their cohort of African Americans presenting with cardiac amyloidosis, 23% had the V122I point mutation; moreover, the authors of this study reported that the risk for cardiac amyloid in African Americans over the age of 60 was four times greater than Caucasians.

Patients with the V122I point mutation present predominantly with cardiomyopathy (Table 2) due to pervasive amyloid infiltration of the heart[40].
Consequently, disease treatment is aimed mainly at managing the symptoms of cardiac impairment. If eligible, patients may receive a heart transplant or combined heart and liver transplantation[36]. Median survival in untreated patients with ATTRm due to V122I is 27 months [51].

Current treatment options for ATTRm include liver transplantation, removing the source of the aberrant TTR protein, as well as small molecule drug treatments. Several of these small molecule therapeutics are currently in clinical trials treating ATTRm patients and preliminary results are promising[28], [52], [53].
Figure 4. Phenotype of ATTRm amyloidosis mutations.

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Table 2. Clinical phenotype and ages of onset associated with amyloidogenic forms of transthyretin (TTR).

<table>
<thead>
<tr>
<th>TTR variant</th>
<th>Phenotype</th>
<th>Age of onset (years)</th>
</tr>
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<tbody>
<tr>
<td>wt</td>
<td>Cardiomyopathy</td>
<td>&gt;60</td>
</tr>
<tr>
<td>L55P</td>
<td>Cardiomyopathy, Peripheral neuropathy,</td>
<td>&lt;30</td>
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<tr>
<td></td>
<td>Autonomic neuropathy</td>
<td></td>
</tr>
<tr>
<td>V122I</td>
<td>Cardiomyopathy</td>
<td>&gt;60</td>
</tr>
<tr>
<td>V30A</td>
<td>Cardiomyopathy, Autonomic neuropathy</td>
<td>30-40</td>
</tr>
</tbody>
</table>
In addition to the many TTR variants that can form amyloid and lead to systemic amyloidosis, the wild-type TTR protein can also misfold and cause disease despite the absence of a gene mutation[54]. The disease associated with wild-type TTR is referred to as ATTR wild-type amyloidosis, or ATTRwt, which until recently was known as senile systemic amyloidosis (SSA)[55]. Initially, the disease was considered a localized form of amyloidosis which exclusively featured heart involvement and was thus, originally termed senile cardiac amyloidosis (SCA). In the early 1980s, Pitkanen et al. first described a systemic distribution of amyloid deposits that occurred in multiple organs throughout the body; the term senile systemic amyloidosis (SSA)[55] was coined. The authors reported that histological screening of tissues from patients with SSA showed amyloid deposits in the lung, liver, and kidney even though the majority of cases demonstrated that the heart was extensively infiltrated.

ATTRwt amyloidosis is an irreversible and life-threatening late onset disease with a median survival of less than 5 years[56]–[58]. The disease has been reported almost exclusively in men above the age of 60 years[57], [59], [60]. Cardiomyopathy is the predominant clinical feature in ATTRwt (Figure 4)[57] and patients frequently present with symptoms of congestive heart failure at time of diagnosis. Echocardiographic evaluation usually reveals thickening of the left ventricular wall and/or valve, and an increase in the interventricular septal diameter. Atrial fibrillation is also a common characteristic[59], [61], and almost half of patients present with carpal tunnel syndrome (CTS)[57].
While once considered a rare disease, several reports have recently suggested that ATTRwt amyloidosis may be largely unrecognized and therefore, under-diagnosed. The prevalence of the disease is unknown, but may be higher than previously appreciated[62]. In fact, it is now believed that 25% of elderly men over the age of 80 have cardiac amyloid deposits composed of wild-type TTR[32].

Unfortunately, there is no cure currently available for patients with ATTRwt amyloidosis; treatment involves management of symptoms and palliative care. The absence of treatments or therapeutic options for individuals afflicted with this disease represents a major unmet need for this patient population. In addition, there is an urgent necessity for biomarkers of disease and indicators of progression; the diagnosis of ATTRwt amyloidosis is particularly challenging due to the fact that the amyloidogenic protein lacks any mutations or detectable abnormalities.

1.5 Transthyretin and the heart

The cardiac amyloidoses are the leading cause of infiltrative cardiomyopathy (CMP)[63] and can be divided into two types: immunoglobulin light chain or primary amyloidosis (AL) and the TTR-associated amyloidoses (ATTRm and ATTRwt). Both AL and ATTR amyloidosis are confirmed by Congo red staining of a biopsy from the affected organ and identification of the amyloid deposited protein is accomplished by immunochemical or mass spectrometric analyses. In cases of ATTRm amyloidosis, serum screening for the variant TTR protein can be achieved using isoelectric focusing (IEF) which has 96% sensitivity and 100% specificity; the presence and identity of the TTR gene mutation are determined using direct DNA sequence analysis[64]. For
ATTRwt, the diagnosis is more challenging; criteria include ruling out the presence of AL and ATTRm amyloidosis, and proof that wild-type TTR is the major component of the amyloid fibrils usually through mass spectrometric testing.

The occurrence of TTR amyloid deposits severely impacts the structure and proper functioning of the heart (Figure 5). Echocardiographic imaging in patients with ATTR amyloidosis frequently shows an increased interventricular septal thickness (IVST) in the absence of hypertension[59]. Patients also present with systolic dysfunction, lower left ventricular ejection fraction (LVEF) and a high frequency of intracardiac thrombosis[65], [66]. By electrocardiography, low-QRS voltage and arrhythmia[67] are commonly observed; laboratory values of brain natriuretic peptide (BNP) and cardiac troponin (c-Tn I) are elevated and seem to correlate with decreased survival although the exact relationship remains to be determined[58].
Figure 5. Progression of ATTR cardiac amyloid disease parameters.

As cardiac ATTR amyloidosis progresses, BNP and troponin levels rise whereas ejection fraction (EF) and functional status of the heart quickly fall. Reproduced with permission from Springer Science and Business Publishing (Castano et al., 2004).
1.6 Current treatment and alternative strategies

Treatment options for patients with TTR amyloidosis have been limited and are focused on either controlling the symptoms or interrupting the process of amyloid deposition in target organs. The management of symptoms can range from painkillers and diuretics to prophylactic pacemaker placement in patients with CMP. Orthotopic liver transplantation (OLT), used to treat ATTRm amyloidosis, is aimed at halting or slowing amyloid infiltration by removing the source of the variant TTR protein[68]. In addition, if the heart is severely affected, a patient may also receive an orthotopic heart transplant (OHT) or in some cases a combination of both OLT and OHT.

It is important to note that several groups have reported signs of renewed cardiac infiltration in ATTR patients after liver transplantation[69], [70] and the development of amyloid in domino liver recipients much more rapidly than anticipated. Okamoto et al. described continued development of arrhythmia in ATTRm patients following OLT; the authors reported that OLT did not prevent the development of heart arrhythmia in their cohort of FAP and in most cases pacemaker insertion was necessary[71]. Olofsson et al. also reported a progression of CMP after liver transplantation in their study of Portuguese patients with FAP[72]. The probability of survival, 5 years after OLT, was 77%[70]; survival, 2 years after OHT, was 67% in ATTRm and 100% in ATTRwt patients.

While liver transplantation is aimed at slowing disease progression by removal of the amyloidogenic protein, an alternative therapeutic strategy seeks to halt the amyloid process by stabilizing the native tetrameric form of circulating TTR. The mechanism of TTR amyloid formation is widely believed to feature an initial tetramer dissociation step.
Like thyroxine, compounds that bind to TTR in regions of the dimer-dimer interface could potentially block the propensity of TTR to be amyloidogenic. Several compounds that reportedly act as TTR tetramer stabilizers are tafamidis, epigallocatechin gallate (EGCG), and diflunisal[41], [52], [73]–[75]. Tafamidis, 1-(3,5-dichlorophenyl)-1,3-benzoxazole-6-carboxylic acid, is a drug designed specifically to bind TTR in the thyroxine-binding clefts and stabilize the tetrameric form of the protein[73]. Clinical trials of tafamidis have shown effective in vivo maintenance of native, serum circulating TTR in patients with FAP; however, data from these studies also indicated that the drug failed to halt disease progression as worsening of neurologic functions occurred in the treatment groups[52], [53]. The long-term tolerability and efficacy of tafamidis have yet to be determined and a Phase III trial by Pfizer is currently underway. EGCG is an abundant catechin found in green tea that has been shown to bind TTR and prevent aggregation in vitro[74]. Diflunisal is a salicylic acid derivative that is classified as a non-steroidal, anti-inflammatory drug (NSAID). The ability of diflunisal to act as a TTR tetramer stabilizer was studied as part of the research presented in this thesis and a further description of the drug will follow.

Another approach to treating patients with ATTR amyloidosis targets the amyloid fibrils that constitute the deposits. Doxycycline, tauroursodeoxycholic acid (TUDCA), and EGCG are being investigated for their potential to degrade amyloid fibrils. The combination of doxycycline and TUDCA, an anti-oxidant, has been shown to lower TTR amyloid deposition in mice[76]. Moreover, in addition to stabilizing tetrameric TTR,
EGCG also appears to be capable of digesting preformed TTR amyloid fibrils in vitro[77].

Since both wild-type and variant forms of TTR are amyloidogenic, it seems likely that factors extrinsic to TTR structural features, e.g. other plasma circulating or tissue specific proteins, may be involved in the formation of amyloid fibrils and deposits. The extracellular chaperone, clusterin, is one example of an extrinsic factor that has recently been implicated in ATTR amyloidosis pathogenesis[75], [78]. Research into understanding the potential roles of such extrinsic factors and the protective effects of natural binding partners of TTR are of interest as results of these studies could lead to discovery or design of anti-amyloid agents.

1.7 Potential therapeutics

Currently, there are a number of research initiatives aimed at developing an efficacious treatment for TTR-associated forms of amyloidosis. The treatment strategies are varied and target different steps in the process of TTR amyloid formation. Agents that bind to TTR in the thyroxine pocket, stabilize tetrameric TTR and thus, inhibit amyloid oligomer and fibril formation[79]. Diflunisal and tafamidis are examples of this approach[80]. Alternatively, fibril degradation has been explored as a means of ridding the organ of damaging amyloid deposits; doxycycline is a drug with reported fibril disrupting activity. In addition, heparan sulfate proteoglycans, commonly found in amyloid deposits, have been the focus of anti-amyloid agent development. Specifically, sulfated glycosaminoglycans (GAGs) have been targeted for their role in amyloidosis as they enhance amyloid deposition. Compounds such as Kiacta®, are being tested as small
molecules that compete with GAGs to bind amyloid proteins and thus, prevent amyloid infiltration[81]. Diflunisal, doxycycline, and Kiacta® were used in our studies and are detailed further in subsequent paragraphs.

As mentioned, diflunisal (Figure 6a) is a salicylic acid derivative classified as a non-steroidal anti-inflammatory drug (NSAID). Diflunisal was previously shown in biophysical studies to stabilize the native state of TTR and slow amyloid fibril formation[75], [82]. In addition, efficacy of the drug was reported to be directly related to the relative thermodynamic stabilities of the various TTR mutants[83]. Under the leadership of Dr. John Berk at the Boston University Amyloidosis Center, a two-year, multicenter, international, randomized, placebo-controlled clinical trial studying the effect of diflunisal in patients with ATTRm amyloidosis has been conducted; findings from the study demonstrated that diflunisal, a repurposed drug, significantly reduced disease progression in a cohort of FAP patients[28].

Doxycycline, α-6-deoxy-oxytetracycline (Figure 6b), is a tetracycline antibiotic which was originally produced to fight bacterial infections such as Staphylococcus and Enterococcus[84]. In recent years, however, repurposing of the antibiotic has led to studies demonstrating an ability of the drug to disrupt fibrils in FAP mouse models[76], [85], [86]. Moreover, several groups have reported that doxycycline is capable of attenuating protein aggregation in an αB-crystallin cardiac proteinopathy mouse model[80] and reducing fibril formation in an AL amyloidosis mouse model[87].

Kiacta® (Figure 6c) is a glycosaminoglycan mimetic also known by the generic name, eprodisate. Originally, the drug was proposed as a potential anti-amyloid
compound to treat amyloid A (AA) or secondary amyloidosis by Kisilevsky et al.[88].
Kiacta® is believed to act as a glycosaminoglycan (GAG) mimetic, specifically
competing with the GAG structures in amyloid deposits and preventing further deposition
of amyloidogenic material. This drug is currently being studied in an ongoing, multi-
center, international clinical trial as a potential treatment to arrest renal function decline
in AA amyloidosis (NCT01215747).
Figure 6. Chemical structures of potential small molecule therapeutics.
(a) Diflunisal (2’,4’-difluoro-4-hydroxybiphenyl-3-carboxylic acid), (b) doxycycline (α-6-deoxy-oxytetracycline), and (c) Kiacta®, also known as eprodisate (disodium 1,3-propanedisulfonate).
1.8 Retinol-binding protein

A natural binding partner of TTR, retinol-binding protein (RBP) is a 21 kD monomeric protein that exists as one of five isoforms. RBP4 is the most abundant and the only secreted isoform; it circulates in the bloodstream and binds approximately 50% of all circulating TTR at a 1:1 molar ratio[29]. The binding of TTR to RBP4 occurs at the C-terminus of the RBP molecule through non-covalent interactions[89]. There are two potential RBP binding sites on TTR and while the stoichiometry of the TTR-RBP complex in circulation is believed to be 1:1, some in vitro studies have demonstrated the formation of a 1:2 species with both available sites on TTR occupied (Figure 7)[90], [91]. However, in this latter situation, the binding of the first RBP molecule appears to have a negative cooperative effect; the affinity of the TTR tetramer for binding a second RBP molecule was shown to be significantly lower[92], [93].

RBP4 functions as the only carrier of all-trans retinol (Vitamin A) in the circulation; transport of the vitamin is from the liver, where both RBP4 and TTR are synthesized [16], to target tissues throughout the body. When bound to retinol, the RBP molecule is referred to as holo-RBP, whereas the unbound form is termed apoRBP [29]. Retinol is an extremely hydrophobic molecule and thus, association with RBP occurs within the beta barrel structure of the RBP molecule[89], [94]. The RBP4-TTR-retinol triad is known as the retinol transport complex[95]. All components in the retinol transport complex benefit from molecular interactions with these partners. The binding of retinol to RBP4 gives RBP4 a higher binding affinity for TTR[95]. TTR binding to RBP4 prevents glomerular filtration of RBP4 by the kidneys[96]. RBP4 binding to TTR
in turn significantly stabilizes the native tetramic state of TTR[29]. A paper by Kelly and White in 2001[97] showed that RBP binds both wild-type and L55P TTR *in vitro* and stabilized the tetrameric form of TTR. Thus, RBP appeared to prevent disassembly of TTR into monomers, the key step in the TTR fibril formation pathway. In addition, the authors showed that the presence of RBP slowed down amyloid fibril formation *in vitro* and enhanced small molecule inhibitor activity of flufenamic acid and diclofenac.
Figure 7. Retinol-binding protein–transthyretin (RBP-TTR) complex.

The ribbon diagram shows the TTR tetramer (blue) bound to two RBP molecules (red).

Each RBP molecule has one retinol molecule (yellow) bound inside its beta barrel structure. White JT et al., PNAS (2001)[97]. Copyright (2001), National Academy of Sciences, U.S.A.
1.9 Research goal, hypothesis, and specific aims

The goal of the research presented in this dissertation was to further understand the pathologic effect of soluble amyloidogenic forms of TTR on the heart and to explore the protective potential of several agents.

We hypothesized that pre-fibrillar, oligomers of TTR would be cardiotoxic and that this cytotoxic effect contributed to cardiac amyloid pathology in ATTR amyloidosis (Figure 8). Further, we postulated that this cardiotoxic effect could be inhibited by agents, namely diflunisal, doxycycline, Kiacta®, and RBP through interactions with native and non-native forms of TTR. Specifically, we posited that soluble, aggregated TTR would be toxic to cultured cardiac cells, i.e. cause decreased cell survival and increased myocardial stress. Using purified recombinantly-generated TTR proteins, we tested our hypothesis by creating amyloidogenic TTR oligomers in vitro, testing the effect of oligomeric TTR on cultured rat and human cardiac cells, and investigating the cell rescue potential of the proposed inhibitory agents.

The specific aims of this research were as follows:

Specific Aim 1: To develop a method for reproducibly creating soluble amyloidogenic TTR in a time-efficient manner using physiological conditions.

Specific Aim 2: To treat cardiac cells (rat cardiomyoblasts, human iPSC-derived cardiac cells) with oligomeric, pre-fibrillar TTR and assess the cellular response both morphologically and chemically.

Specific Aim 3: To examine the potential of diflunisal, doxycycline, Kiacta®, and retinol-binding protein (RBP) to inhibit the cardiotoxic effect of TTR oligomers.
1.10 Significance

The TTR-associated amyloidoses are a group of rare, but aggressive and fatal diseases with few treatment options available to patients. While the clinical presentations in ATTR amyloidosis have been extensively described, little is known about the precise mechanism of the disease. While the presence of amyloid deposits may cause mechanical disruption of cellular and tissue function, it is generally held that these are not the disease-causing entity. Over the past ten years, research in the field has shifted from the tissue deposits to earlier stage forms of the amyloid protein and understanding the damaging impact of soluble, pre-fibrillar forms of TTR. In the studies presented in this dissertation, the goal was to characterize the toxic effects of TTR oligomers using cell-based models. Furthermore, since both ATTRwt and ATTRm frequently feature infiltrative cardiomyopathy and cardiac amyloid, we chose to study the effects of amyloidogenic forms of TTR on two cardiac cell types, rat cardiomyoblasts and human iPSC-derived cardiac embroid bodies (cEBs). Results from these studies should further the present understanding of the pathobiology of ATTR amyloidosis as we believe the mechanism includes cardiotoxicity induced by pre-fibrillar forms of TTR.

In addition to the incomplete understanding of disease mechanism, treatment options and efficacious therapies for patients with ATTR amyloidosis are lacking. There is also a necessity for disease markers and prognostic indicators in ATTRwt amyloidosis, a disease that is particularly difficult to detect in the early stages. These unmet needs prompted us to investigate several potential inhibitory agents, as well as study the nature of retinol-binding protein (RBP) in our TTR-amyloid patient population.
Figure 8. TTR amyloid cascade hypothesis.
CHAPTER 2. METHODS

2.1 Chemicals and reagents

All chemicals and reagents were of the highest grade available. Phosphate buffered saline (PBS), methanol (MetOH), hydrogen peroxide (H₂O₂), dimethyl sulfoxide (DMSO), penicillin-streptomycin (Pen-Strep), L-glutamine, and ammonium persulfate (APS) were all purchased from Fisher Scientific (Fairlawn, NJ). Gelatin, bovine serum albumin (BSA), Congo red, Thioflavin T, doxycycline hyclate, and LB broth were purchased from Sigma (St. Louis, MO). Glutaraldehyde and diflunisal were purchased from MP Biomedicals (Santa Ana, CA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Flowery Branch, GA). Primary antibodies were purchased from DAKO (Troy, MI). All secondary antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX). Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Corning (Corning, NY) and Tryp-LE from Invitrogen (Carlsbad, CA). Non-essential amino acids (NEAA) were obtained from CellGro (Corning, NY). Kiacta® was obtained from Piramal Healthcare Ltd. (Aurora, Ontario, Canada).

2.2 Cell culture experiments

H9C2 rat cardiomyoblasts (ATCC CRL-1446) were grown in 75 cm² flasks containing DMEM supplemented with 10% FBS, 100 units/mL Pen-Strep, and 2 mM L-glutamine. Cells were cultured at 37 °C and 5% CO₂. To passage confluent cells, medium was aspirated off and cells were washed with PBS. Cells were detached with the addition of 1 mL Tryp-LE and this was followed with a 5 minute incubation at 37 °C.
When detachment was complete, 7 mL of fresh medium was added and the cells were transferred into new T75 flasks at a 1:4 or 1:8 ratio (mL cell suspension: mL medium).

2.3 Generation of cardiac embryoid bodies from induced pluripotent stem cells

Human cardiac embryoid bodies (cEBs) were derived from induced pluripotent stem cells (iPSCs). The iPSCs were from two sources, commercially obtained dermal fibroblasts from a 27-year old heterozygous for the TTR-L55P mutation, and blood mononuclear cells (MNCs) from a healthy individual (labeled as BU6).

A 7-day protocol was used to differentiate the iPSCs into cardiac cells. On day 0, iPSCs that had been grown up to 80% confluence in 6-well plates were washed with PBS, treated with 500 μL dispase for 2 minutes, and washed again with PBS. One mL of cEB growth medium (DMEM, 20% FBS, 1% NEAA, 1% L-glutamine, 100 mM 2-mercaptoethanol, 2 ng BMP4, 1 μM RhoK) was added to each well and cells were dissociated using a cell scraper. The cell/medium mixture was transferred to 35 mm diameter dishes (Stemcell Technologies, Vancouver, Canada) and brought to a final volume of 2 mL medium per dish. After 24 hours of incubation at 37 °C, low O₂ and 5% CO₂ (day 1), the cells were switched into cEB medium containing BMP4, ActA and bFGF. Medium was refreshed again on day 4. On day 7, 12-well plates were prepared by coating them with 0.1% gelatin in H₂O for 10 minutes. The cEBs were collected from the 35mm dishes, washed with PBS, transferred into the coated 12-well plates, and supplemented with cEB medium containing no cytokines.
To assess cell viability, we used the ApoTox-Glo™ Triplex Assay (Promega, Madison, WI). This assay detects live cells by using their protease activity to cleave the fluorogenic substrate, glycyl-phenylalanyl-aminofluorocoumarin (GF-AFC). The substrate is taken up by intact cells and cleaved by cellular proteases. This enzymatic activity generates free AFC with a fluorescent signal that has an excitation wavelength at 400 nm and emission wavelength at 505 nm. Twenty μL of the GF-AFC compound was added to wells in a black 96-well plate and incubated for 30 minutes at 37 °C, 5% CO₂. Fluorescence was measured at 400nm (excitation) and 505nm (emission).

2.5 Cross-linking protocol

Ten μL of 25% glutaraldehyde was added to 100 μL TTR protein sample (0.4 mg/mL) and incubated at room temperature for 3 minutes. The reaction was quenched by adding 5 μL of 7% (w/v) NaBH₄ in 0.1 M NaOH. Cross-linked samples were analyzed by SDS-PAGE electrophoresis as described in 2.8.

2.6 Immunohistochemistry and Congo red staining

Paraffin blocks containing the tissue of interest were frozen overnight and placed in a shallow bath of ammonia water immediately before sectioning. Using a microtome, sections were cut to 5 μm or 8 μm in thickness for immunohistochemical or Congo red staining, respectively. Sections cut from the paraffin block as a ‘ribbon’ were gently placed on the water surface in the bath and maintained at 45 °C. Using tweezers, sections were separated from one another and floated onto glass barrier microscope slides (Biogenex, San Ramon, CA) or positively charged slides for Congo red staining. Slides
containing the sections were heated at 65 °C in an oven for at least one hour; this allowed the tissue to adhere to the glass surface and prevented loss of sections during the histologic treatment process. Sections were deparaffinized in two changes of xylene (4 minutes each) and subsequently hydrated in two changes of 100% ethyl alcohol and 95% ethyl alcohol. Following three rinses in dH₂O, slides were placed in an H₂O₂/MetOH solution (200 mL MetOH, 50 mL 3% H₂O₂) for 10 minutes.

To unmask antigen for better recognition by the antibodies, slides were placed in Antigen retrieval Citra plus solution (Biogenex) and heated to 95 °C for 8 minutes. Slides were cooled to room temperature for 20 minutes and placed in an automated immunohistochemical staining instrument (DAKO). To prevent drying, slides were immediately covered with wash buffer (DAKO).

The autostainer was loaded with appropriate staining solutions. After the program run, slides were counterstained with Harris-modified hematoxylin (Harris-modified hematoxylin with 1% glacial acetic acid) rinsed, dipped in 0.25% acid alcohol (0.25% HCl in 70% ethyl alcohol) two times and quickly washed under running tap water. This was followed by a rinse in 1% ammonium water for 20 seconds; dehydration of the sections was accomplished with 95% and 100% ethanol, and lastly xylene. Sections of the slides were protected with coverslips applied using Permount mounting medium (Fisher).

2.7 Fluorescent microscopy

H9C2 cells (3000 cells/well) were plated onto sterile glass coverslips pre-coated with 0.2% gelatin in 6-well plates. The cells were allowed to adhere and settle onto the
coverslips for 24 hours at 37 °C and 5% CO₂, prior to treatment. Following experimental
treatment protocols of 72 hours, media was aspirated and cells were washed in three
changes of PBS. Using a 4% formaldehyde solution, cells were fixed for 15 minutes at
room temperature, washed three times with PBS, and permeabilized with 0.1% Triton-X
for 5 minutes. Following fixation and permeabilization, cells were blocked in 3% BSA
overnight at room temperature. The next day, cells were washed four times with PBS
and incubated with a 1:200 dilution in PBS of primary antibody (polyclonal goat-anti-
human TTR) for one hour at 4 °C. The antibody solution was removed and cells were
again washed three times with PBS. Secondary antibody (polyclonal donkey-anti-goat-
IgG, FITC-labeled) at a 1:100 dilution in PBS was added to the cells which were then
incubated for another hour at 4 °C in the dark.

For visualization of actin filaments within the cells, coverslips were removed
from the 6-well plate, placed in a humid chamber, and stained with 100 nM rhodamine
phalloidin (Cytoskeleton Inc., Denver, CO) for 30 minutes in the dark at room
temperature. Following three final washes with PBS, the coverslips were counterstained
for DNA and mounted onto microscope slides using slow-fade DAPI mountant (Life
Technologies, Carlsbad, CA). An Olympus BX60 fluorescent microscope was used for
visualization; picture images were taken using a QImaging Retiga 2000R camera and
software.
2.8 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

2.8.1. Manual gel electrophoresis

Protein samples were mixed at a 1:1 dilution with loading buffer (50 mM tris-HCl pH6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue) supplemented with 1% 2-mercaptoethanol. Samples were boiled for 5 minutes, briefly spun at 10,000 rpm, and loaded into 1 mm thick 10% SDS-PAGE gels with 5% stacking gels for electrophoretic separation. Protein standards (Spectra Broad Range Protein Ladder, Pierce) were run in parallel for reference.

2.8.2. Automated (Pharmacia PHAST system) gel electrophoresis

For protein separation on precast 4-15% gradient gels, the automated Phast System (GE, Fairfield, CT) was used. Precast gels were placed in the system, making sure to pipette 90 µL H₂O onto the surface in order to avoid air bubbles. Next, SDS buffer strips were placed on either end of the gel to prevent dehydration during electrophoresis. Protein samples were mixed with 2x loading buffer at a 1:1 ratio and boiled as described in 2.8.1. One µL samples were loaded into one of six wells on a plastic comb as quickly as possible to avoid evaporation.

2.9 Western blot analysis

For Western blot analysis, proteins were initially separated by SDS-PAGE and subsequently transferred onto a nitrocellulose membrane using a wet transfer method that proceeded overnight in transfer buffer (1.66 g tris base, 7.2g glycine, 100 mL MetOH, final volume 500 mL, pH 8.4) at 4°C (BioRad). The next day, the membrane was soaked in blocking buffer (10% non-fat dried milk in TBST) for 1 hour at room temperature,
washed with TBST, and incubated with primary antibody in 5% milk for two hours at room temperature. For TTR detection, a polyclonal rabbit anti-human TTR antibody (DAKO) was used at 1:20,000 dilution in 2.5% BSA in PBS. A mouse anti-human polyclonal RBP antibody (Santa Cruz) at 1:1000 dilution in 2.5% BSA in PBS was used for RBP analyses. Following three TBST washes of the membrane, the appropriate secondary antibody, either 1:10,000 goat anti-rabbit IgG-HRP or 1:10,000 goat anti-mouse IgG-HRP, was added for detection of TTR or RBP, respectively. After a 1 hour incubation, blots were washed and developed using the ECL Plus Western blotting detection system (Pierce).

2.10 Congo red shift assay

To assess aggregation of TTR, a Congo red shift assay was performed. An initial stock solution of 1 mM Congo red in 40% EtOH was used in these studies. TTR protein samples that had been incubated for one week at 80 °C in 1.5 mL Eppendorf tubes were spun down at 25,000 x g for 10 min. Supernatant was discarded and the pellet was resuspended in 10 μM Congo red working solution (10 μM Congo red, 5mM KH₂PO₄, 150 mM NaCl, pH 7.5). Samples were transferred into cuvettes and absorbances were recorded at 477 nm and 540 nm in a Cary 300 UV-Vis spectrophotometer (Agilent). A red shift, i.e. a shift towards the longer wavelength end of the spectrum, indicates that the dye is binding to protein and provides evidence for the presence of pre-fibrillar aggregates and/or amyloid fibrils[6].
2.11 Thioflavin T analysis

TTR protein (0.4 mg/mL) was heated to 80 °C over the course of several hours. At various time points, the amount of soluble TTR aggregates was assessed by thioflavin T (ThT) fluorescence measurements. ThT was dissolved in 100% EtOH at a concentration of 10 mM and filtered through a 0.22 µm syringe filter to remove any particulates. The solution was diluted in PBS to make a stock solution of 20 µM ThT. For ThT fluorescence measurements, 100 µL of TTR sample was combined with 100 µL ThT stock solution; the final ThT concentration was 10 µM. The 200 µL mixture was transferred into a black 96-well plate and ThT fluorescence was measured in a FluoroMax spectrofluorometer (Horiba Jobin, Kyoto, Japan) at an excitation wavelength of 440 nm and emission wavelength of 482 nm using a 5 nm slit width. Samples were measured in triplicate and measurements were normalized to a blank solution of ThT.

2.12 Expression and purification of recombinant transthyretin protein

Recombinant TTR proteins with N-terminal 6-histidine-tags were expressed in E.coli M15 [pREP4] cells using the pQE30 expression vector[98]. To grow up the E.coli, flasks of LB broth (each 500 mL) were autoclaved, supplemented with 12.5 mg kanamycin and 50 mg ampicillin, inoculated with a single isolated bacterial colony, and cultured overnight at 225 rpm at 37 °C.

The following day, 0.5 liters of LB broth was inoculated and incubated 37 °C with shaking at 225 rpm. This was continued until the broth solution exhibited an optical density (OD) of 0.6 at 600 nm. Expression of rTTR was induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 1 mM. Following 4 hours
of incubation at 37 °C, the media was centrifuged in an SLA-1500 rotor at 6,000 rpm for 20 minutes at 4 °C.

An immobilized-metal ion affinity column (IMAC) method was used to purify the bacterially-generated TTR. Once the cells were pelleted, the supernatant was discarded; pellets were resuspended in IMAC cell lysis buffer (50 mM NaH$_2$PO$_4$ 2 H$_2$O, 300 mM NaCl, 10 mM imidazole, pH 8.0) at 3 mL per gram wet weight. Solutions were supplemented with lysozyme at 1 mg/mL and incubated on ice for 30 minutes. Following incubation, the cells were sonicated in 15 minute intervals until the solution appeared clear and had changed to a darker brown color, indicating cell lysis. This sonication procedure was performed on ice to prevent overheating. The lysed cell mixture was centrifuged in an SS-34 rotor at 9,000 rpm for 30 minutes at 4 °C. The supernatant was filtered through 0.22 µm syringe-filters.

A 3 mL IMAC Ni-NTA (nickel-nitrilotriacetic acid) sepharose column (Qiagen) was prepared and equilibrated in several rinses of PBS. Up to 20 mL of cleared lysate was allowed to run through the column, allowing the rTTR protein to bind to the nickel resin through its 6 histidine tag. The column was rinsed with IMAC wash buffer (50 mM NaH$_2$PO$_4$ 2 H$_2$O, 300 mM NaCl, 20 mM imidazole, pH 8.0) until OD$_{280}$ nm was zero. Recombinant TTR protein was eluted from the column using IMAC elution buffer (50 mM NaH$_2$PO$_4$ 2 H$_2$O, 300 mM NaCl, 250 mM imidazole, pH 8.0); fractions with an OD$_{280}$ nm > 0.1 were collected and pooled. The pooled fractions containing rTTR protein were dialyzed into PBS using 7,000 MWCO Slide-A-Lyzer cassettes (Thermo Scientific) and the PBS was changed every 30 minutes for at least 2 hours.
Protein concentration was determined by measuring the absorbance at 280 nm and calculating with the following formula: \( A = \varepsilon \lambda C \), where \( A \) is absorbance at \( \text{OD}_{280 \text{ nm}} \), \( \varepsilon \) is the extinction coefficient for TTR = 77,600 M\(^{-1}\) cm\(^{-1}\)[99], \( \lambda \) is the cell length of the cuvette, and \( C \) is the molar concentration of the rTTR protein solution. Protein was immediately flash frozen in a mixture of dry ice and acetone, and transferred to a -150 °C freezer for long-term storage.

2.13 Serum retinol-binding protein 4 measurement by enzyme-linked immunosorbent assay

To measure circulating levels of RBP4 in patient serum samples, a sandwich enzyme-linked immunosorbent assay (ELISA) was developed. High-binding, flat bottom 96-well plates (Nunc) were coated with 1:500 mouse anti-human RBP4 monoclonal antibody (R&D) and stored overnight at 4 °C. The coating antibody was removed and plates were washed three times with PBS containing 0.1% tween using a Biotek ELx405 automated plate washer. Plate wells were blocked with 1% BSA for one hour at room temperature and washed three times with PBS containing 0.1% tween-20. RBP4 standard protein (R&D) and serum dilutions (1:100,000) were prepared in 0.2% BSA, 0.25% tween and loaded onto plates; all samples were run in triplicate. The plates were incubated for 2 hours at room temperature with shaking at 500 rpm. Following another wash, 1:4,500 biotinylated polyclonal mouse anti-human RBP4 antibody was added and plates were incubated for 2 hours at room temperature with shaking at 500 rpm. The plates were washed and streptavidin-HRP was added; a 30 minute incubation at room temperature with shaking at 500 rpm followed. After another wash step, TMB substrate
was added and plates were incubated in the dark at room temperature for 25 minutes. This reaction was quenched with 1.5 N H₂SO₄ and plates were read at 465 nm and 590 nm (background) using a Biotek ELx800 plate reader. Background values were subtracted from 465 nm measurements and triplicate values were averaged. Data were analyzed using unpaired two-tailed t-tests with Mann-Whitney U test. Statistical significance was p-value < 0.05.

2.14 RNA isolation and quantitative polymerase chain reaction

Cells, plated in a 12-well plate, were washed three times with PBS and 350 µL RLT (Qiagen) lysis buffer was added to each well. The cell lysate solutions were homogenized and transferred to 2 mL conical bottom sample tubes. The tubes were loaded into a Qiacube automated RNA isolation system and RNA was purified from cell lysates using the RNeasy Plus Mini kit (Qiagen). Isolated RNA was removed from the Qiacube and quantified prior to cDNA synthesis. cDNA was synthesized from 250 ng of RNA template using the QuantiTect Reverse Transcription kit (Qiagen) according to the manufacturer instructions. Briefly, genomic DNA (gDNA) was removed prior to cDNA synthesis by addition of gDNA removal buffer to 250 ng RNA. The mixture was incubated at 42 °C for 2 minutes. Immediately after gDNA removal, reverse transcription master mix containing reverse transcriptase and primers (obtained from the Dr. George Murphy Laboratory, see Table S1 for sequences) was added to the reaction tubes. The reaction was incubated for 15 minutes at 42 °C and inactivated at 95 °C. For qPCR, SYBR® Green PCR Master Mix (Applied Biosystems) was mixed with the appropriate primers (Table S1) and added to 7.25 ng cDNA in 96-well reaction plates.
The plates were loaded into a StepOne Real-time PCR machine (Applied Biosystems). All samples were run in duplicate and normalized to GAPDH. The Student’s t-test statistical analysis was used to determine significance. Statistical significance was p-value < 0.05.

2.15 Circular dichroism spectroscopy

Secondary structure and folding of proteins was studied using a Jasco J-815 spectropolarimeter. Briefly, protein solutions were prepared at 0.4 mg/mL in PBS and transferred to a 0.1 cm quartz cell. Far-UV spectra were recorded at 25 °C in triplicate with monitoring between 200 and 250nm (far UV) using a 0.1 nm bandwidth. To assess changes in the secondary protein structure during thermal denaturation or melting, the sample was heated from 25 °C to 98 °C at a rate of 1 °C/min and the CD signal was recorded at 213 nm every minute. Subsequent sample cooling from 98 °C to 25 °C was also recorded and analyzed. Data were normalized to protein size (residue number) and concentration, and plotted as mean residue molar ellipticity (MRE).

2.16 Study populations

Patient sera, tissue samples, and clinical data were obtained from the Boston University Amyloidosis Center repository with the approval of the Institutional Review Board at the Boston University Medical Campus in accordance with the Declaration of Helsinki. Patient groups included patients with ATTRm, ATTRwt, or AL amyloidosis. Inclusion criteria for the patient study groups were male gender, aged 60 years or older, and triglyceride levels < 200 mg/dL. A triglyceride cut-off value of 200 mg/dL was chosen based on previous reports showing a correlation of increased RBP4 levels to
obesity[100]. Control sera from healthy individuals, which were age- and gender-
matched to our patient groups, were obtained from BioReclamation (Westbury, NY).
Since BMI values were not available on all study patients, triglyceride levels were
considered in order to rule out a potential confounding variable. A diagnosis of TTR
amyloidosis was established by Congo red histology and immunohistochemistry or mass
spectrometric analysis, isoelectric focusing (IEF), and genetic testing by DNA
sequencing. AL amyloidosis was confirmed by bone marrow biopsy and the presence of
a monoclonal immunoglobulin light chain in the serum and/or urine.

A patient was considered to have cardiomyopathy or cardiac involvement when
presenting with an abnormal echocardiogram, increased left ventricular mass (LVM),
lowered ejection fraction (EF), and left ventricular wall thickening > 12 mm. Patient
laboratory data that was evaluated included brain natriuretic peptide (BNP), cardiac
troponin (cTn-I), VO₂ (metabolic stress test), and uric acid. These values were obtained
from the Boston University Amyloidosis Center clinical database.
CHAPTER 3. IN VITRO GENERATION OF AMYLOIDOGENIC TRANSTHYRETIN OLIGOMERS UNDER PHYSIOLOGIC CONDITIONS

3.1 Introduction

As was previously described, there has been an increasing focus on the pre-fibrillar soluble TTR oligomers believed to be the toxic species in amyloidosis; this is in contrast to the previously held belief that the mature fibrils were the sole cause of disease manifestations[20]. The aim of our studies was to investigate the effect of TTR pre-fibrillar species on cells. Our plan was to examine cellular viability following direct exposure of cells to soluble amyloidogenic forms of TTR. Thus, we needed to develop a method that would allow us to generate oligomeric TTR under conditions that were close to physiologic and within a reasonable time frame. In addition, it would also be ideal to minimize manipulation or alteration of the TTR proteins that were being used. Previously reported TTR fibril formation assays all presented major disadvantages or were not suitable for our planned cell-based studies. One method that has been used extensively generates TTR oligomers and amyloid fibrils under acidic conditions (pH ≤ 4.5)[39], [47], [101]. This protocol was developed for in vitro biochemical and biophysical analyses of TTR aggregates and fibrils. For our studies, this method was not an option as treating the cells with acidic solutions of protein would undoubtedly cause damage unrelated to amyloidogenic TTR.

Some research groups have attempted to use physiological conditions for TTR aggregate and fibril formation. Sousa et al.[102] incubated TTR-L55P protein at 37 °C and pH 7.0 for 15 days; the authors of this study observed fibrils by transmission electron
microscopy (TEM) on day 15 and aggregates after just 24 hours. Moreover, Azevedo et al. observed TTR-A25T fibril formation after 15 days at pH 7.3 and 37 °C [43].

Based on these studies, our aim was to create an assay at a neutral pH, i.e. pH 7.4, using thermal denaturation (80 °C) to form aggregates in a shorter time interval than the previously described methods.

Our interest in understanding how TTR amyloid affects the heart led us to study TTR proteins that have a strong association with cardiac amyloid disease and are reported to be most amyloidogenic based on in vitro studies. In this dissertation research, we focused on the wild-type protein and three TTR variants L55P, V30A, and V122I (Table 1).

3.2 Thermal denaturation forms high molecular weight transthyretin aggregates

The first step in our studies was to produce each TTR protein (wt, L55P, V30A, V122I) in abundant amounts and purified to homogeneity. It was also critical to create proteins that exhibited structural characteristics identical to the native protein. Expression of rTTR protein was accomplished using an E.coli bacterial system established in our laboratory[98] and purification of the histidine-tagged protein was performed using immobilized metal ion column chromatography (IMAC), as was described in Chapter 2.

The purity of recombinant proteins was assessed by SDS-PAGE run under reducing conditions; a representative example is shown in Figure 9a. Protein staining with Coomassie blue indicated a predominant species with an apparent molecular weight
of 14 kD and a minor one at 55 kD; these results were as expected for monomeric and tetrameric TTR, respectively.

Confirmation of native conformation, i.e. proper folding and correct secondary structure, was analyzed by circular dichroism spectroscopy (CD). Far UV (195-240 nm) spectra were recorded and an example demonstrating the analysis is shown in Figure 9b. The measurements indicated that rTTR (wt shown as an example) protein was properly folded and displayed high beta sheet content, as is expected in neutral PBS buffer.

Having confirmed the purity and structural integrity of the rTTR proteins, we proceeded with our method development (Figure 10) for generating TTR oligomers. Solutions of TTR at 0.4 mg/mL in PBS (pH 7.4) were freshly prepared prior to each experiment. This concentration of TTR was chosen because it represents a physiologic level; the normal range for serum-circulating TTR is 0.2 – 0.45 mg/mL[26], [27].

Unfolding and aggregation of the protein samples were induced using a thermal denaturation assay in which the proteins were incubated at 80 °C. After 0, 24, 48, 120 or 144 hours of incubation, the proteins were cross-linked with 25% glutaraldehyde and run on 10% SDS-PAGE gels to assess the aggregated state of the protein.

Representative images of the gels showing results obtained for wt and L55P TTR, the most unstable mutant of the three studied variants, are illustrated in Figure 11. The results showed that L55P rapidly aggregated into high molecular weight (HMW) soluble aggregates with an apparent molecular weight > 260 kD. Within 24 hours of the 80 °C treatment, there was little evidence of tetrameric TTR providing evidence for dissociation of the native protein. By 48 hours at 80 °C, a decrease in the HMW species was
observed on the gels and this suggested that the aggregated forms of TTR had become insoluble and were precipitating out of solution.

In contrast to L55P TTR, the wt tetramer, as expected, proved to be more resistant to the heat denaturation process. Results from the electrophoretic analysis suggested that a minimal amount of aggregated (HMW) wt TTR was formed by 24 hours, but thereafter was present and remained visible for several days. This is in contrast to the L55P HMW species which were no longer observed by 48 hours, most likely due to formation of large insoluble aggregates. When compared to L55P TTR, the wt protein, overall, showed much less loss of tetramer or native state protein in the heat denaturation process during the 144 hour course of incubation. These data provide evidence consistent with the literature that wt TTR is structurally more stable than L55P[47].
Figure 9. SDS-PAGE and far-UV circular dichroism of wild-type recombinant TTR.

(a) SDS-PAGE gel of wt rTTR protein with a predominant band at 14 kD (monomer) and minor band at 55 kD (tetramer) on a 10% acrylamide reducing gel. The gel was stained with Coomassie blue dye. (b) Far UV spectrum recorded between 195 and 240 nm in PBS at room temperature showing expected folding and secondary structure (high beta sheet content) of the wt rTTR protein.
Figure 10. Schema for *in vitro* formation and analyses of TTR oligomers.

The experimental design for development and validation of our thermal denaturation assay is shown. TTR protein at 0.2 mg/mL in PBS was incubated at 80 °C. Aliquots of the protein solution were removed at timed intervals (0, 4, 24, 48, 72, 144 hours) for analyses by 1) cross-linking proteins and SDS-PAGE, 2) Congo red shift, and 3) Thioflavin T assay.
Figure 11. Formation of high molecular weight aggregates by 24 hours at 80 °C.
Aggregates formed in the thermal denaturation assay were analyzed by cross-linking and SDS-PAGE. The results with wt and L55P TTR proteins are shown in the left and right panels, respectively. In each gel, a molecular weight ladder was run in the first left lane; standard proteins had apparent molecular weights of 10, 15, 25, 35, 40, 50, 70, 100, 140, 260 kD shown from the bottom to the top of the gel. From left to right on both gels, samples of TTR removed during the 80 °C incubation at times 0, 24, 48, 120, and 144 hours are shown. The apparent molecular weights of tetrameric or native TTR and high molecular weight species were observed at 55 kD and > 260 kD, respectively. Protein samples were run on 10% SDS-PAGE gels and visualized with Coomassie blue.
3.3 High molecular weight aggregates formed are pre-fibrillar oligomers

Once it was established that HMW TTR aggregates could be successfully generated with our heat denaturation protocol, we wished to characterize these species and confirm that they were in fact intermediates on pathway to amyloid fibril formation. To assess the amyloidogenicity of the aggregates, we used Congo red, a diazo dye described in Chapter 1. As mentioned, this is a dye that has the unique ability to intercalate between the beta sheets of amyloid. Congo red can be used as a histologic stain to detect the presence of amyloid fibrils in tissues or as an indicator of aggregates in an absorbance shift assay[6]. In the latter assay, a solution containing amyloid aggregates can be assessed by observing a difference (spectral shift) in the wavelengths at maximum absorbance ($\lambda_{\text{max}}$) of the free and amyloid-bound dye. This assay is a common way to measure amyloid protein aggregation.

Wild-type, V30A, and V122I TTR proteins were incubated for one week at 80 °C and subsequently mixed with Congo red. The absorbance spectra were recorded between 300 and 700 nm. As can be seen in Figure 12, a red shift in $\lambda_{\text{max}}$ was observed for each of the three TTR proteins compared to the Congo red control. The $\lambda_{\text{max}}$ of the free dye is 486.0 nm; spectral shifts to 487.0, 491.0, and 492.0 nm occurred in the wt, V30A, and V122I TTR samples, respectively. These data presented evidence supporting the formation of amyloidogenic aggregates in the TTR thermal denaturation assay. As expected, the wild-type TTR exhibited a minimal red shift indicating lower levels of aggregate formation. Interestingly, V122I TTR showed the largest red shift indicating
that over the course of seven days this protein formed more amyloidogenic species compared to the other variants tested.

To confirm that the L55P TTR HMW aggregates were on pathway to form amyloid, we used Thioflavin T, a benzothiazole dye which also has been used to detect amyloid[7]. We chose to use this assay, as opposed to the previously described Congo red shift assay, because we anticipated that L55P, a very unstable variant, would form aggregates and oligomers very early in the thermal denaturation process. Since the Congo red shift assay requires a significant amount of aggregation for detection, we chose to use the Thioflavin T assay which can detect earlier stage (within hours) soluble oligomer formation. L55P TTR protein was incubated at 80 °C for approximately 4 hours. Measurements of ThT fluorescence on TTR samples removed at 0, 45, 60, 120, and 200 minutes during the thermal denaturation assay are shown in Figure 13. ThT fluorescence increased within one hour of heating and indicated that HMW TTR species were being formed. These data indicated that the HMW aggregates seen by SDS-PAGE analysis (Figure 11) were amyloidogenic in nature.
Figure 12. Congo red spectral shift analysis of thermally denatured TTR samples.

The absorbances of wt, V30A, and V122I TTR solutions (1mg/ml), incubated for one week at 80 °C, were measured in the wavelength range between 450 and 550 nm. The final Congo red concentration in each sample was 10 μM. Measurements were made in a 1 cm cuvette. A control solution containing only Congo red yielded a maximum absorbance at a wavelength ($\lambda_{\text{max}}$) of 468.0 which is consistent with the reported value of the free dye. All three TTR proteins showed spectra with shifts in the $\lambda_{\text{max}}$ of Congo red. Values for the $\lambda_{\text{max}}$ of individual proteins and Congo red alone are shown in the table.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Absorbance $\lambda_{\text{max}}$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Congo red alone</td>
<td>486.0</td>
</tr>
<tr>
<td>wt TTR</td>
<td>487.0</td>
</tr>
<tr>
<td>V30A TTR</td>
<td>491.0</td>
</tr>
<tr>
<td>V122I TTR</td>
<td>492.0</td>
</tr>
</tbody>
</table>
Figure 13. ThT fluorescence analysis of thermally denatured TTR-L55P.

The L55P TTR protein (0.4 mg/mL) was incubated at 80 °C for 200 minutes. Aliquots were collected from the incubation at 0, 45, 60, 120, and 200 minutes and mixed with ThT (final concentration of 10 μM ThT), then analyzed for fluorescent output. Fluorescence was measured at excitation and emission wavelengths of 440 and 482 nm, respectively. Samples were all measured in triplicate; each datapoint shown on the graph represents the mean of the triplicate readings along with standard error bars of variation.
3.4 Summary and conclusions

In this chapter, we have detailed the development of a reproducible method to create soluble, high molecular weight aggregates of wt and variant TTR proteins. We have provided evidence showing that heat denaturation at 80 °C is a rapid and effective means of creating oligomeric forms of TTR that are precursors of amyloid fibrils.

Initially, we used SDS-PAGE and CD analyses to verify the generation of homogeneous preparations of recombinant wt and variant TTR proteins that were properly folded and exhibited native structural characteristics. Focusing on wt and L55P, we showed by glutaraldehyde cross-linking and SDS-PAGE analysis that thermal denaturation at 80 °C yielded high molecular weight TTR aggregates. The amyloidogenic TTR variants, V30A and V122I, were also studied in these experiments and results will be detailed in Chapter 4.

We conclude from the data presented in this section that 80 °C treatment of TTR under physiologic conditions of concentration (0.2 mg/mL), salt (PBS), and pH (7.4) generates amyloidogenic TTR aggregates by 4 hours. In addition, this is a reproducible and time efficient assay that will be appropriate for use in the planned cell-based studies undertaken to gain a fuller understanding of the effects of amyloidogenic TTR species on cardiac cell viability.
CHAPTER 4. CARDIAC CELL RESPONSE TO TRANSTHYRETIN

OLIGOMERS

4.1 Introduction

With an established method in place to create amyloidogenic TTR oligomers, the next step was to examine the effect of these on cardiac cells. The goal was to measure the effect of TTR oligomers on cell viability. As was detailed in Chapter 3, the thermal denaturation studies were performed in a time interval of 144 hours; however, oligomer formation was observed as early as 24 hours. In designing the cell-based studies, we specifically wanted to focus on earlier stage oligomers for several reasons. Previous studies had suggested that earlier, smaller oligomeric forms of TTR were cytotoxic compared to larger aggregates and pre-fibrillar species, which elicited a far less damaging response. For example, Sörgjerd et al. reported that only small TTR oligomers > 250 kD but < 1000 kD were toxic to SH-SY5Y neuroblastoma cells as measured in an MTT assay. Moreover, the authors demonstrated that TTR aggregates > 1000 kD were significantly less toxic[20]. Findings published by Krishnan et al. using the prion-determining region of yeast Sup35 similarly concluded that earlier stage oligomers were the toxic entities[103]. These previous findings, taken together with our SDS-PAGE results described in Chapter 3, led us to focus our studies on TTR oligomers formed in the initial four hours of thermal denaturation at 80 ºC.

There have been numerous studies that have previously reported a toxic effect of small soluble TTR oligomers on cells. However, a majority of these investigations have focused on FAP-related TTR variants and were almost exclusively limited to studies
using neuronal cell lines as model systems. For instance, pre-fibrillar TTR-V30M
oligomers were shown to be cytotoxic to the IMR-32 neuroblastoma[104] and SH-SY5Y
cells[105]. The goal of the studies presented in this dissertation was to further understand
cardiac amyloid diseases associated with TTR. To this end, we chose to use TTR
proteins linked to major cardiac involvement featuring cardiomyopathy and sought to
establish a cardiac cell-based model for study of these cardiac amyloidoses.

Specifically, we opted to study wild-type TTR associated with age-related or
senile systemic amyloidosis, and three TTR variants, L55P, V30A, and V122I,
responsible for familial cardiac amyloidosis. Studies of these variants, particularly in
cardiac cell systems, are limited. Two recent reports examined TTR-V122I cell toxicity
in experiments with human cardiomyocytes. However, the TTR to which the cells were
exposed was never characterized and it is unclear whether the protein was in the native or
an aggregated state[106], [107]. The TTR-V30A variant has been shown to cause
cytotoxicity in neuroblastoma cells[33], [48]. Sousa et al. demonstrated a cytotoxic
effect of L55P aggregates in transgenic mice[108]. In addition, neurotoxicity due to
L55P aggregates was observed in SH-SY5Y neuroblastoma[109] and rat Schwannoma
(RN22) cells[110].

To establish a cell model that would allow us to gain insight into TTR-associated
cardiac amyloid pathology, we initially chose to use rat cardiomyoblasts (H9C2). This is
a cell line that has been used extensively to study cytotoxicity and cell viability, and is
considered a most stable and reliable cardiac cell model[111]–[114]. A second model
system that was utilized our subsequent studies consisted of human cardiac emboid bodies (cEBs) differentiated from patient-derived iPSCs.

For comparison to TTR, we chose to use bovine serum albumin (BSA) as a control protein in all studies. BSA is a globular, non-amyloidogenic protein that is approximately 66 kD, a size similar to tetrameric TTR (55 kD). BSA, as a protein control, would allow us more confidence in attributing effects from oligomeric TTR to the amyloidogenic species and not merely to non-specific aggregates.

4.2 Some aggregated forms of transthyretin are toxic to rat cardiac cells

Early stage oligomers were formed using the thermal denaturation assay in which a physiologic concentration (0.4 mg/mL) of TTR in PBS at pH 7.4 was heated at 80 °C for 4 hours. Following respective 0 or 4 hour incubations, samples were cross-linked in 25% glutaraldehyde, separated on 10% SDS-PAGE gels, and stained with Coomassie blue. As can be seen in Figure 14a, the results for wt TTR showed a loss of tetramer content (T) and slight increase in intensity of the monomeric band (M) after the 4-hour thermal denaturation treatment; this was likely due to the molecular destabilization of the tetramer with concomitant dissociation to monomer. In contrast, native TTR-L55P dissociated rapidly and aggregated into HMW oligomers (H) within 4 hours of the 80 °C treatment. Very little monomer was observed in the heated sample, but an intensely stained protein band was noted at the top of the gel and indicated that aggregated forms of TTR (H) were present. TTR-V30A and -V122I both showed an increase in monomeric content after thermal denaturation, as well some HMW oligomer formation (H). Unlike the wt and L55P samples, there was an increased appearance of dimeric TTR
(D) in the heated V30A and V122I samples consistent with the occurrence of tetramer dissociation. Interestingly, a 4-hour heat treatment of the BSA (B) control protein appeared to induce aggregation and loss of the native protein (Figure 14b). The loss of the 66 kD protein (B) and appearance of a strongly intense protein band at the top of the gel (H) indicated thermally-induced denaturation and aggregation of BSA.

H9C2 cells grown to confluence or an approximate density of 20,000 cells/well were exposed to untreated (80 °C for 0 hours) or heat-denatured (80 °C for 4 hours) TTR protein samples; cell-based testing included the four TTR proteins (wt, L55P, V30A, and V122I), BSA as a control protein of similar size to native TTR, and staurosporine as a positive indicator of apoptosis[115], [116]. Protein samples (0.4 mg/mL) were each added to the cardiomyoblast cells at a 1:1 ratio with complete medium; thus, cells were treated with TTR at a final concentration of 0.2 mg/mL, a representative level of the protein in patient sera[51], [117]. All experiments were repeated three times.

Following initiation of treatment, cells were allowed to grow for an additional 72 hours in the presence of TTR protein or the respective negative and positive controls. After the 3-day exposure interval, cell viability was assessed using the ApoToxGlo (Promega) assay. Samples from 3 separate experiments were analyzed in triplicate. The results (Figure 15) demonstrated that cardiac cell viability was significantly lowered in cells treated with the L55P and V30A samples containing oligomeric forms of TTR (t = 4 hours); p-values were 0.0043 and 0.0159, respectively. In contrast, treatment with V122I and wt TTR, as well as the BSA control, had minimal to no effect on cell viability. It is interesting to note that the HMW species of BSA (Figure 14) formed after a 4-hour heat
treatment, unlike the TTR HMW species, were not toxic to the cells. Staurosporine, as expected, induced apoptosis.
Figure 14. SDS-PAGE demonstration of TTR oligomer formation in 4 hour denaturation assay.

(a) TTR proteins heated to 80 °C were electrophoretically analyzed for evidence of tetramer dissociation and aggregation. Aliquots of wild-type (wt), L55P, V30A, and V122I were removed at 0 and 4 hours of heat treatment; results are shown from left to right. Wt showed minimal tetramer (T) loss and a slight increase of monomer (M), while L55P tetramer loss was observed by 4 hours along with the appearance of a high molecular weight species (H). Both V30A and V122I showed an increased amount of monomer (M) by 4 hours along with the presence of dimer (D), as well as some tetramer (T) and HMW (H). (b) The control protein, BSA (B), also showed the presence of HMW (H) aggregates in the 4-hour heat-treated sample. Proteins were run on 10% SDS-PAGE gels and stained with Coomassie blue.
Rat cardiomyoblasts (H9C2) were exposed to staurosporine (SSP) as a positive control for apoptosis or TTR proteins (0.2 mg/mL) in native (untreated, 80 °C for 0 h) or oligomeric (80 °C for 4 h) form. Cell viability was measured after 72 hour exposure using the ApotoxGlo assay (Promega). Treatment of the cells with L55P and V30A TTR oligomers significantly lowered viability with p = 0.0043 and 0.0159, respectively. Data were normalized to vehicle control results and are shown as averages of the triplicate samples from three separate experiments.
4.3 L55P transthyretin oligomers are internalized by rat cardiomyoblasts

Having observed a negative effect of TTR oligomers on cell viability, we wished to further explore the mechanism of this cytotoxicity. There have been numerous published studies suggesting that some cells have the ability to internalize certain forms of TTR and that specific cellular environments could promote TTR aggregation[118–120]. However, these previous studies mainly focused on neuronal and other non-cardiac cell lines. In addition, these reports demonstrated internalization of wt TTR, often in its native form and there is no information regarding variant internalization. Fleming et al. reported on megalin-mediated native wt TTR internalization by neuroblastoma cells[118], and Sousa et al. observed native wt TTR internalization in hepatocytes[120]. An earlier study in 1990, observed native wt TTR binding to and internalization by hepatocytes, neuroblastoma, and lung cells[119]. Furthermore, Leung et al. demonstrated that L55P ATTR iPSC-derived neuronal-lineage cells internalized wt and L55P recombinant human TTR proteins[121]. An interesting study, published by Misumi et al. in 2013, described wt and V30M aggregate internalization by fibroblasts; however, the authors made the interesting observation that soluble native TTR protein was not taken up by the cells. In addition, the aggregates reportedly were localized specifically to lysosomes within the fibroblasts[122].

To investigate whether our observation of TTR cytotoxicity was partially explained by internalization, cardiomyoblast cells were fixed, permeabilized, and stained following a 3-day exposure to the wt and L55P TTR oligomers. We chose to test and compare these two TTR proteins since the strongest toxic effect was observed with the
L55P oligomers and little to no cell response was seen with the wt heat-treated sample. TTR was visualized using a FITC-tagged anti-human TTR polyclonal antibody. DAPI stain was used to detect the cell nuclei and Rhodamine phalloidin allowed examination of F-actin filaments. As can be seen in Figure 16, only the cells treated with TTR-L55P oligomers (L55P t=4 at 80 °C) displayed a striking and distinct internalization pattern of the protein (e), whereas cells exposed to vehicle (a), wt TTR (b,c) or native L55P (d) showed no immunoreactivity to the fluorescently labeled anti-TTR antibodies. Images are shown at 10x magnification. The punctuate, vesicular staining pattern seen in the cells treated with TTR-L55P oligomers is more apparent at a higher (40x) magnification as shown in Figure 17. These results suggest that wt TTR in native and heat-denatured form is not taken up by cardiomyoblasts cells; alternatively, if wt TTR is internalized, the protein may be removed so rapidly that it does not affect cell viability. Conversely, our data provides strong evidence that L55P TTR oligomers are taken up by and retained within the cardiomyoblasts for the 3-day duration of the experiments.

The striking high quantities of internalized L55P TTR oligomers and distinct staining pattern led us to wonder whether further aggregation of TTR was occurring within vesicles. A paper, published in 2009 by Hu et al., described such an observation; the authors reported internalization of Aβ protein by neuroblastoma cell (SH-SY5Y) endosomes or lysosomes and the subsequent formation of higher molecular weight aggregates which occurred in a time-dependent manner[123]. Based on this information, we stained cells after 24 or 48 hours of exposure to TTR oligomers, to more closely follow any changes in the aggregated state of L55P. As shown in Figure 18, the L55P
TTR oligomers appeared to increase in amount and aggregate size in a time dependent manner.
Figure 16. TTR-L55P oligomers are internalized by cardiomyoblasts.
Rat cardiomyoblast cells (H9C2) were treated with (a) PBS vehicle, (b) native wt TTR (untreated, 0h at 80 °C), (c) heat-denatured wt TTR (4h at 80 °C), (d) native L55P (untreated, 0h at 80 °C), or (e) oligomeric L55P TTR (4h at 80 °C). The duration of treatment was 72 hours and throughout the interval, cell cultures were maintained at 37 °C in 5% CO₂. A FITC-tagged anti-human TTR polyclonal antibody was used to detect TTR; DAPI stain allowed visualization of the cellular nuclei, and Rhodamine phalloidin was used to observe F-actin filaments. Images were taken at 10x magnification using an Olympus BX60 scope. Scale bar represents 100 µm.
Figure 17. *Internalized TTR-L55P oligomers display a punctate cellular pattern.*

Higher magnification (40x) of Figure 16e showing the results from rat cardiomyoblasts (H9C2) treated with oligomeric TTR-L55P (4 h, 80 °C) for 72 hours. Cells were maintained at 37 °C in 5% CO₂ for the duration of the experiment. A FITC-tagged anti-human TTR polyclonal antibody was used to detect TTR; DAPI stain allowed visualization of the cellular nuclei and Rhodamine phalloidin was used to observe F-actin filaments. TTR oligomers appeared to be contained within the cytoplasm in round, vesicular type strictures; this result yielded a punctate staining pattern. Images were taken using an Olympus BX60 scope. Scale bar represents 100 µm.
Figure 18. TTR-L55P oligomers aggregate within cells in a time-dependent manner. Rat cardiomyoblasts (H9C2) were exposed to (a) PBS vehicle, (b) L55P oligomers (4 h, 80 °C) for 24 hours, or (c) L55P oligomers (4 h, 80 °C) for 48 hours. Cell cultures were maintained at 37 °C in 5% CO₂ throughout the duration of the experiment. A FITC-tagged anti-human TTR polyclonal antibody was used to detect TTR; DAPI stain allowed visualization of the cellular nuclei and Rhodamine phalloidin was used to observe F-actin filaments. By fluorescent microscopy, we observed cellular uptake of TTR oligomers by 24 hours; at 48 hours, internalization appeared to be continuous as an increased amount of TTR was visualized. All images were taken at 10x magnification using an Olympus BX60 scope. Scale bar represents 100 µm.
4.4 Oligomeric transthyretin induces alterations in human cardiac cell stress markers

A collaborative effort with the laboratory of Dr. George Murphy provided the opportunity to enhance our cell-based studies. Using a patient-specific disease model, we were able to further our testing of the effects of amyloidogenic forms of TTR on human cardiac cells. Dr. Murphy and colleagues have developed a method of establishing patient-specific cell lines from mature somatic patient cells such as skin or peripheral blood[121]. Specifically, somatic cells are initially reprogrammed into pluripotent cells using a set of four transcription factors (Oct4, Klf4, Sox2, cMyc) that are introduced using retroviral vectors. Following this step, the aforementioned factors can be excised resulting in induced pluripotent stem cells (iPSC) that share many characteristics with embryonic stem cells (ESC). Cardiac cells can then be generated from the iPSC lines. The creation of cardiac cell lines from patient specimens represented an opportunity to further investigate the effects of amyloidogenic forms of TTR in a novel and cutting-edge experimental model.

To validate our data showing the cytotoxic effects of TTR oligomers on rat cardiac cells and to put our studies more in the context of ATTR amyloidosis, we sought the use of this human cell system. Fibroblasts obtained from an individual with L55P ATTR amyloidosis were reprogrammed into iPSCs and subsequently differentiated into cells that grow as cardiac embryoid bodies (cEBs) using a seven day protocol as described in Chapter 2. In addition, iPSCs derived from a healthy human subject (coded as BU6) were studied and served as a non-ATTR control. Similar to the rat cardiomyoblast
experiments, we treated cEBs with either heat-denatured wt or oligomeric L55P; in addition, heat-denatured BSA served as a negative control for these studies. After a 3-day exposure to protein samples, the cEBs were lysed and RNA was extracted from the cell lysates using a Qiacube automated RNA isolation protocol. After RNA quantification, cDNA was synthesized and prepared for qPCR experiments. For our qPCR analysis, several stress and cell cycle markers were analyzed and included matrix metallopeptidase 9 (MMP9), cardiac troponin (cTnT), heme oxygenase 1 (HO1), heat shock protein 27 (Hsp27), and p21. Each of these markers has been implicated in cell stress and/or cardiac amyloid pathology as detailed in the following paragraph. Data was normalized to GAPDH expression and triplicate measurements were performed.

MMP9, also known as gelatinase B, is an enzyme that can degrade the extracellular matrix (ECM) and is involved in myocardial remodeling[124], [125]. Reactive oxygen species (ROS), as well as myocardial infarction, have been shown to increase MMP9 expression[126]. Troponin expression has been shown to be increased in the failing heart[127] and circulating troponin levels were found to be elevated in ATTRwt patients[56]. HO1, a stress response protein, has been shown to be induced in response to stress and increased expression of the enzyme is considered to be protective[128], [129]. Increased expression of Hsp27 has been inversely correlated with cell proliferation[130], shown to inhibit apoptosis during chemical stress, and reported to enhance degradation of ubiquitinated protein[131]. P21 expression is induced upon DNA damage through both p-53 dependent and independent transcription activation pathways. Overexpression of p21 inhibits cell proliferation and promotes cell cycle arrest[132].
Treatment of the control cEBs (BU6) with native and heat-denatured forms of wt and L55P TTR samples yielded varying, but non-significant differences in the expression analyses. It is interesting to note that MMP9 levels for all samples were higher than the vehicle (PBS) control; this could imply that exposure to any TTR induced a response or pathway that led to elevated amounts of MMP9. In addition, cardiac troponin seemed to be negatively affected by the exposure to TTR-L55P regardless of oligomeric status (Figure 19). Unfortunately, due to a limited number of experimental data points, we were not able to perform statistical analysis to determine the significance of these findings.

The stress marker, HO1, did not show any significant changes in expression level when comparing the different treatments. Even though HO1 levels in the PBS and wt TTR (80 °C for 0h) samples appeared to be lower, these were single measurements and therefore, we could not draw any significant conclusions. Hsp27 expression was higher across all treatments compared to the vehicle control, but these were data from a single experiment and limit our interpretation. Similarly, p21 showed no changes amongst treatments; both wild-type and L55P proteins elicited similar expression levels. These data may indicate that healthy cEBs were minimally affected by the presence of native or amyloidogenic forms of TTR.

In addition to studying healthy subject-derived cEBs, we repeated these experiments in cEBs derived from an individual with ATTRm amyloidosis, heterozygous for the L55P mutation. Cells were treated with wt and L55P forms of TTR as previously; the effect of aggregated BSA (80 °C for 4 h) was also studied. Expression levels were
normalized to GAPDH and all samples were analyzed in triplicate. The results are shown in Figure 20. As expected, both PBS and BSA treatment translated to the similar levels of expression across all five markers except in the case of Hsp27. This observation may be attributed to the lack of multiple measurements of the BSA time point. Even though multiple experiments were run and treatments were run in duplicate, low RNA yield led to the loss of several of the replicates. Since BSA aggregates were considered a negative control, we expected these levels to be comparable to vehicle treatment.

Cells treated with native wt TTR (80 °C for 0h) showed a slight increase in MMP9 expression; however, due to the data variation, as indicated by the error bars on the graph, the validity of this result remains undetermined and whether this increased expression is indeed a significant cellular response warrants further study. Troponin, Hsp 27, and p21 expression levels did not appear to change following cell exposure to native, denatured and oligomeric forms of wt and L55P proteins. These data imply that early stage forms of non-native and oligomeric TTR do not induce cellular stress at a level or in a mechanism that can be followed with these markers.
Figure 19. Stress marker expression levels in control human cardiac cells vary in response to transthyretin treatment.

Expression levels of MMP9, troponin, HO1, Hsp27, and p21 in control cEBs (BU6) following exposure to vehicle (PBS), native wt TTR (untreated, 80 °C for 0 h), heat-denatured TTR (80 °C for 4 h), native L55P TTR (untreated, 80 °C for 0), or oligomeric L55P TTR (80 °C for 4 h) were measured by qPCR. Samples were run in triplicate, averaged, and subsequently normalized to GAPDH.
Figure 20. Stress marker expression levels in human ATTR-L55P cardiac cells vary in response to transthyretin treatment.

Expression levels of MMP9, troponin, HO1, Hsp27, and p21 in ATTR-L55P cEBs following exposure to vehicle (PBS), native wt TTR (untreated, 80 °C for 0 h), heat-denatured TTR (80 °C for 4h), native L55P TTR (untreated, 80 °C for 0), or oligomeric L55P TTR (80 °C for 4h) were measured by qPCR. Samples were run in triplicate, averaged, and subsequently normalized to GAPDH.
Interestingly, an increased expression of HO1 was observed for cells exposed to oligomeric TTR-L55P (80 °C for 4h, Figure 20). This result was particularly striking since all other treatments yielded near equal or lower expression levels for the native (80 °C for 0h) and denatured or oligomeric (80 °C for 4h) forms of TTR, respectively. This increased expression could indicate an early stage of the stress response in these cells, as HO1 is considered an early and high sensitivity stress response marker[133], [134]. Significance however remains to be determined as multiple measurements are needed.
4.5 Summary and conclusions

The thermal denaturation of wild-type and variant TTR proteins led to formation of non-native and early stage oligomers which we were able to characterize by SDS-PAGE analyses. Oligomerization was observed with the L55P, V30A, and V122I TTR proteins which all showed decreased tetramer content and concomitant increases of TTR monomer and oligomers following 4 hours of heating at 80 °C. The most dramatic results were obtained with the L55P variant which is widely held to be an extremely unstable variant[47]. Conversely, wild-type TTR was only slightly denatured following heat treatment.

Exposure of H9C2 rat cardiomyoblasts to denatured forms of TTR led to a significant decrease in cell viability, especially in cells treated with oligomeric TTR-L55P and TTR-V30A. Interestingly, even though V30A and V122I samples from the heat denaturation assay appeared to contain equivalent amounts of oligomers, based on SDS-PAGE analyses, the V122I oligomers did not affect cell viability; this was in sharp contrast to the V30A results. These observations are consistent with several previously reported studies (discussed in Chapter 1) which implied that variants with lower stabilities in biochemical and biophysical in vitro studies are featured in ATTRm amyloidosis with earlier ages of onset and more aggressive disease progressions. As was shown in Table 1, TTR-V30A and TTR-L55P are associated with very early ages of onset compared to most other amyloidogenic TTR variants. Conversely, wild-type and V122I demonstrate a high degree of tetrameric stability and these TTR proteins are linked to amyloid diseases that feature much later ages of onset, usually beyond 60 years.
Results from our SDS-PAGE analysis and cell viability studies were consistent with the proposed correlation between TTR tetramer stability and disease phenotype.

We expanded our cellular studies to explore internalization of TTR oligomers as a cause of cytotoxicity. Indeed, we observed high levels of oligomeric L55P internalization. Interestingly, this cellular uptake of TTR seemed to be time-dependent; TTR aggregation appeared to be further propagated once the protein was within the cell, a process that was monitored over the course of 48 hours. This could also explain why even after 48 hours, some cells contained more aggregates compared to others; in a subset of cells, a more efficient seeding and aggregate growth process may have occurred. We also observed a distinct granular and vesicular staining pattern of internalized L55P oligomers when viewed at higher magnification (40x). A particularly striking observation was the apparent expansion of L55P aggregates from initial, localized, smaller inclusions within the cell cytosol to large globules present throughout a majority of the cytoplasm. This progressive infiltration was so extensive that entire cells appeared to be saturated with oligomeric TTR.

Based on the results in our rat cardiomyoblast model, we concluded that only the L55P oligomers, due to their extreme amyloidogenic nature, were recognized and internalized by cells. Once internalized, the oligomers further associated to form aggregates of increasing size, either within an endosome or lysosome. Moreover, this process was continuous and ultimately yielded a high concentration of aggregates that were cytotoxic, led to cell death, and resulted in the lowered cell viabilities observed in our studies.
In the studies of healthy subject-derived cardiac emboid bodies (BU6) treated with TTR oligomers, we observed little to no change in the expression levels of several stress and cell cycle markers. Some fluctuations in MMP9 expression were noted in cells treated with denatured forms of TTR when compared to the PBS vehicle control. Furthermore, cardiac troponin expression appeared to be lower in cells treated with both native and oligomeric TTR-L55P. Alternatively, in studies of the patient-derived ATTR-L55P cardiac emboid bodies, treatment with oligomeric L55P led to a striking increase in HO1, indicating that a stress response had been triggered in these cells.

The gene expression level data were less dramatic and striking compared to the cell viability results obtained in the studies of rat cardiomyoblasts. This could be partially attributed to the fact that cEBs are a more robust cell type, and are grown as very dense three-dimensional cultures. The 3-dimensional nature of this cell culture system (versus the monolayer grown cardiomyoblasts) may impede uptake of L55P TTR oligomers, i.e. make it difficult for TTR oligomers to penetrate the cellular masses. Thus, we would expect that a cytotoxic effect would be attenuated. In contrast, a monolayer of rat cardiomyoblasts should be much more susceptible to the cytotoxic effects of TTR proteins; hence, one would predict that a stronger response would be elicited from cells that are more accessible and vulnerable to treatment.
CHAPTER 5. POTENTIAL INHIBITORS OF TRANSTHYRETIN CARDIOTOXICITY

5.1 Introduction

Having demonstrated that some variant TTR oligomers cause a cytotoxic response in cardiac cells, we wished to study several chemical and biological agents as inhibitors of this negative effect. We chose to focus on three small molecule drugs, diflunisal doxycycline, and Kiacta®; in addition, we investigated the ability of retinol-binding protein (RBP), a natural binding partner of TTR, to block TTR cardiotoxicity.

Diflunisal was studied based on previously published *in vitro* work demonstrating the ability of the drug to stabilize native (tetrameric) TTR through binding in the T₄ attachment sites[75], [135] and encouraging results from a recent clinical trial[28]. As previously described (Chapter 1), the antibiotic doxycycline has been shown to disrupt TTR fibrils in both FAP and AL mouse models[80], [84], [87]. To our knowledge, doxycycline has never been studied in TTR aggregation models and has only been considered as a fibril disruptor. We chose to study the effect of this drug on early protein aggregation, encouraged by one previous report that suggested a potential role for doxycycline as an inhibitor of αB crystallin aggregation[80]. Moreover, concurrent with the cellular studies detailed in Chapter 4 and in parallel with biophysical analyses performed in our laboratory, the opportunity to explore the inhibitory potential of a novel compound, Kiacta®, arose through an ongoing collaboration with the pharmaceutical company, Neurochem Inc. Kiacta® had formerly been shown to inhibit fibril
polymerization and amyloid deposition[81]. The effects of the compound on TTR aggregation and cytotoxicity were unknown.

Our investigations also included studies aimed at exploring the role of RBP, a natural binding partner of circulating TTR, in ATTR amyloidosis. As mentioned (Chapter 1), RBP4 is the only secreted RBP isoform and normally is present in the bloodstream complexed to TTR; the binding of RBP4 to TTR stabilizes the native form (tetramer) of the latter protein and prevents disassembly into monomers[96], [97]. These data suggest that the biological role of RBP4 directly affects TTR amyloidogenicity; indeed, RBP4 may act as an anti-amyloid agent. A perturbation of the RBP4-TTR association might offer a partial explanation of why ATTR disease usually features delayed onset of symptoms. Thus, we sought to characterize RBP4 in ATTR amyloidosis to further define the mechanism of disease.

5.2 Doxycycline inhibits transthyretin-induced cytotoxicity

The ability of doxycycline to block the toxic effect of oligomeric TTR on cardiac cells was tested. Briefly, TTR proteins (0.4 mg/mL) were thermally denatured for 4 hours at 80 °C in the presence of 50x molar excess doxycycline. As before, we included untreated (0 hour, no heat treatment) and 4-hour heat-treated TTR samples for comparison. Sample solutions were mixed 1:1 with complete medium and applied to the rat cardiomyoblast (H9C2) cultures; the cells were treated for 72 hours and then assessed for cell viability. Viability was measured using the ApotoxGlo assay (Promega) and data were normalized to vehicle control. Samples were tested in triplicate and each experiment was repeated three times. Data from all experiments were averaged and
significance calculated by two-tailed Student’s t-test and Mann-Whitney post-test. A p-value < 0.05 was considered significant.

The results of these experiments are represented by histograms and shown in Figure 21. Cells treated with TTR-L55P or TTR-V30A thermally denatured in the presence of doxycycline (80 °C for 4 h + Dox), showed a significant improvement in cell viability compared to cells exposed to oligomers formed in the absence of drug (80 °C for 4 h). P-values were equal to 0.0357 for both comparisons (TTR-L55P or TTR-V30A vs. TTR-L55P or TTR-V30A + Dox). While the damaging consequence of oligomeric L55P on cells appeared to be lessened by doxycycline, the presence of drug did not totally abolish the effect (90% of control). However, while TTR-V30A oligomers decreased cell viability, the presence of doxycycline abrogated this effect as demonstrated by cell viability (80 °C for 4 h + Dox) nearly equal to the control group (80 °C for 0 h). In contrast, doxycycline showed no effect in the cells treated with the wt or V122I TTR proteins.

These results suggested that the presence of doxycycline in TTR solutions during the thermal denaturation process either partially prevented or slowed the formation of toxic oligomers species. Alternatively, doxycycline may have induced the degradation, disassembly, or restructuring of the oligomeric species thus, rendering them no longer toxic to cells. The ability of doxycycline to block cytotoxicity appeared to be TTR variant specific, as little to no effect was observed in cells treated with either the wt or V122I TTR samples.
Figure 21. Doxycycline abrogates the cytotoxic effect of some transthyretin variants.

Rat cardiomyoblast (H9C2) cells were treated with staurosporine (SSP), native TTR (80 °C for 0 h), or oligomers (80 °C for 4 h); final TTR concentrations were 0.2 mg/mL. Viability measurements of cells after 72 hours of treatment were performed using the ApotoxGlo (Promega) assay. Compared to the oligomer-treated cells (80 °C for 4 h), cells exposed to TTR-L55P or TTR-V30A samples with 50x molar excess doxycycline (80 °C for 4 h + Dox) showed significantly increased cell viability (p = 0.0357 for both comparisons). Results were normalized to vehicle control and are shown as averages of triplicate samples in three experiments. Significance was calculated by two-tailed Student’s t-test and Mann-Whitney post-test; p-value < 0.05 was considered significant.
5.3 Doxycycline partially inhibits formation of transthyretin oligomers

It was important to biochemically characterize the effect of doxycycline on the thermal denaturation of TTR. In parallel with the cell-based studies, we analyzed the oligomeric forms of TTR generated by heat treatment in the presence of doxycycline using chemical cross-linking and electrophoretic analysis. Briefly, TTR samples, with and without drug, were heated (80 °C) for 4 hours, cross-linked with glutaraldehyde, and analyzed by SDS-PAGE. As in other experiments, BSA was similarly treated and served as a control. The SDS-PAGE results for TTR and BSA are shown in Figure 22.

The presence of doxycycline during heat treatment of wild-type TTR (Figure 22a) did not prevent the loss of tetrameric TTR (T). Moreover, the unanticipated appearance of a high molecular weight (H) protein band (not observed in the adjacent lane containing thermally denatured TTR, no doxycycline) indicated that tetrameric TTR was less stable in solution with drug. One possible explanation for this finding may be inappropriate storage of the protein test solution; the wt TTR sample, to which doxycycline was added, had been stored at 4 °C for 4 hours. Sörgjerd et al. have shown that storage of TTR at 4 °C leads to aggregation of the protein[20]. Of note, these aggregated species of wt TTR, only faintly visible on the SDS-PAGE gel, were likely low in abundance or not cytotoxic as there was no detectable effect of this TTR on cell viability (Figure 21). In contrast, L55P oligomer formation was drastically reduced in the presence of doxycycline even though the drug did not prevent the loss of L55P TTR tetramer (T); the absence of tetramer and appearance of monomer (M) and dimer (D) suggested that native TTR had undergone nearly complete dissociation. The effect of
doxycycline in heated samples of V30A was loss of TTR tetramer (T) and dimer (D) content in the absence of high molecular weight aggregate (H) formation. TTR-V122I showed a decreased amount of high molecular weight oligomers (H) in the presence of doxycycline, as well as a loss of tetramer (T) and dimer (D); a slight increase in monomeric species (M) was also observed. Interestingly, the presence of doxycycline also seemed to prevent the aggregation of non-amyloidogenic BSA protein (Figure 22b).

Taken together, these data suggest that doxycycline does not bind to and stabilize tetrameric TTR, as monomers and/or dimers from dissociation of the intact protein were clearly visible. One inference from the results is that the drug interacts with non-native TTR intermediate conformations formed in the transition between monomer and high molecular weight species. Doxycycline appeared to promote tetrameric dissociation while preventing formation of the high molecular weight soluble oligomers possibly by stabilizing the monomers and interfering in self-association of the subunits.

Our hypothesis that doxycycline does not interact or bind to tetrameric TTR, but instead acts on the smaller monomeric and dimeric species, was supported by circular dichroism analyses (Figure 23). Using wild-type and L55P, mixtures of each TTR with doxycycline were analyzed for structural changes in the far UV range (195 – 250 nm). The spectral signal of each mixture was compared to a computed signal obtained by summing the separate CD signals of TTR protein and doxycycline. The mixture and calculated spectra for wt TTR and doxycycline were superimposable; a near perfect overlap of signals was also seen for L55P and doxycycline. These data provided strong evidence that no secondary structural changes in TTR occurred when drug was present;
these results imply that there was no interaction between tetrameric TTR and doxycycline.
Figure 22. The presence of doxycycline during thermal denaturation of transthyretin partially prevents formation of high molecular weight oligomers.

The characterization of TTR dissociation following 4 hours at 80 °C in the presence or absence of 50x molar excess doxycycline (Dox) was accomplished using SDS-PAGE. From left to right, wt TTR showed tetramer (T) loss and a slight increase in oligomers (H) in the presence of drug. L55P showed complete abolishment of high molecular weight species (H). Both V30A and V122I showed a decrease in dimer (D) and tetramer (T) content. (b) BSA in native (B) and high molecular weight (H) forms are shown before (0) and after (4) heating; heating in the presence of doxycycline resulted in a diminished amount of large aggregates (H). Protein samples were run on 10% SDS-PAGE gels and stained with Coomassie blue.
Figure 23. Doxycycline and tetrameric transthyretin do not interact as evidenced by circular dichroism spectroscopy.

Far-UV spectra were generated for mixtures of doxycycline and TTR, wild-type TTR (top panel), and TTR-L55P (bottom panel); monitoring was performed at room temperature between 195 and 250 nm. TTR solutions were 0.2 mg/mL in PBS and contained 50x molar excess of doxycycline. Additive signals (blue) were computed by summing the individual TTR and doxycycline CD signals. The near indistinguishable overlap in the additive and mixture curves for both wt and L55P demonstrates little to no interaction between doxycycline and native (tetrameric) TTR.
5.4 Internalization of transthyretin oligomers is inhibited by doxycycline

In order to further understand how doxycycline lessened the cytotoxic effect of oligomeric TTR, we extended our internalizations studies to include samples with and without drug. Briefly, rat cardiomyoblast (H9C2) cells were treated with TTR protein (0.2 mg/mL) that had been thermally denatured in the presence (50x molar excess) or absence of doxycycline. Following a 3-day exposure, the cells were fixed, permeabilized, and stained for TTR oligomers, nuclei, and F-actin filaments using FITC-tagged anti-human TTR antibody, DAPI stain, and rhodamine phalloidin, respectively.

As shown in Figure 24a, vehicle treated cardiomyoblasts appeared healthy with abundant F-actin filaments (red) and well-defined nuclei (blue); no TTR (green) was observed. As in prior experiments, cells treated with L55P oligomers showed heavy staining for TTR within the cells (Figure 24b); this result indicated that cellular uptake of TTR oligomers had occurred. Retention of TTR oligomers within the cells appeared to be in vesicular type structures or organelles. In stark contrast, the cells that were exposed to the thermally denatured preparations of L55P and doxycycline (Figure 24c) showed little to no TTR oligomers within the cells.

These results with drug were consistent with our previous studies showing that the presence of doxycycline drastically reduced the formation of L55P oligomers by thermal denaturation. It seems reasonable to assume that TTR internalization was not detected due to the scarcity of oligomers in the treatment sample as a consequence of doxycycline. In addition, this effect of doxycycline translated to an improvement in cell viability when present with TTR. Taken together, the cell viability, electrophoretic, circular dichroism,
and fluorescence microscopy data suggest that 1) doxycycline inhibits the formation of high molecular weight oligomers of TTR-L55P possibly through stabilizing interactions with the monomers, and 2) protects cells from the cytotoxic effect of TTR aggregates which may be mediated through aggregate internalization.
Figure 24. Presence of doxycycline during thermal denaturation significantly reduces internalization of TTR-L55P oligomers.

Rat cardiomyoblasts (H9C2) were treated with (a) vehicle, (b) L55P previously heated for 4 h at 80 °C, or (c) L55P + doxycycline previously heated for 4 h at 80 °C; the duration of treatment was 72 hours during which cells were incubated at 37 °C in 5% CO₂. TTR oligomers (green) appear to be contained within the cytoplasm in round, vesicular type structures which yields a punctate staining pattern. Results for TTR (green), nuclei (blue), and F-actin (red) are shown. Images were taken at 40x magnification using an Olympus BX60 scope. Scale bar represents 100 µm.
5.5 Kiacta® and diflunisal do not inhibit transthyretin-related cytotoxicity

As with the studies of doxycycline, we sought to determine whether Kiacta® and diflunisal could block the cardiotoxic effects of oligomeric TTR. Briefly, rat cardiomyoblast cells were seeded in 96-well plates and exposed to wt or L55P TTR proteins (0.2 mg/mL), that had been previously heated (80 °C) for 0 or 4 hours in the presence (10x or 100x molar excess) or absence of drug. Results obtained from the cell viability experiments, shown in Figure 25, demonstrated that Kiacta® had no effect on cells treated with the wt or L55P TTR samples. In addition, it appeared that the presence of diflunisal in the L55P oligomer samples (80 °C for 4 h + diflunisal 1:10 or 1:100) actually had a slightly adverse effect on cell viability. The diflunisal data are most likely a consequence of structural changes in the drug induced by the 80 °C heat treatment; indeed, we found published thermal analysis data demonstrating that decarboxylation of diflunisal occurs upon heating[136].

It is important to note that neither heat-denatured wt nor L55P TTR (80 °C for 4 h) elicited a change in the viability of the cardiac cells. This did not appear to be a technical problem with the assay as the cells treated with staurosporine (SSP), a positive control for apoptosis, showed a dramatic decrease in cell viability, i.e. a reduction in viability of almost 90%. Furthermore, the absence of decreased cell viability in cells treated with L55P oligomers (80 °C for 4 h), as had been observed in repeated earlier experiments, was unexpected. This result may have been due to the use of cells that were passaged a greater number of times and over a longer period of time, compared to cells used in the previous experiments. We concluded that this cell line, H9C2, undergoes
alterations as passage number increases, i.e. the cells become more desensitized over time. Our conclusion is strengthened by other studies with H9C2 cells that use consistently low passage numbers in cell death and apoptosis experiments[137], [138].

Biochemical analyses of the wt and L55P samples, heat-denatured in the presence or absence of Kiacta®, were performed using SDS-PAGE (Figure 26). With wild-type TTR, no differences were observed in the samples with and without drug. Interestingly, we did observe an effect of Kiacta® in the L55P TTR oligomer (H) sample. TTR-L55P, heated for 4 h in the absence of Kiacta®, contained high molecular weight oligomeric species (H), as previously demonstrated. However, when TTR-L55P was thermally denatured in the presence of 50x molar excess of Kiacta®, little to no high molecular weight species were apparent on the SDS-PAGE gel. These observations imply that Kiacta® may, in fact, inhibit the formation of TTR-L55P oligomers.
Figure 25. Effect of Kiacta® and diflunisal on the transthyretin-induced cytotoxic response of cardiac cells.

Rat cardiomyoblast cells were treated with 0.2 mg/mL TTR (wild-type or L55P), that had been previously incubated for 0 or 4 hours at 80 °C in the presence or absence of Kiacta® or diflunisal (1:10 or 1:100 molar ratio of protein to drug). No changes in viability were noted in cells treated with the heated (80 °C for 4 h) wild-type TTR alone or in the presence of either concentration of Kiacta®; the results for L55P were similar. For cells treated with samples containing diflunisal, a decrease in cell viability was observed; however, this result was most likely due to heat-induced structural changes in diflunisal which likely altered the properties of the drug. Cell viability is shown as % of control (PBS). Staurosporine (SSP) served as a positive control for apoptosis.
Figure 26. Kiacta® inhibits transthyretin oligomer formation.

Wild-type and L55P TTR protein samples were heated for 4 hours at 80 °C in the presence or absence of Kiacta® at 50x molar excess. Samples were cross-linked with 25% glutaraldehyde and analyzed by SDS-PAGE (10%). In the wild-type samples with and without Kiacta®, no significant differences were noted; tetrameric (T) TTR was most abundant with little evidence of the high molecular weight (H) form. Analysis of the TTR-L55P samples with and without drug suggested that the presence of Kiacta® inhibited formation of TTR oligomers as a decrease in the high molecular weight (H) species was observed.
5.6 Retinol-binding protein slows the rate of transthyretin aggregation

Retinol-binding protein (RBP) is a natural partner of TTR and normally circulates as a ternary complex of TTR-RBP-vitamin A[89]. Previous studies have shown that RBP, in the absence of vitamin A and referred to as apoRBP, reportedly has a lowered affinity for TTR compared to holoRBP, i.e. RPB complexed to retinol[139]. This may, in part, be due to conformational changes that occur in RBP upon binding to vitamin A making the protein structure of RBP more favorable for interactions with tetrameric TTR. Raghu et al. have reported a much higher binding affinity of holoRBP for native TTR compared to apoRBP (8×10^{-6} (K_{d}) M^{-1} vs. <8×10^{-6} (K_{d}) M^{-1} to no binding)[29]. This information, along with data showing that RBP stabilizes the tetrameric form of TTR[97], prompted us to investigate the effect of RBP on in vitro TTR aggregation. We hypothesized that holoRBP would inhibit formation of oligomeric TTR.

To test our theory, we analyzed solutions of TTR with and without holoRBP; holoRBP was prepared immediately prior to these experiments by incubation of the protein in an excess of vitamin A (all-trans retinol). Protein solutions of TTR-L55P, holoRBP, and TTR-L55P + holoRBP were prepared in PBS and heated to 80 °C for 1 hour. The mixture containing TTR-L55P and holoRBP was prepared with a 1:1 molar ratio of the two proteins to represent the physiologic situation. TTR-L55P was chosen for these experiments because it is a highly unstable variant. Whereas wild-type TTR has a melting temperature > 98 °C, L55P will begin to unfold at 65 °C. Thus, we felt that TTR-L55P would be easily and more rapidly destabilized, and most appropriate for our in vitro studies. To assess progression of TTR aggregation, we monitored protein
absorbance changes in aliquots removed at 10 minute intervals from each sample, over the course of 60 minutes (1 hour). Absorbance was measured at two wavelengths, 280 and 340 nm. For each sample, the aggregation rate was represented as an aggregation index (AI) and calculated with the following formula:

\[ AI = 100 \times \left( \frac{A_{340nm}}{A_{280nm}} - A_{340nm} \right) \]

where \( A_{340nm} \) and \( A_{280nm} \) are absorbance values recorded at 340 and 280 nm, respectively.

The results of this experiment are shown in Figure 27. The data demonstrate that the TTR-L55P (black) had the quickest rate of aggregation, as was expected; by 20 minutes, the AI was approximately equal to 3 and increased to 4 by 60 minutes. For holoRBP (red), rapid aggregation was also observed with an AI of about 2 by 20 minutes and 3.5 by 60 minutes. This was an anticipated result as holoRBP is a small protein which is denatured at 80 °C. In contrast, the aggregation rate for the 1:1 mixture of TTR + holoRBP (blue) was significantly lower than either protein alone, with AI = 1.5 at 20 minutes and AI = 2 by 60 minutes.
Figure 27. Stabilization of transthyretin by retinol-binding protein during thermal denaturation.

Solutions of TTR, holoRBP, and TTR + holoRBP (1:1 molar ratio) were heated for 60 minutes at 80 °C to induce protein aggregation. Protein absorbance measurements were recorded on aliquots removed at approximately 10 minute intervals from each sample. The rate of aggregation was expressed as an aggregation index (AI), calculated from absorbance measurements recorded at wavelengths of 280 and 340 nm. TTR (black) showed the quickest rate of aggregation, AI = 3 at 20 min and AI = 4 at 60 min; holoRBP (red) also demonstrated aggregate formation, but at slower rate than TTR (AI = 2 at 20 min and AI = 3.5 at 60 min). In contrast, the aggregation rate in the 1:1 (molar ratio) mixture of TTR:holoRBP (blue) was significantly lower with AI = 1.5 at 20 min and AI = 2 at 60 min.
5.7 Retinol-binding protein is not complexed to transthyretin in cardiac amyloid deposits

Our *in vitro* data provided evidence that RBP slowed the aggregation of thermally-denatured TTR; this finding implied that RBP was stabilizing native TTR. Therefore, we hypothesized that TTR found in amyloid deposits would no longer be bound to its natural and stabilizing binding partner, holoRBP. To investigate our theory, we performed immunohistochemical staining for RBP and TTR in autopsied heart samples heavily infiltrated with TTR amyloid. Formalin-fixed, paraffin-embedded cardiac tissues were sectioned and used in these analyses; the presence of amyloid deposits in all tissue sections was confirmed by Congo red staining and resultant green birefringence by polarized light microscopy.

Specifically, we used serial sections of autopsied heart tissue from a patient who had been diagnosed with ATTR-L58H amyloidosis (*Figure 28*). In a Congo red treated section, heavily stained areas of tissue were observed by standard light microscopy (*Figure 28a*); a polarized view of the same field (*Figure 28b*) exhibited ‘apple green’ birefringence thus, confirming the presence of amyloid in the section. Immunohistochemical staining of a serial section with anti-human TTR antibodies (*Figure 28c*) demonstrated that TTR was the amyloid protein constituent of the deposits. For our RBP analyses, we tested several anti-human RBP antibodies. Initial attempts at immunohistochemical staining rendered results that suggested non-specific antibody binding to the tissue. One antibody preparation, appeared to recognize the cellular isoform (RBP1, also known as CRBP) and not the serum isoform (RBP4) which we were
investigating (Figure 28d). Of note, the cellular form has been shown to be strongly up-regulated during myocardial stress[140]. Subsequent testing was performed with an RBP4-specific antibody (Abcam, ab48624); the immunohistochemical results with this antibody showed minimal cellular staining (little background) and there was no evidence of RBP in TTR amyloid deposits (Figure 28e). Thus, our immunohistochemical staining using antibodies against RBP4 and TTR demonstrated that TTR was present in the amyloid deposits, whereas RBP4 was absent. This finding indicated that the two proteins were no longer associated and implies that the formation of TTR amyloid may, in part, be due to a decrease in the stabilizing effect of RBP on native (tetrameric) TTR.

5.8 Serum retinol-binding protein is present in amyloid-infiltrated heart tissue

Using an antibody that specifically recognized serum RBP4 and did not react with other cellular isoforms of the protein, we extended our immunohistochemical analyses to include heart tissue sections from cases of ATTRwt and AL amyloidosis. Liver tissue sections from a patient with AL amyloidosis were used as positive controls since the liver is the main production site of RBP4; transplanted heart tissue sections from a patient with ATTR-T60A amyloidosis who had undergone cardiac transplantation and received a ‘non-amyloid’ heart eight years prior to death were used as a ‘non-amyloid’ control. As expected, our positive control (liver tissue) stained strongly for RBP4 (Figure 29a) and could be easily observed at 10x magnification. Although our negative control (Figure 29b), a section not treated with primary antibody, appeared negative to the naked eye, we did observe a faint background staining which was granular in appearance at 40x magnification (Figure 29d). The brown stained material was identified as lipofuscin
(with expert input from Dr. Carl O’Hara), a cellular waste resulting from oxidative reactions that accumulates within the lysosomes; the presence of lipofuscin is considered a hallmark of aging[141], [142]. Lipofuscin staining was observed in ATTRm (Figure 29f, i) and AL heart tissue sections (Figure 29h, k). Interestingly, RBP4 staining was noted in the transplanted ‘non-amyloid’ heart (Figure 29c); at 40x magnification (Figure 29e), this staining for RBP4, not due to lipofuscin, appeared to be located in the cytoplasm of the myocytes. Particularly intriguing was the appearance of localized areas of staining, in regions of slight collagen infiltration and early signs of ischemia.

In addition to the transplant heart, wild-type ATTR patient tissue also stained strongly for RBP4 around amyloid infiltrated vasculature (Figure 29 g, j). Further histologic analysis of the ATTRwt heart tissue was performed (Figure 30a). Amyloid deposition in the vasculature was confirmed by Congo red staining in near serial sections of the tissue (Figure 30b, c). Of note, all sections shown in Figure 30 were from the same tissue block; however, the section used for RBP4 immunohistochemical testing (Figure 30a) was an earlier cut in the series, whereas Congo red staining (Figure 30b) was performed on a section cut approximately 50 um later. The tissue architecture in these sections was not identical; however, based on the location within the tissue, it seemed likely that we were viewing the same blood vessel in the center of the image. As with the transplanted heart tissue section, the ATTRwt tissue appeared to exhibit early hallmarks of ischemia characterized by wavy fibers and variation in cell size and shape.

Our results suggest that these two hearts, possibly infiltrated by early-stage wild-type
TTR amyloid, appear to have RBP4 present in tissue directly surrounding the infiltrated area.
Figure 28. Histological and immunohistochemical analyses of ATTR-L58H heart tissue.

Serial sections of autopsied heart tissue from a patient with ATTR-L58H were stained with Congo red and viewed by (a) standard and (b) polarized light microscopy. Proof of amyloid was demonstrated by the appearance of (a) salmon-colored deposits that exhibited (b) green birefringence on polarization. Immunohistochemical staining with (c) anti-human TTR antibody confirmed the presence of TTR in the amyloid deposits, whereas (d) anti-human RBP1 antibody treatment showed strong reaction with intracellular protein, and (e) anti-human RBP4 antibody staining was negative in the deposits indicating that there was no co-localization of RBP with TTR amyloid. All images are at 10x magnification. Scale bar represents 100 µm.
Figure 29. Immunohistochemical testing for serum retinol-binding protein in control and amyloid tissues.

Tissue sections of autopsied liver and heart tissues were analyzed for the presence of RBP4 by immunohistochemical staining. Shown are (a) positive control – liver tissue, the site of RBP4 production, (b) negative control – heart tissue not treated with primary RBP4 antibody that showed faint granular staining identified as lipofuscin, (c) transplanted heart tissue exhibiting strong staining for RBP4 located in an area with collagen infiltration, (d) negative control boxed area in panel (b) at 40x magnification, (e) transplanted heart tissue boxed area in panel (c) at 40x magnification, (f) ATTRm heart staining negative, (g) ATTRwt heart staining positive around vessel, and (h) AL heart staining negative. Unless otherwise indicated, images are shown at 10x magnification. Scale bars represent 100 µm.
Figure 30. Histological and immunohistochemical analyses of ATTRwt heart tissue.

Near serial sections of autopsied heart tissue from a patient with ATTRwt amyloidosis were stained with Congo red and compared to the previous RBP4 immunohistochemical testing results. (a) Immunohistochemical analysis (from Figure 29j) for RBP4 indicated the presence of the protein in areas proximal to a blood vessel. (b) Congo red staining showed strong areas of dye binding around the myocytes and blood vessel by standard light microscopy and (c) green birefringence by polarized view. Images are 10x magnification.
5.9 Serum retinol-binding protein concentrations are varied in ATTR amyloidosis

Based on our immunohistochemical evidence that TTR protein in ATTRm amyloid deposits was not bound to RBP4, we theorized that RBP4 levels in ATTR sera would be decreased and correlated to increased amounts of unbound TTR. Abnormally low serum levels of RBP4 in patients with TTR-associated amyloidosis could potentially explain increased native TTR destabilization leading to the formation of TTR amyloid fibrils.

With samples from the Amyloidosis Center serum repository obtained under protocols (IRB# H-22838 and H-28609) approved by the Boston University School of Medicine Institutional Review Board, we developed an ELISA to measure serum RBP4 concentrations in several patient and control groups. Plate to plate reproducibility was confirmed and inter-and intra-plate variability values (CV) were below 10. The goal was to assess and compare the serum levels of RBP4 in the following age-matched groups: 1) healthy controls, 2) ATTRwt (wild-type TTR-associated) amyloidosis, 3) ATTRm (mutant TTR-associated) amyloidosis featuring cardiomyopathy (CMP), 4) AL (immunoglobulin light chain-associated) amyloidosis with CMP, and 5) non-amyloid with CMP (sera kindly gifted by Dr. Flora Sam). The results of the ELISA measurements are shown in Figure 31. RBP4 levels were significantly higher in the ATTRwt (47,000 vs. 38,000 ng/mL, p = 0.03) and AL groups (50,000 vs. 38,000 ng/mL, p = 0.04) when compared to controls. In contrast, RBP4 was significantly lower in ATTRm with CMP (30,000 vs. 38,000 ng/mL, p = 0.02) compared to controls. Serum RPB levels were significantly different in ATTRm and AL (50,000 vs.30,000 ng/mL, p =
0.005), as well as in ATTRwt and ATTRm (47,000 vs. 30,000 ng/mL p > 0.0001). The CMP non-amyloid serum samples showed increased RBP4 serum levels compared to controls (41,000 vs. 38,000 ng/mL); however, due to low number of samples and large amount of error this difference was not deemed significant.

These data demonstrated varied concentrations of RBP4 among the amyloid groups, all significantly different compared to controls. It was particularly intriguing to us that the TTR-associated amyloidosis groups, ATTRwt and ATTRm, showed opposite trends in serum RBP4 levels. These results prompted us to ask whether these changes in RBP4 serum levels were reflected or correlated in any way to circulating TTR levels. Our aim was to measure TTR concentrations in the same sets of sera and the development of a TTR ELISA was undertaken.
Figure 31. Levels of retinol-binding protein in control and amyloid patient sera measured by ELISA.

Using an ELISA developed as part of this thesis research, RBP4 levels (ng/mL) were measured in serum samples from several age-matched groups: healthy controls, ATTRwt (wild-type TTR-associated) amyloidosis, ATTRm (mutant TTR-associated) amyloidosis featuring cardiomyopathy, AL (immunoglobulin light chain-associated) amyloidosis with cardiomyopathy, and non-amyloid with cardiomyopathy (CMP no amyloid) sera kindly provided by Dr. Flora Sam. Compared to the control group, RBP4 was significantly higher in ATTRwt (p = 0.03) and AL (p = 0.04), but significantly lower in ATTRm (p = 0.02). In addition, RBP4 levels were significantly different between ATTRwt and ATTRm (p < 0.0001), as well as ATTRm and AL (p = 0.005). Sera were measured in triplicate; n represents the number of sera tested in each group.
5.10 Transthyretin enzyme-linked immunosorbent assay development

To further understand the RBP4 data in the context of TTR amyloid diseases, we sought to measure circulating TTR levels in patient sera. Initially, two commercial kits (Abcam) were used in a pilot study of a small collection of samples; however, the inter-plate variability was incredibly high (CV > 20) and inconsistent (non-reproducible) inter-assay results were obtained for some, but not all, serum samples. Concentration values did appear to be within the accepted limits for serum TTR, 200 – 450 µg/mL for normal, < 200 µg/mL for ATTR[27], [51], and data from the commercial ELISA kit (Figure 32) showed a striking difference between the control and ATTRm sera (190,000 vs. 350,000 ng/mL, p = 0.04). However, the group numbers were low and are a limitation in this analysis. Concerned with the reliability of these data, we investigated other commercially available TTR ELISA kits and found that all required extremely high dilutions of serum (1:100,000); this would require numerous dilution steps and increase the probability of variability and no doubt increase chance of error. Thus, we attempted to establish an ELISA in our laboratory that would be accurate and reproducible in measurements of serum TTR concentrations.

The initial step in development of the TTR ELISA was validation of coating and detection antibody dilutions; optimal antibody concentrations for coating and detection were 1:500 and 1:8,000, respectively. Absorbances were measured at 465 and 570 nm. Moreover, we found casein to be the most efficient blocker, minimizing background signal to near-zero values. TTR, purified from pooled sera (Sigma) was used for standard curve construction; signal to noise ratios were > 100. A representative standard curve for
the TTR ELISA is shown in Figure 33. The data display the optimal characteristics for a standard curve; it is typical in sigmoidal shape with low background and allows for a sizeable distribution of concentration measurements, spanning an appropriate linear concentration range of 10-100 ng/mL between 0.5 and 3.0 absorbances. Surprisingly, the initial ELISA testing of patient sera yielded little to no TTR signal in any of the samples; low absorbance values indicated that TTR in the serum samples was not being captured and/or detected by the antibodies. Concentrations of the standard solutions containing purified TTR, however, were always accurately and reproducibly generated. We concluded that TTR, in a complex milieu like serum, is less available for immune complex formation possibly due to epitope masking or crowding effects that prevent antibody recognition.
Figure 32. Serum transthyretin levels in control and ATTR sera.

Using a commercially available ELISA kit (Abcam), TTR levels (ng/mL) were measured in serum samples (previously analyzed for RBP4, Figure 31) from several age-matched groups: healthy controls, ATTRwt (wild-type TTR-associated) amyloidosis, and ATTRm (mutant TTR-associated) amyloidosis featuring cardiomyopathy. Compared to the control group, TTR was significantly lower in ATTRm (p = 0.04). TTR levels in ATTRwt were higher than ATTRm and lower than control, but not significantly different from either group. Sera were measured in triplicate and triplicate values were averaged; n represents the number of sera tested in each group.
Figure 33. Transthyretin ELISA standard curve.

Standard curves were produced using commercially available TTR (Sigma) purified from pooled sera. Serial dilutions (dilution steps of 3x) of a stock solution (1 mg/mL) of TTR protein were made and run in triplicate. Absorbances were measured at 465 and 570 nm (background) and change in optical density (Delta OD) was calculated by subtracting background values. Representative of multiple standard curves that were generated, these data show low background and a sigmoidal distribution of values with linearity in the concentration range between 10 and 100 ng/mL. Standard protein was diluted in 0.1% casein buffer supplemented with Tween-20.
5.11 Summary and conclusions

Doxycycline

The presence of doxycycline nearly abolished internalization of TTR-L55P TTR oligomers. We concluded that this was due to significant inhibition of TTR-L55P aggregation and oligomer formation by the presence of 50x molar excess drug. Doxycycline appeared not to interact with the native wt or L55P tetrameric TTR, since our electrophoretic studies demonstrated that presence of doxycycline did not inhibit denaturation of the native protein (tetramer dissociation). The drug did appear to halt monomeric aggregation and resultant progression into HMW formation. Our conclusion that doxycycline did not act on the tetramer was further strengthened by circular dichroism studies demonstrating doxycycline did not interact with native wt or L55P TTR tetramers.

Kiacta® and diflunisal

In our cell viability studies, we did not observe any significant effect of Kiacta® when present in the TTR samples placed on the cells. However, in this set of experiments, we did not observe the toxic effects previously noted with TTR-L55P oligomers. The discrepancy between experiments may lie in variable cell characteristics arising from increased passage number. Cells used in Kiacta® testing had been passaged 20 times more than those used in previous studies. We concluded that the higher passage number rendered the cells less sensitive to the toxic effect of oligomeric TTR. Interestingly, SDS-PAGE analysis provided evidence that Kiacta® did affect TTR-L55P
TTR oligomer formation by either preventing aggregation of the subunits and progression to oligomers or by degrading the oligomeric species formed.

In our studies with diflunisal, we found that the presence of the drug in the L55P TTR oligomer samples lowered cellular viability slightly. This effect most likely was the result of structural changes in the drug induced by heating of the samples at 80°C.

*Retinol-binding protein*

In aggregation studies, we showed that RBP slows the oligomerization of denatured TTR-L55P. By immunohistochemical analysis, we demonstrated that RBP4 protein was present and localized to specific areas in autopsied heart tissues from ATTRm (transplanted) and ATTRwt cases. In both of these tissue samples, we observed the RBP4 staining in areas characterized by early ischemic changes such as wavy fibers and collagen infiltration between cardiomyocytes. In the ATTRwt heart, RBP4 was observed in concentrated regions of the tissue surrounding an amyloid infiltrated blood vessel. In addition, in the ATTRm transplanted heart, no amyloid was detected by light microscopic examination of Congo red stained sections; however, the appearance of early stage collagen infiltration was noted which frequently is an early sign of ischemia and may indicate an area that eventually will show amyloid fibril deposition. We believe that the staining of RBP4 occurred in areas proximal to the blood pool (vasculature) where cardiac damage/remodeling could have indicated a site suitable for amyloid infiltration. We theorized that RBP4 presence in the periphery could point towards RBP4 transport of TTR to the heart and subsequent dissociation of the two proteins.
Our immunohistochemical findings were consistent with a recently published case report showing the evolution of ATTRwt disease in a 72 year old patient over a 15 year period[143]. Specifically, the patient was diagnosed with wild-type ATTR and received a ‘non-amyloid’ heart at transplant. Within eight years, the patient showed amyloid deposition which gradually increased over time. MRI also revealed intracardiac fibrotic remodeling in the interventricular septum and echocardiography confirmed septal wall thickening. Though we did not observe any amyloid infiltration in our ‘non-amyloid’ transplant heart sample, the observation of slight collagen infiltration led us to believe that, similar to the case report, this tissue had been in the process of undergoing cardiac changes which may have included early ischemia and amyloid infiltration.

By ELISA, we found that circulating RBP4 levels were significantly increased in ATTRwt and AL amyloidosis; lower concentrations were present in ATTRm amyloidosis featuring cardiomyopathy. A truer interpretation of these data likely will be possible with matching TTR concentrations and evaluation of TTR:RBP4 values in the patient sera. We believe that lowered RBP4 levels should correlate to higher amounts of unbound (RBP4-free) TTR, available to become destabilized and form amyloid fibrils. This hypothesis was partially supported by our immunohistochemical results demonstrating TTR deposits with no bound RBP4 in heart tissue from a case of ATTR-L58H (Figure 28).

The increased RBP4 levels in ATTRwt patient serum was a surprising observation. One explanation is that wild-type TTR, unlike the mutant forms, maintains a normal sequence that favors interactions with RBP4, thereby preventing elimination of
RBP4 through renal filtration and higher circulating levels of the protein. This might also partially explain why RBP4 was observed in ATTRwt, but not ATTRm (ATTR-L58H) heart tissue. TTR and RBP4 could have remained in complex longer and RBP4 could have transported the TTR to the heart tissue where it dissociated, allowing TTR to deposit and showing RBP4 in the periphery of the amyloid deposit.

**TTR ELISA development**

We were unable to measure TTR in patient sera as our attempts to establish an ELISA method were unsuccessful. We believe this was due, in part, to the complex nature of serum with TTR epitope masking from crowding effects or possibly through steric hindrance of bound RBP that shielded TTR protein from capture and detection with the ELISA antibodies. A steric hindrance effect has been suggested by Liz et al. in a report of *in vitro* studies showing RBP4 binding to TTR blocking a proteolytic site on the latter protein[144]. In addition, it has been well established that RBP4-TTR binding results in a highly stable complex; the strength of this interaction is evident in studies of RBP4-TTR showing that dissociation requires alkaline (pH 10) conditions and additionally that the complex is readily co-immunoprecipitated with both RBP and TTR antibodies[145].
CHAPTER 6. DISCUSSION

6.1 Transthyretin oligomers cause cellular toxicity through internalization and intracellular aggregation

The major goal of this work was to study the effect of amyloidogenic TTR on cardiac cells with a focus on soluble, aggregated TTR forms that were early stage precursors of the amyloid fibrils. The first task was to establish a method for creating TTR oligomers that was reproducible, time-efficient, and suitable for the planned cell-based studies. We sought a protocol that would induce TTR denaturation and aggregation under conditions that would nearly replicate the in vivo situation, i.e. physiologic pH, ionic strength, and TTR protein concentration. Furthermore, it was important that solutions with TTR oligomers be appropriate for direct delivery to the cells; thus, a method with limited sample manipulation was also required. To this end, we generated a rapid and effective protocol using heat treatment at 80 ºC to thermally denature and aggregate TTR proteins. Our method did not require the use of acidic (pH ≤ 4.5) buffers, a common condition used to form in vitro TTR oligomers and amyloid fibrils[47], [101]. Even though wild-type TTR exhibits extraordinary structural stability with a high melting point temperature (Tm > 98 ºC), we knew that partial perturbation of the quaternary structure of the protein, i.e. denaturation of the tetramer, was needed to promote amyloid oligomerization based on the published work of Kelly and Colon[101]. Using circular dichroism analysis, the authors demonstrated that dissociation of the tetrameric TTR was key, as secondary and most of the tertiary structure of the protein was retained during aggregation and amyloid fibril formation.
For our studies, we used highly purified recombinantly-generated TTR proteins. Wild-type and several TTR proteins associated with cardiac amyloid disease were tested. The mutant forms included L55P, V30A, and V122I. Using the thermal denaturation method of a 4-hour heat treatment at 80 °C, we generated oligomers for each type of TTR. We confirmed the formation of TTR oligomers as high molecular weight species (> 250 kD) present on SDS-PAGE gels. In addition, using Congo red shift measurements and Thioflavin T binding, we showed that these oligomers formed amyloid fibrils if heated for longer time intervals.

In cell-based experiments, we observed a significant cytotoxic response in rat cardiomyoblasts that were treated with heat-denatured TTR-L55P or TTR-V30A samples containing high molecular weight (> 250 kD) oligomeric species. In contrast, the wild-type and V122I treatments appeared ineffective. This variation in toxicity of the TTR proteins seemed to correlate to the inherent structural instability/stability of the variants; numerous biochemical and biophysical studies have demonstrated that TTR-L55P and TTR-V30A are destabilized more easily than the V122I or wild-type proteins[42], [49], [146]. Indeed, the L55P and V30A variants are associated with more aggressive and earlier onsets of disease, possibly due to their structural instabilities[23], [41]–[45].

The toxic response of rat cardiomyoblasts to oligomeric TTR seemed, in part, due to internalization of the amyloidogenic forms; moreover, oligomers taken up by the cells appeared to increase in size, suggesting continued intracellular aggregation. We observed this time-dependent aggregate growth process by fluorescent microscopy. Interestingly, the cells only responded to and internalized the L55P oligomers. This may
be due to the fact that these oligomers have a structure or conformation that was recognized by certain receptors on the cellular membrane. Further testing is required to confirm whether internalization was in fact receptor-mediated. To our knowledge, these studies demonstrating cellular uptake of soluble, pre-fibrillar TTR-L55P and further propagation of the aggregation process within the cell, are novel. The cell staining results demonstrated a pattern indicating that internalized oligomers were localized to the endosomal or lysosomal compartments of the cell. However, at this stage, we can only conjecture that aggregation was occurring within these organelles. Further studies using fluorescent markers for endosomes and lysosomes would provide evidence in support of this hypothesis. Understanding and characterizing the complete pathway of TTR oligomer recognition, internalization, intracellular trafficking, and retention by cardiac cells may further our understanding of ATTR pathobiology.

Interestingly, in our human cardiac cell studies, the qPCR expression data pointed to a potential early stress response in the iPSC-derived cardiac emboid bodies (cEBs). In this series of experiments, we dosed both healthy subject-derived, as well as ATTR-L55P patient-derived cEBs with wild-type and L55P TTR oligomers. We measured several cell cycle and stress markers including matrix metallopeptidase 9 (MMP9), cardiac troponin (cTnT), heme oxygenase 1 (HO1), heat shock protein 27 (Hsp27), and p21. Each of these markers has been implicated in cell stress and/or cardiac amyloid pathology. In analyzing our results from the healthy subject-derived cEBs that were treated with TTR oligomers, we observed little to no change in expression levels of most of these markers. Some fluctuation in MMP9 expression levels were measured in cEBs
treated with TTR oligomer when compared to PBS vehicle control. Additionally, cardiac troponin expression appeared lowered in cells treated with native, as well as oligomers of TTR-L55P. The results from our studies of the patient-derived ATTR-L55P cEBs demonstrated that treatment with L55P oligomers led to a striking increase in HO1, indicating an early stress response in these cells. Further studies of additional cell markers that signal early stress response such as other heat shock proteins would be of interest. Moreover, extending the exposure time of cells to TTR oligomers may induce a stronger and more significant stress effect clearly seen in measurements of marker expression.

Our studies provide unique insight into how TTR oligomers could potentially exert their toxic effects on cellular viability and may provide a clue in understanding the mechanism of TTR-related amyloid diseases. We have produced and studied TTR oligomers that were shown to be on pathway to amyloid fibril formation (Congo red shift and Thioflavin T assays), i.e. these soluble TTR aggregates are amyloid fibril precursors. While the study of TTR toxic oligomers is novel and exiting, it is important to note that the presence of TTR oligomers has yet to be demonstrated in vivo and may suggest that these are short-lived, metastable species or that an alternative pathogenetic mechanism is in play.

6.2 Doxycycline interacts with destabilized transthyretin, inhibits oligomerization, and prevents cardiac cell toxicity

We demonstrated that cellular toxicity induced by TTR-L55P and TTR-V30A oligomers could be prevented when doxycycline was present in combination with TTR
during the thermal denaturation process. These findings are both new and exciting for several reasons. Our data not only suggest a potential role for doxycycline as a therapeutic in ATTR amyloidosis, but provide evidence in support of a novel mechanism of action. Thus far, the antibiotic has only been reported to be a amyloid fibril disruptor[76], [85]–[87]; there have been no studies testing doxycycline interactions with soluble, pre-fibrillar forms of amyloid (Figure 34). In addition, it has been reported that doxycycline reduces MMP expression in human aortic smooth muscle cells which could be of interest in further studies of MMP expression in TTR oligomer-treated cardiac cells.

Unlike other small molecules that stabilize native TTR by occupying the T₄-binding pockets of the protein, doxycycline does not bind to the tetramer. In fact, based on our CD and SDS-PAGE studies, it appears that the drug mechanism of action involves stabilizing interactions with TTR monomers that block formation of HMW species. Alternatively, it is possible that doxycycline binds to the HMW species of heat-denatured TTR and this interaction facilitates dissociation of the aggregates to dimers and monomers (Figure 34). The exact form of TTR, to which doxycycline binds, remains undetermined. Structural studies (CD and mass spectrometry analyses) and binding assays (ELISA, surface plasmon resonance) of drug with TTR monomers, tetramers, and HWM oligomers would aide in further understanding how doxycycline exerts this seemingly beneficial action.

The striking absence of TTR-L55P oligomer internalization in the presence of doxycycline indicated several important and related points. Doxycycline did indeed reduce the presence of TTR oligomers; thus, TTR forms present in the cell treatment
sample included only monomers and dimers, but no tetramers or HMW species. As there was no evidence of TTR uptake by the cells and no change in cell viability in samples with doxycycline, we conclude that oligomers are the cytotoxic form of TTR. Further, we believe that cellular toxicity is elicited in a mechanism mediated by internalization, and doxycycline can inhibit this process by preventing TTR aggregate formation. To our knowledge, this is the first time a strategy suggesting the use of doxycycline as an agent for therapeutic targeting of early stage TTR amyloid formation has been proposed.

6.3 Retinol-binding protein

*Retinol-binding protein and transthyretin*

We showed that RBP stabilizes the TTR tetramer. In our thermal denaturation studies, the presence of RBP slowed the rate of TTR aggregation; this result occurred only when RBP was pre-incubated with vitamin A. As other studies have described, vitamin A is a key element of the RBP-TTR binding complex[29], [94]. Furthermore, ELISA analysis of serum RBP4 levels in control and amyloid groups showed significant differences. Remarkably, ATTRwt and ATTRm results showed dissimilar trends when compared to healthy age- and gender-matched controls. Specifically, ATTRwt levels were significantly increased and ATTRm RBP4 serum concentrations were significantly decreased compared to controls. While these data are intriguing, it is important to note that a limitation of this study was the small sample size of each group. However, these trends, if upheld in a larger series, could prove useful as a diagnostic indicator.

The analysis of serum RBP4 concentrations does not provide us with direct information about TTR stabilization by RBP. However, based on previous reports[90],
and our *in vitro* findings that RBP stabilizes and slows down the aggregation of TTR, we theorized that changes in RBP levels could lead to *in vivo* changes in TTR tetramer stability. Assuming that TTR and RBP4 can form complexes at 1:1 or 1:2 molar ratios of the two proteins, it would be important to measure serum TTR concentrations in ATTR and calculate circulating TTR/RBP4 ratios. We did attempt to measure serum TTR levels by ELISA for this purpose; unfortunately, we were unsuccessful in our attempts to establish a reproducible and accurate ELISA method. Our inability to develop a TTR ELISA may possibly be due to the complex nature of serum, i.e. masking of TTR epitopes through crowding or steric hindrance of ligands or other factors. Without TTR values it is unclear if circulating RBP4 levels alone would be helpful in differentiating between ATTR disease types (ATTRwt vs. ATTRm) or for monitoring disease progression and outcome. Expansion of these studies to larger group numbers would establish the importance of this protein as a disease biomarker.

We propose roles for RBP4 as both a biomarker candidate and a potential therapeutic that can stabilize free TTR (*Figure 34*) and prevent dissociation of tetrameric TTR into monomers. Since RBP4 is a natural binding partner of TTR with no toxic effects *in vivo*, it would be interesting to study supplementation of the protein or stimulating transcription of the protein as potential treatment strategies. We also suggest that evaluation of RBP:TTR circulating ratios may prove to be a useful diagnostic tool and possibly serve to provide novel insight into disease progression and survival.
**Retinol-binding protein and vitamin A status**

Based on our holoRBP-TTR aggregation index results, we hypothesized that vitamin A concentrations would show a positive correlation to TTR-RBP levels in the patient sera. We sought to analyze vitamin A levels in the matched serum samples. The gold standard for vitamin A measurement is quantification of total liver reserves, a procedure that is not an option in living patients. Serum retinol levels are an indirect reflection of total vitamin A in the liver and accurate quantification of the vitamin is challenging as levels are homeostatically controlled (50-200 μg/dL)[147]. However, normal homeostasis of vitamin A concentration is interrupted in several conditions where decreased levels (< 0.7 μg/dL) have been reported[148]–[151]. Based on these reports, it seemed that measuring the circulating levels of vitamin A, except in cases of acute vitamin A deficiency, would be difficult to accurately interpret and provide limited insight. An assay, recently reported to be a ‘valid’ method for indirectly assessing vitamin A levels, relies on data obtained from mixtures of an isotopically-labeled form of the vitamin (¹³C or ²H-labeled retinyl acetate) with the normal blood pool[149]. In addition to being time-consuming and costly, this dilution method requires the use of fresh blood samples. Thus, vitamin A quantification in sera from our RBP studies proved to be beyond the scope of our resources.

In addition, we attempted to study the effects of vitamin A, specifically all-trans-retinol, in our TTR oligomerization and cell-based toxicity studies. The biochemical nature of vitamin A presented challenges in the handling and utilization of the compound in our planned *in vitro* experiments. An extremely hydrophobic molecule, vitamin A is
insoluble in water and glycerol; moreover, the compound is rapidly oxidized on exposure to air and rendered chemically inactive by UV light. Therefore, we made sure to limit exposure to light in all our experiments and used a large excess of vitamin A in our \textit{in vitro} studies to compensate for any loss or inactivation of the vitamin.

\textit{Retinol-binding protein in tissue}

We were initially puzzled by the results from our immunohistochemical analyses of RBP4 in amyloid heart specimens. Congo red analyses demonstrated the presence of amyloid deposits in the ATTR\textsubscript{wt} and ATTR\textsubscript{m} cardiac tissue sections; there was no evidence of amyloid in the ‘non-amyloid’ heart transplant sample. The appearance of RBP4 in the ATTR\textsubscript{wt} and transplant heart tissues, but not the ATTR\textsubscript{m} sample was an unanticipated result. Moreover, when present, RBP4 appeared to be localized to specific areas surrounding, what we believed were, ischemic areas and early stage amyloid infiltrated areas of the tissue. The transplant tissue used in our study was from a patient with ATTR-T60A amyloidosis who had undergone concurrent liver and heart transplantations; the specimen examined was from the ‘new’ and ‘non-amyloid’ heart obtained at autopsy 8 years post-transplantation. We posit that, had a section of this heart been available from a later time point, amyloid deposition would have been observed.

Even though amyloid was not apparent in the transplant heart (by Congo red staining), the detection of RBP4 seems consistent with findings in a recent case report\cite{143}. The case describes a male patient who had received a heart transplant to treat his severe, and fairly early onset (age 57), wild-type ATTR disease (diagnosed by Congo red histology and mass spectrometric protein sequencing). Repeated
immunohistological staining of heart tissue, over a 15 year post-transplant period, revealed small deposits of amyloid after 8 years; the authors estimated that the deposits reflected a < 2% amyloid load in the tissue. In addition, the amount of amyloid (amyloid load) was shown to steadily increase over the following 5 years; magnetic resonance imaging (MRI) revealed intra-cardiac fibrotic remodeling in the interventricular septum and echocardiography confirmed septal wall thickening within the transplanted heart. Though we did not observe any amyloid infiltration by Congo red staining, the observation of slight collagen infiltration led us to believe that, similar to the case report, the transplanted heart used in our study may have been undergoing cardiac changes including early ischemia and amyloid infiltration.

It would be of great interest and value to clarify the findings from our studies and the previously mentioned report[143]. This could be accomplished with analyses of tissue samples obtained from a group of ATTRm transplanted patients at post-transplantation follow-up over an interval, preferably > 5 years; Congo red and immunohistochemical staining for RBP4, as well as TTR, on these tissues would extend our understanding of amyloid development and progression. Indeed, the transplant heart that we studied appeared to exhibit features of early ischemia, i.e. collagen infiltration, which we think could suggest pre-amyloid infiltrative changes. Together, these results may indicate that the occurrence of RBP4 is specific to hearts with wild-type TTR amyloid deposits. We believe that the presence of RBP4, in regions surrounding the amyloid deposits, implies a role in the transport to and localization of TTR in the amyloid heart deposits. Specifically, in ATTRwt amyloidosis, we posit that TTR is carried to the
heart through the blood stream complexed to RBP4. Through signaling or receptor-mediated targeting, RBP4 becomes dissociated from the TTR-RBP complex allowing tetrameric TTR to become destabilized, internalized, and able to form amyloid aggregates within the cell.

We did not observe RBP4 in the heart tissues containing amyloid deposits from cases of ATTRm and AL amyloidoses. This may be due to weaker interactions between RBP4 and variant forms of TTR, or at least a decreased binding affinity between TTR-L58H (tested case) and RBP4. For the AL sample, the absence of detectable RBP4 was expected as there is no known association between immunoglobulin light chain and the binding partner of TTR. Our conclusion is that the role of RBP4 in the mechanism of TTR amyloidogenesis is varied in ATTRwt and ATTRm amyloid diseases, and we hypothesize that the different pathways may be related to dissimilarities in disease onset and progression.

Proposed mechanism

We propose different mechanisms of amyloidogenesis in ATTRwt and ATTRm diseases, as shown in Figure 34. We believe that wild-type TTR and RBP4 are complexed in circulation and that RBP4 targets circulating TTR to the heart in ATTRwt amyloidosis. Once in the heart, RBP4 dissociates from the TTR-RBP4 complex allowing the protein to deposit in tissue, as was demonstrated in our immunohistochemistry studies. The wild-type TTR protein, now lacking its binding partner, dissociates into monomers which enter the heart cell. In contrast, we posit that ATTRm proteins form weakened interactions with RBP4 in circulation, leading to a loss of RBP4 through
kidney filtration. The ATTRm protein becomes destabilized and starts to form soluble oligomers in circulation, which then are targeted to the heart through other signaling pathways, correlating with earlier age of onset. Both the wild-type and mutant TTR protein species aggregate intracellularly, as we observed by fluorescent microscopy. The internalized pre-fibrillar oligomers lead to cellular toxicity and increased HO1 and p21 expression, as was shown in our qPCR studies. The cells eventually undergo cell death and rupture due to the increasing intracellular stress with the amyloidogenic material deposited in the extracellular space, ultimately forming amyloid deposits. Doxycycline (Dox) may act as a TTR monomer stabilizer preventing subunit unfolding and aggregation; alternatively, the drug may interact with oligomeric (HMW) forms of TTR and cause dissociation of the aggregates to monomeric and dimeric forms of the protein. Our conclusion from the experimental results reported in this dissertation is that the role of RBP4 in the mechanism of TTR amyloidogenesis is varied in ATTRwt and ATTRm amyloid diseases, and we posit that the different pathways may be related to dissimilarities in disease onset and progression.

6.4 Future directions

We hypothesize that doxycycline prevents aggregation of TTR-L55P by stabilizing subunit (monomers or dimers) forms of the protein or destabilizing soluble aggregated (HMW) species. If the latter is the case, the dissociated forms of TTR must remain small enough to be effectively removed, possibly through renal filtration if in the bloodstream, and at the cellular level in forms that do not affect cell viability. To further understand the nature of doxycycline action, a study of cells treated with TTR oligomers
and doxycycline could be performed to characterize the species found excreted by cells. If our theory holds true, we would expect higher amounts of small low molecular weight soluble TTR species to be present in the supernatants of cells exposed to the oligomer-doxycycline mixture. For the drug studies, it would also be important to expand the study of doxycycline to include a compliment of drug dosage experiments. We used 10x and 50x molar excesses of drug, but accurately determining optimal concentrations and differing concentration effects are crucial in promoting this as a therapeutic agent in ATTR amyloidosis.

To further explore the effect of diflunisal on the viability of cells, a modification of the protocol we used to generate TTR oligomers is needed. Our heat treatment method using 80 °C over the course of 4 hours proved inappropriate for diflunisal; the drug seems chemically unstable at 80 °C at least in the buffering conditions that were used. Thus, a method to denature TTR and form oligomers in the presence of diflunisal should involve heating at temperatures < 80 °C; however, this likely will require a longer incubation period, i.e. > 4 hours, and is a considerable disadvantage. In addition, based on our studies, we have no reason to believe that Kiacta® would be useful in treatment of patients with ATTR amyloid disease.

Future studies should also include extension and modification of the cell-based experiments. The absence of effects in the ‘older’ (greater passage number) H9C2 cells suggests that further studies be performed on cells that have undergone consistent and lower (< 20) number of passages. In addition to the rat cardiomyoblast experiments, further expansion of the iPSC-derived cEB experiments should include qPCR analyses of
a timed series of stress marker expression levels. Increasing the number of experiments testing cell response to TTR oligomer treatments with and without drug (doxycycline in particular) would render more statistical power in calculating significant differences. In addition, we propose studying doxycycline in this patient-specific cellular model.

In moving forward with the RBP4 studies, circulating TTR values should be obtained to gain insight into circulating TTR-RBP4 complexes and further explore the potential of using these values as predictive or prospective indicators. Moreover, it would be particularly interesting, as mentioned previously, to further investigate the presence or absence of RBP4 in heart tissue from cases of ATTRwt and ATTRm amyloidoses. Finally, obtaining a measurement of vitamin A status on patients with ATTR might help us to uncover a role of this molecule in amyloid disease development and/or progression related to RBP-TTR complex formation; the correlation of vitamin A with RBP-TTR complex instability and ATTR disease status would likely have diagnostic and therapeutic implications.

6.5. Summary statement

Our overall goals were to: 1) create and use a cell-based model that closely mirrored the in vivo pathobiology of ATTR amyloidosis, 2) further define the mechanism of cardiac TTR-associated amyloid at the cellular level, and 3) uncover potential therapeutic agents that could interrupt the amyloid process. Specifically, we designed studies aimed at investigating the effects of soluble amyloidogenic forms of TTR on heart cells and exploring the use of several agents as inhibitors of TTR-induced cytotoxicity. We developed a method to created soluble TTR oligomeric species, as demonstrated by
Congo red, Thioflavin T, and SDS-PAGE analyses. Furthermore, we showed that TTR-L55P oligomers are toxic to cardiomyoblasts through a pathway that includes internalization and progressive intracellular aggregation. Our data showed that doxycycline could block the cytotoxic effect of TTR oligomers suggesting a potential therapeutic role for the drug in preventing *in vivo* deposition of amyloid. By immunohistochemistry, we found that RBP4 was present in ATTRwt and ‘non-amyloid transplant heart tissues; the transplant heart sample was from an ATTRm case and obtained 8 years post-surgery. RBP4 was localized to areas containing amyloid or in the case of the transplant tissue, regions that appeared to be injured, i.e. displaying ischemic damage, collagen infiltration, and early hypereosinophilia. Measurements of circulating RBP4 concentrations demonstrated significant differences of levels in the ATTR and age-matched, healthy controls groups, as well as in ATTRwt vs. ATTRm. We propose a role for RBP4 as a potential biomarker and/or therapeutic agent based on results from our ELISA, immunohistochemical, and aggregation studies.

In conclusion, the studies reported in this dissertation support the notion that small soluble oligomeric TTR species are a cause of toxicity and cellular damage in ATTR forms of systemic amyloidosis, and that TTR destabilization and localization in ATTRm and ATTRwt diseases are the result of two distinct and separate pathways. Moreover, we propose the idea that doxycycline may have utility as a possible therapeutic agent to target toxic amyloidogenic TTR. Lastly, we have refueled the interest in the potential of RBP4 as both a biomarker and therapeutic strategy in the ATTR amyloid diseases.
Figure 34. Schema of TTR amyloid formation pathway and interactions of retinol-binding protein and doxycycline.

We believe that RBP4 targets circulating TTR to the heart in ATTRwt amyloidosis; once in the heart, the TTR–RBP4 complex dissociates allowing tetrameric TTR to become destabilized. In ATTRm, the TTR–RBP4 complex may already be dissociated in the circulation, and TTR is targeted to the heart through other signaling pathways. Once internalized by a cardiac cell, the wild-type or mutant TTR protein species aggregate intracellularly and cellular stress levels rise, demonstrated by increased HO1 and p21 expression. Eventually the heart cell dies, secreting the amyloidogenic material into the extracellular space. Doxycycline may act as a TTR monomer stabilizer preventing subunit unfolding and aggregation; alternatively, the drug may interact with oligomeric forms of TTR and cause dissociation of the aggregates.
APPENDIX

Supplemental data

Table S1. SYBR Green quantitative PCR primers.

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List of Journal Abbreviations

Am Heart J. ................................................................. American Heart Journal
Am. J. Med.............................................................. The American Journal of Medicine
Am. J. Transplant.................................................. American Journal of Transplantation
Ann. Med. ................................................................ Annals of Medicine
Ann. Neurol ................................................................ Annals of Neurology
Annu. Rev. Biochem............................................ Annual Review of Biochemistry
Appl. Microbiol. .................................................... Applied Microbiology
Arch. Intern. Med. ............................................. Archives of Internal Medicine
Biochem J................................................................. Biochemical Journal
Cell Metab. ............................................................ Cell Metabolism
Circ. Heart Fail. .................................................. Circulation: Heart Failure
Cli. Genet. ............................................................. Clinical Genetics
Crit. Care Med. ...................................................... Critical Care Medicine
Exp. Mol. Med. ...................................................... Experimental & Molecular Medicine
FEBS J .......................................................... Federation of European Biochemical Societies Journal
Heart Fail. Rev. .................................................. Heart Failure Reviews
Int. J Cancer ........................................................ International Journal of Cancer
J. Am. Coll. Cardiol. ........................................... Journal of the American College of Cardiology
J. Biol. Chem ..................................................... Journal of Biological Chemistry
J. Clin Invest. .................................................. Journal of Clinical Investigation
J. Control. Release ................................................. Journal of Controlled Release
J. Med. Chem. .......................................................... Journal of Medicinal Chemistry
J. Mol. Med.......................................................... Journal of Molecular Medicine
J. Neurol. Sci.................................................... Journal of Neurological Sciences
J. Neurosci......................................................... Journal of Neuroscience
J. Pathol. Bacteriol ........................................... The Journal of Pathology and Bacteriology
JAMA ............................................................... Journal of the American Medical Association
Lab Invest. .......................................................... Laboratory Investigation
Liver Transpl. ....................................................... Liver Transplantation
Mod. Pathol. ....................................................... Modern Pathology
Mol. Cancer Ther................................................. Molecular Cancer Therapeutics
Mol. Cell. Biochem. .............................................. Molecular and Cellular Biochemistry
Nat. Neurosci ....................................................... Nature Neuroscience
Nat. Rev. Drug Discov ........................................... Nature Reviews Drug Discovery
Nat. Rev. Neurosci ................................................ Nature Reviews Neuroscience
Neurosci. Res ...................................................... Neuroscience Research
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*Boston University School of Medicine*
Advisor: Prof. Jan Krzysztof Blusztajn
Project: Studying the effect of BMP9 as a cholinergic trophic factor on the expression of various genes in the basal forebrain cholinergic neurons in wildtype and transgenic mice.
Techniques: Cell culture, RNA isolation, ribogreen RNA quantification, RT-PCR, gel electrophoresis, primer design.

**Graduate Laboratory Rotation** January-March 2010

*Amyloidosis Center*
*Boston University School of Medicine*
Advisor: Prof. Lawreen H. Connors
Project: Studying the effect of oligomeric species of transthyretin on rat cardiomyoblasts.
Techniques: Cell culture, cell viability assay, protein purification by affinity chromatography, gel electrophoresis, circular dichroism.
Graduate Laboratory Rotation  
*Boston University School of Medicine*  
September-December 2009  
Advisor: Dr. Joel M. Henderson  
Project: Visualizing isolated glomeruli treated with cytoskeleton-active agents.  
Techniques: Transmission electron microscopy (TEM), immunofluorescent staining, cryosectioning

Undergraduate Research  
*Department of Experimental Vascular Medicine*  
*Academic Medical Center (AMC-UvA)*  
*Amsterdam, The Netherlands*  
April - July 2009  
Advisors: Prof. Joost C. M. Meijers and Dr. Pauline F. Marx  
Project: Undergraduate senior thesis studying the role of thrombin-activatable fibrinolysis inhibitor (TAFI) in AD brain.  
Techniques: Cell culture, protein purification by affinity chromatography, gel electrophoresis, cell proliferation assay, fluorescence microscopy, FACS analysis, high-pressure liquid chromatography (HPLC), endogenous thrombin potential (ETP) assay, clot lysis assay.

Undergraduate Research  
*Department of Experimental Therapy*  
*Netherlands Cancer Institute (NKI-AvL)*  
*Amsterdam, The Netherlands*  
June - July 2008  
Advisors: Prof. Adrian Begg and Dr. Sari Neijenhuis  
Project: Studying targeted radiosensitization of cells expressing truncated DNA polymerase  
Techniques: Cell culture, clonogenic survival assays, sister chromatid exchange (SCE) analysis

Undergraduate Research  
*Department of Experimental Immunology*  
*Academic Medical Center (AMC-UvA)*  
*Amsterdam, The Netherlands*  
July 2007  
Advisor: Dr. Cristina Lebre  
Project: Studying immunohistochemical and immunofluorescent staining techniques to identify various immune cells and cytokines in patient blood.  
Techniques: Immunohistochemical staining, PBMC isolation, immunofluorescent staining.
PRESENTATIONS


Biomolecular Characterization of Transthyretin Oligomeric Interactions with the Molecular Chaperone Clusterin. Michael J. Greene, Elena Klimtchuk, Clarissa M. Koch, David C. Seldin and Lawreen H. Connors. XIIIth International Symposium on Amyloidosis, Groningen, The Netherlands 2012


PUBLICATIONS


Cardiotoxicity of pre-fibrillar transthyretin oligomers and attenuation by doxycycline. Koch CM, Klimtchuk E, Seldin DC, Connors LH. Proceedings of the XIIIth International Symposium on Amyloidosis 2013

TEACHING

Tutor

Graduate course MS 131 Medical Immunology
Boston University School of Medicine, Boston MA

Tutoring Coordinator 1st year medical students

Graduate course MS 131 Medical Immunology
Boston University School of Medicine, Boston MA
Teaching Assistant
Graduate Course FC706 Molecular Metabolism
Boston University School of Medicine, Boston MA

LANGUAGES

English – Native language
German – Native language
Dutch – Native language
French – Proficient