2022

Optimization of the structure of TTR Ligands for Half-life Extension (TLHE)

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A Thesis Submitted to the

Office of Research and Graduate Studies

In Partial Fulfillment of the

Requirements for the Degree of

Master of Science

Thomas J. Long School of Pharmacy
Pharmaceutical and Chemical Sciences

University of the Pacific
Stockton, California

2022

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DEDICATION

This thesis is dedicated to the people who have supported me through my entire education. Especially thanks to my parent's mother Qiu Ouyang and father Yongsheng Jiang.

ACKNOWLEDGEMENT

I would like to thank my advisor and mentor Dr. Mamoun Alhamadsheh. They are no words to express my gratitude for his constant guidance and support what he made is more than a research advisor. I am greatly benefited from his teaching, advice, and guidance on a number of academic, prosomal, professional, and carrier choices. I like to thank Dr. Park and Dr. Zhao for serving on my committee. I would like to thank Toufiq for teaching peptide and chemical synthesis. I am grateful to my colleagues Fang, Dengpan, Arindom, Tuhin for their encouragement and support. Last and most of all I would like to thank my parents for their unconditional love and support.

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OPTIMIZATION OF THE STRUCTURE OF TTR LIGANDS FOR HALF-LIFE EXTENSION (TLHE)

Abstract

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2022

Many potential therapeutic agents face challenges for their clinical development due to short circulation half-life. As a result, prolonging the half-life of therapeutic drugs in circulation while preserving their hydrophilicity and small size will be a key step toward more effective and safe pharmacological molecules. Our lab developed a new approach for enhancing the safety and efficacy of therapeutic agents. By endowing therapeutic agents with a hydrophilic small molecule (a derivative of the clinical candidate, AG10) which reversibly binds to the serum protein transthyretin (TTR), the half-life of the therapeutic agent should be extended by binding to the TTR in serum. We termed this technology TTR Ligand for half-life extension (TLHEs). The approach involved using TLHE, which binds with TTR by high specificity and affinity. Our group has already shown that this technology extends the half-life of peptides, small molecules, and proteins without seriously affecting their binding activity towards their receptor and efficacy. As we are expanding the applicability of TLHE to extend the half-life of hydrophobic moieties, increasing the polarity of the TLHE linker could be beneficial to maintain overall hydrophilicity. Our main objective here is to see the effect of TTR binding affinity and selectivity of TLHE in serum when we attach a hydrophilic glutamic acid in the TLHE linker

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CHAPTER 1: GENERAL INTRODUCTION

Transthyretin

A 55 kDa homo-tetrameric protein named Transthyretin (TTR) is present mainly in plasma and cerebrospinal fluid. TTR is produced by the liver and secreted into the blood. Its function is to work as a backup carrier for thyroxine (T_4) and the leading carrier of holo retinol-binding protein (RBP) in blood. The crystalline structure of the transthyretin is shown in (Figure.1) [1] [2] [3]. TTR has a tremendous medical and scientific interest because it is one of more than 100 blood proteins whose aggregation causes diseases like amyloid fibril formation in the nervous system, soft tissue, and solid organs by getting toxic function mechanism [3]. Aggregation, dissociation, and misfolding of TTR are known to be primarily responsible for different amyloid-related diseases like familial amyloid polyneuropathy (FAP), senile systemic amyloidosis (SSA), and familial amyloid cardiomyopathy (FAC).

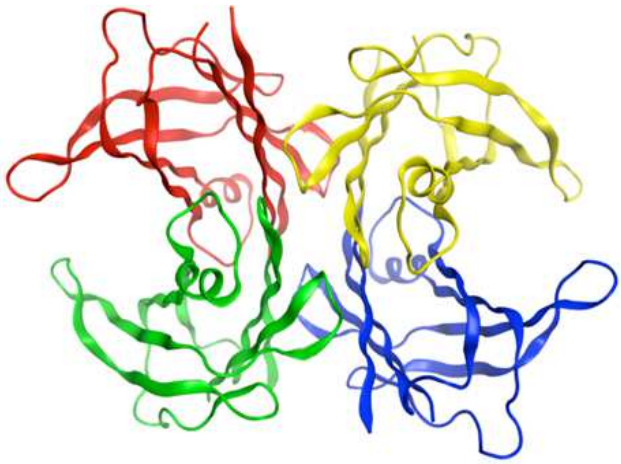


Figure 1 . Crystal structure image of Transthyretin

TTR in the plasma and TTR in the brain is mainly synthesized in the liver and choroid plexus, respectively. TTR is also known to be produced at lower amounts in several other tissues such as islets of Langerhans of the pancreas, intestine, retinal pigment epithelium of the eye, and meninges [4]. Hepatocytes of the liver produced and secreted the TTR found in the blood. For healthy adults TTR concentrations normally range between 0.2 – 0.4 mg/mL (3.5 – 7 μM) [5]. Significant reduction in plasma TTR level occurs due to livers participation in acute-phase response to injury, malnutrition, or chronic inflammation. Therefore, TTR levels in the blood are used as a nutritional marker and indicative of inflammatory status in the clinic. That's why TTR level determination could be helpful in clinical settings where comprehensive and detailed nutritional assessment such as evaluation of albumin, C-reactive protein, and α1-acid glycoprotein is difficult to obtain [6].

Structure of TTR

Human TTR comprises four identical 127 amino acid residue subunits (~14 kDa for each subunit) that make up an extensive β-sheet structure [7]. TTR's four identical subunits (or monomers) form an internal channel at the weaker dimer–dimer interface, allowing two thyroid hormone (T_4) molecules to attach to the resulting tetramer. [4] [7]. Due to the availability of two other T_4 transporter proteins in the blood, the T4 binding sites in humans are largely unoccupied (<1 % T_4 bound), and among those that are occupied, only one site is filled due to negative cooperativity in the binding of the second T_4 transport protein.[8] [9]. Additionally thyroid hormones (T_4) along with its metabolites and several other pharmacologic agents, natural products like nonsteroidal analgesic drugs, plant flavonoids, and inotropic bipyridines, are capable competitors for T4 binding site to TTR and greater binding affinities than T4 [4] [10].

The Wild-type TTR (WT-TTR) structure of humans was one of the first proteins determined by X-ray crystallography [2]. As of June 2017, approximately 267 reported TTR crystal structures and their variants were listed in Protein Data Bank (PDB, www.rcsb.org) for five species of protein (human, rat, mouse, chicken, and fish). It was found through research that the amino acid sequence of human WT- TTR is about 85% identical to various species [11].

TTR's Function and Clinical Importance

According to a recent study, TTR's most significant function is to transfer T_4 and retinol. On the other hand, TTR may have a key physiological role in proteolysis and the transport of other ligands [12]. TTR is not the principal transporter of T_4 (less than 1 % bound) which is because at least two other T_4 transport proteins are present in blood naming thyroxine-binding globulin (TBG) and albumin thus the T_4 binding sites remain largely unoccupied in humans [13] [14]. However, TTR is a major transporter of T_4 in both humans and rats in CSF. Interestingly, TTR is the primary transporter of T_4 in rat plasma unlike in human plasma. Due to the negative cooperativity between the two T_4 binding pockets, every TTR tetramer can carry only one T_4 molecule at a time [15]. Vitamin A (retinol) circulates in the bloodstream, bound to retinol-binding protein (holo-RBP), which is present in a complex with TTR. This association facilitates RBP release from its site of synthesis in the endoplasmic reticulum and prevents renal filtration of RBP [16] [17]. To form the stable complex with TTR, the presence of retinol bound to RBP is required. [18]. According to structural studies, it was observed that the RBP binding site on TTR is orthogonal to TTR's T_4-binding sites (Figure.1.1). Due to the limited RBP concentration available in plasma, RBP binds to TTR in a 1:1 molar ratio. Recently, TTR has been shown to have a protective effect against amyloid-beta (Aβ) deposition in Alzheimer's disease (AD). It was found that TTR could bind and cleave soluble Aβ and prevent invitro Aβ amyloid fibril formation [19]. TTR cleaves Aβ at

different positions resulting in shorter Aβ peptides showing lower amyloidogenic potential. Additionally, TTR was able to degrade aggregated forms of Aβ and may contribute to the maintenance of normal Aβ levels in the brain.

Kinetic stabilizers of the native tetrameric structure of TTR by interallelic trans suppression in compound heterozygote patients, that carry both the destabilizing V30M as well as a second, disease suppressing mutation (T119M) [20], or by a small molecule which occupies the T4-binding sites, raises a dissociative transition state energy and prevents amyloidosis (Figure 1.2). A recent clinical trial of a kinetic TTR stabilizer named tafamidis indicates a significant slowdown of the progression of early-stage neuropathy in FAP patients [21]. Currently, there are only very few FDA-approved drugs for the treatment or prevention of FAC or SSA. As a result, the majority of patients' treatment is limited to symptomatic alleviation. For hereditary TTR amyloidosis, the best way to get rid of the altered protein is to replace the liver. For a subset of FAC patients, combined liver and heart transplantation is used as a palliative therapy [22]. Organ transplantation carries significant risks and costs, and recipients must take immunosuppressive medicines for the rest of their lives, resulting in morbidity. Because of these factors, a pharmaceutical treatment for TTR cardiomyopathy is urgently needed.

We identified a number of powerful and structurally very diverse TTR kinetic stabilizers using high-throughput screening (HTS) for TTR ligands. Our lab utilized the previous compounds as precursors for structure-activity relationship studies and made a series of analogs, one of which that best hit from HTS named AG10 [3] is a highly effective and selective stabilizer of TTR.

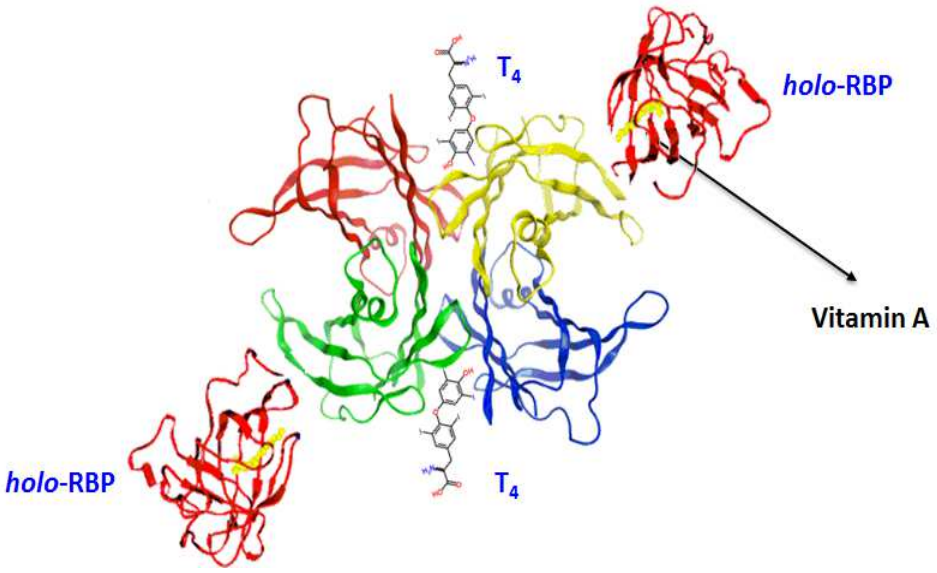


Figure 2. TTR bound to T_4 and holo-Retinol-binding protein (holo-RBP)

TLHE Approach to Enhance the Half-Life of the Peptide

TTR is a 55 kDa homo-tetramer that is secreted into the bloodstream by the liver and secreted into the CSF by the choroid plexus (TTR concentration in serum is 5 M) [23]. It has a half-life of 48 hours in vivo. The primary and secondary function of TTR in humans is to transport holo-retinol binding protein and T_4 binding protein in the blood respectively. Because of its small size (21 kDa), the Apo-retinol binding protein has a low binding affinity for TTR and is excreted rapidly in the kidneys with a half-life of 3 hours [23]. The reversible connection between holo-retinol binding protein and TTR in blood prevents the holo-retinol binding protein from being filtered by the glomerulus, thus extending its circulation half-life (11 hrs). Based on this natural observation, our laboratory already established that conjugating a peptide to selective TTR-binding ligands will help the peptide conjugate bind reversibly to TTR and help in extending the *in vivo* circulation half-life of therapeutic peptides (Figure.3.1). The intrinsic activity of the peptide conjugates is unaffected by their reversible binding to TTR [24]. Several AG10-linker modified

analogs (TLHEs) have already been produced in our lab and are utilized for half-life extension of peptide, small molecule, and protein [25].

Our approach of employing a TTR ligand for half-life extension (TLHE; 500 Da) should not impair peptide or small molecule's binding affinity to extracellular receptors. TLHE are conjugated to peptides by a short linker system that will give TLHE-peptide conjugate the capability to bind in reversible to T_4 binding sites of TTR [25]. This will increase the peptide's in vivo half-life by shielding it from protease and lowering glomerular filtration due to the gain of large hydrodynamic size when attached with TTR. Most importantly, due to its reversible binding to TTR, the binding affinity of the peptide conjugated TLHE to its target receptor would not be adversely affected.

Our Hypothesis to Optimize the Structure of TLHE

Our hypothesis's main goal is to apply our TLHE technique to a wide spectrum of therapeutic agents. Many clinically used therapeutic agents especially chemotherapeutics are lipophilic in nature, which makes them prone to passive diffusion into the cell membrane (despite being ligand-targeted therapeutics e.g. Adcetris). This leads to nonspecific binding to lipoproteins, lipid bilayers, and scavenger receptors for nonpolar therapeutics causing off-target toxicity. The overall polarity of a molecule can be greatly influenced by linker (TLHE) composition. This may lead to alterations in the molecule's properties, relating to tissue distribution, toxicity, PK, and in vivo efficacy. Therefore, we hypothesize that incorporating a more hydrophilic linker in our TLHEs would allow us to compensate for the hydrophobicity exerted by conjugating our TLHEs to lipophilic therapeutic agents. We also hypothesize that the addition of hydrophilic spacers within the TLHE linker will exert overall hydrophilicity and binding.

Evolution of TLHEs

The crystal structure of AG10 bound to TTR [3] (Figure 1.3) along with the design of our previous TLHE [25], and *in silico* modeling studies served as a blueprint for the design of new generation hydrophilic TLHE linkers.

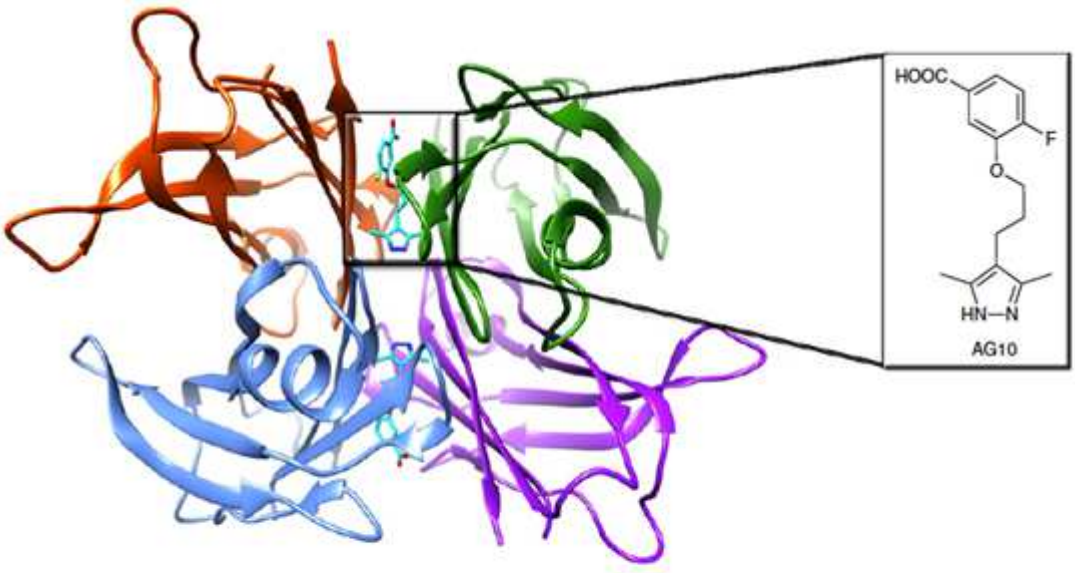


Figure 3. Crystal structure of TTR bound to AG10, with monomers colored individually and a box showing a close-up view of AG10 bound in one of the two TTR T_4 pockets (PDBID: 4HIQ)[3]

Development of the first TLHE (TLHE1) was designed and synthesized by two modifications to AG10. Firstly, the fluorine atom on AG10 was removed (Compound 2, Figure 1.4. b); and it was found that this modification maintained the binding affinity and selectivity of AG10 for TTR. Secondly, by attaching a lipophilic five-carbon spacer with an alkyne on the terminal at the *meta*-position of AG10. Because it does not participate in any important interactions within the T_4 binding site of TTR and is located outwards of the T_4 pocket, this position was determined to be appropriate for spacer attachment (Figure 1.4.b). Thus, the spacer will extend the T_4 binding pocket without considerable steric impact on TTR residues at the periphery of the T_4 pocket. Neither pyrazole ring nor the carboxyl group of AG10 was changed since they are required to form two hydrogen bonds with Ser117 and 117' and two salt bridges with Lys15 and 15' of TTR monomers respectively (Figure 1.4.a).

TLHE1 showed a high binding affinity (K_d = 42 nM and K_d = 32 nM, evaluated by SPR and ITC, respectively) and selectivity for TTR (70% binding to TTR in serum, evaluated by covalent probe selectivity assay). Although the activity of TLHE1 is 10-fold less than AG10 (K_{d1} = 4.8 nM; 98% binding to TTR in serum), it is still higher than the clinical candidate of TTR amyloidosis, tafamidis. Our group has previously determined the PK parameters and cytotoxicity of TLHE1, and they correlate well with those of AG10.

In order to functionalize TLHE1 with a payload like a peptide which will be clear from the TTR binding pocket, and will not interfere with peripheral residues of TTR, the distances from the *meta*-position on the phenyl ring carbon, to two residues at the outermost of the binding pockets were previously determined by modeling studies (50.8 Å and 26.4 Å). In addition, the shortest possible distance from the meta-position on compound **2**, to the T_4 binding site entrance was also determined (14.4 Å). Our group has also previously determined the minimum distance for the length of the spacer (distance to the ridge of the narrow side of top ellipse ~17.3 Å), which implied that the spacer should be as long as 17±3 Å (Figure 7c). Subsequently, TLHE2 was developed by our group with a spacer that is ~20 Å in length (Figure 1.4.d).

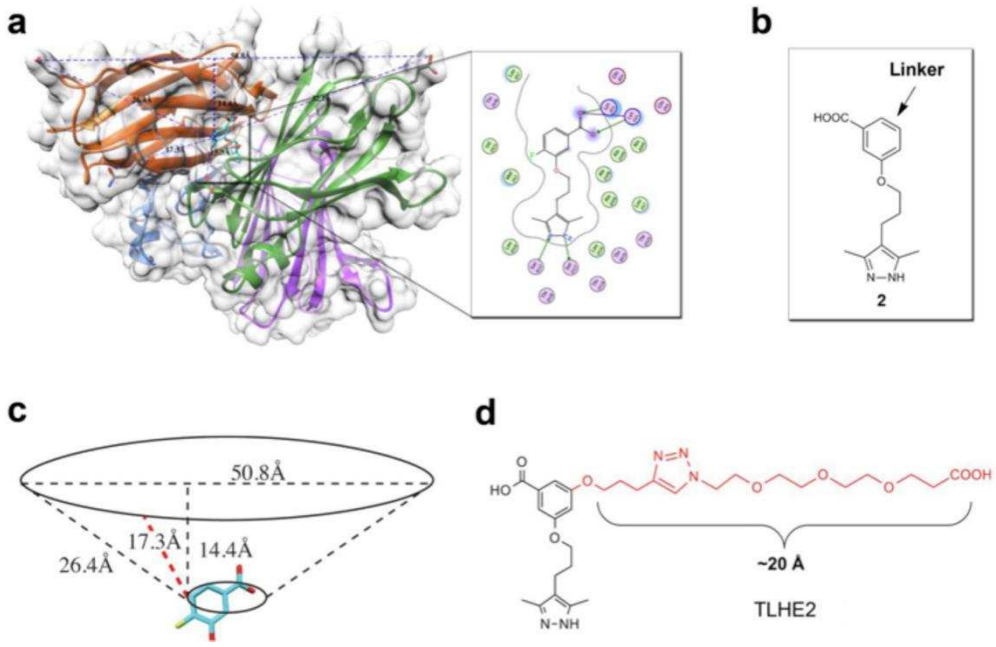


Figure 4. Structure of TTR bound to AG10, *in silico* modeling of linker length. (a) Ribbon diagram with transparent surface of TTR and a close-up top view of AG10 (shown as stick) bound in one of the two TTR T4 binding sites (pdb id: 4HIQ) [3]. The interaction between AG10 and TTR monomers (expanded box) through two H-bonds with Ser117 and 117' and two salt bridges with Lys15 and 15'. (b) Structure of AG10 analog **2** with potential sites for linker attachment. The position is pointing out towards the solvent, thus attaching a spacer will project it outside of the T4 binding pocket. (c) The distance from the meta-position on the phenyl ring carbon of AG10 analog 2 required to determine optimum linker length. (d) Chemical structure of TLHE2. TLHE2 has a linker length of ~20 Å sufficient to clear out of the TTR T4 binding sites and potentially be functionalized by payload

Design and Development of A New Generation of Hydrophilic TLHE

The assessment of the positions in TLHEs that is amendable for spacer modification was performed by rational design and guided by *in silico* modeling studies. This *in silico* study identified possible interactions that could be formed between our new hydrophilic spacers and the T_4 pocket of TTR. We hypothesized that a possible way to increase the hydrophilicity of TLHE spacers is by incorporating an amine group (which will be protonated under physiological pH) at the terminal end of the linker. Using insights for the interaction of T_4 with TTR, in addition to *in silico* modeling studies, we hypothesized that the incorporated amine group in TLHE could

potentially participate in a new ionic interaction (salt bridges) with the two new glutamic acid residues (*Glu54/Glu54'*) adjacent to the surface of TTR (Figure.1.5). The modeling studies also showed that this interaction does not interfere with the primary interactions between AG10 and TTR. Therefore, we anticipate this modification will have the dual advantage of increasing the binding affinity of the new TLHEs to TTR and increasing the selectivity for TTR in serum (as conferred by increasing the overall hydrophilicity).

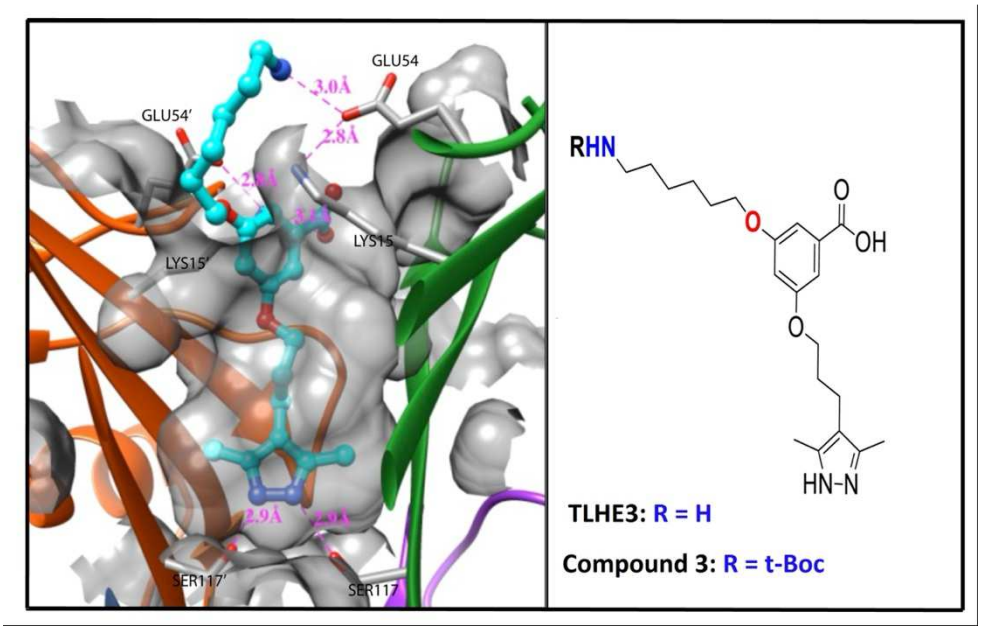


Figure 5. Close up view of modeled TLHE3 bound in one of the two TTR T_4 pockets with structure of TLHE3. Compound 3 is the t-Boc protected version of TLHE3.

To test the formation of the putative salt bridge between TLHE3 and *Glu54* of TTR, we also designed and synthesized compound **3** (Figure 1.5) where the amine group of TLHE3 is protected with *tert*-Butylcarbonly (*t*-Boc). The *t*-Boc group would preclude the formation of a salt bridge between compound **3** and *Glu54* which should result in a much lower binding affinity of compound **3** compared to TLHE3. Evaluation of the binding affinity and selectivity of compound

3 and TLHE3 to TTR in buffer and serum showed much lower TTR binding of compound **3** compared to TLHE3. This finding supports the creation of a salt bridge between TLHE3's amine and TTR's Glu54. In order to further increase the hydrophilicity of TFMs, and to implement a spacer extension system, a short PEG spacer was conjugated to the amine group of TLHE3. In a previous study, the spacer extension effect was described, by designing TLHE4; where 2 PEG units are attached to the amine of TLHE3 and designed TLHE5, where 3 PEG units are attached to the amine of TLHE3 (Figure 1.6). An azido group is added to each spacer at the terminal end for "click coupling" with an alkyne group on the therapeutic agent.

To further improve the hydrophilicity and see the effect in terms of binding with TTR in serum, the aim of my project was to introduce a glutamic acid in TLHE. This should serve two purposes; increase the hydrophilicity of TLHE by incorporating the side chain carboxylic acid of glutamic acid (should be ionized at physiological pH). In addition, the alpha carboxylic acid of glutamic acid should allow us to conjugate various molecules to the new TLHE.

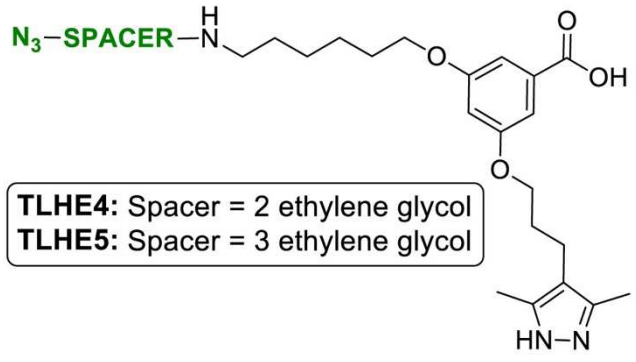


Figure 6. Chemical structures of chemically synthesized TLHE4 and TLHE5.

CHAPTER 2: CHEMICAL SYNTHESIS OF LIGAND 6 AND LIGAND 7

Materials and methods

Human plasma pre-albumin (human TTR) was purchased from Sigma, both covalent and non-covalent probes were synthesized in our lab. All other starting materials for the synthesis were purchased from Chemimpex, Sigma, and Fisher. The solvents used were ACS grade solvents were purchased from Fisher and other reagents were purchased from sigma Aldrich and Acros and used in the reaction without further purifications. Thin-layer chromatography (TLC) comprising 0.2mm POLYGRAM® SIL silica gel plates were used with fluorescent indicator UV254 using UV light as a visualization aid. Normal phase flash column chromatography was used for purification of compounds comprises of Davisil® silica gel (100-200 mesh, Fisher Scientific), and HPLC analysis was performed on a Waters™ Alliance 2790 system attached to Waters™ 2990 PDA detector operating UV ranges from 200 – 400 nm. An empower 2.0 data acquisition system software was used for quantification purposes. The specification of the HPLC column is Waters™ XBridge C18 column with L1 packing (4.6 X 150 mm, 5µm) The UV absorbance was recorded at 254 nm.

Synthesis scheme of TLHE ligand 6 and ligand 7

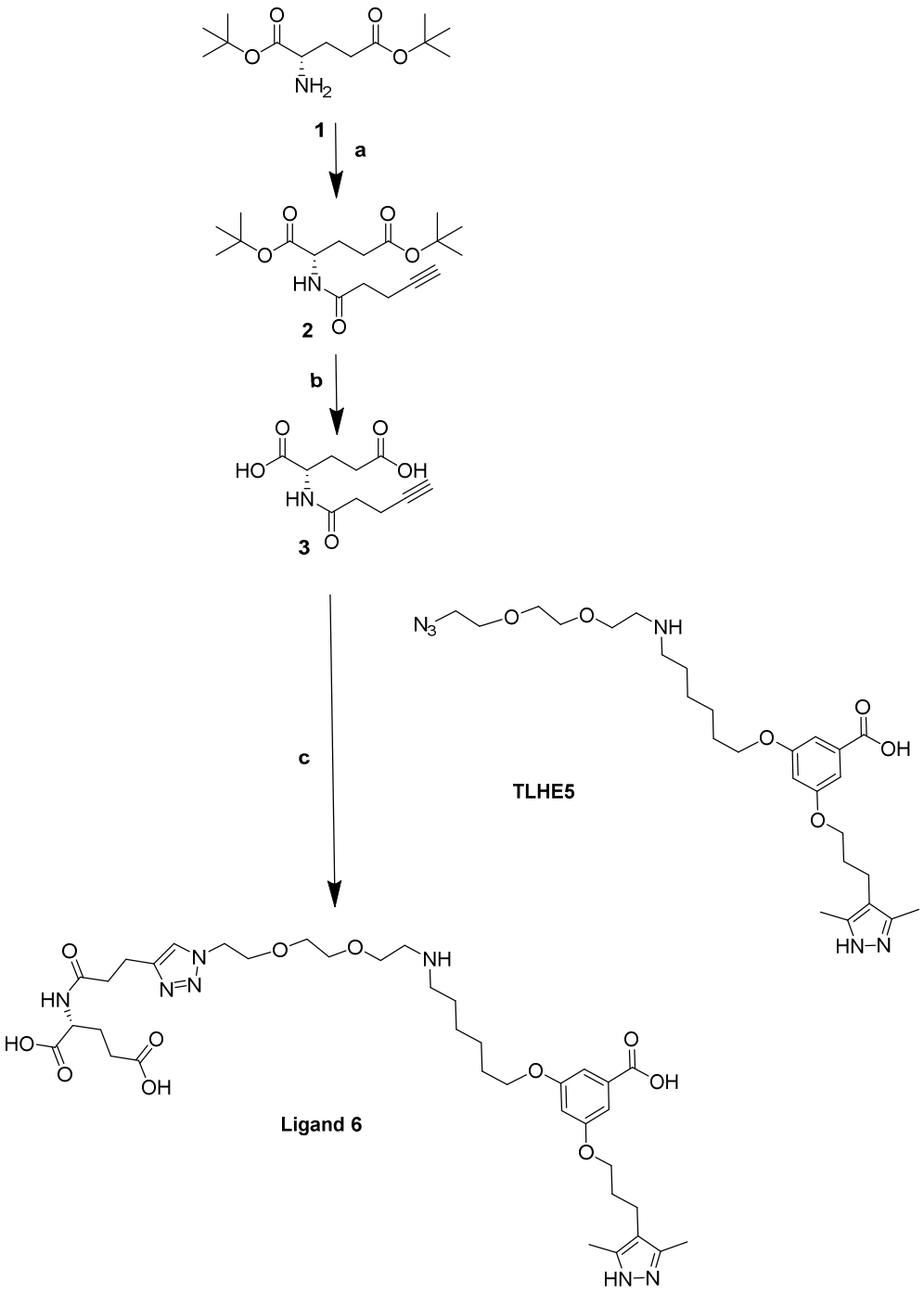


Figure 7. Synthetic scheme of Ligand 6. a) HOBt monohydrate, EDCI, DIPEA, DCM, room temperature (rt), 16 h; b) TFA in CH_2Cl_2, (1:4 ratio), rt, 2h; c) TLHE5, $CuSO_4$, sodium ascorbate, of H_2O/THF (2:1), rt, 3 h.

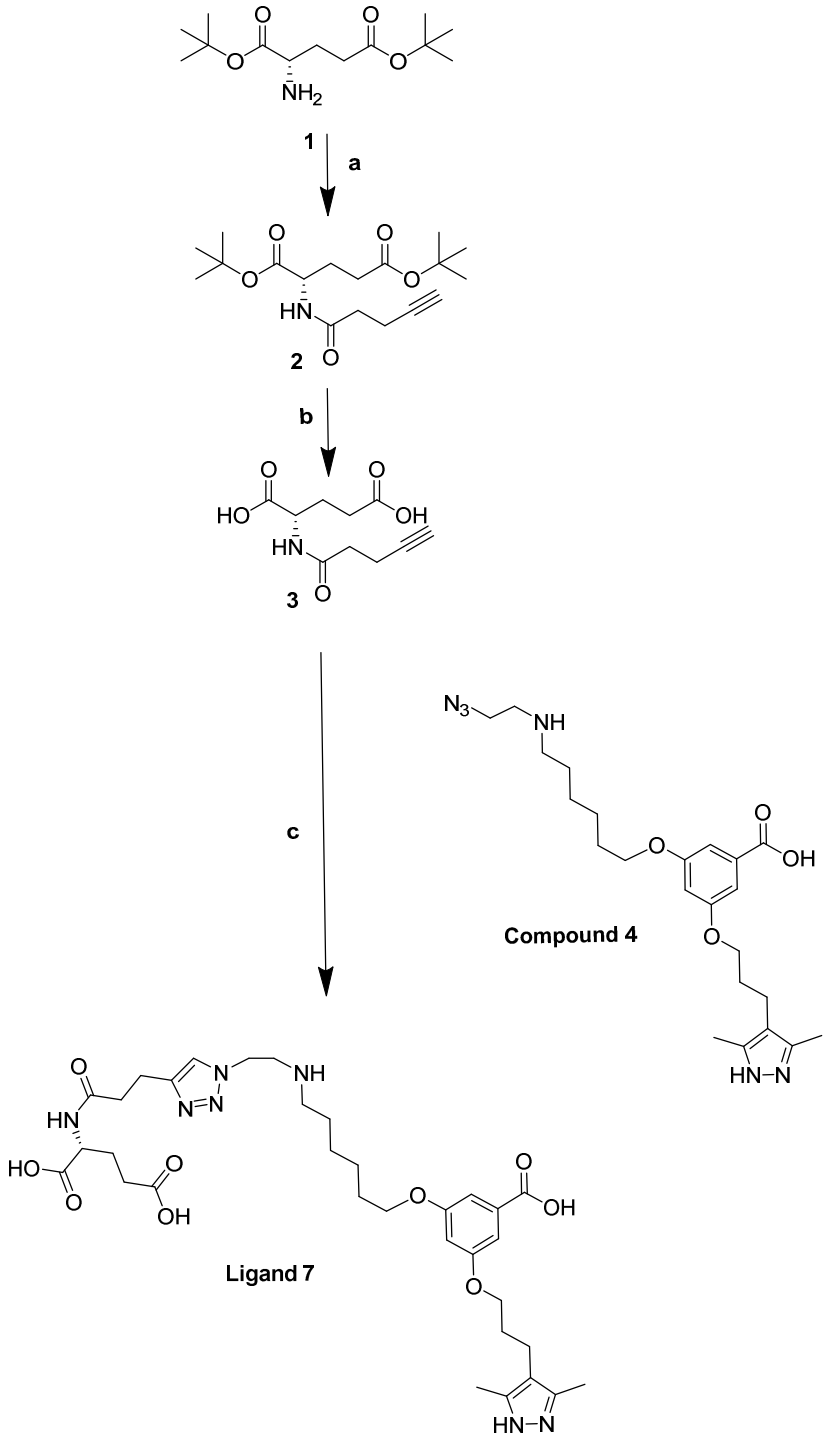
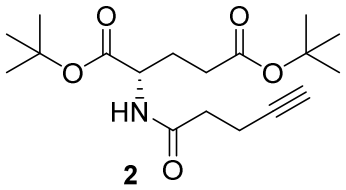
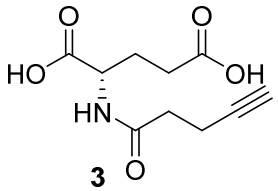


Figure 8. Synthetic scheme of Ligand 7. a) HOBt monohydrate, EDCI, DIPEA, DCM, room temperature (rt), 16 h; b) TFA in CH_2Cl_2, (1:4 ratio), rt, 2h; c) Compound 4, $CuSO_4$, sodium ascorbate, of H_2O/THF (2:1), rt, 3 h.



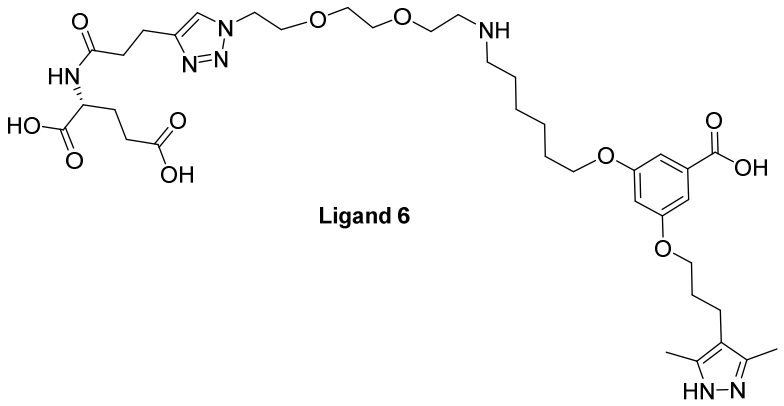
2

Synthesis of compound (2). To a solution of L-glutamic acid di-tert-butyl ester hydrochloride (100 mg, 0.34 mmol, 1 equiv) in DCM (10 ml) 4-Pentynoic acid (40 mg, 0.41 mmol, 1.2 equiv), HOBt monohydrate (50.2 mg, 0.37 mmol, 1.1 equiv), EDCI (71.3 mg, 0.37 mmol, 1.1 equiv and DIPEA (166 ul, 1.01 mmol, 3 equiv) was added. The reaction was flushed with nitrogen and stirred overnight. The crude reaction mixture was concentrated under reduced pressure and the reaction mixture was separated by flash column chromatography (silica gel, 1-10% EtOAc/hexanes system) to afford compound **2** (47mg, 41% yield).



3

Synthesis of compound (3). To a solution of **2** (23.5 mg, 0.07 mmol, 1 equiv) was added a mixture containing TFA and CH_2Cl_2, (1:4 ratio) (2 ml), and the reaction was stirred at room temperature for 3 h. The solution was concentrated under reduced pressure and proceed to the next step without further purification.



Ligand 6

Synthesis of Ligand 6. TLHE5 was reported elsewhere. The click (CuAAC) coupling was carried out by reacting TLHE5 (25.13 mg, 0.046 mmol, 1 equiv), Compound 3 (15.9 mg, 0.07 mmol, 1.5 equiv), $CuSO_4$ (2.87 mg, 0.0115 mmol, 0.25 equiv), and sodium ascorbate (4.5 mg, 0.023 mmol, 0.5 equiv) in a mixture of H_2O/THF (2:1) (3 ml). The reaction mixture was stirred at room temperature for 3 h. The solution was concentrated under reduced pressure and purified by preparative HPLC to afford Ligand 6 (3.27 mg, yield 8%). We check the mass to confirm identification and analytical HPLC for purity. (95% purity by HPLC measured at wavelength 254 nm): tR (column) (C18) = 18.91 min; ESI-MS: Exact mass calcd for $C_{37}H_{55}N_7O_{11}$ [M+H]+ 774.4 ; [M+2H]2+ 387.2. Found: 774.6, 387.8.

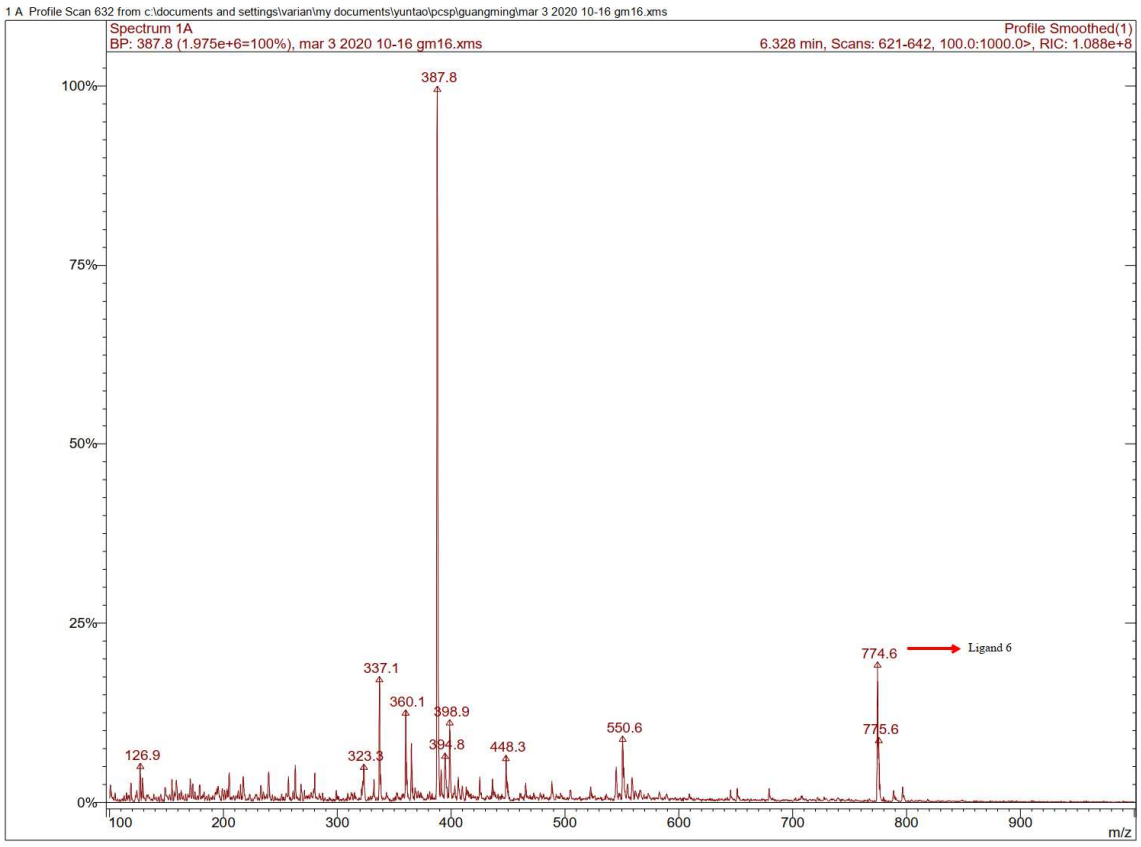


Figure 9. MS Data showing Ligand 6 (M.WT: 773.4)

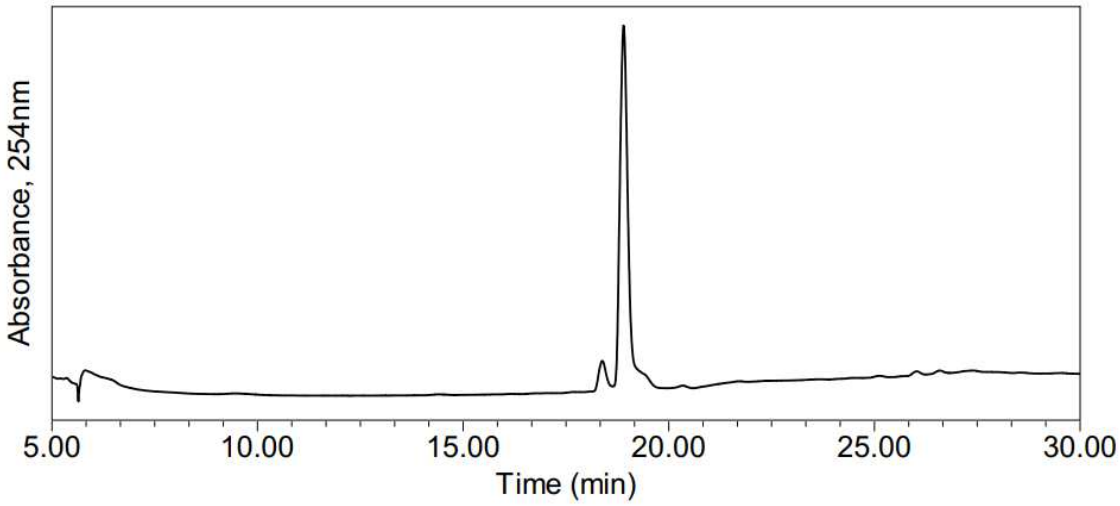
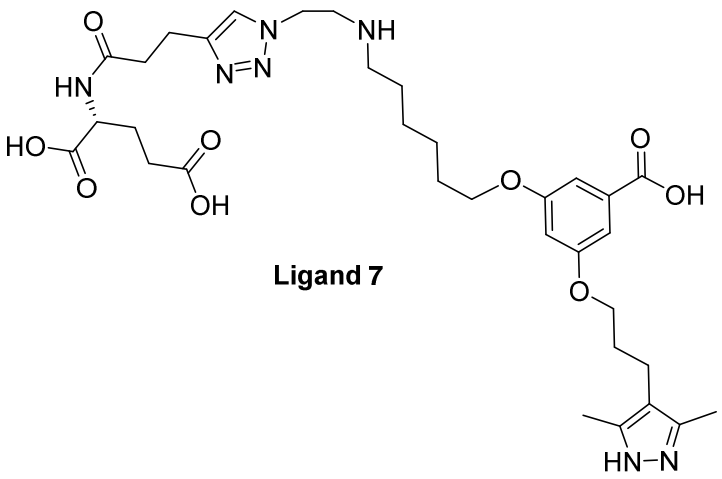


Figure.10. HPLC trace of TLHE Ligand **6** (>95% purity)



Ligand 7

Synthesis of Ligand 7. Compound 4 was reported elsewhere. The click (CuAAC) coupling was carried out by reacting Compound 4 (11.9 mg, 0.026 mmol, 1 equiv), Compound 3 (8.85 mg, 0.039 mmol, 1.5 equiv), $CuSO_4$ (1.7 mg, 0.0065 mmol, 0.25 equiv), and sodium ascorbate (2.7 mg, 0.013 mmol, 0.5 equiv) in a mixture of H_2O/THF (2:1) (3 ml). The reaction mixture was stirred at room temperature for 3 h. The solution was concentrated under reduced pressure and purified by preparative HPLC to afford Ligand 7 (12 mg, yield 58%). We check the mass to confirm identification and analytical HPLC for purity. (95% purity by HPLC measured at wavelength 254 nm): tR (column) (C18) = 18.56 min; ESI-MS: Exact mass calcd for $C_{33}H_{47}N_7O_9$ [M+H]+ 686.77; [M+Na]+ 708.76; [2M+2H]2+ 1373.54 Found: 686.3, 708.5, 1372.

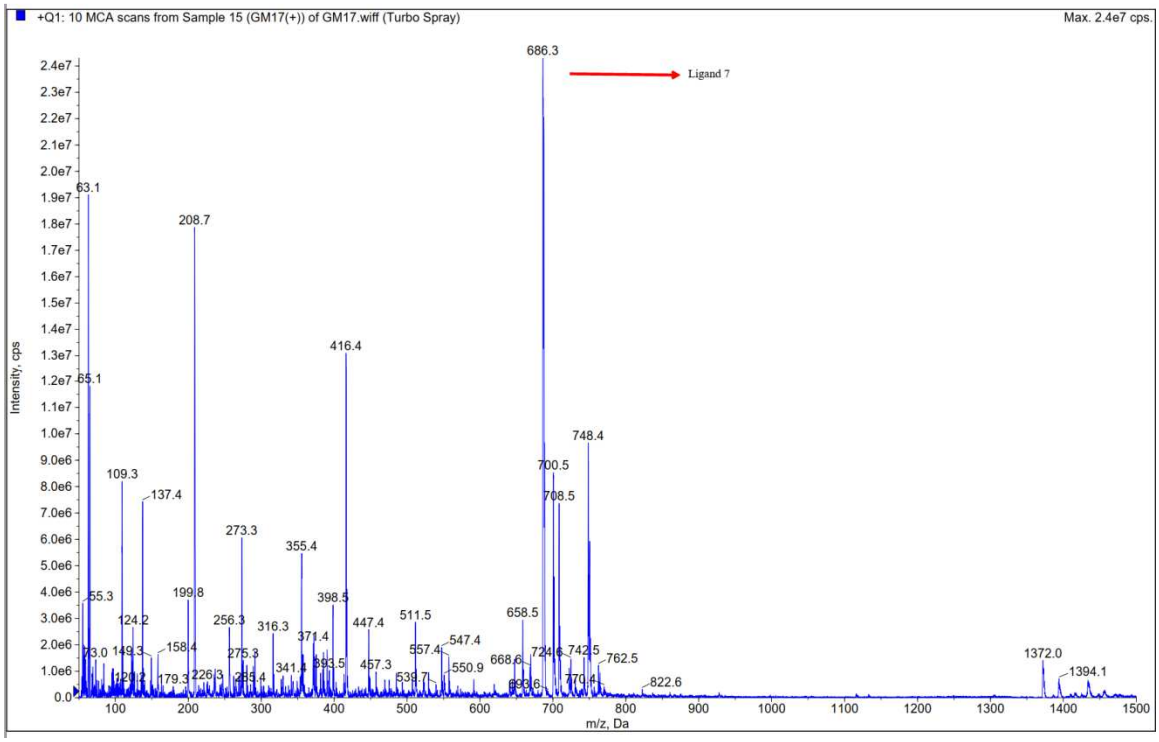


Figure.11. MS Data showing TLHE Ligand **7** (M.WT: 685)

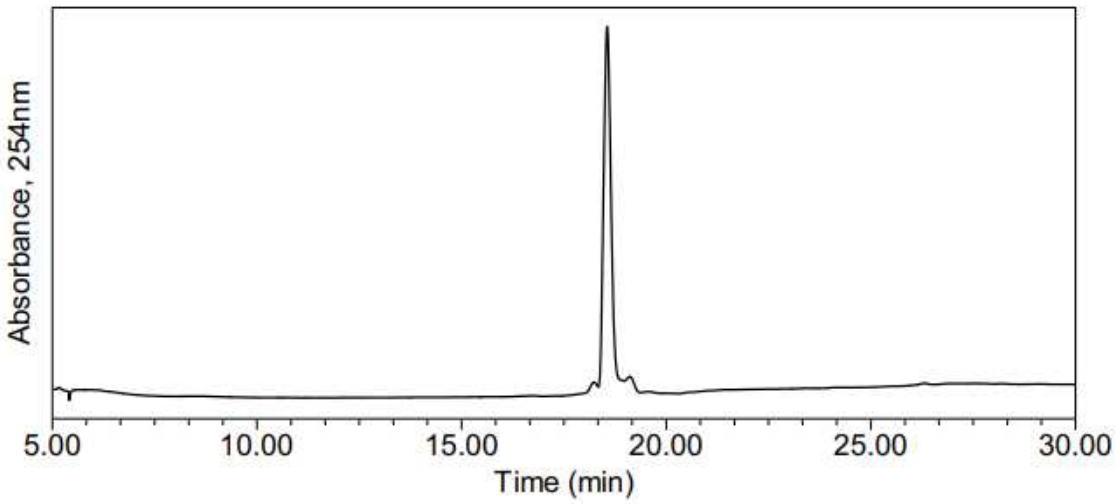


Figure 12. HPLC trace of TLHE Ligand 7 (>95% purity)

Evaluation of the Binding Affinity and Selectivity of Ligand 6 and 7 to TTR in Buffer and Serum

As previously stated, the composition of a spacer can have a significant impact on a molecule's tissue distribution, toxicity, and PK in vivo. From different studies, it is speculated that conjugation of spacers and molecules to TLHEs is likely to compromise their binding affinity and selectivity to TTR. The enhancement of half-life is dependent on the enhanced binding affinity and selectivity of our TLHEs to TTR according to our hypothesis. In order to ensure their efficacy, we first determined the binding affinity and selectivity of Ligand 6 and Ligand 7 to TTR in buffer and serum respectively. The binding affinity of our new ligands in buffer was evaluated by our group's established Fluorescence Polarization (FP) assay. This is a competitive ligand binding assay that measures ligand binding affinity (Kd) to TTR based on change of fluorescence by the influence of ligands (test compound), demonstrating ligand's ability to displace a fluorescent probe (FP-probe; Figure 2.6) from the TTR T4-binding sites. The advantages of FP assay are that the assay is simple and straightforward which only needs small amounts of test compound, TTR, and FP-probe. SpectraMax M5 microplate reader was used to measure fluorescence changes The apparent binding constant was calculated as the mean for duplicate experiments and the best data fit was determined by the R^2 value. Serial dilutions of Ligand 6 and 7 (20 mM to 0.01 mM) were mixed to FP-probe and TTR in assay buffer (final volume 25 mL). The FP assay was carried out as described above. The binding affinity and selectivity of the test compounds to TTR were measured by their capability to compete for the *covalent* probe that binds to TTR in human serum as reported earlier [26]. An aliquot human serum (Sigma–Aldrich) was mixed with test compounds so that the final concentration in each compound would be 10 μM and the probe would be 3.6 μM. Microplate spectrophotometer reader (Molecular Devices SpectraMax M5) measures

the fluorescence changes (λex = 328 nm and λem = 384 nm) in every 15 min using for 6 hrs. at 25o C.

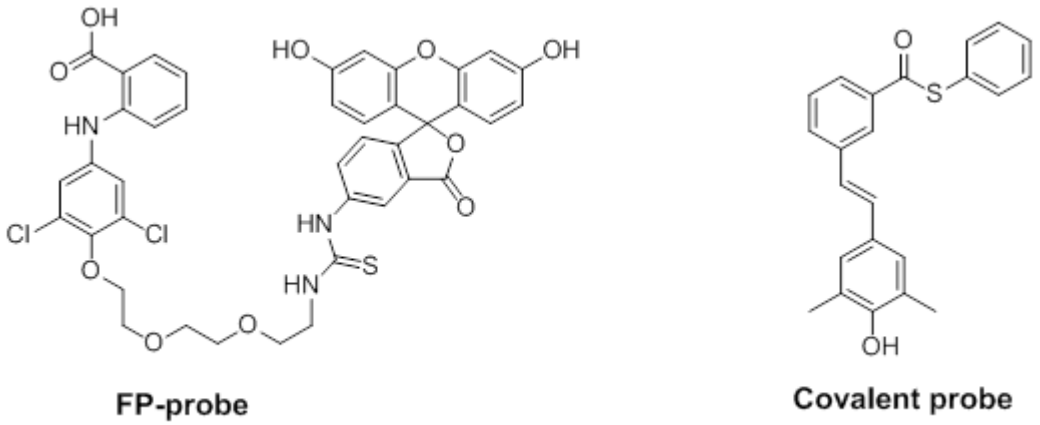


Figure 13. Chemical structures of Fluorescence Polarization (FP)-probe and covalent fluorescence probe.

CHAPTER 3: RESULTS

Binding Affinity of Ligand 6 and 7 to TTR in Buffer

The binding affinities of the Ligand 6 and 7 (Figure.3.1) to TTR were evaluated using our established fluorescence polarization (FP) assay [27]. The Fluorescence Polarization assay is a competitive assay that allows for the measurement of ligand binding to TTR depending on their capacity to replace a fluorescent probe [27] from TTR T4-binding sites. All test compounds should be able to bind to TTR (purified from human plasma) at 10 μM. Ligand 6 and 7 were assayed in a multi-point dose-response FP assay (concentration range between 20 and 0.01 μM). The binding affinity for Ligand 6 (Kd = 305 nM) and Ligand 7 (Kd = 279.6 nM) are shown in Figure 3.1.

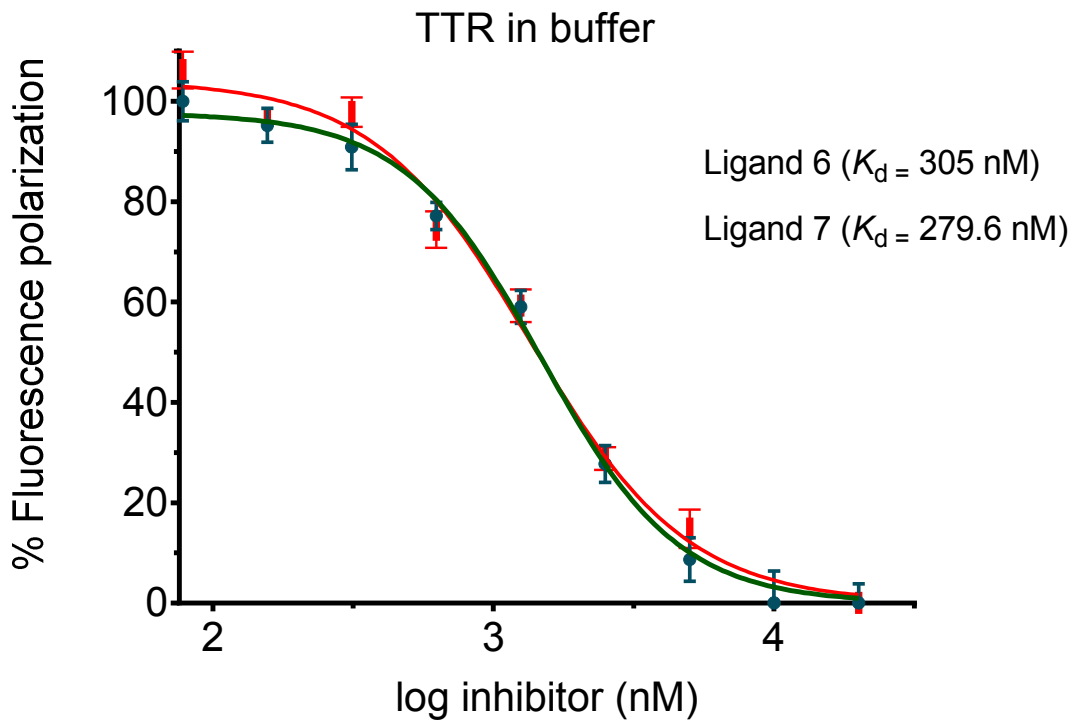


Figure 14. Evaluation of TLHE Ligand binding to TTR in buffer by Fluorescence Polarization. Competition of FP-probe from TTR by increasing concentrations (20 to 0.01 μM) of Ligand 6 and Ligand 7. The binding constant for Ligand 6 (K_d = 305 nM) and Ligand 7 (K_d = 279.6 nM) were calculated using the Cheng−Prusoff equation from IC50 values. Each point shows the mean ± SD of three replicates.

Binding Selectivity of Ligand 6 And 7 to TTR in Human Serum

The small molecule ligands must selectively bind to TTR in the presence of more than 4,000 other serum proteins. We examined the selectivity of Ligand 6 and 7 for TTR in human serum employing a ligand competition assay using a *covalent* probe [26]. The reported *covalent* probe binds selectively to TTR in human serum and then covalently modifies Lysine 15 residue, creating a fluorescent conjugate. The general principle of this fluorescence-based competition assay is that the candidate non-covalent kinetic stabilizers compete with the probe for the T_4 binding pocket, reducing the fluorescence generated by amide bond formation with TTR. Ligands that bind selectively to TTR in serum decrease the binding of the covalent probe to TTR, thus

decreasing the fluorescence signal [26]. In this assay all the test compounds (10 μM) were mixed with human serum (TTR concentration ~5 μM) in the presence of (3.6 μM) probe concentration. Ligand 6 and 7 performed better in this assay in serum (70% and 73% TTR occupancy, respectively) compared to tafamidis (~50% TTR occupancy) (Figure 3.2 and Figure 3.3).

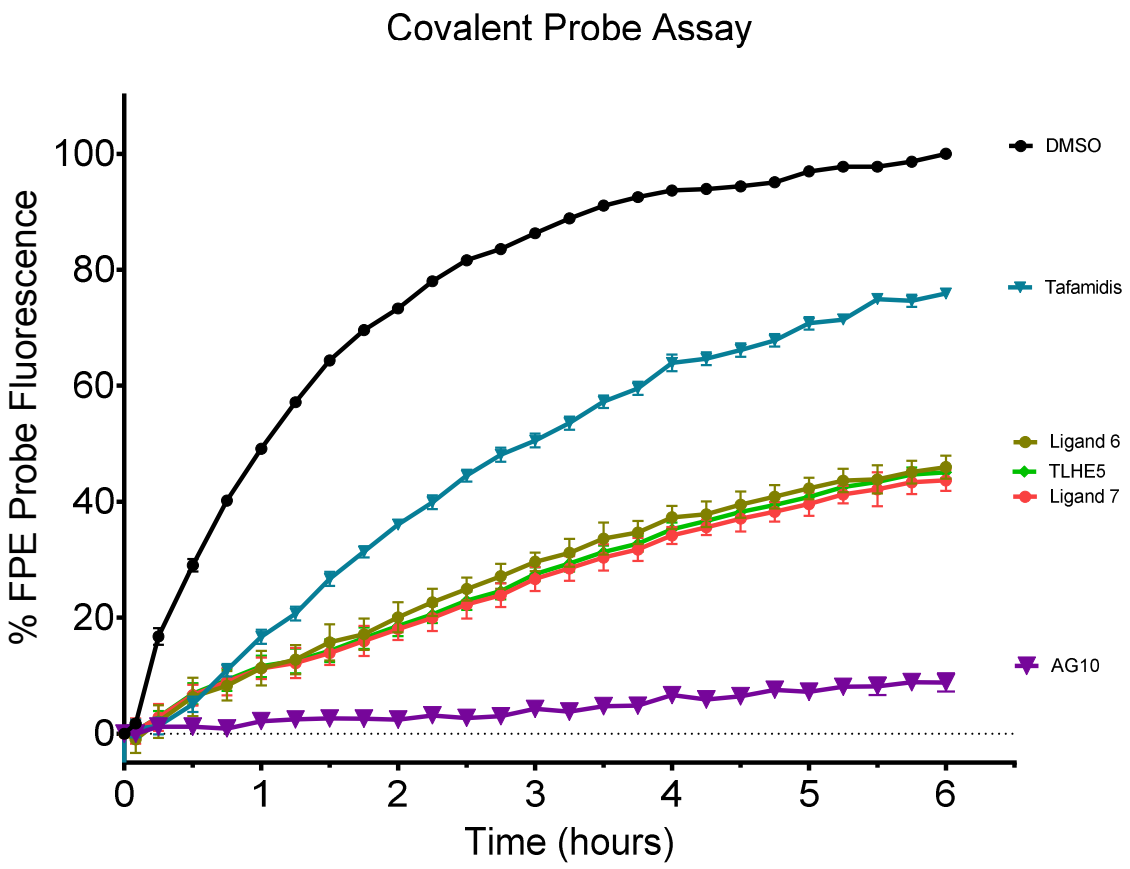


Figure 15. Ligand 6 and 7 bind selectively to TTR in human serum. Fluorescence change caused by modification of TTR in human serum by *covalent* probe monitored for 6 hours in the presence of probe alone (black circles) or probe and TTR ligands (colors).

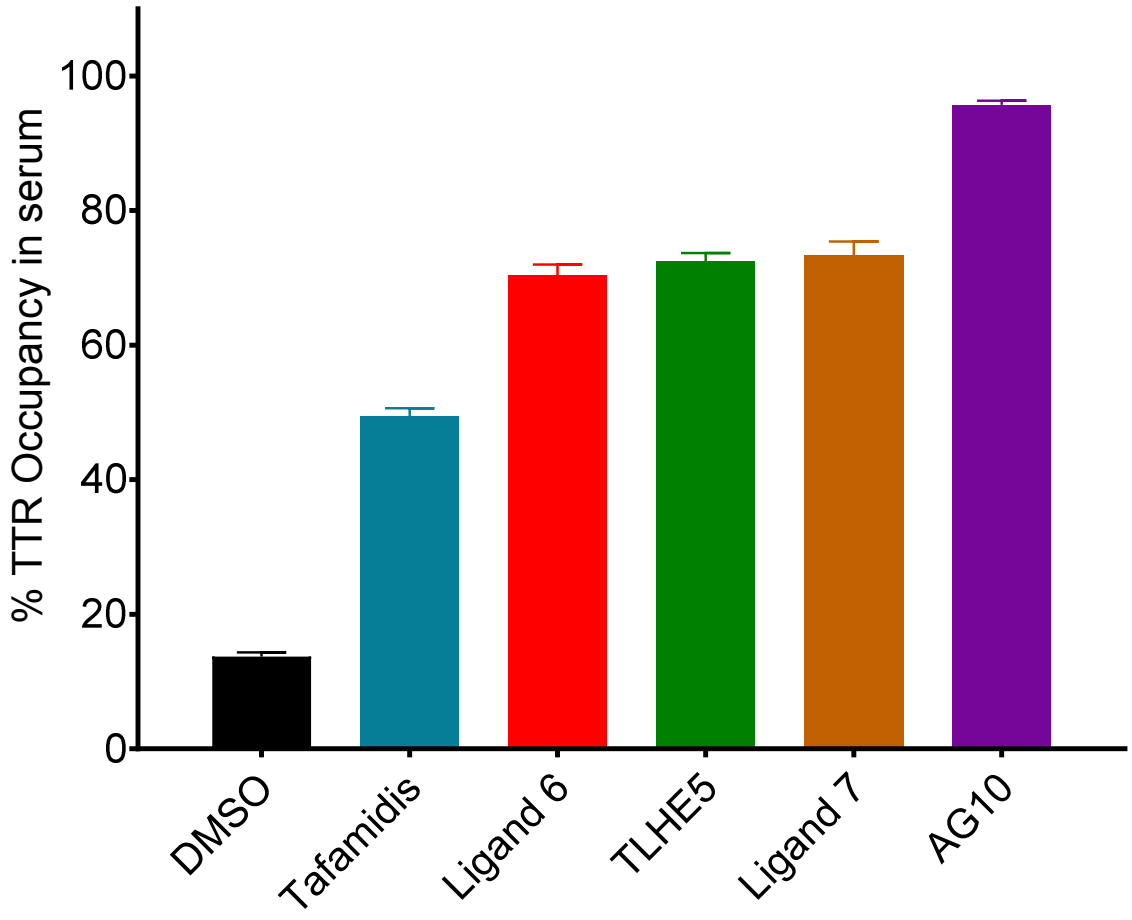


Figure 16. Percentage of test compound binding to TTR in human serum using *covalent* probe assay after 3 hours.

CHAPTER 4: DISCUSSION & CONCLUSION

For big peptides and proteins, there are a variety of appealing technologies that improve the half-life, such as conjugation to PEG and albumins. However, owing to the steric barrier, these techniques have some drawbacks, such as non-biodegradability, immunogenicity, and low binding affinity with target receptors. On the other hand, chemotherapeutic agents are generally hydrophobic in nature. Due to their nonpolar nature, the hydrophobic chemotherapeutic drug can easily be distributed to tissues irrespective of healthy and tumor cells resulting in off-target toxicity. Our group already published that increasing hydrophilicity of the chemotherapeutic agents by linker system can reduce penetration in healthy cells thus preventing nonselective toxicity. Therefore, the TLHE system has the potential to optimize the hydrophilicity as well as half-life extension of small molecules, peptides, and proteins without affecting the binding with their target. In addition to that, our approach offers a number of additional advantages over other strategies. Our technology involves a simpler chemical conjugation of the molecules of interest to TLHE, and the products are homogenous, easily purified, and characterized in harsh conditions like HPLC. The newest addition of this TLHE approach (e.g., Ligand 6 and Ligand 7) could maintain the hydrophilicity of the overall conjugated molecules in addition to half-life extension. These features could be useful for preventing off-target penetration of lipophilic drugs conjugated to TLHE especially hydrophobic anticancer payload. Here our data reveal, the addition of polar groups like glutamic acid on our TLHE does not hamper its binding affinity and selectivity towards TTR.

The affinity and selectivity of newly synthesized glutamic acid-containing TLHE ligands to TTR in human serum exceeds that of the FDA-approved TTR kinetic stabilizer drug, tafamidis. Ligand 6 and ligand 7 have similar binding affinity to previous TLHEs. The new ligand 6 and ligand 7 will be a starting point for further evaluation by conjugation to

hydrophobic drug molecules. The future study would be to see whether any improvement of hydrophilicity and binding affinity when this ligand 6 and ligand 7 will attach with highly nonpolar anticancer or other drugs of potential half-life issue.

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