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Synthesis of AG10 analogs and optimization of TTR ligands for Half-life enhancement (TLHE) of Peptides

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SYNTHESIS OF AG10 ANALOGS AND OPTIMIZATION OF THE STRUCTURE
OF TTR LIGANDS FOR HALF-LIFE ENHANCEMENT (TLHE) FOR PEPTIDES

by

Raghavendra Jampala

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Thomas J. Long School of Pharmacy and Health Sciences
Pharmaceutical and Chemical Sciences

University of the Pacific
Stockton, California

2017

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DEDICATION

To my Parents, Aruna, Sudarshan Babu and my brother Rahul

ACKNOWLEDGEMENT

First and foremost, I would like to thank my advisor and mentor Dr. Mamoun M. Alhamadsheh. They are no words to express my gratitude for his constant guidance and support he is more than a research advisor. I am greatly benefited from his teaching, advice and guidance on number of academic, prosomal, professional and carrier choices. His attention to detail and his vision of seeing a big picture always helped to understand the basics and big picture of the projects. I have always enjoyed working in his lab and hope I can follow what I have learned from him in coming years. I like to thank Dr. Park, Sravan, Rohit kumar and Fang for their support and teaching me the techniques for Pharmacokinetic studies and I am great full to my colleagues Sravan, Mark, Arindom, Wabel, Tuhin and Dengpan for their hard work and contribution towards my research.

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Synthesis of AG10 analogs and optimization of the structure of TTR Ligands for Half-life Enhancement (TLHE) for peptides

Abstract

by

Raghavendra Jampala

UNIVERSITY OF THE PACIFIC

2017

The misassembly of soluble proteins into toxic aggregates, including amyloid fibrils, underlies a large number of human degenerative diseases. Like cardiac amyloidosis, which is most commonly, caused by aggregation of Immunoglobulin (Ig) light chains or transthyretin (TTR) in the cardiac muscle, represent an important and often underdiagnosed cause of heart failure. TTR-mediated amyloid cardiomyopathies are chronic and progressive conditions that lead to arrhythmias, biventricular heart failure, and death. As no Food and Drug Administration-approved drugs are currently available for treatment of these diseases, the development of therapeutic agents that prevent TTR-mediated cardiotoxicity is desired. AG10 is a potent and selective kinetic stabilizer of TTR. AG10 prevents dissociation of TTR in serum samples obtained from patients with amyloid cardiomyopathy. The oral bioavailability and selectivity of

AG10, makes it a very promising candidate to treat TTR amyloid cardiomyopathy. Understanding the reason behind the potency of AG10 would be beneficial for designing stabilizers for other amyloid diseases. This would be possible by designing and synthesizing structural analogues of AG10. Here we report the synthesis, characterization and analysis of AG10 analogs and compare the *in vitro* activities of the synthesized analogs.

The tremendous therapeutic potential of peptides has not been fulfilled and potential peptide therapies that have failed far outnumber the successes so far. A major challenge impeding the more widespread use of peptides as therapeutics is their poor pharmacokinetic profile, due to short *in vivo* half-life resulting from inactivation by serum proteases and rapid elimination by kidneys. Extending the *in vivo* half-life of peptides is clearly desirable in order for their therapeutic potential to be realized, without the need for high doses and/or frequent administration. Covalent conjugation of peptides to macromolecules (e.g. polyethylene glycol or serum proteins such albumin) has been the mainstay approach for enhancing the *in vivo* half-life of peptides. However, the steric hindrance and immunogenicity of these large macromolecules often compromises the *in vivo* efficacy of the peptides. Recently, our laboratory established the first successful reversible method of extending the half-life of peptides using serum protein Transthyretin (TTR). The approach involved the use of a TTR Ligand for Half-life Extension ((TLHE-1) which binds to TTR with high specificity and affinity. We have shown that our technology extends the half-life of multiple peptides without seriously affecting their activity. Our main objective here is to modify the structure of the TLHE1 using linkers with different length and composition to optimize its affinity and selectivity for TTR in human serum.

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Chapter 1.General Introduction

1.1 Transthyretin

Transthyretin (TTR) is a 55kDa homo-tetrameric protein present in both plasma and cerebrospinal fluid. TTR is synthesized by the liver and secreted into the blood, where it acts as a backup carrier for thyroxine (T4) and primary carrier of holo retinol-binding protein (RBP). The crystalline structure of transthyretin is showed in (Figure.1) [1, 2, 3]. TTR invokes great scientific and medical interest as it is one of more than 30 proteins whose aggregation could cause disease by a gaining toxic function mechanism leading to amyloid fibril formation, in nervous system, soft tissue, and solid organs [3]. TTR aggregation, dissociation, and misfolding are known to be primarily responsible for several amyloid-related diseases like familial amyloid polyneuropathy (FAP), familial amyloid cardiomyopathy (FAC) and senile systemic amyloidosis (SSA).

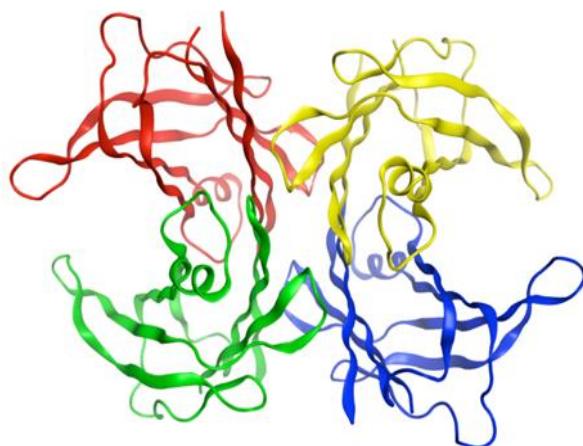


Figure.1: Crystal structure image of Transthyretin

TTR in plasma is primarily synthesized in the liver, while TTR in the brain is synthesized by choroid plexus. TTR is also synthesized at lower amounts in several other tissues such as retinal pigment epithelium of the eye, islets of Langerhans of the pancreas, intestine and meninges [4]. TTR present in blood is produced and secreted by the hepatocytes in the liver. Normal TTR concentrations in healthy adults range between 0.2 – 0.4 mg/mL (3.5 – 7 µM) [5]. When the liver is participating in acute phase response to injury, malnutrition, or chronic inflammation there is a significant decrease in plasma or blood TTR levels. Hence, TTR levels are used as a marker for nutritional and inflammatory status in the clinic especially in settings where it is difficult to obtain a more detailed and comprehensive nutritional assessment such as evaluation of albumin, α 1-acid glycoprotein, and C-reactive protein [6].

1.2 Structure of TTR

Human TTR is composed of four identical 127 amino acid residue subunits (~14 kDa) that form an extensive β -sheet structure [7]. The four identical subunits (or monomers) of TTR assemble to form an internal channel at the weaker dimer–dimer interface where two T_4 molecules could bind on the resulting tetramer [4, 7]. Recent studies have revealed that due to the presence of two other T_4 transport proteins in blood these T_4 binding sites remain largely unoccupied in humans (<1 % T_4 bound) and among those occupied, only one site is filled as there is negative cooperativity in the binding of the second T_4 [8, 9]. In addition to thyroid hormones (T_4) and its metabolites, several other pharmacologic agents and natural products, such as plant flavonoids, nonsteroidal analgesic drugs, and inotropic bipyridines, are strong competitors for T_4 binding to TTR and have binding affinities greater than T_4 [4, 10].

The structure of human wild-type TTR (WT-TTR) was one of the first proteins to be determined by X-ray crystallography [2]. Currently (as of June 2017), there are about 267 reported TTR crystal structures and their variants listed in the Protein Data Bank (PDB, www.rcsb.org) representing five species of protein (human, rat, mouse, chicken, and fish).

Studies have shown that the amino acid sequence of human WT- TTR is about 85 % identical to that from various other species [11].

1.3 Function and clinical importance of TTR

The most important role of TTR is the transport of T₄ and retinol. However, recent studies have indicated that TTR may also play an important physiological role in proteolysis and transport of other miscellaneous ligands [12]. TTR is not the primary transporter of T₄ (less than 1 % bound), due to the presence of two other T₄ transport proteins in blood, thyroxine-binding globulin (TBG) and albumin, the T₄ binding sites remain largely unoccupied in humans [13, 14]. However, in CSF, TTR is a major transporter of T₄ in both humans and rats. Interestingly, unlike in human plasma, TTR is the major transporter of T₄ in rats. As there is negative cooperativity between the two T₄ binding pockets, each TTR tetramer carries no more than one T₄ molecule at a time [15]. Retinol or vitamin A circulates in blood, bound to retinol binding protein (*holo*-RBP) which is found in complex with TTR. Evolution has proposed this association to facilitate RBP release from its site of synthesis in the endoplasmic reticulum and to prevent renal filtration of RBP [16, 17]. The presence of retinol bound to RBP is essential for the formation of a stable complex with TTR [18]. Structural studies revealed that the RBP binding site on TTR is orthogonal to TTR T₄-binding pockets (Figure.1.1). In plasma, RBP binds to TTR in 1:1 molar ratio due to limited concentration of RBP available. Recently, TTR has shown to have protective effect against amyloid beta (A β) deposition in Alzheimer's disease (AD). Studies have shown that TTR could bind and cleave soluble A β and prevents the A β amyloid fibril formation *in vitro* [19]. TTR cleaves A β in multiple positions generating shorter A β peptides displaying lower amyloidogenic potential. Also, TTR was able to degrade aggregated forms of A β and may contribute to maintenance of normal A β 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). Recently, clinical trial on tafamidis a TTR kinetic stabilizer, indicates that it slows the progression of early stage neuropathy in FAP patients [21]. Currently, there are no FDA-approved drugs for the treatment or prevention of FAC or SSA. As a result, therapy for most patients is confined to symptomatic reliefs. The treatment of choice to remove the mutated protein is by replacing the liver for hereditary TTR amyloidosis. Combined liver and heart transplantation is performed as a palliative measure for a subset of FAC patients [22]. The risk and cost of organ transplantation is substantial and transplant patient requires lifelong use of immune-suppressive drugs leading to morbidity. Due to these circumstances; there is an urgent need for a pharmacologic treatment of TTR cardiomyopathy.

We used high-throughput screening (HTS) for TTR ligands, which enabled the identification of a variety of potent and structurally very different TTR kinetic stabilizers. Our lab used the previous compounds as a starting point for structure-activity relationship studies and synthesized a series of analogs, one of which the best hits from HTS named AG10 [3] is a highly effective and selective stabilizer of TTR. Understanding the reason behind the potency of AG10 would be beneficial for designing stabilizers for other amyloid diseases. This would be possible by designing and synthesizing structural analogues of AG10. Here we report the synthesis of AG10 analogs (Figure.2) and the evaluation of their *in vitro* activity in stabilizing TTR in comparison to tafamidis and AG10.

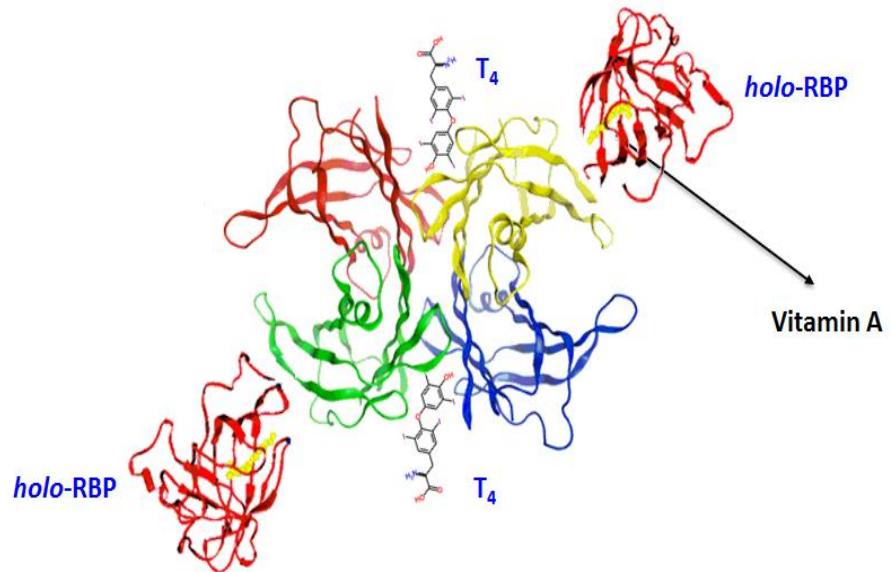


Figure 1.1: TTR bound to T₄ and holo-Retinol-binding protein (holo-RBP).

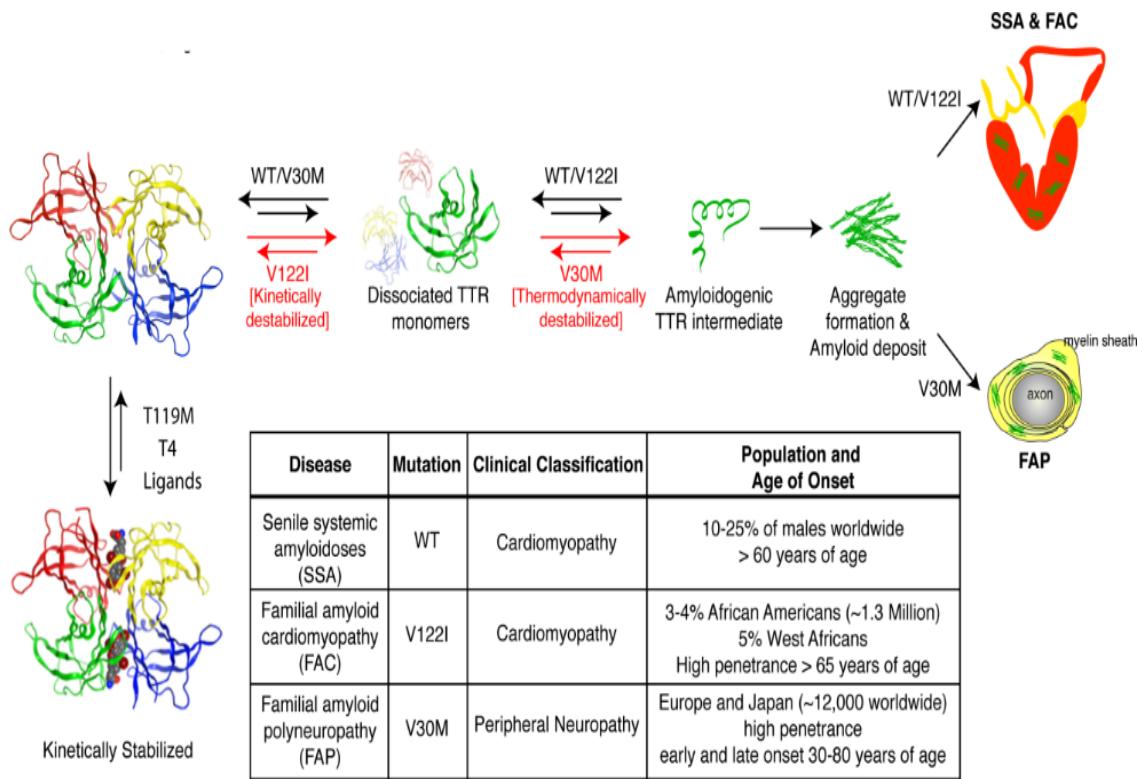


Figure 1.2: The TTR amyloidogenesis cascade and a table summarizing TTR-mediated amyloidoses. TTR amyloidoses require rate-limiting tetramer dissociation to dimers, followed by dissociation into monomers before partial unfolding of monomers yields the aggregation-prone amyloidogenic intermediate. The amyloidogenic intermediate can misassemble to form a variety of toxic aggregates, including amyloid fibrils. Disease-associated destabilizing mutations can kinetically or thermodynamically destabilize TTR. Kinetic stabilization can be achieved through trans-allelic suppression with the kinetically stabilized T119M-TTR, binding to T₄ or other small molecules (Lower Left).

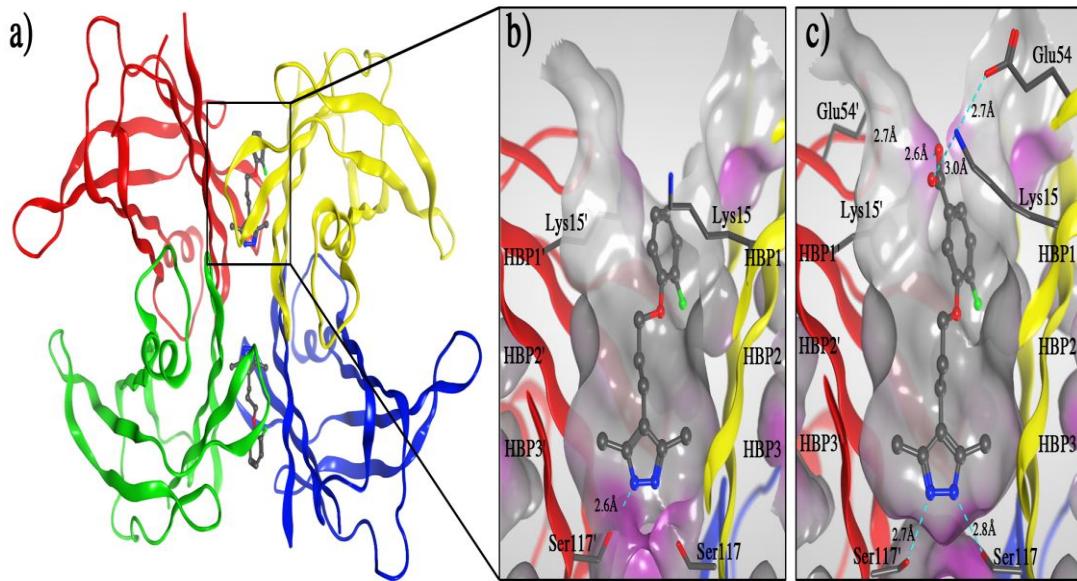


Figure 1.3: Crystal structures of TTR ligand complexes. (A) Quaternary structure of 1 bound to WT-TTR shown as a ribbon representation with monomers colored individually. (B) 1 in complex with WT-TTR (3P3T). (C) AG10 in complex with V122I-TTR showing the introduced carboxylate making electrostatic salt bridge interactions with Lys15 and Lys15'. Close-up views of one of the two identical T₄-binding sites in a ribbon depicted tetramer colored by chain. A “Connolly” molecular surface was applied to residues within 10 Å of ligand in the T₄-binding pocket hydrophobic (grey), polar (purple). The innermost halogen binding pockets (HBPs) 3 and 3' are composed of the methyl and methylene groups of S117/117', T119/119', and L110/110'. HBPs 2 and 2' are made up by the side chains of L110/110', A109/109', K15/15', and L17/17'. The outermost HBPs 1 and 1' are lined by the methyl and methylene groups of K15/15', A108/108', and T106/106'. Hydrogen bonds shown in light blue dashed lines, with the atomic distances labeled in Å.

Chapter 2. Synthesis of AG10 Analogs

2.1 Methods and Materials

Pre-albumin from human plasma (human TTR) was purchased from Sigma, probe was synthesized in lab. All other starting materials for the synthesis of AG10 analogs were purchased from Alfa Aesar and Accela. The solvents used were ACS grade purchased from Fisher Scientific, and reagents were purchased from Aldrich and Acros, and used without further purifications. Reactions were monitored using thin-layer chromatography (TLC) carried out on 0.2mm POLYGRAM® SIL silica gel plates with fluorescent indicator UV254 using UV light as a visualizer. For purification normal phase flash column chromatography was carried out using Davisil® silica gel (100-200 mesh, Fisher Scientific), High-resolution mass spectra (HRMS) were determined by JEOL AccuTOF DART using helium as an ionization gas and HPLC analysis was performed on a Waters™ Alliance 2790 system attached to Waters™ 2990 PDA detector operating between the UV ranges of 200 – 400 nm. A empower 2.0 data acquisition system was used for quantification purposes. A Waters™ XBridge C18 column with L1 packing (4.6 X 150 mm, 5 μ m) was used at ambient temperature. The UV absorbance was recorded at 280 nm and 254 nm.

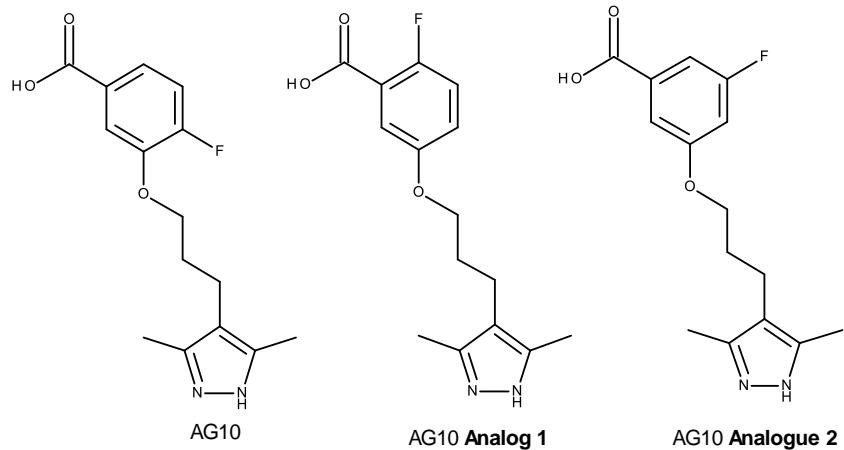


Figure.2: Structure of AG10, AG10 **Analog 1** & AG10 **Analogue 2** showing difference in the position of fluorine.

2.2 Chemical Synthesis of AG10

The chemical synthesis of AG10 is very similar to what was reported earlier for AG10 [3]. The only difference is using different fluoro-aromatic ring as starting materials.

2.3 Chemical Synthesis of AG10 Analog 1

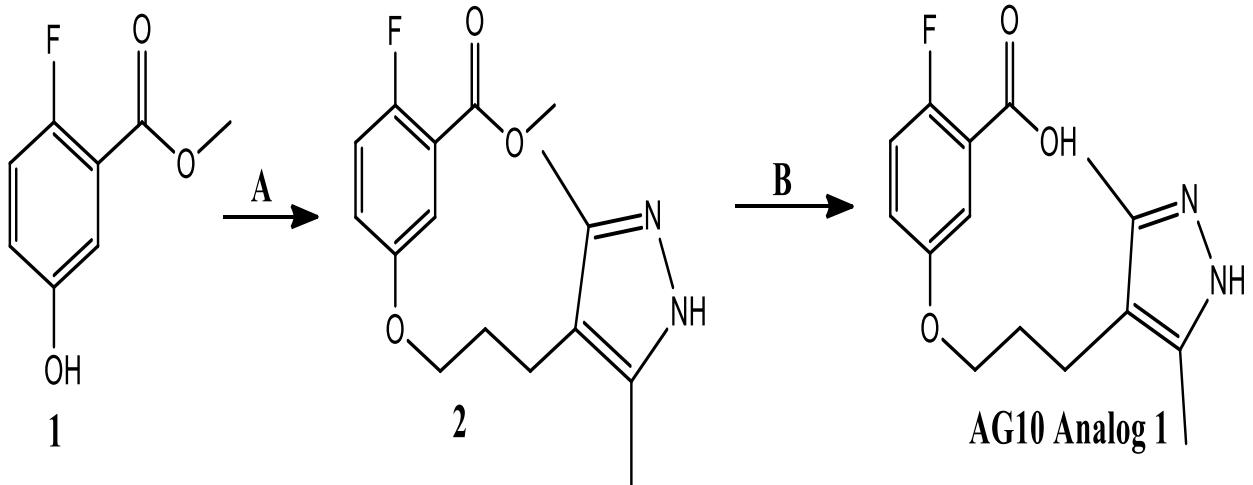


Figure.2.1: Synthesis of AG10 **Analog 1**. **A)** Methyl-2-fluoro-5-hydroxybenzoate, 4-(3-bromopropyl)-3,5-dimethyl-1*H*-pyrazole, K_2CO_3 , DMF, room temperature, 18-20 hrs; **B)** NaOH, water, MeOH, 50°C reflux, 14 hrs.

Methyl 5-(3-bromopropoxy)-2-fluoro-benzoate (2): the synthesis scheme of AG10 **Analog 1** is depicted in (Fig. 2.1). To a solution of methyl 2-fluoro-5-hydroxybenzoate **1** (50 mg, 0.59 mmol, 1 equiv) and 4-(3-bromopropyl)-3,5-di-methyl-1H-pyrazole (87 mg) in DMF (2 mL) was added K₂CO₃ (101 mg, 1.42 mmol, 2.5 equiv). The reaction mixture was stirred at room temperature (r.t.) for 16 hrs. The mixture was diluted with EtOAc (1.5 L), washed with brine (3x0.5 L) and dried with Na₂SO₄. The solution was filtered and concentrated. The residue was purified by flash column chromatography (silica gel, 1-10 % EtOAc/hexanes) to afford compound **2** (77 mg, 82 % yield).

5-[23]-2-fluoro-benzoic acid (AG10 analog 1); to a suspension of **2** (77 mg, 0.33 mmol, 1 equiv) in a mixture of MeOH (1.5 mL) and water (500 μL), NaOH.H₂O (21 mg, 0.50 mmol, 2 equiv) was added. The reaction mixture was stirred at 50°C reflux for 14 hrs. The mixture was extracted with EtOAc (3 x 30 mL) and the combined organic extracts were dried over anhydrous sodium sulfate and concentrated *in vacuum*. The crude product was subjected to flash column chromatography (silica gel, 10-50 % MeOH/CH₂Cl₂) to give of **AG10 analog 1** (40 mg, 71% yield) as a white solid (>98 % purity by HPLC).

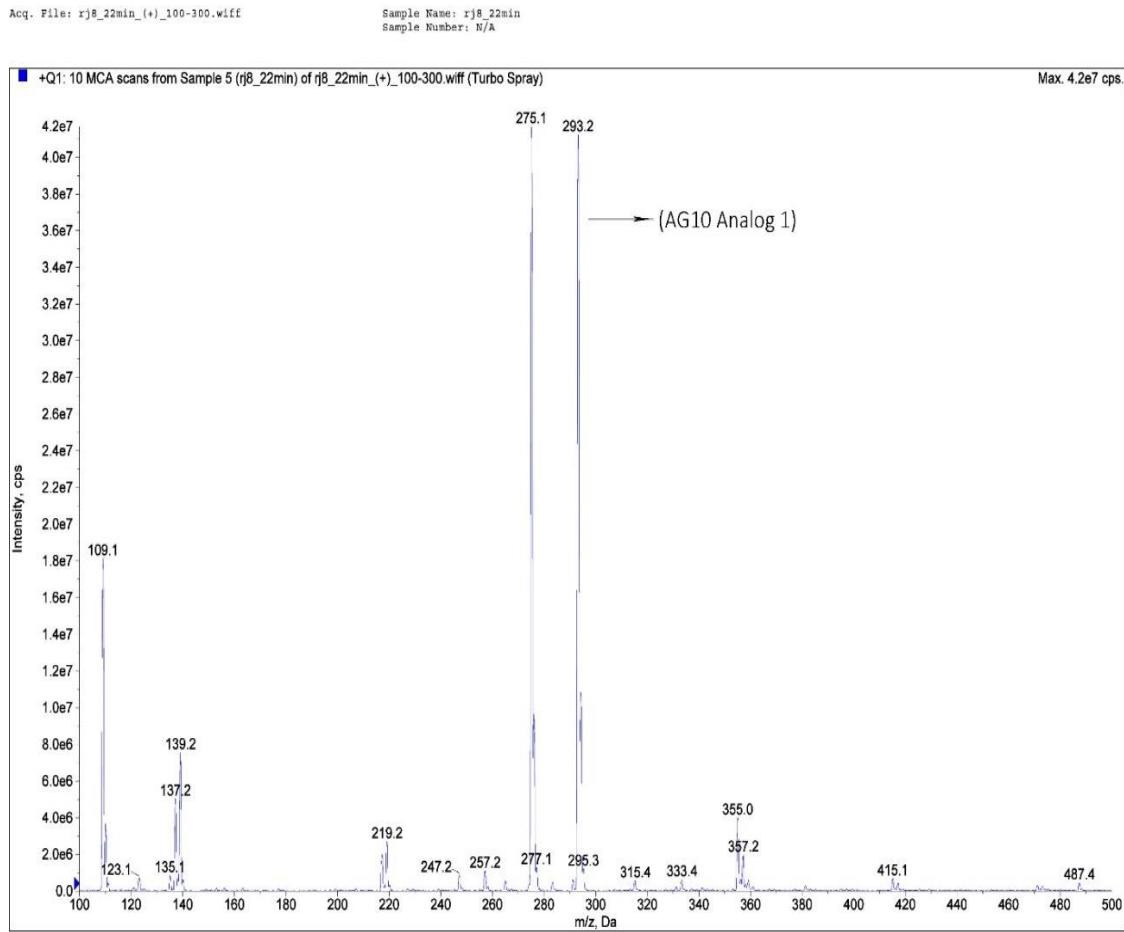


Figure 2.2. MS Data showing AG10 Analog 1 (M.WT: 292).

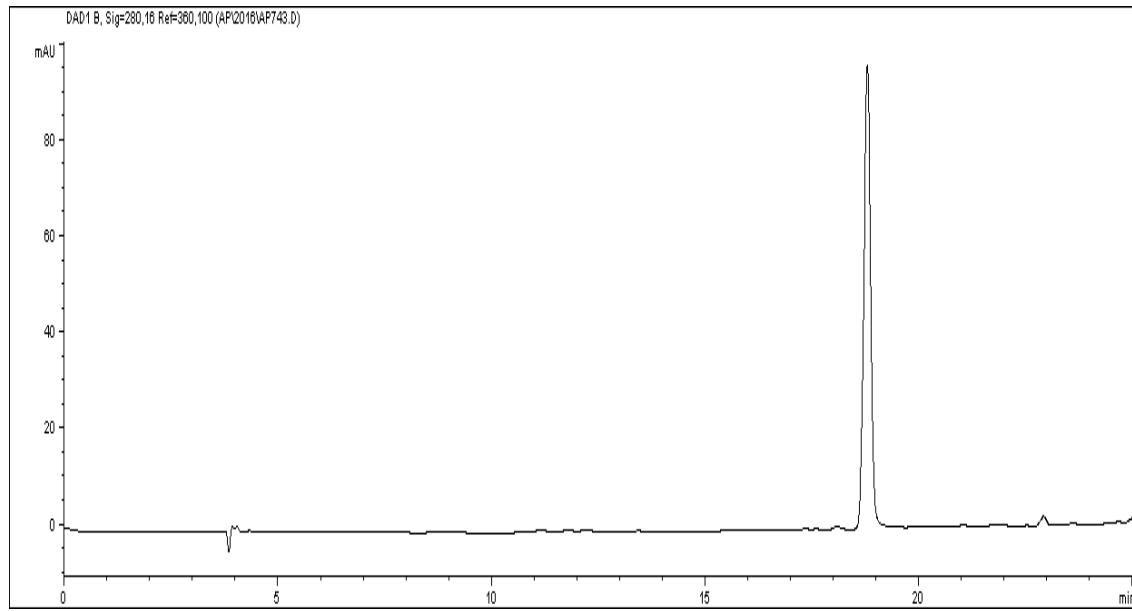


Figure 2.3: HPLC trace of AG10 Analog 1 (>98% purity).

2.4 Chemical Synthesis of AG10 Analog 2

The synthesis scheme of AG10 **Analog 2** is depicted in Figures 2.4.

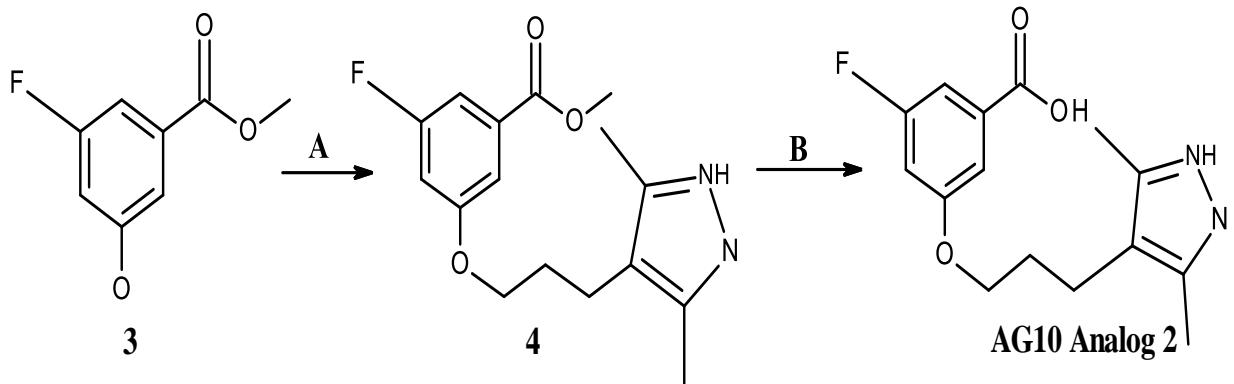


Figure 2.4: Synthesis of AG10 **Analog 2**. **A)** Methyl-3-fluoro-5-hydroxybenzoate 4-(3-bromopropyl)-3,5-dimethyl-1H-pyrazole, K₂CO₃, DMF, rt, 18-20 hrs; **B)** NaOH, water, 50°C reflux, 14 hrs.

methyl 3-[3-(3,5-dimethyl-1H-pyrazol-4-yl)propoxy]-5-fluoro-benzoate (4). To a solution of methyl 4-fluoro-3-hydroxybenzoate **3** (101mg, 0.59 mmol, 2.5 equiv) and 4-(3-bromopropyl)-3,5-di-methyl-1H-pyrazole (87 mg) in DMF (2 mL) was added K₂CO₃ (101mg, 1.42 mmol, 2.5 equiv). The reaction mixture was stirred at room temperature (r.t.) for 16 hrs. The mixture was diluted with EtOAc (1.5 L), washed with brine (3x0.5 L) and dried with Na₂SO₄. The solution was filtered and concentrated. The residue was purified by flash column chromatography (silica gel, 1-10 % EtOAc/hexanes) to afford compound **4** (4.21 g, 82 % yield).

3-(3-(3,5-Dimethyl-1H-pyrazol-4-yl)propoxy)-4-fluorobenzoic acid (AG10 analog 2). To a suspension of **4** (121.25 mg, 0.40 mmol, 1 equiv) in a mixture of MeOH (1.5 mL) and water (500μL), NaOH.H₂O (33.24 mg, 0.50 mmol, 2 equiv) was added. The reaction mixture was

stirred at 50°C reflux for 14 hrs. The mixture was extracted with EtOAc (3 x 30 mL) and the combined organic extracts were dried over anhydrous sodium sulfate and concentrated *in vacuo*. The crude product was subjected to flash column chromatography (silica gel, 10-50 % MeOH/CH₂Cl₂) to give of **AG10 analog 2** (85 mg, 71% yield) as a white solid (>98 % purity by HPLC).

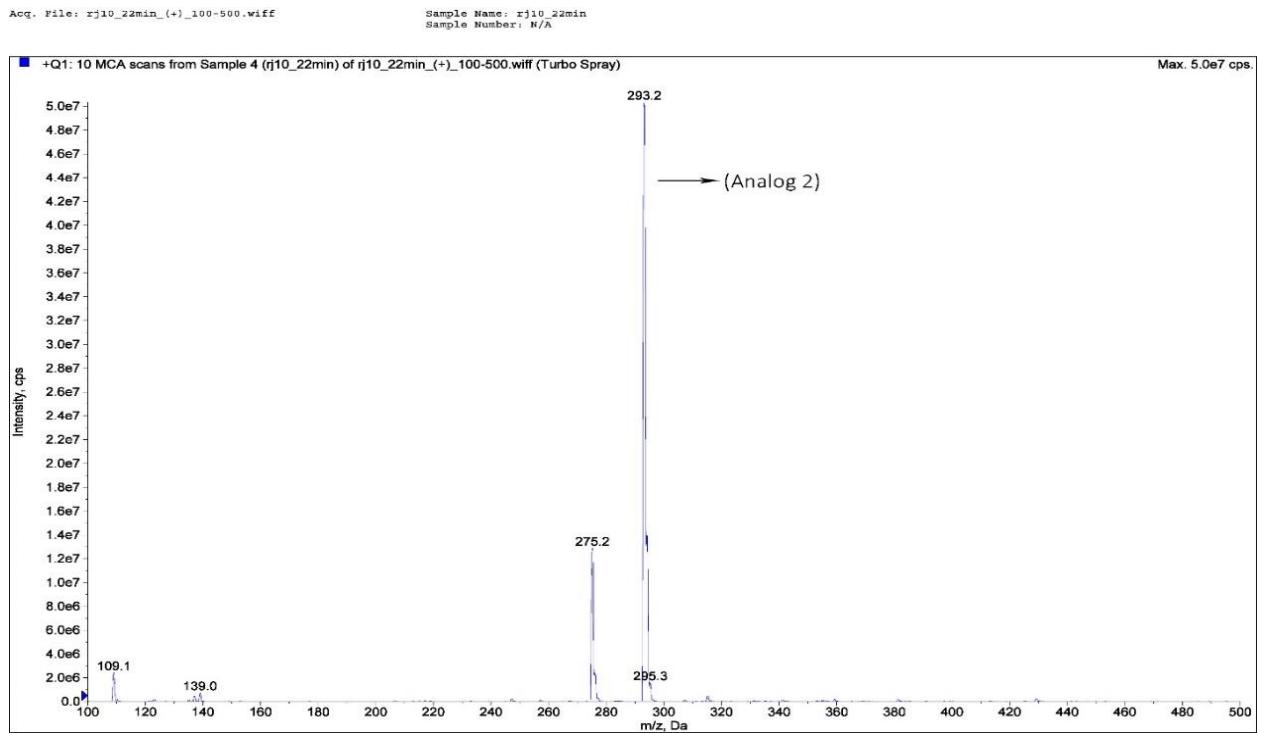


Figure.2.5: MS Data showing AG10 Analog 2 (M.WT: 292).

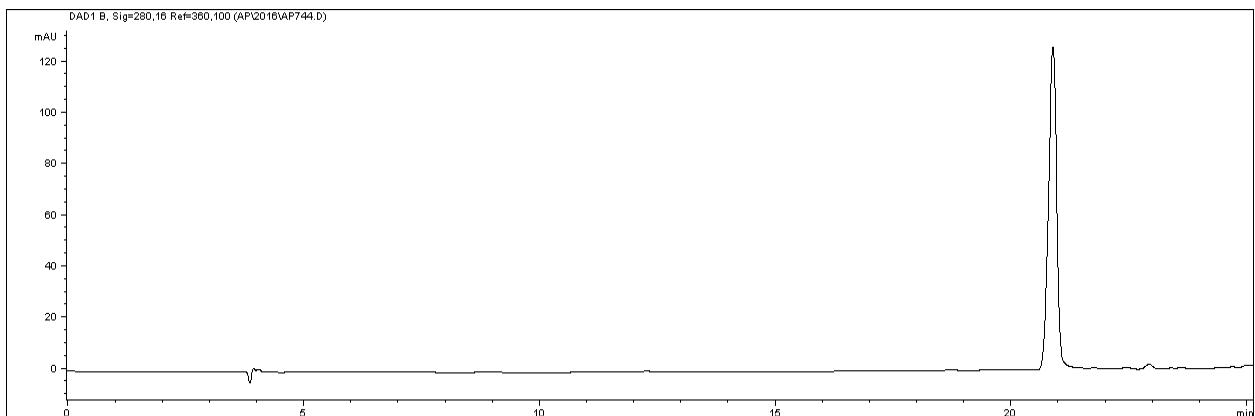


Figure 2.6: HPLC trace of AG10 Analog 2 (>98% purity).

2.5 Fluorescence Polarization (FP) Binding Assay

Determination of FP Probe Displacement by TTR Ligands. The affinity of test compounds to TTR was determined by their ability to displace FP probe form TTR using our recently developed assay [25]. The apparent binding constant was reported as the mean for triplicate experiments and the best data fit was determined by R^2 value.

Determination of IC₅₀ using the FP assay; Serial dilutions of **AG10** analogs and tafamidis (40 μ M to 0.003 μ M) were added to a solution of FP-probe and TTR in assay buffer (25 μ L final volume). The FP assay was then carried out as described above.

2.6. Serum TTR selectivity Assay

The binding affinity and selectivity of the compounds to TTR was determined by their ability to compete for *covalent* probe binding to TTR in human serum as previously reported [26]. An aliquot (98 μ L) of human serum (Sigma–Aldrich) was mixed with 1 μ L of test compounds (1.0 mM stock solution in DMSO, final concentration: 10 μ M) and 1 μ L of probe (0.36 mM stock

solution in DMSO: final concentration: 3.6 μM). The fluorescence changes ($\lambda_{\text{ex}} = 328 \text{ nm}$, $\lambda_{\text{em}} = 384 \text{ nm}$) were monitored every 15 min using a microplate spectrophotometer reader (Molecular Devices SpectraMax M5) for 6 hrs at 25°C.

2.7 Results

2.7.1 Binding affinity of AG10 Analogs to TTR

The binding affinities of the AG10 analogs (Fig. 2) to TTR were evaluated and compared to AG10 using our established fluorescence polarization (FP) assay [25]. The FP assay is a competitive assay that allows for the measurement of ligand binding to TTR based on their ability to displace a fluorescent probe [27] from the TTR T₄-binding sites. All test compounds were able to bind to TTR (purified from human plasma) at 10 μM . AG10 and new AG10 analogs, were assayed in a multi-point dose-response FP assay (concentration range between 40 and 0.00244 μM). The binding affinity of **AG10** to WT-TTR (apparent binding constant, $\text{IC}_{50} = 87.39$, $R^2 = 0.996$) were approximately same than that of AG10 analogs ($\text{IC}_{50} = 99.6$ and 94.5, $R^2 = 0.998$ and 0.981) (Fig. 2.7).

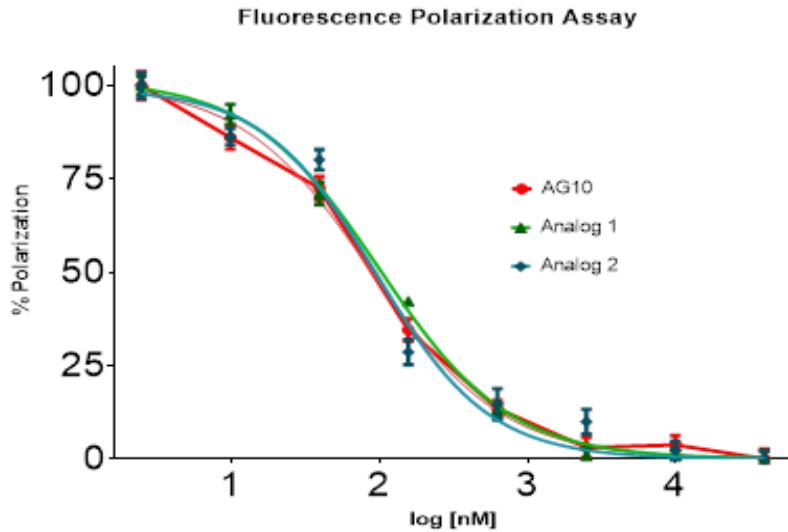


Figure.2.7: Evaluation of AG10 analogs binding to TTR in buffer by FP. Competition of FP-probe from TTR by increasing concentrations (40 to 0.00244 μ M) of AG10 ($IC_{50} = 87$, $R^2 = 0.996$) and AG10 analogs ($IC_{50} = 99.6$ and 94.5, $R^2 = 0.998$ and 0.981). Each point shows the mean \pm SD of three replicates.

Table.2.1: IC_{50} value of compounds in Fluorescence polarization.

Compound Name	IC_{50} Value (nM)	K_d value (nM)
AG10	87.39	18.01
AG10 Analog 1	99.6	20.53
AG10 Analog 2	94.5	19.48

2.7.2 Binding selectivity of AG10 analogs to TTR in human serum

To stabilize TTR tetramers in FAC and SSA patients, small molecules must selectively bind to TTR in the presence of more than 4,000 other serum proteins. We examined the selectivity of AG10 analogs for TTR in human serum employing a ligand competition assay using a *covalent* probe (Fig. 2.8) [23]. The *covalent* Probe binds selectively to TTR in serum and then covalently modifies K15 residue, creating a fluorescent conjugate. Ligands that bind selectively

to TTR in serum decrease the binding of probe to TTR, decreasing the fluorescence [27]. However, in addition to selectivity, this assay depends also on the relative affinity of probe and the test compound for the TTR binding sites, especially the second binding site (given that generally $K_{d2} > K_{d1}$). Nevertheless, empirically, the results of the probe competition assay at 3 hr correlate well with other direct measures of selectivity. The compounds (10 μ M) are mixed with human serum (TTR concentration ~5 μ M) in the presence of probe (3.6 μ M). AG10 analogs performed better in this assay in serum (42% probe binding for Analog 1 and 41% probe binding for Analog 2 after 6 hrs) compared to tafamidis (84% probe binding after 6 hrs) (Fig. 2.8). AG10 was more selective to TTR (12% probe binding after 6 hrs) than the two new analogs.

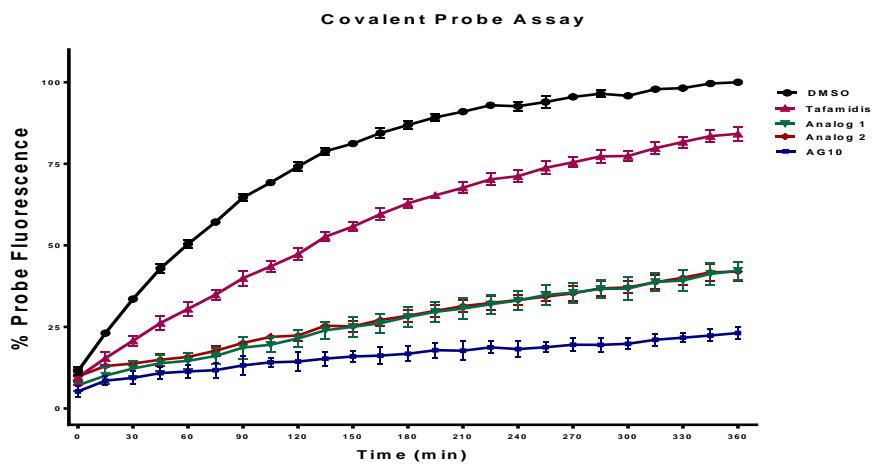


Figure 2.8: AG10 Analogs binds selectively to TTR in human serum. Fluorescence change caused by modification of TTR in human serum by covalent probe monitored for 6 hrs in the presence of probe alone (black circles) or probe and TTR ligands (colors).

Table 2.2: Percentage of test compound binding to TTR in human serum using *covalent* probe assay after 3 hrs.

Test Compound	% Binding to TTR
DMSO	0%
Tafamidis	16%
AG10 Analog 1	71.6%
AG10 Analog 2	72%
AG10	92%

2.8 Discussion

Genetic and pharmacologic data have indicated that kinetic stabilization of tetrameric TTR can slow down disease onset and/or progression [28, 29, 21]. Ligands that bind tightly to the T₄ binding site of TTR reduce tetramer dissociation and subsequent amyloidogenesis. The selectivity, affinity and efficacy of TTR stabilizers could be optimized if a compound were able to bind to both the inner cavity and the periphery of the T₄ binding site. This would be especially critical for compounds aimed at stabilization of disease-associated TTR variants. Using AG10 as the starting point, we designed two new analogs by changing the position of fluorine atom [Figure 2]. We hypothesized that the fluorine atom might have an important role in the binding of AG10 analogs to TTR, because the halogen of the AG10 fits in the halogen binding pocket (HBP) when AG10 occupies the T₄ binding pocket of TTR. Guided by structural information on TTR ligands [25], we designed and synthesized two AG10 analogs and tested them for affinity, selectivity, and stabilization of TTR. Among all the analogs, AG10 was the most potent compound [3]. The new Analogs 1 and 2 displayed higher binding affinity and selectivity to TTR than tafamidis. However, AG10 was more selective TTR binder than the new analogs.

2.9 Conclusion

The affinity and selectivity of AG10 and newly synthesized AG10 analogs to TTR in human serum exceeds that of the FAP drug, tafamidis. The binding selectivity and affinity of

AG10 was much better when compared to AG10 analogs, but still AG10 analogs showed a much better affinity and selectivity when compared with tafamidis. The new AG10 analogues will be a starting point for further evaluation. Understanding the reason behind the higher potency of AG10 compared to these analogs would be beneficial for designing stabilizers for other amyloid diseases.

Chapter3. Optimization of the structure of TTR ligands for half-life extension (TLHE) of Therapeutic Peptides

3.1 Introduction

3.1.1 Peptides. Peptides are small chain of amino acids connected by a peptide bond. Distinguished from proteins based on size, peptides have approximately 50 or less amino acids [30]. They are synthesized by simple solid phase peptide synthesis where a covalent bond is formed when a carboxyl group of the C-terminal from one amino acid reacts with amine group of the N-terminal from another amino acid, in peptides the shortest peptide is known as a dipeptide and longest one know as a polypeptide. Therapeutic peptides are often used in treating a wide range of disorders, such as cancers, diabetes etc [24, 31]. Their high selectivity, potency, and safety compared with small molecules, make peptides attractive drug candidates.

Limitations to Use Peptides

Most peptides have short *in vivo* half-life (2–30 min) owing to enzymatic degradation by proteases and fast renal clearance (molecules <30 kDa are excreted rapidly by glomerular filtration). Therefore, extending the *in vivo* half-life of peptides is clearly desirable for their therapeutic potential to be realized without the need for high doses and frequent administration.

Strategies to Enhance the Half-life of peptides and their Limitations

There are various methods in the market for increasing the half-life of peptides, the most commonly used strategies are as follows [32]:

PEGylation

This method is most commonly used for enhancing the half-life of peptides, where a native peptide is modified by conjugation to a polyethylene glycol (PEG) [33]. The peptide which was conjugated to the PEG acquires the molecular size of the PEG, escapes excretion by glomerular filtration, and escapes the sensitivity of the proteases from degrading which helps in increasing blood circulation half-life of the peptides.

Limitations of PEGylation

They are a number of drawbacks for PEGylation. For example, PEG is non-biodegradable and repeated administration of these PEGylated peptides accumulates the PEG in kidneys and choroid plexus. The PEG generates anti-PEG antibodies [33] which increases the clearance of PEGylated peptides from the body.

Fusion Protein

Fusion proteins are created when two or more genes originally coded for different proteins are translated into a single or multiple polypeptides, whose function is derived from the original protein. In this case, a peptide is genetically fused with human serum albumin (HAS; 67 kDa) [34] to increase the circulation half-life of the peptides.

Limitations of Fusion Protein

The most common problems for this approach are that it has lower affinity towards its target receptor, shows long terms immunological toxicities [34] and has stability issues for storing and transporting.

Lipidation

Lipidation is a process where a hydrophobic fatty acids are conjugated to the peptide of interest [35, 36]. This results in increasing the circulation half-life of the peptides by allowing it to bind to human serum albumin.

Limitations of Lipidation

The most common limitations for this method are that it shows lower binding towards its receptors [35, 36], has some stability issues which make storage and transportation difficult.

3.2 Conjugation to Transthyretin Ligands

Transthyretin is a 55 kDa homo-tetramer, liver secrets transthyretin into blood, choroid plexus secrets transthyretin in CSF (hTTR concentration in serum is ~ 5 μ M) [37] and has *in vivo* half-life of 48 hrs. The primary function of hTTR in human is to transport holo-retinol binding protein and secondary function is to transport T₄ binding protein in blood. The Apo-retinol binding protein which has low binding affinity for hTTR due to the relative small size (21 kDa) undergoes fast renal excretion having half-life of 3 hrs [37]. The reversible association between the holo-retinol binding protein and hTTR in blood prevents the glomerular filtration of holo-retinol binding protein which results in enhances the circulation half-life (11 hrs). Based on this natural observation, our laboratory previously demonstrated that conjugation of peptides to a selective hTTR-binding ligands will help the peptide conjugate to bind reversibly to hTTR and helps in increasing the *in vivo* half-life of therapeutic peptides (Figure.3.1). Due to their reversible binding to hTTR, the intrinsic activity of the peptide conjugates are not adversely affected [38].

Our laboratory developed a potent and selective small molecule hTTR ligand (AG10). At 10 μ M, almost all of AG10 was bound to hTTR in human serum [3, 39]. Our lab also successfully developed several AG10-linker modified analogs (TLHEs) which are used for half-life extension [39].

3.3 Our Approach

A major limitation for the above strategies to enhance the half-life of peptides is that the steric hindrance of macromolecules often harms the binding affinity of peptides to its extra-cellular receptors, which undermine the therapeutic potency of peptides. Our approach of using TTR ligand for half-life extension (TLHE <500 Da) should not affect the binding affinity of peptides to its extra-cellular receptors (Figure 3.1). TLHE are conjugated to peptides through a short linker that will give TLHE-peptide conjugate the ability to bind reversibly to T₄ binding sites of endogenous hTTR [39]. This will increase the *in vivo* half-life of the peptide by protecting against protease and by decreasing glomerular filtration. Importantly, due to its reversible binding to hTTR, the binding affinity of the peptide conjugate to its target receptor would not be adversely affected.

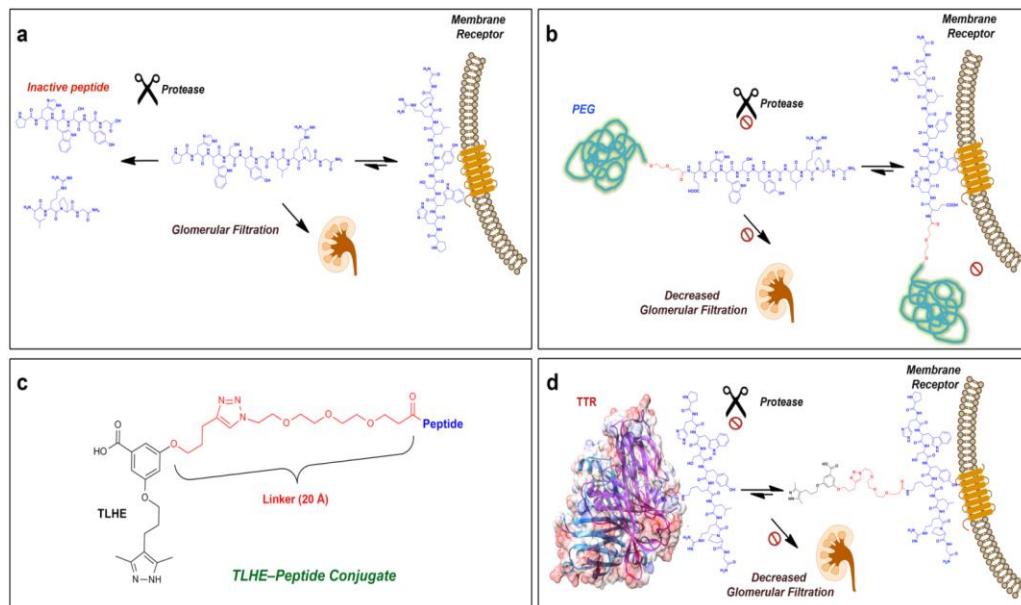


Figure 3: Current approaches for enhancing the *in vivo* $t_{1/2}$ of peptides and our new hTTR based approach. (a) Most peptides have short *in vivo* $t_{1/2}$ (2–30 minutes) due to enzymatic degradation by proteases and fast renal excretion by glomerular filtration. (b) Covalent conjugation of peptides to macromolecules such as PEG and HSA enhances the *in vivo* $t_{1/2}$ of peptides by sterically protecting the peptide from proteases and by increasing the hydrodynamic size of the peptide and therefore decreasing its renal excretion. (c) Conjugation of peptides to TTR ligands for half-life extension (TLHEs) (<500 Da), through a short linker will form TLHE—peptide conjugates. (d) The TLHE—peptide conjugate can bind reversibly to the T₄ binding sites of endogenous hTTR (shown as ribbon diagram with transparent surface).

3.4 Development of AG10-Linkers for Conjugating Peptides

Our lab had made slight change by removing the fluorine atom of AG10 and attaching a short linker resulting in TLHE1 (Figure 3.2). TLHE1 maintained good binding affinity and selectivity to hTTR [39]. My project involved the synthesis of three new TLHEs (TLHE 2, 3, and 4; Figure 3.3). We found that TLHE1 has the best binding affinity and selectivity for hTTR. For our approach to work in vivo, TLHE1 and its peptide conjugates should be able to selectively bind to hTTR in the presence of more than 4,000 other human proteins [39]. The binding selectivity of the TLHE-linker peptide conjugate to hTTR in human serum was evaluated using a well-established hTTR serum covalent-probe selectivity assay. The lower performance of TLHE1 compared to AG10 was due to the lower binding affinity of TLHE1 and possibly some binding to other serum proteins. Nevertheless, the activity of TLHE1 in this assay was better than that of the clinical candidate, tafamidis. TLHE1 was stable in serum for at least 48 hrs and has very low cytotoxicity [39]. Therefore, TLHE is a very good candidate for conjugation to peptides.

Our modeling suggested that lowering the size of linker to TLHE1 to of~20Å should be sufficient to clear the hTTR T₄ binding sites and potentially be functionalized with peptides. To test our hypothesis we conjugated TLHE1 to three different length linkers. The resulting three TLHEs (TLHE2, TLHE3, and TLHE4) were conjugated to the endogenous peptides, gonadotropin-releasing hormone (GnRH; 10 amino acid peptide hormone). All Three conjugates displayed good binding selectivity for hTTR in human serum.

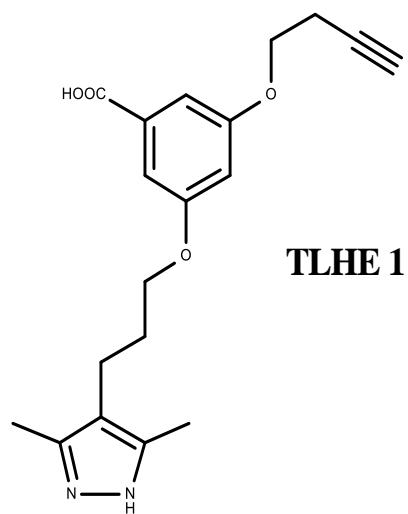


Figure.3.1: Structure of TLHE1

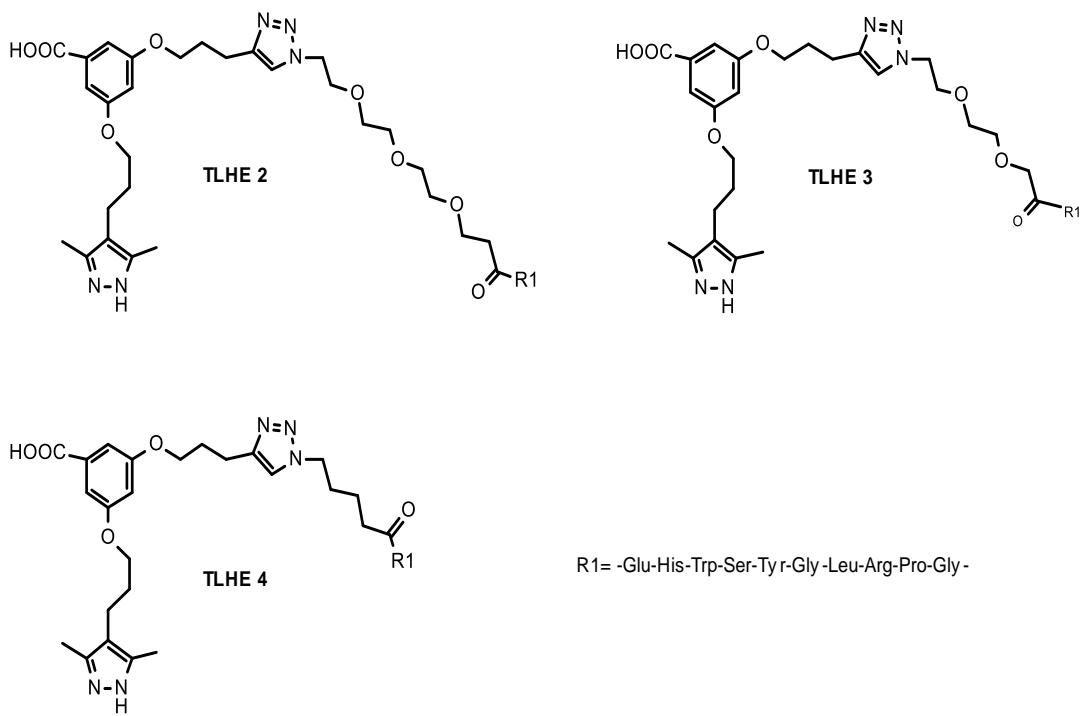


Figure.3.2: TLHE-Peptide conjugates with different chain linkers.

3.5 Methods & Materials

3.5.1 Materials

All the peptides, and linkers are synthesized in lab as reported and their reactions were carried out under a nitrogen or argon atmosphere and anhydrous conditions, unless noted. The solvents used were ACS grade purchased from Fisher Scientific, and reagents were purchased from Aldrich and Acros, and used without further purifications. Reactions were monitored using thin-layer chromatography (TLC) carried out on 0.2mm POLYGRAM® SIL silica gel plates with fluorescent indicator UV254 using UV light as a visualizer. For purification normal phase flash column chromatography was carried out using Davisil® silica gel (100-200 mesh, Fisher Scientific). High resolution mass spectrometry (HRMS) were used to determine the exact mass of synthesized conjugates and peptides by JEOL AccuTOF DART using helium as an ionization gas and polyethylene glycol (PEG) as an external calibrating agent. The human serum was obtained from Sigma, the probe and AG10 was synthesized as reported in reference [3].

3.6 Chemical synthesis of TLHE1

The synthesis scheme of **TLHE1** is depicted in Figure 3.4 and was synthesized as in reference [39].

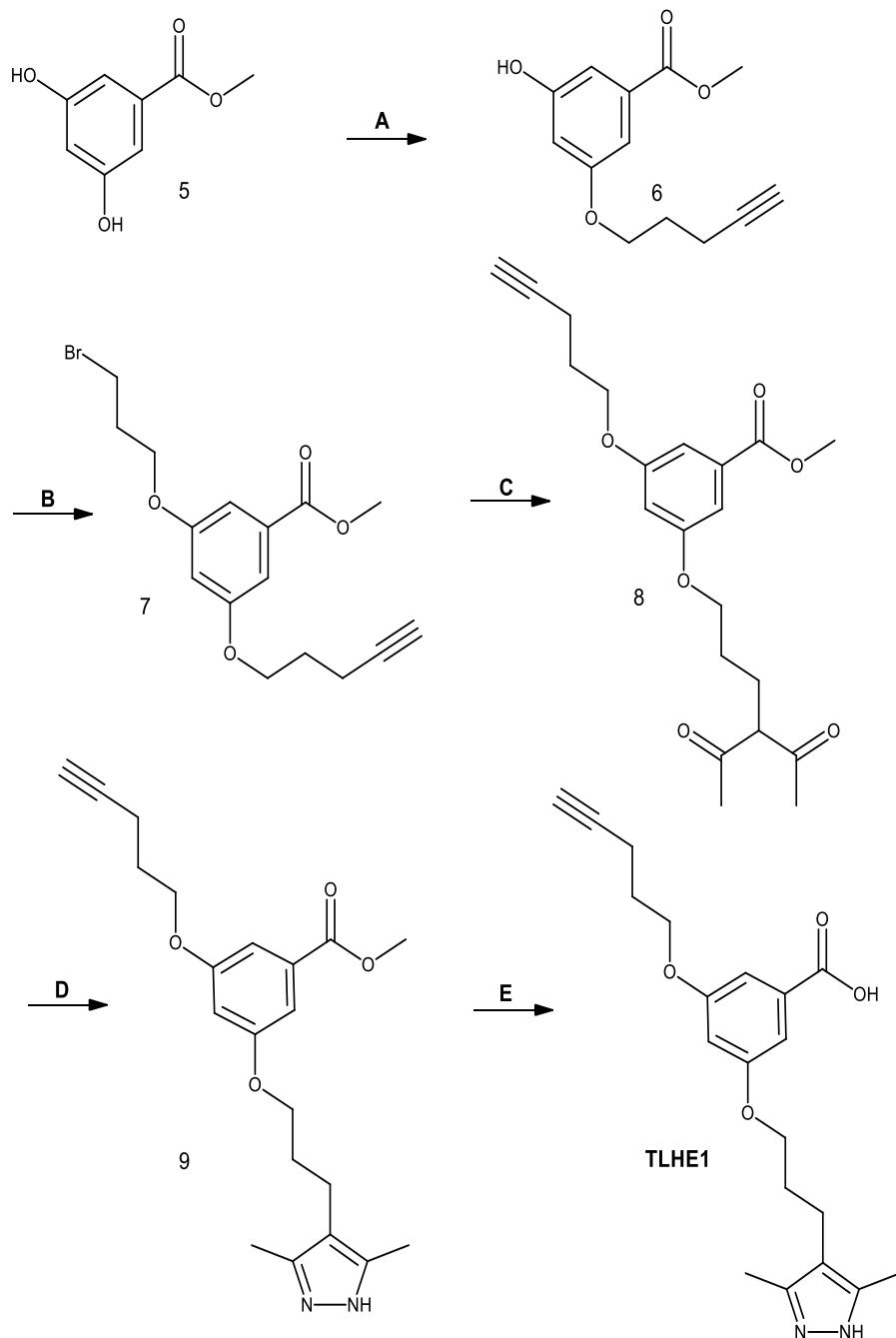


Figure 3.3: Synthesis of **TLHE1**. **A)** 1,3-dibromopropane, K_2CO_3 , DMF, room temperature, 16 hrs; **B)** Acetylacetone, 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU), benzene, room temperature, 3 days; **C)** hydrazine hydrate, ethanol, 90°C, 4 hrs; **D)** NaOH, THF, water, room temperature, 14 hrs [39]. **Methyl 3-**

hydroxy-5-pent-4-yloxy-benzoate. To a solution of methyl 3, 5-dihydroxybenzoate (**5**) (6.60 g, 38.93 mmol, 1 equiv) and pent-4-ynyl-4-methylbenzene sulfonate (9.27g, 88.2 mmol, 5 equiv) in Acetonitrile (60 mL) was added K₂CO₃ (2.59 g, 16.6 mmol, 2.2 equiv) and KI (311mg, 22 equiv). The reaction mixture was stirred at room temperature (r.t.) for 16 hrs. The mixture was diluted with EtOAc (1.5 L), washed with brine (3x0.5 L) and dried with Na₂SO₄. The solution was filtered and concentrated. The residue was purified by flash column chromatography (silica gel, 1-60 % hexane/DCM) to afford compound (4 g, 82 % yield); to the above dried compound add 6N HCL (2mL) and THF (2.0mL) and put it under reflux of 50°C for 3.5 hrs then washed with 250mL water and 500mL EtOAC and collect EtOAC mixture, dry to form compound **6**.

Methyl 5-[5-hydroxy-4-(1-hydroxyethyl)hexoxy]-2-pent-4-yloxy-benzoate (7). To a solution of methyl 5-hydroxy-2-pent-4-yloxy-benzoate (**6**) (2.92 g, 3.6 mmol, 1 equiv) and 1,3-dibromopropane (6.67 mL, 18.2 mmol, 5 equiv) in DMF (40 mL) was added K₂CO₃ (2.08 g, 4.36 mmol, 2.4 equiv). The reaction mixture was stirred at room temperature (r.t.) for 16 hrs. The mixture was diluted with EtOAc (1.5 L), washed with brine (3x0.5 L) and dried with Na₂SO₄. A solution of (**7**) (840 mg, 2.36 mmol, 1 equiv) in benzene (2 mL) was added dropwise to a solution of acetyl acetone (1.79 mL, 4.73 mmol, 2 equiv) and DBU (2.638 mL, 4.73 mmol, 2 equiv) in benzene (28 mL). The reaction mixture was stirred at r.t. for 3 days. The mixture was filtered and concentrated. The residue was purified by flash column chromatography (silica gel, 1-10 % EtOAc/hexanes) to afford compound (**8**) which was used in the next step directly. Hydrazine hydrate (456 mL, 6.73 mmol, 2.5 equiv) was added to a solution (**8**) in ethanol (15 mL) and the reaction was heated under reflux for 4 hrs. The reaction was concentrated and purified by flash column chromatography (silica gel, 1-20 % MeOH/CH₂Cl₂) to afford compound (**9**) (288 mg, 35% yield) in two steps;

3-[3-(3,5-dimethyl-1H-pyrazol-4-yl)propoxy]-5-pent-4-yloxy-benzoic acid (TLHE1). To a suspension of (**9**) (100 mg, 0.0946 mmol, 1 equiv) in a mixture of NaOH.H₂O (11.89 mg, 0.189

mmol, 2 equiv) was added to the MeOH (4mL). The reaction mixture was stirred under reflux of 50°C for 12 h after which it was cooled to room temperature. The mixture was fractionated with EtOAc and MeOH at different concentrations and finally run a thin layer chromatography after conforming the fraction with compound dry the fraction. The crude product was subjected to flash column chromatography (silica gel, 10-50% MeOH/CH₂Cl₂) to give of **TLHE1** (30 mg, 32% yield) as a white solid (95% purity by HPLC)

3.7 Chemical synthesis of GnRH analog 1 (TLHE2-GnRH)

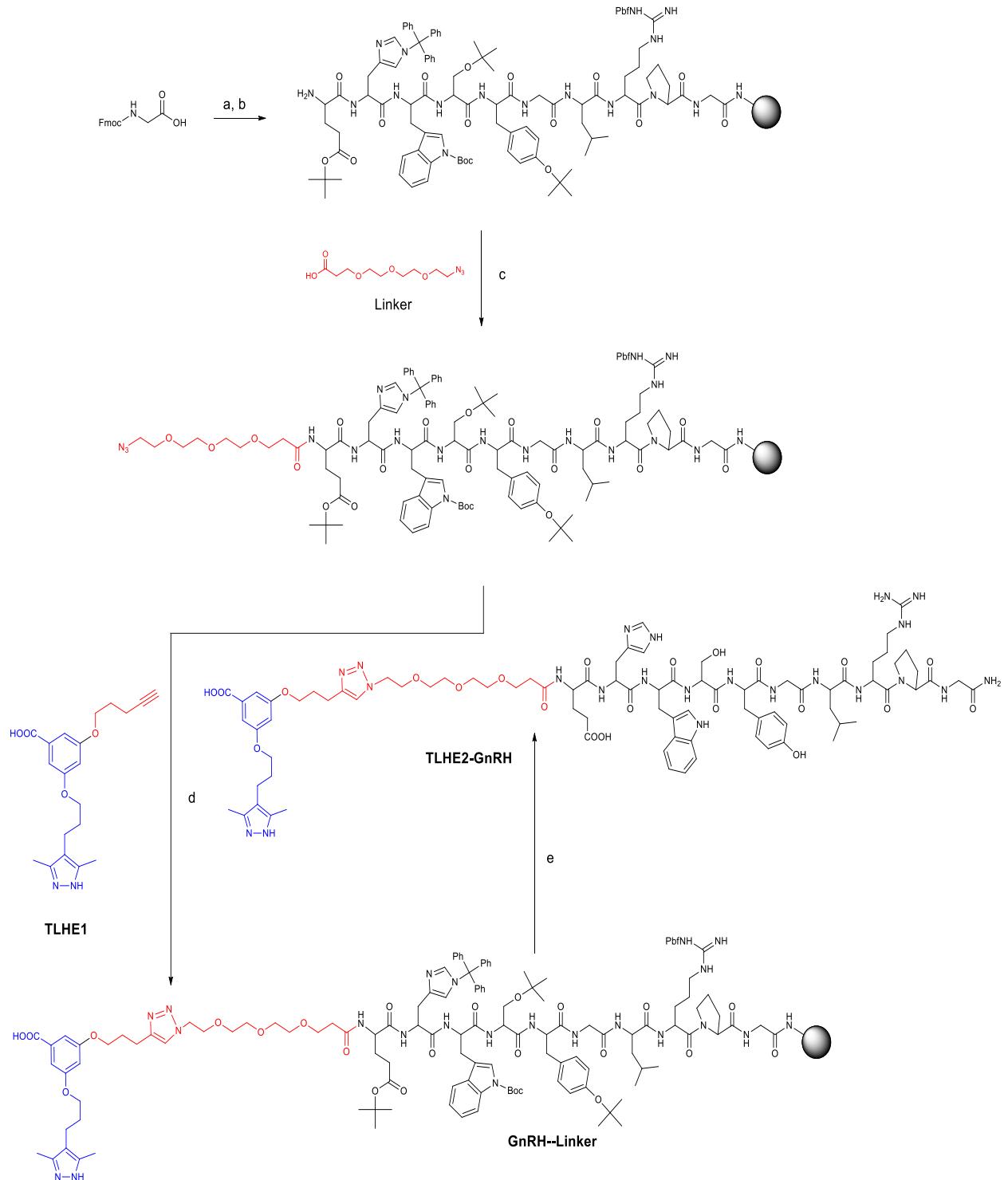


Figure 3.4: Synthesis of TLHE2-GnRH. Reagents and conditions: **a**) Rink amide resin, DIPEA, DCM, 16 hrs; **b**) Fmoc SPPS (all L-amino acids); **c**) Linker, HATU, HOEt, DIPEA, DMF, 24 hrs; **d**) TLHE1, Cu, sodium ascorbate, DMF/piperidine (4:1), 16 hrs; **e**) TFA, phenol, H_2O , and TIS (88:5:5:2 ratio) 3 hrs.

Synthesis of GnRH

The GnRH Peptide was synthesized using standard solid phase synthesis utilizing Fmoc/tBu protocols. All amino acids used for synthesis are L-Amino acids. The synthesis was carried out on a rink amide MBHA resin (NovobioChem #855003, 0.79 mmol/g). For the resin loading step, the resin (250mg, 0.1975 mmol) was reacted with Fmoc-Gly-OH () in DMF (3mL) and DIC (0.137 mL, 0.875 mmol). The reaction mixture was shaken for 5hrs, the peptides were built by coupling Fmoc protected (L) amino acid monomers to the first amino-acid attached to the rink amide resin using DIC, HOBT, in DMF and shaken for 2hrs. Once the GnRH deca-peptide synthesis was completed, it was dried and used for conjugating the linker.

Synthesis of GnRH-Linker

The azide PEG-linker 3[2-[2-(2- azidoethoxy)ethoxy]ethoxy] propanoic acid was used directly after synthesis from 3[2-[2-(2-Hydroxyethoxy)ethoxy]ethoxy]propanoic acid tert-butyl ester taken with Triethylene glycol (104.45 g) with THF (40mL). Then NaOH (5.581 g) mixed with deionized water (30 mL) are added to the round bottom flask at 0°C, the reaction mixture was stirred for 15 min. PTsCl dissolved in THF (100mL) was added dropwise and stirred for 2hrs at 0°C. The reaction mixture was washed with water/EtOAc in a separation flask. The EtOAc was collected concentrated, flash column was done using MeOH/DCM (50:50) which formed purified (2-[2-(2-hydroxysulphonylethoxy) ethoxy] ethanol). The product was further synthesized by mixing in DMF (30mL) under nitrogen. Sodium Azide is added and refluxed for half-hour. Then 200mL of water was added to the reaction and (50mL) of EtOAc was added multiple time to extract compound it was concentrated and purified using a flash column to separate pure compound. Ethanol, 2-[2-(2-azidoethoxy)ethoxy] (200mg) was further dissolved with acetone (12mL) and kept at 0°C, Chromium oxide (521.1mg) in 1.5m of sulfuric acid (6mL) was slowly added at 0°C, then the reaction is slowly changed to room temperature for overnight. Finally isopropanol was added to quench the reaction. The final compound (2-[2-(2- azidoethoxy) ethoxy] acetic acid) is dried and stored.

The GnRH peptide-linker was synthesized in a similar way to what described above for GnRH synthesis. The only difference is that we used glutamic acid instead of pyro-glutamic acid the N-terminus. The azide PEG linker (37.5 mg, 0.198 mmol) is activated in HATU (150.6 mg, 0.396 mmol), HOBT (53.58 mg, 0.396 mmol), DIPEA (51.2 mg, 0.396 mmol) and DMF (3mL). All the reagents are added to the GnRH peptide before the product was cleaved from the resin, the reaction mixture was shaken for 20 hrs.

Synthesis of TLHE2-GnRH

The click reaction was carried out by the resin bound GnRH-linker (0.132 mmole), 3-[3-(3, 5-dimethyl-1H-pyrazol-4-yl)propoxy]-5-pent-4-yloxy-benzoic acid (34 mg, 0.132mmol), copper iodide (75mg, 0.132mmol), Sodium ascorbate (78.4 mg, 0.132mmol), DMF/piperidine (4:1) 3mL, the mixture was transferred into the reaction flask under nitrogen gas and reaction was left for 16 hrs in room temperature. The product was then cleaved from the resin including the deprotonation of groups present in the side chains groups was performed by treating with cleavage cocktail which contains TFA, phenol, water, and TIS (88:5:5:2) ratio, shaken with this cocktail for 1 to 1 ½hr. After cleavage the resulting product was collected in cold ether and washed multiple times with ether and finally centrifuged to separate the GnRH peptide-linker-conjugate in the form of precipitate, the precipitate was dried and purified using preparative HPLC using (50:50) of buffer for injecting the peptide conjugate using buffer solvent A and B (buffer A: water, methanol, TFA at ratio 95:5:0.1) and (buffer B: methanol, water, TFA at ratio 95:5:0.1) using C18 column.

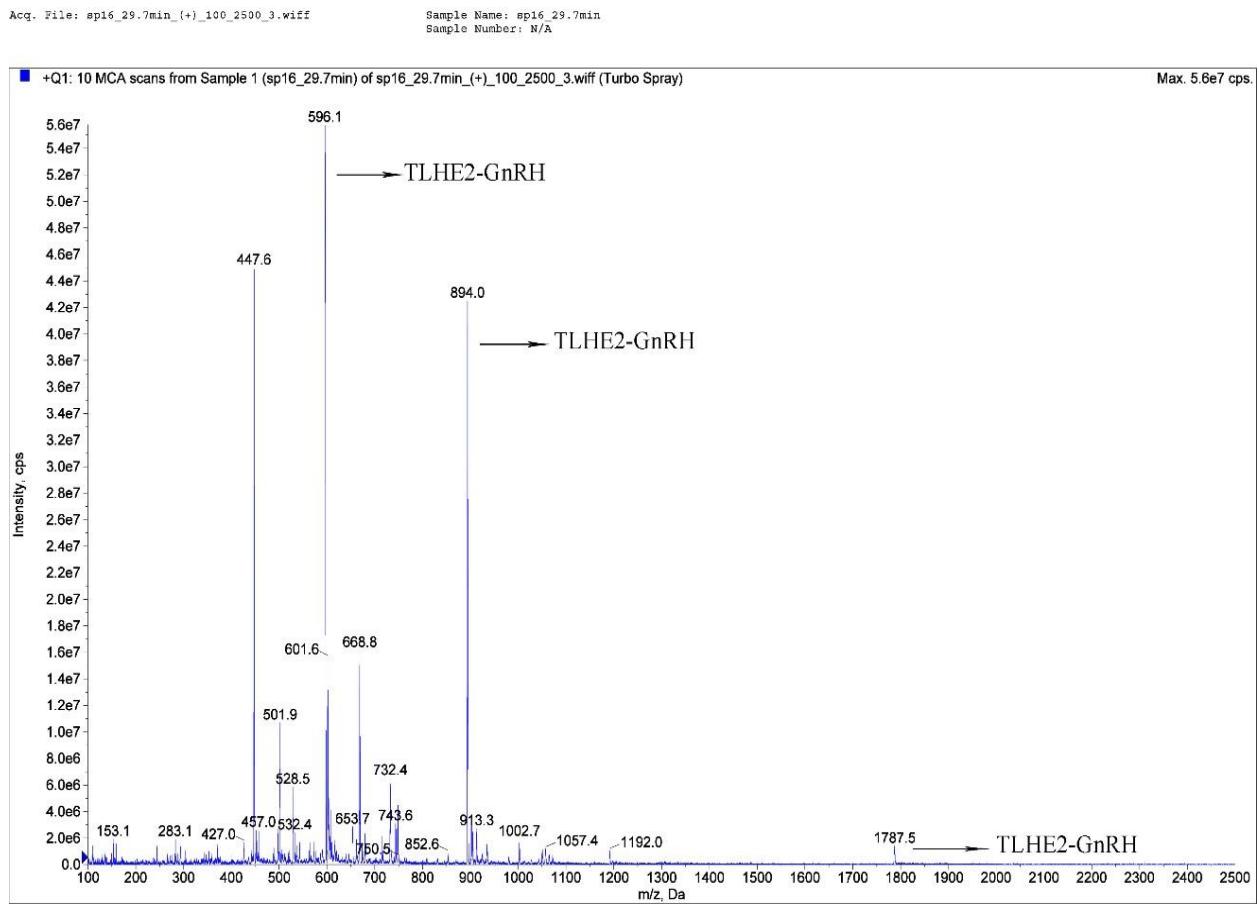


Figure 3.5: MS data showing **TLHE2-GnRH** (M.Wt: 1787.5).

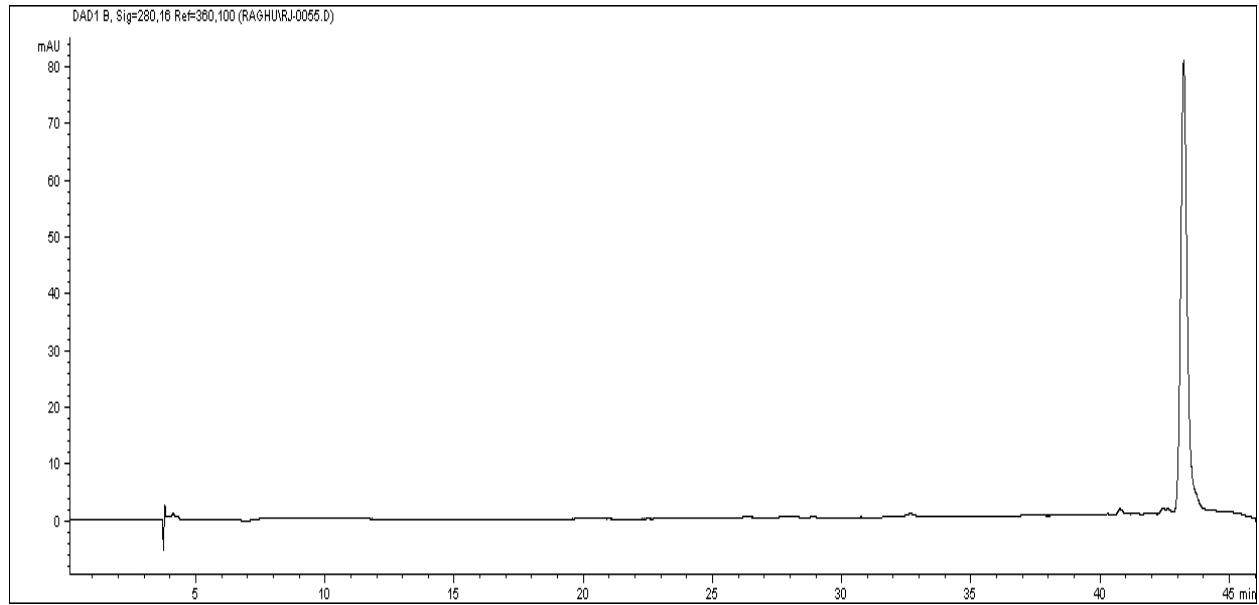


Figure 3.6: HPLC trace of **TLHE2-GnRH** (>98 % purity).

3.8 Chemical synthesis of GnRH analog 2 (TLHE3-GnRH)

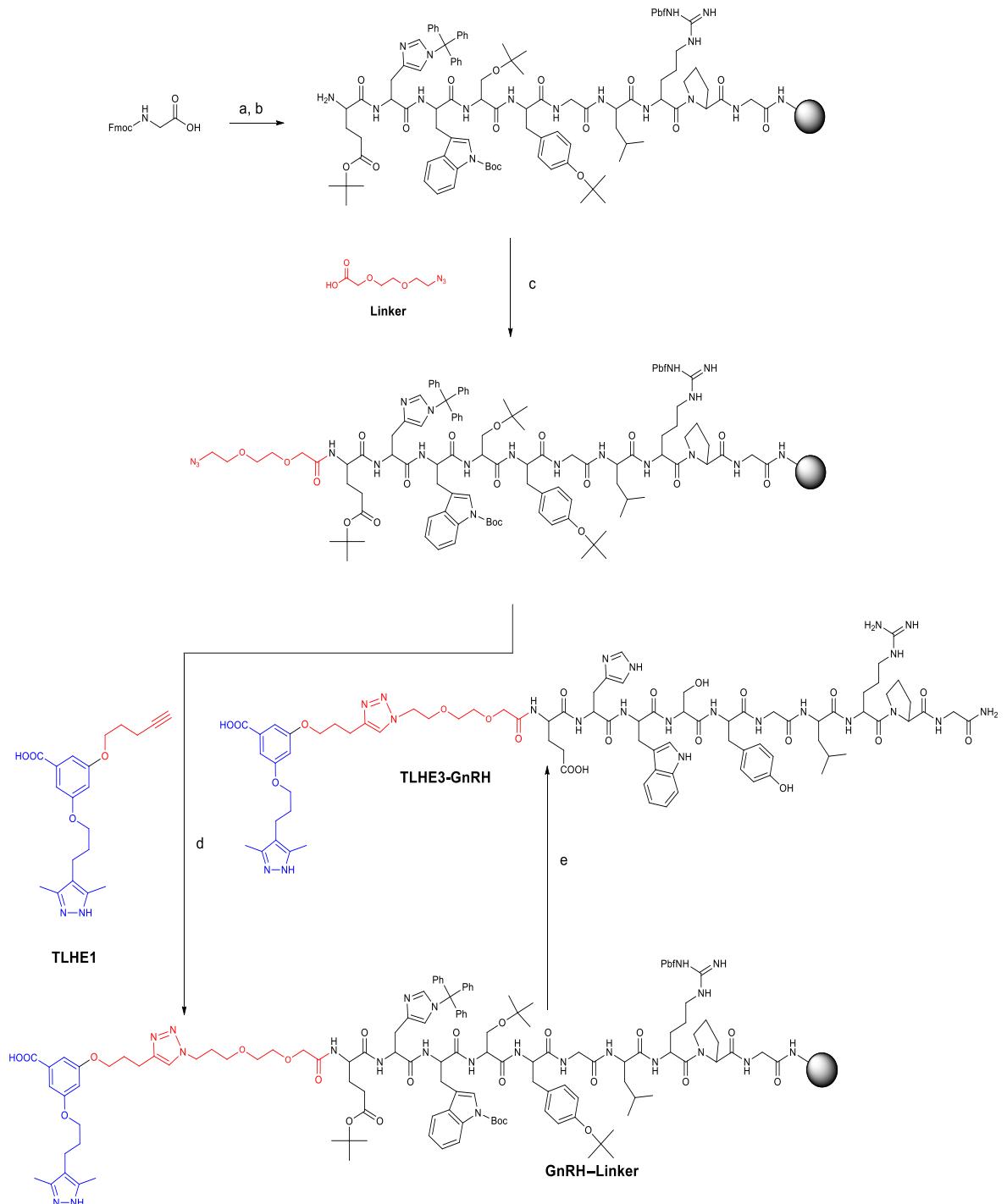


Figure 3.7: Synthesis of TLHE3-GnRH. Reagents and conditions: **a**) Rink amide resin, DIPEA, DCM, 16 h; **b**) Fmoc SPPS (all L-amino acids); **c**) Linker, HATU, HOEt, DIPEA, DMF, 24 h; **d**) TLHE1, CuI, sodium ascorbate, DMF/piperidine (4:1), 16 h; **e**) TFA, phenol, H₂O, and TIS (88:5:5:2 ratio) 3h.

Chemical Synthesis of GnRH

The synthesis of the peptide is same as previously explained in (3.7)

Synthesis of GnRH-Linker

The azide PEG-linker (2-[2-(2- azidoethoxy) ethoxy] acetic acid) was used directly after synthesis from (2-[2-(2-hydroxyethoxy) ethoxy] ethanol) taken with T-glycol (104.45 g) with THF (40mL). Then NaOH (5.581 g) mixed with deionized water (30 mL) was slowly added to a round bottom flask at 0°C, the reaction mixture was stirred for 15 minutes. PTScl dissolved in THF (100mL) was added dropwise and stirred for 2hrs at 0°C. The reaction mixture was washed with water/EtOAc in a separation flask, The EtOAc was collected concentrated, and flash column was done using MeOH/DCM (50:50) and which formed purified (2-[2-(2-hydroxysulphonylethoxy) ethoxy] ethanol). The product was further synthesized by mixing in DMF (30mL) under nitrogen Sodium azide was added and refluxed for half-hour, then 200mL of water was added to the reaction and (50mL) of EtOAc was added multiple time to extract compound it was concentrated and purified using a flash column to separate pure compound. Ethanol, 2-[2-(2-azidoethoxy)ethoxy] (200mg) was then dissolved with acetone (12mL) and kept at 0°C, chromium oxide (521.1mg) in 1.5M sulfuric acid (6mL) was slowly added at 0°C. The reaction then gradually changed to room temperature for overnight. Finally isopropanol was added to quench the reaction. The final compound (2-[2-(2- azidoethoxy) ethoxy] acetic acid) was dried and stored.

The GnRH peptide- Linker was synthesized in a similar way to what described above for the GnRH synthesis. The only difference is that we used glutamic acid instead of pyro-glutamic acid at N-terminus. The azide PEG linker (37.5 mg, 0.198 mmol) is activated in HATU (150.6 mg, 0.396 mmol), HOBT (53.58 mg, 0.396 mmol), DIPEA (51.2 mg, 0.396 mmol) and DMF (3mL). All the reagents are added to the GnRH peptide before the product was cleaved from the resin, the reaction mixture was shaken for 20 hrs.

Synthesis of TLHE3-GnRH

The click reaction was carried out by the resin bound GnRH-linker (0.132 mmole), 3-[3-(3, 5-dimethyl-1H-pyrazol-4-yl)propoxy]-5-pent-4-yloxy-benzoic acid (34 mg, 0.132mmol), copper iodide (75mg, 0.132mmol), Sodium ascorbate (78.4mg, 0.132mmol), DMF/piperidine (4:1) 3mL, the mixture was transferred into the reaction flask under nitrogen gas and reaction was left for 16 hrs in room temperature. The product was then cleaved from the resin including the deprotonation of groups present in the side chains groups was performed by treating with cleavage cocktail which contains TFA, phenol, water, and TIS (88:5:5:2) ratio, shaken with this cocktail for 1 to 1 ½hr. After cleavage the resulting product was collected in cold ether and washed multiple times with ether and finally centrifuged to separate the GnRH peptide-linker-conjugate in the form of precipitate, the precipitate was dried and purified using preparative HPLC using (50:50) of buffer for injecting the peptide conjugate using buffer solvent A and B (buffer A: water, methanol, TFA at ratio 95:5:0.1) and (buffer B: methanol, water, TFA at ratio 95:5:0.1) using C18 column.

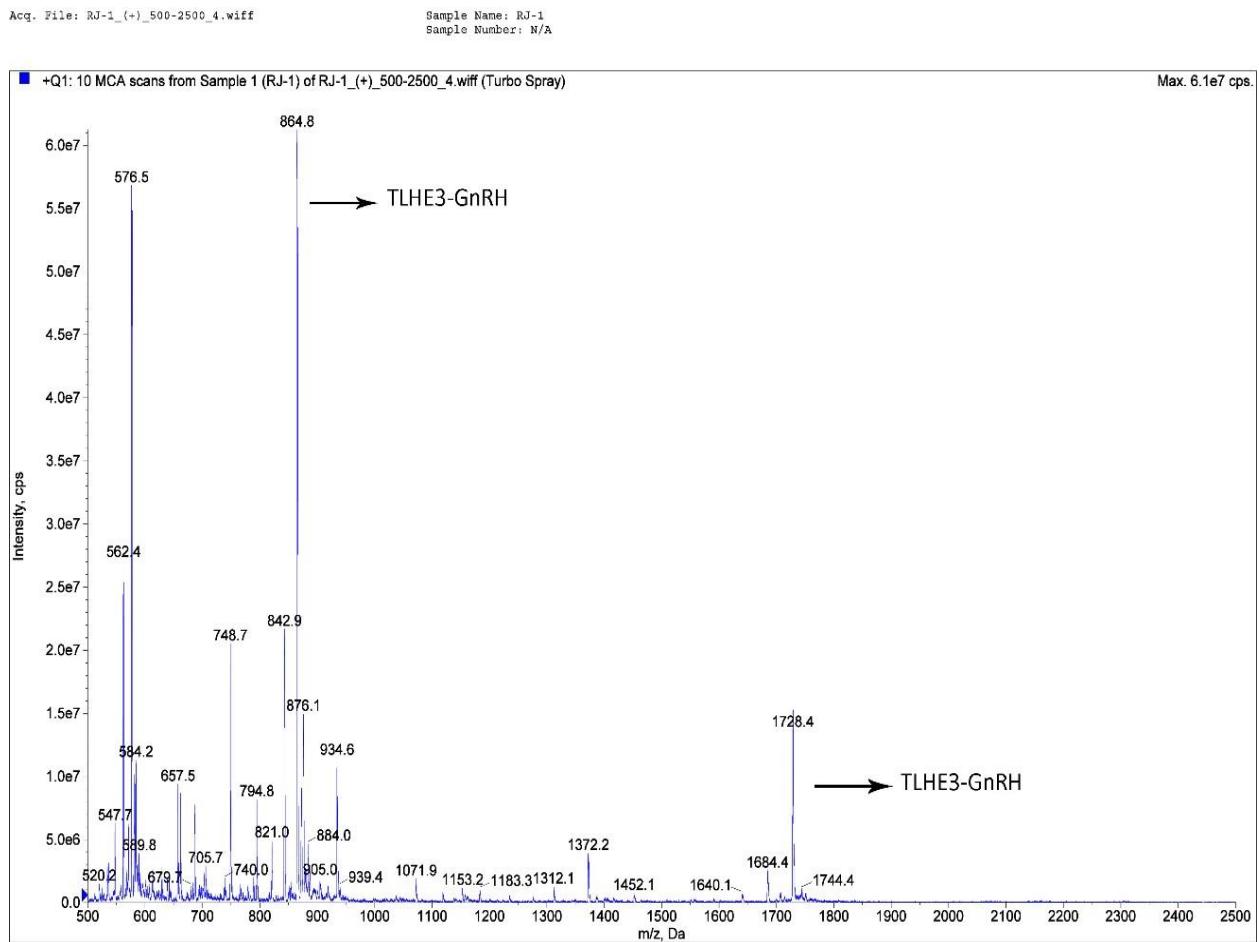


Figure 3.8: MS data showing TLHE3-GnRH (M.Wt: 1728).

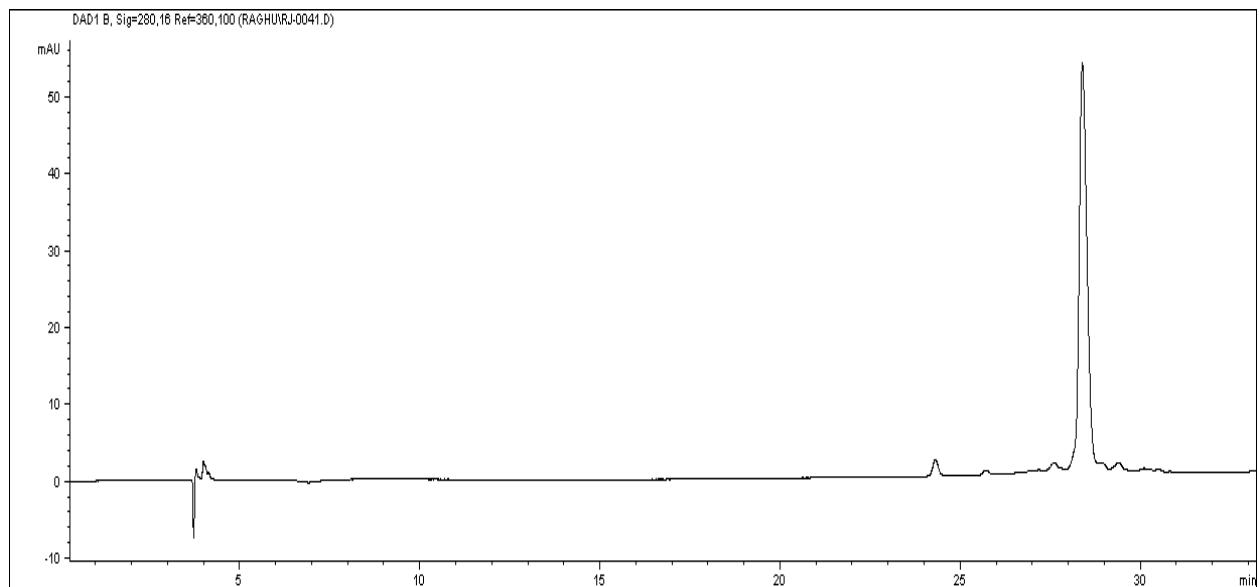


Figure 3.9: HPLC trace of TLHE3-GnRH (>99.20 % purity).

3.9 Chemical synthesis of TLHE4-GnRH

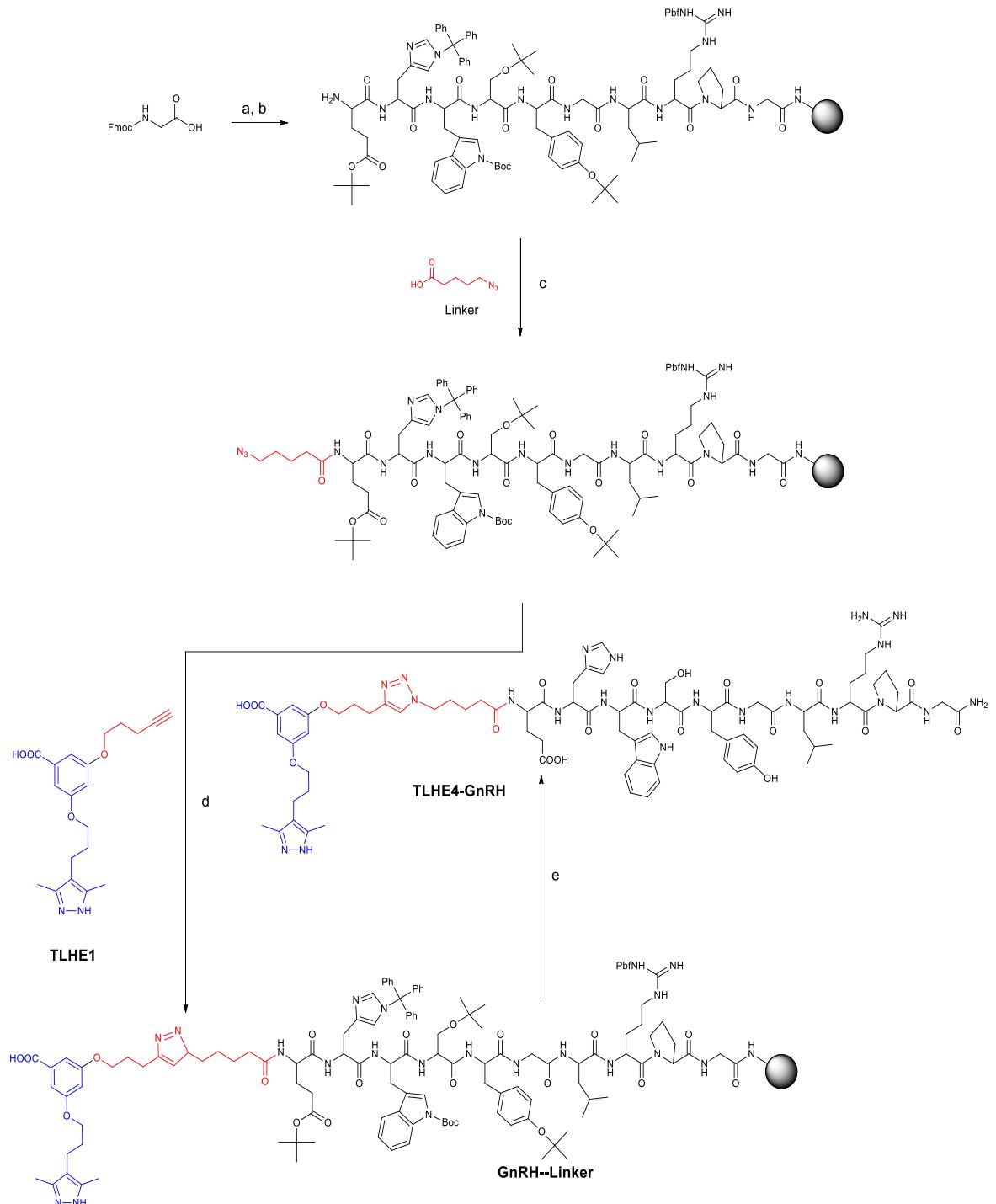


Figure 3.10: Synthesis of TLHE4-GnRH. Reagents and conditions: **a)** Rink amide resin, DIPEA, DCM, 16 h; **b)** Fmoc SPPS (all L-amino acids); **c)** Linker, HATU, HOEt, DIPEA, DMF, 24 h; **d)** TLHE1, CuI, sodium ascorbate, DMF/piperidine (4:1), 16 h; **e)** TFA, phenol, H₂O, and TIS (88:5:5:2 ratio) 3 hrs.

Synthesis of GnRH

The synthesis of the peptide is same as previously explained in (3.7). **Synthesis of GnRH-Linker**

The azide PEG-linker (5-Azido pentaonic acid) was ordered from Sigma Aldrich (CAS Number 79583-98-5), and The GnRH peptide- Linker is synthesized in a similar way to what described above for GnRH synthesis, the only difference was here we are using glutamic acid instead of pyro-glutamic acid at N-terminus. The azide PEG linker (27.91mg, 0.065 mmol) is activated in HATU (74.14mg, 0.065 mmol), HOBT (29.86 mg, 0.065 mmol), DIPEA (16.79 mg, 0.065 mmol) and DMF (3mL). All the reagents are added to the GnRH peptide before the product was cleaved from the resin, the reaction mixture was shaken for 20 hrs.

Synthesis of TLHE4-GnRH

The click reaction was carried out by the resin bound GnRH-linker (0.132 mmole), 3-[3-(3, 5-dimethyl-1H-pyrazol-4-yl)propoxy]-5-pent-4-yonoxy-benzoic acid (34 mg, 0.132mmol), copper iodide (75mg, 0.132mmol), Sodium ascorbate (78.4mg, 0.132mmol), DMF/piperidine (4:1) 3mL, the mixture was transferred into the reaction flask under nitrogen gas and reaction was left for 16 hrs in room temperature. The product was then cleaved from the resin including the deprotonation of groups present in the side chains groups was performed by treating with cleavage cocktail which contains TFA, phenol, water, and TIS (88:5:5:2) ratio, shaken with this cocktail for 1 to 1 ½hr. After cleavage the resulting product was collected in cold ether and washed multiple times with ether and finally centrifuged to separate the GnRH peptide-linker-conjugate in the form of precipitate, the precipitate was dried and purified using preparative HPLC using (50:50) of buffer for injecting the peptide conjugate using buffer solvent A and B (buffer A: water, methanol, TFA at ratio 95:5:0.1) and (buffer B: methanol, water, TFA at ratio 95:5:0.1) using C18 column.

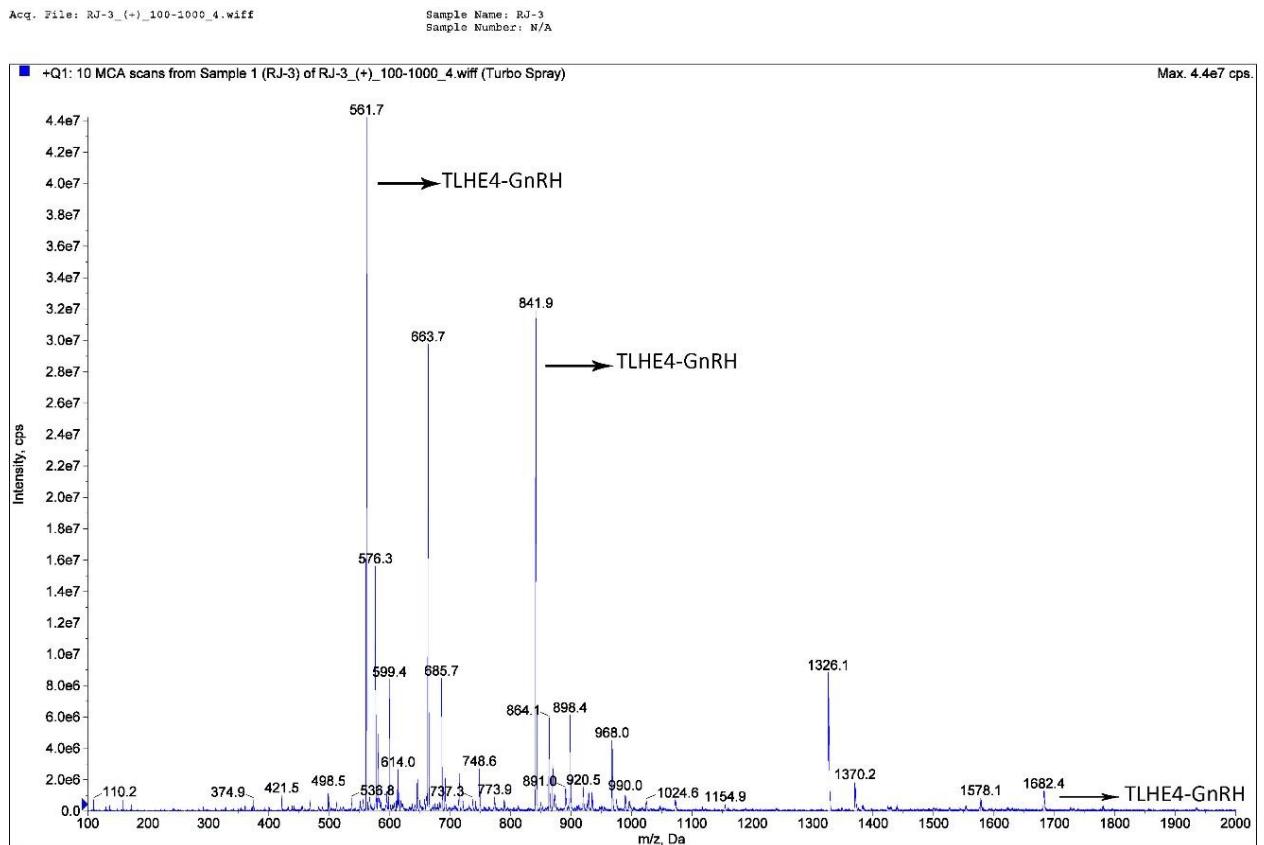


Figure 3.11: MS data showing TLHE4-GnRH (M.Wt: 1682.4).

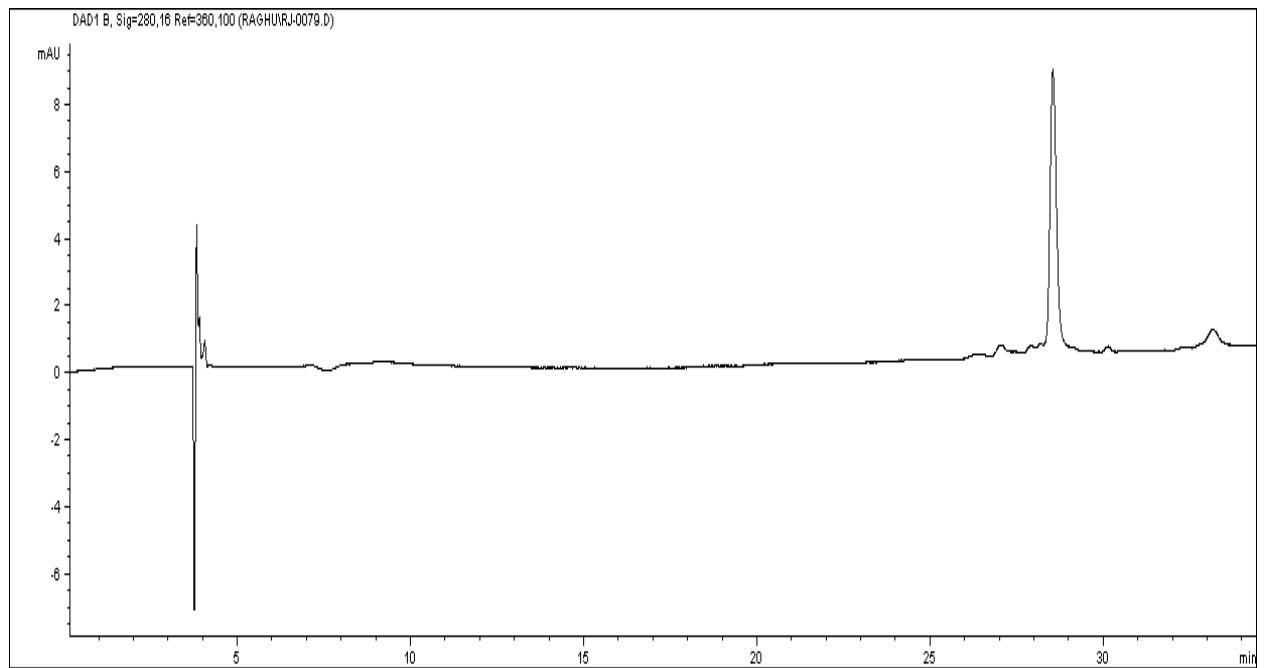


Figure 3.12: HPLC trace of TLHE4-GnRH (>97 % purity).

3.10 Results

3.11 TLHE-GnRH Conjugates Bind Selectively to WT-TTR in Human Serum

The selectivity of TLHE-GnRH conjugates to hTTR in human serum was evaluated using a well-established hTTR serum covalent-probe selectively assay [3, 39, 23] as shown in (Fig.3.14) The lower performance of TLHE-Peptide analogs **TLHE2-GnRH**, **TLHE3-GnRH**, and **TLHE4-GnRH** (47%, 46%, and 36% binding to hTTR in serum) compared to AG10 ($K_d = 13.8 \text{ nM}$; 87% binding to hTTR in serum) was due to the lower binding affinity of conjugates to hTTR and possibly some binding to other serum proteins. However, the binding affinity and selectivity of TLHE-GnRH conjugated in this assay were better than that of the clinical candidate, tafamidis (16% binding to hTTR in serum).

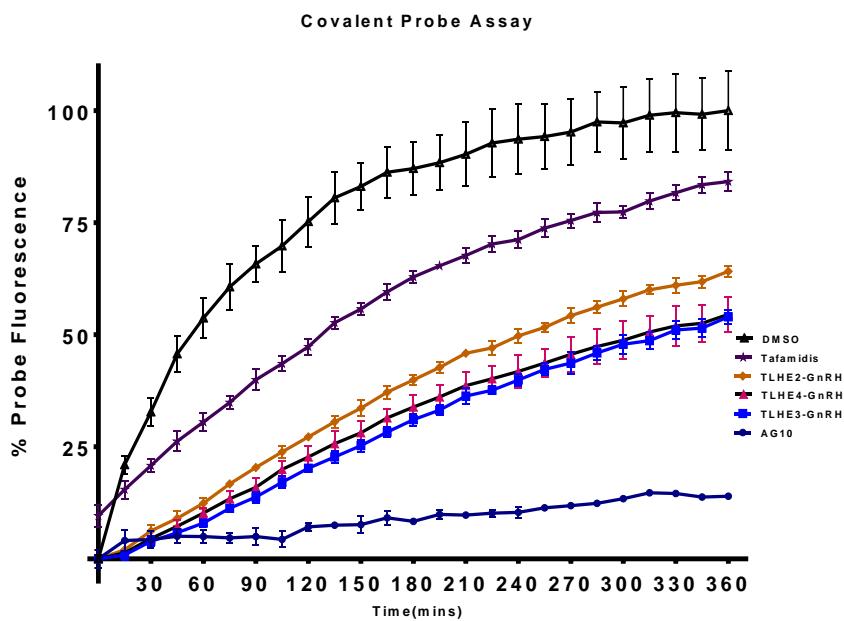


Figure 3.13: Fluorescence change caused by covalent probe (hTTR concentration, $\sim 5 \mu\text{M}$) by covalent probe monitored for 6 h in the presence of covalent probe alone (black circles) or covalent probe and GnRH attached hTTR ligands (colors; $10 \mu\text{M}$). The lower the binding and fluorescence of covalent probe, the higher binding selectivity of ligand to hTTR. Each bar shows the mean \pm SD of three replicates. **Table 3.1:** Showing the percentage of TLHE-GnRH binding to hTTR in human serum using covalent probe Assay after (3 hrs).

Compound Name	% Binding to TTR
DMSO	11%
Tafamidis	30%
TLHE2-GnRH	61%
TLHE4-GnRH	67%
TLHE3-GnRH	69%
AG10	94%

3.12 hTTR Protected TLHE-GnRH Conjugates against Serum Protease

We tested the ability of hTTR to protect TLHE-GnRH conjugates against proteolytic hydrolysis in human serum. The stability of the conjugates was evaluated in human serum (hTTR conc. ~5 μM). It was determined that the GnRH peptide had the lowest stability in serum (no amount detected after 8 hrs). Attaching three different linkers to GnRH (GnRH-linker) enhanced their stability. However they still are considered small molecules which are rapidly excreted by the kidneys. Therefore, we conjugated linker-GnRH to the TLHEs which has a good binding affinity with TTR, to see whether it protects the peptide from protease and glomerular filtration by increasing the molecule's size. After performing the serum protease stability assay we found that the stability of the conjugates was enhanced and GnRH-conjugates were protected against serum protease degradation in comparison to GnRH (Fig.3.15 and Table- 3.2).

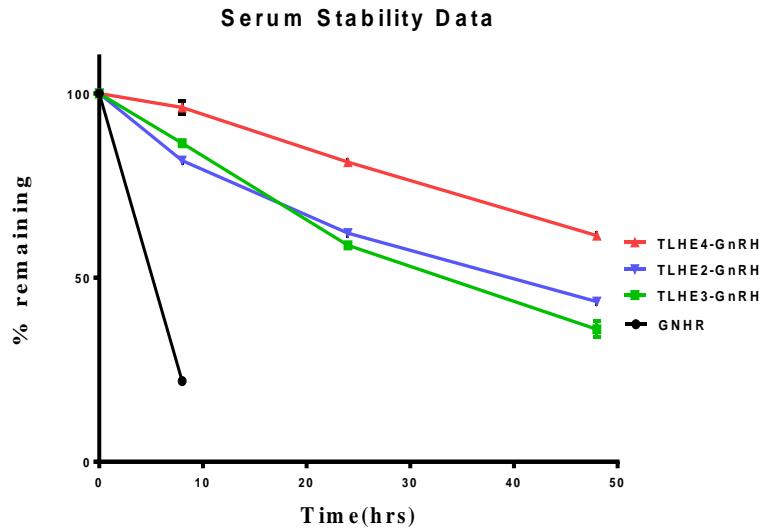


Figure 3.14: hTTR protected TLHE4-GnRH 62%, TLHE2-GnRH 38%, and TLHE3-GnRH 36%, against proteolytic hydrolysis in human serum. Test compounds (5 μ M) were added to serum and to serum. The amounts of compounds remaining in serum were quantitated at indicated time points. Each point shows the mean \pm SD of three replicate.

Table 3.2: Showing the percentage of the THLE-GnRH conjugates remaining in human serum after 48 hrs of incubation.

Compound Name	% Remaining in serum after 48 hrs
GnRH	0%
TLHE2-GnRH	38%
TLHE3-GnRH	36%
TLHE4-GnRH	62%

3.13 Binding affinity of TLHE-GnRH conjugates to TTR

The binding affinity of TLHE-GnRH conjugates to TTR at physiological pH was evaluated using our established fluorescence polarization (FP) assay [25]. The FP assay is a competitive assay that allows measurement of ligand binding to TTR based on their ability to displace a fluorescent probe [27] from the TTR T₄-binding sites. All test compounds were able to bind to TTR (purified from human plasma) at 10 μM. The TLHE-GnRH conjugates and AG10 were then assayed in a multi-point dose-response FP assay (concentration range between 40 and 0.02 μM). The binding affinity of AG10 to TTR (apparent binding constant, IC₅₀ = 85.36 nM, R² = 0.991) was better than the GnRH- analogs and TLHE ligands. The binding affinity of TLHE1 and to TTR (TLHE1; IC₅₀ = 204.3 nM, R² = 0.9905). The binding affinity of GnRH- analogs to WT-TTR were as follows: TLHE2-GnRH (IC₅₀ = 1047.64 nM, R²=0.99); TLHE3-GnRH (IC₅₀ = 1370.16 nM, R²=0.98); TLHE4-GnRH (IC₅₀ = 1505 nM, R²=0.9815) (Figure.3.16 and table 3.16.1).

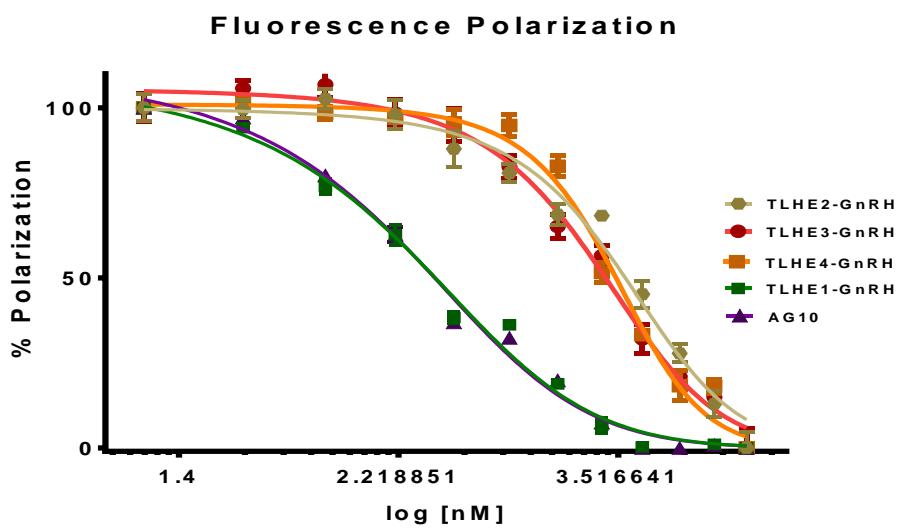


Figure.3.15: Evaluation of ligand binding to TTR in buffer by FP. Competition of FP-probe from TTR by increasing concentrations (40 and 0.02 μM) of TLHE-GnRH conjugates: TLHE3-GnRH (IC₅₀ = 1047.64 nM, R²=0.99); TLHE2-GnRH (IC₅₀ = 1370.16 nM, R²=0.98); TLHE4-GnRH (IC₅₀ = 1505 nM, R²=0.9815).

Each point shows the mean \pm SD of three replicates. **Table 3.3:** IC₅₀ value for binding affinity of TLHE-GnRH conjugates to TTR using Fluorescence polarization

Compound Name	IC ₅₀ (nM) Value	K _d (nM) Value
AG10	85.36	17.6
TLHE1	90	18.55
TLHE2-GnRH	1370.64	285.45
TLHE3-GnRH	1047.16	234.2
TLHE4-GnRH	1505.28	310.30

3.14 Discussion

There are various attractive technologies like conjugation to PEG and albumins for large peptides and proteins, which enhance the half-life of peptides. However; these strategies have some limitations, such as non-biodegradability, immunogenicity and low binding affinity with the target receptors because of the steric hindrance. Therefore, the TLHE1 system has the potential to optimize half-life extension of peptides without affecting the binding with their receptors. In addition, our approach offers a number of additional advantages over other strategies. Our technology involves a simple chemical conjugation of the peptide to TLHE1, and the products are homogenous, easily characterized and purified in harsh conditions like HPLC.

GnRH agonist is a ten amino acid peptide used to treat prostate and breast cancers, as well as fertility disorders yet it has a small half-life. Our THLE technology increased the half-life of GnRH-analogs by improving their binding affinity and selectivity to hTTR.

3.15 Conclusion

Conjugation of peptides and proteins to PEG and albumin are considered as an attractive technology, But the TLHE system has additional advantages for enhancing the half-life of peptides. Besides maintaining the potency, our technology provides a number of advantages over traditional genetic fusion, PEGylation and human serum albumin conjugations. Our approach

involves in a simple chemical conjugation of peptides, linkers and TLHEs, and the products are homogenous and can be easily purified and characterized using harsh conditions such as HPLC. The nature of synthesis offers flexibility of attachment sites and incorporates the unnatural amino acids or non-peptidic functionality into the peptide. Unlike HSA peptide conjugation, where the three-dimensional structure of the fusion partner need to be maintained, but conjugation to TLHEs results in stable product that do not need refrigeration, which decreases the cost of production, transportation and storage. Because of the small size we anticipate our conjugates penetrate solid tumors efficiently. Due to non-peptidic nature and small size, it is unlikely that TLHEs will cause any immunogenic response.

From the preliminary screening of the synthesized TLHE-GnRH conjugates, we can conclude that lowering the linker length provide better selectivity and protection against serum protease cleavage.

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