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Bifunctional Coumarin Derivatives that Inhibit Transthyretin Amyloidogenesis and Serve as Fluorescent Transthyretin Folding Sensors †

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Abstract

We describe a coumarin derivative that is non-fluorescent in aqueous buffers and that very selectively binds to transthyretin (TTR) in complex biological environments potently inhibiting TTR amyloidogenesis while also exhibiting sensitive off-on fluorescent sensing of the properly folded quaternary structure of TTR.

Fluorescent biosensors to detect and image particular proteins in living cells are increasingly used within the fields of biological and medical research.¹ The development of specific small molecule fluorescent sensors of enzymes and particularly non-enzymatic proteins remains challenging.

Transthyretin (TTR) is a 55-kDa homotetrameric protein composed of four 127-amino acid β -sheet-rich subunits (Figure 1A). TTR is secreted from the liver and the choroid plexus into the blood and the cerebrospinal fluid, respectively.² TTR transports both the thyroid hormone thyroxine (T₄) and holo-retinol binding protein. The energetically weaker dimer-dimer interface, bisected by the crystallographic C₂ axis, creates the two T₄-binding pockets

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(Figure 1A).³ In the blood, more than 99% of the T₄-binding sites within TTR are unoccupied because of the presence of two major T₄ carrier proteins, thyroid binding globulin and albumin.^{2a} TTR is one of more than 30 amyloidogenic human proteins that can misfold and misassemble into cross β -sheet rich amyloid fibrils and other aggregate structures leading to human amyloid diseases such as senile systemic amyloidosis, familial amyloid cardiomyopathy, familial amyloid polyneuropathy, and central nervous system selective amyloidosis.⁵ Herein, we sought to discover coumarin derivatives that inhibit TTR amyloidogenesis and serve as fluorescent folding sensors for tetrameric TTR upon binding, i.e., bifunctional coumarin derivatives.

Recently, we systematically optimized the typical substructures of TTR kinetic stabilizers that exhibit structural complementary to the T₄-binding sites and ameliorate the TTR amyloidoses by slowing rate-limiting tetramer dissociation and monomer misfolding of TTR, inhibiting amyloidogenesis.^{3a, 6} While substantial genetic and biochemical evidence support the hypothesis that the process of amyloidogenesis causes the TTR amyloidoses, it is not clear how the process of amyloidogenesis leads to the loss of post-mitotic tissue.^{2a, 7} Therefore, the development of a simple, but robust fluorescence method for detection and visualization of natively folded TTR in complex biological systems is desirable.

Previously, we reported a family of fluorogenic stilbenes that selectively bind to and then covalently modify TTR, creating a bright blue fluorescent conjugate.⁸ However, their application for imaging is limited by their short excitation and emission wavelengths (λ_{ex} = 328 nm, λ_{em} = 430 nm). In this study, we create TTR kinetic stabilizers utilizing a coumarin scaffold found in a variety of natural products that exhibit anticancer, anticoagulant and antimutagenic activities. Coumarins possess desirable photophysical attributes including high quantum yields, long excitation and emission wavelengths and environmentally sensitive fluorescence.⁹ Using a *trans* alkene or alkyne linker to extend the π system, we introduced a 3,5-dimethyl-4-hydroxyphenyl ring at the 3-position of the coumarin substructure, a ring known to preferentially bind to the inner subsite of the T₄-binding sites of TTR^{6b} (Figure 1B). In this study, we designed and synthesized coumarins that would bind to the T₄-binding sites within TTR and function both as TTR amyloidogenesis inhibitors and as sensors that become fluorescent upon binding to correctly folded tetrameric TTR, ideally in the context of a complex biological environment.

As the primary filter for the identification of bifunctional coumarins, we first evaluated the ability of the coumarin derivatives **1-10** to inhibit wild type (WT)-TTR amyloidogenesis using the established acid-mediated fibril formation assay, as potent inhibition requires high affinity TTR binding.^{6a} Briefly, a candidate coumarin derivative (7.2 μM , the minimum concentration required to occupy the two T₄-binding sites) was incubated with a physiologically relevant concentration of WT-TTR (3.6 μM) for 30 min and then TTR aggregation was initiated by adjusting the pH to 4.4 (a denaturation stress). The extent of TTR fibril formation was quantified by measuring sample turbidity after 72 h of incubation at 37 °C, a period over which aggregation is nearly complete in the absence of inhibitor. Inhibitor potency was expressed as percentage fibril formation by WT-TTR, which in the absence of inhibitor is assigned to be 100%. Coumarins **1**, **5**, **6**, and **10** all proved to be

potent inhibitors of WT-TTR aggregation, allowing between 3% and 9% of WT-TTR fibril formation (Figure 2).

As the secondary filter for the identification of bifunctional coumarins, the spectral properties of the coumarin analogs were evaluated by comparing the excitation and fluorescence emission spectra in phosphate buffer vs. bound to WT-TTR in phosphate buffer (Table S1). Coumarins **5** and **10** showed large increases in their fluorescence emission upon binding to WT-TTR (Figures 3 and S1A, respectively, blue traces), but barely detectable fluorescence when incubated alone in buffer (Figures 3 and S1A, respectively, red traces). Coumarins **1** and **6** exhibited weak fluorescence in complex with WT-TTR and thus were eliminated from further consideration as bifunctional molecules. Incubation of **5** (7.2 μ M) with WT-TTR (3.6 μ M) for 10 min revealed a 35-fold increase in fluorescence intensity, compared to **5** alone in phosphate buffer (Figure 3 and S1B), and a slight red shift ($\sim$ 10 nm) in the emission maximum. For **10**, the change in fluorescence intensity was smaller (20 fold) upon binding to WT-TTR, but still substantial (Figure S1A and C). That **5** is an environmentally-sensitive fluorophore was revealed by the red shift in its fluorescence maximum and its intensity decrease as the polarity of the solvent was changed from the nonpolar methylene chloride to increasingly polar mixtures of methylene chloride and methanol (Figure S2). The OFF to ON fluorescence switching when **5** and **10** bind to WT-TTR is likely a consequence of occupancy of the more hydrophobic T₄-binding sites and decreased water-mediated hydrogen bonding to the coumarins. The fluorescence emission change of **5** was not detectable in the presence of the monomeric variant of TTR (M-TTR)¹⁰ (Figure 3), suggesting that **5** reports only on the tetrameric folded structure of WT-TTR.

To assess the binding selectivity of the bifunctional coumarins in complex biological environments, such as human blood plasma that contains 4000+ proteins,¹¹ compounds **5** or **10** (10.8 μ M) were incubated with human blood plasma (TTR concentration is 3.6-5.4 μ M) at 37 °C for 24 h and then TTR, along with any bound coumarin, was immunocaptured using a Sepharose resin-conjugated anti-TTR antibody. After dissociation of TTR from the resin at high pH, HPLC analysis revealed that the ratio of TTR to bound **5** or **10** in human blood plasma was 1.21 and 0.54, respectively, out of maximum of 2, indicating high binding selectivity to TTR of **5** in a complex biological system (Figure S3A). To put these numbers in perspective, the ratio of WT-TTR bound to tafamidis, the drug approved to treat familial amyloid polyneuropathy is 0.81.¹² We also demonstrated that the expression and folding of WT-TTR in *E. coli* could be monitored by **5**, reflecting on its selectivity. While **5** exhibits no significant fluorescence background in *E. coli* transfected with WT-TTR before IPTG induction, strong fluorescence is observed after IPTG induction, reflecting TTR production, folding and tetramer formation (Figure S3B).

Since **5** fluoresces more strongly than **10** when bound to tetrameric WT-TTR (Figure 3), we evaluated whether **5** would be useful for assessing the quaternary structural changes exhibited by WT-TTR upon urea denaturation (previously assessed by measuring tryptophan fluorescence¹³). The fraction of WT-TTR tetramer retained as a function of increasing urea concentration was evaluated using tetrameric TTR-**5**_{n 2} fluorescence (Figures 4 and S4A). The fluorescence of **5** in the absence of TTR is not affected by increasing urea concentrations (Figure S4B). The concentration of urea at the midpoint of TTR tetramer-

monomer equilibrium (C_m) assessed by TTR- 5_n 2 fluorescence afforded a value of 3.3 M, in excellent agreement with the value obtained from the unfolding of TTR monomer measured by tryptophan fluorescence (C_m for WT-TTR = 3.4 M)¹³.

Since compound **5** was the most promising bifunctional compound to emerge from this study, the crystal structure of the complex between **5** and WT-TTR, WT-TTR- 5_n 2, was determined to 1.13 Å resolution (Figure 5 and Table S2). The 3,5-dimethyl-4-hydroxyphenyl substructure occupies the inner T₄-binding cavity, as expected from analogous stilbene structures.^{3a} The methyl substituents extend into the two symmetry-related halogen-binding pockets (HBP) 3 and 3'. The 4-OH substituent makes bridging hydrogen bonds with the Ser117 and Ser117' side-chain hydroxyls of adjacent TTR subunits. The *trans* -CH=CH- linker places the coumarin ring in the hydrophobic environment of HBP1/1'. K15 and K15' form salt bridging interactions with E54 and E54' which may enable water-mediated interactions with the 7-hydroxyl group of the coumarin, although this is not observable in the structure.

In conclusion, we have identified two coumarin derivatives that bind selectively to tetrameric TTR and become fluorescent in complex biological environments, such as those found in *E. coli* and in human blood plasma. Bifunctional coumarin derivatives **5** and **10** are useful not only as TTR kinetic stabilizers (amyloidogenesis inhibitors), but also as fluorescent TTR native quaternary structure sensors.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

(A) Crystal structure of the TTR tetramer (PDB 2ROX)⁴ with thyroxine (T₄) binding pockets at the weaker dimer-dimer interface, bisected by the crystallographic C₂ axis (B) Chemical structure of coumarin derivatives synthesized.

Fig. 2.

Evaluation of the potency of coumarin derivatives **1-10** as amyloid fibril inhibitors Percent (%) values represent the extent of in vitro WT-TTR fibril formation in the presence of inhibitors **1-10**.

Fig. 3. Fluorescence spectra of **5** observed after 10 min of incubation with WT-TTR (blue trace) and monomeric M-TTR (solid black trace).

Fig. 4. Evaluation of the thermodynamic stability of WT-TTR (3.6 μ M) as a function of urea concentration using fluorogenic **5** (7.2 μ M) to assess the folded tetramer–monomer equilibrium.

Fig. 5.

Crystal structure of WT-TTR in complex with **5** (PDB 4KY2). A Connolly molecular surface representation (grey = hydrophobic; purple = polar) depicts the hydrophobicity of the binding site. Figure generated using MOE (2011.10, Chemical Computing Group).