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Research article

Thyroxine metabolite-derived 3-iodothyronamine (T1AM) and synthetic analogs as efficient suppressors of transthyretin amyloidosis

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ABSTRACT

Aggregation and fibrillization of transthyretin (TTR) is a fatal pathogenic process that can cause cardiomyopathic and polyneuropathic diseases in humans. Although several therapeutic strategies have been designed to prevent and treat related pathological events, there is still an urgent need to develop better strategies to improve potency and wider applicability. Here, we present our study demonstrating that 3-iodothyronamine (T1AM) and selected thyronamine-like compounds can effectively prevent TTR aggregation. T1AM is one of the thyroid hormone (TH) metabolites, and T1AM and its analogs, such as SG2, SG6, and SG12, are notable molecules for their beneficial activities against metabolic disorders and neurodegeneration. Using nuclear magnetic resonance (NMR) spectroscopy and biochemical analysis, we confirmed that T1AM analogs could bind to and suppress acid-induced aggregation of TTR. In addition, we employed computational approaches to further understand the detailed mechanisms of the interaction between T1AM analogs and TTR. This study demonstrates that T1AM analogs, whose beneficial effects against several pathological processes have already been proven, may have additional benefits against TTR aggregation and fibrillization. Moreover, we believe that our work provides invaluable insights to enhance the pleiotropic activity of T1AM and structurally related analogs, relevant for their therapeutic potential, with particular reference to the ability to prevent TTR aggregation.

1. Introduction

Thyroid hormones (THs) are essential signaling molecules that modulate diverse physiological and pathological processes [1,2]. Upon secretion from the thyroid, the predominant forms of THs are L-thyroxine (T4) and 3,3′,5-triiodo-L-thyronine (T3), of which T3 is the active form that mediates most of the TH-related physiological processes. In 2004, however, it was discovered that in human blood and tissues, there is another active endogenous molecule whose physiological activity is mostly antagonistic to T3, and subsequent mass-spectrometric analyses determined its chemical structure as a mono-iodine-containing thyronamine, 3-iodothyronamine (T1AM) [3]. In contrast to the classical signaling targets of T3, such as the nuclear thyroid hormone receptor, the major target of T1AM was identified as trace amine-associated receptor families (such as TAAR1), thus mediating different signaling pathways from those of T3 [4].

The discovery of T1AM and its distinctive signaling pathways has attracted considerable attention owing to its potential as a novel therapeutic intervention for various human diseases [1,5,6]. In particular, several research groups have identified that T1AM and its analogs are effective not only for metabolic disorders, but also for neurodegenerative diseases (NDDs) [5,7,8]. A series of reports have demonstrated that the neuroprotective effects of T1AM and its analogs are mediated by an increase in ERK1/2 phosphorylation and transcription factor c-fos expression [5], by modulation of SIRT6 and autophagy induction [7], or by interplay with the histaminergic system [8,9]. Notably, Chiellini et al.

Abbreviations: THs, Thyroid hormones; T4, L-thyroxine; T3, 3,3′,5-triiodo-L-thyronine; T1AM, 3-iodothyronamine; TTR, transthyretin; PDB, Protein Data Bank; ThT, thioflavin T; WT, Human wild-type; Aβ, amyloid-β; AD, Alzheimer's disease; NMR, nuclear magnetic resonance; ATTR, TTR amyloidosis; MD, Molecular dynamics.

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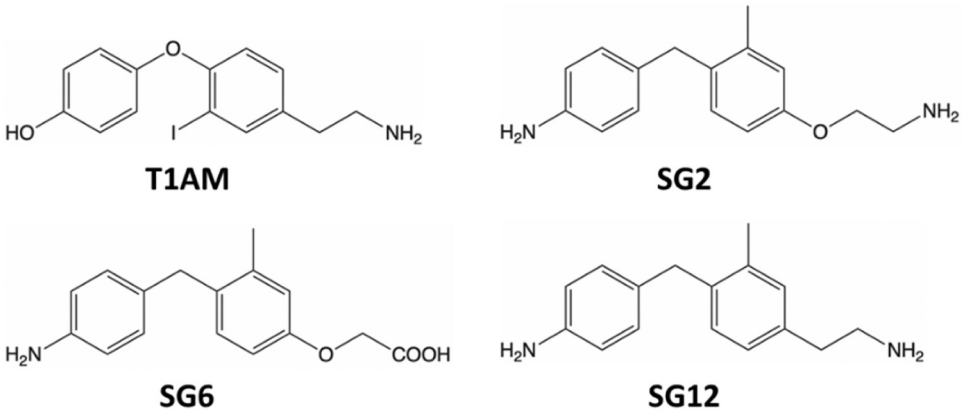

Fig. 1. Structures of endogenous 3-iodothyronamine (T1AM) and synthetic thyronamine-like analogs, SG2, SG6, and SG12.

developed a 3-methylbiaryl-methane analog of T1AM, SG2, and its potential oxidative metabolite SG6, and found that they exhibited therapeutic activities against NDDs [5,10,11]. In addition, an aspect of considerable importance in the development of drugs intended for the treatment of CNS disorders is to know the effective delivery of these agents across the blood brain barrier (BBB). In a recent study, di Leo et al. [6] investigated the permeability of an in vitro model of BBB to T1AM, showing compelling evidence that T1AM was able to efficiently cross the BBB.

One of the advantageous features of TH analogs as drugs is their multifunctionality, which simultaneously target various physiological processes. T1AM can target multiple receptors and channels, for example, TAAR families (TAAR1, TAAR5, and TAAR8), ADRA2A, TRPM8, TRPV1, and F_0F_1-ATPase, implying that similar multifunctionality is also expected for T1AM analogs [11]. However, there are several TH transporter proteins, such as thyroxine-binding globulin, transthyretin (TTR), and albumin [12], suggesting that T1AM analogs may interact with these proteins and affect their physiological features.

In the present study, we investigated the possible beneficial effects of T1AM and its analogs on TTR. TTR is a transporter of thyroxine and a holo-retinol-binding protein. In its native state, TTR maintains a well-folded tetrameric complex, in which two hydrophobic binding pockets for T4 are constructed [13,14]. Notably, in addition to its physiological importance, TTR has attracted enormous attention because of its intrinsic ability to aggregate and form amyloid fibrils. TTR amyloidosis (ATTR) is known to be caused by wild-type protein (often called senile systemic amyloidosis, as it mainly occurs at the age of 70 years) or by a single amino-acid substitution, such as V30M, L55P, and V122I, which often results in amyloid fibril deposition in the heart or peripheral nervous system (thus referred to as familial amyloid cardiomyopathy or polyneuropathy) [15–18]. Previous in vitro and in vivo studies have shown that the amyloidogenic propensity of TTR correlates with its tetramer stability; dissociation into monomers and the subsequent formation of misfolded species is a rate-limiting step of TTR aggregation and fibril formation [17,19]. Based on these observations, it was reasoned that molecules that can bind to the hydrophobic T4 binding pocket may stabilize the tetrameric state of TTR and inhibit its aggregation [20]. This idea was indeed followed by the successful development of the drug tafamidis, whose inhibitory effect on ATTR was validated in clinical trials [21,22].

However, even after this great success, the need to develop further optimized therapeutic molecules for ATTR still exists because of the occasional limited efficacy and reduced capability of tafamidis to be distributed in the brain or eyes [23,24]. Therefore, in the present study, we aimed to assess whether T1AM and its analogs, SG2, SG6, and SG12 (Fig. 1), interact with TTR and exert an additional role as a suppressor of ATTR. These T1AM analogs were selected based on their structural resemblance and functional efficacy, while pertaining differences in their functional groups. This series of ligands is therefore suitable to investigate the role of specific chemical moieties in interacting with TTR and suppressing its aggregation. To this end, we combined nuclear magnetic resonance (NMR) spectroscopy and computational simulation approaches to investigate the interactions between TTR and T1AM analogs at atomic resolution. We also validated the efficacy of T1AM analogs as stabilizers of the TTR tetramer with in vitro thioflavin T (ThT) fluorescence and turbidity measurements. Our results indicate that T1AM analogs are promising stabilizers of the TTR tetramer. In particular, considering their multi-functionality toward metabolic and neurodegenerative disorders, T1AM and its analogs may provide a novel and powerful therapeutic strategy for various related diseases.

2. Materials and methods

2.1. Drugs

T1AM and tafamidis were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Cayman Chemical (Ann Arbor, MI, USA), respectively, and the thyronamine-like compounds, SG2, SG6, and SG12, were kindly provided by Prof. Rapposelli [5,10]. Aliquots were stored at − 20 °C in DMSO as a 200 mM stock solution and diluted to the desired final concentration in the assay media.

2.2. Protein sample preparation

Human wild-type (WT), V30M, and L55P TTR proteins were recombinantly expressed in *Escherichia coli* and purified using previously described procedures [25]. Briefly, the pQE30 vector (Qiagen, Hilden, Germany) and *E. coli* M15(pREP4) competent cells were used for protein expression. To complement the relatively large size (~55 kDa) of tetrameric WT TTR complex for NMR measurements, we employed the fractional deuteration procedure, where M9 minimal media made with 99 % deuterium oxide and supplemented with 3 g/L [U-^{13}C]-D-glucose and 0.5 g/L $^{15}NH_4Cl$ (Cambridge Isotope Laboratories, Tewksbury, MA, USA) was used for protein production. Subsequently, the purification procedures were composed of sonication and centrifugation for cell debris removal, anion exchange chromatography (HiTrap Q HP; Cytiva, Marlborough, MA, USA), and the final size-exclusion chromatography (HiLoad 16/600 Superdex 75 pg; Cytiva); the latter two chromatographic procedures were conducted with the ÄKTA FPLC system (Cytiva). The fractions from the chromatographic runs were monitored using SDS-PAGE for purity. The final pure fractions were pooled, flash-frozen with liquid nitrogen, and stored in a − 80 °C freezer until use. We confirmed the integrity of the final TTR samples with mass-spectrometry and NMR; the mass-spectrometric analysis showed no indication of modification, and the NMR spectra obtained with our samples fit well with the deposited data in BMRB (see below).

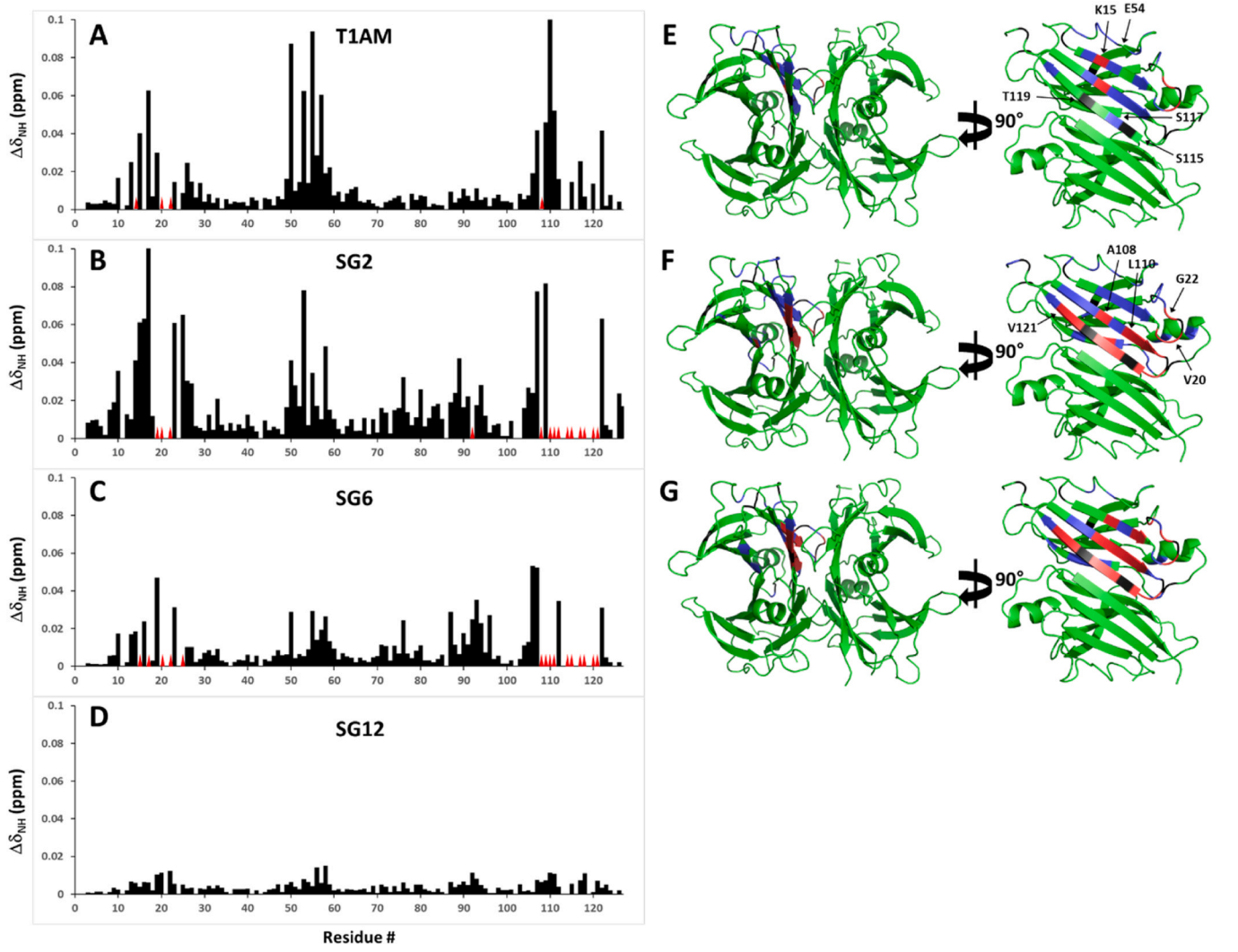

Fig. 2. Results of NMR signal perturbation induced by titrations of T1AM analogs onto TTR. One-fold equivalent (vs. the concentration of TTR monomer) of T1AM and its analogs were added to the sample of [frac-^{2}H; U-^{13}C; U-^{15}N]-TTR, and the 2D ^{1}H–^{15}N HSQC and 3D HNCA spectra were obtained to monitor the signal perturbations of TTR. (A-D) Perturbation of the backbone ^{1}H–^{15}N HSQC signals by the titration of T1AM analogs was plotted using the following equation: $\Delta\delta_{NH}$ (ppm) = $[(\Delta\delta_N/5)^2 + (\Delta\delta_H)^2]^{1/2}$ (see Methods for details). The residues whose signals disappeared beyond detection during titration are indicated by red triangles. (E-G) Structural model of TTR (PDB ID: 2ROX) colored according to the extent of signal perturbations. Residues whose signals were significantly perturbed are colored as follows: red, signals that disappeared during titration; blue, signals that exhibited significant shifts ($\Delta\delta_{NH} > 0.02$ ppm); black, signals not assigned residues and prolines. On the left is the native tetrameric model, while only half of the tetrameric model is shown on the right for better presentation. The colors are shown at one subunit for simplicity. Note that the same figure was not included for the result of SG12 due to the lack of noticeable signal perturbation.

2.3. NMR spectroscopy

For NMR experiments, we used an Avance III HD 850 MHz NMR spectrometer equipped with a cryogenic HCN probe (Bruker, Billerica, MA, USA). Fractionally deuterated and uniformly ^{13}C- and ^{15}N-labeled ([frac-^{2}H; U-^{13}C; U-^{15}N]) WT-TTR were prepared at a concentration of 200 μM in a buffer consisting of 50 mM MES pH 6.5, 100 mM NaCl, 5 mM dithiothreitol, 0.01 % NaN_3, and 7 % D_2O. The sample volume was adjusted to 300 μL to be accommodated in a 5-mm Shigemi tube (Sigma-Aldrich). Resonance assignment information was obtained from BMRB accession numbers 5507 and 27514 [26,27]. For NMR data acquisition, the TopSpin 3.2 software package (Bruker) was used, and we employed the POKY software suite for NMR data analysis [28].

Titration experiments were conducted by serially adding T1AM analogs (dissolved in DMSO-d_6) to the [frac-^{2}H; U-^{13}C; U-^{15}N]-WT-TTR sample and collecting 2D ^{1}H–^{15}N TROSY-HSQC spectra. We repeated the same titration experiments with tafamidis (dissolved in DMSO-d_6) for comparison with those of T1AM analogs. To clarify the signal assignment of the 1:1 mixture of TTR and the T1AM analog, 3D TROSY-HNCA spectra were also obtained. Notably, we confirmed that the NMR spectra did not exhibit any noticeable signal perturbation upon the addition of the same amount of DMSO-d_6 (without T1AM analogs) to the TTR sample. The signal perturbation plot was obtained by calculating $\Delta\delta_{NH}$ for each signal upon addition of 1-fold T1AM analogs using the following equation: $\Delta\delta_{NH} = [(\Delta\delta_N/5)^2 + (\Delta\delta_H)^2]^{1/2}$, where $\Delta\delta_N$ and $\Delta\delta_H$ are the signal movements in ppm in the NMR spectra of ligand-free and bound WT-TTR.

Finally, to test whether tafamidis competes with SG2 or SG6 for the same binding site of TTR, we added 1-fold tafamidis to the samples of [frac-^{2}H; U-^{13}C; U-^{15}N]-WT-TTR containing 2-fold SG2 or SG6, and collected 2D ^{1}H–^{15}N TROSY-HSQC spectra.

2.4. Aggregation assay

The aggregation propensity of TTR samples (WT, V30M, and L55) in the absence or presence of T1AM analogs was evaluated using an

aggregation assay under mildly acidic conditions [29,30]. TTR samples, which were prepared at a concentration of 64 μM in PBS buffer (pH 7.4; 10 mM phosphate, 140 mM NaCl, and 2.7 mM KCl), were first treated with either DMSO or five-fold T1AM analogs (T1AM, SG2, SG6, or SG12) dissolved in DMSO. These TTR-T1AM analog mixtures were subsequently mixed in a 1:1 ratio with acetate buffer (200 mM sodium acetate pH 4.2, 100 mM KCl, and 1 mM EDTA). The mixture was then incubated at 37 °C without agitation.

Aggregation was monitored by ThT fluorescence (and turbidity measurements for WT TTR) using a Tecan Spark™ 10 M microplate reader (Männedorf, Switzerland). To measure ThT fluorescence, the samples were first diluted to a final concentration of 4 μM TTR with a buffer consisting of 200 mM Tris (pH 8.0) and 150 mM NaCl, and 400 μL of this mixture was then mixed with 2 μL of 2 mM ThT solution in a buffer consisting of 200 mM Tris (pH 8.0) and 150 mM NaCl. The measurements were performed in triplicate with the samples filled in a 96-well black-wall microplate (excitation and emission wavelengths were 440 nm and 482 nm, respectively). To measure turbidity, the sample was transferred into a 96-well transparent microplate, and the optical density at 330 nm was obtained in triplicate. The measurement results were averaged and plotted in the figures along with their standard deviation as an error bar. The reproducibility of the measurement results was confirmed by repeating the same experiment two to three times.

2.5. Molecular docking and molecular dynamics (MD) simulation

Protein preparation and molecular docking were performed in the TTR-T4 complex of the Protein Data Bank (PDB) [31] coded with PDB ID 2ROX [14] using the Chemscore function of the GOLD program, as previously validated [32]. MD simulations were performed to test the stability of the predicted pose using AMBER Version 16 (San Francisco, CA, USA) [33]. The TTR complexes, derived from the best scored docking poses of SG2, SG6, SG12, T1AM, and tafamidis as a control were placed in a parallelepiped water box using an explicit solvent model (TIP3P) and solvated with a 10 Å water cap. For system neutralization, sodium ions were used as counterions. Three steps of minimization were carried out before 70 ns of MD simulation: optimization of the solvent, relaxation of the side chains of the protein and finally of the ligand. We used the particle mesh Ewald electrostatic and periodic boundary conditions. For MD trajectory, minimized structures as the starting conformations were used. We set the time step of the simulations to 2.0 fs with a cutoff of 10 Å for the non-bonded interaction, and employed SHAKE to keep all bonds involving hydrogen atoms rigid. Constant-volume periodic boundary MD was run for 500 ps along with the temperature increase from 0 to 300 K. Subsequently, constant-pressure periodic boundary MD was run for 69.5 ns at 300 K. We used the Langevin thermostat to keep the temperature of the system constant, sustaining all the α carbons and ligand with 10 kcal of constant harmonic force for the first 1.6 ns. After this, we relaxed the ligand for additional 1.6 ns, and ran the last simulation without any constraint for the remaining 66.3 ns. The trajectory was collected in one frame per every 100 ps of the simulation, generating 700 frames for all complexes. General Amber force field parameters were assigned to the ligands, and partial charges were calculated using the AM1-BCC method. The set of the 30 closest water molecules to the ligand was determined using the closest command in the CPPTRAJ module [34] of AMBER 16. The solvent molecules were set automatically by distance, and the water closest for each frame was recorded. The MD trajectories were examined with the MD Movie tool of Chimera [35] and the CPPTRAJ module. The Molecular Mechanics Poisson-Boltzmann Surface Area (MMPBSA) approach [36] was used to calculate binding free energy of the complexes, applying the MMPBSA routine [37]of AMBER to 100 frames extracted from stable simulation trajectories. The contribution of each binding site residue to ligand stabilization in the same trajectory was also analyzed, using the PAIRWISE command in the CPPTRAJ module, which writes out van der Waals energy (EVDW) and electrostatic energy (EELEC) of non-bonded interactions. Results are reported as the sum of EVDW and EELEC in kcal/mol.

3. Results

3.1. NMR titration experiments of TTR with T1AM analogs

We first examined whether T1AM analogs directly interact with TTR. To this end, we collected a series of 2D ^{1}H–^{15}N TROSY-HSQC and 3D TROSY-HNCA spectra of the [frac-^{2}H; U-^{13}C; U-^{15}N]-TTR samples titrated with increasing amounts of ligands; that is, T1AM, SG2, SG6, and SG12 (Fig. 1). Notably, all the ligands incurred NMR signal perturbation, albeit in a different fashion (Fig. 2 and S1). Upon addition of 1-equivalent of T1AM to TTR, signals corresponding to the residues V14, V20, G22, and A108 disappeared beyond detection, whereas signals for M13, K15, L17, A19, I26, S50, G53, L55-G57, T59, I107, A109-L111, S117, and V122 shifted significantly ($\Delta\delta_{NH} > 0.02$ ppm; the average plus one standard deviation value from the results of SG6 titration). SG2 induced consistent, yet more pronounced signal perturbation: the signals for A19, V20, G22, E92, A108, L110-S112, Y114, S115, S117, T118, A120, and V121 disappeared with 1-fold addition of SG2, and the signals for C10, V14-L17, S23, A25-N27, F33, S50, E51, G53, L55, L58, K76, K80, H88, E89, A91, V94, Y105-I107, A109, V122, and K126 shifted significantly. The 1-fold addition of SG6 resulted in the disappearance of the signals for K15, L17, V20, G22, A25, A108-L111, Y114, S115, S117, T118, A120, and V121, whereas the signals for V16, A19, S23, S50, L55, L58, K76, F87, E92-V94, T96, T106, I107, S112, and V122 were significantly shifted. In contrast, the overall signal perturbation was greatly diminished in the titration results of SG-12; the 1-fold addition to TTR only caused minimal signal shifts without any disappearance. We also observed consistent changes in the signal intensity upon ligand titration; as a representative example, the signal intensity change of the residue A109 is shown in Fig. S2.

To compare the ligand binding of T1AM and its analogs, we examined whether each added ligand dissociates from TTR using a simple buffer exchange procedure. By comparing the 2D ^{1}H–^{15}N TROSY-HSQC spectra before and after buffer exchange, we could easily distinguish the ligand-free state of TTR from the ligand-bound state. From these trials, we confirmed that T1AM and SG12 could be easily removed from the TTR sample, whereas extensive buffer exchange could not dissociate the SG2- and SG6-TTR complexes (Fig. S3). This result indicated that the binding interactions of TTR with SG2 and SG6 were sufficiently stable, whereas those of T1AM and SG12 were not.

Overall, ligand titration experiments showed that the addition of SG2 and SG6 resulted in the most severe signal perturbations (Fig. 2). Notably, SG6 exhibited more signal broadening than SG2, while signal movements were more noticeable in the SG2 titration than in SG6. In contrast, T1AM or SG12 caused only modest or minimal signal changes, respectively.

Moreover, we tested whether tafamidis competes with T1AM analogs for the binding site on TTR. The 2D ^{1}H–^{15}N TROSY-HSQC spectra of the TTR samples, which mixed first with either 2-fold SG2 or SG6, and subsequently with 1-fold tafamidis, exhibited the same spectral feature with that of the TTR sample containing 1-fold tafamidis (Fig. S4). This indicates that the affinity of tafamidis for TTR was stronger than those of SG2 and SG6, and T1AM and its analogs bind to TTR with the overlapped binding site of tafamidis.

3.2. Aggregation assay of TTR in the presence of T1AM analogs

Next, we tested the efficacy of T1AM analogs in inhibiting the aggregation of TTR. The aggregation of TTR was induced by lowering the pH of the protein sample to 4.4, and the suppressive effects of T1AM analogs were tested by inducing TTR aggregation in the absence or presence of 5-fold ligands [29,38]. Subsequent aggregation was