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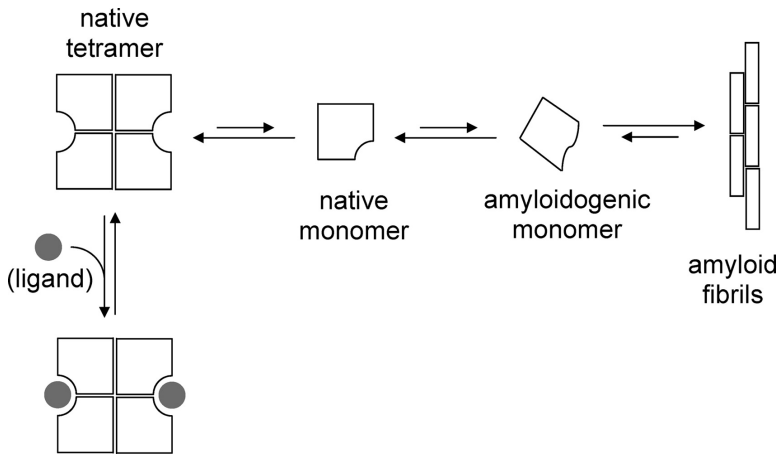

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Kinetic Assay for High-Throughput Screening of In Vitro Transthyretin Amyloid Fibrillogenesis Inhibitors

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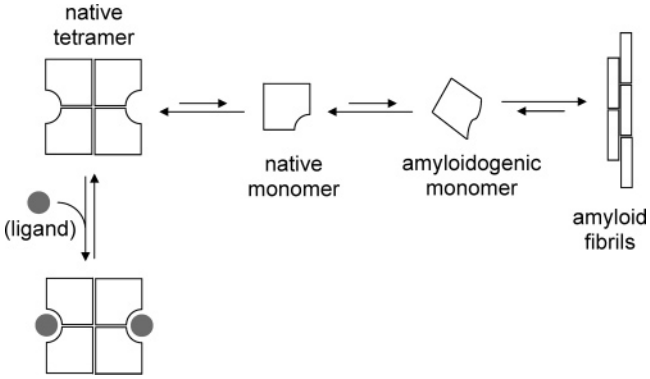
Stabilization of tetrameric transthyretin (TTR) by binding of small ligands is a current strategy aimed at inhibiting amyloid fibrillogenesis in transthyretin-associated pathologies, such as senile systemic amyloidosis (SSA) and familial amyloidotic polyneuropathy (FAP). A kinetic assay is developed for rapid evaluation of compounds as potential in vitro inhibitors in a high-throughput screening format. It is based on monitoring the time-dependent increase of absorbance due to turbidity occurring by acid-induced protein aggregation. The method uses the highly amyloidogenic Y78F mutant of human transthyretin (heterogously expressed in *Escherichia coli* cells). Initial rates of protein aggregation at different inhibitor concentrations follow a monoexponential dose−response curve from which inhibition parameters are calculated. For the assay development, thyroid hormones and nonsteroidal antiinflamatory drugs were chosen among other reference compounds. Some of them are already known to be in vitro inhibitors of TTR amyloidogenesis. Analysis time is optimized to last 1.5 h, and the method is implemented in microtiter plates for screening of libraries of potential fibrillogenesis inhibitors.

Introduction

Amyloid fibrillogenesis is a central event in the pathogenesis of many diseases, including neurodegenerative disorders such as Alzheimer's, Parkinson's, and Huntington's diseases; spongiform encephalopathies; and familial amyloidotic polyneuropathies. All of them are characterized by formation of supramolecular protein aggregates containing whole or partially processed misfolded proteins that form insoluble fibrilar deposits with a very characteristic molecular structure, usually called amyloids.[1−4] Transthyretin (TTR) has been implicated in pathologies such as senile systemic amyloidosis (SSA) and familial amyloidotic polyneuropathy type I (FAP-I).[5−8] The latter is an autosomal dominant lethal disease in which amyloid fibrils are mostly constituted by mutant TTR variants and may affect individuals beginning in their 20s.[9] Its amyloid deposits are found extracellularly, mostly in peripheral nerves, heart, kidneys, and vitreous humor.

Human transthyretin (hTTR) is a 55-kDa homotetrameric serum protein (0.1−0.4 mg·mL^{-1}) with a high proportion of β-sheet structure and a main role in transporting thyroid hormones (T4 and T3) as well as retinol-binding protein.[10] The 3D structure[11,12] reveals the assembly of two dimers that form a central hydrophobic channel containing two symmetrical T4 binding sites with different dissociation constants, reflecting negative cooperativity.[13]

Scheme 1. Model of hTTR Amyloid Fibril Formation and Stabilization of the Tetramer by Ligand Binding



Amyloid fibril formation is initiated by TTR tetramer dissociation into dimers and monomers that evolve to a misfolded or non-native monomer intermediate (Scheme 1). Depending on its thermodynamic stability, the native monomer produces partially unfolded amyloidogenic monomeric species that start an intermolecular aggregation process, probably involving a number of states leading to mature fibrils.[14−16] Recent studies showed that TTR intermediates (protofibrils) rather than mature fibrils are toxic in culture and may play a role in pathogenesis.[17] Therefore, therapeutic strategies are nowadays mainly focusing on either tetramer stabilization or preventing the formation of the toxic intermediates.[18,19] Stabilization of the tetramer in the asymp-

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tomatic phase seems to be a preferred option for a therapeutic strategy. Since T4 is known to stabilize the tetrameric form of TTR and a number of compounds have already shown in vitro inhibitory effect of amyloid fibril formation,[20−24] small-molecule ligands to the T4 binding sites of the tetramer could be considered potential candidates for future drugs aimed at SSA and FAP.

Amyloidogenesis assays are based on the in vitro detection of fibril formation by different techniques.[25] In the case of TTR in vitro fibrillogenesis, many different assays have been employed, such as thioflavin-T fluorescence and Congo red staining,[26−28] electron microscopy and atomic force microscopy,[15] filter retardation assays,[29] mass spectrometry,[30] and turbidimetric methods.[20,22,24,31,32] The latter are straightforward methods to monitor fibril formation and evaluate the effect of TTR ligands as inhibitors of the aggregation process by measuring changes in absorbance at 330 nm. Wild type hTTR amyloid fibril formation is highly dependent on pH, with maximal occurrence at pH 4.4 for protein solutions of 0.2 mg·mL^{-1} at 37 °C. More amyloidogenic hTTR variants, such as V30M and L55P, aggregate readily at higher pH values. Even though the mechanisms of in vivo amyloid formation are still unknown and, thus, the involvement of acid catalysis is not clear, acid-induced aggregation has proven to be useful for biophysical studies of tetramer stability and aggregation pathways, as well as for implementing in vitro assays to evaluate fibril formation inhibitors.[26,33,34]

Common turbidimetric assays for testing potential amyloidosis inhibitors measure the extent of fibrilogenesis in the presence and absence of inhibitor after a 72-h incubation period. Long incubation times are required with wt TTR to discriminate between inhibitors, thus reducing the capacity for a high-thoughput screening and limiting the assay to large reaction volumes to minimize solvent evaporation, otherwise important when miniaturizing the assay. We report here the use of a naturally occurring and highly amyloidogenic hTTR variant, the Y78F mutant expressed in recombinant *E. coli* cells, in a turbidimetric assay to monitor fibril formation. The method evaluates initial rates of amyloid aggregation, and it is implemented in a high-throughput format for rapid screening of series of small compounds as potential amyloidogenesis inhibitors.

Results and Discussion

In commonly used protocols for turbidimetric monitoring of in vitro fibrils formed by wt hTTR,[26] amyloidogenesis is induced by lowering the pH of the medium to 4.0−4.5. When applying these methods for testing potential amyloidosis inhibitors, the extent of amyloid fibrils formation is quantified spectrophotometrically (by absorbance at $\lambda = 340$ nm) after a 72-h incubation period for a battery of concentrations of each inhibitor. Subsequently, the inhibitory potency of the tested compounds is evaluated as the percentage of absorbance reduction of the inhibitor-containing samples when compared with the inhibitor-free control sample. The limitations of these methods are that long incubation times are required to obtain enough sensitivity to discriminate between inhibitors and that kinetic information of the initial steps of fibrillogenesis is lost.

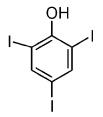
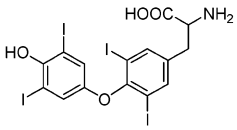
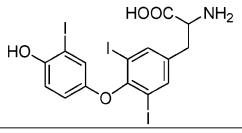
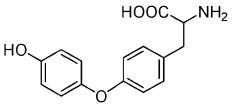
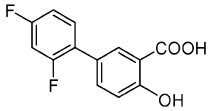
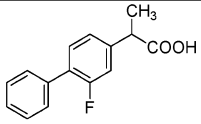
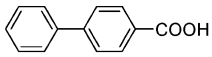
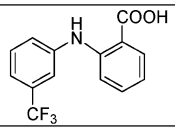
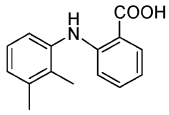
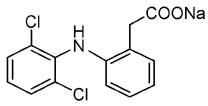
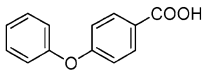
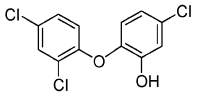
To analyze the effect of small-molecule inhibitors on the initial kinetics of protein fibrillogenesis, reduce the analysis time, avoid artifacts derived from a long nonsterile incubation time, and implement the method in a high-throughput format for rapid screening of libraries of compounds as potential amyloid fibril inhibitors, we optimize here a variant of these common turbidimetric assays.

Assay Development. Among the different hTTR mutant proteins, the Y78F variant was chosen because it was proven to be more amyloidogenic in vitro than the wt hTTR.[35] Y78F forms fibrils at pH 7.0 and 37 °C in aging experiments, as studied by electron microscopy,[36] thus suggesting that this mutant can be a good candidate for a more rapid and sensitive turbidimetric screening test. Compounds **1**−**12** (Table 1) were selected to develop the kinetic assay and validate the methodology for further library screening. Some of them are known to be ligands of tetrameric hTTR and to behave as inhibitors of fibril formation. Selected compounds include triiodophenol (**1**); thyroid hormones (**2**, **3**);[31] nonsteroidal antiinflammatory drugs (NSAIDs), such as diflunisal (**5**), flurbiprofen (**6**), flufenamic acid (**8**), mefenamic acid (**9**), and diclofenac (**10**);[20,22,32,37] the antiseptic triclosan (**12**);[38] and model biaryl compounds **7** and **11**, which are not classified in any therapeutic category but are chosen as potential TTR ligands with related structures to those reported in ref 39.

Turbidity Assay at 72 h. To compare protein performance, the commonly used 72-h assay was conducted with both the more amyloidogenic Y78F mutant and the wt protein. The resulting data for the whole series of reference compounds is shown in Table 2. As opposed to the behavior of the wt hTTR, fibril formation by the Y78F mutant is almost unaffected by the presence of inhibitors after 72-h incubation. These results seem to indicate that, although the inhibitors may exert differential stabilizing effects on the tetrameric protein and reduce fibril formation at initial stages, the more amyloidogenic character of the Y78F mutant rapidly leads to aggregation by displacing the equilibrium toward fibril formation after long (72-h) incubation time. If so, Y78F hTTR seems an appropriate mutant to develop the assay aimed to monitor the initial rate of fibril formation at short incubation time for a rapid screening of potential inhibitors.

Kinetic Turbidity Assay (Initial Rates). To develop such an assay, the pH of the medium and protein concentration (Y78F mutant) were optimized to fulfill high sensitivity and short analysis time. Different protein concentrations close to the physiological hTTR concentration in plasma (0.1−0.4 mg·mL^{-1}) were tested, selecting 0.4 mg·mL^{-1} as a concentration high enough to obtain good signals in a wide range of pH values (data not shown). Time courses of fibril formation for Y78F at 37 °C at different pH values are presented in Figure 1. Amyloidogenesis is too slow at pH values higher than 6.0 to be used in a throughput screening. Despite the fact that pH 4.0 renders the greatest amyloidogenecity, it is too close to pH 3.9, the pH below which amorphous aggregation has been proposed to occur to a significant extent.[26] In compromising some sensitivity but avoiding amorphous aggregation, pH 4.2 was finally selected to obtain fast initial rates of fibril formation. In such

Table 1. Inhibitors of Y78F-hTTR Amyloid Fibril Formation Used in This Study and Inhibition Parameters by the Turbidimetric Kinetic Assay

	Inhibitor	Structure	A [a] (UA·h^{-1})	B (UA·h^{-1})	C (μM^{-1})	IC_{50} [b] (μM)	Ψ [c] (UA·h^{-1}·μM^{-1})	RA (%) [d]
1	Triiodophenol		0.012±0.002	0.198±0.004	0.239±0.017	3.2	0.047	95
2	L-Thyroxine (T4)		0.031±0.004	0.126±0.005	0.093±0.01	10.5	0.012	80
3	L-Triiodothyronine (T3)		0.084±0.008	0.077±0.008	0.055±0.013	>50	0.004	45
4	D,L-thyronine (T0)		0.556±0.004	0.082±0.005	0.17±0.03	>50	0.014	13
5	Diflunisal		0.021±0.009	0.144±0.008	0.052±0.008	16.3	0.008	87
6	Flurbiprofen		0.12±0.05	0.09±0.05	0.04±0.05	>50	0.001	44
7	Biphenylcarboxylic acid		0.121±0.006	0.082±0.007	0.090±0.023	>50	0.007	40
8	Flufenamic acid		0.035±0.007	0.173±0.007	0.091±0.009	10.0	0.016	84
9	Mefenamic acid		0.036±0.003	0.122±0.006	0.229±0.041	3.6	0.035	77
10	Diclofenac		0.095±0.02	0.061±0.005	0.93±0.25	>50	0.057	40
11	4-Phenoxybenzoic acid		0.084±0.02	0.125±0.02	0.035±0.012	51	0.004	60
12	Triclosan		0.125±0.005	0.076±0.007	0.12±0.03	>50	0.009	40

[a] Parameters obtained from fitting the data "initial rates of fibril formation (v_0)" vs "inhibitor concentration ([I])" to eq 1. [b] Concentration of inhibitor at which the initial rate of fibril formation is one-half that at [I] = 0. [c] $\Psi = -dv_0/d[I]$ at [I] = 0; see text. [d] RA(%) = 100B/(A + B): percent reduction of fibril formation rate at high inhibitor concentration relative to the rate at [I] = 0.

conditions, sensitivity is high enough to detect decreases of initial rates of amyloidogenic aggregation in the presence of inhibitors. This is shown in Figure 2, where time courses of amyloidogenesis of Y78F−hTTR at pH 4.2 and 37 °C at different concentrations of L-thyroxine (T4) ranging from 0 to 40 μM are plotted. It is important to note that in using

Table 2. TTR Amyloidogenesis Inhibition Activity of Selected Compounds Using the Turbidity Assay at 72 h with the wt and Y78F Mutant Proteins, and Inhibition Parameters from the Kinetic Assay

	72-h end-point assay		kinetic assay[b]	
inhibitor	FR (%)[a] wt	FR (%)[a] Y78F	IC_{50} (μM)	RA (%)
1	42	10	3.2	95
5	32	0	16.3	87
8	31	0.4	10.0	84
2	30	9	10.5	80
9	30	0.4	3.6	77
11	30	n.d.	>50	40
6	32	6	>50	44
12	31	n.d.	>50	40
10	25	0	>50	40
7	19	6	>50	40
3	17	3	>50	45
4	n.d.	0	>50	13

[a] FR (%): fibril reduction in the presence of 10 μM inhibitor relative to the same assay in the absence of inhibitor. FR (%) = $(\Delta Abs_0 - \Delta Abs_i)/\Delta Abs_0$, where ΔAbs_0 is the absorbance change without inhibitor, and ΔAbs_i is the absorbance change in the presence of 10 μM inhibitor after 72 h. [b] Inhibition parameters from the kinetic assay using the Y78F mutant. Data from Table 1.

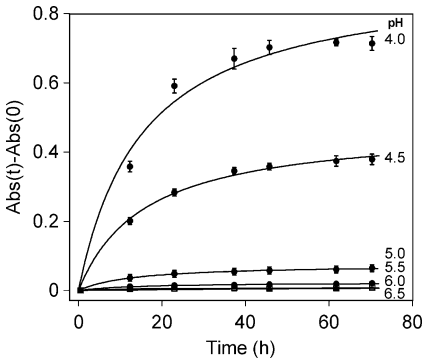


Figure 1. Time course of fibril formation (Y78F-hTTR mutant, 0.4 mg·mL^{-1}) at different pH values in 200 mM buffer, 100 mM KCl, and 1 mM EDTA at 37 °C. Buffers used for acid-induced amyloidogenesis were 200 mM acetate (pH 4−5), 100 mM acetate + 100 mM phosphate (pH 5.5−6), and 200 mM phosphate (pH > 6.5).

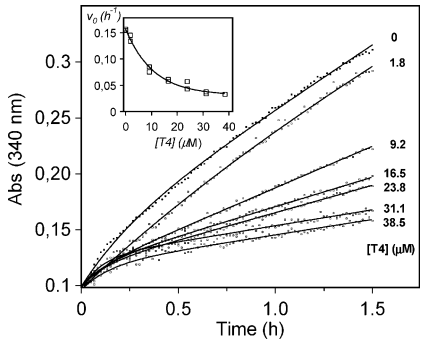


Figure 2. Time course of Y78F-hTTR fibril formation at pH 4.2, 37 °C in the presence of different concentrations of L-thyroxine (T4) as monitored by absorbance at 340 nm at different concentration. Inset: plot of initial rates of fibril formation vs T4 concentration. Data were fitted to eq 1.

this protocol, the incubation time required for the analysis of any effects of the inhibitor on amyloid fibril formation is reduced from 72 to 1.5 h.

Implementation of this kinetic assay, in which absorbance at 340 nm is continuously monitored, requires agitation of the reaction mixture to avoid deposition of aggregates. Parallel experiments with and without shaking are summarized in Figure 3, and it is concluded that no significant effect due to shaking is observed under the experimental conditions used.

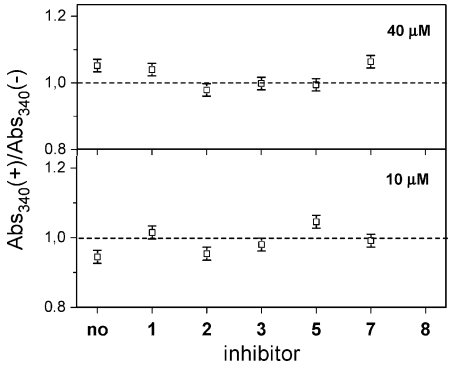


Figure 3. Effect of agitation in the turbidity assay. The increment of absorbance at 340 nm after 1.5 h of incubation in the presence of two concentrations of inhibitor (10 and 40 μM) are determined without and with shaking (15 s every min). $\Delta Abs(+)/\Delta Abs(-)$ is the ratio of absorbance increments after 1.5 h of incubation with (+) and without shaking (−). *x* axes, inhibitor number; no, protein without inhibitor.

A wavelength of 340 nm was chosen for turbidity measurements because it is the common wavelength used in most of the reported 72-h end-point assays. Colored compounds in the screening library may, in principle, interfere at this wavelength; however, the compounds with some absorbance at 340 nm here analyzed (compounds **2**, **8**, and **9**) showed the same molar absortivity values for free and protein-bound forms. Although the absorbance value at 340 nm is slighly increased, the kinetic method evaluates changes of absorbance with time, and the slope Abs vs time is unaffected by absorbance ligand, since it is a constant value. However, this may not be always true, and a higher wavelenth (i.e., 400 nm) may be more appropriate, depending on the compounds analyzed.

Repeatability of the assay was determined with T4 as inhibitor at three different concentrations with six repetitions. Variation coefficients of initial rates of fibril formation were 0.7% at 0 μM, 1.5% at 16 μM, and 1.8% at 40 μM T4.

General Assay Procedure for High-Throughput Screening. After setting the different parameters discussed above, the assay was adapted for running in a high-throughput screening mode. For this purpose, a 96-well microplate is used with a final assay volume of 200 μL. Seven concentrations of inhibitor are tested, ranging from 0 to 40 μM, allowing the analysis of up to 14 compounds per plate in a 1.5-h assay. After following the general procedure indicated in the Experimental Section, time-course curves such as the ones shown in Figure 2 are obtained from which the initial rates of fibril formation (v_0) are calculated as the slopes of the linear increase of absorbance. When plotting the initial rates vs inhibition concentration (Figure 2, inset), an exponential decay is obtained with all inhibitors analyzed. Data were fit to eq 1,

$$v_0 = A + Be^{-C[I]} \quad (1)$$

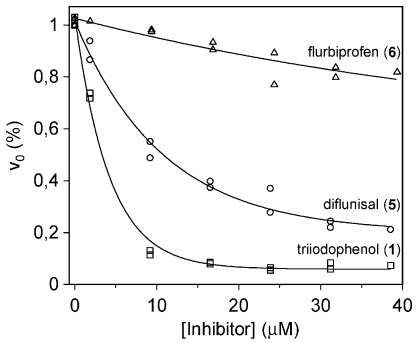


Figure 4. Kinetic turbidity assay with Y78F-hTTR. Relative initial rates of fibril formation (v_0, %) are plotted against inhibitor concentration for compounds **1**, **5**, and **6**. Experiments were performed in duplicate. Data were fitted to eq 1, and fitted parameters are given in Table 1.

where v_0 is the initial rate of fibril formation (in absorbance units per hour, AU·h^{-1}), and [I] is the concentration of the inhibitor (μM). Fitted parameters are A (AU·h^{-1}), residual aggregation rate at high concentration of inhibitor; B (UA·h^{-1}), amplitude or maximum decrease of initial rate of fibril formation; and C (μM^{-1}), the exponential constant. A + B is equal to the initial rate of fibril formation under the assay conditions in the absence of inhibitor. Meaningful parameters to evaluate the potency of a compound as fibril formation inhibitor are the following:

IC_{50}: concentration of inhibitor at which the initial rate of fibril formation is one-half that without inhibitor.

$\Psi = -dv_0/d[I]$ at [I] = 0: slope of the curve at [I] = 0, or decrease of initial fibril formation rate with inhibitor concentration at low [I], reflecting the sensitivity of the aggregation process to the inhibitor.

RA(%) = 100B/(A + B): percent reduction of fibril formation rate at high inhibitor concentration relative to the rate at [I] = 0. For those compounds in which RA < 50%, the IC_{50} value cannot be estimated, since the amyloidosis rate is not reduced to 50% at the maximum inhibitor concentration assayed. In these cases, an $IC_{50} > 50$ μM is given.

A good inhibitor according to this assay would be one with low IC_{50}, high Ψ value, and a RA value close to 100%. Results of evaluation of the inhibition properties of reference compounds **1**−**12** are summarized in Table 1.

Evaluation of Compounds 1−12. Figure 4 shows examples of the data obtained for a typical potent inhibitor (triiodophenol **(1)**, IC_{50} 3.2 μM, RA 95%) with a greater effect as fibril formation inhibitor than the natural thyroxine (T4); a weak inhibitor, such as diflunisal (**5**); and a poor inhibitor, such as flurbiprofen (**6**), with an RA value of 44%.

Results of the kinetic assay using Y78F-hTTR correlate well with the data obtained running the 72-h turbidity assay with the wt protein (Table 2). That is, compounds with an $IC_{50} < 50$ μM in the kinetic assay (Y78F mutant) follow the same ranking when ordered using either RA% values (kinetic assay) or FR (%) values at 10 μM inhibitor concentration (72-h end-point assay, wt TTR). Thus, the order is as follows: **1** > **5** ≥ **8** ≥ **2** ≥ **9** > **11**. Moreover, poor inhibitors according to the kinetic assay ($IC_{50} > 50$ μM, RA < 50%)are also poor inhibitors in the 72-h assay (FR ≤ 30%) at 10 μM concentration.

In summary, we report here a kinetic assay that monitors amyloid fibril formation by measuring the time-dependent increase of absorbance due to aggregation (turbidity). It is a reproducible method that uses the highly amyloidogenic Y78F-hTTR mutant under conditions that allow rapid screening of transthyretin amyloid fibrillogenesis inhibitors, and it is able to discriminate between inhibitors with IC_{50} values lower than 50 μM. Statistically, the assay has good quality for high-throughput screening, with Z′-factor values between 0.85 and 0.95 for all inhibitors analyzed.

Testing for amyloid fibril formation inhibition is a widespread strategy in the search for potential drug candidates for amyloid diseases. The test here proposed fulfills a first selection criterion that is to choose effective candidate molecules with an in vitro activity. Another important characteristic of a selection test at the early stages of the drug development process is speed and capacity to handle many molecules at a time. Both requirements are also met by the kinetic test here reported, which thus improves more time-consuming turbidimetry versions. Active compounds should then be analyzed in further detail, including binding studies to the tetramer; stabilization of the tetramer; selective binding to hTTR and not to other major plasma thyroxine-binding proteins in an unspecific fashion (such as albumin and thyroxine-binding-globulin); and eventually, evaluation of the effect in decreasing the amount of conformational intermediates in the amyloidogenic process that may be responsible for the toxic response.

Experimental Section

Recombinant Proteins. Recombinant wild-type hTTR was produced using a pET expression system. The hTTR cDNA contained in the pHPA27 expression vector (based on a pIN-III−OmpA1 vector)[40] was subcloned into pET-38b(+) (Novagene) as a *Nde*I/*Avr*II fragment to yield the phTTRwt-I plasmid that expresses hTTR intracellularly under the control of the T7-promoter. The expressed protein only contains an additional methionine on the N terminus if compared to the mature natural human protein sequence. The mutant Y78F was prepared by total PCR site-directed mutagenesis using the phTTRwt-I plasmid as a template and the QuickChange kit (Strategene). Both wild type and Y78F hTTR proteins were expressed in *Escherichia coli* BL21-(DE3) cells harboring the corresponding plasmid. Expression cultures in LB medium containing 50 μg/mL kanamycin were grown at 37 °C to an optical density (at 600 nm) of 0.6, then induced by addition of IPTG (1 mM final concentration), grown at 37 °C for 20 h, and harvested by centrifugation (13700*g* for 15 min). After cell lysis by sonication, intracellular proteins were fractionated by ammonium sulfate precipitation. The hTTR-containing fraction precipitated between 55 and 85% ammonium sulfate. The precipitate was dissolved in 20 mM Tris, pH 7.2, 0.1 M NaCl and dialyzed against the same buffer. It was applied to a Q-Sepharose High Performance (Amersham Biosciences) anion exchange column and eluted with a linear gradient 0.1−0.5 M NaCl in 20 mM Tris pH 7.2. hTTR-enriched fractions were

dialyzed against 5 mM Tris pH 7.2, 2.5 mM NaCl; lyophilized; and redisolved in a small volume of buffer (10 mM Tris pH 7.2, 0.1 mM NaCl). The protein was further purified by gel filtration chromatography on a Superdex 75 prep grade column (Amersham Biosciences) and eluted with 10 mM Tris pH 7.2, 0.1 M NaCl. Purest fractions were combined and dialyzed against 20 mM phosphate buffer pH 7.6, 100 mM KCl and stored at 4 °C. The purity of protein preparations were >95% as judged by SDS−PAGE. Average production yields were 150−200 mg of purified protein per liter of culture. Protein concentration was determined spectrophotometrically at 280 nm using calculated ϵ values of 17780 M^{-1} cm^{-1} for wt and 16500 M^{-1} cm^{-1} for Y78F.

Inhibitors. Triiodophenol (**1**), biphenylcarboxylic acid (**7**), 4-phenoxybenzoic acid (**11**), and triclosan (**12**) were from Aldrich. L-Thyroxine (**2**), L-triiodothyronine (**3**), D,L-thyronine (**4**), diflunisal (**5**), flurbiprofen (**6**), flufenamic acid (**8**), mefenamic acid (**9**), and diclofenac (**10**) were from Sigma.

Stocks of compounds assayed as inhibitors were dissolved in DMSO (spectrophotometry grade from Sigma) at 1.5 mM concentration. Working solutions were prepared by diluting the stock solution 1:4 in H_2O/DMSO (2:1). In all cases, DMSO concentration was adjusted to 5% (v/v) in the final reaction assay mixture.

Kinetic Turbidity Assay. Protein (Y78F hTTR) stock: 4 mg/mL in 20 mM phosphate, 100 mM KCl, pH 7.6. Incubation buffer: 10 mM phosphate, 100 mM KCl, 1 mM EDTA, pH 7.6. Dilution buffer: 400 mM sodium acetate, 100 mM KCl, 1 mM EDTA, pH 4.2. Protocol for one inhibitor: 20 μL of Y78F-hTTR stock is dispensed into 7 wells of a 96-well microplate. Different volumes of working inhibitor solution are added to give final concentrations ranging from 0 to 40 μM, and the final DMSO content of each well is adjusted to 5% by adding the corresponding volume of a H_2O/DMSO (1:1) solution. Incubation buffer is then added up to a volume of 100 μL. The plate is incubated at 37 °C in a thermostated microplate reader with orbital shaking 15 s every minute for 30 min. A 100-μL portion of dilution buffer is dispensed to each well, and the mixture is incubated at 37 °C with shaking (15 s every min) in the microplate reader. Absorbance at 340 nm is monitored for 1.5 h at 1 min intervals. Data are collected and analyzed using Microsoft Excel software. All assays are done in duplicate.

The quality of the assay for high-throughput screening is evaluated by means of the statistical Z′-factor, defined as[41]

$$Z' = 1 - \frac{(3\mathrm{SD}_{c^+} + 3\mathrm{SD}_{c^-})}{|\mathrm{mean}_{c^+} - \mathrm{mean}_{c^-}|}$$

where c^+ is the highest inhibitor concentration (40 μM), and c^- is the control (0 μM inhibitor), "mean" is the mean value of the initial rate of amyloidogenesis, and SD is the standard deviation.

Turbidity Assay (72-h Incubation). Either Y78F or wt hTTR were incubated with the inhibitor under the same conditions described above. After acid induction (addition of dilution buffer), samples were incubated without shaking for 72 h at 37 °C and then homogenized by mixing to resuspend any fibrils present. Turbidity was measured at 340 nm and normalized to amyloidogenesis in the absence of inhibitor.

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