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Comparative *in vitro* and *ex vivo* activities of selected inhibitors of Transthyretin aggregation - relevance in drug design

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Running title: Inhibitors of TTR aggregation

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Abbreviations used:

FAP- Familial amyloidotic polyneuropathy

TTR- Transthyretin

TTR WT- Transthyretin wild-type

TTR V30M- Transthyretin with a valine substituted by a methionine at position 30

TTR L55P- Transthyretin with a leucine substituted by a proline at position 55

TTR Y78F- Transthyretin with a tyrosine substituted by a phenylalanine at position 78

IEF- Isoelectric focusing

TEM- Transmission electron microscopy

AA- amyloid A associated amyloidosis

Abstract

Destabilization of the tetrameric fold of transthyretin (TTR) is important for aggregation of the protein which culminates in amyloid fibril formation. Many TTR mutations interfere with tetramer stability increasing the amyloidogenic potential of the protein. The vast majority of proposed TTR fibrillogenesis inhibitors are based on *in vitro* assays with isolated protein, limiting their future use in clinical assays. We studied TTR fibrillogenesis inhibitors by a cellular system that produces TTR intermediates/aggregates in the media.

Plasmids carrying wild-type TTR, V30M or L55P cDNA were transfected into a rat Schwannoma cell line and TTR aggregates investigated in the media by a dot blot filter assay followed by immunodetection. Results showed that, in 24 hours, TTR L55P forms aggregates in the media, whereas, up to 72 hours, wild-type TTR and V30M do not. A series of 12 different compounds described in the literature as *in vitro* TTR fibrillogenesis inhibitors, were tested for their ability to avoid L55P aggregate formation; in this system, 2-((3, 5-Dichlorophenyl) amino) benzoic acid, benzoxazole, 4-(3, 5-difluorophenyl) benzoic acid and triiodophenol were the most effective inhibitors, as compared to the reference iododiflunisal, previously shown by *ex vivo* and *in vitro* procedures to stabilize TTR and inhibit fibrillogenesis. Among these drugs, 2-((3, 5-Dichlorophenyl) amino) benzoic acid and triiodophenol stabilized TTR from heterozygotic carriers of V30M in the same *ex vivo* conditions as those used previously for iododiflunisal.

The novel cellular based test herehein proposed for TTR fibrillogenesis inhibitor screens avoids not only lengthy and cumbersome large scale protein isolation steps but also artifacts associated with most current *in vitro* first-line screening methods, such as those associated with acidic conditions and the absence of serum proteins.

Keywords: Transthyretin; amyloid; iododiflunisal; aggregation; anti-amyloidogenic drugs.

Introduction

Familial amyloidotic polyneuropathy (FAP) is an autosomal dominant disorder characterized by the extracellular deposition of Transthyretin (TTR) amyloid fibrils. This systemic disease has a special involvement of the peripheral nerve. Over 80 TTR mutations have been related to amyloid deposition and disease [1], being the most frequent a substitution of a methionine for valine at position 30 - V30M. TTR is a homotetrameric protein produced in the liver and in the choroid plexus of the brain and functions as a transporter of T₄ and vitamin A.

The knowledge acquired in the last years on structural and biochemical features characteristic of amyloidogenic intermediates, has paved the way for the investigation of drugs capable of interfering early on the amyloidogenic pathway. For TTR related amyloidosis, the first step of a rational intervention is to obstruct tetramer dissociation and thus, prevent potential aggregation prone species from being generated. A second target is to inhibit the association between conformational intermediate species that lead to fibril elongation. Following these principles, several authors have reported the identification of compounds capable of inhibiting TTR fibril formation [2-6]. However, most of these fibril inhibition studies were performed under non-physiological acidic conditions believed to induce rapid conformational changes and no specific distinction was made regarding the molecular species affected by those drugs. Furthermore, these *in vitro* tests were performed in the test tube using prokaryotic rather than eukaryotic systems for the synthesis of human TTR. Cellular systems represent a more physiological *in vitro* approach to study inhibitors of amyloidogenesis which avoids isolation of the target protein. Here, we describe, for the first time, a TTR based cellular system for the screening of amyloid inhibitors. Using this system we also report comparative results of activity of a series of drugs referred to in the literature as inhibitors of TTR fibril formation.

Materials and methods

Vector constructions

Vectors p169ZT carrying human wt TTR (p169ZT-hTTRwt) and p169ZT carrying human TTR L55P (p169ZT-hTTR55) were generated as previously described [7]. We further produced a construct carrying the V30M TTR mutation by site directed mutagenesis using the Quickchange mutagenesis kit (Stratagene) following the manufacturer's instructions and the appropriate primers: 5'-AATGTGGCCATGCATGTGTTC-3' and 5'-GAACACATGCATGGCCACATT-3'.

Transfection and TTR expression in cell culture

Rat Schwanomas (RN22) (American Type Cell Collection) were stably co-transfected with each of the TTR cDNA constructs (WT, V30M or L55P) plus a plasmid with resistance to neomycin (pLNeo, kindly provided by Dr. Paulo Vieira, Pasteur Institute) following the CaPO₄-DNA precipitate method [8]. Briefly, cells were grown to approximately 30-50% confluence in 10 cm dishes (Costar). 25 µg of the appropriate purified cDNA TTR plasmid plus 5 µg of purified pLNeo plasmid were resuspended in 500 µl of 250 mM CaCl₂ and diluted with 500 µl 2X HEPES-buffered saline (2X HEBS). The CaPO₄-DNA precipitate was allowed to form by standing the mixture 20 min at room temperature and was subsequently added to the cells. After 5 hours incubation, cells were washed twice with phosphate-buffered saline (PBS) and fed with 10 mL of DMEM (Gibco BRL) supplemented with 10% FCS and 1% pen/strep (complete medium). 24 hours later, transfection medium was replaced by complete medium supplemented with G418 (1 mg/mL). Resistant colonies arose approximately after 7 days of selection and were isolated and separated over the course of the following weeks.

TTR secretion was tested by quantitative enzyme-linked immunosorbent assay (ELISA) [9]: 96 well plates (Maxisorp-Nunc) were coated overnight at 4°C with polyclonal rabbit anti-human TTR (1:500 dilution, DAKO) and blocked with 5% skim milk in PBS; conditioned media was applied to the wells for 1 h at room temperature; as secondary antibody peroxidase conjugated anti-human TTR (The Binding Site; 1:500 dilution in PBS-0.05% Tween 20 (PBS-T)) was used. Development was performed with ABTS (Sigma) / H₂O₂ (Merck). TTR concentration in cell media was calculated from a standard curve ranging from 5 to 340 ng/mL.

Since the vector used is under the control of the metallothionein promoter, experiments were performed in presence of 100 μM ZnSO_4 in the cell culture media in order to induce TTR synthesis.

Immunoprecipitation and Mass spectrometry analysis

Zinc treated cells expressing TTR were grown to approximately 100% confluence in complete medium supplemented with G418 which was then replaced by ^{14}C -Leucine containing medium, and collected 4 hours later. Cells were harvested and then lysed with 1 ml of lysis solution (30 mM Tris pH 7.5, 300 mM NaCl, 1% Triton x-100, 10 μM PMSF), centrifuged 30 minutes at 16,000 g, the pellet discarded and the supernatant collected.

Both medium and cell supernatant were incubated with rabbit anti-human TTR (1:20 dilution, DAKO) and kept overnight at 4°C under stirring. Next, 150 μl of protein A Sepharose beads (62.5 $\mu\text{g}/\mu\text{l}$ in 30 mM Tris pH 7.5) (CL-4B, Pharmacia) were added and allowed to mix for 1 hour at room temperature with agitation. The beads were washed 3 times with washing solution (30 mM Tris pH7.5, 300 mM NaCl, 1% Triton x-100) and the complex eluted with SDS loading buffer (62.5 mM Tris, pH 6.8; 2% SDS; 10% glycerol; 5% 2-mercaptoethanol). Finally, samples were fractionated on a 15% polyacrylamide gel under denaturing conditions (SDS-PAGE) and the gel was processed for autoradiography.

In some experiments, cells were grown in medium supplemented with 1% serum and G418, for 24 hours; the medium was collected and processed for immunoprecipitation as above. After separation in a SDS-PAGE gel, proteins were stained following a silver stain protocol. The TTR band was excised from the gel and digested with trypsin. MALDI mass spectroscopic analysis was performed on a PerSeptive Voyager mass spectrometer in the linear mode [10].

Dot blot filter assay for aggregate detection and drug assessment

Transfected and non-transfected cells were treated with ZnSO_4 in complete medium supplemented with G418 for 3 days, grown to about 80% confluence and then incubated with serum-free medium supplemented with ZnSO_4 for different periods of time. For the screening of drugs, cells were incubated with different concentrations of each compound for 48 hours, prior to the addition of ZnSO_4 , and then for further 3 days simultaneously with ZnSO_4 . At that point, cells reached about 80% confluence and were incubated with serum-free medium supplemented with ZnSO_4 and the desired drug, for 24 hours. TTR in the

medium was quantified by ELISA as described above, and 500 ng were applied onto a 0.2 μm pore cellulose acetate membrane filter (Schleicher & Schuell) using a manifold system (Gibco BRL) under vacuum to separate soluble proteins from aggregates. The filter was washed 3 times with PBST and processed for TTR immunodetection. Briefly, the membrane was blocked with 5% skim milk in PBS for 1 hour at room temperature, and then incubated with 1:500 dilution of rabbit anti-human TTR anti-serum (DAKO) for another hour; anti-rabbit HRP (1:500) was used as secondary antibody. Detection was performed with enhanced chemiluminescence (ECL, Amersham). Dot blot quantification was performed using the ImageQuant program. Experiments were repeated at least three times in duplicate and data shown is representative of the obtained results.

Chemical compounds studied for interaction with TTR

Triiodophenol (TIP), Flufenamic acid (Fluf), Diflunisal (Dif), Diclofenac (Diclof), Resveratrol (Resv), 2-((3, 5-Dichlorophenyl) amino) benzoic acid (DCPA), Dinitrophenol (DNP), Diethylstilbestrol (DES) and Genistein were from Sigma. The diflunisal derivative iododiflunisal (*Mr* 376.1) was synthesized as part of a screening program for TTR polymerization inhibitors carried out at IIQAB-CSIC, Barcelona, and at the University of Oviedo. After synthesis, the compounds were purified by HPLC (99.9% purity) and characterized fully by NMR, MS and elemental analysis. The synthetic procedure will be reported elsewhere; details are available from G. Valencia on request. Benzoxazole (Benzo), Diclonisal (Diclon) and 4-(3, 5-difluorophenyl) benzoic acid (DFPB) were also synthesized at IIQAB-CSIC and were previously described by other authors (see table 1). The compounds were weighed and dissolved at approximately 10 mg/ml in DMSO and used at a final concentration of 1 μM .

Recombinant protein

Recombinant TTR Y78F were produced in a bacterial expression system using *Escherichia coli* BL21 [11] and purified as previously described [12]. Protein concentration was determined using the Lowry method [13].

Western blotting

In some experiments, after application of the medium on the membrane filter, each dot was cut and boiled in SDS loading buffer. The removed material was run on a 15% SDS

denaturing acrylamide gel and then transferred to a nitrocellulose membrane (Amersham). The membrane was blocked with 5% skim milk in PBS for 1 hour at room temperature and then incubated with 1:500 dilution of rabbit anti-human TTR anti-serum (DAKO) for another hour at room temperature; as secondary antibody (1:5000 dilution) peroxidase-conjugated anti-rabbit IgG (Amersham) was used. Development was performed with 3', 3'-diaminobenzidine (DAB) (Sigma).

Transmission Electron Microscopy

Inhibition of fibrillogenesis by selected drugs was evaluated using TTR Y78F which aggregates under physiological conditions (PBS, pH 7.4) after 2 days at 37°C. For negative staining, 5 µl of these preparations were adsorbed to glow-discharged carbon collodion film on 200-square mesh copper grids for 1 min. The grids were washed with deionized water and stained with 1% uranyl acetate (Fluka). Specimens were visualized with a Zeiss electron microscope operating at 60 kV and grids were exhaustively examined.

Assessment of tetrameric TTR stability by isoelectric focusing (IEF) in semi-denaturing conditions

The conditions used for the IEF of plasma TTR were described previously by Altland *et al* 1981 and Almeida *et al* 2004 [14, 15]. Briefly, 30 µl of plasma from a TTR V30M heterozygotic carrier were incubated at 37°C for 1 h, with 5 µL of a 10 mM solution of the compound to be tested. The preparations were then subjected to native polyacrylamide gel electrophoresis to isolate TTR. The gel band containing TTR was excised and subjected to IEF in a gel with 4 M urea and 5% ampholytes pH 4-6.5, at 1200 V for 6 hours. Proteins in the gel were stained with Coomassie blue. These semi-denaturing conditions allow the visualization of bands corresponding to the TTR monomer and tetramer and also to an oxidized form of the monomer. Gels were scanned (hp Scanjet 4470c; Hewlett Packard) and subjected to densitometry using the ImageQuant program. Experiments were repeated at least three times and data shown is representative of the obtained results.

Statistical analyses

All data examined were expressed as mean \pm S.E. Comparison between groups was made using the Student's t test. A p value of less than 0.05 was considered statistically significant.

Results

TTR Expression

To set up a cellular system for the production of TTR aggregates, cells were transfected with different human TTR constructs (p169ZT-hTTRWT, p169ZT-hTTRV30M, and p169ZT-hTTRL55P). Demonstration of TTR secretion and quantification of TTR levels in conditioned media was performed by quantitative ELISA after 24 hours in serum-free conditions. Cells were first treated with 100 μ M ZnSO₄ for 3 days to produce TTR. The media was then replaced by serum-free media for 24 hours and TTR in the media was quantified by ELISA as detailed in materials and methods. The interval range of TTR expression differed; for TTR WT varied from 100-300, was 350-1000 for TTR V30M and 300-900 for TTR L55P (expressed in ng/ml)/10⁶ cells/24 hours).

Protein expression and degradation was also assessed by immunoprecipitation on both media and cell lysates. Figure 1 A shows TTR from media and cell lysates (pellet), separated under denaturing conditions (SDS-PAGE) after immunoprecipitation with anti-human TTR antibody and reveals that most of the produced protein is secreted into the medium, as soluble non-degraded material. To further confirm the identity of each TTR variant expressed and secreted by the cells, we performed mass spectrometry analysis of the protein in the conditioned media of transfected cells. The results are displayed in figure 1 B which highlights the particular peaks corresponding to each mutation, thus confirming the expected TTR variant in each transfection. As shown in the left panel of figure 1-B2, a peptide of 2181 mass, representing residues 16-35 of TTR but with methionine replacing valine, was detected in the preparation deriving from TTR V30M transfected cells, not present in the preparation of the right panel (deriving from TTR L55P transfected cells), which has the corresponding non-mutated, normal 2149 mass peptide. Conversely, in the preparation deriving from TTR L55P transfected cells (right panel), a mutated peptide of 3126 mass, representing residues 49-76 of TTR but with proline replacing leucine, was detected, which was absent from the V30M preparation; instead, a normal, non-mutated peptide of 3142 is observed.

Production of amyloidogenic TTR species

We next investigated the ability of the different constructs to induce the production of amyloidogenic species. Cells were grown in serum free medium for 24, 48 or 72 hours and

then the media was analyzed for the presence of aggregated material retained by a 0.2 μm filter in a dot blot assay. While TTR aggregates were not detected in media from cells transfected with constructs carrying the TTRWT or TTR V30M cDNA even after 72 hours of cell culture, TTR L55P protein material was partially retained by the membrane filter as early as 24 hours of culture (figure 2A), suggesting the formation of oligomers or aggregates.

The dotted area was analyzed by SDS-PAGE gel; the proteins were then transferred to a nitrocellulose membrane followed by immunodetection against TTR (figure 2B), revealing the intact TTR monomer; under these conditions we did not have evidence for aggregate formation since SDS disrupted those aggregates. TTR, even under denaturing conditions does not completely dissociate into monomers, originating a second band corresponding to the dimer with approximately 32 kDa of apparent mass.

Comparative effects of Diflunisal and Iododiflunisal on TTR L55P aggregation

We next evaluated this system in the screening of drugs capable of interfering with TTR fibrillogenesis. Very recently, it has been reported that an iodinated diflunisal (Dif) derivative, iododiflunisal (I-Dif), was very potent as a TTR fibrillogenesis inhibitor, and as a stabilizer of the TTR tetrameric fold when compared with diflunisal [15] as assessed by *in vitro* and *ex vivo* approaches, respectively. In this study, we started by studying in our cellular system the effect of different I-Dif concentrations (0.01- 10 μM) on TTR aggregation. As depicted in figure 3A, I-Dif inhibited L55P aggregate formation at concentrations between 0.1-10 μM .

We next compared the inhibitory activity on TTR L55P fibrillogenesis of diflunisal and iododiflunisal at three different concentrations : 0.1, 1 and 10 μM . Results are displayed in figure 3B and showed that iododiflunisal was able to inhibit aggregate formation at 0.1 μM whereas for diflunisal, the same effect was only observed at 10 μM concentration. Thus, I-Dif presented a 100 fold increase in inhibitory activity when compared to diflunisal. These observations confirmed the above referred data on the much higher capacity of iododiflunisal over diflunisal to prevent TTR fibrillogenesis

It is noteworthy that some variation occurred in these assays. However, the relative intra-assay activities between different drugs and between drugs and control maintained significance, despite inter-assay fluctuations. For example, 0.1 μM of I-Dif inhibited L55P fibril formation by 25% as displayed in figure 3A, but by 90% as shown in figure 3B. Yet,

statistical significance is observed with the referred concentration of I-Dif in both experiments, when compared with the respective control.

Comparative aggregate inhibitory activities of selected compounds

Following the results described above and to further assess the usefulness of the cellular model for screening purposes, we continued our studies on this cellular system with different drugs. We evaluated a series of compounds selected based on their ability to inhibit amyloid fibril formation according to literature data (for references see Table 1), which in most cases used isolated recombinant TTR upon acidification to pH 4.4 and monitorization of turbidity at 400nm. A further criteria for lead compound selection was individual products representative of different therapeutic categories such as, for instance, non-steroid anti-inflammatory drugs (NSAIDs) (diflunisal, diclofenac, flufenamic acid) or compounds sharing molecular structural similarities with those (DCPA, DFPB, diclonisal). Other small organic compounds also referred to as good amyloid inhibitors such as plant compounds (genistein and resveratrol), DES, benzoxazole and triiodophenol were also selected for testing. Thus, compounds listed in table 1 were tested at 1 μ M concentration and results are shown in figure 4. Different levels of inhibition were evident when compared to the iododiflunisal control (Figure 4, histogram). Among the compounds tested, triiodophenol (TIP) showed an inhibitory capacity similar to that of I-Dif. (~57% inhibition). Three of the compounds analyzed revealed to be more efficient than iododiflunisal: 2-((3, 5-Dichlorophenyl) amino) benzoic acid (DCPA), 4-(3, 5-difluorophenyl) benzoic acid (DFPB) and Benzoxazole (Benzo), inhibiting aggregate formation in more than 80%. For the other assessed compounds including DES, resveratrol, diflunisal, DNP, genistein, diclofenac, diclonisal and flufenamic acid, no inhibitory effect was evident when compared to the control. Conditioned media incubated with compounds just prior application onto the membrane showed a signal equivalent to conditioned media alone (not shown) ruling out a possible effect of the compounds on the immunoassay.

Transmission electron microscopy

To compare the results obtained in the cellular model, we analyzed by TEM the effect of six of the drugs under study: Genistein, TIP, DES, DCPA, DFPB and benzoxazole. This study was performed with a different TTR variant: TTR Y78F which is very prone to aggregation as compared with the L55P mutant. The soluble Y78F variant aggregates in about 48 hours

at pH 7.4 as documented by Almeida *et al.*, 2004 [15] who observed that upon incubation with I-Dif no aggregates were visible and only round particles resembling the native TTR were present; in contrast, incubation with diflunisal or diclofenac did not prevent aggregate formation [15].

Figure 5 is representative of the results obtained in the present study and shows a complete inhibition of aggregate and fibril formation with DFPB and benzoxazole (lower panels); DCPA was only able to partially inhibit aggregate and fibril formation (upper right panel). TIP inhibited aggregate and fibril formation whereas the addition of genistein and DES resulted in no visible effect by TEM (not shown).

In vitro and *ex vivo* activities of selected compounds

The same panel of compounds was further tested *ex-vivo* for their effect on TTR stability by IEF analysis under semi-denaturing conditions, namely in the presence of 4M urea. To perform the assays we used basically the same conditions as in previous studies [15]. Plasma from a heterozygotic carrier of TTR V30M was incubated with each of the compounds referred to in the legend of figure 6 and, following IEF, the bands corresponding to the TTR tetramer and monomer were visualized, and the tetramer/monomer ratio measured. Under the conditions used, TTR presents a characteristic pattern of two main bands, representing monomers (normal and oxidized form) and several bands of lower pI, representing different forms of tetramers.

As compared to iododiflunisal, a similar stabilization effect was obtained with DCPA and with TIP, not evident for DFPB and benzoxazole under the conditions used. All the other compounds without inhibitory TTR fibrillogenesis activity in the cellular assay failed to stabilize V30M *ex vivo*.

Discussion

The process that converts a soluble protein into amyloid is complex and not fully understood. TTR amyloid deposits have been suggested as the causative agent of FAP. However, the finding that asymptomatic individuals carrying the TTR V30M mutation show a positive reaction for inflammatory markers and present non fibrillar TTR deposits in the nerve raised the possibility that species besides mature fibrils could be involved in the disease [16]. In fact, *in vitro* studies showed that TTR L55P aggregates are toxic to a Schwannoma cell line, inducing caspase-3 activation, while long fibrils do not produce significant caspase-3 activation. These observations suggest that a major target on FAP therapy is to inhibit the formation of intermediate species and that comprehension of how native TTR converts into toxic species is of major importance.

Initially, most *in vitro* studies on TTR amyloidogenesis were performed by acidifying the protein [2, 3, 5, 6], using an environment certainly different from *in vivo* conditions. More recently, TTR variants such as Y78F, which are able to produce aggregates and fibrils at physiological pH have benefited research on anti-amyloidogenic drugs and disease mechanisms by avoiding pH related artifacts. In both cases (with and without acidification), most studies are still conducted with purified recombinant proteins [2-6] and thus, such approaches lack factors that occur *in vivo*. Thus, a cellular system producing the amyloidogenic protein would represent an improved approach to study effects of modulating factors and drugs on fibrillogenesis. In fact, different cellular models have been generated in order to study amyloidogenesis. A cell culture system for generation of amyloid A associated amyloidosis (AA) was established using murine peritoneal macrophages after repeated injections of Hammersten casein [17]. Staining with congo red, thioflavin T, and anti-AA revealed amyloid like structures associated with macrophage clusters. Such systems can be useful in studies on the influence of chaperon molecules and other factors on the formation and degradation of amyloid fibrils. Similarly, transfected cells with presenilin 2 (PS2) gene were used to examine the processing and degradation pathways of PS2 [18]. Furthermore, different cellular models have been created in order to study many different aspects of Alzheimer's disease [19, 20, 21]. Here we report on the establishment of a cellular system of transfected RN22 (rat Schwannoma) with WT- TTR, V30M and L55P mutants. The latter was shown to form species that were retained by a filter, in contrast to the WT and V30M proteins.

Although the most prevalent mutation responsible for the pathology, V30M does not show a very high amyloidogenic potential *in vitro*, since amyloid fibrils are only obtained after acidification. Furthermore, crystal X-ray studies [22] revealed a structure similar to the WT counterpart and the differences found could not explain a significant instability of the protein. Thus, *in vivo*, this mutation plays a very important role in the pathology of FAP but *in vitro*, the protein does not lend itself to study by the assays described here.

One of the advantages of this *in vitro* cellular system is that it measures aggregation under good physiological conditions because: 1) uses an eukaryotic system for the production of TTR; 2) the secreted protein is not isolated avoiding modifications, such as oxidations that might influence aggregation; 3) aggregation occurs in the cell media, under physiological pH and in the presence of serum proteins. None of the previous “in vitro” available methodologies to screen for TTR inhibitors take into consideration these aspects, as TTR aggregation is measured on isolated recombinant TTR isolated from bacteria and in the absence of serum proteins.

When the 12 compounds classified in the literature as good TTR fibrillogenesis inhibitors were tested on the cellular system, having iododiflunisal as the reference compound, only DCPA, benzoxazole, DFPB, and TIP, were able to prevent L55P aggregate formation in the conditioned media, which reflects the above mentioned limiting conditions, such as the aggressive environment imposed by acidification, the exclusive use in most instances of WT rather than mutant TTR and the absence of serum proteins [23, 24, 4]. Regarding the ultrastructural analysis, DCPA did not show an inhibitory effect as high as DFPB and benzoxazole, indicating that the Y78F mutant may not be as sensitive to this drug as TTR L55P and V30M are.

Stabilization of tetrameric V30M TTR by iododiflunisal was previously shown in *ex vivo* tests using plasma from hetero and homozygous carriers. The sample was directly analyzed without previous treatment with cross-linkers and did not suffer further processing such as immunoprecipitation. 4 M urea allowed discrimination of susceptibility to tetramer dissociation into monomeric species, directly visualized on the IEF gel without the use of TTR antibodies for quantification of the tetramer/monomer ratio. We also did not observe a stabilizing effect of benzoxazole and DFPB by *ex vivo* testing under the conditions used; this fact is suggestive that these 2 drugs either inhibit TTR fibrillogenesis by mechanisms other than tetramer stabilization, such as inhibition of polymerization of soluble

intermediary species generated after tetramer dissociation; alternatively they might have a more effective anti-fibrillogenic effect in L55P and in Y78F.

At any rate, testing of the V30M variant is most pertinent in studies searching for TTR anti-fibrillogenic drugs as besides being the most common mutant found in FAP, further application of the drug under study encompasses *in vivo* testing in animal models which are available for this mutant [7].

The main conclusions of this screening work indicate that: (i) DCPA and TIP are the best stabilizers of V30M tetramers in plasma from carriers of this mutant, and clearly inhibit aggregation in the cellular system; also, DFPB displayed anti-aggregation properties for L55P and Y78F but did not stabilize V30M tetramers, possibly by acting through mechanisms other than tetrameric stabilization. Therefore, iododiflunisal, DCPA and TIP are promising for the treatment of V30M associated FAP but need to undergo further drug development stages to overcome their toxicity. (ii) Compounds with virtually null stabilization of tetrameric structure (as assessed *ex vivo*), such as flufenamic acid, diclofenac, resveratrol, ginestein, diflunisal, and DES clearly had no effect on TTR aggregation in cell culture and thus further testing them may not be worthwhile.

The cellular model here described represents a step forward in the study of events occurring very early in TTR amyloidogenesis and of factors/drugs affecting such process; moreover, our cellular model supplies a unique tool to test drugs independently of the mechanism behind their action, i.e., tetramer stabilization, inhibition of aggregation and others. We believe this system will have important implications in future drug screening and design while constituting an important tool for the selection of compounds that merit preclinical testing.

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Table 1- Compounds tested for the inhibition of aggregate formation by TTR L55P expressing cells.

Compound	reference
Triiodophenol (TIP)	[25]
Flufenamic acid (Fluf)	[4]
Diflunisal (Dif)	[5]
Diclofenac (Diclof)	[2]
Resveratrol (Resv)	[2]
2-((3,5-Dichlorophenyl)amino)benzoic acid (DCPA)	[26]
Dinitrophenol (DNP)	[27]
Benzoxazole (Benzo)	[6]
Iododiflunisal (I-Dif)	[15]
Diethylstilbestrol (DES)	[28]
Diclonisal (Diclon)	[23]
4-(3,5-difluorophenyl)benzoic acid (DFPB)	[23]
Genistein	[29]

Legends to figures

Figure 1–

A: Immunoprecipitation analysis of RN22 cells media and pellet from: non-transfected cells (**NT**); transfected with plasmids for TTR WT (**WT**), V30M (**V30M**) and L55P (**L55P**).

B: Mass spectrometry analysis of immunoprecipitated TTR from conditioned media of TTR V30M and L55P transfected cells. After immunoprecipitation, proteins were separated in a SDS-PAGE gel which was silver stained (**B1**): molecular weights (MW); standard TTR (TTR std); non-transfected cells (NT); cells transfected with plasmids for TTR V30M (V30M) and L55P (L55P). The bands corresponding to the TTR protein (arrow) were cut, digested by trypsin and analyzed by mass spectrometry (**B2**), revealing specific peptides for TTR V30M (left panel) and for L55P (right panel).

Figure 2– Aggregation in conditioned media of RN22 cells

A: Dot blot filter assays of media from: non-transfected RN22 cells or transfected with plasmid for WT and TTR mutants (**WT, V30M and L55P**). TTR was quantified in the medium by ELISA and 500 ng were applied in the filter, followed by TTR immunodetection.

B: Western blotting of the material retained on the membrane filter, revealing the presence of TTR (**lane1**); TTR standard (**lane 2**). In both lanes the TTR monomer and dimer are observable; Standard molecular weights (MW).

Figure 3–

A- Effect of different concentrations of I-Dif in L55P aggregate formation.

Conditioned media containing 500 ng of total TTR of cells expressing TTR L55P treated with different concentrations of I-Dif (0.01, 0.02, 0.1, 0.5, 1, 5 and 10 μ M) or non-treated (0 μ M I-Dif) was applied onto an acetate cellulose membrane filter, followed by immunodetection of TTR aggregates with a specific anti-human TTR antibody, and quantified using ImageQuant program. * $p < 0.02$.

B- Dot blot showing the effect of three different concentrations of I-Dif and Dif in L55P aggregate formation. The experiment was performed as in **A** and reveals that I-Dif has an ability of preventing L55P aggregate formation approximately 100X higher than Dif.

Histogram: densitometry of dot blot showing % of inhibition of aggregate formation by each compound, compared to the control (0 % inhibition, no drug added). * $p < 0.03$.

Figure 4- Screening for TTR anti-amyloidogenic drugs.

Filter assays of the conditioned media from TTR L55P cells incubated with different drugs at 1 μM . Conditioned media of cells incubated with I-Dif, TIP, showed significant less aggregates than control. DCPA, DFPB and benzoxazole showed the highest inhibitory effect whereas all the other 8 compounds tested produced no effect. **Histogram:** densitometry of dot blot showing % of inhibition of aggregate formation by each compound, compared to the control (0 % inhibition, no drug added). * $p < 0.006$.

Figure 5- Morphological analysis by TEM

TTR Y78F incubated 48 hours at 37°C evolved from round particles to fibrils (control, top left panel). When incubated with DFPB or benzoxazole fibril formation was completely inhibited (bottom panels) whereas incubation with DCPA produced only a partial inhibition (top right panel)

Figure 6- Stabilization of tetrameric V30M TTR

Plasma from a heterozygotic carrier of TTR V30M was incubated with different drugs under study. TTR was then separated by PAGE and the isolated protein was analyzed by IEF. The different molecular species visualized in the IEF gel after Coomassie Blue staining are indicated. In the presence of I-Dif, DCPA and TIP stronger bands corresponding to TTR tetramers are visualized and the monomer bands strongly reduced or absent. **Histogram:** densitometry of IEF of isolated TTR V30M incubated with the different compounds and expressed in tetramer/monomer ratio. * $p < 0.006$.

Figure 1

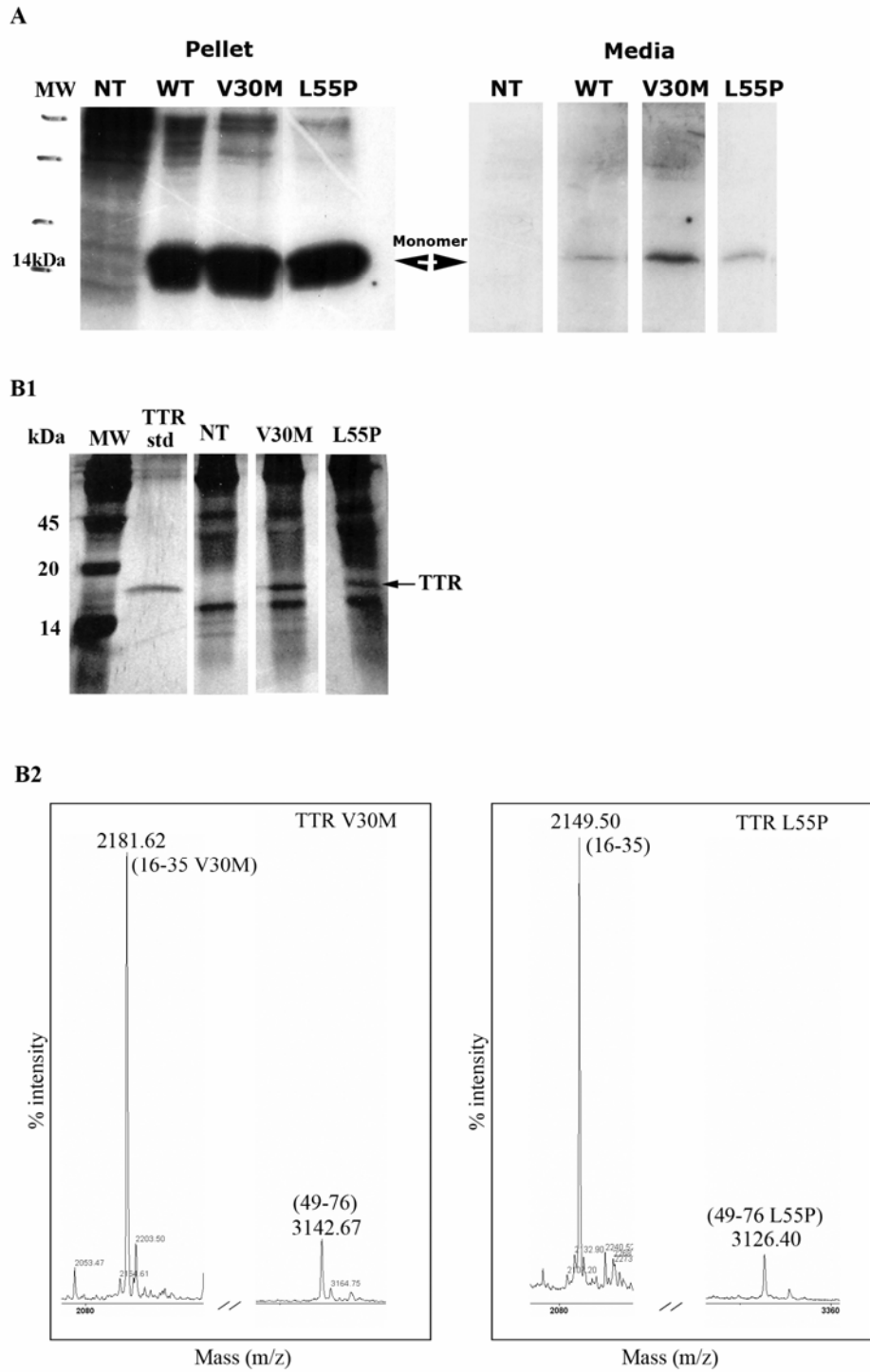


Figure 2

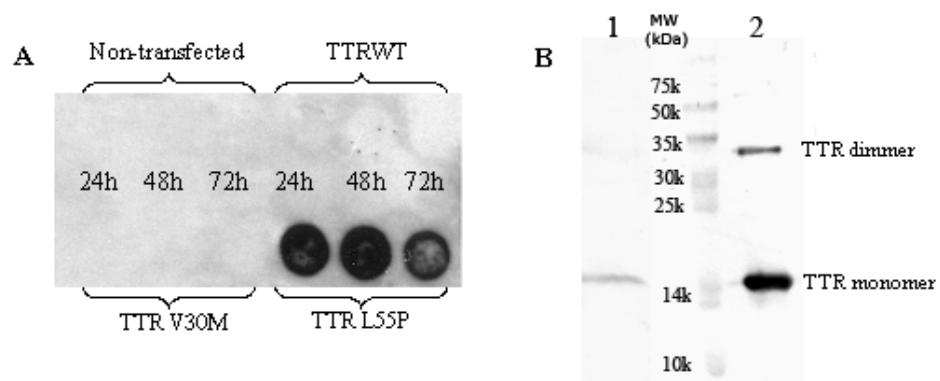


Figure 3

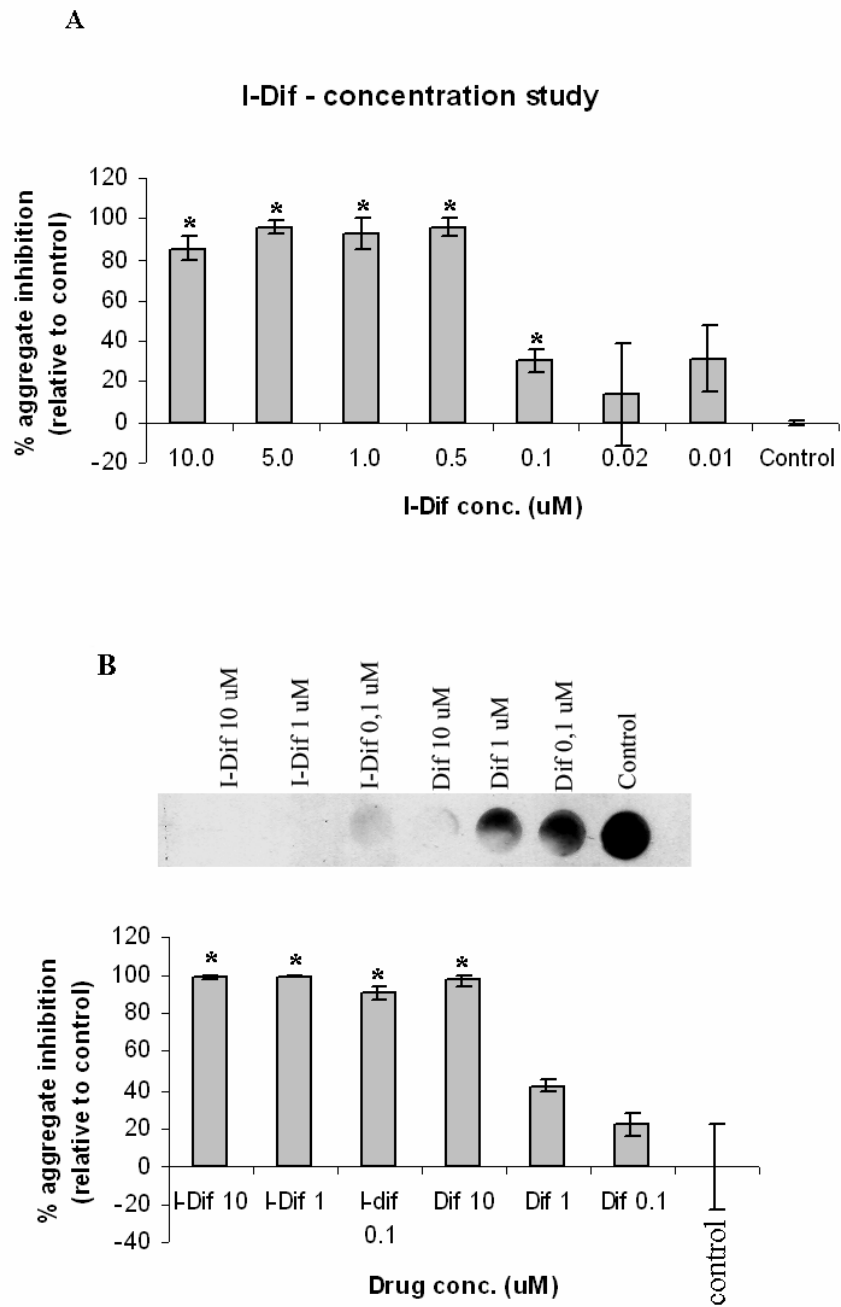


Figure 4

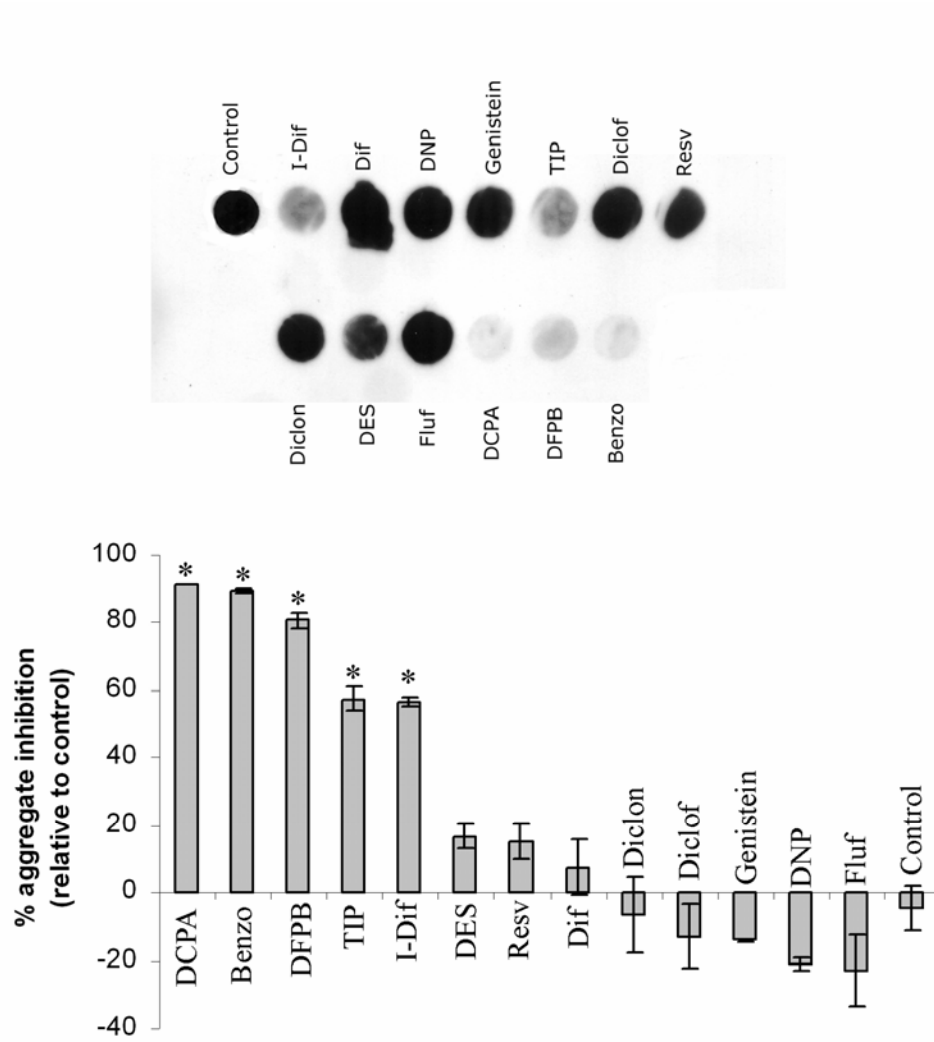


Figure 5

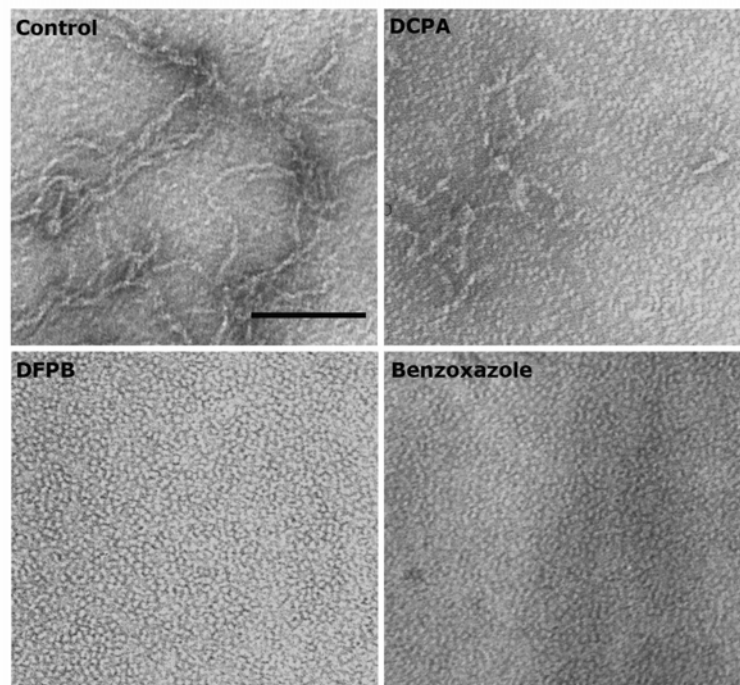


Figure 6

