Neurobiology of Disease 120 (2018) 118–125

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

Assessment of the effects of transthyretin peptide inhibitors in *Drosophila* models of neuropathic ATTR

Lorena Saelices[a,*], Malgorzata Pokrzywa[b], Katarzyna Pawelek[b], David S. Eisenberg[a]

[a] Howard Hughes Medical Institute, UCLA-DOE Institute for Genomics and Proteomics, Department of Biological Chemistry, Molecular Biology Institute, UCLA, 611 Charles Young Dr. East, Los Angeles, 90095, CA, USA
[b] Airoptic Sp. z o.o., Ul. Rubiez 46 H, 61-612 Poznań, Poland

ARTICLE INFO

Keywords:
Neuropathology
Amyloidosis
Transthyretin
Drosophila
Peptide inhibitors

ABSTRACT

Transthyretin amyloidosis (ATTR) is a fatal disease caused by the systemic aggregation and deposition of transthyretin (TTR), a blood transporter that is mainly produced in the liver. TTR deposits are made of elongated amyloid fibrils that interfere with normal tissue function leading to organ failure. The current standard care for hereditary neuropathic ATTR is liver transplantation or stabilization of the native form of TTR by tafamidis. In our previous work, we explored an additional strategy to halt protein aggregation by capping pre-existing TTR fibrils with structure-based designed peptide inhibitors. Our best peptide inhibitor TabFH2 has shown to be effective at inhibiting not only TTR aggregation but also amyloid seeding driven by fibrils extracted from ATTR patients. Here we evaluate the effects of peptide inhibitors in two *Drosophila* models of neuropathic ATTR and compared their efficacy with diflunisal, a protein stabilizer currently used off-label for the treatment of ATTR. Our peptide inhibitor TabFH2 was found the most effective treatment, which resulted in motor improvement and the reduction of TTR deposition. Our *in vivo* study shows that inhibiting TTR deposition by peptide inhibitors may represent a therapeutic strategy for halting the progression of ATTR.

1. Introduction

Transthyretin amyloidosis (ATTR) is caused by the deposition of amyloid fibrils of transthyretin (TTR), a transporter of thyroxine and retinol in the blood and cerebrospinal fluid (Costa et al., 1978; Sletten et al., 1980; Westermark et al., 1990). Both mutant and/or wild-type TTR are found in these deposits, which are made of elongated, resilient amyloid fibrils. Although TTR deposits in virtually every organ, ATTR patients commonly exhibit progressive cardiomyopathy and/or polyneuropathy that lead to disability and death (Galant et al., 2017). In hereditary ATTR, deposition and disease progression is accelerated by TTR mutations thereby resulting in earlier onsets (Hurshman Babbes et al., 2008). The most common form of hereditary amyloidosis, ATTR-V30M, is associated with familial amyloid polyneuropathy (FAP) that typically manifests in the third or forth decade of life, with or without cardiac involvement (Gertz et al., 2015).

The current standard of care for hereditary ATTR cases is liver transplantation. Because TTR is mainly synthesized in the liver, this procedure replaces the circulating mutated TTR with the more stable wild-type TTR (Benson, 2012). Liver transplantation has prolonged the life of many ATTR patients, with the most favorable prognosis for FAP-V30M cases (Benson, 2013). However, this drastic procedure is not recommended for cases of advanced age, longstanding disease, or with cardiac involvement, factors that have been associated with higher post-surgical mortality (Carvalho et al., 2015). In patients with a significant cardiac involvement, amyloid derived from wild-type TTR produced by the transplanted liver may continue to add to existing cardiac deposits, leading to accelerated deposition and heart failure (Liepnieks and Benson, 2007). Liver transplantation remains the only specific therapy for a limited number of ATTR patients in many countries.

More recent efforts are focused on the stabilization of the native form of TTR by small compounds as a method to inhibit protein aggregation (Miroy et al., 1996). The functional native form of TTR is a tetramer made of two homo-dimers that form a hydrophobic tunnel in which thyroxine binds as it is transported (Nilsson et al., 1975). The aggregation of transthyretin to form amyloid fibrils starts by the dissociation of the tetramer into monomers that unfold to expose amyloidogenic segments (Foss et al., 2005). Kelly and coworkers found that compounds bound to the thyroxine site stabilize the tetramer, thereby inhibiting protein aggregation (Miroy et al., 1996; Baures et al., 1998). Out of those studies, a stabilizing compound known as Tafamidis was

* Corresponding author.
E-mail address: lorenasaelices@mbi.ucla.edu (L. Saelices).

https://doi.org/10.1016/j.nbd.2018.09.007
Received 9 July 2018; Received in revised form 20 August 2018; Accepted 6 September 2018
Available online 10 September 2018
0969-9961/

discovered and is now approved in Europe, Japan, Mexico, Argentina and other countries for FAP patients (Bulawa et al., 2012). Some other compounds have shown promising results in the stabilization of TTR *in vitro* and were tested in clinical trials. This is the case for diflunisal, a non-steroidal anti-inflammatory drug that is currently being used off-label for the treatment of cardiac amyloidosis (Castano et al., 2012).

In two recent studies, we have developed and optimized peptide inhibitors that inhibit both transthyretin aggregation *in vitro* as well as amyloid seeding catalyzed by ATTR *ex-vivo* amyloid fibrils (Saelices et al., 2015; Saelices et al., 2018a; Saelices et al., 2018b). We first discovered that there are two amyloidogenic segments of TTR that drive protein aggregation: β-strands F and H (Saelices et al., 2015). In that study, we determined the atomic structures of these two segments in their amyloid form, which allowed us to design specific peptide inhibitors of F and H β-strands self-association. Later we further optimized these inhibitors to inhibit amyloid seeding driven by ATTR *ex-vivo* amyloid fibrils (Saelices et al., 2018a).

Drosophila melanogaster has recently emerged as a convenient model for human transthyretin deposition disorder (Pokrzywa et al., 2007; Berg et al., 2009; Pokrzywa et al., 2010; Andersson et al., 2013; Iakovleva et al., 2015). The overexpression of several familial and engineered amyloidogenic variants of human TTR in neurons results in TTR deposition in the brain, fat body and glia, atrophy of wings, locomotor impairment and shortened lifespan. In this manuscript, we evaluate the efficacy of our peptide inhibitors and the stabilizing compound diflunisal in two *Drosophila* models of ATTR. We found that the treatment of diseased flies with our optimized peptide inhibitor results in motor improvement and a reduction of TTR deposition.

2. Materials and methods

2.1. Antibodies

Antibodies used were rabbit anti-human transthyretin polyclonal antibody (DAKO, Agilent Technologies; 1:2000), anti-human transthyretin monoclonal antibody mAb 15, obtained from Prof Erik Lundgren, Umea University, Sweden (Goldsteins et al., 1999; 0.2 μg/ml) and horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (DAKO, Agilent Technologies; 1:5000).

2.2. Drosophila stocks

The formation of intracellular amyloid aggregates in thoracic adipose tissue and brain glia in ATTR models of the fruit fly results in an abnormal wing posture and motor defects (Pokrzywa et al., 2007; Pokrzywa et al., 2010; Iakovleva et al., 2015). Several ATTR models are available to be tested in flies; here, the focus was on flies carrying the TTR familial mutant V30M (Iakovleva et al., 2015) (abbreviated V30M), and the amyloidogenic mutant V14N/V16E (Pokrzywa et al., 2007) (abbreviated TTR-A). Transgenic lines were generated in the w1118 strain. Two transgenes for the human TTR gene UAS-TTRV30M and UAS-TTRV14N/V16E (abbreviated UAS-TTR-A) were expressed under control of pan-neuronal GAL4 driver (nSyb-GAL4) to drive expression in all types of post-mitotic neurons. Genotypes: w; +; UAS-TTRV30M /nSyb-GAL4 (Iakovleva et al., 2015), or w; +; UAS-TTRV14N/V16E/nSyb-GAL4 (Pokrzywa et al., 2007); wild-type Oregon R strain was obtained from Drosophila Bloomington Stock Center (BDSC #6361, Indiana University) and used as healthy controls in crosses with the nSyb-GAL4 driver line (w; +; +/nSyb-GAL4).

2.3. Fly rearing and drug feeding

Flies were kept at 60% humidity at 20 °C under a 12:12 h light:dark cycle (8 a.m. to 8 p.m. daily) until fly eclosion and at 29 °C post-eclosion. This temperature shift was adopted to lower the expression of nSyb-GAL4 driver during development before adding the tested compounds. The crossings were reared in bottles containing standard *Drosophila* food (corn meal, corn syrup solids, yeast, water, and agar). Newly eclosed female flies (10 flies per vial) were transferred into 5 ml ventilated vials (75 × 13 mm, polystyrene tubes with archiving caps with filter, Sarstedt, Nümbrecht, Germany), containing low-melt fly food and tested compounds according to the formula developed by Markstein et al. for mixing drugs in low volumes (Markstein et al., 2014). Briefly, the food was prepared with distilled water containing 2% (w/v) autoclaved yeast, 7% (v/v) corn syrup liquids, and 1.5% (w/v) agarose (composed of 1 part standard agarose to 11 parts low-melt agarose). The food was mixed as a liquid with drugs at 37 °C. The resulting food and compound mixtures solidified at 30 °C into soft fly edible gels. Peptides were synthesized at > 97% purity from GL Biochem (Shanghai) Ltd. (Shanghai, China). Purity and molecular weight were confirmed by MALDI-TOF and reversed phase HPLC. The sequences of our peptides are: TabFH1 is an equimolar cocktail of RRRRPFHEHA(*N*-methyl)EVVFTA and RRRRPYSYSTT(*N*-methyl)AVVTN; TabFH2 is an equimolar cocktail of RRRRHVAHPFV(*N*-methyl)EFTE and RRRRSYVTNPTSY(*N*-methyl)AVT, as previously described (Saelices et al., 2018a). All peptides were dissolved in 0.22 μm filtered water, first to 5 mM stock solution. These working solutions were further diluted in fly food prior to use to final concentration: 100 μM or 300 μM. Flies were fed compounds present in fly food after adult flies eclosure (developmental stages excluded) until death. The food intake in TTR-A flies during active feeding periods was 33,65 nl/h, measured after undisturbed food intake allowed for 2 h. Fresh food containing the compounds was changed every second or third day and the number of dead flies was recorded. Peptide stability in fly food was confirmed by HPLC.

2.4. Analysis of locomotor abilities of treated flies

All female flies were pooled and randomized. Ten flies of each genotype or drug treatment were placed in plastic vials in replicates, gently tapped down to the bottom and allowed to climb. Several motor skills were analyzed in a climbing assay at various time points along with survival analysis. (i) Mean distance or mean length of trajectories corresponding to one fly in a vial in mm. (ii) Mean velocity of 10 flies moving in one vial in mm/s. (iii) Motion represents the percentage of flies being in movement in one vial. A fly is considered in movement when its velocity is equal to or higher than 2.5 mm/s. (iv) Maximum velocity of flies moving in one vial in mm/s. (v) Mean length of trajectory among all detected flies in one vial in mm. (vi) Total distance or the sum of all trajectory lengths in one vial in mm. Recordings of 10-s fly movements were acquired in duplicates and analyzed with FlyTracker hardware and software (Pokrzywa et al., 2017). Data analysis was performed in real time and again in the offline mode.

2.5. Analysis of TTR deposition in head homogenates from treated flies

For every condition, 20 heads were homogenized in 100 μl Triton X-100 buffer (1%Triton X-100, 1× PBS, pH 7,6) containing a protease inhibitor cocktail (general use, Amresco) on ice. Samples were mixed gently and centrifuged for 20 min at 15000 ×*g* at 4 °C. This process was done twice and the supernatant was collected and saved as the soluble fraction (SF). The pellet was resuspended in 100 μl of 50 mM Tris pH 7,6, 4% SDS, gently vortexed and saved as the insoluble fraction (IF). Both SF and IF samples were boiled separately for 10 min. Samples were sonicated for 10 min and centrifuged at 15000 ×*g* at room temperature for 10 min. Both supernatants were collected and saved (SF-S1 and IF-S1, respectively). The pellet resultant from SF was resuspended in 100 μl of 50 mM Tris pH 7,6, 4% SDS (SF-P1). The pellet resultant from IF was resuspended in 50 μl of 50 mM Tris-HCl pH 7,6, 175 mM NaCl, 5 mM EDTA, 5% SDS, 8 M urea, and vortexed for 1.5 h (IF-P1). SF-S1 and SF-P1 were mixed and loaded together. IF-S1 and IF-P1 were mixed and loaded together. 4 x LDS sample buffer and DTT containing