Review

Inhibition of the Amyloidogenesis of Transthyretin by Natural Products and Synthetic Compounds

Takeshi Yokoyama^a and Mineyuki Mizuguchi^{*,a,b}

^aGraduate School of Medicine and Pharmaceutical Sciences, University of Toyama; 2630 Sugitani, Toyama 930–0194, Japan; and ^bGraduate School of Innovative Life Science, University of Toyama; 2630 Sugitani, Toyama 930–0194, Japan.

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Hereditary transthyretin (TTR)-related amyloidosis is caused by mutations in the *TTR* gene. The mutations destabilize the tetramer and/or monomer of TTR, and thus the stabilization of TTR is a key strategy for the treatment of TTR-related amyloidosis. In this review, we summarized the natural products and synthetic compounds that have been shown to inhibit the amyloidogenesis of TTR. The stabilizers and/or the amyloid fibril disrupters isolated from natural sources may become lead compounds for the treatment of TTR-related amyloidosis.

Key words transthyretin; crystal structure; amyloidosis; natural product; stabilizer; amyloid fibril disrupter

1. INTRODUCTION

Transthyretin (TTR) is a β -sheet-rich homo-tetrameric protein whose subunit is composed of 127 amino acid residues. The vast majority of TTR is produced by the liver and is secreted into the blood.¹⁾ TTR is also expressed in the choroid plexus of the brain, the retinal pigment epithelium of the eye, and the α -cells of pancreatic islets.²⁾ TTR transports retinol *via* binding to holo-retinol binding protein in the blood. TTR also binds and transports thyroxine (T4) in the blood and cerebrospinal fluid.¹⁾ The TTR tetramer has two thyroxine-binding channels in the dimer–dimer interface (Fig. 1a). However, more than 99% of the T4-binding sites in TTR are unoccupied in the blood, since T4 predominantly binds to thyroid binding globulin and albumin. The majority of the T4-binding sites in TTR are also unoccupied in the cerebrospinal fluid because of the low concentration of T4, although TTR is the main carrier of T4 in the cerebrospinal fluid.¹⁾

2. TTR-RELATED AMYLOIDOSIS

TTR causes transthyretin-related amyloidosis (ATTR), including familial amyloid polyneuropathy (FAP), familial amyloid cardiomyopathy (FAC), central nervous system selective amyloidosis (CNSA), and senile systemic amyloidosis (SSA).^{2,3)} Hereditary ATTR (FAP, FAC, and CNSA) is an autosomal dominant disease that is caused by mutations in the *TTR* gene. Almost all of the patients are heterozygotes, namely, their TTR tetramers are composed of mutant and/or wild-type subunits.¹⁾ To date, more than 130 mutations have been identified in the *TTR* gene, and most of them are associated with the onset of ATTR.²⁾ Hereditary ATTR is a progressive and devastating disease. It can be seen in people as young as their late twenties, and the average life expectancy after the onset of symptoms is about 10 years.^{2,4)} The clinical features of ATTR include sensorimotor polyneuropathy, autonomic dysfunction, heart and kidney failure, gastrointestinal tract disorders, vitreous opacity, cerebral infarction, and dementia.^{2,5,6)} Amyloid fibrils of TTR are deposited in the nerves,

heart, kidney, gastrointestinal tract, retina, leptomeninges, etc.^{2,6)}

The TTR tetramer is highly stable under physiological conditions, but its dissociation to monomers and subsequent partial unfolding results in aggregation of the amyloidogenic intermediate, leading to the accumulation of amyloid fibrils and oligomers that ultimately cause the clinical symptoms of ATTR.¹⁾ The mutations associated with ATTR induce subtle structural changes that destabilize the TTR tetramer and/or monomer, thereby promoting dissociation into monomers and/or subsequent partial unfolding. The most frequent mutant associated with ATTR is V30M-TTR, which is found worldwide as well as in endemic areas in Portugal, Japan, and Sweden.⁵⁾ The V30M mutation makes TTR less stable and more susceptible to tetramer dissociation into monomers.⁷⁾ Therefore, stabilization of the tetramer is a key strategy for the treatment of ATTR.

Stabilization of the TTR tetramer and the inhibition of TTR amyloidogenesis can also be achieved by small molecules that bind to the T4-binding sites of TTR^{8–10)} (Figs. 1a, e). A number of structurally diverse small molecules that stabilize the TTR tetramer, and thereafter inhibit the resulting amyloidogenesis, have been reported (Fig. 2). This review describes the biophysical properties of natural products and synthetic compounds that inhibit the amyloidogenesis of TTR.

3. NATURAL PRODUCT INHIBITORS OF ATTR

Investigation of the interactions of TTR with natural products was already underway when, in 1978, it was demonstrated that TTR was associated with ATTR.¹¹⁾ At that time, TTR was known as T4-binding prealbumin (TBPA), and the research was conducted from the point of view of the interaction between the constituents of blood and various drug ingredients. In 1963, salicylic acid was indicated to competitively bind to TTR with T4 by a competitive binding assay using isotopic labeled T4.¹²⁾ In 1986, phloretin, which is a natural polyphenol chalcone found in apple tree leaves, was also shown to bind to TTR in a competitive manner with T4.¹³⁾ In 1994, retinoic

*To whom correspondence should be addressed. e-mail: mineyuki@pha.u-toyama.ac.jp

Fig. 1. (a–d) X-Ray Crystal Structures of TTR in Complex with (a) T4 (PDB ID: 2ROX), (b) Retinoic Acid (PDB ID: 1TYR), (c) EGCG (PDB ID: 3NG5) and (d) γ -Mangostin (PDB ID: 4Y9E)

Four subunits of TTR are designated as A–D and colored yellow, magenta, cyan and green, respectively. The stabilizers are represented as a stick model with a semi-transparent surface. Chloride ions are represented as orange spheres. (e, f) Schematic view of the interactions of TTR with (e) T4 and (f) γ -mangostin. The carbon atoms of subunits B and D and stabilizers are colored magenta, green and white, respectively. Iodine atoms of T4 are colored grey. Black dashed lines indicate hydrogen bonds, CH $\cdots$ Cl $^-$ bonds or OH $\cdots$ Cl $^-$ bonds.

Biography

Takeshi Yokoyama graduated from the School of Engineering Science, Osaka University (2001), and the Graduate School of Integrated Science, Yokohama City University (2006). He conducted postdoctoral research at the Department of Biology and Biochemistry, University of Houston, TX, U.S.A. (2006–2008), and at the Frontier Research Center for Applied Atomic Sciences, Ibaraki University (2008–2011). In 2011, he became assistant professor at the Laboratory of Structural Biology, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama. Currently, he is working on neutron protein crystallography of the protein–inhibitor complex, focusing on the role of hydrogens, and on drug discovery for disease-related proteins such as transthyretin, kinase and bromodomain.

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Fig. 2. Chemical Structures of the TTR Amyloidogenesis Inhibitors

The compounds in group A have been shown to stabilize the TTR tetramer (TTR stabilizer). The compounds in group B were shown to exhibit both TTR stabilization and TTR amyloid fibril disruption activities in the present study (dual inhibitors). The compounds in group C have been shown to disrupt the TTR amyloid fibril (fibril disrupters).

acid, which is a metabolite of retinol, was shown to bind to TTR in a competitive manner with T₄,¹⁴⁾ and in the following year, the X-ray crystal structure of TTR in a complex with retinoic acid was solved at 1.8 Å resolution¹⁵⁾ (Fig. 1b). This crystallographic analysis provided the first picture of TTR in a complex with a natural product other than T₄. In 1996, it was elucidated that the binding of T₄ stabilizes the TTR tetramer and inhibits the amyloid fibril formation of TTR.⁸⁾ By this discovery, the search for TTR stabilizers that bind to the T₄-binding site of TTR and stabilize the TTR tetramer became an efficient strategy for TTR amyloidosis therapy.¹⁶⁾ In 1998, 77 compounds containing natural flavonoids and anthraquinones were screened for their inhibitory activity against TTR amyloid fibril formation.⁹⁾ At that time, it was understood that the common structural feature of TTR stabilizers, which are able to bind to the T₄-binding site of TTR, was a two aromatic-ring substructure with a linker. In 2005, the xanthonoids from *Calophyllum teysmannii* var. *inophylloide* were shown to bind to

TTR in a competitive manner with T₄, and the binding modes were predicted by docking simulation.¹⁷⁾ In terms of xanthone derivatives, we identified γ-mangostin, which is found in the pericarp of mangosteen, as a TTR amyloid fibril formation inhibitor.¹⁸⁾ The crystallographic analysis of TTR and the γ-mangostin complex revealed a novel binding mode; that is, the binding of γ-mangostin was associated with the binding of a chloride ion, suggesting that γ-mangostin is a novel TTR amyloidogenesis inhibitor which cooperatively inhibits TTR amyloid fibril formation with the help of chloride ions (Figs. 1c, f). In 2014, we also found that caffeic acid phenethyl ester (CAPE) from New Zealand propolis inhibits TTR amyloid fibril formation.¹⁹⁾ Some compounds found in common and functional foods have also been identified as TTR amyloidogenesis inhibitors. Curcumin from turmeric and xanthohumol from hops were shown to inhibit TTR amyloid fibril formation in 2009 and 2010, respectively.^{20,21)} In addition, flavonoids should be introduced as a representative compound group that

Fig. 3. (a–g) Competitive Binding Assays of the Selected Compounds with Fluorescent Probes

The titration and fluorescence experiments were performed as described previously.¹⁸⁾ Briefly, the compounds were titrated into a 2 μ M TTR solution in the order of the fluorescent probe and the natural products (1–50 μ M), and fluorescence spectra were recorded at each step. The measurement conditions (titrated compound, fluorescent probe and excitation wavelength) were (a) psoromic acid, 5 μ M resveratrol, 320 nm, (b) usnic acid, 5 μ M resveratrol, 320 nm, (c) gossypol, 5 μ M resveratrol, 320 nm, (d) honokiol, 5 μ M ANS, 360 nm, (e) hematoxylin, 5 μ M ANS, 360 nm, (f) rottlerin, 40 μ M ANS, 280 nm, and (g) magnolol, 5 μ M ANS, 360 nm. The decrease in fluorescence intensity indicates that the selected compounds competitively displaced the probes from the T4-binding sites. (h) TTR amyloid fibril disaggregation assays. Amyloid fibril disaggregation assays were performed as described previously.¹⁹⁾ Briefly, preformed TTR amyloid fibrils were incubated in the presence of 10 or 50 μ M of the compounds for 5–6 h, and the extent of amyloid fibril formation was quantified using a thioflavin T assay (excitation: 440 nm; emission: 484 nm).

exhibits inhibitory activity for TTR amyloid fibril formation. In 2005, genistein, which is an isoflavone found in soybeans, was shown to inhibit TTR amyloid fibril formation.²²⁾ In 2012, it was elucidated that the inhibitory potency of luteolin was stronger than that of genistein.²³⁾ In 2014, we found that glabridin, which is a prenylated isoflavan from licorice, is an inhibitor of TTR amyloidogenesis, and the inhibitory potency of glabridin is similar to that of diflunisal.²⁴⁾ X-ray crystallo-

graphic analysis revealed that the interactions between TTR and glabridin consist mainly of hydrophobic interactions. The characteristic structural change occurred on A108, which is formed by CH $\cdots\pi$ interaction with the A-ring of glabridin. It was suggested that the stable binding of glabridin would be achieved by the induced fit.

As described above, several natural products that bind to the T4-binding site have been discovered. Meanwhile, an al-

losteric ligand has also been discovered. A 2009 study indicated that (–)-epigallocatechin-3-gallate (EGCG) from green tea binds to TTR in an uncompetitive manner with T4 and inhibits TTR amyloid fibril formation.²⁵⁾ In the following year, the X-ray crystal structure of TTR in complex with EGCG was determined at 1.7 Å resolution, and it was revealed that six EGCG molecules bind to the surface of the TTR molecule but not the T4-binding site²⁶⁾ (Fig. 1c). The authors suggested that the binding to the molecular surface induces non-toxic aggregation of TTR and consequently inhibits the amyloid fibril formation.

As one of the therapeutic strategies for TTR amyloidosis, amyloid fibril disrupters, which directly disaggregate amyloid fibrils, have been developed. It has been determined that doxycycline, a tetracycline antibiotic, dissolves TTR amyloid fibrils.²⁷⁾ Although the mechanism by which the fibril disruption occurs is not fully understood, it is proposed that amyloid fibril disrupters bind uniformly on the fibers, which leads to disruption at multiple sites.²⁸⁾ Several compounds have been shown to disaggregate various amyloid fibrils other than TTR amyloid fibrils. The typical structure of an amyloid fibril disrupter is a multi-cyclic system such as doxycycline. Considering that the T4-binding site accepts tricyclic compounds, such as γ -mangostin, it is conceivable that there is also a dual inhibitor which inhibits TTR amyloidogenesis by both stabilizing the TTR tetramer and disaggregating TTR amyloid fibrils. Indeed, some biphenyl ether compounds have been shown to exhibit TTR stabilization and fibril disruption activities.²⁹⁾ Therefore, we performed a limited screening to discover the dual inhibitors from 26 natural products. The screening revealed that psoromic acid, usnic acid, gossypol, honokiol, hematoxylin, rottlerin and magnolol inhibited amyloid fibril formation (Table 1). These compounds were tested to determine whether they competitively bound to the T4-binding site; this was done using fluorescent probes such as 1,8-anilinonaphthalene-8-sulfonic acid (ANS) or *trans*-resveratrol. The competitive binding assays indicated that all but hematoxylin competitively bound to the T4-binding site with ANS or resveratrol (Figs. 3a–g).

In order to analyze their amyloid fibril disaggregation activity, pre-formed amyloid fibrils were incubated in the presence of the compounds, and the amounts of amyloid fibrils were quantified. Gossypol, hematoxylin and rottlerin disrupted the pre-formed amyloid fibrils (Fig. 3h). Based on the above observations, we concluded that gossypol and rottlerin are dual inhibitors, psoromic acid, usnic acid, honokiol, and magnolol are TTR stabilizers, and hematoxylin is an amyloid fibril disrupter. The disaggregation activity of rottlerin is not surprising, since it was shown that rottlerin dissolved the amyloid fibrils of lysozymes. It is possible that the T4-binding site of TTR may not accept a large molecule such as rottlerin. It is also possible that rottlerin binds to the allosteric site, and stabilizes the TTR tetramer in that manner, as with EGCG. The binding of rottlerin to the allosteric site may cause a decrease in the binding affinity at the T4-binding site.

4. DRUGS FOR ATTR

In addition to the natural products described above, Kelly and colleagues have discovered that several non-steroidal anti-inflammatory drugs have potential as inhibitors of TTR

Table 1. Limited Screening against Amyloidogenesis of V30M-TTR

Compound name	Inhibition ratio (%)	Compd. Conc. (μ M)	IC ₅₀ (μ M)
Diffunisol	97	40	6.3
Bilobalide	–1.6	80	
Boldin	–11	40	
Colchicine	12	40	
Psoromic acid	92	40	8.9
Piperine	16	40	
Usnic acid	77	20	7.7
Cryptotanshinone	8.2	32	
Gossypol	94	40	9.9
Honokiol	95	40	8.5
Nalidixic acid	–1.6	40	
Oridonin	–5.3	80	
Parthenolide	–9.3	40	
Silibinin	12	40	
Sinomenin	3.6	40	
Tanshinone I	14	40	
Tetrandrine	1.9	40	
Hematoxylin	78	40	25
Limonin	9	32	
Rottlerin	70	40	23
Magnolol	81	40	19
Magnolignan C	13	80	
Coumestrol	11	20	
Brefeldin A	15	20	
Coptisine	12	20	
Emodin	9.1	20	
Auraptene	–6.7	20	

The TTR amyloid fibril formation assay was performed according to the previous study.³⁴⁾ Briefly, V30M-TTR was incubated in the presence of the compounds at pH 4.5 for 4 d, and then the amyloid fibrils were quantified using thioflavin-T by fluorometry (excitation: 440 nm; emission: 484 nm).

amyloidogenesis.^{30–33)} The non-steroidal anti-inflammatory drug diflunisal, which has already been approved as a prescription drug in more than 40 countries, binds to the T4-binding sites of TTR, stabilizes the tetramer, and inhibits the amyloidogenesis^{4,9,33,34)} (Fig. 2). Clinical trials have been conducted to assess the efficacy of diflunisal on the progression of ATTR.^{2,3,5)} In addition, a new drug, tafamidis, has been developed for the treatment of FAP. Tafamidis binds with high affinity to the T4-binding pockets of the TTR tetramer, leading to stabilization of the tetramer and inhibition of the amyloidogenesis³⁵⁾ (Fig. 2). Tafamidis has been approved in Europe, Japan, Mexico, and Argentina, but only for the treatment of early-stage FAP.^{2,36)} However, tafamidis was unable to halt the progression of the disease in patients with advanced FAP caused by the V30M mutation.³⁷⁾ Therefore, the development of other drugs is of great importance for the treatment of ATTR. TTR stabilizers and/or amyloid fibril disrupters isolated from natural sources may become lead compounds for the treatment of ATTR.

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Conflict of Interest The authors declare no conflict of interest.

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