Suppression of amyloid β fibril growth by drug-engineered polymorph transformation

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Abstract

Fibrillization of the protein amyloid β is assumed to trigger Alzheimer's pathology. Approaches that target amyloid plaques, however, have garnered limited clinical success and their failures may relate to the scarce understanding of the impact of potential drugs on the intertwined stages of fibrillization. Here we demonstrate that bexarotene, a T-cell lymphoma medication with anti-amyloid activity both *in vitro* and *in vivo*, suppresses amyloid fibrillization by promoting an alternative fibril structure. We employ time-resolved *in situ* atomic force microscopy to quantify the kinetics of growth of individual fibrils and supplement it with structure characterization by cryogenic electron microscopy. We show that fibrils with structure engineered by the drug nucleate and grow substantially slower than "normal" fibrils; remarkably, growth remains stunted even in drug-free solutions. We find that the suppression of fibril growth by bexarotene is not due to the

drug binding to the fibril tips or to the peptides in the solution. Kinetic analyses attribute the slow growth of drug-enforced fibril polymorph to the distinctive dynamics of peptide chain association to their tips. As an additional benefit, the bexarotene fibrils kill primary rat hippocampal neurons less efficiently than normal fibrils. In conclusion, the suggested drug-driven polymorph transformation presents a mode of action to irreversibly suppress toxic aggregates not only in Alzheimer's, but also potentially in myriad diverse pathologies that originate with protein condensation.

Introduction

The accumulation of fibrils and plaques of the protein amyloid β (Aβ) in patients' brains is deemed a trigger of Alzheimer's disease (AD) and the related cerebral amyloid angiopathy (CAA) (1-3). Current approaches to delay or reverse fibrillization (4-7), however, have accumulated limited success in the clinic (8-11), which has been construed as a suggestion that alternative pathophysiological pathways may be preferred as intervention targets (9,12-14). A recent clinical study, however, found a correlation between the brain loci of entangled tau, activated microglia, and Aβ accumulation and concluded that accumulated Aβ may potentiate the activation of microglia and then synergistically cooperate with the activated microglia to promote the propagation of tau tangles and increased severity of cognitive symptoms (15). Notably, a substantial fraction of potential AD drugs currently in phase 3 clinical trials aim at Aβ fibrils and plaques (16-28).

In view of this recent evidence, the limited success to-date of the clinical approaches based on suppression of Aβ fibrils and plaques may be partially attributed to the insufficient insight into how potential drugs interfere in the complex network of intertwined processes that generate

amyloid aggregates (13,29,30). In the first step of fibrillization, several peptide monomers jointly search for a stable structure (31). This step is identified as nucleation and it spawns a population of small aggregates that may already be neurotoxic (1). The assembled nuclei grow into linear fibrils by adding peptide chains from the solution (32,33). As the fibrils prolongate, they may branch by secondary nucleation or fracture to release peptide oligomers, which, in turn, boost nucleation (13,34). This complex network of events can generate vast amounts of fibrils and oligomers that spread disease throughout patients' brains. Drugs, such as bexarotene, a cutaneous T cell lymphoma medication (35) with anti-amyloid activity both *in vitro* and *in vivo* (30,36), may intervene at any of the constituent processes. They may bind to peptide monomers (Fig. 1A) or oligomers (Fig. 1B) (30) and delay or fully stunt further aggregation. They may cap the tips (Fig. 1C) or affix to the sides of growing fibrils to inhibit growth and delay branching and fragmentation.

To unveil the molecular mechanism employed by a drug to interfere in amyloid aggregation we focus on the 40-residue Aβ isoform (Aβ40). Aβ40 dominates the amyloid plaques in the meninges of AD patients (37,38) and has been found in plaques extracted from patients with both pre-symptomatic and symptomatic AD (3,39). Aβ40 also accumulates in the brain vasculature, where it causes CAA (38). We focus on the growth rates of individual fibrils, which we quantify by time-resolved *in situ* atomic force microscopy (AFM). The kinetic data afford the opportunity to identify the steps of the molecular mechanism of peptide incorporation at the fibril tips and evaluate the governing thermodynamic and kinetic parameters (40,41). In contrast to previous AFM work, we base our conclusions on statistics over multiple fibrils and emphasize reproducible behaviors. We extend the kinetics measurements to fibril dissolution in peptide-free solutions. Finding the lowest concentration for net fibril growth defines the fibril solubility and quantifies the equilibrium constant for fibril growth and the related standard free energy for fibrillization. We

supplement the kinetic insights with fibril structure characterization by cryogenic electron microscopy. To elucidate how the findings of the chemical mechanism of Aβ fibrillization may fit in the context of brain damage and disease we measure the toxicity of fibrils formed in the presence of bexarotene to neurons.

Results and discussion

Passivity of bexarotene to the fibril tips

To monitor the evolution of fibrillization and the overall impact of bexarotene, we employed the fluorescence intensity emitted at 488 nm by the dye Thioflavin T (ThT) in the presence of amyloid β-sheets, which is expected to scale the total amyloid mass (42). The initial period of negligible fluorescence from Aβ40 solutions with added ThT (Fig. 1D) manifests slow nucleation (43,44). The addition of bexarotene further extends this period (Fig. 1D), announcing that bexarotene suppresses Aβ40 fibril nucleation (45,46). Spectroscopic tests reveal (Fig. S2) that bexarotene does not perceptibly bind to Aβ40 monomers (Fig. 1A), analogously to its undetectable binding to the similar Aβ42 (47,48); notably, the Aβ40 sequence replicates the first 40 aminoacid residues of Aβ42 and both Aβ42 and Aβ40 are unstructured (3). With this, the observed nucleation delay by bexarotene implies that the drug interacts with the only other Aβ40 species present during nucleation, Aβ40 peptide oligomers (Fig. 1B), analogously to its association to Aβ42 oligomers (30).

The upturn of fluorescence intensity (Fig. 1D) that follows the period dominated by nucleation reports contributions to fibril mass from concurrent fibril growth, branching, and fragmentation that are hard to deconvolute. To judge whether bexarotene inhibits the first of these

steps, fibril growth, we turn to time-resolved *in situ* atomic force microscopy (AFM) (41) (Fig. 2A - D). We deposit fibrils preformed in the absence of bexarotene on mica and monitor the growth of the fibril tips along the fibril axis with respect to immobile reference points (Fig. 2B) in Aβ40 solutions with known concentrations. We evaluate the fibril growth rate as the slope of the time correlation of the fibril tip displacement (Fig. 2C) (41). Our previous examination of this method (41) revealed that the measured fibril growth rates and solubilities were similar to those determined from fibrils suspended in solution (33). This agreement signifies that interactions with the substrate that may strain the fibrils or assist the supply of monomers to the fibril tips do not modify the growth rates (41). The addition of bexarotene at three concentrations up to 1 μM (which exceeds the concentration of fibril tips by many orders of magnitude) does not modify the average growth rates of fibrils nucleated in the absence of this drug (Fig. 2D). Fibril growth comprises association of peptide chains to fibril tips. The passivity of bexarotene to fibril growth concurs with the lack of bexarotene - Aβ40 peptide binding (Fig. S2) and also advocates that bexarotene interactions with the tips or alternative sites of the fibrils are weak (Fig. 1C).

Bexarotene modifies the structure of the fibrils nucleated in its presence

AFM and cryo-EM characterization of the morphology of fibrils nucleated in the presence and absence of bexarotene advance that bexarotene not only delays fibril nucleation (Fig. 1D) but also drives the assembly of a distinct Aβ40 fibril structure. Aβ40 assembles into fibrils with diverse structures (often called polymorphs), in which the peptide chains fold into unique conformations (32,37,49-51). The divergent molecular arrangements of the polymorphs dictate specific mesoscopic morphologies identifiable by AFM and cryo-EM. AFM measures the fibril thickness as the maximum separation of the AFM probe from the substrate, averaged from 10 scans perpendicular to the fibril axis (Fig. 3A). Statistics over 60 fibrils nucleated in the presence of

bexarotene (Fig. 3C) and 68 fibrils nucleated without the drug (Fig. 3B) reveal that bexarotene fibrils—with average thickness 4±1 nm—are, on the average, thinner than the normal fibrils, whose average thickness is 5±1 nm (Fig. 3D and Table S1).

Cryo-EM (Fig. 4A – F) demonstrates that both types of fibrils appear as twisted flat strips and affords the opportunity to quantify two distinguishing characteristics of the fibril morphology: the crossover distance and the fibril width (Fig. 4F, inset). The cryo-EM micrographs (Fig. 4A – D) establish that the examined normal fibrils structure rather uniformly, with an average crossover distance of ca. 140 nm and average fibril width of ca. 8 nm (Fig. 4A,E,F). By contrast, the dominant subpopulation of the bexarotene fibrils exhibits a substantially shorter average crossover distance of ca. 30 nm (Fig. 4E, Table S1) and an exaggerated average width of ca. 10 nm (Fig. 4F, Table S1); a minority subpopulation presents morphological characteristics similar to those of normal fibrils, whose nucleation may have been unaffected by bexarotene. The distributions of the widths at the crossover points of both normal and bexarotene fibrils (Fig. S3) approximately match the respective distributions of AFM-determined thicknesses (Fig. 3D); the two measures roughly correspond to the same fibril dimension owing to the distinctive perspectives of the two methods (Fig. 4F, inset). Interestingly, lone bexarotene fibrils feature variable crossover length that might expose the merging of two independently nucleated fibrils (Fig. 4D).

Distinct molecular mechanism of growth of fibrils with bexarotene – engineered structure

The rate of fibril growth as solute peptides associate to the fibril tip and its correlations with the concentrations of the peptide and a denaturant present sensitive indicators of the molecular-level processes that comprise growth (40,41). Fibrils with bexarotene-enforced structure exhibit unique kinetics of growth and dissolution, which combine features shared with normal fibrils (41)

and dramatically divergent behaviors. Time-resolved *in situ* AFM images (Fig. 5A) reveal that bexarotene fibrils grow at steady rates (Fig. 5C) when exposed to solutions of Aβ40 without bexarotene. In further analogy to normal fibrils (41), when submerged in a buffer with no Aβ40, bexarotene fibrils steadily shorten (Figs. 5B and S4), reporting the release of peptide chains from the fibril tips into the solution. The line connecting the negative rate of fibril dissolution and the positive growth rate crosses the line of zero growth (Fig. 5E) at C_e, the concentration at which the fibrils are in equilibrium with the solution, which is often called solubility (40,41). The solubility of bexarotene fibrils, 0.33±0.06 μM (Table S2), is somewhat lower than the solubility of normal fibrils, 0.44±0.07 μM (40,41). The growth rate of bexarotene fibrils at $C_{A\beta40}$ = 1 μM is faster than the respective rate for normal fibrils (Fig. 5E). Importantly, adding bexarotene to the Aβ40 solution does not inhibit or promote the growth of fibrils with structure imposed by bexarotene (Fig. 5D). The preserved growth rate in the presence of bexarotene announces that the drug is indifferent to the tips or any other features of the bexarotene fibrils involved in growth—just as to normal fibrils (Fig. 2D).

The growth of bexarotene fibrils at $C_{A\beta40}$ greater than 3 μM is insensitive to increasing $C_{A\beta40}$ and substantially deviates from the growth of normal fibrils (Fig. 5E). We relate the saturating dependence of the growth rate on $C_{A\beta40}$ to our previous finding that the incorporation of a solute peptide into the fibril tip occurs in two steps (40). First, an incoming peptide associates to a complex occupying the fibril tip and composed of one or more chains that have conformations distinct from those in the fibril bulk. In the second step one of the peptides within that complex rearranges to the bulk fibril structure (Fig. 5F) (40). The similarities and differences of this scenario to a “lock-and-dock mechanism” put forth by simulations (52-55) are discussed in ref. (40).

We assume that a similar two-step pathway guides peptide association to bexarotene fibril tips. In the absence of an atomic structure of the bexarotene fibrils, complete molecular modeling of the relevant dynamics that may detail the peptide association to a complex, in which the peptide chains assume conformation distinct from the one in the fibril bulk, and then its transformation to the native structure, as done for normal fibrils (40), would be challenging. Instead, we analyze the $R(C_{A\beta 40})$ correlations for bexarotene and normal fibrils to reveal that essential features of the two steps differ for the two fibril structures. Dimers and higher order oligomers, extant in Aβ solutions, reside in equilibrium with a majority of monomers (56,57). Our previous results inform that normal Aβ40 fibrils grow by sequential addition of monomers (40,41) and bolster the assumption that bexarotene fibrils also grow by monomer association. We model the two-step incorporation of Aβ40 monomers M into fibril tips T with a Michaelis–Menten-type sequence of two reactions, as in ref. (33), $M + T \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} MT \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} T'$, where k_i are the respective rate constants, MT denotes the intermediate complex at the tip, and T' are tips with an added peptide (Fig. 5F). Mass preservation relates the fibril average growth rate R to the rate of consumption of monomers $-\frac{d[M]}{dt}$, $R = -\frac{a}{C_T}\frac{d[M]}{dt}$ (see Supplementary text), where a is the contribution of one peptide chain to the fibril length and C_T is the total concentration of fibril tips. We exploit that the solubility C_e relates to the equilibrium constants K, of the entire process, and K_i, of the two constituent reactions, and the rate constants k_i, $C_e \cong [M]_e = K^{-1} = K_1^{-1}K_2^{-1} = \frac{k_{-1}k_{-2}}{k_1 k_2}$ (the first equality reflects the dominance of monomers in the solution (56,57) and the second is afforded by the reversibility of fibril growth and the equality $[T] = [T']$ (40)), and arrive (see Supplementary Text) at

$$R = a\left(\frac{k_1 k_2 (C_{A\beta 40} - C_e)}{k_1 C_{A\beta 40} + k_{-1} + k_2 + k_{-2}}\right).$$

At low $C_{A\beta40}$ or if the reactions that involve the intermediate complex MT are fast, $k_1 C_{A\beta40} < k_{-1} + k_2 + k_{-2}$. We retrieve a linear growth rate law $R = ak_a(C_{A\beta40} - C_e)$, where $k_a = k_1 k_2 (k_{-1} + k_2 + k_{-2})^{-1}$, which describes the $R(C_{A\beta40})$ correlation for normal fibrils (40,41) (Fig. 5E). In cases where the intermediate complex restructures slower than it sheds monomers to the solution, $k_2 + k_{-2} \ll k_{-1}$ and $k_a = K_1 k_2$. The saturating $R(C_{A\beta40})$ branch for bexarotene fibrils at $C_{A\beta40}$, for which R of normal fibrils increases linearly with $C_{A\beta40}$ (Fig. 5E), announces that $k_1 C_{A\beta40}$ supersedes $k_{-1} + k_2 + k_{-2}$ at much lower $C_{A\beta40}$ than during growth of normal fibrils owing to disparate values of the rate constants k_i. The discrepancy between the rate constants for bexarotene and normal fibrils argues that the unique structure of the bexarotene fibrils (Figs. 3, 4) dictates an intermediate complex with distinct properties that may relate to a unique arrangement of the constituent peptide chains.

The response of the rates of growth of bexarotene fibrils to the addition of urea concurs with the conclusion of distinct properties of the complex at their tips. Owing to its favorable interaction with the amide groups of the peptide backbones (58), urea denatures most proteins (59) and weakens the contacts that support the structures of amyloid fibrils (60). Urea impacts the growth of normal fibrils in two ways. It boosts the solubility by stabilizing the solute peptide chains and accelerates fibril growth by impairing the contacts that uphold the complex at the fibril tips (40). For bexarotene fibrils, the narrow range of the ascending branch of the $R(C_{A\beta40})$ correlation precludes inferences on how urea affects fibril solubility. In the saturated branch of $R(C_{A\beta40})$ urea does not appear to affect the fibril growth rate $R_{max} = ak_2$. The constant k_2 characterizes the rate of restructuring of a peptide chain from the intermediate complex to the conformation in the fibril bulk. Its insensitivity to urea for bexarotene fibrils advocates that their intermediate complex

primarily relies on bonds that weakly respond to urea (61) (Table S2), in sharp contradistinction with normal fibrils (40).

The fibrils with bexarotene – engineered structure show reduced neurotoxicity

Neurotoxicity is a cardinal feature of amyloid fibrils, in which the known Aβ40 fibril polymorphs severely diverge (32,49,50). We test whether the distinct structure of the fibrils nucleated in the presence of bexarotene may motivate higher toxicity to neurons, which would, at least partially, negate the benefit of their slow growth. We quantify the survival of primary rat hippocampal neurons after exposure for 24 and 48 hours to five Aβ40 specimens: freshly prepared Aβ40 solution, fibrils grown in the presence and absence of bexarotene, and the respective fibrils' supernatants (Fig. 6A). We select the concentration of the Aβ40 solution, 2 μM, near the solubility of normal fibrils, 0.44 μM (40,41), to approximately match the concentration of the supernatants and to minimize the probability of fibrillization. We prepare fibrils analogously to the fibrils used in AFM growth experiments and structure determinations and separate them from their respective supernatants by centrifugation (Fig. 6A). The fibril equilibration time, 24 hours (Fig. 6A), is substantially longer than the time, 10 to 14 hours, over which fibrillization ceases as the solution concentration approaches the solubility (Fig. 1D). The selected long equilibration time allows the fibrils to capture most of the Aβ40 amounts that exceed the solubilities of normal and bexarotene fibrils. We suspend the fibrils in the neuron culture medium and treat the neurons with aliquots that contain fibril mass sufficient to bring the final Aβ40 concentration to ca. 10, 20 and 40 μM. To quantify neuron survival, we add to the treated neurons 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), a yellow dye (32). Mitochondrial dehydrogenases produced from viable neurons reduce MTT to purple formazan, which is insoluble in the culture medium. After three – hour incubation with MTT, we remove the cell culture medium to eliminate its

absorbance and add a buffer that dissolves the formazan crystals into a purple-colored solution (Fig. 6B). The absorbance of this solution at 570 nm quantifies the formazan concentration and the fraction of live neurons (Fig. 6B).

The measurements of the formazan absorbance demonstrate that primary rat hippocampal neurons are largely indifferent to Aβ40 peptide solutions (Fig. 6C). By contrast, both normal and bexarotene fibrils kill the neurons (Fig. 6C). Surprisingly, the supernatants kill neurons more potently than the fibrils themselves. The greater toxicity of the supernatants may be due to Aβ40 peptide oligomers (13,29,31,56,62-66) present in the solution after fibrillization ceases or released during centrifugation. Notably, the fibrils added to the rat neurons were from freshly solubilized centrifugation pellets and did not contain any supernatant. Thus, the concentration of bexarotene, which may promote neurogenesis (67), in the fibril suspension is negligible. More neurons survive after 48-hour exposure to bexarotene-generated than to normal fibrils. The distinctions between the toxicities of two types of amyloids after 24-hour exposure to fibrils and after exposure to supernatants for both 24 and 48 hours are not statistically significant (Fig. 6C and Tables S3, S4). Importantly, these statistics declare that amyloids with bexarotene-sanctioned structure are not more toxic to primary rat hippocampal neurons than their normal equivalents.

Conclusions

The responses to bexarotene of the structure and growth rate of Aβ40 fibrils reveal that a drug may suppress fibrillization even if it does not bind to fibrils. We demonstrate that compounds that have a potential as drugs may engineer a distinct fibril structure, whose growth stalls owing to a unique intermediate state for incorporation of solute peptides. Suppression of fibril growth by drug-induced polymorph transformation may present a superior mode of drug action: since the

fibril structure is selected during nucleation and persists during growth, fibrils with drug-enforced structure grow more slowly than normal fibrils even in the absence of the drug in the environment that hosts the fibrils. We show that a drug-enforced fibril structure may carry a supplementary advantage of lower neurotoxicity. The reengineering of the fibril structure by bexarotene exposes the rich variety of fibrillization pathways that may be targeted by strategies to suppress and reverse amyloid fibrillization as a route to treating Alzheimer's and other neuropathies.

Experimental procedures

A summary of experimental procedures is provided below with additional details included in the Supporting Information

Solution Preparation.

Fibril growth buffer was prepared by dissolving sodium phosphate monohydrate (Mallinckrodt) in DI water to a final concentration of 40 mM and adjusting pH to 7.4 using NaOH. Bexarotene stock solution was prepared by dissolving bexarotene (Sigma Aldrich) in DMSO (dimethyl sulfoxide, Sigma Aldrich) to a final concentration of 0.2 mM.

Aβ40 expression and purification.

We followed published methods to express and purify Aβ40 (68). We used Western blot and liquid chromatography–mass spectrometry (LC-MS) to confirm the identity of the purified peptides (Fig. S1, A to D).

Thioflavin T (ThT) fluorescence assay.

The stock solution of ThT was prepared by dissolving ThT (Sigma Aldrich) in ethanol (Decon Labs), and filtering through 0.22 mm PES syringe filter. To monitor the fibril formation, Aβ40 peptides were diluted to 50 μM in fibril growth buffer in a black 96-well plate with clear bottom. For control experiment, ammonium acetate solution in a volume equivalent to the peptides volume was added to the growth buffer instead of Aβ40 peptides. To monitor the effect of bexarotene on Aβ40 fibrillization, bexarotene was added to diluted Aβ40 solution at final desired concentrations. ThT then was added to the Aβ40, Aβ40-bexarotene, and control solutions to a final concentration of 35 μM. The ThT fluorescence signal was measured at 37 ºC (with mixing every 5 minutes) every 15 minutes with excitation and emission at 442 nm and 488 nm, respectively, for a time period of 15 hours using SpectraMax Gemini EM Microplate Reader (Molecular Devices). The sigmoid curves were obtained by subtracting the absorbance of the control and normalizing all the data points to their maximum value.

Complexation of Aβ40 with bexarotene.

To test whether Aβ40 peptides form complex with bexarotene, Aβ40 peptide solution (in ammonium acetate) with initial volume of 600 μL and concentration of 156 μM was titrated with bexarotene by adding 20 μL aliquots of a solution of 142 μM bexarotene in ammonium acetate. This addition was repeated 20 times. The absorbance of the Aβ40-bexarotene mixture at 280 nm was compared to the sum of the individual absorbance of bexarotene and Aβ40 peptides at the corresponding concentrations. The agreement of the two absorbance values at all concentrations indicates that no complex was formed (Fig. S2, C and D).

Preparation of normal and bexarotene fibrils.

Bexarotene fibrils grew at the same condition as normal fibrils (41). These first-generation fibrils then were used as seeds for the second-generation fibrils. The stock Aβ40 peptides were diluted to 50 μM in the growth buffer with 10% v/v of the first-generation fibrils, and subjected to the same growth conditions as the first-generation fibrils for another 24 hours.

Time resolved in situ atomic force microscopy (AFM).

To prepare samples for AFM growth rate measurements, 2 μL of second-generation normal (or bexarotene) fibrils was added to the fibril growth buffer. Urea (Sigma Aldrich) was also added to the solution, to a final concentration of 1 M, if needed based on the experiments. The diluted fibrils solution then was sonicated (6 W output) for 2 minutes with 15-second intervals on ice, and was kept at 27 °C for at least 15 minutes to equilibrate the temperature. Then Aβ40 peptides were added to the fibril solution at a desired final concentration and the total volume of the solution was adjusted to 1 mL.

Multimode atomic force microscope (Nanoscope VIII or IV, Bruker) in tapping mode was used to monitor the growth of fibrils. To collect images 500 μL of the prepared sample was injected into the AFM liquid cell over freshly cut mica (Ted Pella Inc.) attached to a 15 mm metal disk (Ted Pella Inc.) and to avoid any leakage, an O-ring was inserted firmly to the liquid cell. The temperature in the liquid cell reached equilibrium of 27.0 ± 0.1°C within 15 minutes, higher than room temperature (ca. 22°C), due to heating by the AFM scanner and laser (69-71). Height, amplitude, and phase images were collected in image sizes ranged from 2 μm × 2 μm to 8 μm × 8 μm, and scan rates between 3 and 3.5 s^{-1} in most images.

To study the effect of bexarotene on normal and bexarotene fibrils growth rates (Figs. 2D and 5D), first, a fibril suspension at 3 μM Aβ40 peptide concentration was prepared as described above, without bexarotene, and the fibril growth was monitored with AFM in tapping mode for 15 to 30 minutes. Then the solution in the liquid cell was replaced with a fresh solution containing the same concentration of Aβ40 peptides and desired concentration of bexarotene, with a volume adjusted to 1 mL by the fibril growth buffer. Image collection was continued with the new solution to measure the growth rates of Aβ40 normal and bexarotene fibrils after addition of bexarotene at different concentrations.

A sequence of 10-16 images were collected to measure the displacement of each fibril end from an immobile reference point using Nanoscope analysis or ImageJ and determine the growth rates. The reported growth rates at each Aβ40 concentrations were represented by the average of 20 to 40 fibril ends growth rate measurements. The correlation between the growth rates of the opposing ends of bexarotene fibrils suggests asymmetric growth (Fig. S5, A to F), in contrast to the symmetric growth of normal fibril ends (41).

To obtain the thickness distribution, the heights of 68 and 60 normal and bexarotene, respectively, fibrils were measured. The thickness of each fibril was determined by the average height of at up to 10 cross sections along the fibril length. The thickness remains unchanged along the fibril axis and growth rates of normal and bexarotene fibrils have no correlations with the fibril thickness (Fig. S6, A to C).

Cryogenic electron microscopy (cryo-EM) imaging.

To prepare normal and bexarotene fibrils sample for cryo-EM, 20 μL of second-generation fibrils was added to the fibril growth buffer at a final volume of 500 μL and sonicated (6 W output)

for 1 minute with 15-second intervals on ice. Aβ40 peptide solution was added to the fibril suspension to a final concentration of 50 μM. The sample was sonicated in a water bath sonicator (Cole-Parmer Ultrasonic Bath, Cole-Parmer) for one minute to eliminate fibril clumping. Images were collected with a Titan Krios G3i electron microscope (ThermoFisher Scientific) operating at 300 keV and equipped with a GIF Quantum LS energy filter (Gatan) and a K3 direct electron detector camera (Gatan). We used 20 eV energy slit width during data acquisition. Total electron dose/image was ~40 electrons/$Å^2$. Image pixel size was 1.1 Å on the specimen scale for normal fibrils, and 0.8 Å for bexarotene fibrils.

The crossover distances and fibrils widths were measured from 59 micrographs for normal fibrils, and 67 micrographs for bexarotene fibrils using Fiji software. Several individual measurements were taken from each micrographs. In total, 227 crossover distances, 228 fibril widths at the widest points between crossovers, and 292 widths at the crossovers were measured for normal fibrils; 247 crossover distances, 262 fibril widths at the widest points between crossovers, and 291 widths at the crossovers for bexarotene fibrils.

Neurotoxicity assay.

Primary embryonic rat hippocampal neurons (Sprague Dawley embryonic day-18 rats, Thermo Fisher Scientific) were cultured in neurobasal medium (Thermo Fisher Scientific), supplemented with 2% v/v B-27 supplement (Thermo Fisher Scientific), 0.5 mM glutamine (Thermo Fisher Scientific), 25 μM L-glutamate (Fisher Scientific) (only up to day 4 of incubation), and 1% v/v antibiotic-antimycotic (Sigma Aldrich). The cells were seeded in a 96-well plate coated with 50 μg/mL poly-D-lysine (Thermo Fisher) to achieve 3×10^4 cells per well, and incubated at 37°C in a humidified atmosphere of 5% CO2 for 7 days (the medium was replaced every 3 days)

before exposure to Aβ40 fibrils. Images of cultured neurons were obtained (EVOS M7000 Florescence Microscopy, Thermo Fisher) to ensure neural network was formed.

Statistical tests for similarity between groups of data on Figs. 2D, 3D, 4E, 4F, 5D, 6C, and S3.

To test if the growth rates of the normal and bexarotene fibrils in absence and presence of bexarotene at the specified concentrations (Figs. 2D and 5D) are statistically identical, one-way analysis of variance (one-way ANOVA) was employed which compares the variance between each group to the variance within each group.

For normal fibrils, the F-value is 0.32, smaller than critical F-value 2.69 resulted from 4 groups of 34, 29, 27, and 24 individual measurements with a 95% confidence interval. The p-value is 0.81, greater than the α-value of 0.05. The F-value and p-value indicate the null hypothesis is true, which means the mean values are the same for all independent groups. Therefore, the growth rates of normal fibrils are not affected by bexarotene at all indicated concentrations.

We also performed Kruskal-Wallis test for the data in Fig. 2D (Table S5). The obtained p-value of 0.75 is greater than the α-value 0f 0.05 and suggests that the null hypothesis that the mean ranks of the groups are the same is true. This result advocates that bexarotene does not affect the growth rates of normal fibrils, consistent with ANOVA test results.

For bexarotene fibrils, the F-value from two groups of 24 and 36 individual measurements is 0.19, smaller than critical F-value 4.01, and the p-value is 0.66, greater than the α-value of 0.05 (95% confidence interval), suggesting that the two groups are statistically identical. This ratifies that bexarotene does not affect the growth rates of bexarotene fibrils.

The same one-way ANOVA tests were performed for normal and bexarotene fibrils thicknesses measured by AFM, fibril widths, and widths at the crossovers (Table S3). In all cases F-values are greater than critical F-values and p-values are smaller than the α-value of 0.05 (95% confidence interval), rejecting the null hypothesis which suggests the two groups are statistically different.

The two-way ANOVA test was performed for the neurotoxicity measurements. Two categories of normal and bexarotene fibrils have three concentrations of fibrils (10, 20, and 40 mM) each (Table S4). The p-value for normal and bexarotene fibrils categories is greater than α-value of 0.05 for 24-hour treatment and smaller than the α-value of 0.05 for 48-hour treatment which means the neurotoxicity of normal and bexarotene fibrils are statistically identical after 24 hours, while, different after 48 hours. The p-values for comparisons between fibrils concentrations are greater than the α-value of 0.05 for both 24 and 48-hour treatment, suggesting that increasing fibril concentration does not affect the neurotoxicity of fibrils. Also, the p-values for interaction between two categories are greater than the α-value of 0.05 for both 24 and 48-hour treatment, which means null hypothesis cannot be rejected and there is no correlation between neurotoxicity of normal and bexarotene fibrils, and fibril concentrations.

Data availability

The datasets generated during and analysed during the current study are available from the corresponding author upon reasonable request.

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Author contributions:

P.G.V. and S.M. conceived this work, designed experiments, and analyzed data; S.M. and Y.X. expressed and purified Aβ40 and carried out AFM measurements of fibril growth; P.G.V. developed a kinetic model of fibril growth; S.M., M.B.S, and E.V.O. characterized fibril structure; S.M., P.K., and M.O. measured amyloid species toxicity; and P.G.V. and S.M. wrote the paper. All authors discussed the results and commented on the manuscript.

Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

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Figure Captions

Fig. 1. Bexarotene effects on Aβ40 fibrillization kinetics. **(A – C)** Schematic illustrations of binding of bexarotene (purple) to an Aβ40 monomer (gold) (**A**) and an Aβ40 oligomer (**B**) and capping of the fibril tip **(C)**. **(D)** The evolution of Thioflavin T (ThT) fluorescence at 488 nm in 50 μM solutions of Aβ40 in the absence and presence of bexarotene at 17 and 50 μM. Inset: the chemical structure of bexarotene.

Fig. 2. Bexarotene effects on fibril growth. **(A)** Schematic of *in situ* AFM imaging of a fibril deposited on a substrate and submerged in an Aβ40 solution. **(B)** AFM images of Aβ40 fibrils recorded *in situ* during growth in a 3 μM Aβ40 solution at indicated times after the first image. Yellow arrows point to immobile reference points; navy arrows indicate growing fibril tips. **(C)** The displacements of six fibril tips, depicted with distinct symbols, growing in 3 μM solutions measured from sequences of AFM images as in B. The slopes of the best linear-regression fits define the growth rate of the monitored fibril. **(D)** Jitter plots of the rates of fibril growth in a 3 μM solution after adding bexarotene at indicated concentrations C_{Bex}. About 30 measurements, similar to those illustrated in C, are shown for each C_{Bex}. Crosses mark the slowest and fastest growth rates, black spheres indicate the average values, and capped vertical bars display the standard deviations of the data sets. Lower, median, and upper horizontal lines represent 25, 50, and 75% of the data points, respectively. ANOVA and Kruskal-Wallis tests (Tables S3, S5) support the hypothesis that the average growth rates in presence of 0.1, 0.5, and 1.0 μM bexarotene are equal to those measured in the absence of bexarotene.

Fig. 3. AFM characterization of the morphology of normal and bexarotene fibrils. **(A)** Schematic of the AFM measurement of fibril thickness *h* as height deviation from the substrate. **(B, C)** AFM images of normal (**B**) and bexarotene (**C**) fibrils. **(D)** Thickness distributions of normal (red, back row) and bexarotene (purple, front) fibrils growing at $C_{A\beta 40}$ = 3 μM obtained from, respectively, 68 and 60 thickness measurements by AFM.

Fig. 4. Cryo-EM characterization of the morphology of normal and bexarotene fibrils. **(A-D)** Cryo-EM micrographs of normal (**A**) and bexarotene (**B-D**) fibrils. The crossover points are indicated by arrows. **(E, F)** Distributions of crossover distances (**E**) and fibril widths (**F**) for normal (red, back row) and bexarotene (purple, front) fibrils. Inset: A schematic of a twisted fibril with arrows delineating thickness, crossover distance, fibril width, and width at crossover point. ANOVA tests (Table S3) for the distributions in E and F support the hypotheses that the average thicknesses, in E, and fibril widths, in F, for normal fibrils are distinct from the respective characteristics of bexarotene fibrils.

Fig. 5. The growth of bexarotene fibrils. **(A, B)** *In-situ* AFM images of bexarotene fibrils growing in a 10 μM Aβ40 solution (**A**) and dissolving in a peptide-free solution (**B**). Yellow arrows indicate immobile reference points and navy arrows point to fibril tips that grow or dissolve. **(C)** Evolutions

of the displacements of six bexarotene fibril tips at $C_{A\beta40}$ = 10 μM. **(D)** Jitter plots of the growth rates of bexarotene fibrils at $C_{A\beta40}$= 3 μM in the absence and presence of 1 μM bexarotene. ANOVA tests (Table S3) support the hypothesis that the two average growth rates are equal. **(E)** Correlations between $C_{A\beta40}$ and the growth rates of normal fibrils (red, data from ref. (41)) and bexarotene fibrils in the presence of 1 M urea (blue) and in urea-free solutions (purple). Error bars indicate standard deviations from the averages of 20-30 measurements for each composition and fibril type. Solid lines depict best fits to a kinetic model; horizontal dashed lines are extensions for high $C_{A\beta40}$. **(F)** Schematic illustration of the association of an Aβ40 monomer, M, green, to a complex at the fibril tip with a conformation distinct from the one in the fibril bulk, T, red, followed by the integration of one peptide chain into the fibril to produce a longer fibril T'. Aβ40 chains with native contacts in the fibril bulk are drawn in blue.

Fig. 6. Neurotoxicity of normal and bexarotene fibrils. **(A)** Schematic of the isolation of normal and bexarotene fibrils from their respective supernatants. **(B)** Schematic illustration of the MTT assay to measure the toxicity of Aβ40 fibrils to primary rat hippocampal neurons. An optical micrograph of a live neuron is shown. **(C)** Neuron survival fractions after exposure to solutions of Aβ40 peptide, supernatants separated from the respective fibrils, and fibrils at three total peptide concentrations for 24 and 48 hours. Red bars indicate normal fibrils and supernatant and purple, bexarotene fibrils and supernatant. Control (navy) is only neurobasal medium. The volume of the added supernatant was equal to that for the 20 μM fibril suspension. The determinations were performed three times. Error bars indicate the standard deviations from the average fractions of surviving neurons. The parameters of the ANOVA tests of the similarity between the toxicities of normal and bexarotene fibrils and their supernatants are presented in Tables S3 and S4.