



An aminosterol breaks the autocatalytic cycle of A β ₄₂ aggregation and protects cell membranes from its soluble aggregates

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Aberrant aggregates of the 42-residue form of the amyloid- β peptide (A β ₄₂) are cytotoxic in Alzheimer's disease (AD). Cost-effective and chronically safe disease-modifying therapeutics are needed to address the AD medical emergency worldwide. To increase our understanding of the mechanisms of A β ₄₂-induced cytotoxicity and to investigate clinically relevant aminosterols, we study the impact of claramine on the aggregation kinetics and properties of A β ₄₂ aggregates, as well as the ability of these proteotoxic species to bind and disrupt cell membranes. Whereas previously studied aminosterols accelerated A β ₄₂ aggregation, we show that claramine potently inhibits A β ₄₂ amyloid fibril formation. We find that claramine stabilizes soluble A β ₄₂, speeding up primary and secondary nucleation into species with antiparallel β -sheet structure that are elongation incompetent, thereby depleting A β ₄₂ monomers from the aggregation reaction. This steroid-polyamine also dissociates A β ₄₂ fibrillar aggregates, resulting in the abrogation of the autocatalytic capacity of A β ₄₂ fibrils, and it also inhibits the aggregation of a tau fragment relevant to AD. Upon exposure of human neuroblastoma cells to stabilized A β ₄₂ oligomers, claramine effectively neutralized A β ₄₂ oligomer-induced cytotoxicity by preventing their binding to cell membranes. Owing to the unique mechanism of action of aminosterols to reduce the toxicity of soluble A β ₄₂ aggregates by protecting cell membranes, and the newly characterized ability of claramine to inhibit A β ₄₂ fibril formation and dissociate fibrillar A β ₄₂ resulting in the interruption of the positive feedback loop in A β ₄₂ aggregation, our findings further emphasize the relevance of this family of natural products as potential treatments for AD and other protein misfolding diseases.

amyloid | chemical kinetics | drug discovery | Alzheimer's | protein misfolded oligomers

About two-thirds of the approximately 57 million dementia cases worldwide are caused by Alzheimer's disease (AD), a neurodegenerative disorder associated with the deposition of misfolded proteins in the brain (1, 2). In AD, the tau protein aggregates into hyperphosphorylated neurofibrillary tangles within neurons, and the amyloid- β peptide (A β) accumulates into amyloid plaques in the extracellular space of the brain parenchyma (3, 4). In both cases, normally soluble monomeric A β and tau misfold and self-associate to form aberrant deposits largely composed of amyloid fibrils. These highly ordered structures exhibit a signature cross- β core composed of layers of hydrogen bonds between adjacent β -sheets arranged parallel and in-register down the long axis of the fibril, making these structures remarkably stable (4). Many amyloid fibrils, including those of tau, A β , α -synuclein, and several others, have well-characterized autocatalytic properties, where fibril surfaces can promote the conversion of monomers to form new oligomers, some of which undergo substantial structural rearrangement and elongate to form new fibrils (5). This process results in the establishment of a positive feedback loop and rationalizes the progressively increasing rate of protein deposition over the course of AD (6).

Given the relevance of A β aggregation in the etiology and pathogenesis of AD, intense efforts are underway to develop drug strategies that can prevent the toxicity associated with the aberrant formation of A β aggregates. Among the most promising treatments for AD are the US Food and Drug Administration (FDA)-approved humanized monoclonal IgG1 antibodies Lecanemab (Leqembi[®]) and Donanemab (Kisunla[™]), designed to bind soluble protofibrillar and insoluble, modified, N-terminal truncated forms of A β , respectively (7, 8). These antibodies reduce the levels of amyloid plaques in the human brain alongside slowing down the rate of cognitive decline in patients with early-stage or mild

Significance

Alzheimer's disease is characterized by the aberrant self-assembly of the A β ₄₂ peptide into amyloid fibrils. These ordered aggregates can self-replicate through the feedback mechanism of surface-catalyzed secondary nucleation. Clinically relevant aminosterols were previously found to protect cell membranes from neurotoxic soluble A β ₄₂ aggregates, but they also accelerated A β ₄₂ fibril formation. Herein, we characterize the aminosterol claramine for its ability to similarly protect cell membranes from soluble aggregates. However, this small molecule markedly inhibits A β ₄₂ aggregation by stabilizing soluble A β ₄₂ species with antiparallel β -sheet structure and disassembles A β ₄₂ fibrils, which results in the near complete breakage of the autocatalytic capacity of fibrillar A β ₄₂. Claramine also inhibits tau aggregation. These findings suggest a promising small-molecule therapeutic intervention strategy against Alzheimer's disease.

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cognitive impairment caused by AD (7, 8). These successes provide hope for the field and society. Alongside a significant body of genetic, biochemical, and animal studies (4, 9–11), they also support the amyloid hypothesis. However, notable challenges persist, such as the effectiveness of passive immunotherapy treatments in later disease stages, the substantial financial costs of long-term patient treatment, and the rates of adverse side effects of treatment (7). Further, a key problem is that the accumulation of aggregated forms of A β can precede the appearance of clinical manifestations of AD by 10–20 years, necessitating early therapeutic interventions during preclinical stages of disease (12) where the efficacy, cost, and risks associated with long-term passive immunotherapy treatments are not well characterized. Therefore, the long-term administration of safe, cost-effective, and efficacious treatments is urgently needed.

In an effort to develop such treatments, we previously found that aminosterols have unique anti-amyloid properties that make them promising potential treatments for AD (13), Parkinson's disease (PD) (14, 15), and potentially other disorders related to proteotoxicity (16). The two most studied aminosterols, originally isolated from the gastrointestinal tract of dogfish sharks, are squalamine and trodusquemine. These molecules are bile salts with a cholestane-like steroid ring, with hydroxyl and sulfate groups at C-7 and C-24, respectively, coupled to a polyamine moiety at C-3 (17). Squalamine and trodusquemine were initially found to effectively inhibit the aggregation, fibril formation, and toxicity of α -synuclein, which is central to the onset and development of PD, by inhibiting lipid-induced nucleation, fibril amplification, and, importantly, by preventing the binding and toxicity of stabilized soluble oligomeric aggregates of α -synuclein to cell membranes (14, 15). These aminosterols were then found to neutralize the toxicity of stabilized oligomeric soluble aggregates of the 40-residue form of A β (A β ₄₀), A β ₄₂, and the model *Escherichia coli* protein HypF-N (13, 18, 19), through the unified mechanism of preventing or displacing the binding of cytotoxic aggregates of misfolded proteins from cell membranes (17, 19).

Orally administered squalamine targets α -synuclein, which aggregates in the enteric neurons of the gut and accumulates in other body parts, including the central nervous system (20). A phosphate salt of squalamine (ENT-01) corrected intractable constipation in two PD clinical trials (RASMET, Phase 2a, NCT03047629 and KARMET, Phase 2b, NCT03781791). ENT-01 increased the number of weekly spontaneous bowel movements and also improved Mini-Mental State Examination (MMSE) scores and reduced incidences of psychosis when dementia and psychosis were present as comorbidities (20). PD mouse studies showed squalamine acts locally to displace α -synuclein aggregates from enteric neurons, restoring normal electrical activity and correcting bowel dysmotility. The reactivated enteric neurons resume communication with the brain via the vagus nerve ("the gut–brain axis"), with observed health benefits, such as normalization of circadian rhythm and improvement in memory and mood (21). Trodusquemine has also been administered intravenously in a phase 1 trial and demonstrated improved insulin sensitivity in obese patients (22).

While trodusquemine and squalamine reduce A β ₄₂ soluble aggregate toxicity to cells through their protective effects on cell membranes (17, 19), trodusquemine also causes dose-dependent increases in the rate of A β ₄₂ aggregation by enhancing the rate of secondary nucleation (13). Squalamine and closely related derivatives similarly accelerate A β ₄₂ aggregation (17, 19). As studies using chemical kinetics have shown that the mechanism by which small molecules influence A β ₄₂ aggregation can change dramatically even with subtle changes to their molecular structures (23, 24), we began

a search for aminosterols that could retain the unique ability to prevent the binding and toxicity of soluble A β ₄₂ aggregates to cell membranes without enhancing the rate of A β ₄₂ aggregation. In the present work, we screened a panel of available aminosterols, including synthesized aminosterol derivatives and the commercially available small molecule claramine. We investigated their influence on the aggregation mechanism of A β ₄₂ and the binding and cytotoxicity of its resulting oligomeric aggregates to cell membranes. For the molecules that were tested, only claramine demonstrated unique beneficial effects against A β ₄₂, as: 1) it inhibits fibril formation by stabilizing soluble A β ₄₂ into an antiparallel β -sheet structure, thereby depleting monomer from the system, and remodels fibrillar forms of A β ₄₂ to eliminate its ability to self-replicate, 2) it retains the ability to protect cell membranes from cytotoxic soluble aggregated A β ₄₂ species, like other aminosterols, and 3) it inhibits tau aggregation. Collectively, this aminosterol highlights a promising therapeutic approach to target the toxicity inherent to AD.

Results

Claramine Inhibits A β ₄₂ Aggregation. Claramine shares a cholesterol backbone with other aminosterols, but the hydroxyl group on claramine is present at C-3 instead of C-7 and the spermine polyamine moiety of claramine is at C-6 instead of C-3 (Fig. 1A). Unlike other aminosterols, claramine also does not contain a negatively charged sulfate group at C-24 on the long aliphatic chain substituent on the cyclopentyl ring of the cholesterol backbone. This unique structure makes claramine easier to synthesize than trodusquemine (25), with a two-step reductive amination process using 6-ketocholestanol with a polyamine followed by sodium borohydride reduction over a period of two days with higher yield and lower cost (25).

Analytical frameworks using chemical kinetics have revealed that A β ₄₂, which is produced by the sequential cleavage of the amyloid precursor protein (APP) by β - and γ -secretases, aggregates through a series of interconnected microscopic steps that can be resolved from macroscopic observables (4, 26, 27), to include primary nucleation (k_n), secondary nucleation (k_2), and elongation (k_+) (27–30). The secondary nucleation step occurs when fibril surfaces autocatalytically convert monomers to new oligomeric species, some of which can detach, rearrange, and elongate to form additional fibrillar aggregates (31). Secondary nucleation thus creates a positive feedback loop, where the rate of fibril formation increases over time, and manifests in vitro as a sigmoidal aggregation curve encompassing: 1) a lag phase, 2) a rapid growth phase, and 3) a plateau phase, where all these macroscopic phases include a combination of the various microscopic steps to different proportions (4). We began by leveraging this chemical kinetic framework to quantify the effect of claramine on the rate of A β ₄₂ fibril formation to determine its impact on the microscopic steps that govern A β ₄₂ aggregation, including the rate constants k_n , k_2 , and k_+ (24). Experimentally, we accomplished this by adding different concentrations of fibrils at the beginning of an aggregation assay, where higher concentrations of seeds bypass both k_n and k_2 and only k_+ is rate-limiting, and lower concentrations of seeds only bypass k_n and both k_2 and k_+ are rate limiting (3).

We initially used thioflavin T (ThT) fluorescence to monitor the conversion of size exclusion chromatography (SEC)-purified monomeric A β ₄₂ to mature amyloid fibrillar aggregates over time, as fibrils are bound by ThT with a markedly higher affinity than monomers or oligomeric species (32). The quiescent aggregation of 2 μ M A β ₄₂ (in 20 mM sodium phosphate buffer, 200 μ M EDTA, pH 8.0, 37 °C) was followed by unseeded, low-seeded

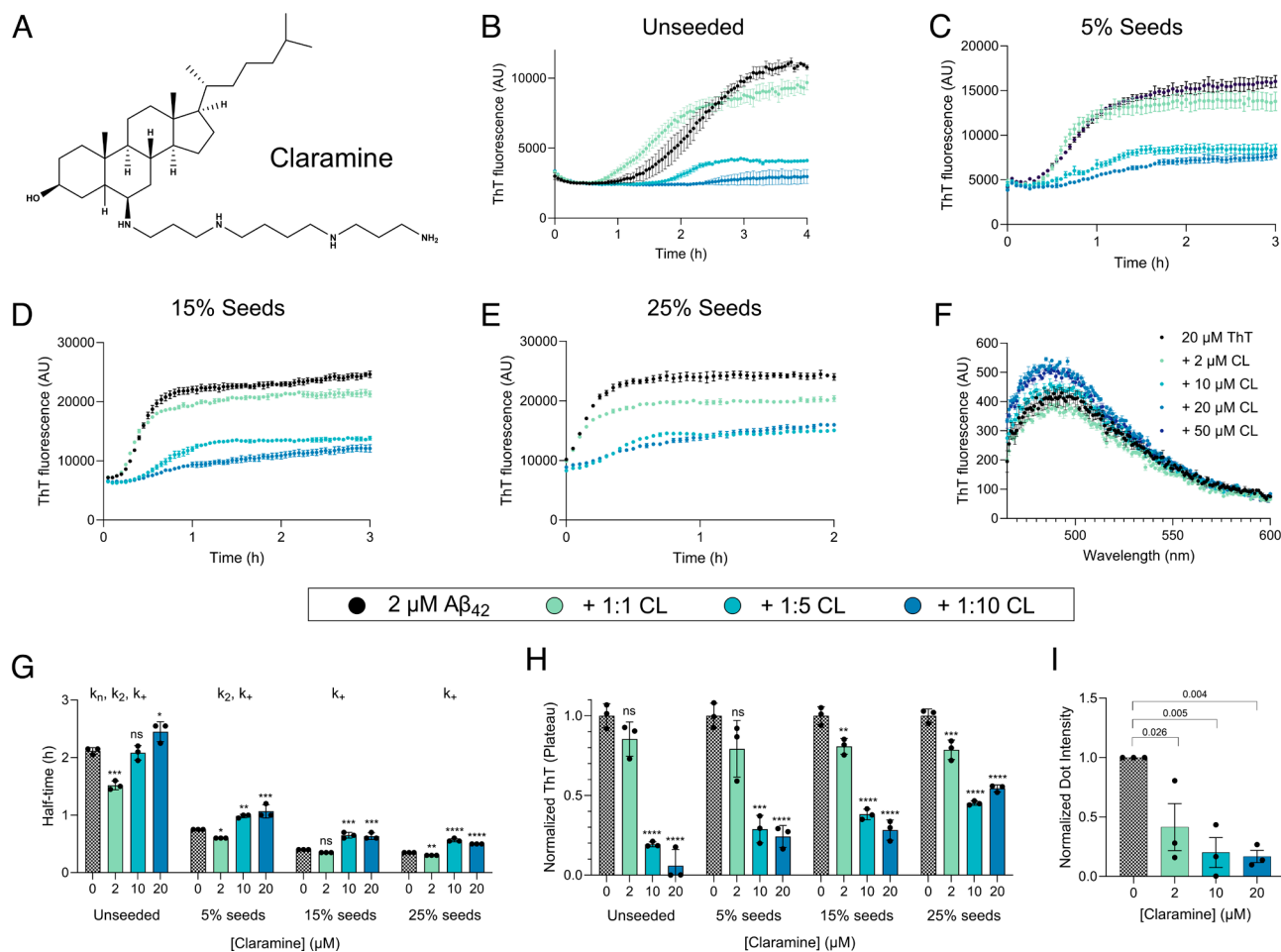


Fig. 1. Claramine inhibits Aβ₄₂ aggregation. (A) Chemical structure of claramine (CL). (B) Kinetic profiles of 2 μM Aβ₄₂ in the absence (black) or presence of 1:1 to 1:10 molar ratios of Aβ₄₂ to claramine (corresponding to 2 to 20 μM CL, shown in different colors). (C–E) Kinetic profile of the above conditions with 5, 15, and 25% fibril seeds added at the start of the aggregation reaction to assess the impact of claramine on specific microscopic steps governing Aβ₄₂ aggregation. (F) ThT was incubated at 37 °C for 15 min in the absence and presence of claramine at concentrations ranging from 2 to 50 μM. No clear changes in the ThT emission spectra were observed upon the introduction of claramine to the system. (G and H) For all kinetic profiles in panels B–E, the half-time of aggregation (G) and maximum ThT fluorescence signal at the end of the aggregation reaction (e.g., in the plateau phase) (H) are shown. Plateau ThT calculations are shown as the increase relative to the initial baseline ThT signal at $t = 0$ h. (I) Dot blot assays performed at the end of the unseeded aggregation reaction ($t = 4$ h) using the fibril-specific OC antibody in the absence of ThT. In all panels, error bars represent the SEM of three technical replicates. In panels (G–I), data were compared within each group by one-way ANOVA followed by Dunnett's post comparison. ns, *, **, ***, and **** indicate not significant, $P < 0.05$, 0.01, 0.001, and 0.0001, respectively, relative to Aβ₄₂ in the absence of claramine.

(5%, in monomer equivalents), and high-seeded (15 and 25%) experiments in the absence and presence of claramine at ratios of Aβ₄₂ to claramine of 1:1, 1:5, and 1:10 molar equivalents (Fig. 1 B–E).

The unseeded Aβ₄₂ aggregation measurements showed that claramine weakly accelerates fibril formation at equimolar ratios and slows aggregation at higher concentrations of this aminosterol with dose dependence (Fig. 1B). The seeded Aβ₄₂ aggregation experiments in the presence of claramine with 5, 15, and 25% preformed fibrils (in monomer equivalents) showed similar results to the unseeded assays, including a dose-dependent decrease in aggregation for 1:5 and 1:10 molar ratios of Aβ₄₂ to claramine in all the seeded assays (Fig. 1 C–E).

To check that claramine was not causing the above effects by interfering with ThT, we performed experiments with 20 μM ThT in the presence of claramine (Fig. 1F). There were no observed overt changes to the emission spectra of 20 μM ThT (as used in the kinetic assays) in the absence of protein and in the presence of up to 50 μM claramine. To further assess the possibility that the macroscopic changes to Aβ₄₂ aggregation above with claramine were driven by competition of the small molecule with ThT for fibril surfaces rather than a change in the aggregation reaction, we

conducted experiments with 5, 10, and 20 μM concentrations of ThT and the above ratios of Aβ₄₂ to claramine. Aggregation traces were altered in highly similar ways by claramine for the various tested ThT concentrations (SI Appendix, Fig. S1), supporting that the relevant factor driving the change in aggregation behavior is the ratio of Aβ₄₂ to claramine and not ThT to claramine. Finally, similar inhibitory effects for claramine were observed when Aβ₄₂ aggregation was monitored using Congo red, a direct diazo dye widely used by pathologists to stain amyloid in tissue sections, in the presence of comparable ratios of Aβ₄₂ to claramine (SI Appendix, Fig. S2).

Analysis of the half-times of Aβ₄₂ aggregation across all the kinetic experiments showed the dose-dependent decrease in aggregation rate at higher concentrations of claramine (Fig. 1G). In all the unseeded (Fig. 1B) and seeded experiments (Fig. 1 C–E), a clear and dose-dependent decrease in the plateau ThT fluorescence signal was observed at the end of the aggregation reaction (Fig. 1H), with the unseeded and 5% seeded experiments showing a dramatic decrease in plateau ThT fluorescence induced by claramine. This change became less pronounced with increasing concentrations of fibrillar seeds, as expected considering the initial fibrillar mass differences. In particular, relative to the signal for

A β_{42} fibrils formed in buffer alone, the unseeded experiments showed a decrease in plateau ThT fluorescence to a final content of $85 \pm 6\%$ ($P = 0.147$), $19 \pm 1\%$ ($P < 0.001$), and $5 \pm 3\%$ ($P < 0.001$) for the 1:1, 1:5, and 1:10 molar ratios of A β_{42} to claramine, respectively. With 25% fibril seeds introduced at the start of the aggregation reaction, plateau ThT fluorescence decreased to a final content of $79 \pm 4\%$ ($P < 0.001$), $45 \pm 1\%$ ($P < 0.001$), and $55 \pm 1\%$ ($P < 0.001$), all by one-way ANOVA with Dunnett's multiple comparisons test relative to A β_{42} alone) for the 1:1, 1:5, and 1:10 molar ratios of A β_{42} to claramine, respectively. This result suggests a lower concentration of fibrillar aggregates are formed and bind ThT in the presence of increasing concentrations of claramine, and/or that the types of aggregates formed at the end of the aggregation reaction in the presence of claramine differentially bind ThT due to changes in their structure.

Next, we further studied the fibrillar mass fraction at the end of the aggregation assay as a function of claramine concentration. An unseeded aggregation reaction was conducted with identical conditions as above, but in the absence of ThT. Samples were probed at the end of the aggregation reaction for fibrillar A β_{42} ($t = 4$ h, as observed in Fig. 1*B* and also confirmed in real-time using a tracer sample containing ThT in adjacent wells of the 96-well plate). We quantified the relative abundance of A β_{42} fibrils formed in the absence and presence of claramine using the OC-fibril-specific antibody (33), which has low and negligible affinity for monomeric and oligomeric A β_{42} , respectively, per the manufacturer's specifications. The quantity of OC-reactive A β_{42} decreased when formed in the presence of claramine with a well-defined dose dependence (Fig. 1*I*), to a final content of $41 \pm 20\%$ ($P = 0.026$), $20 \pm 13\%$ ($P = 0.005$), and $17 \pm 5\%$ ($P = 0.004$), one-way ANOVA with Dunnett's multiple comparisons test relative to A β_{42} alone) for the 1:1, 1:5, and 1:10 molar ratios of A β_{42} to claramine, respectively.

Analysis of the unseeded plateau ThT results (Fig. 1*H*) and fibril-specific immunoblots (Fig. 1*I*) yielded IC₅₀ values for claramine against fibril formation of $4.1 \mu\text{M}$ (1:2.05 ratio of A β_{42} to claramine, 95% CI: 2.6 to $6.4 \mu\text{M}$) and $1.8 \mu\text{M}$ (~1:0.9 ratio of A β_{42} to claramine, 95% CI: 0.6 to $4.0 \mu\text{M}$), respectively, showing these results are in good agreement. These also suggest claramine is effective at inhibiting fibril formation at concentrations similar to prototypical highly effective small-molecule inhibitors of A β aggregation in vitro like (–)-epigallocatechin 3-gallate (EGCG) (IC₅₀ ~ $2.4 \mu\text{M}$), curcumin (IC₅₀ ~ $13.3 \mu\text{M}$), and resveratrol (IC₅₀ ~ $15.1 \mu\text{M}$), although these IC₅₀ values were determined in different labs and with different A β preparation techniques (34). Collectively, our data support the conclusion that claramine can change the relative aggregate concentration of A β_{42} .

AFM, SEC, and FTIR Analysis of the Effect of Claramine on A β_{42} Aggregate Structure and Morphology. At different timepoints in ThT-free aggregation reactions, aliquots of the samples were deposited on a freshly cleaved mica surface functionalized with 0.5% (v/v) (3-aminopropyl)triethoxysilane (APTES) (35). The mica surface is negatively charged, and functionalization with APTES imparts a positive charge at the near neutral pH values that were used herein. Moreover, at this pH, A β_{42} is negatively charged, and the APTES–A β_{42} interaction promotes its binding to the mica surface for subsequent imaging. Of note, aminosterols have been shown to bind negligibly to mica functionalized with APTES (13, 19), since they have positive charges at this pH. Claramine, in particular, has an expected net charge of +4, relative to +3 for trodusquemine and +2 for squalamine.

By using high-resolution and phase-controlled atomic force microscopy (AFM), we characterized in detail the morphology of

the aggregates of A β_{42} formed with and without claramine. To accomplish this, we kept sample deformation to less than 10% during imaging by maintaining a constant regime of low tip–sample interaction (13). A β_{42} deposited at the half-time of its aggregation reaction ($t = 2$ h for $2 \mu\text{M}$ A β_{42} at 37°C) showed numerous oligomeric and fibrillar aggregates, ranging in height from circa 2 to 8 nm (see color scales, Fig. 2*A* and *C*). The addition of a 1:10 molar ratio of A β_{42} to claramine under the same conditions stabilized the formation of only oligomeric aggregates, ranging in height from circa 2 to 8 nm (Fig. 2*B* and *C*). The same samples were also deposited at the end of the A β_{42} aggregation reaction ($t = 4$ h for $2 \mu\text{M}$ A β_{42} at 37°C). In the absence of claramine, only fibrillar aggregates were predominantly observed, with cross-sectional heights ranging from circa 2 to 6 nm (Fig. 2*D* and *F*). In the presence of claramine, however, only small globular aggregates could be found at the end of the aggregation reaction, the largest of which were about 4 nm in cross-sectional height (Fig. 2*E* and *F*). Several areas of the deposited specimens were analyzed during image acquisition, and thread-like fibrillar aggregates were not observed in the claramine-containing samples (Fig. 2*B* and *E*). These results further support the conclusion that claramine prevents the growth of mature amyloid fibrillar aggregates. Of note, these results are distinct from past AFM studies on trodusquemine and squalamine, which, when added to monomeric A β_{42} , caused the formation of shorter and wider A β_{42} fibrils (13, 19).

We then sought to further investigate the structural features of the A β_{42} aggregates stabilized by claramine using SEC by fast protein liquid chromatography (FPLC). First, $5 \mu\text{M}$ monomeric A β_{42} was incubated at 37°C for 30 min in the absence and presence of a 1:10 molar ratio of A β_{42} to claramine, an early timepoint where the lag phase for $5 \mu\text{M}$ A β_{42} is expected to be about to end (reference data are shown in *SI Appendix*, Fig. S3*A*). Reaction mixtures were separated using SEC by FPLC and the absorbance at 280 nm was monitored. For A β_{42} alone, a peak was observed at the elution volume of ~15 mL, as expected for monomeric A β_{42} (Fig. 2*G*). A low absorbance peak at ~6 mL was also recorded, arising from oligomeric or fibrillar A β_{42} aggregates. The addition of claramine to the reaction mixture resulted in a decrease in the monomeric peak as the reactive flux shifted toward the stabilization of soluble oligomeric A β_{42} species, as observed by a relatively broad peak at ~9 mL (Fig. 2*G*).

To better understand the nature of the stabilized aggregates formed at the end of the aggregation reaction by claramine, we also conducted Fourier transform infrared spectroscopy (FTIR) analyses. $5 \mu\text{M}$ A β_{42} in the absence or presence of a 1:10 molar ratio of A β_{42} to claramine was aggregated until both samples had entered the plateau phase, as assessed using separate tracer samples containing ThT. Based on observations that claramine-stabilized A β_{42} can be pelleted similarly to stabilized A β_{40} oligomers and fibrils (36), aggregation mixtures were then collected and centrifuged for 15 min at $18,000\times g$, and resuspended in $10 \mu\text{L}$ pure water such that the final concentration of A β_{42} was $275 \mu\text{M}$ (in monomer equivalents). Second derivative analyses showed that the presence of a 10-fold molar excess of claramine stabilized A β_{42} species with markedly increased antiparallel β -sheet structure (Fig. 2*H*). This peak was not observed when fibrils were formed in the absence of claramine (Fig. 2*H*) or in presence of a 1:1 molar ratio of A β_{42} to claramine (*SI Appendix*, Fig. S4), which correlates well to the observed kinetic changes (Fig. 1) and provides molecular level insight as to why molar excess concentrations of claramine are necessary to observe these macroscopic and microscopic changes to A β_{42} aggregation. Considering pathologically relevant fibrils are parallel and in-register and that prefibrillar aggregated

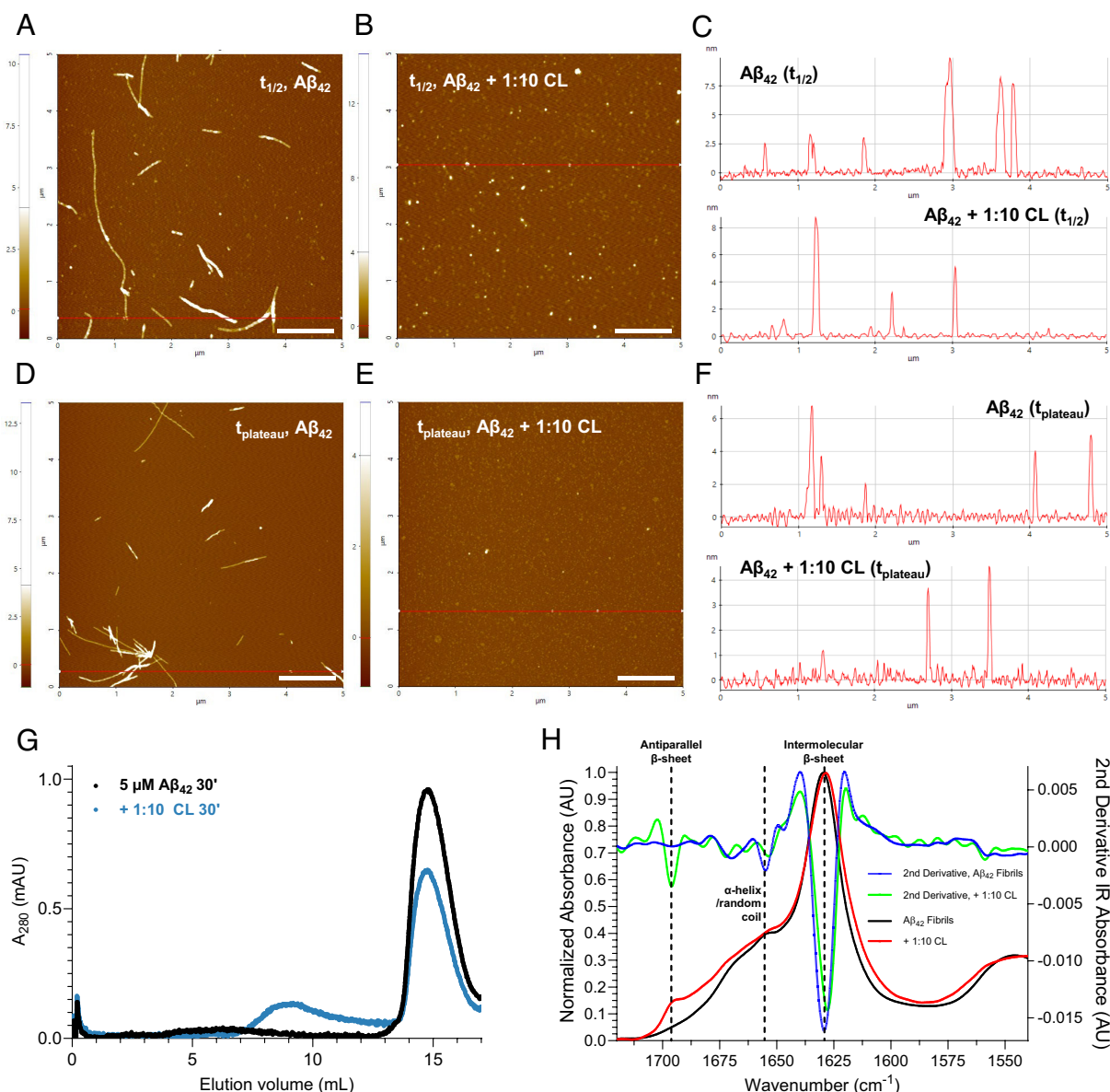


Fig. 2. Claramine prevents Aβ₄₂ fibril formation. (A and B) High-resolution, phase-controlled AFM images for samples prepared at the half-time of the 2 μM monomeric Aβ₄₂ aggregation reaction at 37 °C (t = 2 h) in the absence (A) or presence (B) of a molar 1:10 ratio of Aβ₄₂ to claramine. (C) Red lines through each color plot correspond to the shown cross-sectional height profiles. (D and E) AFM images for samples prepared at the end of the aggregation reaction when all traces had reached their plateau (t = 4 h) in the absence (D) or presence (E) of a 1:10 molar ratio of Aβ₄₂ to claramine. (F) Cross-sectional height profiles for (D and E). (Scale bars, 1 μm.) (G) SEC by FPLC of 5 μM Aβ₄₂ incubated for 30 min at 37 °C in the absence or presence of a 10-fold molar excess of claramine. (H) FTIR spectra and associated 2nd derivative for Aβ₄₂ aggregated in the absence or presence of a 10-fold molar excess of claramine.

Aβ₄₂ must rearrange substantially from antiparallel (or parallel, but out-of-register) to parallel β-sheet structure to grow into fibrillar species (3), the stabilization of soluble Aβ₄₂ by claramine into a kinetically arrested antiparallel β-sheet conformation suggests a molecular basis for how this aminosterol inhibits the Aβ₄₂ aggregation reaction.

Claramine Can Dissociate Fibrillar Aβ₄₂. We next checked whether this aminosterol can disaggregate preformed fibrils of Aβ₄₂. First, we followed the quiescent aggregation of 5 μM Aβ₄₂ using ThT. Once fibrils finished forming, as manifested by a plateau in the ThT signal, concentrated aliquots of claramine were added and mixed gently such that the final concentration of claramine in each reaction mixture ranged from 0 to 50 μM without diluting the fibrils and ThT by more than 10% (SI Appendix, Fig. S5A). Upon the addition of claramine, the ThT signal rapidly dropped within 5 min of exposure to the molecule to 93 ± 1% (P = 0.073), 79 ±

2% (P < 0.001), and 77 ± 2% (P < 0.001) with 1, 25, and 50 μM claramine, respectively, relative to 98 ± 1% in the absence of the aminosterol (SI Appendix, Fig. S5B). The signal then continued to decrease slowly until a new plateau was established approximately 1 h after the addition of claramine, with a final relative ThT value of 89 ± 2% in the absence of claramine and 77 ± 5% (P = 0.070), 56 ± 1% (P < 0.001), and 64 ± 5% (P = 0.002, all by one-way ANOVA with Dunnett's multiple comparisons test relative to the condition without claramine) for 1, 25, and 50 μM claramine, respectively (SI Appendix, Fig. S5C). We note that only quiescent aggregation was followed to avoid fibril fragmentation that can be induced by orbital shaking.

We then tested the impact of claramine on a lower concentration (2 μM) of preformed Aβ₄₂ fibrils. These complementary measurements similarly show the addition of claramine to fibrils decreased the ThT value over time and with dose dependence (SI Appendix, Fig. S5D and E). We also executed dot blot assays

with the anti-amyloid fibrils OC antibody under identical conditions as above using 2 μM fibrils but without ThT, finding that claramine reduced the content of fibrillar $\text{A}\beta_{42}$ over time (SI Appendix, Fig. S6).

Next, we conducted additional dot blot experiments using the OC antibody for 2 μM $\text{A}\beta_{42}$ supplemented with 25% fibril seeds and 0 to 20 μM claramine (analogous to Fig. 1E, but in the absence of ThT) (SI Appendix, Fig. S5F). Aliquots of the various aggregation reactions were deposited at the beginning of the aggregation reaction (5 min with the time to deposit, at room temperature), after 1 h at 37 $^{\circ}\text{C}$ (when the $\text{A}\beta_{42}$ alone sample had reached its plateau), and after 2 h at 37 $^{\circ}\text{C}$ (when all samples had plateaued). Claramine reduced the quantity of fibrils at all three time points measured (SI Appendix, Fig. S5G and H). In the presence of 10 and 20 μM claramine at the latter two timepoints, the quantity of fibrils detected was lower than the amount present at the start of the aggregation reaction for $\text{A}\beta_{42}$ alone.

To test whether claramine could dissociate $\text{A}\beta_{42}$ fibrils over time if introduced multiple times to the system, we used the previously described conditions with 5 μM $\text{A}\beta_{42}$ and added fresh aliquots of claramine (10 μL for each addition, 11x concentrated stock relative to the final reported concentration) after a new equilibrium plateau was established. Consistent with progressive fibril dissociation, we found the addition of fresh claramine to the equilibrated system resulted in a decrease in ThT binding with a well-defined dose dependence over the three tested additions (Fig. 3A and B). Specifically, adding claramine at a concentration of 1 μM dropped the ThT signal corresponding to fibril mass to

$92 \pm 7\%$ after one addition ($P = 0.508$), $73 \pm 3\%$ after the second addition ($P = 0.006$), and $63 \pm 2\%$ after the third and final addition ($P = 0.001$, one-way ANOVA relative to the $\text{A}\beta_{42}\text{-H}_2\text{O}$ control). For 50 μM additions of claramine, the signal dropped to $63 \pm 1\%$ after the first addition ($P = 0.006$), $56 \pm 1\%$ after the second addition ($P = 0.006$), and $42 \pm 1\%$ after the third and final addition ($P < 0.001$, one-way ANOVA relative to the $\text{A}\beta_{42}\text{-H}_2\text{O}$ control). Disaggregation was not observed using comparable concentrations of squalamine, a structurally similar aminosterol that can accelerate $\text{A}\beta_{42}$ aggregation (17, 19), added under the same conditions (Fig. 3C and D).

$\text{A}\beta_{42}$ Aggregates Formed with Claramine Have Reduced Seeding Capacity. Given the unique structural and morphological properties of $\text{A}\beta_{42}$ aggregates formed in the presence of claramine, we next sought to investigate the seeding ability of such aggregates. First, we followed the fibril formation process using 5 μM SEC-purified monomeric $\text{A}\beta_{42}$ in the absence and presence of up to 50 μM claramine (SI Appendix, Fig. S3A), which corresponds to a 1:10 molar ratio of $\text{A}\beta_{42}$ to claramine as in the previous experiments. In direct agreement with the above kinetic results, we observed low concentrations of claramine slightly accelerated $\text{A}\beta_{42}$ aggregation, whereas higher concentrations of claramine delayed markedly the half-time of aggregation (SI Appendix, Fig. S3B) and led to the stabilization of $\text{A}\beta_{42}$ species with reduced plateau ThT binding (SI Appendix, Fig. S3C).

Once all samples entered the plateau phase of aggregation at $t = 2.25$ h under these conditions, we collected the aggregates and

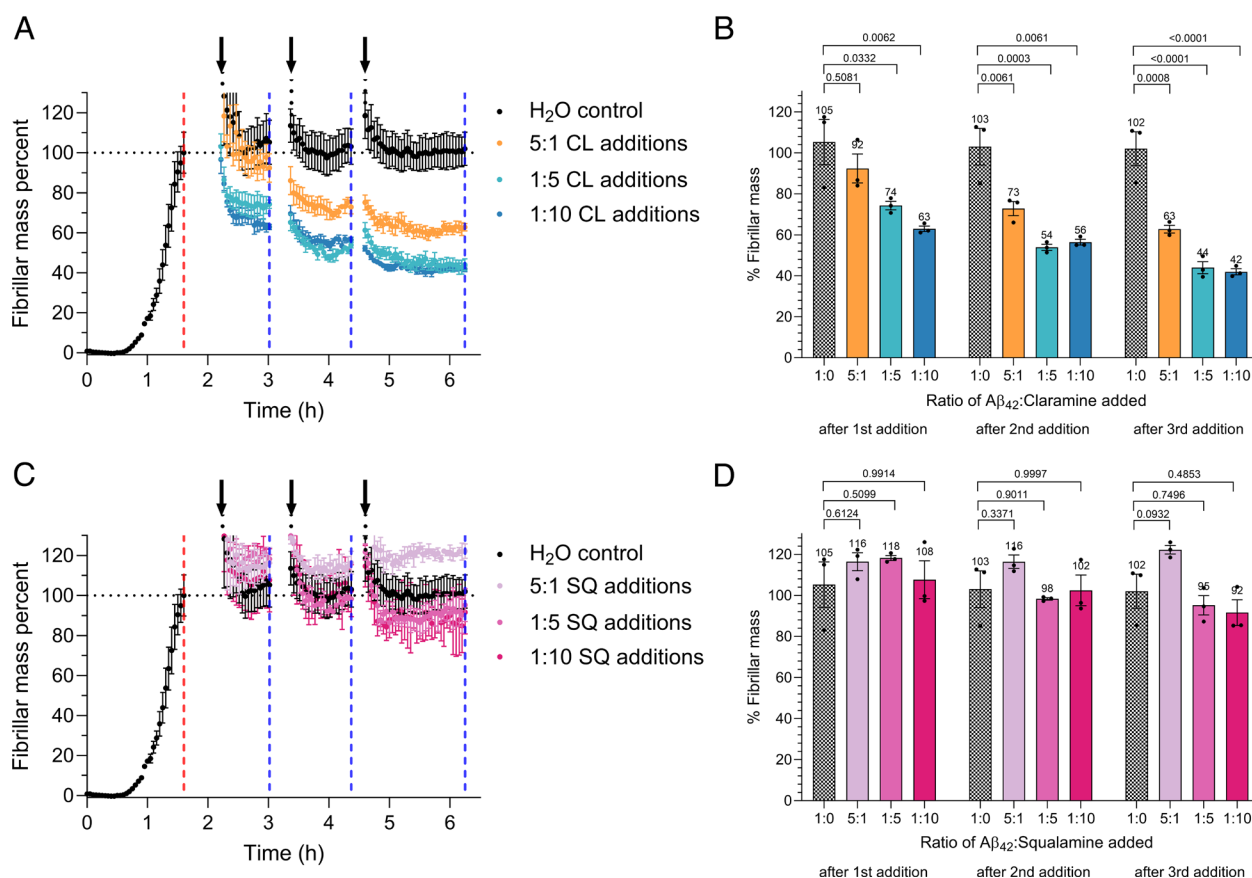


Fig. 3. Repeated claramine exposure causes the progressive dissociation of $\text{A}\beta_{42}$ fibrils. Fibrils were formed at a concentration of 5 μM as monitored using ThT, and the plate reader was paused (red dashed line). Concentrated aliquots of claramine (A and B) or squalamine (C and D) were then introduced at the indicated times (see arrows), such that they were added when the ThT signal had plateaued. The molar ratios of $\text{A}\beta_{42}$ to molecule are listed. (B and D) Quantifications of the relative fibril masses from the ThT signal at the timepoints indicated (dashed blue lines), shown relative to the initial signal for the control (dashed red line). In all panels, error bars represent the SEM of three technical replicates. Data were compared within each group by one-way ANOVA followed by Dunnett's post comparison, as shown.

tested their ability to seed the aggregation of new monomeric A β_{42} (SI Appendix, Fig. S3D). Specifically, we followed the aggregation of 2 μ M SEC-purified monomeric A β_{42} , in the absence of added claramine, supplemented with 2% (SI Appendix, Fig. S3E), 5% (SI Appendix, Fig. S3F), and 25% (SI Appendix, Fig. S3G) seeds (in monomer equivalents) having been formed in the absence and presence of 25:1 to 1:10 molar ratios of A β_{42} to claramine. Across all seeding conditions, fibrils formed in the presence of low concentrations of claramine (e.g., 25:1 and 2.5:1 ratios of A β_{42} to claramine) maintained their seeding capacity, showing templating efficacy at the level of A β_{42} fibrils alone. As the ratio of claramine to A β_{42} increased during the first fibril formation process, we observed a clear decrease in the rate of the second A β_{42} aggregation process, corresponding to a marked decrease in seeding capacity, as analyzed by half-time (SI Appendix, Fig. S3H) and ThT plateau (SI Appendix, Fig. S3I). The reduction in half-time and final plateau ThT signal in the seeded kinetic traces are consistent with a reduction in fibril mass induced by the inclusion of claramine during the unseeded aggregate formation process and the formation of seeding-incompetent oligomeric forms of A β_{42} with an antiparallel β -sheet structure, as observed with AFM and FTIR.

To further assess seeding capacity and ensure residual, unbound claramine free in solution at the end of the aggregation reaction was not driving the observed macroscopic changes to seeding capacity, we repeated the above seeding capacity experiments with a centrifugation step under two procedures: 1) claramine was added at the start of the aggregation reaction, and 2) claramine was added to preformed fibrils. For the first procedure, 5 μ M monomeric A β_{42} was aggregated in the presence of 0 to 50 μ M claramine for $\sim$ 2 h. Fibrils or claramine-stabilized A β_{42} species were collected, centrifuged, and their pellets resuspended in phosphate buffer to generate aggregated stocks that were formed in the presence of 1:0, 1:1, 1:5, or 1:10 molar ratios of A β_{42} to claramine. Conditions were prepared with 2, 5, or 10% preformed seeds, 2 μ M monomeric A β_{42} in the absence of claramine, and 20 μ M ThT. In agreement with the above seeding capacity experiments, the ability of A β_{42} aggregates to passively self-replicate was

dose-dependently attenuated when seeds were formed in the presence of increasing concentrations of claramine, where A β_{42} species formed in the presence of a 1:10 molar ratio of A β_{42} to claramine had minimal seeding capacity and their aggregation profile approached that of 2 μ M A β_{42} in the absence of seeds (Fig. 4A–D). For the second procedure, claramine was added to preformed A β_{42} fibrils rather than from the beginning of the aggregation reaction and incubated at 37 $^{\circ}$ C for 2 h. Similarly, fibrils or claramine-stabilized A β_{42} species were collected, centrifuged, and their pellets resuspended in phosphate buffer to produce aggregates that were generated in the presence of 1:0, 1:1, 1:5, or 1:10 molar ratios of A β_{42} to claramine (Fig. 4E–H). Again, conditions were prepared with 2, 5, or 10% preformed seeds, 2 μ M monomeric A β_{42} without claramine, and 20 μ M ThT. Fibrils incubated with a 10-fold molar excess of claramine had negligible seeding capacity. Collectively, these experiments show that claramine remodels A β_{42} aggregates structurally and, consequently, eliminates the templating ability of conventional A β_{42} aggregates.

Claramine Inhibition of A β_{42} Aggregation Is Driven by Its Stabilization of A β_{42} Oligomeric Species. The macroscopic ThT data produced by unseeded and various seeded assays can be fit to analytical solutions using the master equation formalism to quantify the apparent rate constants (k^{app}) governing A β_{42} aggregation, including for k_n , k_2 , and k_+ (defined above), in the absence and presence of additives like small molecules (3). The observation that fibril yield is reduced significantly by claramine at the concentrations examined (Fig. 1) raises the hypothesis that claramine promotes catalytically the formation of stable oligomers or binds and stabilizes nonfibrillar forms of A β_{42} , such as monomers and/or nonfibrillar oligomeric intermediates. In so doing, it may effectively reduce the free A β_{42} monomer concentration, which can be expected to reduce the rates of all reaction steps.

We first tested whether monomer depletion could explain the observed inhibition in the 25% seeded kinetic experiment (Fig. 1E), where aggregation kinetics are dominated mainly by fibril elongation. We fitted the standard kinetic model for A β_{42}

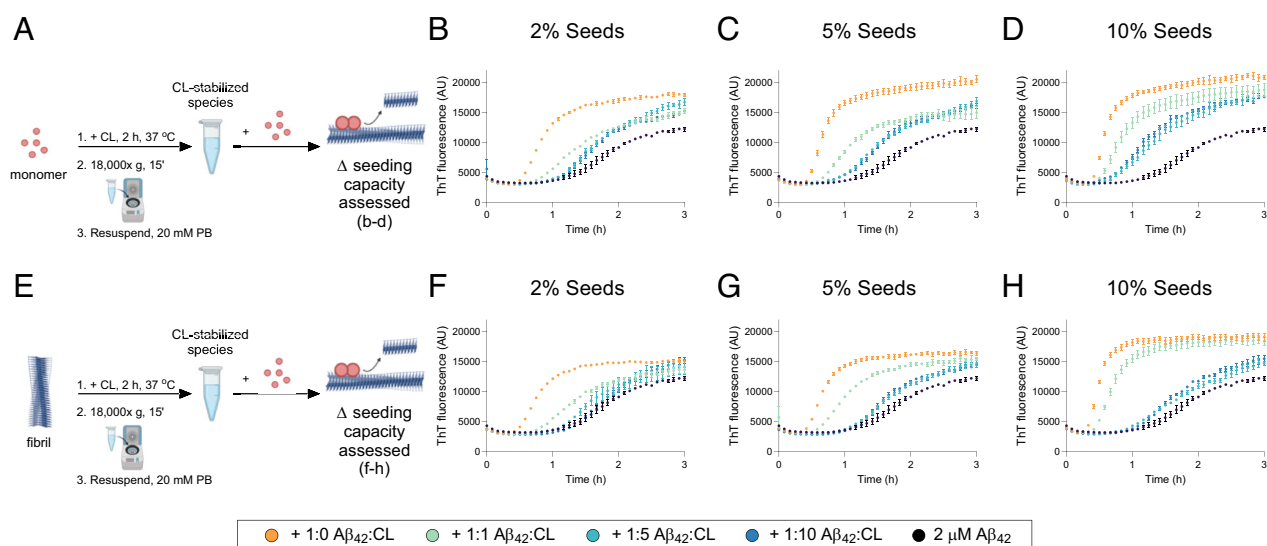


Fig. 4. A β_{42} aggregates formed in the presence of claramine and fibrils exposed to claramine after their formation show attenuated ability to passively self-replicate. (A) Incubation conditions for 5 μ M monomeric A β_{42} with 0 to 50 μ M claramine. After 2 h at 37 $^{\circ}$ C when all samples had entered their plateau phase of aggregation, samples were centrifuged at 18,000 $\times$ g for 15 min and resuspended in 20 mM phosphate buffer (PB) to remove any unbound claramine. (B–D) Aggregates formed in the presence of 1:0, 1:1, 1:5, or 1:10 molar ratios of A β_{42} to claramine were then used to assess the aggregation of 2 μ M A β_{42} containing 2% (B), 5% (C), or 10% (D) preformed fibril seeds (in monomer equivalents). (E) Incubation conditions for 5 μ M A β_{42} fibrils with 0 to 50 μ M claramine. After 2 h at 37 $^{\circ}$ C, samples were centrifuged at 18,000 $\times$ g for 15 min and resuspended in 20 mM phosphate buffer to remove any unbound claramine. (F–H) Fibrils that were incubated in the presence of 1:0, 1:1, 1:5, or 1:10 molar ratios of A β_{42} to claramine were then used to assess the aggregation of 2 μ M A β_{42} containing 2% (F), 5% (G), or 10% (H) preformed fibril seeds (in monomer equivalents). In all experiments, 2 μ M A β_{42} in the absence of seeds is shown for reference. In all panels, error bars represent the SEM of three technical replicates. Panels A and E were created with BioRender.com.

aggregation (31) to these data using AmyloFit (26), assuming no change in rate constants upon claramine addition but allowing each claramine concentration to result in a different effective initial $A\beta_{42}$ monomer concentration, m_0 . This gives good fits (Fig. 5A); moreover, we found that the fitted m_0 values correspond well to the reduction in yield as reported in Fig. 1H by the plateau ThT value.

We then tested whether this monomer depletion could accurately capture the effect of claramine on the 5% seeded reaction, where secondary (but not primary) nucleation becomes important. We found that the same model using the previously fitted m_0 values as constants yield poor fits under these conditions (Fig. 5B). This is potentially consistent with claramine promoting the formation of stable oligomeric intermediates of secondary nucleation rather than binding to monomers, thereby stabilizing them and possibly also increasing their formation rate, which would in turn increase the rate constant of secondary nucleation. To test this mechanism, we performed a second fit, still using these m_0 values, but in which k_2 was allowed to vary (Fig. 5C), finding this gives good fits with a moderate increase in k_2 as claramine concentration increases.

Finally, we investigated the unseeded time course data, where primary nucleation becomes important. If the oligomer stabilization hypothesis is correct, claramine will likely promote primary nucleation even more strongly than secondary nucleation since the free energy barrier for primary nucleation in the absence of claramine is higher than for secondary nucleation. To test this possibility, we first fitted the unseeded data using the k_2 and m_0

values determined previously and did not allow k_n to vary (Fig. 5D). This analysis yielded poor fits. By comparison, allowing k_n to vary yielded good fits (Fig. 5E), and as predicted, the corresponding k_n values increased more strongly with increasing claramine concentration compared to the k_2 values.

Thus, the observed reduction in monomeric $A\beta_{42}$ protein available for fibril formation through oligomer stabilization (Fig. 1) drives a significant part of the inhibitory effect seen at higher seed or claramine concentrations. In the absence of seeds and at low claramine concentration, the acceleration in the kinetics did not overtly change the maximum kinetic slope (Fig. 5D and E), instead decreasing only the lag time, strongly implying primary nucleation is promoted. This, in turn, almost certainly requires oligomer formation to be promoted, which should also increase secondary nucleation, although potentially less strongly. This is in agreement with the AFM and SEC by FPLC data (Fig. 2A–G). Thus, claramine promoting the formation of stable oligomers either mechanistically or by binding to them more strongly than to other species can, in principle, explain all observed kinetic phenomena.

Oligomer binding by claramine also can rationalize the observed ability of claramine to disaggregate mature fibrils. At equilibrium, $A\beta_{42}$ fibrils coexist with a constant concentration of free monomeric and oligomeric $A\beta_{42}$, and under standard in vitro conditions, fibril elongation and depolymerization can be considered in equilibrium. When added, claramine stabilizes soluble protein species and thereby reduces the free monomer concentration so that the depolymerization rate exceeds that of the elongation rate,

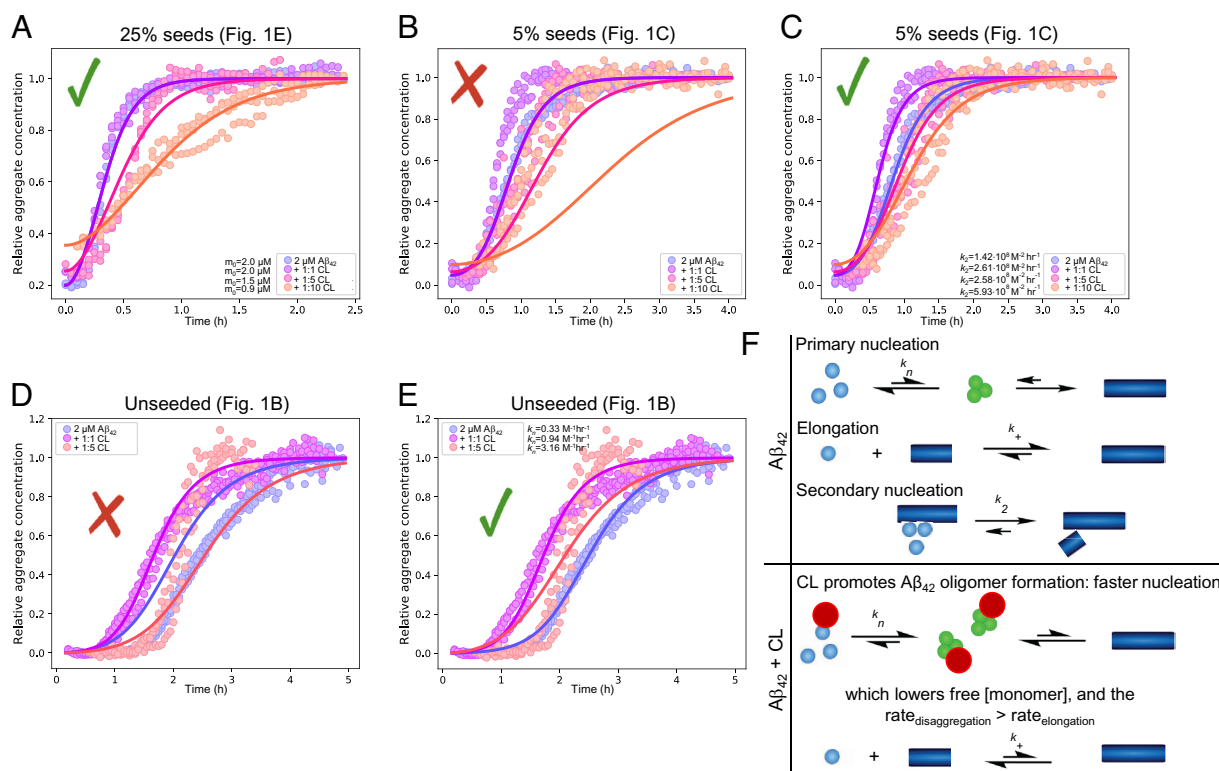


Fig. 5. Claramine stabilizes soluble forms of $A\beta_{42}$. As claramine disaggregates fibrillar $A\beta_{42}$, we postulated that molar excess concentrations of aminosterol would stabilize nonfibrillar forms of $A\beta_{42}$. (A) Normalized kinetic profiles of 2 μM $A\beta_{42}$ aggregation supplemented with 25% fibril seeds in the absence (blue) and presence of increasing concentrations of claramine (represented in different colors). Solid lines indicate theoretical predictions based on kinetic fitting (Results); the data are well described by fitting for initial monomer concentration (m_0), and resulting m_0 values are shown. (B) Normalized kinetic profile of 2 μM $A\beta_{42}$ aggregation supplemented with 5% fibril seeds; model using the previously fitted m_0 values as constants yields poor fits. (C) Second model for the data in panel B in which k_2 is allowed to vary gives good fits with a moderate increase in k_2 as claramine concentration increases, and resulting k_2 values are shown. (D) Normalized unseeded kinetic profiles of 2 μM $A\beta_{42}$ aggregation with and without claramine. Fitting of the unseeded data using the k_2 and m_0 values determined previously and not allowing k_n to vary resulted in poor fits. (E) Varying k_n resulted in a good fit, showing k_n undergoes a relatively large increase with increasing claramine concentration compared to the k_2 values. Resulting k_n values are shown. (F) Claramine promotes the formation of soluble $A\beta_{42}$ oligomers, reducing free monomer concentration, causing the rate of depolymerization to exceed that of elongation rationalizing the observed disaggregation effect of claramine on insoluble $A\beta_{42}$.

causing fibrils to disaggregate (Fig. 5*F*). We note that we cannot rule out an active catalytic effect of claramine on the disaggregation process, although such an effect is not required to explain all the data.

Kinetic Analysis of A β ₄₂ Aggregation Reveals Squalamine Potentiates Secondary Nucleation. While it is known that squalamine can accelerate A β ₄₂ aggregation leading to the formation of fibrils that are shorter and wider (13, 19), seeded experiments to fully derive the mechanism of its influence on A β ₄₂ had not been conducted. We therefore characterized seeded A β ₄₂ aggregation with squalamine to resolve its mechanism, as was done previously with trodusquemine (13), which also enabled us to have an internal control to ensure the changes induced by claramine are distinct from past aminosterols. We repeated the aforementioned kinetic framework with identical aggregation conditions for squalamine (*SI Appendix*, Figs. S7 and S8). Similarly to trodusquemine, we observed that the squalamine aggregation data were very well described by an increase in k_1 , k_2 in the unseeded assay (*SI Appendix*, Fig. S7 *B* and *C*), a large increase in k_2 in the low-seeded experiments (*SI Appendix*, Fig. S7 *D* and *E*), and a small increase in k_1 in the high seeded experiments (*SI Appendix*, Fig. S7 *F* and *G*). The derived k_2^{app} and k_1^{app} values from the seeded experiments are in good agreement with the observed k_1 , k_2^{app} values from the unseeded experiments, suggesting the model is of good quality. Therefore, like trodusquemine, squalamine accelerates A β ₄₂ aggregation predominantly by increasing the rate constant for secondary nucleation, and claramine is distinctly different from both these past aminosterols in its mechanism of action against A β ₄₂ aggregation.

Claramine Reduces A β ₄₂ Oligomer Toxicity By Neutralizing Their Binding to Cell Membranes. Knowing that squalamine and trodusquemine can prevent the binding and toxicity of soluble oligomers of several types, including those of A β , to neuronal cells (17), we next measured the effects of claramine on the toxicity of stabilized A β ₄₂ oligomers to cultured SH-SY5Y human neuroblastoma cells. A β ₄₂ oligomers were formed as A β -derived diffusible ligands (ADDLs) from recombinant A β ₄₂ (37), similarly to our past studies (13), and we explored cell health and the affinity of oligomeric aggregates for cell membranes (Fig. 6*A*). We began by investigating cellular metabolic function as a measure of viability using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. Across five biologically independent experiments from unique aggregation reactions and cell preparations, we observed cells treated with 3 μ M oligomers (in monomer equivalents) for 24 h had an average viability of $63 \pm 3\%$ of untreated cells (mean $\pm$ SEM, $P < 0.001$, Student's t test), while the coinubation of oligomers with 5 and 10 μ M concentrations of claramine increased viability to $71 \pm 3\%$ ($P = 0.066$) and $79 \pm 2\%$ of untreated cells ($P = 0.001$ by one-way ANOVA followed by Dunnett's multiple comparison test relative to cells treated with oligomers), respectively (Fig. 6*B*). Cells were also exposed to the highest concentration of claramine alone, 10 μ M, with no significant change in viability observed (Fig. 6*B*, $P = 0.714$, Student's t test). This is consistent with past studies, where 10 μ M claramine was nontoxic to SH-SY5Y cells, and greater concentrations slightly reduced cell viability (16).

We next sought to determine the ability of claramine to prevent or displace oligomer binding to cell membranes, which is a well-characterized mechanism of action for squalamine and trodusquemine against multiple types of oligomers, including those of A β ₄₂ (17). Samples were prepared as above and exposed to cells for 15 min, after which cells were labeled with wheat

germ agglutinin (WGA) to visualize cell membranes (red channel), and 6E10 sequence-specific antibodies were then used to label all A β ₄₂ bound to cell membranes (green channel). Relative to cells treated with 3 μ M A β ₄₂ oligomers only, cells coexposed with A β ₄₂ oligomers and 5 and 10 μ M concentrations of claramine showed reduced membrane binding to $87 \pm 4\%$ ($P = 0.057$) and $42 \pm 2\%$ of treated cells ($P < 0.001$ by one-way ANOVA followed by Dunnett's multiple comparison test relative to cells treated with oligomers), respectively (Fig. 6 *C* and *D*). Given claramine becomes slightly toxic to SH-SY5Y cells at concentrations greater than 10 μ M, we only probed up to 1:3.33 molar ratios of A β ₄₂ to claramine. This may explain why oligomer binding and toxicity were not fully neutralized in this case by claramine. Collectively, the cell results indicate claramine, like previously studied aminosterols, can protect cell membranes from the binding of A β ₄₂ oligomers, which in turn reduces their cytotoxicity.

Claramine-Stabilized Aggregates Maintain Their Toxicity, if Claramine Does Not Bind the Membrane. Finally, we assessed the cytotoxicity of the aggregates stabilized in the presence of claramine. We formed the claramine-stabilized species as described in the seeding capacity experiments (Fig. 4). Claramine was added from 1:0 to 1:10 molar ratios of A β ₄₂ to claramine at the beginning of the aggregation reaction (denoted "from monomer," *SI Appendix*, Fig. S9*A*) or to preformed fibrils of A β ₄₂ (denoted "to fibrils," *SI Appendix*, Fig. S9*B*). Samples were centrifuged, the supernatants were removed to eliminate soluble molecules including claramine, and pellets were resuspended in cell culture medium. The same treatment procedure was followed as in the above MTT assays, and the toxicities of the claramine-stabilized species were not significantly different than unmodified A β ₄₂ fibrils for any of the conditions (assessed by one-way ANOVA with Dunnett's multiple comparisons test relative to A β ₄₂ fibrils, *SI Appendix*, Fig. S9). These results suggest that the various claramine-stabilized species have similar degrees of cytotoxicity as unmodified fibrillar A β ₄₂. Importantly, dissociation of fibrillar A β ₄₂ into smaller claramine-stabilized species did not significantly increase its toxicity. We note that by removing free, unbound claramine in these experiments, we also negated the ability of claramine to enter cell membranes and protect them from the binding and cytotoxicity of protein aggregates through the well-documented displacement-based mechanism of aminosterols (13–19).

Claramine Inhibits AD Tau Aggregation. Previously investigated aminosterols have demonstrated the ability to modulate the aggregation of different amyloidogenic proteins in unique ways. In particular, squalamine and trodusquemine inhibited and accelerated α -synuclein and A β ₄₂ aggregation (17), respectively, and claramine is known to inhibit α -synuclein aggregation within liquid condensates (38). We therefore explored the quiescent aggregation kinetics of an ultrapure tau fragment containing residues 304–380 of the longest 441-residue isoform of tau (4R tau), which aggregates spontaneously with a mechanism dominated by secondary nucleation once a low, but critical concentration of tau fibrils have formed (39). This fragment contains the ordered part of the tau fibril (residues 306–378) and aggregates much more readily than full-length 4R tau due to its decreased intrinsic solubility (39). 10 μ M SEC-purified monomeric tau fragment was incubated with 0 to 10 μ M concentrations of claramine at 37 °C, and claramine was observed to dose-dependently inhibit tau aggregation (*SI Appendix*, Fig. S10). While the precise mechanism by which claramine inhibits tau aggregation remains to be determined, these results suggest claramine can target the

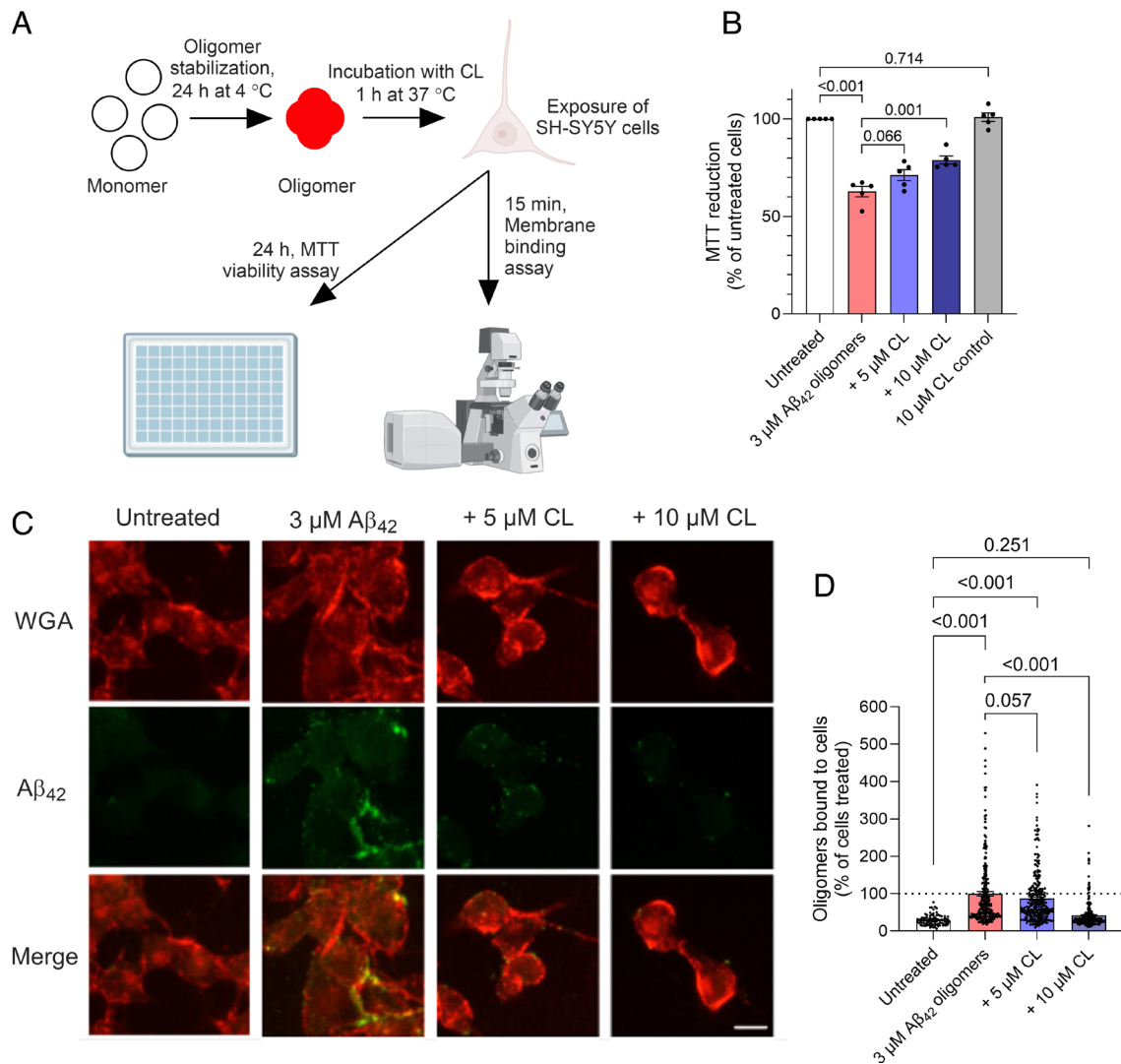


Fig. 6. Claramine reduces A β_{42} oligomer cytotoxicity and cell membrane binding. (A) Experimental design schematic created with BioRender.com. (B) Effects of claramine (CL) on the A β_{42} oligomer-induced decrease of MTT reduction. Oligomers (3 μ M in monomer equivalents, red bar) were incubated in the absence or presence of 5 or 10 μ M CL (blue bars) for 1 h at 37 °C and incubated with cells for 24 h. Cells were also treated with corresponding maximum concentrations of preincubated claramine (gray bar). A total of 60,000 cells were analyzed per condition corresponding to $n = 6$ technical replicates. Data shown for $n = 5$ biologically independent experiments. (C) Representative confocal scanning microscopy images of cells treated for 15 min with oligomers (3 μ M) in the absence or presence of 5 and 10 μ M CL. Red and green fluorescence indicate the labeled cell membranes with wheat germ agglutinin (WGA) and the A β_{42} oligomers, respectively. (Scale bar, 30 μ m.) (D) Semiquantitative values of green fluorescence signals corresponding to the colocalization of oligomers and cell membranes. For each condition containing oligomers, a total of 275 data points were analyzed in $n = 2$ independent experiments. In all panels, error bars represent SEM, and cells treated with only oligomers or claramine were compared to untreated cells using Student's t test, as indicated. Samples containing oligomers were analyzed by one-way ANOVA followed by Dunnett's multiple comparison test relative to cells treated with oligomers or untreated cells, as indicated.

aggregation of both A β_{42} and tau, which may be the two most relevant biomolecules in AD (3).

Discussion

Aminosterols have relevant anti-aggregation properties for several amyloidogenic proteins. Squalamine and trodusquamine are potent inhibitors of α -synuclein aggregation and toxicity (14, 15, 17). They both prevent the binding to cell membranes and resulting toxicity of a variety of protein misfolded oligomers, to include those of α -synuclein, A β_{40} , A β_{42} , and HypF-N (13–15, 17–19, 40). Detailed structural studies on trodusquamine showed that it readily inserts into model neuronal membranes and the plasma membranes of SH-SY5Y cells, and it can modulate the physical states of lipid membranes (41). The spermine moiety that is positively charged positions itself on the surface of the external hydrophilic face of bilayers sticking out of the membrane plane, while

the steroid part of the aminosterol extends downward at an angle of 55° with respect to the normal of the bilayer plane and spans from the external hydrophilic layer down to the interface between the hydrophilic and hydrophobic layers (41). This results in resistance to oligomer binding and toxicity, driven in large part by 1) an increase in indentation force at the bilayer, 2) a lower negative surface charge, and 3) a redistribution of monosialotetrahexosyl-ganglioside 1 and cholesterol molecules (17, 40, 41). While trodusquamine effectively reduced A β_{42} toxicity to cells through the above mechanisms, it also increased the rate of A β_{42} aggregation in vitro by augmenting the secondary nucleation microscopic step (13, 17). Similar effects were observed for squalamine herein. Although it has been postulated that bypassing the highly cytotoxic soluble aggregate intermediate state by speeding up fibril formation may be sufficient to alleviate toxicity overall by decreasing the lifetimes of the most cytotoxic aggregates (13, 42, 43), the efficacy of this strategy is yet to be validated clinically, and other

therapeutic approaches, such as passive immunotherapy (7, 8) or small-molecule mediated inhibition of aggregation (23), appear to us as more mechanistically appealing.

The results of the present study show that claramine can go beyond these limitations, as this aminosterol molecule can inhibit amyloid fibril formation for $A\beta_{42}$. Moreover, it can disassemble $A\beta_{42}$ fibrils and inhibit amyloid fibril formation of tau, in addition to confirming its ability to prevent $A\beta_{42}$ soluble aggregate toxicity by protecting cell membranes (summarized in *SI Appendix, Fig. S11*). Our results revealed claramine stabilizes $A\beta_{42}$ soluble species, as manifested by a large drop in the concentration of free monomer available for aggregation (Fig. 5*A*) and the clear presence of stabilized oligomers observed by AFM (Fig. 2*B* and *E*) and SEC with FPLC (Fig. 2*G*), which causes a modest dose-dependent increase in k_2 (Fig. 5*C*) and a relatively larger increase in k_n compared to k_2 (Fig. 5*E*). While it increases these nucleation rates, molar excess concentrations of claramine stabilize $A\beta_{42}$ species that have antiparallel β -sheet structure, as shown with FTIR spectroscopy (Fig. 2*H*), and do not participate in surface-catalyzed secondary nucleation, thereby breaking the $A\beta_{42}$ catalytic cycle responsible for its passive self-replication (Fig. 4 and *SI Appendix, Fig. S3*).

Small molecules that can disrupt $A\beta_{42}$ fibrils are of considerable therapeutic interest. The polyphenol EGCG provided an initial example of an inhibitor of the aggregation of multiple proteins with the ability to remodel and disaggregate their fibrillar forms, resulting in the formation of off-pathway, stabilized oligomeric species for $A\beta$ (44), α -synuclein (44), and tau (45), though its poor pharmacokinetic properties limited its therapeutic use (45, 46). Claramine can similarly inhibit the aggregation of multiple types of proteins given a recent report on its inhibition of α -synuclein aggregation within liquid condensates (38) and our findings herein that it inhibits tau aggregation.

While claramine studies in humans have not been conducted, studies in mice observed a therapeutic dose of 5 mg/kg injected intraperitoneally to be safe and, like trodusquemine, claramine could cross the blood–brain barrier (25). Claramine has also been shown to access the brain when delivered by the intranasal route to the mouse at concentrations that partially correct insulin resistance (47). While aminosterols demonstrate great potential as AD/PD drugs because of their ability to incorporate into cell membranes and improve their resistance to misfolded protein oligomers, they also act on other targets, including pathways for anxiety (48), insulin resistance (49), tissue regeneration (50), and malignancy (51). Of note, inflammation activates protein tyrosine phosphatase 1B (PTP1B), and trodusquemine, an effective PTP1B inhibitor, demonstrated efficacy in preventing memory impairment, inflammation, and hippocampal neuronal loss in both sexes of the aggressive transgenic hAPP-J20 mouse model of AD that has $A\beta$ pathology (52). Claramine is known to similarly effect PTP1B (25). It is likely, therefore, that aminosterols act on pathways beyond specifically $A\beta_{42}$ to prevent the harmful impact of AD and aging, in general, on the cell. Future work will aim to address whether this multiplicity of targets and mechanisms of action are beneficial for AD and will provide greater clarity into the mechanisms by which $A\beta$ is toxic and can drive the onset and progression of AD. They will also reveal what role aminosterols, their derivatives, or their use as combinatorial therapeutics may play in the treatment of AD.

Conclusions

The aggregation of $A\beta_{42}$ in AD is characterized by two particularly deleterious aspects: 1) $A\beta_{42}$ can passively self-replicate using its fibril surfaces, creating a positive feedback loop that increases the

rate of $A\beta_{42}$ fibril formation over time, and 2) $A\beta_{42}$ aggregation continuously generates highly cytotoxic soluble aggregates that bind extensively to cell membranes and cause neuronal dysfunction and loss. Claramine corrects these aberrant processes central to AD. By reducing fibril formation, dissociating $A\beta_{42}$ fibrils, and forming claramine-stabilized aggregates that are incompetent for seeding, this small molecule attenuates the ability of $A\beta_{42}$ fibrils to self-replicate. Moreover, claramine prevents the membrane binding and resulting toxicity of soluble $A\beta_{42}$ aggregates to cells and inhibits tau aggregation. Owing to these synergistic achievements, our results prompt further studies to investigate the therapeutic potential of claramine in the setting of AD.

Methods

Detailed materials and methods are in *SI Appendix*.

Reagents. Claramine trifluoroacetate salt (>98%) was acquired from Sigma-Aldrich. The trifluoroacetate (TFA) counterion was not responsible for the observed aggregation changes induced by claramine (*SI Appendix, Fig. S12*).

Protein Preparation. 1 mL of 6 M guanidine hydrochloride was added to 1 mg of >97.0% pure $A\beta_{42}$ lyophilized peptide and purified using SEC.

Aggregation Assays. Samples containing $A\beta_{42}$ monomers (2 to 5 μ M), fibrils (2 to 25%), ThT, Congo red, and small molecules, where relevant, were prepared on ice in Protein LoBind Eppendorf tubes and measured in Corning 3881 plates. Fibril formation was monitored by X34 fluorescence for an ultrapure fragment containing residues 304–380 of tau441 (39).

Dot Blot Assays. Membranes were probed with 1:1,000 diluted rabbit anti-amyloid fibrils OC polyclonal antibody and 1:5,000 IRDye® 680RD Goat anti-rabbit IgG secondary antibody. Representative images are shown in *SI Appendix, Fig. S13*.

AFM. Solutions containing aggregated $A\beta_{42}$ were deposited on mica positively functionalized with (3-aminopropyl)triethoxysilane. Imaging was conducted on a Park NX10 Atomic Force Microscope operating in noncontact mode.

FTIR. Spectra were recorded on a Nicolet iS20 FTIR with a resolution of 4 cm^{-1} and 512 scans and were smoothed (19 points, 9.160 cm^{-1}) prior to taking the second derivative using a 2nd order, 13-point Savitzky–Golay filter. Reference spectra for claramine alone at 1 mM are shown (*SI Appendix, Fig. S14*).

Cell Culture. Human SH-SY5Y neuroblastoma cells (ATCC, VA) were maintained in a 5% CO_2 -humidified atmosphere at 37 °C and grown until they reached 80% confluence for a maximum of 20 passages (13, 53).

MTT Reduction. $A\beta_{42}$ oligomers prepared according to Lambert's protocol (54) were incubated with or without increasing concentrations of claramine for 1 h at 37 °C and added to the cell culture medium of SH-SY5Y cells for 24 h. Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay (55).

Oligomer Binding to the Cell Membrane. SH-SY5Y cells were treated for 15 min with $A\beta_{42}$ oligomers in the absence or presence of claramine. Cells were counterstained with Alexa Fluor 633-conjugated wheat germ agglutinin. $A\beta_{42}$ was detected with 1:800 diluted mouse monoclonal 6E10 anti- $A\beta$ antibodies and subsequently 1:1,000 diluted Alexa Fluor 488-conjugated anti-mouse secondary antibodies.

Statistics. Comparisons were performed by one-way ANOVA followed by Dunnett's post comparison test or an unpaired, two-tailed Student's *t* test in GraphPad Prism 10.3.

Data, Materials, and Software Availability. All study data are included in the article and/or [supporting information](#).

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