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RESEARCH ARTICLE

Masking the transmembrane region of the amyloid β precursor protein as a safe means to lower amyloid β production

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Abstract

Introduction: Reducing brain levels of both soluble and insoluble forms of amyloid beta (Aβ) remains the primary goal of most therapies that target Alzheimer's disease (AD). However, no treatment has so far resulted in patient benefit, and clinical trials of the most promising drug candidates have generally failed due to significant adverse effects. This highlights the need for safer and more selective ways to target and modulate Aβ biogenesis.

Methods: Peptide technology has advanced to allow reliable synthesis, purification, and delivery of once-challenging hydrophobic sequences. This is opening up new routes to target membrane processes associated with disease. Here we deploy a combination of atomic detail molecular dynamics (MD) simulations, living-cell Förster resonance energy transfer (FRET), and in vitro assays to elucidate the atomic-detail dynamics, molecular mechanisms, and cellular activity and selectivity of a membrane-active peptide that targets the Aβ precursor protein (APP).

Results: We demonstrate that Aβ biogenesis can be downregulated selectively using an APP occlusion peptide (APPOP). APPOP inhibits Aβ production in a dose-dependent manner, with a mean inhibitory concentration (IC_{50}) of 450 nM toward exogenous APP and 50 nM toward endogenous APP in primary rat cortical neuronal cultures. APPOP does not impact the γ-secretase cleavage of Notch-1, or exhibit toxicity

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toward cultured primary rat neurons, suggesting that it selectively shields APP from proteolysis.

Discussion: Drugs targeting AD need to be given early and for very long periods to prevent the onset of clinical symptoms. This necessitates being able to target Aβ production precisely and without affecting the activity of key cellular enzymes such as γ-secretase for other substrates. Peptides offer a powerful way for targeting key pathways precisely, thereby reducing the risk of adverse effects. Here we show that protecting APP from proteolytic processing offers a promising route to safely and specifically lower Aβ burden. In particular, we show that the amyloid pathway can be targeted directly and specificically. This reduces the risk of off-target effects and paves the way for a safe prophylactic treatment.

KEYWORDS
Alzheimer's precursor protein, amyloid beta, molecular dynamics, protein folding, transmembrane domain

1 | BACKGROUND

Alzheimer's disease (AD) is the most common form of dementia, the fifth leading cause of death globally,[1] and rapidly becoming the leading cause of death in the developed world.[2] A defining diagnostic marker of AD is an abundance of senile plaques, brain lesions formed chiefly of aggregated amyloid beta (Aβ) peptides,[3] which are generated from Aβ precursor protein (APP) following consecutive proteolytic cleavages by the Aβ cleavage enzyme-1 (BACE1) and the γ-secretase complex.

Despite previous failures of immunologically based therapeutics targeting Aβ directly, two recently U.S. Food and Drug Administration (FDA)–approved drugs, aducanumab (EMERGE) and BAN2401 (Clarity AD), harness antibodies to target Aβ aggregates and soluble Aβ protofibrils, respectively. Both immunotherapies result in strong reduction in brain plaque load over baseline.[4,5] In both the EMERGE and Clarity AD trial this was associated with a statistically significant decrease in the rate of cognitive decline for the group on the highest dose (10 mg/kg body weight once a month for EMERGE and 10 mg/kg body weight every other week for Clarity AD).[6,7] These studies demonstrate the therapeutic potential of Aβ-focused treatments.

Approaches targeting Aβ production, such as small molecule antagonists of BACE1, have shown promise at reducing amyloid burden in animal models. Treating AD model mice with the BACE1 inhibitor NB-360 resulted in a 12-fold reduction in the formation of new amyloid plaques when administered prior to the onset of pathology.[8] However, inhibition of BACE1, and of γ-secretase, is generally found to be toxic,[9] as both process a range of other substrates that are critical for normal neuronal functioning.[10] This limits the maximum dose that can be administered safely to levels that are insufficient to show sufficient therapeutic benefit. Consequently, NB-360 becomes less effective with disease progression and can only slow, but not arrest, plaque growth.[8] Despite being viable, fertile, and appearing overtly normal, a detailed examination of BACE1-deficient mice has revealed many central defects due to the loss of BACE1, including memory deficits and reduced dendritic spine density in the hippocampus.[11,12] Furthermore, clinical trials of a BACE1 inhibitor were halted as it was found to exacerbate cognitive deficits in AD subjects.[13]

Any pharmaceutical AD therapy will require chronic administration over many years to prevent irreversible loss or damage to brain tissues. This highlights the pressing need to develop new therapeutics that can selectively target key processes in the pathogenesis of AD, providing cognitive benefit at non-toxic doses.

Here we demonstrate that Aβ production can be attenuated selectively with high specificity by shielding the parent molecule, APP, from proteolysis. APP is a type I, single pass, transmembrane protein with a large N-terminal region connected to a smaller C-terminal region by a 25-residue transmembrane domain (TMD). Within the TMD there are two highly conserved dimerization motifs: GxxxGxxxG and GxxxA. The γ-secretase cleavage site is located around the alanine residue of the GxxxA motif (Figure 1A). This motif forms the interface of a dimeric APP-TMD structure determined via solid-state nuclear magnetic resonance (NMR) in detergent micelles.[14,15] In addition, the cryo-EM (electromyographic) structure of the γ-secretase/APP complex revealed that a monomeric APP-TMD fragment resides in the proteolytic cleft of presenilin-1, the catalytic subunit of γ-secretase.[16] These data suggest that only monomeric APP-TMDs can enter the proteolytic cleft of γ-secretase, and thus binding a peptide specifically to the APP-TMD will protect it from proteolysis, and prevent Aβ production.

The key goal of this work was to revive the momentum behind secretase inhibitors that showed great potential in reducing Aβ burden, but ultimately failed due to off-target toxicity. Here we show that targeting APP directly with a cleavage-site masking peptide, APPOP, can provide an alternative route to selectively and specifically block γ-secretase activity toward APP, without affecting its activity for other essential cellular substrates (e.g., the Notch receptor family). Our key focus is validating the therapeutic target on APP, identified as the GxxxA motif within the TMD, and the molecular mechanism underpinning activity of

APPOP, identified as site-specific oligomerization with the APP-TMD. This lays the foundation for further pre-clinical efficacy and toxicity studies.

RESEARCH IN CONTEXT

1. **Systematic review**: The existing literature was surveyed using standard databases (e.g., PubMed). Based primarily on a nuclear magnetic resonance (NMR) structure and the presence of dimerization sequence motifs, this body of work proposed that native amyloid beta (Aβ) precursor protein (APP) forms stable dimers in the membrane. However, in vitro work and recent Cryogenic electron microscopy (electromyographic) structural evidence suggest that the cleavable form of APP is monomeric.
2. **Interpretation**: Our work shows that APP is primarily. In addition, we demonstrate a route to prevent APP proteolysis by γ-secretase that is specific to APP and non-inhibitory to other substrates. The results are consistent with previous findings.
3. **Future directions**: Our work outlines an alternative and safe route toward specific modulation of amyloid-β levels. Further work will explore the role of precise levels of Aβ in AD pathogenesis and disease progression. Translation as a therapeutic will require demonstration of safety and efficacy in vivo. Specifically, solubility, stability, and pharmacokinetic challenges will necessitate a suitable nanoformulation.

2 | METHODS

2.1 | Molecular dynamics simulations

All simulations were performed and analyzed using GROMACS (www.gromacs.org)[17] and HIPPO beta (www.biowerkzeug.com), using the CHARMM36 all-atom force field[18] and TIP3P water.[19] Electrostatic interactions were computed using particle-mesh-Ewald (PME), and a cutoff of 10 Å was used for the van der Waals interactions. Bonds involving hydrogen atoms were restrained using LINCS.[20] Simulations were run with a 2 fs time-step, and neighbor lists were updated every five steps. All simulations were performed in the Constant particle number (N), pressure (P), and temperature (T) (NPT) ensemble, with water, lipids, and the protein coupled separately to a heat bath with T = 50–120°C and a time constant $\tau_T = 0.1$ ps using a velocity rescale temperature thermostat. Atmospheric pressure of 1 bar was maintained using the Parrinello—Rahman semi-isotropic pressure coupling method with compressibility $\kappa_z = \kappa_{xy} = 4.6 \cdot 10^{-5}$ bar^{-1} and time constant τ_P = 1 ps. To improve sampling we employed an elevated temperature simulation protocol. For transmembrane segments, such as the APP-TMD segment studied here, this produces quantitatively accurate folds and transfer-free energies.[21–23]

2.2 | APP-TMD surface density and apparent FRET efficiency measurements

The Fully Quantified Spectral Imaging (FSI) method is utilized in this work to measure the surface densities, and the apparent Forster Resonance Energy Transfer (FRET) efficiencies of an mTurquoise (APP-TMD-mTurquoise) and Yellow-Fluorescent-Protein (APP-TMD-YFP) labelled APP-TMD. The FSI methodology has been described previously in detail.[24] The total apparent FRET efficiencies of oligomers of donor-labeled and acceptor-labeled proteins are calculated using the kinetic theory of FRET formalism.[25,26] The key equations and methodology are summarized in the Supplementary Material.

2.3 | Concentration-dependent modulation of Aβ production

An SH-SY5Y cell line stably expressing human APP was seeded in 12-well plates and grown to confluence. Cells were then treated with APPOP by addition to the culture media at concentrations ranging between 0 and 10 μM or with vehicle (DMSO) and incubated overnight. Culture media were collected after 18 h and levels of secreted Aβ determined using A$\beta_{1\text{-}40}$ enzyme-linked immunosorbent (ELISA) (#KHB3481, R&D systems). Controls received vehicle only and vehicle concentrations (1% DMSO) were held constant across all treatment conditions.

2.4 | Effects on endogenous neuronal Aβ production in primary rat brain cortical cultures

Neuronally enriched rat primary cortical cultures were generated from E18 Sprague Dawley rat embryos and maintained in vitro in NeuroBasal Medium supplemented with B27, 2 mM L-glutamine, 100 IU penicillin, 100 μg/mL streptomycin (all culture reagents were obtained from Invitrogen Life Sciences [ThermoFisher] as described previously.[27] After 21 days in culture, cells were treated with the peptide at a range of concentration up to 10 μM (as indicted), or vehicle (1% DMSO) overnight and endogenously produced rat A$\beta_{1\text{-}40}$ measured by ELISA assay (ThermoFisher: #KMB3441).

2.5 | Cell viability

Cell viability was determined by measuring intracellular ATP levels normalized to total protein content. Cells were seeded as described earlier. Following treatment and removal of culture media, 6.5% trichloroacetic acid (TCA) in phosphate-buffered saline (PBS) was

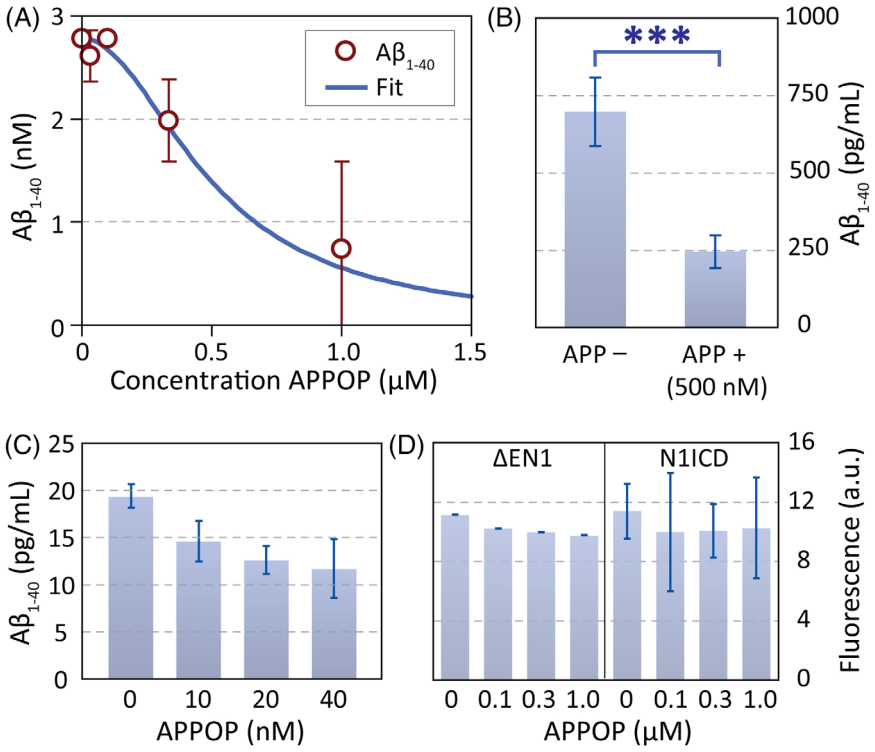

FIGURE 1 APP membrane domain structure and Aβ genesis. (A) Aβ is released from the membrane after consecutive proteolysis of APP by β- and γ-secretases. The proteolytic target sites on APP are indicated by arrows; the glycine and alanine residues of the GxxxA motif are highlighted in red. (B) Atomic detail structure of the APP membrane domain in a lipid bilayer determined via an unbiased folding MD simulation. (C) Center of mass distance (GxxxA/GxxxA dimer: $r = 7$ Å, red arrow). (D) Snapshots of the three types of dimers found, together with a cluster analysis that reveals the populations of the most common dimeric structures. The GxxxA/GxxxA dimer (1, red) is the most populated and compact single cluster, followed by closely associated clusters (2, green), and loosely attached dimers (3, blue). (E) Dimer PMF, highlighting the relative energies of the three types of dimers. The lowest free-energy state is associated with loosely bound dimeric states (3). Aβ, amyloid beta; APP, amyloid beta precursor protein; APP, amyloid beta precursor protein; MD, molecular dynamics; PMF, potential of mean force.

applied to cells to precipitate ATP. After removal of TCA, total cell protein was collected by the addition of 0.5 M NaOH.

For intracellular ATP quantification, samples were dispensed into white, solid-bottomed 96-well plates in duplicate alongside standards of known ATP concentration (Sigma, A26209). Reconstituted crude firefly lantern extract solution (Sigma FLE250) was injected into each well and luminescence detected using a CLARIOstar plate reader (BMG Labtech, UK).

Protein concentration was determined using a Pierce bicinchoninic acid assay (BCA) Protein Assay kit (Thermo Scientific), and the assay was performed according to manufacturer's instructions. ATP concentrations were calculated from a blank-corrected standard curve ($R^2 \geq 0.995$) and normalized to protein concentration.

3 | RESULTS

3.1 | The structure and dynamics of the APP-TMD in a lipid bilayer

We used unbiased long-timescale molecular dynamics (MD) simulations,[28,29] to determine the native APP-TMD structure in a lipid bilayer and explore the dimerization dynamics and structural features of the dimerization interface. Starting from a fully extended configuration, APP-TMD rapidly forms a stable membrane–spanning helix, with both dimerization motifs fully buried in the membrane (Figure 1B). The helicity was confirmed experimentally using circular dichroism (CD) spectroscopy, which also revealed that membrane-embedded APP-TMD is stable against thermal denaturation, with no loss of helicity even at 95°C (extended data Figure 1).

We determined the monomer-dimer equilibrium and conformational ensemble using 100 μs unbiased assembly MD simulations (Figure 1C), which captured multiple dimerization events and the equilibrium ensemble of monomeric and dimeric conformers. Of these, only the dimer with the interface formed by the GxxxA motif had significant compactness and stability, whereas other dimers are short-lived random collisions (Figure 1D). Cluster analysis reveals that monomeric APP-TMD is the dominant state with a population of >83%, whereas GxxxA and GxxxGxxxG dimers have populations of 1.6% and 0.03%, respectively. The remaining dimeric population are short-lived random collisions. Thus the GxxxA interface dimer, which contains the γ-secretase cleavage site, is the dominant dimeric form, but with a free energy ≈1 kcal/mol higher than the monomeric state.

Analysis of the GxxxA dimer packing interface found in the simulations reveals a contact area of 420 ± 60 Å^2, similar to the 470 ± 30 Å^2 found in the detergent micelle NMR structures, with relatively minor changes in packing residues (extended data Figure 2).

These data suggest that in its native state the overwhelming majority of APP-TMDs are susceptible to γ-secretase proteolysis.

3.2 | An APP occlusion peptide (APPOP) induces dose-dependent reduction of Aβ production

To explore the possibility of protecting APP from γ-cleavage we generated a solubilized synthetic peptide containing the APP-TMD with additional N- and C-terminal lysine residues: KKKKGAIIGLMVGGVVIATVIIITLVMLKKKK. This APP occlusion peptide (APPOP) is designed to autonomously insert into cell membranes and form heterodimers

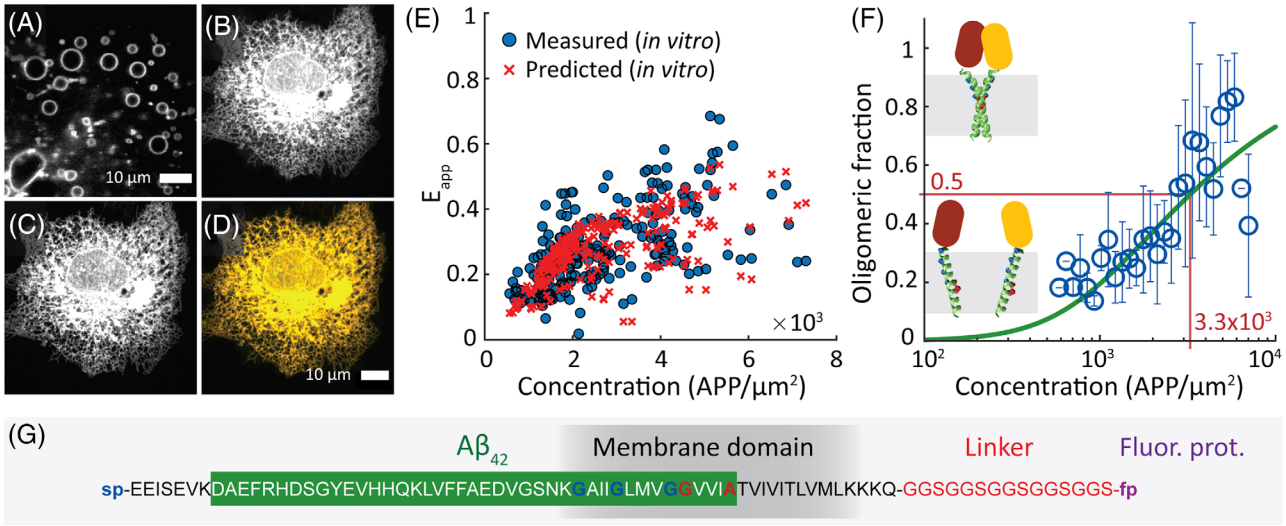

FIGURE 2 APPOP efficacy and selectivity in vitro. (A) Dose-dependent reduction of Aβ levels in SH-SY5Y cells, stably transfected to express APP, upon titration with APPOP. (B) Reduction in Aβ production levels upon administration of a single 500 nM dose of APPOP in SH-SY5Y cells, transiently transfected to express APP, $^{***}p \leq 0.001$. (C) Dose-dependent reduction of native Aβ production in primary rat cortical neuronal cultures upon titration of APPOP. (D) CBF1 luciferase-based reporter gene activity induced by γ-secretase-dependent, ΔEN1, and γ-secretase-independent, N1ICD, Notch-1 constructs upon APPOP titration. Each of the assays were performed a minimum of three times, $N = 3$, with $n = 3$ technical replicates per condition. Data shown in A–C are the average of three assays. (C) Shows a single, representative, reporter gene assay. Error bars in all panels show standard deviation. APPOP, amyloid beta precursor protein occlusion peptide.

with the TMD region of APP with high specificity, thereby reducing Aβ production.

Protection of APP from proteolytic processing was evaluated by co-incubation of cells with APPOP in three systems: (1) a SH-SY5Y cell line stably expressing wild-type (WT) human APP, (2) SH-SY5Y cells transiently transfected with APP, and (3) WT primary rat cortical neurons natively expressing endogenous APP.

Aβ production as a function of APPOP concentration was measured using ELISAs that detect A$\beta_{1\text{-}40}$ after titration of cells with 10 to 1000 nM APPOP. Inhibition of A$\beta_{1\text{-}40}$ production is dose dependent, with a 50% reduction of Aβ production at ≈450 nM APPOP in both stably (Figure 2A) and transiently (Figure 2B) transfected SH-SY5Y cell. In primary rat cortical neurons, which naturally expressing APP at much lower levels that the transfected SH-SY5Y cells, A$\beta_{1\text{-}40}$ production can be reduced by ≈40% with 40 nM APPOP (Figure 2C).

3.3 | APPOP does not inhibit γ-secretase activity for Notch-1

To evaluate the specificity of APPOP for protecting APP we used a Notch-1 reporter assay. Notch-1 is a highly conserved receptor essential for intercellular communication that requires cleavage by γ-secretase for canonical Notch signal to occur. Direct inhibition of γ-secretase impairs Notch signaling, which is to be avoided given the fundamental roles of Notch signaling in the developing and mature brain.

The reporter assay utilizes a firefly luciferase reporter gene driven by a promoter harboring multiple consensus Centromere Binding Factor 1 (CBF-1) transcription factor binding sites to which the fully cleaved Notch-1 intracellular domain (ICD) binds to activate transcription. We activated this reporter with two Notch ICD constructs: N1ICD, the fully cleaved form of the Notch-1 ICD and ΔEN1, a Notch-1 ICD construct including the transmembrane membrane region that requires γ-secretase cleavage for transcriptional activity.

Titration with APPOP (Figure 2D) did not impact the signaling ability of either ΔEN1 or N1ICD, demonstrating that γ-secretase activity is unaffected by APPOP. This suggests that APPOP selectively protects APP from γ-cleavage without impacting γ-secretase directly.

3.4 | APPOP toxicity

Cells treated with APPOP appear visually healthy (extended data Figure 3). Toxicity was assessed by measuring the total protein to ATP content of cells treated with APPOP (extended data Figure 3). This showed no statistically significant changes in intracellular ATP, protein content, or the ratio of ATP to protein, for both rat primary neurons and SH-SY5Y cells. Thus APPOP results in no detectable toxicity over medium for concentrations up to 300 nM in rat primary neurons and up to 750 nM in SH-SY5Y cells, the highest doses used, respectively.

3.5 | APP-TMD oligomeric form in live cells

To investigate the mechanism of APP-TMD protection by APPOP we measured the strength of the APP-TMD interaction in the endoplasmic reticulum (ER) membrane of live cells by quantitative Förster

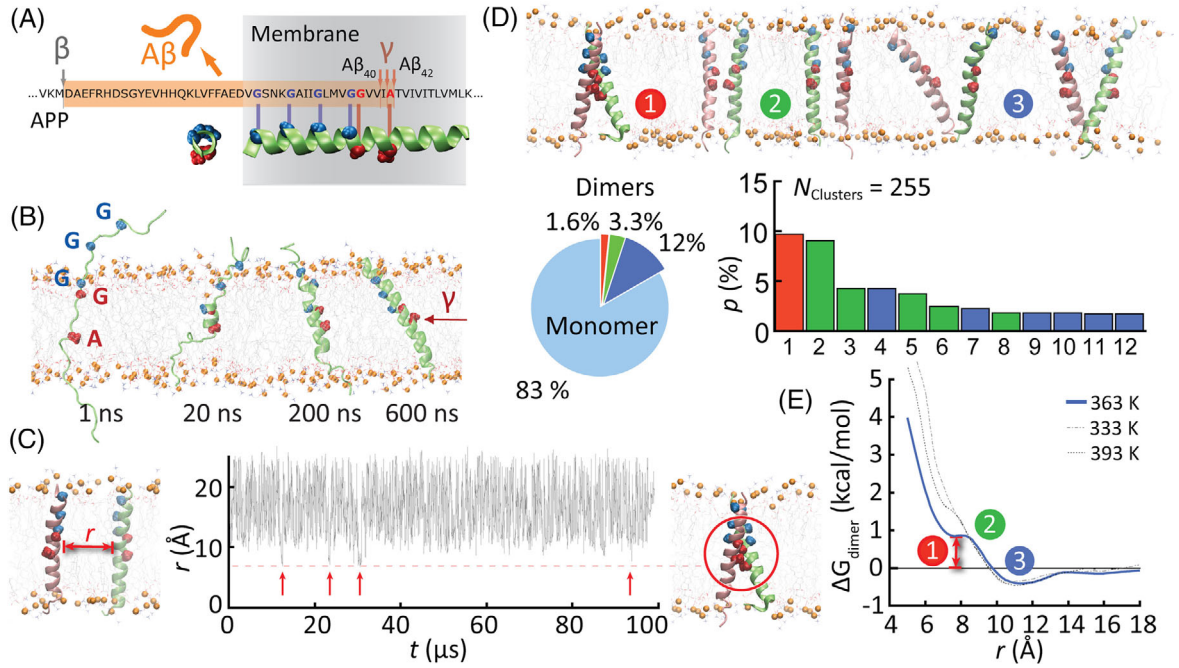

FIGURE 3 Measurement of APP-TMD oligomerization. (A) ER-derived large intracellular vesicles containing YFP-labeled APP-TMD constructs. (B) Cellular location of YFP-labeled APP-TMD. (C) Cellular location of mCherry-labeled Sec61β. (D) Overlay of YFP-labeled APP-TMD and mCherry-labelled Sec61β. (E) Measured and predicted apparent energy transfer (E_{app}) from the in vitro experiment. (F) Oligomeric fraction determined from the vesicles in (A) as a function of APP-TMD concentration. Error bars show the standard deviation. (G) APP-TMD-FP constructs designs. sp, APP signal peptide; fp, fluorescent protein.

resonance energy transfer (FRET) using the FSI methodology.[24] Membranes with a locally flat topology are required for protein surface density measurements with the FSI method. Thus we utilized hypotonic treatment to create ER-derived Large Intra-Cellular Vesicles (LICVs)[30] in COS7 cells transiently transfected to co-express APP-TMD constructs tagged with mTurquoise (the FRET donor) and yellow fluorescent protein (YFP) (the FRET acceptor) (Figure 3A).

Co-localization of the fluorescently labeled APP-TMD constructs with mCherry-labeled Sec61β translocons demonstrates successful membrane integration (Figure 3B-D). A total of 123 cells were imaged at room temperature with the OptiMis spectral imaging microscope in two separate experiments, yielding 240 data points from ER LICV membranes. The data were then fit with a thermodynamic model for APP membrane dimerization (Figure 3E, see Methods for details). The best-fit dimerization model indicates a weakly favorable association between APP-TMDs in the ER membrane, with a Gibbs free energy of association of -3.0 ± 0.1 kcal/mol. With this dimerization affinity, surface densities of the APP-TMD will need to exceed 3300 segments/μm^2 for greater than 50% of the proteins to be in an oligomeric state (Figure 3F). The best-fit donor-acceptor pair-wise FRET efficiency is high: 0.87 (0.79, 0.94), indicating that the donor and the acceptor fluorophores are located in proximity to oligomeric APP-TMDs. Construct designs were validated by sequencing (Figure 3G).

These data suggest that the vast majority of APP-TMDs are susceptible to γ-secretase proteolysis, in agreement with the MD simulations.

4 | DISCUSSION

Despite the continued, and much publicized, failure of amyloid-targeting therapies to show any substantial patient benefit in clinical trials, genetic and experimental data support the idea that lowering Aβ levels in brain is the key therapeutic goal when attempting to address AD.[31] Although it is not clear whether in the sporadic form of AD the increased amyloid burden in brain is due to an overproduction of Aβ, a shift in relative amounts of Aβ species 1–40 and 1–42, or a failure of clearance mechanisms, a reduction in total Aβ production is still thought to hold the potential to provide patient benefit. Thus identifying a safer means to modulate Aβ production remains a key milestone toward developing an effective treatment for AD.

APP dimerization has been proposed as a possible factor in modulating protease processing. The APP-TMD, in particular, contains three GxxxG/A motifs that enable close association of membrane-spanning helices and are commonly found in single-span membrane receptors that require dimerization of the membrane domain for signal transduction. Indeed, SDS-PAGE analysis found WT APP-TMD to form a stable dimer[32] and solution NMR studies established that the dimer interface is formed by the GxxxA motif.[14]

Of interest, familial AD mutations are clustered at both this motif (e.g., Iranian, T714A; Austrian T714I, German V715A; French V715M) and other residues forming the dimeric interface (e.g., Florida I716V; London V717I; Indiana V717F), suggesting a possible role of APP-TMD dimerization in AD pathogenesis. Mutational studies aimed at

disrupting the APP-TMD dimer interface observed shifts in the ratios of different Aβ lengths, but did not significantly affect overall production levels.[33] Further studies established that only the monomeric form of the APP-TMD can be processed by γ-secretase,[34] with the recent structure of a single APP-TMD chain trapped in the proteolytic cleft of γ-secretase providing strong structural support.[16]

These studies suggest that targeting the APP-TMD, rather than γ-secretase may provide a more selective and thus safer route to specifically modulate APP processing and Aβ production. Indeed, the present study demonstrates that selective occlusion of the γ-secretase cleavage site on APP, in the form of a solubilized APP-TMD, is able to reduce Aβ production in rat primary neurons and neuronal phenotype SH-SY5Y cells. A similar approach has been shown previously to reduce γ-cleavage of HEK293 cells expressing Swedish APP mutants, with solubilized APP-TMDs containing the Indiana mutation (V717F) providing the strongest reduction in Aβ production.[32] Here we further show that the Notch-1 processing is unaffected using this approach, suggesting that solubilized APP-TMD selectively protects APP. This resolves the main challenge associated with γ-secretase inhibitors, which is their lack of specificity toward the many other biologically important substrates of the enzyme complex. Despite these encouraging results, a key shortcoming of the current solubilized APP-TMD design is its poor solubility. This makes long-term dose–response and toxicity studies unreliable and will need to be addressed in future pre-clinical studies, either via re-engineering of the APP-TMD sequence or encapsulation into a formulation better suitable for delivery.

Previous studies,[32] as well as the NMR structure,[14] have suggested that APP-TMDs are predominantly dimeric. However, we show here, using a novel intracellular FRET assay in live cells, that APP-TMDs are predominantly monomeric and located primarily in the ER (Figure 3B). This shows that APPOP is able to cross the plasma membrane, which suggests it may be suitable for brain delivery. Unbiased atomic detail simulations of the folding and dimerization process confirm the APP-TMD monomer as the predominant equilibrium state (Figure 1).

Although the effect of APPOP appears directly linked to APP membrane dimerization, a previous study found that P8, a highly charged fragment (sequence: DEEEDEEL) corresponding to residues 66–73 of the presenilin-1 N-terminal domain, can also inhibit Aβ production, albeit via strong and specific binding to the APP ectodomain.[35] Presenilin-1 is the catalytic component of the γ-secretase complex, suggesting that P8 also acts by blocking APP reaching the proteolytic site of the y-secretase complex. This suggests that APP dimerization is sufficient, but not necessary, to block Aβ production, and that other binding sites on APP can also protect it from γ-secretase. Similar to the current study, secretase activity was found to be unaffected. In a follow-up study the same authors demonstrated the clinical potential of P8 by exploring pharmacokinetics and administrative routes in vivo, with subcutaneous delivery being the most promising route.[35]

Finally, a very recent related study investigated a small peptide fragment derived from APP, sAPPα, which contains the sequence segment cleaved by BACE1.[36] The authors report reduced production of Aβ in vitro, as well as reduced Aβ deposition, tau phosphorylation, and neuro-inflammation, and rescuing of behavioral deficits in vivo. This study suggests that protecting APP via peptide binding or sandwiching of peptides at the interface between APP and the proteolytic enzyme seems to work for both secretases involved in Aβ production. Together, these prior studies highlight the relevance of the use of APP-targeting peptides in blocking Aβ production as a viable approach for the treatment of AD.

In summary, protecting APP from proteolysis offers an elegant alternative route to quantitatively and selectively modulate Aβ production with unprecedented specificity. This opens a new avenue for generating novel, potentially safe, and inexpensive peptide-based therapeutics that can reduce Aβ levels. Apart from the clinical potential, this approach also provides an exciting means to specifically regulate Aβ levels in experimental models, which will be of broad utility for AD research in general. The next steps toward translation will require development of a suitable APPOP formulation and thorough toxicological evaluation.

The present study has focused on demonstrating the validity of a novel peptide-based methodology for reducing Aβ production through in vitro studies. Use has been made of primary rat cortical neurons, which retain their in vivo characteristics better than continuous cell lines, and undifferentiated SH-SY5Y cells stably expressing APP as disease models.[36] Although these techniques are suited for the drug discovery stage, the next steps toward APPOP translation will require a thorough in vivo evaluation of efficacy of Aβ regulation and cognitive benefit, as well as demonstration of safety and tolerability. These data are essential for de-risking further pre-clinical evaluation toward phase I trials.

For APPOP this will require overcoming the pharmacological and delivery challenges generally associated with peptides. Although peptides offer superior targeting precision compared to small molecules, pharmacokinetics and plasma stability are generally poor, and delivery options are often limited. Hydrophobic peptides that naturally reside in membranes, such as APPOP, pose a further challenge due to their extremely poor solubility, which results in immediate precipitation at the injection site upon intravenous administration (the half life of APPOP in plasma is estimated to be on the order of a second), This precludes in vivo studies, as the peptide will not be able to reach the brain microvasculature, even if injected directly into circulation.

Fortunately the last 20 years have seen tremendous progress in the design of biocompatible nanocarriers that can be precisely tailored to encapsulate poorly soluble therapeutics, making them suitable for intravenous administration.[37] We have recently demonstrated that this approach can deliver a poorly soluble hydrophobic peptide that preferentially targets and perforates cancer cell membranes. In this study a specially designed nanocarrier enabled successful intravenous delivery of the therapeutic peptide for xenograft studies demonstrating good in vivo efficacy.[38] Two FDA-approved block-co-polymers, PLGA and PEG, were used to encapsulate the peptide in ultra-small 20-nm diameter nanoparticles, which typically circulate for days, greatly improving pharmacokinetics, preventing precipitation, and protecting the therapeutic peptide en route to target tissues.

A similar approach is anticipated for APPOP. Once available, this formulation will enable an in vivo pilot study to (1) demonstrate that

effective Aβ modulation can be achieved in vivo and is sufficient to arrest plaque formation in AD animal models, (2) determine the potential cognitive benefit associated with arresting Aβ production via APPOP, and (3) assess basic safety and tolerability demonstrating that this type of therapeutic is suitable for long-term use. With these data in hand, APPOP will be suitably de-risked for thorough pre-clinical evaluation.

AUTHOR CONTRIBUTIONS

Ayesha Khan and Martin B. Ulmschneider designed the study. Ayesha Khan designed the APPOP segment. Christopher R. King developed the in vitro APP-TMD-FP constructs and carried out intra-cellular FRET. Ayesha Khan, Martin B. Ulmschneider, and Jakob P. Ulmschneider carried out molecular dynamics simulations. Richard Killick designed the in vitro assays and carried out dose–response and cell-viability studies. Daniel Wirth, Dominique Hoogland, and Kalina Hristova carried out and analyzed FRET experiments. All authors analyzed the data. Ayesha Khan, Richard Killick, and Martin B. Ulmschneider wrote the paper with input from all authors.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest. Author disclosures are available in the supporting information.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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