A “Common-Precursor” Protein Mimetic Approach to Rescue Aβ Aggregation-mediated Alzheimer’s Phenotypes

Ryan A. Dohoney\textsuperscript{1,2}, Johnson A. Joseph\textsuperscript{1,2}, Charles Baysah\textsuperscript{1,2}, Alexandra G. Thomas\textsuperscript{2,3}, Apshara Siwakoti\textsuperscript{2,3}, Tyler D. Ball\textsuperscript{1,2}, Sunil Kumar\textsuperscript{1,2,4}

\textsuperscript{1}The Department of Chemistry and Biochemistry, \textsuperscript{2}The Knoebel Institute for Healthy Aging, \textsuperscript{3}The Department of Biological Sciences, University of Denver, Denver, CO 80210

\textsuperscript{4}Correspondence: sunil.kumar97@du.edu
ABSTRACT

Aberrant protein-protein interactions (aPPIs) are associated with an array of pathological conditions, which make them important therapeutic targets. The aPPIs are mediated via specific chemical interactions that spread over a large and hydrophobic surface. Therefore, ligands that can complement the surface topography and chemical fingerprints could manipulate aPPIs. Oligopyridylamides (OPs) are synthetic protein mimetics that have been shown to manipulate aPPIs. However, the previous OP library used to disrupt these aPPIs was moderate in number (~30 OPs) with very limited chemical diversity. The onus is on the laborious and time-consuming synthetic pathways with multiple chromatography steps. We have developed a novel chromatography-free technique to synthesize a highly diverse chemical library of OPs using a “common-precursor” approach. We significantly expanded the chemical diversity of OPs using a chromatography-free high-yielding method. To validate our novel approach, we have synthesized an OP with identical chemical diversity to a pre-existing OP-base potent inhibitor of Aβ aggregation, a process central to Alzheimer’s disease (AD). The newly synthesized OP ligand (RD242) was very potent in inhibiting Aβ aggregation and rescuing AD phenotypes in an in vivo model. Moreover, RD242 was very effective in rescuing AD phenotypes in a post-disease onset AD model. We envision that our “common-precursor” synthetic approach will have tremendous potential as it is expandable for other oligoamide scaffolds to enhance affinity for disease-relevant targets.
INTRODUCTION

Protein-protein interactions (PPIs) are essential for the regulation of many biological processes."1-12 Consequently, aPPIs can lead to numerous pathological conditions, including infectious diseases, cancer, and amyloid diseases."1-13 Therefore, aPPIs are biologically compelling targets for drug discovery and the specific targeting and modulation of aPPIs are considered promising therapeutic interventions toward various pathologies. During aPPIs, the chemical interactions between protein interfaces are spread over a large surface area, making them very challenging to modulate with small molecules."3,4,6,12 Peptides are a potential strategy to modulate aPPIs because the large surface area of peptides can complement aPPIs interfaces. However, poor chemical, structural, and enzymatic stability in the cellular milieu and poor cell permeability of peptides limit their pharmaceutical applications in targeting aPPIs."14,15 Therefore, ligands with combined properties of peptides and small molecules could offer a therapeutic solution to modulate these pathological interactions. Oligopyridylamides (OPs) are a class of ligands with a large surface area and synthetically tunable side chains, which mimic the topography and side-chain residues of proteins such as those present at interfaces of aPPIs."16-35 Accordingly, OPs possess the properties of both small molecules and peptides. OPs have been shown to manipulate multiple aPPIs, which are associated with various diseases, including islet amyloid polypeptide,"18,21,24,29 Aβ peptide,"19,20 and mutant p53,"23 which are associated with type 2 diabetes (T2D), Alzheimer’s disease (AD), and cancer, respectively. OPs have shown desirable pharmaceutical properties in terms of their high enzymatic stability, no cytotoxicity, and high efficacy and potency in mouse models for other amyloid targets because of the non-proteogenic side chains and an aromatic backbone scaffold."23 The OPs tested previously against various targets were limited in the chemical diversity of side chains and no efforts were directed to optimize the binding affinity and specificity for OPs against their pathological targets. These limitations for OPs are a consequence of the tedious multistep and chromatography-based synthetic steps."16-35 The synthesis of one tripyridyl with three chemically distinct side chains requires a total of 14 synthetic steps with 11 chromatography steps."16-24 Because of the very long, chromatography-based, and complex synthetic route, a very small number of side chains were used to synthesize OPs Consequently, a very small number of tripyridyls with limited chemical diversity of the side chains were synthesized (~30)."16-35 The small number of OPs and the limited chemical diversity of side chains on OPs limit their potential to achieve high specificity and activity against their pathological targets. From the first report on OPs 20 years ago until now, no synthetic modifications have been carried out in OPs to synthesize a larger library with higher chemical diversity of the side chains."16-35
In this report, we have utilized a novel “common-precursor” approach to generate an OP library with large chemical diversity of the side chains. Our approach is completely chromatography-free and relies on a common precursor, to synthesize an OP library with chemically diverse side chains. Using this approach, we have synthesized an OP library with a very diverse chemical space on the side chains. To demonstrate the feasibility of our approach, we have synthesized an OP (RD242) with identical side chains to a pre-existing OP (ADH-41) potent antagonist of Aβ aggregation, a process critical to the etiology of AD. The process of the aggregation and the accumulation of Aβ into fibers has been proposed to be one of the main causal agents associated with the onset of AD, which is the most common type of neurodegenerative disorder. Aβ peptide is a cleaved downstream product of the neuro-transmembrane protein, amyloid precursor protein (APP). Under pathological conditions, Aβ aggregates into insoluble plaques in the central nervous system and has been linked to impaired neuronal functions and declined cognitive behavior, which are early symptoms of AD. There are ~50 million people suffering from AD worldwide and the number of AD patients is expected to reach ~65 million by the end of this decade. Therefore, there is a pressing need to find drugs that can cure or slow the progression of AD. Therefore, strategies that modulate the aggregation of Aβ could be considered potential therapeutic approaches for the treatment of AD.

We have shown earlier that ADH-41 is a potent antagonist of Aβ aggregation. Similar to ADH-41, the newly synthesized OP (RD242) from our novel method was also a potent antagonist of Aβ aggregation. In this report, we have also tested the effect of RD242 on Aβ aggregation in an in vivo AD model. The OP was highly effective in inhibiting intracellular Aβ aggregation in a C. elegans AD model (GMC101 worm strain). Moreover, the OP was potent in rescuing various AD phenotypes mediated by Aβ aggregation in GMC101 worms. The OP significantly improved the motility rate and attenuated the radical oxygen species (ROS) level in GMC101 worms. The activity of the OP was compared with Bexarotene, which is a very effective ligand in rescuing Aβ aggregation-mediated AD phenotypes in GMC101 worms. The OP ligand demonstrated a greater effect than Bexarotene in rescuing AD phenotypes in GMC101 worms. Additionally, RD242 was effective in suppressing AD phenotypes in a post-disease onset AD model, which makes this ligand very relevant to the current therapies for AD as most of the current therapeutic strategies focus on the treatment after the diagnosis of AD. To the best of our knowledge, this is the first ligand that was able to suppress Aβ aggregation in a post-disease onset in vivo AD model. Our newly synthesized OP could be considered as a template scaffold to develop lead therapeutics to slow the progression of AD symptoms in the post-diagnosed AD model.

Overall, we have developed a novel chromatography-free “common-precursor” based synthetic approach and applied it to an OP scaffold to enhance the size of the library with a more extensive chemical space.
Also, we demonstrated its utility in the identification of potent antagonists of intracellular Aβ aggregation mediated AD phenotypes in both early and post-disease \textit{in vivo} AD models. We envision that this study can be used as a prototype to generate large libraries (with diverse chemical space) of various oligoamide scaffolds. The enhanced diversity of chemical space in oligoamide scaffolds will significantly optimize their specificity and activity for various pathological targets.
RESULTS and DISCUSSION

The novel chromatography-free common precursor approach to generate an OP library

The OPs have previously been shown to act as antagonists of numerous aPPIs, including HIV gp41 oligomerization,22 Bak BH3/Bcl-xL,16,34 p53/HDM2,30 and amyloid proteins.18-21,23,24,27,29,31,35 For most of the pathological targets (protein-protein or protein-nucleic acid interactions), the tripyridyls were the most potent ligands.16-35 However, the total number of tripyridyls synthesized using the literature-based synthetic method was a very small library of tripyridyls (~30) with limited chemical diversity of the side chains.16-35 Moreover, no further studies were carried out to optimize the antagonist activity of tripyridyls for their pathological targets. The main reason behind these shortcomings is that the existing methods to generate OP ligands are very tedious as they require a large number of synthetic steps with multiple chromatography steps (Figure 1, previous work). The synthesis of one tripyridyl with distinct side chains requires a total of 14 synthetic steps and 11 chromatography steps with an overall yield of 0.78%-3.61% (Figure 1a-o, Table 1, the range is attributed to different side chains having different % yields. The overall % yield was calculated by multiplying the yield of each synthetic step).16-35 Also, the reported side chains on tripyridyls were trivial functional groups, including a primary amine, a carboxylic acid, and 7-8 hydrophobic functional groups (Figure 2, previous work).16-35 It is very challenging to synthesize a large library of OPs with more diverse and complex functional groups to mimic the side chains of natural amino acids using the literature-based synthetic method.16-35

We have identified a novel synthetic method to prepare the OP library with a large chemical diversity of the side chains using a chromatography-free method with a significantly lesser number of steps and a much higher % yield (Table 1 for comparison). Our novel method uses common precursors (mono-, di- and tripyridyl) to append the side chain functionalities (Figure 1, current method). The introduction of the side chains and the amide coupling for the elongation of the backbone (from mono- to di- to tripyridyls) were achieved using a completely chromatography-free method. We demonstrate the synthesis of a tripyridyl (RD239DEP) with three distinct side chains as a representative example (Figure 1). We used commercially available 2-chloro-6-methyl-3-nitropyridine as a common precursor to synthesize RD124 by introducing tert-butyl (3-hydroxypropyl) carbamate as a side chain in the presence of NaH (Figure 1p, see supporting information for synthesis). The product did not require column chromatography because the excess reagent [tert-butyl (3-hydroxypropyl) carbamate] was evaporated by lyophilization (See supporting information for synthesis). Subsequently, RD124 was reduced and reacted with 6-chloro-5-nitropicolinoyl chloride to form the dipyridyl (RD129, Figure 1q,r) using a chromatography-free amide coupling. It is worth noting that the amide coupling did not require any column chromatography. In
contrast, the amide coupling used in previous methods to elongate the backbone in oligoamides always required column chromatography to purify the product.\textsuperscript{16-35} Subsequently, RD129 was reacted with butanol in the presence of sodium metal to form the dipyridyl (RD183, Figure 1s) using a chromatography-free method. The dipyridyl (RD183) was reduced and coupled with 6-chloro-5-nitropicolinoyl chloride to form the tripyridyl (RD215) using a chromatography-free amide coupling (Figure 1t,u). The tripyridyl was then reacted with isopropanol in the presence of sodium metal to form the tripyridyl (RD239, Figure 1v), which was deprotected (TFA/DCM/TES) to synthesize the final product (RD239DEP) with distinct side chains using a chromatography-free method (Figure 1w). In comparison to the old method, our new method has several advantages, including high yield, fewer synthetic steps, no column chromatography, and increased chemical diversity (Table 1).

We used a slight modification to the above-mentioned method for the synthesis of the unsaturated (alkyne) or acid-sensitive side chains (Boc protected amine, See supporting information for synthesis, method 2,3). We surmise that the excess NaH (or Na metal) in the reaction mixture might be reacting with the unsaturated alkyne or boc-protected side chains, which give side products in the reaction. Therefore, we tuned the synthetic method to avoid the formation of side products (See supporting information for synthesis, method 2,3). In addition to the primary alcohol, we have also used the common precursor approach to append primary amine-based side chains on pyridyls as well. The pyridyls (mono, di-, or tri-) were treated with primary amines in the presence of a base to append various side chain functionalities on the surface of pyridyls in high yields using a chromatography-free method (Figure 1,2, see supporting information for synthesis, method 4). The excess primary amine and base were dried via lyophilization, which left the final product (pyridyl with amine side chains) in pure form. The appending of the primary amines as side chains on the OPs has also been reported recently by us.\textsuperscript{51} Here, we synthesized an OP library to demonstrate the feasibility of our novel approach (Figure 2). The only difference between the previous OPs and the newly synthesized OPs is the C-terminal functional group, which is a -COOMe in the former and a -Me in the latter (Figure 2). Using this synthetic approach, we can expand the chemical diversity of the side chains of OPs by reacting with various primary alcohols and amines. This approach can be extended to other oligoamide scaffolds yielding a much larger chemical space to identify lead compounds to target various pathological aPPIs.

**Biophysical characterization between RD242 and the Aβ peptide**

We have shown earlier that the tripyridyl, ADH-41 was a potent antagonist of Aβ aggregation, and the side chain functionalities of ADH-41 were essential for its antagonist activity.\textsuperscript{19} Using our novel chromatography-free method, we synthesized a tripyridyl ligand (RD242) whose side chains were identical to the side chains of ADH-41 (Figure 2). Since the side chains were essential for the antagonist
activity, we surmised that RD242 would be a potent antagonist of Aβ42 aggregation (similar to ADH-41). To test the antagonist activity of RD242, a 10 μM solution of Aβ42 in PBS buffer (1 × PBS) was incubated in the absence and presence of RD242 and ADH-41 at an equimolar ratio for 24 h. In the absence of ligands, Aβ42 was aggregated, which was quantified by a high Thioflavin T (ThT) fluorescence signal (Figure 3a), and an abundance of fibers was visualized by transmission electron microscopy (TEM, Figure 3b). At an equimolar ratio, both ligands (RD242 and ADH-41) were potent antagonists of Aβ42 aggregation, represented by a low ThT fluorescence intensity, similar to the control ThT signal (Figure 3a). Also, the TEM image shows no formation of Aβ42 fibers in the presence of RD242 at an equimolar ratio (Figure 3c). Both ligands did not exhibit quenching of the fluorescence intensity of the ThT dye (Figure 3d). Also, we used the heteronuclear single quantum coherence (HSQC) 2D-NMR experiments to identify the binding site of RD242 on the Aβ42 peptide. We used a solution of 20 μM 15N-labelled Aβ42 (10 mM NaPi, pH 7.4) in the absence and presence of RD242 at an equimolar ratio for the HSQC experiment. The changes in the volume of the peaks for amino acid residues of Aβ42 peptide were compared in the absence and presence of RD242 (Figure 3e). The changes in the volume of the amino acid residue peaks of Aβ42 in the presence of RD242 is a consequence of the interaction of Aβ42 with RD242. We observed a decrease in the volume of amino acid residues from E11 to F20 in the presence of RD242 (Figure 3e), suggesting these residues are the potential binding site of RD242 on the Aβ42 peptide. It is important to note that the binding site of RD242 was in very close vicinity to ADH-41 as demonstrated earlier.19 RD242 interacts with the Aβ42 residues spanning from E11 to F20, which have been suggested to play an important role in Aβ fibrillation52. More specifically, a part of the anti-amyloidogenic activity of RD242 is a consequence of the modulation of π–π aromatic interactions between F19 and F20, which has been shown to play a key role in the aggregation of the Aβ peptide (Figure 3e).52 Additionally, RD242 also interacts with S26 residue suggested by the change in the volume of the chemical shift (Figure 3e). It has been suggested that the residue S26 also plays an essential role in facilitating the aggregation of Aβ.53,54 Recently, NMR and computational studies demonstrated that the residue S26 forms H-bonding with other residues, which potentiates the aggregation of Aβ.53,54 Our NMR data suggest that RD242 interacts with various Aβ residues and disrupt various interactions, which are essential for Aβ aggregation.53,54 We also used isothermal calorimetry titration (ITC) to characterize the binding affinity of RD242 for the Aβ peptide. Briefly, a stock solution of 200 μM RD242 was titrated into a solution of 10 μM Aβ40 with constant stirring, which resulted in an endothermic binding profile. The ITC thermodynamic profile was fitted using an independent binding model, which yielded a binding stoichiometry of approximately 1:1 (RD242: Aβ40) and a K_d of 0.84 ± 0.11 μM (Figure 3f). It is also
important to note that the binding affinity of RD242 was in close proximity to ADH-41 ($K_d = 0.7 \pm 0.1 \mu M$)\textsuperscript{19}. The data suggest that RD242 is a potent antagonist of Aβ\textsubscript{42} aggregation and the antagonist activity, binding affinity, and binding site against Aβ\textsubscript{42} were in very close proximity to ADH-41. The data also suggest that the -Me functional group (on the C-terminal of RD242) did not affect the antagonist activity, binding affinity, and the binding site against Aβ\textsubscript{42} aggregation. Clearly, we have synthesized a very similar ligand to ADH-41 without sacrificing the antagonist activity against Aβ\textsubscript{42} aggregation using a very convenient “common precursor” chromatography-free method.

**Rescue effect of RD242 on Aβ aggregation-mediated AD phenotypes in an in vivo model**

We have shown that ADH-41 (Ref. 19 and here) and RD242 (this work) are potent antagonists of Aβ\textsubscript{42} aggregation; however, neither of these tripyridyls (ADH-41 and RD242) were tested for their ability to inhibit intracellular Aβ\textsubscript{42} aggregation in an *in vivo* AD model. Therefore, we tested the antagonist activity of RD242 to inhibit the intracellular aggregation of Aβ\textsubscript{42} in an *in vivo* AD model. We utilized a well-established AD model of *C. elegans* strain (GMC101), which expresses the Aβ\textsubscript{42} peptide in the body wall muscle cells.\textsuperscript{50} In this model, the Aβ\textsubscript{42} peptide aggregates in the muscle cells of GMC101 worms as they grow older. The *C. elegans* models have been used to study mechanisms and therapeutic interventions associated with a plethora of neurodegenerative diseases associated with protein aggregation because of the short lifespan (2-3 weeks), tractability to genetic manipulation, distinctive behavioral and neuropathological defects, and high degree of genetic relevance compared to humans.\textsuperscript{50,55-58} The aggregation of Aβ\textsubscript{42} peptide in the muscle cells of the GMC101 strain potentiates multiple AD phenotypes, including the aggregation of Aβ\textsubscript{42} peptide, elevation in the ROS level, and reduced motility rate.\textsuperscript{50,58} We have shown earlier that ADH-41 can efficiently permeate the cell membrane;\textsuperscript{20} therefore, we envision that the cell permeability of RD242 would be comparable to ADH-41 as both ligands are very similar in chemical properties (two primary amines and one benzyl functional group as side chains and molecular weight). We, therefore, tested the antagonist activity of RD242 against Aβ\textsubscript{42} aggregation-mediated AD phenotypes in GMC101 worms. Moreover, we also compared the rescue effect of RD242 with Bexarotene on AD phenotypes in GMC101 worms under matched conditions. Bexarotene is a potent inhibitor of Aβ\textsubscript{42} aggregation and has shown a remarkable effect on the rescue of AD phenotypes in GMC101 worms.\textsuperscript{50} The GMC101 worms were bleached and synchronized using FUDR (5-Fluoro-2'-deoxyuridine) to avoid offspring, similar to the earlier published results\textsuperscript{50}. In the absence of ligands, we observed a gradual increase in the amount of Aβ\textsubscript{42} aggregates in the muscle cells from day one to day ten of adulthood in GMC101 worms (treated with M9 buffer). The intracellular Aβ\textsubscript{42} aggregates were quantified by treating
the GMC101 worms with five μM NIAD-4 dye using a reported procedure.\textsuperscript{50} The NIAD-4 dye has been shown to stain Aβ\textsubscript{42} aggregates in the worms.\textsuperscript{50} We observed a high fluorescence intensity of NIAD4 dye in GMC101 worms determined by confocal imaging (Figure 3h,k). We treated the GMC101 worms with 30 μM RD242 (in M9 buffer) at the larval stage (L4, day 2) and quantified the aggregates of Aβ\textsubscript{42} from day one to day ten of adulthood (Figure 3g). We observed a very low amount of Aβ\textsubscript{42} aggregates from day one to day ten of adulthood represented by a very low fluorescence signal intensity (Figure 3i,k). We observed a low amount of Aβ\textsubscript{42} aggregates from day one to day ten of adulthood in the presence of Bexarotene as well, similar to the earlier reported work\textsuperscript{50} (Figure 3j,k). The data demonstrate that RD242 is more effective in inhibiting the intracellular aggregation of Aβ\textsubscript{42} in GMC101 worms.

Next, we investigated the effect of RD242 on the ROS level in GMC101 worms due to the aggregation of Aβ\textsubscript{42}. It has been shown that the senile plaques in AD patients consist of Aβ\textsubscript{42} aggregates and multiple metal ions.\textsuperscript{59-65} The complex formation of Aβ\textsubscript{42} aggregates with metal ions facilitates the production of ROS.\textsuperscript{59-65} The generation of ROS oxidizes lipids, proteins, and DNA, which contribute to the etiology of AD.\textsuperscript{59-62} Therefore, we investigated the formation of the ROS in GMC101 worms facilitated by the aggregation of Aβ\textsubscript{42}. The ROS was determined using a fluorescent probe (CM-H2DCFDA), which reacts with intra-worm ROS in GMC101 worms (day ten of adulthood) and produces a green fluorescent signal, whose intensity increases up to 2 h (Figure 3l).\textsuperscript{56,66} In marked contrast, we did not observe any significant production of ROS in GMC101 worms (day ten of adulthood) when treated with 30 μM RD242 at the L4 stage (Figure 3l). The ROS level in GMC101 worms treated with RD242 was similar to the control worms (N2, healthy C. elegans strain) (Figure 3l). We did not observe any significant amount of ROS in the control worms as reported by others as well.\textsuperscript{50} Similarly, we observed a decreased level of ROS in GMC101 worms when treated with 30 μM Bexarotene at the L4 stage (Figure 3l). The data suggest that RD242 is a potent antagonist of intracellular aggregation of Aβ\textsubscript{42} in an \textit{in vivo} model.

Next, we investigated the effect of RD242 on the motility rate of GMC101 worms. The GMC101 worms express Aβ\textsubscript{42} in body wall muscle cells, where it aggregates during the aging process, which lead to a significant decline in the motility rate of GMC101 worms.\textsuperscript{50,67-69} We utilized the ARENA plate reader\textsuperscript{57,70} to measure the motility rate of GMC101 worms in the absence and presence of RD242. The ARENA plate reader is a well-established instrument to measure the motility rate of worms.\textsuperscript{57,70} The GMC101 worms were bleached and synchronized as described in the earlier experiments. We observed a significant decline in the motility rate of GMC101 worms from day two to day 11 of adulthood in comparison to the N2 worms (Figure 4a,b), similar to the earlier reports.\textsuperscript{50,67-69} The GMC101 worms were treated with 30 μM RD242 at the L4 stage and the motility rate was determined from day two to day 11 of adulthood. There was a significant improvement in the motility rate of GMC101 worms in the presence of RD242 (Figure
4a,b). The motility rate for GMC101 treated with 30 μM RD242 was closer to the N2 worms (Figure 4a,b), which indicates that RD242 is very effective in rescuing motility rate by inhibiting intracellular Aβ42 aggregation in GMC101 worms. Under matched conditions, Bexarotene was also very effective in improving the motility rate of GMC101 worms, which has been previously reported as a potent antagonist of Aβ42 aggregation in GMC101 worms. It is important to note that under matched conditions, RD242 was a better ligand than Bexarotene in improving the motility rate of GMC101 worms. We also observed a noticeable effect of RD242 at a lower dose (10 μM) in improving the motility rate of GMC101 worms on the 9th day of adulthood (Figure 4b). Additionally, we also demonstrated that RD-242 is not toxic to C. elegans. We treated the control worms (N2) with 30 µM RD242 on day 2 and then measured the motility rate of N2 worms. We did not observe any significant change in the motility rate of N2 in the absence and presence of RD242 (Figure 4c). Moreover, we also tested the stability of RD242 in the biological milieu. To test the stability of RD242, we used the cell lysate of GMC101 worms (treated with 30 µM RD242 on day 2), which were 10 days old. We used the LC/MS and identified the molecular weight of RD242 in the cell lysate of GMC101 worms (Figure 4d). It clearly suggests that RD242 is stable in the biological milieu. Additionally, we have shown earlier that a very similar ligand (ADH-6) was tested in a mouse model, and it did not display any cytotoxicity. The chemical structure and the physicochemical properties of RD242 and ADH-6 (Table S1) ligands are very similar; therefore, we envision that RD242 will likely behave similarly to ADH-6 in the mouse model. Clearly, the data suggest that RD242 is a potent inhibitor of Aβ42 aggregation and rescues AD phenotypes in a C. elegans AD model without displaying any cytotoxicity.

**Effect of RD242 on the neurodegeneration in a post-disease onset AD model**

The tripyridyl, RD242 was able to rescue AD phenotypes when it was added at the L4 stage to GMC101 worms. However, the effect of RD242 was not tested on AD phenotypes in a post-disease onset AD model. For AD, most of the current therapeutic intervention strategies predominantly rely on the post-disease onset model and the treatment occurs after the diagnosis of AD. During the post-disease onset of AD, the aggregation of the Aβ peptide is facilitated by multiple mechanisms, including the de novo Aβ aggregation, fibers-catalyzed Aβ aggregation, and the prion-like spread of Aβ fibers. Therefore, we decided to assess the effect of RD242 on the post-disease onset AD model. We first assessed the ability of RD242 to inhibit the fibers-catalyzed aggregation of Aβ42. A solution of 5μM Aβ42 peptide was treated with 10% Aβ42 preformed fibers (0.5 μM Aβ42 in monomer). The aggregation profile of fibers-catalyzed Aβ42 peptide resulted in ~2-fold decrease in t50 (time required to reach 50% ThT fluorescence signal) of Aβ42 peptide (Figure 5a, black) in comparison to the de novo aggregation of Aβ42 peptide (Figure 5a, blue). In the presence of RD242 at an equimolar ratio, we observed a low ThT signal, which suggests that RD242 was a potent inhibitor of fibers-catalyzed Aβ42 aggregation (Figure 5a, pink). Additionally, we
used the TEM imaging to validate our ThT results of the fibers-catalyzed aggregation. The fibers-catalyzed aggregation of Aβ42 led to a large quantity of fibers demonstrated using the TEM image (Figure 5b). In marked contrast, there was a significantly less amount of fibers in the fibers-catalyzed Aβ42 aggregation in the presence of RD242 at an equimolar ratio (Figure 5c). Some of the fibers in the presence of RD242 were already present in the solution at the beginning of the experiment due to the preformed fibers added to the solution. Collectively, RD242 is a potent inhibitor of fibers-catalyzed Aβ42 aggregation. We surmise the antagonist mechanism of RD242 is very similar to ADH-41 against the fibers-catalyzed aggregation, where RD242 interacts with Aβ42 and modulate its conformation, which is unable to interact with Aβ42 fibers; therefore, inhibits the fibers-catalyzed aggregation. We have shown earlier that ADH-41 inhibits the fibers-catalyzed aggregation using a similar mechanism. Next, we tested the effect of RD242 in GMC101 worms in a post-disease onset model, which is facilitated by the combination of the de novo and the fibers-catalyzed aggregation of Aβ42. In the GMC101 model, there is an increase in the aggregation of Aβ42 from day five to day ten of adulthood, which is quantified using the NIAD4 dye (Figure 5e,f,h). To test the effect of RD242 on the post-disease onset, we treated GMC101 worms with 30 µM RD242 on the 5th day of adulthood (Figure 5d). The intracellular Aβ42 aggregates (quantified by NIAD4 dye) in GMC101 worms were significantly lower in the presence of RD242 (Figure 5g,h) on day ten of adulthood. The tripyridyl, RD242 suppresses the further progress of Aβ42 aggregates facilitated by de novo and fibers-catalyzed aggregation in GMC101 worms. The data suggest that RD242 was able to suppress the aggregation of Aβ42 peptide when added in a post-disease onset model of AD.
CONCLUSION

In the last 15 years, the aPPIs have transitioned from “undruggable and challenging” to attractive targets because of their role in numerous pathological conditions, including infectious diseases, cancer, and amyloid diseases.\textsuperscript{1-13} Therefore, the manipulation of aPPIs has been considered a key therapeutic approach.\textsuperscript{1-13} To achieve the specific and potent manipulation of aPPIs, the protein surface of aPPIs is required to be complemented with a large surface area and chemical diversity.\textsuperscript{2-6} The use of synthetic protein mimetics is one such therapeutic approach to manipulate the aPPIs. OP is a synthetic protein mimetic scaffold, which has been shown to manipulate various aPPIs.\textsuperscript{16-35} However, one of the main shortcomings of OPs is the limited chemical diversity of side chains presented on their surface. It was a challenging task to enhance the chemical diversity of the side chains because of the tedious synthetic route. Using a novel “common-precursor” chromatography-free approach, we have enhanced the chemical diversity of the side chains of OPs in a very convenient manner. Using this study, we synthesized a potent antagonist OP ligand, which completely suppressed Aβ aggregation, a process central to AD. Very limited success has been reported in slowing the progression of AD or developing a cure. Therefore, there is a pressing need to identify potent leads to cure or slow the progression of AD. We have identified a potent OP ligand with the ability to rescue Aβ aggregation mediated AD phenotypes in a well-established \textit{C elegans} AD model. More importantly, the most potent OP was able to suppress Aβ aggregation in a post-disease onset \textit{C elegans} AD model. This model could be used as a template to identify potent ligands to slow down the progression of AD phenotypes in the post-diagnosed AD model. OPs have shown desirable pharmaceutical properties in mouse models for other amyloid targets due to their non-proteogenic side chains and an aromatic backbone scaffold\textsuperscript{23}. Additionally, the OPs can be synthetically modified to tune the pharmacological properties without sacrificing the antagonist activity.\textsuperscript{51} In the near future, the potent OP ligand identified here will be tested for its ability to cross the blood-brain barrier, which is a requirement for drugs to treat neurological disorders. These studies are underway and will be presented in the near future. The overall study provided a template for potential lead therapeutics for the treatment of AD.

We propose that this study can be used as a prototype to generate large chemical space of the side chains on various oligoamide scaffolds. Another novel aspect of this study is the potential to execute the late-stage diversification of the side chains on OPs to further optimize their binding affinities against pathological targets, which will be explored in the near future. The generation of large chemical space on structured scaffolds is a promising approach for the identification of potent ligands to manipulate aPPIs that are associated with various pathologies. We believe that our “common-precursor” approach will have
a tremendous impact as it is expandable for other oligoamide scaffolds and for a much larger chemical space to identify lead therapeutics for various diseases.
**Figure 1.** The comparison of the synthetic pathway using the literature method and our current approach.

**a, e, h,** Primary alcohol, sodium hydride (NaH, 60% dispersion in mineral oil), toluene (anhyd.), 30 min at 0 °C then 5 h at r.t. **b, f, i,** tributyl(vinyl)tin, Pd(PPh₃)₄, toluene (anhyd.), 110 °C for 12 h. **c, g, j,** KMnO₄, NaHCO₃, acetone, 6 h at r.t. **d,** Mel, K₂CO₃, dimethylformamide (DMF, anhyd.), 4 h at r.t. **k,** Pd/C, H₂ (g), ethyl acetate (EtOAc), 3 h at r.t. **l,** 6-chloro-5-nitro-picolinic acid, 2-chloro-1-methylpyridinium iodide, dichloromethane (DCM, anhyd.), triethylamine (TEA), 5 h reflux at 60 °C. **m,** Pd/C, H₂ (g), EtOAc, 3 h at r.t. **n,** 2-chloro-1-methylpyridinium iodide, DCM (anhyd.), TEA, 5 h reflux at 60 °C. **o,** DCM/trifluoracetic acid (TFA)/triethylsilane (TES) (80:15:5, v/v) 3 h at r.t. **p,** 3-(boc-amino)-1-propanol, NaH (60% dispersion in mineral oil), toluene (anhyd.), 30 min at 0 °C then 24 h at r.t. **q,** Pd/C, H₂ (g), EtOAc/DCM (1:1, v/v), 3 h at r.t. **r,** 6-chloro-5-nitro-picolinic acid, TEA, thionyl chloride (SOCl₂), DCM (anhyd.), 0 °C to r.t. over 1 h. **s,** 1-butanol, sodium metal (Na), tetrahydrofuran (THF, anhyd.), 30 min at r.t. **t,** Pd/C, H₂ (g), EtOAc/DCM (1:1, v/v), 3 h at r.t. **u,** 6-chloro-5-nitro-picolinoyl chloride, NaHCO₃,
DCM, 1 h at r.t. (v) isopropanol, Na, tetrahydrofuran (THF, anhyd.), 1 h at r.t. w, DCM/TFA/TES (60:20:20, v/v), 3 h at r.t.

<table>
<thead>
<tr>
<th>Properties</th>
<th>Old method</th>
<th>Current method</th>
</tr>
</thead>
<tbody>
<tr>
<td>*Total % Yield</td>
<td>0.78%-3.61%</td>
<td>53.3%-79.8%</td>
</tr>
<tr>
<td>Chromatography steps</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>Synthetic steps</td>
<td>14</td>
<td>8</td>
</tr>
<tr>
<td>Total side chains</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>Total tripyridyls</td>
<td>One tripyridyl</td>
<td>Tripyridyl Library</td>
</tr>
</tbody>
</table>

*Table 1.* Comparison of the properties to synthesize a tripyridyl with three distinct side chains using the old method and our new method. *The overall % yield was calculated by multiplying the yield of each synthetic step.*
Figure 2. The comparison of the side chains appended on OPs synthesized via the old method and the new method (current work). The chemical structure of ADH-41 and RD242. The only difference in the synthesis of OPs by both methods is the -COOMe and -Me functional groups on the C-terminal.
Figure 3. The rescue of Aβ42 aggregation mediated AD phenotypes in GMC101 worms by RD242. a, The relative change in the ThT intensity of the aggregation of 10 μM Aβ42 after 24 h in the absence and presence of the indicated ligands at an equimolar ratio. The TEM images of 10 μM Aβ42 in the absence (b) and presence (c) of RD242 at an equimolar ratio. d, The relative change in the ThT (10 μM) intensity in the absence and presence of ligands (10 μM) in 1× PBS. e, Overlay of 2D HSQC (1H, 15N) NMR spectra of 20 μM uniformly 15N-labelled Aβ42 in the absence (red) and presence (blue) of RD242 at an equimolar ratio. f, The ITC thermogram for the titration of a solution of RD242 into Aβ40 where heat burst curves and the corrected injection heats are represented by the upper and lower panels, respectively. The corrected injection heat was fitted using the independent binding site model to yield the binding stoichiometry and binding affinity. g, Schematic of the aging process of GMC101 worms and their treatment with the ligands indicated by the arrow at the L4 stage (day 2). Representative confocal images of GMC101 worms (day 10th of adulthood) with intracellular Aβ42 aggregates stained with an amyloid-specific dye (NIAD-4, red) in the absence (h) and presence of RD242 (i) and Bexarotene (j). k, The fluorescence intensity of NIAD-4 dye treated GMC101 worms in the absence and presence of the indicated ligands on the indicated time points. l, The comparison of the ROS level (at various time points) in various worm strains in the absence and presence of the indicated ligands after treatment with CM-H2DCFDA dye. For the fluorescence intensity experiment, at least 10 worms were used per condition. The data were expressed as mean and error bars report the s.e.m. (n = 3 independent experiments and each experiment consisted of three technical replicates).
Figure. 4. Effect of RD242 on the motility rate of GMC101 worms. a, The comparison of the motility rate of GMC101 and control worms in the absence and presence of the indicated ligands. b, The comparison of the motility rate of GMC101 worms in the presence of the indicated ligands at two different concentrations (30 and 10 μM). c, The motility rate of the control worms (N2) when they were treated with RD242 (30 μM, day 2). d, The LC-MS analysis of the cell lysate of 10 days old GMC101 after treatment with RD242 (30 μM, day 2). The data were expressed as mean and the error bars report the s.e.m. (n = 4 independent experiments and each n consisted of two technical replicates). The statistical analysis was performed using ANOVA with Tukey’s multiple comparison test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Figure 5. The effect of RD242 on the fibers-catalyzed aggregation of Aβ42. a, ThT kinetic assay and TEM images for the fibers (10%, molar ratio) catalyzed aggregation of 5 µM Aβ42 in the absence (b) and presence of RD242 (c) at an equimolar ratio. d, Pictorial representation of the addition of 30 µM RD242 to GMC101 worms in a late-stage onset AD model. Representative confocal images of GMC101 worms on day five (e) and day ten (f) of adulthood with intracellular Aβ42 aggregates stained with NIAD-4 dye. g, A representative confocal image of GMC101 worm on day ten of adulthood with intracellular Aβ42 aggregates in the presence of 30 µM RD242. h, The fluorescence intensity of NIAD-4 dye treated GMC101 worms in the absence and presence of 30 µM RD242. The arrow indicates the late-stage addition
of RD242 to GMC101 worms. For the fluorescence intensity experiment, at least 10 worms were used per condition. The data were expressed as mean and error bars report the s.e.m. (n = 3 independent experiments and each experiment consisted of three technical replicates).
MATERIALS AND METHODS

Synthesis: All reactions containing reagents sensitive to moisture or air were carried out in flame dried glassware under the atmosphere of argon, unless stated otherwise. Anhydrous tetrahydrofuran (THF) was obtained using an Innovative Technology PURESOLV solvent purification system (University of Denver). Anhydrous dichloromethane (DCM), thionyl chloride (SOCl₂), and toluene were obtained from Sigma-Aldrich (Burlington, MA). All general reagents used were purchased from Oakwood chemicals (Estil, SC), Sigma Aldrich (Burlington, MA), or Fischer Scientific (Allentown, PA) in the highest commercial purity available and used without further purification, unless otherwise stated. Reaction progress was monitored by thin-layer chromatography (TLC) carried out on Sorbtech (Norcross, CA) aluminum backed 200 μm silica XG plates w/UV245 and visualized under UV light or by ninhydrin staining (0.3 g Ninhydrin dissolved in 100 mL n-butanol with 3 mL of acetic acid). Filtrations utilizing a silica plug were carried out with standard grade 230 x 400 mesh 60Å Sorbtech (Norcross, CA) silica gel. Yields refer to spectroscopically (1H NMR) or chromatographically homogeneous materials, unless stated otherwise. Nuclear magnetic resonance (NMR) spectra were carried out on a Bruker (Billerica, MA) 500 MHz Ultrashield Plus. Chemical shifts are measured relative to residual solvent peaks as an internal standard set to δ 7.26 ppm for CDCl₃ and δ 2.50 ppm for DMSO-D₆ for ¹H NMR. The following abbreviations are used to describe multiplicities: s = singlet, d = doublet, dd = doublet of doublets, t = triplet, q = quartet, p = pentet, m = multiplet. Lyophilization steps were carried out using a LABCONCO (Kansas City, MO) Freezone 2.5 L, -84 °C freeze dryer under high vacuum reaching a pressure of 0.03 mbar. HRMS spectra were obtained from the University of Illinois Mass Spectrometry Lab (Urbana, IL) or the Latham Lab at the University of Denver (Denver, CO). Several methods of nucleophilic aromatic substitution (SnAr) were utilized in the described procedures, and the method used to obtain each product is indicated in the included table of yields.

Thioflavin T (ThT)-Based Aggregation Assay: Aggregation assays were conducted on a M200PRO plate reader (Tecan, Männedorf, Switzerland). Experiments were conducted in triplicate in a 96-well plate with a final volume of 200 μL per well. Every measurement was an average of 50 readings. The aggregation of 10 μM Aβ₄₂ was initiated by its addition from a stock solution (in DMSO, 0.5-1.0 mM) to 1× PBS buffer along with the ThT dye (10 μM). The aggregation of the Aβ₄₂ peptide was monitored by ThT fluorescence (λₑₓ = 445 nm and λₑₘ = 485 nm). The blank sample contained everything except the Aβ₄₂ peptide. The sample data were processed by subtracting the blank and renormalizing the fluorescence intensity by setting the maximum value to one. The aggregation assay in the presence of small molecules were conducted under the same conditions except that the small molecules were added from a stock solution (1 mM or 10 mM in DMSO) to keep the final concentration of DMSO less than 0.5% (v/v).
molecules were added to the wells with ThT and mixed gently with a pipet before adding the Aβ42 peptide. To keep the conditions identical, an equal amount of DMSO was added to the wells, which contained the Aβ42 peptide. Error bars represent the standard error of the mean (sem) for at least three independent experiments.

**Fibers-Catalyzed Kinetic Assay:** The fibers of Aβ42 were prepared by incubating 200 μM Aβ42 in phosphate buffer for 48 h at r.t. The formation of fibers was confirmed by ThT before storage at 4°C until further use in the fibers-catalyzed aggregation assay. The fibers-catalyzed aggregation assay was started by adding 10% Aβ42 fibers (based on the monomeric Aβ42, v/v) to a solution of 5 μM Aβ42 along with ThT (2.5 μM) in 1× PBS buffer to the 96-well plate. The aggregation of the Aβ42 peptide was monitored by ThT fluorescence (λex = 445 nm and λem = 485 nm). The sample data were processed by subtracting the blank and renormalizing the fluorescence intensity by setting the maximum value to one. The aggregation assay in the presence of small molecules was conducted under the same conditions except that the small molecules were added from a stock solution (1 mM or 10 mM in DMSO) to keep the final concentration of DMSO less than 0.5% (v/v). Small molecules were added to the wells with ThT and mixed gently with a pipet before adding the Aβ42 peptide and Aβ42 fibers. To keep the conditions identical, an equal amount of DMSO was added to the wells, which contained Aβ42 monomer and Aβ42 fibers.

**Transmission electron microscopy:** A solution of Aβ42 (5 μM) was incubated in 1x PBS buffer (150 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, and 2 mM KH2PO4) in the absence and presence of RD242 at an equimolar ratio for 4 days. The aliquots (5 μL) of the solutions were applied on glow-discharged carbon-coated 300-mesh copper grids for 2 min and dried using tissue paper. The copper grids were negatively stained for 60 sec with uranyl acetate (0.75%, w/v). The micrographs were taken on an FEI Tecnai G2 Biotwin TEM at 80 kV accelerating voltages. The TEM experiments were repeated three times (n = 3) independently to ensure the reproducibility of the data.

**Two-Dimensional Heteronuclear Single Quantum Coherence NMR (2D HSQC NMR) Spectroscopy:** Two-Dimensional HSQC NMR experiments were carried out on a 600 MHz Bruker spectrometer. Uniformly labeled 15N-Aβ42 was purchased from reptide (Bogart, GA). A stock solution of 1 mg mL⁻¹ was prepared in 10 mM sodium hydroxide, aliquoted into small fractions, lyophilized, and stored at -20 °C until use. Experiments were carried out in 20 mM NaPi at pH 7.4, prepared in a ratio of 90:10 (H2O:D2O) to ensure Aβ42 remains in the monomeric state. A Stock solution of RD242 was prepared in DMSO-d6 (Cambridge Isotope Laboratories, Andover, MA). For each experiment, a freshly prepared aliquot of 15N-Aβ42 was used to avoid potential complications from the amyloid formation. NMR spectra
were recorded using a fresh sample of 20 μM $^{15}$N-Aβ$_{42}$ in 20 mM NaPi, pH 7.4 in the absence and presence of RD242 at 10 °C with a triple resonance HCN probe.

**ITC Titration:** ITC experiments were performed in a NANO-ITC (TA Instruments, New Castle, DE). A stock solution of RD242 (200 μM in 20 mM NaPi, pH 7.4) was serially added (1.87 μL injections in the rotary syringe, stirring speed = 300 rpm) into a sample cell containing 300 μL of 10 μM Aβ$_{40}$ in the same buffer at 300 s intervals. The heat associated with each injection was calculated by integrating each heat burst curve using NanoAnalyze software (New Castle, DE). The associated heat for each injection was corrected by subtracting heat resulted from the titration of RD242 into buffer under identical conditions. Corrected heats were plotted as a function of the molar ratio of RD242 to Aβ$_{40}$ and fitted using an independent binding site model. No parameter was constrained during the fitting. The final fit data were extracted from the best fit after 10000 iterations.

**Culture methods for *C. elegans* strains:** The N2 (wild-type *C. elegans* Bristol strain), GMC101 (C. elegans model of AD), and *Escherichia coli* OP50 (*E. coli*, uracil requiring mutant) strains were obtained from Caenorhabditis Genomics Center (Minneapolis, MN). The animals were maintained at standard conditions on nematode growth media (NGM) agar (Research Products International, Mt. Prospect, IL) on 60 mm plates (CytoOne, Ocala, FL) using *E. coli* OP50 as the food source. All strains were maintained using previous protocols.$^{50,51,57,58}$ NGM agar plates, M9 buffer (3 g KH$_2$PO$_4$, 6 g Na$_2$HPO$_4$, 5 g NaCl, 1 mL 1 M MgSO$_4$, milli-Q H$_2$O to 1 L), and OP50 solution at 0.5 Optical Density (OD$_{600nm}$) were prepared using previous protocols.$^{50}$

**The motility rate assay for GMC101 strain treated with Ligands:** This experiment was performed based on previously described protocols$^{50}$ with slight modifications. On day one, N2 and GMC101 strains were synchronized using the bleaching process, which involves egg lay$^{50}$ and incubation of the eggs (21 °C, 30 h) on a solution of NGM in 60 mm culture plates (CytoOne, Ocala, FL) with OP50 (350 μL, 0.5 OD$_{600nm}$) as a food source. On day two, the worms were transferred (using M9 buffer) from 60 mm NGM plates to 35 mm NGM plates (CellTreat Scientific, Pepperell, MA) containing 75 μM Fluorodeoxyuridine (FUDR, Sigma Aldrich, St. Louis, MO); to prevent the worm reproduction and ensure that equal ages of worms were used for the experiment) with and without RD242 (10 μM, and 30 μM in DMSO) and Bexarotene (10 μM and 30 μM in DMSO form a stock solution of 10 mM in DMSO, final DMSO conc. >0.2%, v/v) and incubated at 21 °C for 48 h. On day three, fresh stock of OP50 was prepared by diluting 1 μL of OP50 in 5 mL of LB Miller media (Neogen, Lansing, MI) and incubated in a shaking incubator (Eppendorf, Hamburg, Germany) at 37 °C and ~200 rpm for ~24 h. The experiment was conducted using a sterile 24 well plate containing liquid media (1 mL/well). On day four, the liquid media was prepared
with 67.28% (v/v) of M9 buffer, 0.018% (75 μM) of FUDR solution (v/v), 0.1% of 1 M magnesium sulfate (v/v), 0.1% of 1 M calcium chloride (v/v), 2.5% of 1 M potassium phosphate solution (pH 6, v/v), and 30% of 0.5 OD600nm OP50 (v/v). Bexarotene and RD242 (10 μM and 30 μM in DMSO) were reconstituted in the same liquid media for the GMC101 strain of *C. elegans* (for treatment). There were six worm conditions for this experiment including N2 strain (positive control), GMC101 strain (untreated negative control), and GMC101 strain treated with different concentrations of Bexarotene and RD242 as earlier described. There were four technical replicates (four wells) for each worm condition. A total of 50 worms per well were transferred manually into a 24-well plate with a worm pick. Prior to each paralysis assay, the 24-well plate was mechanically tapped for about 30 sec to make the worms more active in the liquid media. The assay was started on day five where 20 activity scores per well were collected using the wMicroTracker Arena plate reader at 21 °C for 1 h per day over a 12 day period. Three biological replicates were performed and the average activity scores per well were reported with the error bars representing the standard error of the mean (s.e.m).

**The quantification of Aβ aggregates in GMC101 worms using NIAD-4 dye**

The GMC101 worms were prepared both in the absence and presence of ligands (Bexarotene and RD242, 30 μM in DMSO) similar to the conditions used for the paralysis assays. The worms were transferred using a worm pick into NIAD-4 solution (5 μM in M9 buffer) and incubated at 21 °C for 4 h at 150 r.p.m on a shaker (Thermo Scientific, Waltham, MA) on day six and day ten. This incubation ensured that the worms were thoroughly stained. Subsequently, they were transferred into NGM plates containing FUDR and incubated again at 21 °C for 24 h to allow the worms to recover through metabolism. At least 10 worms per condition were transferred with a worm pick to a cover slide containing an anesthetic (40 mM sodium azide) and mounted on glass microscope slides (Fischer Scientific, Pittsburgh, PA) containing 2% agarose pads for imaging. The images of the worms were collected using an Olympus Fluoview FV3000 confocal/2-photon microscope (40 x Plan-Apo/1.3 NA objective with DIC capability) on day five and day ten. The images were processed using the OlympusViewer in ImageJ software. The fluorescence intensity for the inclusions of amyloid protein aggregates (in the muscle cells) was quantified using ImageJ software. For each condition, three biological replicates were performed, and the average number of aggregates was reported with the error bars representing the s.e.m.

**The quantification of Aβ aggregates in the post-disease onset AD model**

The GMC101 worms were bleached and synchronized similar to the earlier experiment. On day five, the GMC101 worms were transferred using a worm pick into NIAD-4 solution (5 μM in M9 buffer) and incubated at room temperature for 4 h at 150 r.p.m on a shaker (Thermo Scientific, Waltham, MA). This
incubation ensured that the worms were thoroughly stained. Subsequently, they were transferred into NGM plates containing FUDR and incubated again at 21 °C for 24 h to allow the worms to recover through metabolism. At least 10 worms per condition were transferred with a worm pick to a cover slide containing an anesthetic (40 mM sodium azide) and mounted on glass microscope slides (Fischer Scientific, Pittsburgh, PA) containing 2% agarose pads for imaging. The images of the worms were collected using an Olympus Fluoview FV3000 confocal/2-photon microscope (40 x Plan-Apo/1.3 NA objective with DIC capability) on day five and day ten. The images were processed using the OlympusViewer in ImageJ software. The fluorescence intensity for the inclusions of amyloid protein aggregates (in the muscle cells) was quantified using ImageJ software. The fluorescence intensity for the inclusions of amyloid protein aggregates (in the muscle cells) was quantified using ImageJ software. To test the effect of RD242 on GMC101 worms, the GMC101 worms were treated with RD242 (30 μM in DMSO) on day five of adulthood. The GMC101 worms were stained with NIAD-4 solution on day ten of adulthood in the absence and presence of RD242. The GMC101 worms (at least 10 worms) were then imaged using a confocal microscope on day ten of adulthood in the absence and presence of RD242. The images of the worms were collected using an Olympus Fluoview FV3000 confocal/2-photon microscope (40 x Plan-Apo/1.3 NA objective with DIC capability) on day ten. The images were processed using the OlympusViewer in ImageJ software. The fluorescence intensity for the inclusions of amyloid protein aggregates (in the muscle cells) was quantified using ImageJ software. The fluorescence intensity for the inclusions of amyloid protein aggregates (in the muscle cells) was quantified using ImageJ software. For each condition, three biological replicates were performed, and the average number of aggregates was reported with the error bars representing the s.e.m.

**Measurement of intraworm ROS of GMC101 and N2 worms**

A fluorescent probe 2′,7′-dichlorofluorescein diacetate (H\(_2\)DCFDA) (Cayman Chemical, Ann Arbor, MI) was used to measure the intracellular ROS based on previously established protocols with slight modifications. Both strains, N2 and GMC101 were synchronized and treated (in the absence and presence of 10 μM and 30 μM RD242) as described earlier in the paralysis assay. On day ten, the worms were transferred into 1.7 mL microcentrifuge tubes using M9 buffer (1 mL). The samples were centrifuged for 2 min at 2,500 rpm and 21 °C. Subsequently, 0.8 mL of the supernatant was discarded. After, homogenizing the worm pellet in the remaining solution, 10 μL of the suspension was placed onto a glass slide for counting using an Olympus microscope (SZ-6145, Waltham, MA) in triplicate. The worm solution was then diluted with M9 buffer to approximately 50 worms/10 μL. Each desired well of a Costar 96-well black plate (Corning, Kennebunk, ME) contained M9 buffer (40 μL), worm solution (10 μL), and H\(_2\)DCFDA (50 μL, 50 μM in M9 buffer). For the vehicle, 50 μL of M9 buffer and H\(_2\)DCFDA (50 μL, 50
μM in M9 buffer) were placed in the desired wells. Subsequently, the Costar 96-well plate was gently shaken for 30 sec at 21 °C and the fluorescence intensity was quantified ($\lambda_{ex} = 485$ nm and $\lambda_{em} = 530$ nm) every h for 2 h using the Infinite M200 Pro Plate Reader. This experiment consisted of three biological replicates and five technical replicates. The normalized fluorescence intensities presented are the average values with the error bars representing the s.e.m.

**The identification of RD242 in the cell lysate of GMC101 worms**

The GMC101 were synchronized and treated with 30 μM RD242 as described earlier in the paralysis assay. On day ten, the worms were transferred into 1.7 mL microcentrifuge tubes using M9 buffer (1 mL). The samples were centrifuged for 2 min at 2,500 rpm and 21 °C. Subsequently, the worms were washed five times with 1×PBS buffer. The worms were incubated at -80 °C for 24 h. The worms were dissolved in lysis buffer for 1 h followed by sonication for 30 min. Subsequently, the solution was dried using a lyophilizer and redissolved in a 2 mL solution of water and methanol (50:50, v/v). The solution was then centrifuged for 10 min. at 15,00 rpm. Subsequently, the supernatant was transferred and used for the LC-MS analysis. We repeated the experiment four times with a minimum of 200 GMC101 worms.

**Author Contributions**

S.K. designed and conceived the project with assistance from R.A.D. and J.A.J. The synthesis of the OP libraries was carried out by R.A.D. The biophysical study was carried out by R.A.D. and T.D.B. The *C. elegans*-based *in vivo* experiments, including the confocal imaging, ROS detection, and the motility study were carried out by J.A.J. with assistance from C.B., A. G. T., and A. S. The paper was written by S.K. with assistance from R.A.D. and J.A.J.

**Conflicts of interest**

The authors declare no competing interests

**Acknowledgments**

The authors would like to thank the department of chemistry and biochemistry, The Knoebel Institute for Healthy Aging, and the University of Denver for the startup funds. The authors also thank the PinS program (University of Denver) for awarding a summer undergraduate fellowship to A.S.
References


(52) Gazit E. A Possible Role for Pi-stocking in the Self-Assembly of Amyloid Fibrils. *FASEB J.* 2002, 16(1), 77-83.


(71) Rabinovici G. D. Late-onset Alzheimer Disease. *Continuum (Minneapolis Minn).* 2019, 25(1), 14-33.

