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ACS chemical biology

A High-Throughput Screen for Compounds That Inhibit Aggregation of the Alzheimer's Peptide

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ABSTRACT Aggregation of the Alzheimer's peptide Aβ produces toxic multimeric species that play a key role in the development of Alzheimer's disease. Compounds that inhibit this aggregation may prove useful as therapeutic agents for the prevention or treatment of Alzheimer's disease. Although aggregation inhibitors may already exist in combinatorial libraries, finding these compounds in a cost-effective high-throughput manner poses an enormous challenge. To meet this challenge, we have developed a novel high-throughput screen capable of isolating inhibitors of Aβ aggregation from large libraries of inactive candidates. The screen uses a fusion of Aβ42 to GFP. In the absence of inhibition, the rapid misfolding and aggregation of Aβ42 causes the entire fusion protein to misfold, thereby preventing fluorescence. Compounds that inhibit Aβ42 aggregation enable GFP to fold into its native structure and be identified by the resulting fluorescent signal. By implementing the screen on a pilot library of triazine derivatives, we have identified several putative inhibitors. One of the selected compounds was studied in detail by a series of biochemical and biophysical methods. These studies confirmed that the selected compound inhibits aggregation of synthetic Aβ42 peptide. The fluorescence-based method described here is rapid and inexpensive and can be used to screen large libraries for inhibitors of Aβ42 aggregation and/or amyloidogenesis.

Alzheimer's disease (AD) is estimated to affect nearly 10% of people over the age of 65 (www.alz.org/AboutAD/statistics.asp). As the "baby boom" generation ages and medical advances enable people to live longer, the number of people afflicted by AD is expected to increase dramatically. Given these trends, there is a tremendous need to develop therapeutics that block or reverse this debilitating neurodegenerative disease.

Although a number of drugs are in clinical use for the treatment of AD, most of these compounds target the symptoms of the disease, rather than its underlying molecular cause. Reducing the incidence of AD and slowing its progression will require new drugs that disrupt the underlying molecular etiology of AD. Although the molecular underpinnings of AD are not fully understood, a range of genetic and biochemical studies suggest that aggregation of the Alzheimer's peptide, Aβ, plays a causative role in the development of AD (*1–7*). Therefore, compounds that inhibit production and/or aggregation of Aβ are attractive candidates as therapeutics for the prevention and/or treatment of AD.

Aβ peptides are produced *in vivo* by proteolytic cleavage of the amyloid precursor protein (APP) by β and γ secretases (*1*). Because γ secretase can cleave at several alternative sites, the resulting Aβ peptides vary in length. The most abundant forms found in amyloid plaque are the 40-mer and the 42-mer. Aβ40 is produced in greater abundance; however, Aβ42 aggregates more readily and comprises the major component of amyloid plaque in diseased brains (*8–10*).

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Received for review March 27, 2006 and accepted July 2, 2006.

Published online August 4, 2006

10.1021/cb600135w CCC: $33.50

Each step in the production and aggregation of Aβ can be considered as a target for intervention. These steps include (i) expression of the APP, (ii) proteolytic cleavage of APP into Aβ peptides, (iii) clearance of Aβ peptides from the system, and (iv) aggregation of Aβ into oligomers and insoluble amyloid. Numerous studies, both in academic labs and in the pharmaceutical industry, have targeted the first three steps of this pathway. Although initial results were promising (*11*–*15*), attempts to block expression, cleavage, or clearance have not produced an effective pharmaceutical.

In addition to these upstream processes, efforts have also focused on blocking the aggregation step itself. Although a few aggregation inhibitors have been reported (*16*–*23*), no clinically useful therapeutics have emerged. The discovery of compounds that block Aβ aggregation has been stymied by two major hindrances: First, structure-based rational drug design is precluded by the unavailability of a high-resolution structure. Although structural studies of Aβ have advanced significantly in recent years and models of Aβ amyloid have been built (*24*, *25*), these structures are not of sufficiently high resolution to enable atom-by-atom drug design. Moreover, these structural models are for fibrils of Aβ, rather than the oligomers now assumed to be the toxic species. Second, large-scale screening of combinatorial libraries has been hindered by the unavailability of a cost-effective high-throughput screen for inhibitors of the early steps of Aβ aggregation.

Although several methods to screen for inhibitors of Aβ aggregation have been reported (*26*, *27*), these methods are hampered by several shortcomings: Published methods typically require synthetic Aβ peptide. Because Aβ42 aggregates, synthesis of this 42-residue peptide is laborious and time-consuming. Consequently, synthetic Aβ42 is too expensive to use in screens aiming to analyze large libraries of compounds. In addition to its prohibitive cost, the aggregation of synthetic Aβ42 can also interfere with the efficacy of a screen: Synthetic Aβ42 often contains oligomeric "seeds" that can nucleate further aggregation. Since current models of AD pathogenesis implicate small oligomers on the pathway toward amyloid as the most toxic species (*5*, *6*, *28*–*31*), a screen relying on samples that contain pre-existing seeds may actually miss the most important inhibitors, including those that block the initial formation of soluble Aβ oligomers.

To overcome these challenges, we have developed a novel screen for inhibitors of Aβ42 aggregation. Our screen does not require synthetic Aβ42. Moreover, the new screen uses fluorescence for rapid and high-throughput detection. Here we describe the development of this screen, and its implementation to isolate inhibitors of Aβ42 aggregation from a novel combinatorial library of triazine derivatives.

RESULTS AND DISCUSSION

A High-Throughput Screen for Inhibitors of Aβ Aggregation. A high-throughput screen for inhibitors of Aβ aggregation requires the solubility/aggregation behavior of Aβ to be coupled to a property that can be assayed on many samples in parallel. Such coupling can be achieved by fusing the Aβ sequence to a reporter protein with an observable function that is blocked by Aβ aggregation but enabled by agents that inhibit Aβ aggregation.

Our screen achieves this goal by fusing the sequence of Aβ42 to GFP. Because folding of GFP into its native fluorescent structure occurs slowly (*32*), the fluorescence of the Aβ42–GFP fusion depends on the folding and solubility of the fused Aβ42. Misfolding and aggregation of the Aβ42 sequence cause the entire Aβ42–GFP fusion to misfold prior to formation of the correct fluorescent structure. Inhibitors that retard (or block) Aβ42 aggregation enable GFP to fold into its native structure and can be distinguished by the resulting fluorescent signal.

We chose to work with Aβ42 rather than Aβ40 because the longer peptide is the major component of senile plaque and the ratio of Aβ42/Aβ40 is increased in diseased brains (*8*, *9*). The 42-residue peptide also forms aggregates more rapidly *in vitro* (*10*). In our fusion construct, Aβ42 is separated from the N-terminus of GFP by a linker encoding the sequence GSAGSAAGSGEF (*33*). This sequence was shown previously to be effective in coupling the aggregation state of N-terminal fusions with the fluorescence of GFP (*33*–*35*). (Longer or more disordered linkers are not suitable because they would uncouple the properties of the N-terminal peptide from those of GFP.)

In previous work, we demonstrated that fusions of Aβ42 to GFP do not fluoresce, and expression of the Aβ42–GFP fusion in *Escherichia coli* yields nonfluorescent colonies (*34*, *35*). We used these fusions as an artificial genetic system in *E. coli* to screen for mutations

in Aβ42 that inhibit aggregation (*34*). Nonaggregating mutants were isolated by screening random mutations in Aβ42 for those that produce green fluorescent colonies. The ability of the selected amino acid substitutions to diminish the aggregation of Aβ42 was confirmed by biophysical studies of mutant versions of the synthetic 42-residue peptide.

Just as mutations in the sequence of Aβ42 can retard aggregation by intramolecular effects (*34*), one might expect exogenous compounds to inhibit Aβ42 aggregation by intermolecular effects. To test this hypothesis, we now demonstrate the use of the Aβ42–GFP fusion as a high-throughput screen to search a library of small molecules for inhibitors of Aβ42 aggregation.

In this initial test of the screen (Figure 1), we focus on a library of ~1000 compounds based on the triazine scaffold (*36*, *37*). This library was prepared by varying the substituents at positions *X*, *Y*, and *Z* on the scaffold (Figure 1). The tested compounds were prepared previously for other assays (*37*) or freshly synthesized using a slightly modified method developed in our lab (*36*). The substituents at positions *X*, *Y*, and *Z* are described in our earlier work (*36*, *37*).

To implement the screen, we added *E. coli* cells expressing the Aβ42-GFP fusion protein to 96-well plates. Candidate molecules from the library of triazine derivatives were added to each well, and expression of the Aβ42–GFP fusion protein was induced by addition of isopropyl-β-D-thiogalactopyranoside. Following 3 h of induction, the fluorescence of each well was measured on an automated plate reader. Several wells containing specific triazine derivatives fluoresced at levels significantly above background (Figure 2). Compounds were tested in quadruplicate, and the identification of "hits" was consistent across the four repetitions. The fluorescent hits are considered as putative inhibitors of Aβ42 aggregation.

***In Vitro* Studies Confirm the Activity of a Selected Inhibitor.** The ability of the Aβ42–GFP fluorescence screen to identify potential inhibitors of aggregation is encouraging; however, because the screen relies on several artificial features, one might question whether compounds isolated by this screen actually inhibit aggregation of the Aβ42 peptide in a well-defined biochemical system. The artificial features of the Aβ42–GFP screen include (i) a fusion in which the relevant 42-residue Aβ sequence is only a small fraction of the 292-residue fusion protein and (ii) expression in *E. coli*, which is clearly not the natural system for AD. Consequently, it is essential to verify that fluorescence observed for the Aβ42–GFP fusion expressed in *E. coli* indeed correlates with diminished aggregation of the Aβ42 peptide.

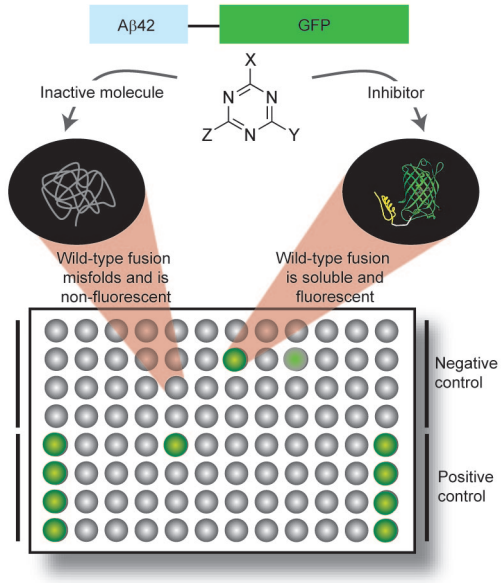

Figure 1. Fluorescence-based screen using the Aβ42–GFP fusion. In the absence of inhibition, the Aβ42 portion of the fusion aggregates rapidly and causes the entire Aβ42–GFP fusion to misfold and aggregate (left). Therefore, no fluorescence is observed. However, inhibition of Aβ42 aggregation enables GFP to form its native green fluorescent structure (right). (The green part of the ribbon diagram shows the structure of GFP; the yellow part is merely a schematic representation of a nonaggregated form of Aβ42.) The triazine scaffold is shown at the center of the figure. Combinatorial diversity was introduced at sites marked *X*, *Y*, and *Z*. A 96-well plate is shown at the bottom of the figure. Compounds were added to each well, followed by *E. coli* cells expressing the Aβ42–GFP fusion. Negative (colorless) and positive (green) controls are shown in the columns on the edges of the plate. For negative controls, no test compounds were added to the wells. For positive controls, the wild-type Aβ42–GFP fusion was replaced with a fusion in which the Aβ42 sequence contained mutations F19S and L34P. This double mutant was shown previously to inhibit aggregation and enable fluorescence of the Aβ42–GFP fusion (*34*).

In earlier work, we demonstrated that mutations in Aβ42 that yield green fluorescence in the context of the Aβ42–GFP fusion expressed in *E. coli* indeed diminish the aggregation of synthetic Aβ42 peptide studied *in*

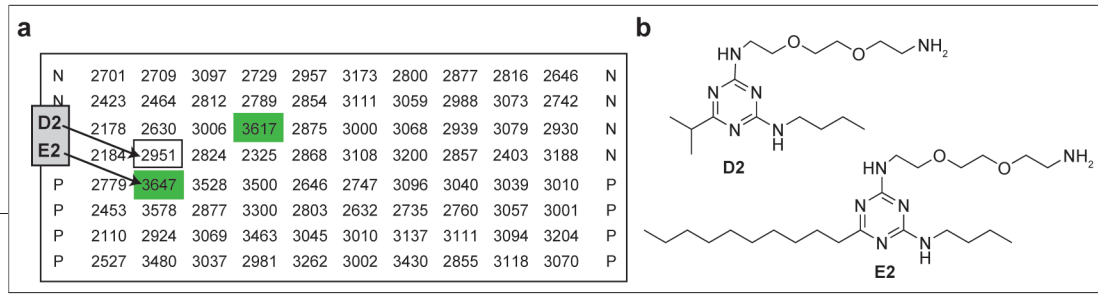

Figure 2. Screening results for the triazine library. a) Digital readout of the fluorescence of *E. coli* cells expressing the Aβ42–GFP fusion in the presence of compounds from a combinatorial library of triazine derivatives. N denotes negative control wells without compound (2706 average, 238 standard deviation). P denotes positive control wells expressing a GFP fusion to the soluble F19S/L34P mutant of Aβ42 (4610 average, 155 standard deviation). Compounds that reproducibly yielded fluorescence signals 3 standard deviations above the average of the negative control are highlighted in green. Compounds E2 (green) and D2 (control) were chosen for further studies. b) Structures of the aggregation inhibitor, E2, and the inactive control compound, D2. (The triazine scaffold of the combinatorial library is shown in Figure 1.)

vitro (*34*). To confirm that this correlation holds not only for mutations in Aβ42 but also for the small molecule inhibitors isolated by our screen, we probed the effects of the selected triazine derivative E2 (Figure 2) and the unselected control D2 (Figure 2) on the aggregation behavior of synthetic Aβ42 peptide.

Soluble monomeric Aβ42 peptide can be prepared using organic solvents, sonication, and filtration (*38*). When such samples are diluted into aqueous buffer, the peptide aggregates into fibrillar amyloid structures, which can be assayed by the binding and resulting fluorescence of thioflavin T (*39*). The rate of Aβ42 aggregation depends on the conditions of the incubation: Under "quiescent" conditions, aggregation is slow, whereas agitation causes Aβ42 to aggregate more rapidly.

We studied the effects of compounds D2 and E2 on the aggregation of Aβ42 under both quiescent and agitated conditions. For the quiescent conditions, synthetic Aβ42 at a concentration of 20 μM was incubated for 2 h in the presence of various concentrations of either D2 or E2. Fibril formation was assayed by the shifted fluorescence of thioflavin T that accompanies binding to fibrils (*39*). Compound E2 inhibits aggregation in a concentration-dependent manner, with an IC_{50} of ~30 μM. At 80 μM, E2 produces nearly complete inhibition of Aβ42 aggregation (Figure 3). In contrast, the control compound D2 shows no inhibitory effect.

Compounds D2 and E2 were also tested for their inhibitory effects under agitated incubation conditions. Here the effect was even more dramatic: While the control compound D2 was inactive, the selected compound E2 caused a 90% reduction in thioflavin T fluorescence at a concentration of only 50 μM (Figure 4). The inhibitory effect of E2 was compared to dopamine and tannic acid, which were shown previously to inhibit Aβ42 aggregation (*40*, *41*). At concentrations of 25 and 50 μM, the inhibitory effect of E2 was similar to, or slightly better than, dopamine or tannic acid (Figure 4).

Despite its inhibitory activity at 25 and 50 μM, E2 at lower concentrations seems to cause a slight increase in amyloid formation (Figure 4). This surprising behavior is reminiscent of the effect of trifluoroethanol (TFE), which inhibits fibrillogenesis at high concentrations but increases the rate of fibrillogenesis when added at low concentrations (*42*). It is not clear whether E2 acts by a mechanism similar to that exerted by TFE on peptide structure.

Finally, the ability of E2 to inhibit the assembly of Aβ42 into amyloid fibrils was assessed by electron microscopy. Aβ42 peptide was incubated for 5 d, either alone or in the presence of compounds D2 or E2. Five days is a relatively long incubation time; in the absence of inhibitors, Aβ42 readily forms visible fibrils after 1 or 2 d (data not shown). Following the 5 d incubation, samples were stained with uranyl acetate and imaged by electron microscopy. The control compound D2 was inactive at all concentrations (Figure 5). Compound E2, however, inhibited fibrillogenesis in a dose-dependent manner. At 50 μM, E2 had no effect, at 100 μM only short fibrils (perhaps "protofibrils") were observed, while at a concentration of 200 μM, compound E2 completely inhibited fibril formation.

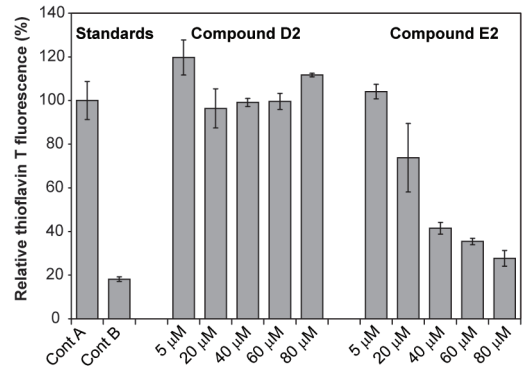

Figure 3. Aggregation of synthetic Aβ42 peptide under quiescent conditions. Amyloid formation was assayed by binding and fluorescence of the diagnostic dye, thioflavin T. Control A is synthetic Aβ42 alone. Control B is buffer alone. Compound E2 inhibits amyloid formation in a dose-dependent manner. The related compound, D2, has no activity. Additional controls showed that in the absence of peptide, compounds D2 and E2 had no effect on ThT fluorescence (data not shown). Fluorescence is shown as a percentage of the control (synthetic Aβ42 alone).

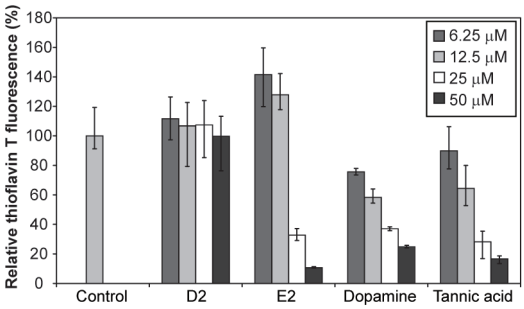

Figure 4. Amyloid formation assayed by thioflavin T fluorescence after incubation under agitated conditions. Compound E2 inhibits amyloid formation in a dose-dependent manner. The control compound, D2, has no activity. The right side of the figure shows results for dopamine and tannic acid, which were shown previously to inhibit Aβ42 aggregation (*40*, *41*). At concentrations of 25 and 50 μM, the inhibitory effect of E2 was similar to, or slightly better than, dopamine or tannic acid. Fluorescence is shown as a percentage of the control (synthetic Aβ42 alone).

These results confirm that the novel fluorescence-based assay using an Aβ42–GFP fusion expressed in *E. coli* can detect compounds that indeed inhibit aggregation and/or amyloidogenesis of the Aβ42 peptide.

Screening for Inhibitors of the Early Steps of Aggregation. An extensive range of genetic and biochemical studies (*1*–*6*) support the "amyloid cascade" hypothesis (*43*), which posits that accumulation of aggregated Aβ initiates a multistep cascade that ultimately leads to AD. While insoluble amyloid plaque has long been thought to play a causative role in AD, recent work suggests that smaller aggregates (Aβ oligomers) on the pathway toward amyloid may in fact be more toxic than insoluble plaque (*5*, *6*, *28*–*31*, *44*, *45*). Although the relative importance of the various stages of aggregation remains a topic of investigation, it is clear that aggregation of Aβ into some form of multimeric complex (ranging from small oligomers to large fibrils) produces toxic species that lead to AD.

Because the exact structure and oligomeric state of the toxic aggregate of Aβ42 are not known, it is important to consider what stages of aggregation might be blocked by compounds scored as "hits" in high-throughput screens. In traditional screens relying on turbidity or binding of thioflavin T, a compound is scored as a hit if it prevents assembly into amyloid fibrils. Since fibrils occur late in the aggregation pathway, a potential disadvantage of these older screens is the likelihood that some compounds isolated by these screens will inhibit the later steps of amyloidogenesis but fail to inhibit the upstream formation of toxic soluble oligomers. A more effective method would screen for compounds that block early misfolding and aggregation without requiring the formation of amyloid fibrils. Our Aβ42–GFP screen for misfolding and aggregation may satisfy this requirement. Although we do not know the exact level of Aβ42 aggregation (dimers? tetramers? hexamers?) that prevents fluorescence of the Aβ42–GFP fusion, it seems likely that the nonfluorescent phenotype of the misfolded aggregate would be apparent at or before the dodecameric stage, which has been proposed to be the toxic species responsible for memory impairment in AD (*31*). Once active inhibitors are isolated, the exact oligomerization stage at which they function and the precise mechanism of their action can be assessed by biophysical studies.

For a screen to find inhibitors of the earliest stages of aggregation, it is important that the compounds being tested are present prior to the initial steps of the aggregation pathway. For screens that relied on synthetic Aβ42 peptide, this posed a serious challenge: Because Aβ42 aggregates so readily, it is difficult to prepare aqueous samples that are entirely free of partially aggregated seeds. The presence of these seeds (which presumably contain oligomers) meant that the species that must be inhibited would have already been present prior to addition of putative inhibitors. Consequently, screens using synthetic Aβ42 peptide could miss the

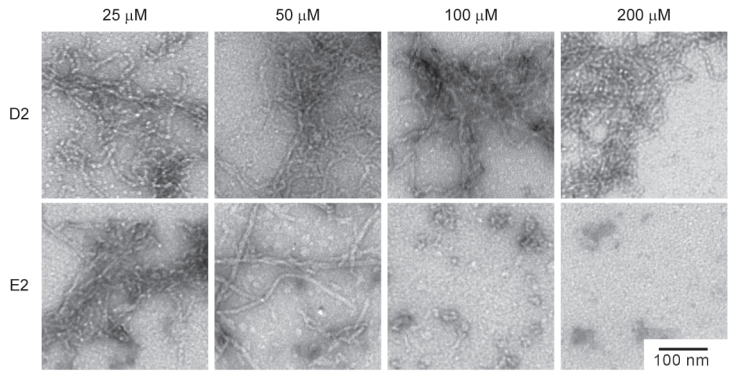

Figure 5. Electron microscopy of fibrils of Aβ42 after incubation with D2 or E2. Synthetic Aβ42 peptide was incubated for 5 d with various concentrations of either D2 or E2. At elevated concentrations, E2 inhibited fibrillogenesis. In contrast, the control compound D2, was inactive at all concentrations.

very compounds that ultimately will provide leads for the development of anti-AD therapeutics. The Aβ42–GFP fusion system overcomes these problems: In the new screen, Aβ42 is *not* present prior to addition of the test compounds; expression of the Aβ42–GFP fusion is induced only *after* the test compounds have been added.

The triazine collection described above was used as a pilot library to demonstrate that the Aβ42–GFP screen can indeed distinguish hits from inactive compounds. Although we are not suggesting that triazine is the optimal scaffold for drug discovery, we note that compound RS-0466, which was shown by Selkoe and co-workers (*46*) to block Aβ oligomerization and rescue long-term potentiation, is also a triazine derivative.

Sensitivity of the Screen. An effective screen must be sensitive enough to detect compounds with relatively low levels of inhibitory activity. This is important for two reasons: First, initial implementation of a screen typically searches for lead compounds, rather than final drugs. Therefore, a screen should be sensitive enough to detect first-generation compounds with only moderate effects on aggregation. (Such leads can be optimized at later stages.) Second, detection of compounds with low activity is important because drugs with modest effects on aggregation may in fact be sufficient to treat AD: In early onset AD caused by familial mutations in APP or in the presenilins, levels of Aβ42 are increased by as little as 30% (*3*). This small increase in Aβ42 can advance the onset of AD by 30–40 years. Therefore, compounds with only moderate inhibitory activity may suffice to delay the onset of AD to the point where it is no longer a major health problem. The Aβ42–GFP fusion system described here has the required level of sensitivity. This was demonstrated explicitly by earlier work using the fusion to screen for mutations in Aβ42 that diminish aggregation (*35*). In that work, we showed that mutations that alter the aggregation rate only moderately are readily detected by changes in the fluorescence of the Aβ42–GFP fusion. Thus, the Aβ42–GFP fusion system is well-suited for the detection of compounds having a range of inhibitory activity.

The Aβ42-GFP fusion system is sensitive to inhibitory effects at sites throughout the length of 42-residue Aβ sequence (*34*). One might be concerned that the presence of a linker following residue 42 would interfere with inhibitory effects on the C-terminal residues of Aβ42, which are known to be important for aggregation (*8–10*, *47*). Our earlier studies, however, demonstrated that the Aβ42–GFP fusion can discriminate small differences in aggregation rates caused by mutations throughout Aβ42, including those at residues 41 and 42 (*34*, *35*).

When screening for compounds that inhibit aggregation, it is important to ensure that the screen does not inadvertently identify generic inhibitors of protein folding. This possibility must be considered because aggregation into β-sheet fibrils and folding into native globular structures are similar processes: Both involve self-assembly of a polypeptide into an ordered structure. Although Aβ42 aggregation is intermolecular and protein folding is intramolecular, the two processes are governed by the same types of interactions (hydrogen bonding, the hydrophobic effect, propensities for secondary structure, side chain packing, *etc.*). Therefore, it is important to establish that a screen for inhibitors of aggregation does not inadvertently identify inhibitors of protein folding, particularly the folding of β-sheet proteins. The Aβ42–GFP fusion system is internally controlled for this possibility. A positive signal (fluorescence) is required to identify hits, and this signal is observed if and only if GFP folds into its native structure. Therefore, generic inhibitors of protein folding will not be isolated by this screen. Moreover, since GFP is a β-sheet protein, generic inhibitors of β-sheet structure will not be isolated. These undesirable effects are "weeded out" by the requirement for correct folding of GFP.

Applications of the Screen. Because the Aβ42–GFP fusion is sensitive enough to detect both low and high levels of inhibitory activity, the screen can be used to determine structure/activity relationships. For example, compounds D2 and E2 are identical at positions *X* and *Y* but differ at position *Z* (Figure 2). By screening collections of molecules that differ at only one position, we have begun to establish the relationship between the substituents at each position and the resulting level of inhibition (Kim *et al.*, unpublished).

AD is one of several diseases involving protein misfolding and aggregation. Others include prion encephalopathies and Huntington's disease (*48*, *49*). The GFP fusion system described here may also be suitable to screen for inhibitors of the aggregation processes that underlie these other diseases.

Certain classes of compounds that successfully inhibit Aβ42 aggregation may nonetheless be missed by our screen. Two examples include compounds that fluoresce at the same wavelength as GFP and compounds

that are toxic to cells. To enable screening of libraries containing green fluorescent compounds, it may be necessary to modify our system for future use with variants of GFP that fluoresce in other parts of the spectrum (*e.g.*, yellow fluorescent protein (*50*, *51*). Cytotoxic compounds will also be missed by our screen; however, this may be advantageous since such compounds are unlikely to be suitable as drugs.

Finally, we note that the initial version of the Aβ42–GFP fluorescent screen described in this work relies on the fusion protein expressed in *E. coli*. Screening in *E. coli* has several advantages: It is fast, inexpensive, and highly reproducible. Moreover, it favors compounds that (i) are nontoxic and (ii) readily penetrate biological barriers. Nonetheless, expression in *E. coli* may also introduce a limitation: To be scored as a hit in this cell-based screen, a compound must enter the bacterial cell. Inhibitors of Aβ aggregation that fail to enter cells will not produce fluorescent signals and will escape detection in this initial version of our screen. The significance of this limitation will depend on the type of library being screened. Some chemical moieties are inherently more likely than others to enter cells (*52*). To effectively screen compounds that do not enter bacterial cells, we recently developed a modified version of the Aβ42–GFP fluorescent screen in which the fusion protein is expressed *in vitro* using a cell-free transcription and translation system (Kim, Wurth, and Hecht, unpublished). This cell-free system readily distinguished between aggregating and nonaggregating mutants of Aβ42. Future work will adapt this cell-free system to screen for small-molecule inhibitors of Aβ42 aggregation.

METHODS

Fluorescent Screen for Inhibitors of Aβ Aggregation. The vector for expressing the Aβ42–GFP fusion was described previously (*34*, *35*). Strain BL21(DE3) *E. coli* cells (*53*) harboring the Aβ42–GFP fusion vector were grown in LB media supplemented with 35 μg mL^{-1} kanamycin. When cultures reached an OD_{600} = 0.8, 100 μL of culture was transferred to the wells of 96-well plates. Candidate compounds from the triazine library were added to each well, and protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM. Samples were incubated with gentle agitation at 37 °C. Following 3 h of incubation, the fluorescence of each well was measured at 512 nm (excitation 490 nm) using an automated plate reader (SpectraMAX Gemini XS, Molecular Devices). To verify that cell densities were consistent across all samples, the OD_{600} was also measured. Compounds were tested in quadruplicate: twice at a final concentration of 30 μM and twice at a final concentration of 100 μM. The identification of hits was consistent across several repetitions. Occasionally, we observed compounds that yielded fluorescent signals below that of the negative control; these compounds may be cytotoxic.

Overall, screening a library of ~1000 compounds required several hours. Scale-up procedures using robotic sample handling will enable screening of much larger libraries on a high-throughput scale.

Synthesis of the Library of Triazine Derivatives. The general design and synthesis of a triazine-based library was reported previously (*36*, *37*). In this solid-phase synthetic approach, three types of building blocks were prepared separately and assembled by chemically orthogonal reactions. The putative inhibitor E2 and the structurally related but inactive compound D2 were resynthesized for further characterization. Data for E2: ^{1}H NMR (400 MHz, MeOH-d_4) δ 3.72–3.63 (8H, m), 3.59 (2H, m), 3.37 (2H, m), 3.12 (2H, t, J = 5.0 Hz), 2.46–2.33 (2H, m), 1.67 (2H, m), 1.56 (2H, m), 1.43–1.24 (16H, m), 0.95 (3H, t, J = 7.5 Hz), 0.89 (3H, t, J = 7.0 Hz). LC–MS (m/z): Calculated for $C_{23}H_{46}N_6O_2$: 438.4. Found: 439.4 $[M + H]^+$. Data for D2: ^{1}H NMR (MeOH-d_4) δ 3.71–3.64 (8H, m), 3.60 (2H, m), 3.38 (2H, m), 3.12 (2H, t, J = 5.0 Hz), 2.65 (1H, m), 1.57 (2H, m), 1.39 (2H, m), 1.21 (6H, d, J = 6.8 Hz), 0.95 (3H, t, J = 7.3 Hz); LC–MS (m/z): Calculated for $C_{16}H_{32}N_6O_2$: 340.3. Found: 341.3 $[M + H]^+$.

Preparation of Synthetic Aβ42 Peptide. Aβ42 peptide (unpurified) was purchased from the Keck Institute at Yale University and purified on a C4 reverse phase column (Vydac). After purification, the peptide was snap-frozen at –75 °C and lyophilized. Monomeric samples were prepared by 15 min of sonication after addition of trifluoroacetic acid (TFA, 1 mg mL^{-1} peptide concentration) (*54*). Residual TFA was removed by addition of hexafluoroisopropanol (Sigma Aldrich) and argon blow.

Thioflavin T Assays. Synthetic Aβ42 peptide was incubated at 20 μM in phosphate buffered saline (PBS, 50 mM NaH_2PO_4, 100 mM NaCl, 0.02% NaN_3) in the presence or absence of candidate inhibitors at various concentrations. Following incubation with or without agitation, thioflavin T was added to a final concentration of 7 μM, and fluorescence was measured at 490 nm (excitation 450 nm).

Electron Microscopy. Aβ42 peptide at a concentration of 20 μM in PBS buffer was incubated in the presence or absence of the test compounds at various concentrations. Following 5 d of incubation at 37 °C under quiescent conditions, Formvar carbon-coated grids were floated on a drop of the sample for 2 min. The grids were blotted using filter paper and then stained for 2 min with freshly made 1% uranyl acetate. Samples were imaged using a Zeiss 912ab electron microscope.

Acknowledgments: We thank M. Bishop for assistance with the electron microscopy, J. Crawford for peptide synthesis, and C. Wurth for initial experiments using the Aβ42–GFP fusion.

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