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RESEARCH ARTICLE

# Development and validation of a yeast high-throughput screen for inhibitors of $A\beta_{42}$ oligomerization

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**SUMMARY**

Recent reports point to small soluble oligomers, rather than insoluble fibrils, of amyloid β (Aβ), as the primary toxic species in Alzheimer's disease. Previously, we developed a low-throughput assay in yeast that is capable of detecting small $A\beta_{42}$ oligomer formation. Specifically, $A\beta_{42}$ fused to the functional release factor domain of yeast translational termination factor, Sup35p, formed sodium dodecyl sulfate (SDS)-stable low-n oligomers in living yeast, which impaired release factor activity. As a result, the assay for oligomer formation uses yeast growth to indicate restored release factor activity and presumably reduced oligomer formation. We now describe our translation of this assay into a high-throughput screen (HTS) for anti-oligomeric compounds. By doing so, we also identified two presumptive anti-oligomeric compounds from a sub-library of 12,800 drug-like small molecules. Subsequent biochemical analysis confirmed their anti-oligomeric activity, suggesting that this form of HTS is an efficient, sensitive and cost-effective approach to identify new inhibitors of $A\beta_{42}$ oligomerization.



## INTRODUCTION

Several aggregated forms of the amyloid β peptide (Aβ), which are generated by proteolytic processing of the amyloid precursor protein (APP), in normal brains and cerebrospinal fluid (CSF) are believed to have a crucial role in the development of Alzheimer's disease (AD) (Hardy and Higgins, 1992; Selkoe, 1991; Younkin, 1995). Although extracellular amyloid plaques and neurofibrillary tangles formed by insoluble fibrils in brains are hallmarks of AD, recent findings suggest that smaller non-fibrillar oligomeric forms of the Aβ peptide are a more likely cause of AD. Indeed, studies in mice as well as mammalian cell culture showed that detergent-stable Aβ oligomers are potent neurotoxins (Dahlgren et al., 2002; Kayed et al., 2003; Lambert et al., 1998; Lesne et al., 2006; Walsh et al., 2002a). Recently, Aβ dimers in AD brain or CSF have been specifically identified as toxic because they (but not Aβ monomers) induce synaptic dysfunction (Klyubin et al., 2008; Walsh et al., 2002a). In addition, oligomer-specific antibodies can reduce the Aβ-induced toxicity of soluble AD brain extract (Gong et al., 2003; Lambert et al., 2001; Lee et al., 2006).

Small molecules that prevent the formation of $A\beta_{42}$ (a 42-residue Aβ protein) aggregates that lead to the formation of large plaques had previously been of interest (De Felice and Ferreira, 2002; Estrada and Soto, 2007; Soto et al., 1998). However, evidence for a pathological role of small soluble Aβ oligomers in early AD development led to the idea that inhibiting the formation of Aβ oligomers is a more promising strategy to prevent or treat AD (Klein et al., 2001; Walsh et al., 2002b). Although the relationship between toxic oligomers, large fibrils and plaques is unclear, at least some oligomers seem not to be precursors of large fibrils. Hence, it is possible that large fibrillar aggregates might help prevent toxic oligomers from forming (Chen et al., 2010; Cheng et al., 2007; Glabe, 2005; Harper et al., 1999; Kayed et al., 2003; Necula et al., 2007a). As a result, the ideal drug candidate might inhibit toxic oligomer formation while not inhibiting large fibril aggregation.

Cell-based assays for drug-like molecules that inhibit $A\beta_{42}$ aggregation are advantageous because toxic compounds are immediately discarded (Bharadwaj et al., 2010; Caine et al., 2007; Kim et al., 2006; Lee et al., 2009; Macreadie et al., 2008). Compounds that inhibit Aβ aggregation have been well studied and some of them also inhibit Aβ oligomerization (Amijee et al., 2009; Amijee and Scopes, 2009; Scherzer-Attali et al., 2010). Such compounds include modified short Aβ peptides, designed to bind to the core region of $A\beta_{42}$ that is involved in fibrillization, e.g. SEN304 (a methylated pentapeptide of $A\beta_{42}$). SEN304 has been reported to inhibit secretion of toxic sodium dodecyl sulfate (SDS)-stable oligomers in 7PA2 cells (Kokkoni et al., 2006). Other compounds that are known to inhibit $A\beta_{42}$ from forming toxic oligomers and that also have a therapeutic effect in AD animal models are: curcumin (Yang et al., 2005), RS-0406 (hydroxyanaline) (Nakagami et al., 2002; O'Hare et al., 2010; Walsh et al., 2005), SEN1269 (hydroxyanaline derivative; Senexis), scyllo-inositol (AZD-103) (McLaurin et al., 2000; McLaurin et al., 2006; Townsend et al., 2006), PBT1 (Clioquinol, 8-hydroxyquinolin) (Hsiao et al., 1996) and PBT2 (a copper/zinc ionophore, 8-hydroxyquinolin) (Adlard et al., 2008; Faux et al., 2010). Both scyllo-inositol (Transition Therapeutics and Elan) and PBT2 (Prana Biotechnology) are currently in clinical trials. Recent work points to compounds that bind to $A\beta_{42}$ as possible inhibitors of $A\beta_{42}$ toxicity (Alavez et al., 2011; Chen et al., 2010; Scherzer-Attali et al., 2010).

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The inhibition of Aβ$_{42}$ oligomer formation is often assayed using pure synthetic Aβ$_{42}$ peptide reconstituted under conditions that favor Aβ$_{42}$ oligomerization over fibrillization. Prevention of oligomer formation is characterized by using Thioflavin T (ThT) and/or antibodies specific for oligomers (Chang et al., 2003; Chromy et al., 2003; Hamaguchi et al., 2009; Necula et al., 2007b; Yang et al., 2005), or by using mammalian cells that overexpress and secrete human Aβ$_{42}$ that forms oligomers in conditioned medium (O'Hare et al., 2010; Walsh et al., 2002a; Walsh et al., 2005).

Here, we develop a yeast in vivo assay that is specific for assessing the inhibition of Aβ$_{42}$ oligomerization activity. Previously, we reported a yeast Aβ oligomerization model in which the formation of SDS-stable low-n oligomers, including dimers, trimers and tetramers, of an Aβ$_{42}$-fusion protein is easily detected (Bagriantsev and Liebman, 2006). Briefly, an Aβ$_{42}$ fusion to the essential functional domain (MRF) of the translational release factor, Sup35 (Derkatch et al., 1996; Ter-Avanesyan et al., 1993), provides an Aβ$_{42}$ aggregation-specific probe tied to a functional Sup35 readout. The Aβ-MRF construct was overexpressed in cells lacking chromosomal *SUP35.* The MRF expressed in the fusion (Aβ-MRF) was shown to have reduced translational termination factor activity by a simple growth test (left panel in Fig. 1). This seems to be due to aggregation of the fusion protein into SDS-stable low-n oligomers reminiscent of the detergent-stable oligomers found in human AD brains (Gong et al., 2003; Klyubin et al., 2008; Shankar et al., 2008) (left panel in Fig. 2A). Indeed, analogous fusions made with an Aβ$_{42}$ aggregation-deficient mutation, F19T, F20T and I31P (Hilbich et al., 1992; Morimoto et al., 2004; Williams et al., 2004), called Aβm2-MRF, retained almost complete translation termination factor activity and formed almost no oligomers (left panel in Fig. 2A) (Bagriantsev and Liebman, 2006).

Using this system, we developed and validated a high-throughput assay to screen small chemicals for an effect on Aβ$_{42}$ oligomer formation. The idea for the primary high-throughput screen (HTS) was to identify small drug-like molecules that restore translation termination activity to the Aβ-MRF strains by inhibiting formation of Aβ-MRF small oligomers, seen as inhibition of growth in media lacking adenine (–Ade) (right panel in Fig. 1). Here we report two drug-like compounds that were selected by the growth assay in a pilot screen. Through biochemical analyses, these compounds were further verified to indeed inhibit Aβ-MRF oligomer formation in vivo. They also inhibited bona fide Aβ$_{42}$ peptide polymerization and fibril formation in vitro. Thus, the yeast HTS growth assay is a promising strategy to screen for inhibitors of Aβ$_{42}$ oligomerization.

## RESULTS

### Characterization of yeast Aβ-MRF aggregation into low-n oligomers

When Aβ-MRF in yeast lysates was treated with 1% SDS at room temperature and resolved by SDS-PAGE, most of the Aβ-MRF was found as low-n oligomers (dimer, trimer, tetramer) and monomers (expected molecular mass 73.7 kDa, but runs as 95 kDa), although a portion of the Aβ-MRF was stuck in the well or appeared as a smear above the oligomers (left panel in Fig. 2A). To investigate whether the SDS-stable oligomers seen were present in native yeast cells or were derived from larger in vivo aggregates that were broken down by the SDS treatment, we examined lysates that were not treated with SDS before loading on the gel (right panel in Fig. 2A). These untreated samples showed similar oligomers and larger material, and this larger material did not break down into oligomers or monomers when they were excised from the gel, treated with 1% SDS at room temperature and run again on a second SDS-PAGE (Fig. 2B). However all forms of the Aβ-MRF were completely converted into monomers by boiling in 2% SDS and β-mercaptoethanol (BME; lower panel in Fig. 2A).

We also examined lysates with non-denaturing PAGE (left panel in Fig. 2C) and size exclusion chromatography (SEC; supplementary material Fig. S1), in which the Aβ-MRF was detected as a complex with 10-15 monomers and monomers and low-n oligomers were not detected. The complex was broken into monomers and low-n oligomers when the native lysate was treated with BME before loading on the gel (middle panel in Fig. 2C). One possibility is that Aβ-MRF monomers and low-n oligomers are cross-linked into, or trapped in, bigger aggregates in the lysate via disulfide bonds. These large aggregates in the lysate might be disaggregated by the reducing agent, BME, possibly returning the Aβ-MRF to the intracellular monomer and oligomer states. Indeed, there are seven cysteine residues in the fusion [five in the release factor (RF) and two in the linkers between Aβ and MRF].

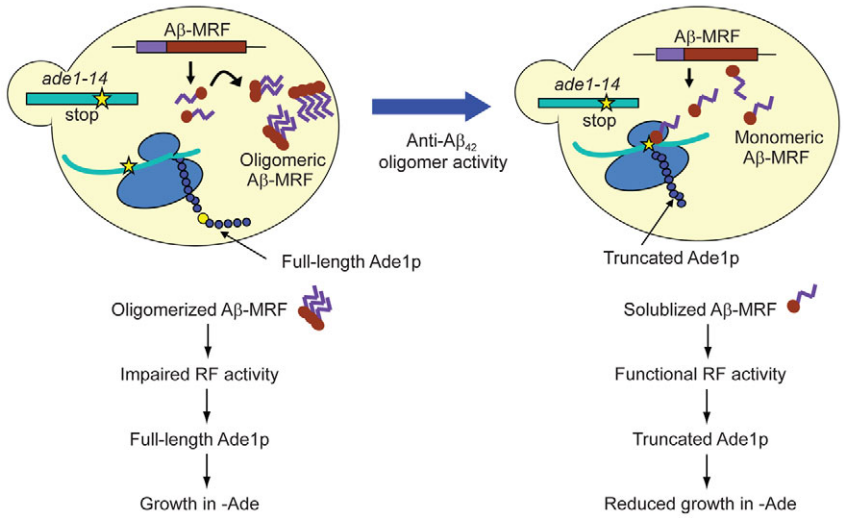


**Fig. 1. Strategy exploiting nonsense suppression of Aβ-MRF in yeast to screen for Aβ-MRF oligomer inhibitors.** To identify compounds that reduce Aβ oligomer formation, we employed a growth assay to screen for small chemical compounds that restore translation termination activity of the oligomerized Aβ-MRF reporter. The cell growth phenotype in this assay requires expression of full-length Ade1p. The assay uses *sup35Δ* cells carrying the *ade1-14* allele with a premature stop codon. The impaired Sup35p translational release factor (RF) activity of oligomerized Aβ-MRF (left) allows read-through of the *ade1-14* premature stop codon, enabling growth on adenine-less media (–Ade). However in the presence of small molecular compounds blocking oligomerization of Aβ-MRF, or in cells expressing Aβ$_{42}$ aggregation-deficient mutations (Aβm2-MRF) (right), the RF activity is restored, preventing stop-codon read-through, so cells cannot grow on –Ade.

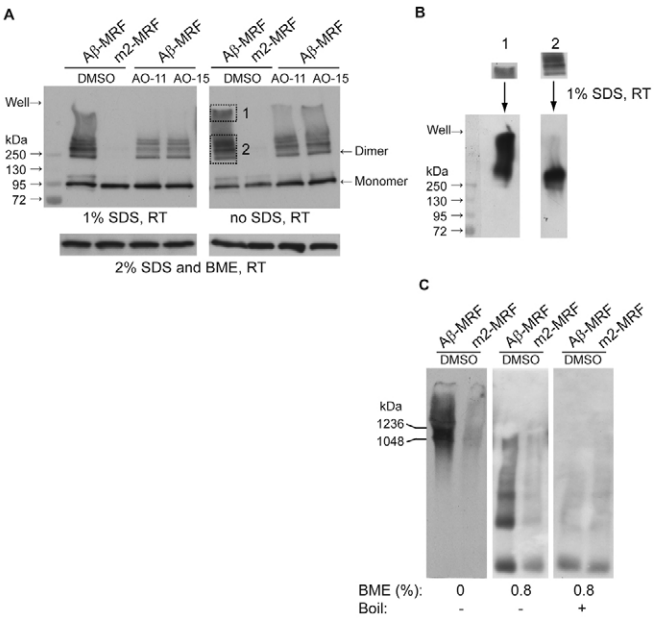


**Fig. 2. Aβ-MRF aggregates into SDS-stable low-n oligomers in yeast.** (A) Detection of Aβ-MRF by western blot analysis. Cells expressing Aβ-MRF or Aβm2-MRF were grown in the presence of DMSO control, or inhibitor candidates (100 μM of AO-11 or AO-15). The lysates were treated with SDS (1%) or without SDS for 7 minutes at room temperature and resolved with SDS-PAGE in which 0.1% SDS was included in the running buffer. Equal expression of Aβ-MRF was confirmed with boiled lysates in the presence of SDS (2%) and β-mercaptoethanol (BME). The prediction of the dimer and trimer Aβ-MRF migration position was based on the migration of the Aβ-MRF monomer (calculated molecular mass 73.7 kDa), which migrated at 95 kDa on PAGE. (B) Detection of SDS-stable Aβ-MRF assemblies. After SDS-PAGE separation of the fusion protein in lysates not treated with SDS, the gel portions marked with rectangles (1 and 2 in A), were excised, equilibrated in SDS sample buffer containing 1% SDS for 7 minutes at room temperature, loaded and resolved on a second SDS-PAGE. (C) Detection of native Aβ-MRF assemblies using blue native PAGE. Yeast lysates were prepared under non-denaturing conditions, treated with, or without, BME (0.8%) and boiled (+) or not boiled (–) before loading on a 3-12% Novex Bis-Tris blue native gel. Marked sizes in kDa were bands of NativeMark protein standards (IgM hexamer, 1236; IgM pentamer, 1048 kDa). Aβ-MRF or Aβm2-MRF were detected in A-C with anti-Sup35-RF antibodies. Each western blot shown is representative of at least three independent trials.

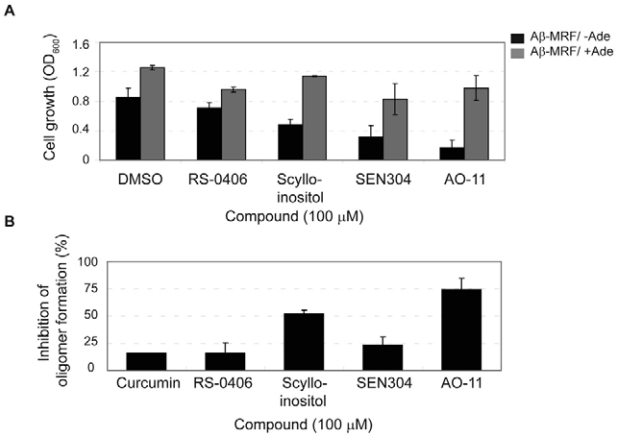


**Fig. 3. Effects of known anti-Aβ-aggregation compounds on Aβ-MRF aggregation in yeast.** (A) Effects of compounds on Aβ-MRF oligomerization (–Ade) and general growth (+Ade). Growth of cells expressing Aβ-MRF was measured at the late logarithmic phase as $OD_{600}$. Data represent the average of three independent experiments and error bars are standard deviation. (B) Effects of compounds on SDS-stable Aβ-MRF oligomer formation. Lysates of Aβ-MRF cells grown in the presence of DMSO or compound (100 μM) were treated with 1% SDS for 7 minutes at room temperature, resolved by SDS-PAGE and immunoblotted with anti-Sup35-RF antibodies. Inhibition of oligomer formation (%) relative to DMSO control (0%) was calculated from the ratio of oligomer and monomer band intensities on western blots and quantified using the Alpha Imager 2200 (Alpha Innotech). Error bars are standard deviation of three blots.

### Testing the yeast assay with known inhibitors

Before examining the effect of compounds in our assay, we made our yeast strain more permeable to chemicals by deleting the *erg6* gene (Dunstan et al., 2002). The altered sterol composition of cell membranes in the *erg6* mutants causes enhanced chemical uptake (Sharma, 2006; Welihinda et al., 1994), and we verified that an *erg6* deletion caused our yeast strain to become more sensitive to cycloheximide (supplementary material Fig. S2).

We tested whether known anti-aggregation compounds, SEN1269, SEN304, RS-0406 and scyllo-inositol, inhibit formation of Aβ-MRF oligomers in our assay by looking for restored Aβ-MRF translation termination activity detected as growth inhibition in media lacking adenine (–Ade) in the presence of 100 μM of the compounds. SEN1269 had no effect on growth inhibition in –Ade media at the various concentrations tested (data not shown). Because SEN304 caused toxicity in +Ade media, we cannot determine whether it restored Aβ-MRF translation termination activity in –Ade media (Fig. 3A). Curcumin and RS-0406 had no significant inhibitory effects on Aβ-MRF oligomerization (Fig. 3B). Scyllo-inositol, however, had a clear effect in our system: growth in –Ade media was inhibited to 50% of the DMSO control without causing toxicity in +Ade media (Fig. 3A). Furthermore, western blot analyses indicate that scyllo-inositol decreased oligomer formation 51.5±7% of that seen with DMSO alone, based on the ratio of oligomers to monomer (Fig. 3B). The recognition of scyllo-inositol as an anti-Aβ$_{42}$-oligomer compound by the yeast system validates it as a promising assay for non-toxic anti-oligomeric compounds. The identification and characteristics of AO-11, which has the most dramatic effects of all the chemicals in Fig. 3, is described below.

### Development of the yeast HTS and a pilot screen for inhibitors of Aβ-MRF oligomerization

We used the growth assay described above to screen for small compounds that block Aβ oligomerization, by selecting for restored Aβ-MRF translation termination activity. Prior to the primary screen, HTS parameters (e.g. shaking level, time of incubation, size of liquid sample and prevention of evaporation) were optimized. The sensitivity, reproducibility and stability of the assay was assessed using a library of pre-fractionated marine bacterial extracts (a gift from William Fenical, Scripps Oceanography, La Jolla, CA) (supplementary material Fig. S3). Positive and negative controls were cells expressing, respectively, the aggregation-deficient mutant Aβm2-MRF or Aβ-MRF.

On the basis of the experimental settings and HTS protocol, a screen with 12,800 compounds was conducted using a sub-library

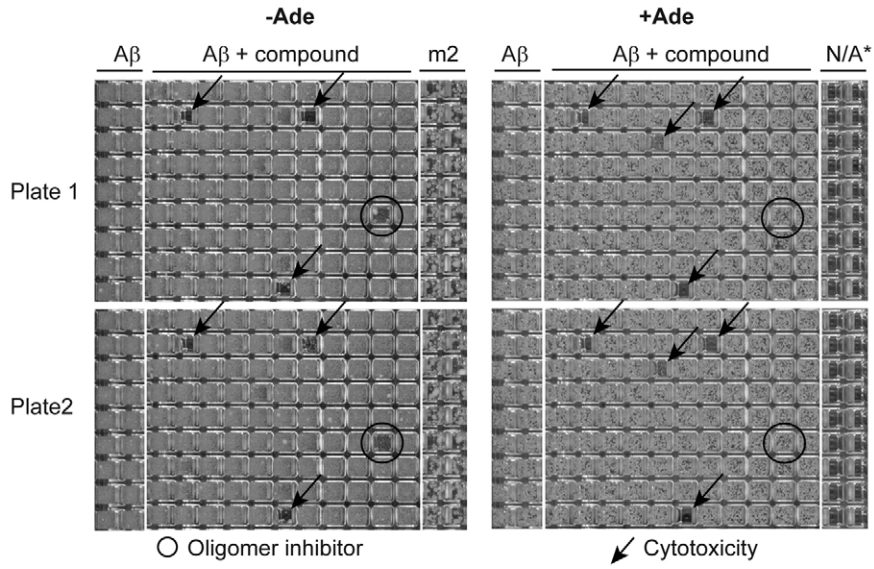


**Fig. 4. Primary growth assay screen.** Yeast expressing Aβ-MRF were inoculated into –Ade or +Ade media in microplate wells with 12,800 compounds at 22 μM. Negative [Aβ-MRF (Aβ)] and positive [Aβm2-MRF (m2)] controls, both lacking compounds, showed a clear easily scored difference in growth in –Ade. All Aβ control wells had diffuse growth throughout the well; all m2 control wells showed absence of growth (blackness) in some position of each well, although small colony 'spots', indicative of genetic revertants, were seen. Candidates in wells marked with a circle look like the m2 growth-inhibited controls in –Ade, but do not inhibit growth in +Ade, and were thus considered to be hits. Although compounds from wells marked with arrows were able to inhibit growth in –Ade, they were eliminated because they were toxic even in +Ade. *Uninoculated (blank) wells.



of the DIVER and CNS sets (ChemBridge, San Diego, CA). Each assay was carried out in duplicate at a single compound concentration (22 μM). We had an initial hit rate of 0.23%: 29 out of 12,800 compounds caused decreased growth in –Ade media without a significant growth decrease in +Ade media in duplicate well plates (Fig. 4).

### Elimination of false positives

We focused on 17 of the 29 compounds that fell into nine structural classes and one miscellaneous group based on their size, shape and the distribution of functional groups (Table 1). These 17 compounds were purchased from the vendor at a purity of 95% and retested in triplicate at the same concentration (22 μM) and conditions as the primary screen. The quality of the test, based on Z-factor statistics (Zhang et al., 1999), confirmed the assay quality to be excellent, reflective of values of >0.8 for both assays calculated from controls. Ten of the 17 compounds inhibited more than 50% growth in –Ade media compared with growth in the presence of DMSO alone. However, six of these compounds were rejected because they also inhibited growth in +Ade media (Table 1 and Fig. 5).

Finally, to eliminate compounds that inhibit growth in –Ade media because they reduce translational read-through of stop codons directly without affecting Aβ-MRF oligomerization, each compound was also tested in cells carrying a missense mutation in *SUP35* (Bradley et al., 2003), which impairs translational termination activity, causing the *ade1-14* premature stop codon to be read through. Two of the remaining four compounds were eliminated because they reduced this read-through (Table 1 and Fig. 5), leaving compounds AO-11 and AO-15 (respectively shown as 11 and 15 in Table 1 and Fig. 5) to be tested further.

### Confirmation of AO-11 and AO-15 activity on inhibition of Aβ-MRF oligomerization

Effects of AO-11 and AO-15 in a dose-dependent assay of Aβ-MRF-associated translational misreading were determined by serially diluting the compounds in DMSO to varying concentrations (0-50 μM) in a 96-well plate format. They showed no or mild growth inhibition in +Ade media, but growth was significantly inhibited in –Ade media as the concentration was increased, suggesting that Aβ oligomer formation was suppressed in a dose-dependent manner (Fig. 6). Estimates for $EC_{50}$ (the concentration of a compound at which 50% of its maximal effect is observed in vivo) obtained using the Enzyme Kinetics module of SigmaPlot (v. 9.01 Systat) (Ratia et al., 2008) were 12.8 and 6.7 μM for AO-11 and AO-15, respectively. The stronger activity of AO-15 represented by the smaller $EC_{50}$ value was partially caused by cytotoxicity of the compound at higher concentrations, whereas AO-11 had no apparent cytotoxicity (Table 1 and Fig. 6).

To directly test whether the restored translational termination factor activity associated with these compounds is correlated with a decrease in the presence of SDS-stable Aβ-MRF oligomers, lysates treated and grown with AO-11 or AO-15 at various concentrations were analyzed (Fig. 7). To determine the level of SDS-stable small oligomers, lysates were treated with 1% SDS for 7 minutes at room temperature, subjected to immunoblot analysis and probed with antibodies against the RF domain of Sup35p (BE4; developed in our laboratory). In cells grown with DMSO only, there were a lot of SDS-stable Aβ-MRF oligomers and much fewer monomers. In the presence of higher concentrations of the compounds, the levels of oligomers decreased and concomitantly the level of monomeric Aβ-MRF increased. AO-11, at 100 μM, decreased oligomer formation about 50% more than the known anti-aggregation compound scyllo-inositol (Fig. 3B). The $EC_{50}$ values for AO-11 and AO-15 are 32.4 and 45.2 μM, respectively. The 2.5- (AO-11) or 6-fold (AO-15) higher $EC_{50}$ values seen here compared with the $EC_{50}$ values for growth inhibition in –Ade media indicate that the growth assays were highly sensitive to the gain of monomers at the expense of oligomers.

The structure of these two new hits is shown in Fig. 8. They both have properties desirable for a CNS drug (Congreve et al., 2003; Rishton, 2003): six or fewer H-bond acceptors (actually three and two); three or fewer H-bond donors (actually zero and one); molecular weight <400 (actually <300); CLogP under 5 (actually 2 and 3) and a topological polar surface area of <75 (actually 37 and

**Table 1. Inhibitory activity of 17 compounds**

| | | | | Growth inhibition[b] | | |
|---|---|---|---|---|---|---|
| | | | | Aβ-MRF | | *sup35* |
| Class | ID (ChemBridge) | Number[a] | $M_r$ | –Ade[c] | +Ade[d] | –Ade[e] |
| 1 | 7653821 | 1 | 304 | Yes | Yes | No |
| | 5318948 | – | – | – | – | – |
| | 6260282 | – | – | – | – | – |
| 2 | 6022040 | 3 | 362 | No | Yes | No |
| | 7693106 | 4 | 260 | Yes | Yes | Yes |
| | 5971293 | – | – | – | – | – |
| 3 | 7664009 | 5 | 303 | No | Yes | No |
| | 7665200 | 6 | 380 | No | Yes | No |
| | 7634421 | – | – | – | – | – |
| 4 | 6626079 | 7 | 273 | Yes | Yes | Yes |
| | 7796937 | 8 | 350 | Yes | Yes | Yes |
| | 6144842 | – | – | – | – | – |
| 5 | 6555779 | 9 | 304 | Yes | Yes | Yes |
| | 7631676 | 10 | 423 | No | Yes | No |
| 6 | **5947437** | **11** | **240** | **Yes** | **No** | **No** |
| | 6325917 | 12 | 254 | Yes | Yes | Yes |
| | 5358273 | – | – | – | – | – |
| | 7735798 | – | – | – | – | – |
| 7 | 6846814 | 13 | 366 | Yes | Yes | Yes |
| | 7765407 | – | – | – | – | – |
| 8 | 5654672 | 14 | 367 | No | No | No |
| | 5133568 | – | – | – | – | – |
| | 6335386 | – | – | – | – | – |
| 9 | 7678698 | 2 | 361 | Yes | No | Yes |
| | 7723544 | – | – | – | – | – |
| | 7649024 | – | – | – | – | – |
| Misc. | **6593214** | **15** | **284** | **Yes** | **No** | **No** |
| | 7540212 | 16 | 283 | No | Yes | Yes |
| | 7760435 | 17 | 216 | Yes | No | Yes |

[a]Numbers used for identification of the 17 compounds focused on in this study, used in Fig. 5. [b]'Yes' indicates that the compound inhibited growth by more than 50% ([c]) and 25% ([d] and [e]) compared with growth of DMSO control in the marked medium. AO-11 and AO-15, which were tested further, are shown in bold. Misc., miscellaneous group.

62). AO-11 was previously patented for use for drug treatment of Duchenne muscular dystrophy [CA2685540 (A1)], whereas there is no previous patent or literature on AO-15.

### AO-11 and AO-15 inhibit in vitro Aβ$_{42}$ polymerization

To further study the activity of the selected inhibitors, electron microscopy (EM) and western blotting were carried out on samples of soluble Aβ$_{42}$ peptide prepared in the presence and absence of AO-11 and AO-15 under defined oligomer-forming conditions (Stine et al., 2003) with slight modifications (see Methods). We tested effects of the inhibitors at 50 μM, when more than 60% of oligomer formation was inhibited in vivo. Soluble recombinant Aβ$_{42}$ peptide was prepared and subjected to polymerization in the presence of DMSO, AO-11 or AO-15 for 24 hours at room temperature without shaking. Aliquots of duplicate samples were taken and examined by EM (Fig. 9A) and SDS-PAGE (Fig. 9B).

In the presence of DMSO (1% v/v, the concentration in which the inhibitors were dissolved), most of the peptides formed large fibrillar networks composed of uniform long fibrils by 24 hours. A clear change in morphology with a reduction in the fibril length and the absence of large fibrillar networks throughout the EM was detected when Aβ$_{42}$ peptides were incubated with AO-11 or AO-15. Particularly with AO-11, which had a stronger effect than AO-15 in vivo, fibrils are difficult to detect and only amorphous precipitates with bumps or nodules were seen. The reduced numbers of fibrils observed by EM correlated with a decrease in SDS-stable large fibrils detected by anti-Aβ antibody (6E10) on western blots of SDS-PAGE performed on parallel samples. These results suggest that AO-11 and AO-15 efficiently inhibited the formation of Aβ$_{1\text{-}42}$ SDS-stable aggregates in vitro.

## DISCUSSION

With accumulating reports pointing to small SDS-stable Aβ$_{42}$ oligomers as a major early culprit in AD pathology, inhibiting their formation has become an attractive therapeutic target. Previous generations of inhibitors of Aβ aggregation were often selected for

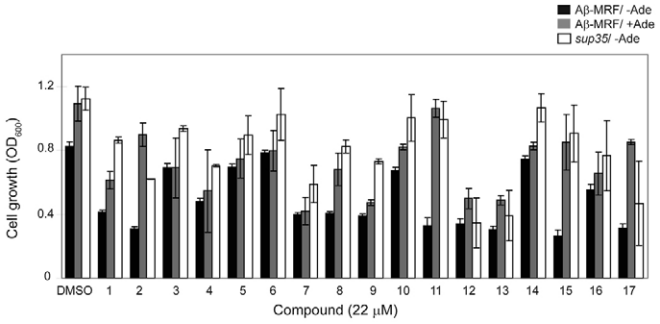


**Fig. 5. Confirmation of primary screen.** The activity of the 17 compounds identified in the primary screen was retested in triplicate. Inhibition of growth in –Ade (black) indicates restored translational release factor activity of Aβ-MRF. Growth on +Ade (gray) indicates absence of compound-associated toxicity. Effects of compounds were also tested on a *sup35* mutant strain lacking Aβ-MRF (white). Compounds 11 (AO-11) and 15 (AO-15) inhibited growth only in –Ade, but not in +Ade, and also had no effect on the growth of the *sup35* mutant, indicating that their ability to restore translational termination activity is not general and is probably caused by reducing Aβ-MRF oligomerization. Cell growth was measured as optical density at 600 nm ($OD_{600}$). This was possible because the filter-sterilized media used here precluded the appearance of revertants because it allowed more rapid growth than the autoclaved media used in the large-scale experiments in Fig. 4. Data are mean values, and error bars represent standard deviation of three replicate assays.

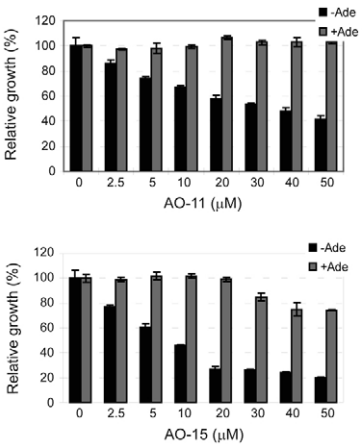


**Fig. 6. Dose-dependent activity of AO-11 and AO-15 on Aβ oligomerization and general cell growth.** Cells expressing Aβ-MRF were grown in –Ade (black) and +Ade (gray) media to late logarithmic phase in the presence of AO-11 or AO-15 at the indicated concentrations. The effect of each compound on Aβ-MRF was measured as $OD_{600}$ and converted into % of growth relative to 0 μM controls. Data represent the average of three replicate experiments and error bars represent the standard deviation.

their ability to inhibit the formation of large Aβ fibrils rather than toxic oligomers. Subsequent attempts to characterize the effects of these compounds on oligomer formation have faced challenges partly due to the myriad of possible in vitro Aβ$_{42}$ assemblies and difficulty in monitoring and characterizing oligomer formation (Bitan et al., 2005; Hepler et al., 2006). Furthermore, because these primary assays were conducted in vitro, many of these compounds were toxic when tested in vivo (Liu and Schubert, 2006).

Our screen is designed to specifically select against Aβ$_{42}$ oligomer formation. Indeed, scyllo-inositol, which is known to inhibit Aβ$_{42}$ from forming toxic oligomers, also inhibited oligomer formation in our assay (Fig. 3). Some small molecules that inhibit Aβ$_{42}$ oligomer formation might also inhibit large fibril formation. Our ultimate goal is to uncover molecules that affect oligomer formation without inhibiting fibril formation. This specificity might be crucial for success in the clinic, because fibril formation might actually be beneficial (Chen et al., 2010; Cheng et al., 2007; Necula et al., 2007a; Treusch et al., 2009).

We found that, in living yeast, Aβ-MRF aggregated into small SDS-stable low-n oligomers such as dimers, trimers and tetramers that look remarkably like the neurotoxic Aβ$_{42}$ oligomers secreted from CHO cells (7PA2) expressing the familial V717F Alzheimer's-disease-causing APP (Walsh et al., 2002a). However, because yeast lysates were treated with 1% SDS before loading on acrylamide gels, the oligomers seen could have been derived from larger in vivo aggregates that were broken down by the room-temperature 1% SDS treatment. To investigate this possibility we omitted the SDS sample treatment and characterized the lysate in gels lacking detergent except for 0.1% of SDS in the running buffer. Interestingly, these untreated samples showed similar oligomers, although some larger material was also stuck in the well (Fig. 2A). The material in the well when cut out and treated with 1% SDS at room temperature did not break into oligomers (Fig. 2B) but, following boiling in 2% SDS, was converted into monomers. We next analyzed Aβ-MRF in lysates on non-denaturing gels in the absence of any detergent. Here, although they ran as 10-15mer sized oligomers, treatment of these lysates with a reducing agent released the Aβ-MRF into low-n oligomers (Fig. 2C). These results suggest that Aβ-MRF exists as low-n oligomers in yeast cells, in which it is likely to be under reduced conditions.

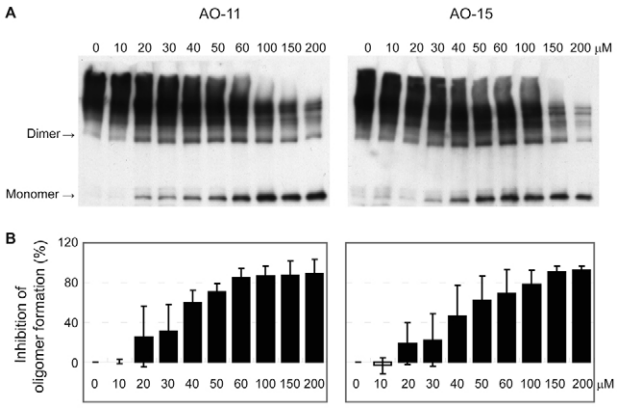


**Fig. 7. AO-11 and AO-15 suppress Aβ-MRF oligomerization in yeast in a dose-dependent manner.** (A) Detection of SDS-stable Aβ-MRF oligomers by western blot analysis. The assay strain overexpressing Aβ-MRF was grown in complex medium in the presence of each compound at the indicated concentrations. Equal amounts of lysate were treated with 1% SDS for 7 minutes at room temperature and analyzed by immunoblotting with anti-Sup35-RF antibodies following SDS-PAGE. Aβ-MRF cells grown with DMSO (0) were used as controls. (B) Dose-dependent inhibition of Aβ-MRF oligomer formation. Intensities of Aβ monomer and oligomer bands in A were quantified using the Alpha Imager 2200 (Alpha Innotech) and were converted into % inhibition of oligomer formation using oligomer to monomer ratios.

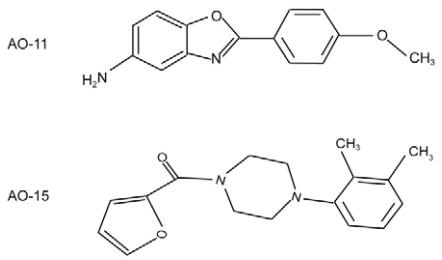


**Fig. 8. Structures of AO-11 and AO-15.** AO-11 is 2-(4-methoxyphenyl)-1,3-benzoxazol-5-amine. AO-15 is 1-(2,3-dimethylphenyl)-4-(2-furoyl) piperazine or Methanone, [4-(2,3-dimethylphenyl)-1-piperazinyl]-2-furanyl.

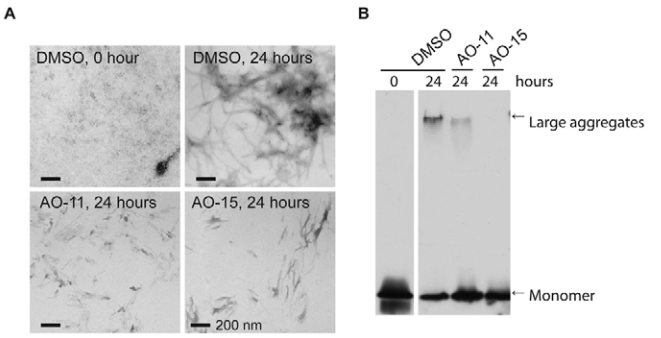


**Fig. 9. Effect of AO-11 and AO-15 on the in vitro polymerization of the Aβ$_{42}$ peptide.** (A) Image of Aβ$_{42}$ incubated for 0 or 24 hours in the presence of DMSO, or for 24 hours in the presence of 50 μM AO-11 or AO-15. A small aliquot was applied to formvar/carbon-coated copper grids and stained with 4% (w/v) aqueous uranyl acetate grids for EM analysis. The images are representative of at least three independent experiments. (B) Western blot analysis of SDS-PAGE performed on parallel samples used in A. A sample of each Aβ$_{42}$ reaction (calculated to be 10 μg) was separated on a 4-20% Tris-Glycine SDS-PAGE gel and probed with monoclonal anti-Aβ antibody (6E10).

In this study, we used a robot to perform our yeast Aβ$_{42}$ oligomerization HTS campaign. Of 12,800 compounds tested, four passed this pilot proof-of-principle primary screen. We eliminated two of the four hits in a secondary screen for false positives that affected the suppression efficiency of a *sup35* mutant (Bradley et al., 2003). The remaining two compounds (AO-11 and AO-15) both significantly decreased Aβ-MRF oligomer formation in vivo and affected Aβ$_{42}$ aggregation in vitro. Although AO-11 and AO-15 seem to affect large aggregate formation as well as oligomerization, our assay should allow the detection of compounds that specifically inhibit oligomerization and not large aggregate formation.

## METHODS

### Yeast strains and media

Assays were performed in L2333, a mutant derivative of 74-D694 (*MATa ade1-14 ura3-52 leu2-3,112 trp1-289 his3-200*) (Chernoff et al., 1993) with a G1256A change in *SUP35* (Bradley et al., 2003), and in Aβ-MRF and Aβm2-MRF yeast also derived from 74D-694 but with a *sup35::LEU2* disruption (Nakayashiki et al., 2001) and respectively containing p1364 (pRS313, *CEN, Ura3, CUP1::* Aβ-*MRF*) or p1541 (pRS313, *CEN, Ura3, CUP1::* Aβ$^{F19,20T/I31P}$-*MRF*) (Bagriantsev and Liebman, 2006). *ERG6* of the ergosterol biosynthetic pathway was deleted (Longtine et al., 1998) in these strains to enhance permeability to small compounds.

Media, cultivation and transformation procedures were standard (Sherman, 2002). Strains were grown in complex medium (YPD), or in synthetic media lacking (–Ade), or including (+Ade) adenine. Expression of Aβ-MRF was driven by the copper-inducible *CUP1* promoter with 50 μM $CuSO_4$.

### Growth assays for the primary screen and validation

Duplicate assays in clear flat-bottom 384-well plates (ScreenMates) in 90 μl used a Tecan Freedom EVO 200 robot (Ratia et al., 2008). Wells were filled in order with 45 μl of assay medium, 0.2 μl of library compounds (10 mM in DMSO) and 45 μl of assay medium inoculated with $1\times10^5$ or $1\times10^4$ cells/well for the –Ade or +Ade assays, respectively, of the assay strain grown without Aβ-MRF overexpression. Each plate contained 32 positive (Aβm2-MRF) and 32 negative (Aβ-MRF) controls with 0.2 μl of DMSO only. Plates sealed half way with parafilm were incubated at room temperature with shaking (900 rpm) until the cultures reached stationary phase (5 days for –Ade; 4 days for +Ade). $OD_{600}$ determined growth.

### Data analysis

Z-factor values (Zhang et al., 1999) were determined from controls using:

$$Z = 1 - \frac{3(\text{SD of positive} + \text{SD of negative})}{|\text{mean of positive} - \text{mean of negative}|},$$

where 'SD of positive' and 'SD of negative' are, respectively, standard deviations of $OD_{600}$ of Aβm2-MRF and Aβ-MRF samples; 'mean of positive' and 'mean of negative' are, respectively, $OD_{600}$ means of Aβm2-MRF and Aβ-MRF samples. Z values greater than 0.5 indicate an excellent assay.

$EC_{50}$ values were estimated by fitting data to $v_i = v_o/(1 + [I]/EC_{50})$ with the SigmaPlot (v. 9.01 Systat) Enzyme Kinetics module, where $v_i$ and $v_o$ are, respectively, the reaction rates in the presence and absence of inhibitor, and $[I]$ is the inhibitor concentration (Ratia et al., 2008).

### SDS and non-denaturing gel electrophoresis and western blot analysis

Preparation of lysates and western blot analysis of Aβ-MRF oligomers were performed as described previously (Bagriantsev and Liebman, 2006). For SDS-PAGE, lysates of late logarithmic cells grown at 30°C in 5 ml +Ade with 50 μM $CuSO_4$ and DMSO or a compound were treated with 1% SDS for 7 minutes at room temperature, resolved on 7.5% Tris-Glycine gels (0% SDS, Bio-Rad) in 0.1% SDS containing Laemmli running buffer and transferred to a PVDF membrane (Bio-Rad) in Laemmli buffer with 0.1% SDS and 15% methanol.

For non-denaturing PAGE, lysates in native sample buffer (Invitrogen) with 0.5% digitonin, 10 mM PMSF, and yeast anti-protease cocktail (Sigma) 1:100 were resolved on a 3-12% Blue native Novex Bis-Tris gel (Invitrogen).

Immunodetection used monoclonal antibodies against the RF domain of Sup35p (BE4; developed by our laboratory). Signals were

## TRANSLATIONAL IMPACT

### Clinical issue

Alzheimer's disease (AD) is the most common form of dementia, currently affecting an estimated 5.2-million people in the United States alone. Although the number of deaths caused by stroke, prostate cancer, breast cancer, heart disease and HIV all decreased from 2000-2008, deaths caused by AD increased by 66%. Furthermore, the increasing age demographic in the United States means that the number of patients with AD is likely to double by 2040. Thus, the need to prevent or more effectively treat the disease is crucial. AD is mainly a sporadic disease, most often appearing in people over 65 years of age. It manifests as a progressive brain disorder that damages and eventually destroys brain cells, leading to loss of memory and cognitive function, and eventually leading to death. Amyloid plaques, composed of the small protein amyloid β ($A\beta_{42}$), and neurofibrillar tangles, composed of Tau protein, are pathological hallmarks of AD. Recent studies indicate that small $A\beta_{42}$ oligomers are the likely neurotoxin that causes AD. Thus, preventing the formation of $A\beta_{42}$ oligomers is a promising approach to prevent or slow AD.

### Results

To identify therapeutic compounds that can prevent or treat AD, the authors developed a high-throughput screen (HTS) for drug-like inhibitors of $A\beta_{42}$ oligomerization that uses yeast expressing $A\beta_{42}$ fused to a reporter. The activity of the reporter in the fusion protein is lost when the $A\beta_{42}$ region causes the protein to aggregate into small oligomers, enabling a screen to detect small molecules that inhibit aggregation, based on restoration of reporter activity. A pilot screen of 12,800 compounds identifies two molecules that restore reporter activity in a dose-dependent manner and that are subsequently validated in secondary genetic screens. The finding that both compounds also inhibit $A\beta_{42}$ oligomer formation shows that the yeast reporter system is a reliable readout for oligomer formation. Both compounds have chemical properties that are desirable for development into central nervous system drugs.

### Implications and future directions

This work confirms the validity of a yeast HTS to identify inhibitors of $A\beta_{42}$ oligomerization. Larger drug and drug-like libraries will now be screened, and the most promising drug candidates will be modified using structure-activity relationship analyses to develop new non-toxic compounds that inhibit $A\beta_{42}$ oligomerization. In future, potential drugs will be tested for effects in models of AD based on higher organisms, paving the way to clinical trials.



detected using a Western-Star chemiluminescence development kit (Applied Biosystems) and quantified using AlphaEaseFC imaging software of Alpha Imager 2200 (Alpha Innotech).

### $A\beta_{42}$ in vitro polymerization and immunoblotting

Polymerization (Stine et al., 2003) was at room temperature with mild shaking for 24 hours followed by sonication after diluting 5 mM $A\beta_{42}$ made from lyophilized recombinant $A\beta_{42}$ peptide (HFIP form, Rpeptide) dissolved in anhydrous DMSO (Sigma) into 40 μM in 10 mM Tris (pH 7.4) in the presence of AO-11, AO-15 or control DMSO. For immunoblotting, 10 μg of polymerized $A\beta_{42}$ was treated with 1% SDS, resolved by SDS electrophoresis in a 4-20% Tris-Glycine gel and detected by anti-Aβ antibodies (6E10, Signet Laboratories).

### Transmission electron microscopy

Samples adsorbed onto 200 hex-mesh formvar/carbon-coated copper grids and stained with 4% (w/v) uranyl acetate were viewed using a JEOL 1200EX transmission electron microscope.

### SEC fractionation and dot blot assay

Total lysate of 200 μl (0.5 mg total yeast protein in native sample buffer) was loaded onto on equilibrated analytical Superose 12 10/300 GL column (GE Healthcare) connected to a liquid chromatography system (Bio-Rad) and eluted with yeast lysis buffer (Bagriantsev and Liebman, 2006) lacking glycerol. Samples from each fraction (20 μl) were spotted onto PVDF membrane that was pre-wet with transfer buffer (25 mM Bicine, 25 mM Bis-Tris, 1 mM EDTA, pH 7.2). The membrane was washed once in TBS (20 mM Tris, 140 mM NaCl, pH 7.6) with 0.1% Tween-20, blocked and immunoblotted with anti-Sup35-RF antibodies.

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#### COMPETING INTERESTS

The authors declare that they do not have any competing or financial interests.

#### AUTHOR CONTRIBUTIONS

S.-K.P. and S.W.L. designed the overall experimental approach and wrote the paper, with S.-K.P. performing most of the experiments. S.D.P. and A.D.M. provided the compound libraries, established the screening protocol, prepared the robot liquid handling system for the HTS screen, and analyzed and placed the hits in chemical family groups. They also edited the manuscript. L.M.J. helped with the in vitro $A\beta_{42}$ polymerizations and size exclusion chromatography, and helped edit the manuscript. M.J.L. helped develop the in vitro biochemical assays and analyzed the data.

#### SUPPLEMENTARY MATERIAL

Supplementary material for this article is available at
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