

Discovery of amyloid-beta aggregation inhibitors using an engineered assay for intracellular protein folding and solubility

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Abstract: Genetic and biochemical studies suggest that Alzheimer's disease (AD) is caused by a series of events initiated by the production and subsequent aggregation of the Alzheimer's amyloid β peptide (A β), the so-called amyloid cascade hypothesis. Thus, a logical approach to treating AD is the development of small molecule inhibitors that either block the proteases that generate A β from its precursor (β - and γ -secretases) or interrupt and/or reverse A β aggregation. To identify potent inhibitors of A β aggregation, we have developed a high-throughput screen based on an earlier selection that effectively paired the folding quality control feature of the *Escherichia coli* Tat protein export system with aggregation of the 42-residue AD pathogenesis effector A β 42. Specifically, a tripartite fusion between the Tat-dependent export signal ssTorA, the A β 42 peptide and the β -lactamase (Bla) reporter enzyme was found to be export incompetent due to aggregation of the A β 42 moiety. Here, we reasoned that small, cell-permeable molecules that inhibited A β 42 aggregation would render the ssTorA-A β 42-Bla chimera competent for Tat export to the periplasm where Bla is active against β -lactam antibiotics such as ampicillin. Using a fluorescence-based version of our assay, we screened a library of triazine derivatives and isolated four nontoxic, cell-permeable compounds that promoted efficient Tat-dependent export of ssTorA-A β 42-Bla. Each of these was subsequently shown to be a *bona fide* inhibitor of A β 42 aggregation using a standard thioflavin T fibrillization assay, thereby highlighting the utility of our bacterial assay as a useful screen for antiaggregation factors under physiological conditions.

Keywords: Alzheimer's disease; aggregation; chemical biology; chemical chaperone; protein misfolding; twin-arginine translocation

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that affects 20–30 million people worldwide.¹ Genetic and biochemical studies suggest that AD is caused by a series of events initiated by the

production and subsequent aggregation of the Alzheimer's amyloid β peptide (A β), the so-called amyloid cascade hypothesis.^{2,3} Accordingly, a logical approach to treating AD is the development of small molecule inhibitors that block the proteases that generate A β from its precursor (β - and γ -secretases) or that interrupt and/or reverse A β aggregation. While there are currently no drugs in clinical use that target these mechanisms, many putative disease-modifying inhibitors are under active development and some have shown *in vivo* efficacy in mouse models and in clinical trials.^{4–8} One of the most notable examples is Neurochem's Alzhemed (tramiprosate) that was developed to

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prevent amyloid formation and deposition in the brain, and thus modify the course of AD. Unfortunately, despite Alzhemed's promise in Phase II clinical trials, the recent U.S. Phase III trial is widely considered to have failed and the EU Phase III trial has been discontinued, thus signifying the urgent need for new inhibitors that can be brought to human clinical trials.

Towards the development of new A β aggregation blockers, a number of methods for screening putative small-molecule inhibitors of A β aggregation have been developed.^{9–12} The majority of these assays have been performed *in vitro* using either turbidity or binding of thioflavin T (ThT) to assay the formation of amyloid fibrils from synthetic A β peptide. However, based on the high cost of generating pure samples of synthetic or recombinant A β peptide, large-scale library screening is cost prohibitive. In addition, there are now numerous reports indicating that small, soluble oligomers of A β , rather than large fibrillar plaques, are the principal cause of synaptotoxicity associated with AD.^{4,13–15} Thus, assays that measure turbidity and binding to ThT, which are inherently based on assembly of A β into amyloid, are unlikely to uncover compounds that affect soluble A β oligomers. Similarly, the rapid aggregation kinetics of A β peptides, especially the 42-residue A β 42 peptide, make it difficult to prepare samples that are entirely devoid of small aggregates. These preexisting “seeds” are known to promote A β aggregation and may therefore prevent the identification of inhibitors that block the earliest stages of aggregation. Finally, inhibitors isolated using these *in vitro* screens may cross-react with cellular components, especially endogenous β -sheet proteins, and induce cytotoxicity.

To address these shortcomings, we have developed a low-cost, bacterial cell-based assay for the isolation of A β aggregation inhibitors. This assay is based on a previously developed genetic selection whereby the folding quality control mechanism of the bacterial twin-arginine translocation (Tat) system was used for the identification of peptides and proteins that fold into a stable and soluble conformation.¹⁶ In this earlier study, we found that the efficiency with which Tat-exported β -lactamase (Bla) chimeras localize in the periplasm, and thus confer ampicillin (Amp) resistance to *Escherichia coli* cells, was dependent on the folding characteristics of the fusion partner polypeptide. By growing cells on increasing concentrations of Amp, we were able to select for protein variants that exhibited higher intracellular stability and were less prone to aggregation. It was observed that the aggregation-prone A β 42 peptide conferred only low levels of resistance to cells when expressed as an ssTorA-A β 42-Bla fusion, where the Tat-dependent signal peptide ssTorA was used to specifically target the chimera to the Tat export pathway. Importantly, screening a library of random A β 42 sequences resulted in the isolation of several solubility-enhanced variants that conferred

resistance to cells on Amp,¹⁶ indicating that factors affecting the solubility of A β 42 are sufficient to confer export via the Tat pathway. Here, we hypothesized that small-molecule inhibitors could similarly affect the solubility of A β 42 in a manner that rescues ssTorA-A β 42-Bla export to the periplasm. To test this, we successfully developed two high-throughput versions of our genetic folding assay that facilitated isolation of A β 42-aggregation inhibitors from a combinatorial library of triazine derivatives without the need for synthetic A β 42. The identification of small molecules that inhibit formation of A β 42 oligomers highlights the utility of our bacterial assay as a useful screen for antiaggregation factors under biologically relevant conditions.

Results

High-throughput strategies for monitoring A β 42 aggregation in living cells

Based on our earlier finding that A β 42 solubility could be effectively discriminated using the Tat quality control mechanism,¹⁶ we hypothesized that small, cell-permeable molecules capable of interfering with wildtype (wt) A β 42 aggregation would promote Tat-dependent export of the ssTorA-A β 42-Bla reporter to the periplasm (see Fig. 1). Such molecules would represent putative inhibitors of A β 42 aggregation and could easily be identified in a high-throughput screening (HTS) approach provided that a robust assay was available for reporting the subcellular location of Bla. One strategy for detecting Bla that has localized in the periplasm is to simply monitor cell viability in liquid medium supplemented with Amp.¹⁶ Indeed, cells expressing ssTorA-A β 42-Bla were much less resistant to Amp relative to cells expressing ssTorA-GM6-Bla (see Fig. 2), where GM6 is a solubility-enhanced version of A β 42 developed previously by Hecht and coworkers¹⁷ that we utilized as a positive control in this study. We found that at Amp concentrations of 400 μ g/mL and above, in 96-well plates, the growth rate of cells expressing ssTorA-GM6-Bla was \sim 16-fold greater than that measured for cells expressing ssTorA-A β 42-Bla [Fig. 2(a)]. Moreover, a 16-fold difference in minimum inhibitory concentration (MIC), where MIC is defined here as the Amp concentration at which cell growth was $<$ 50% of the growth in the absence of Amp, was observed for cells expressing ssTorA-GM6-Bla versus ssTorA-A β 42-Bla ($>$ 800 μ g/mL vs. 50 μ g/mL, respectively). When the inoculation density was decreased 5-fold, a \sim 12-fold difference in growth rate was seen for cells expressing ssTorA-GM6-Bla compared to cells expressing ssTorA-A β 42-Bla at Amp concentrations of 50 and 100 μ g/mL and a 4-fold difference in MIC on Amp was observed (400 μ g/mL vs. 50 μ g/mL, respectively) [Fig. 2(b)]. Importantly, this data indicates that a dramatic increase in Amp resistance is conferred to cells when the solubility of A β 42

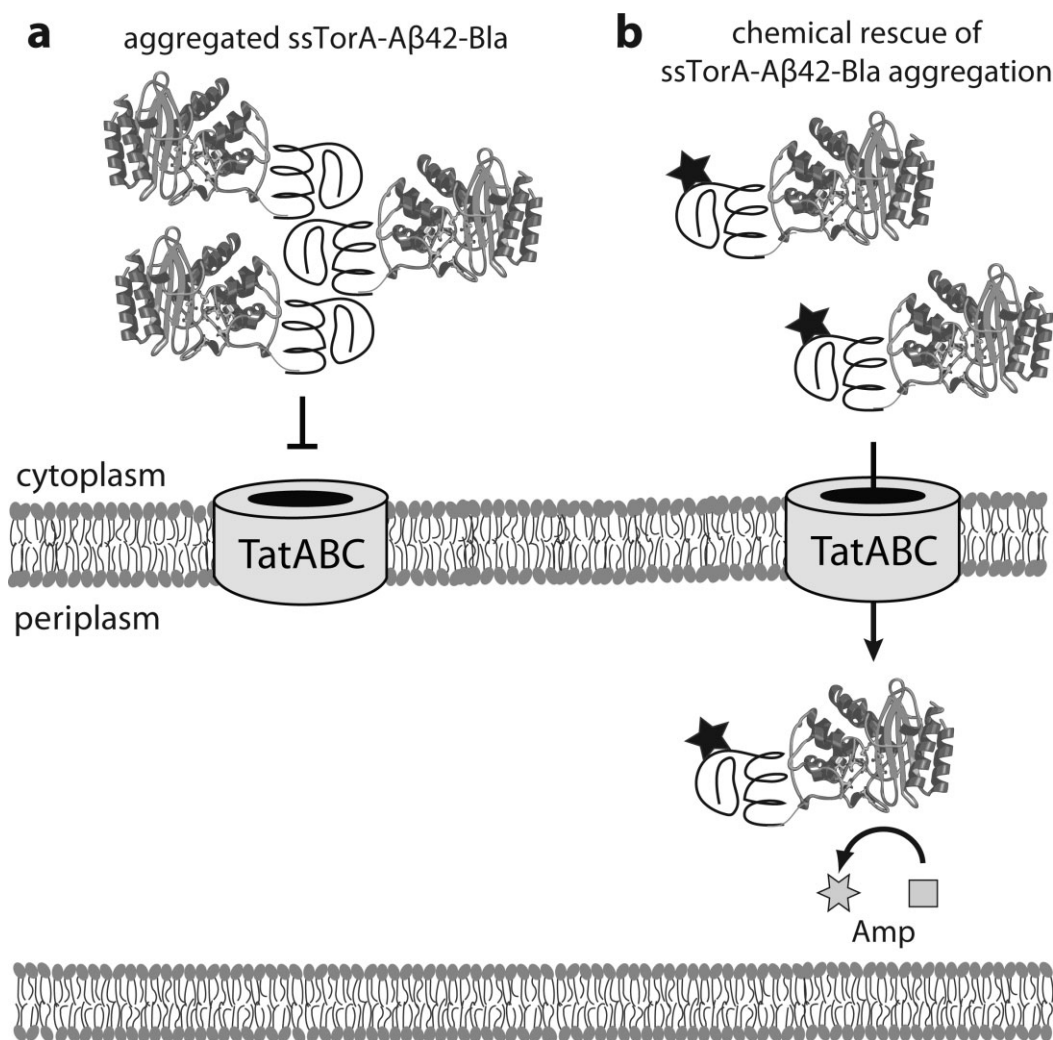


Figure 1. Strategy for isolating A β 42 aggregation inhibitors using the Tat pathway. (a) ssTorA-A β 42-Bla expressed in the cytoplasm readily aggregates via A β 42 self-assembly and thus is not exported by the Tat translocon that recognizes correctly folded proteins. (b) In the presence of small molecules that bind to A β 42 and prevent aggregation, ssTorA-A β 42-Bla remains soluble and is efficiently exported to the periplasm where Bla hydrolyzes Amp.

is enhanced, in this case by genetic mutation. The difference in MIC between soluble and aggregated A β 42-expressing cells is the basis for the HTS growth assay.

As an alternative to using selective pressure to identify aggregation inhibitors, we also explored the development of a fluorescence-based screen that capitalized on our earlier observation that the extent of A β 42 solubility correlated directly to the quantity and activity of Bla enzyme transported to the periplasm via the Tat system.¹⁶ To measure periplasmic A β 42-Bla activity directly in living cells, we employed the fluorogenic Bla substrate CCF2/AM.¹⁸ Intact CCF2/AM is a membrane-permeant ester comprised of two different fluorophores, fluorescein and coumarin, connected via a permissive cephalosporin β -lactam ring. Upon entering cells, host esterases hydrolyze the ester's functional abilities and release the nonpermeant Bla substrate CCF2. Excitation of CCF2 with 409 nm light in the absence of Bla induces FRET within CCF2 and 520 nm light (green) is emitted. After cleavage by Bla, the

acceptor is separated from the donor and emission is at 447 nm (blue). The CCF2/AM assay was originally limited to eukaryotic cells because bacteria such as *E. coli* lacked the esterase activity necessary for activation of the esterified and membrane-permeable CCF2/AM form of the substrate. However, when *E. coli* cells were supplemented with this activity via periplasmic expression of a fungal lipase known as cutinase, periplasmic Bla could be readily detected using CCF2/AM.¹⁹ Indeed, we found that cells expressing *Fusarium solani pisi* cutinase in the periplasm seemed bright green when incubated with CCF2/AM [Fig. 3(a)], indicating that the substrate had been activated inside cells and remained uncleaved. This result also confirmed that the CCF2/AM substrate was capable of crossing the outer membrane and that after activation by cutinase it was effectively trapped inside cells owing to its negative charge. When cutinase and Bla were both colocalized to the periplasm via the addition of N-terminal Sec signal peptides on each, cells emitted

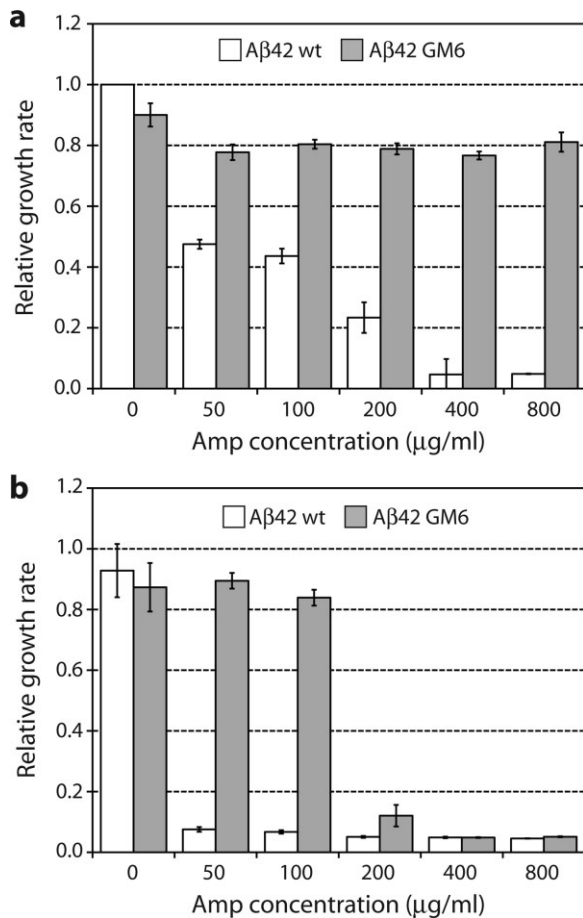


Figure 2. Amp resistance as an indicator of Aβ42 solubility. Inocula of (a) 1:100 or (b) 1:500 grown in the presence of varying Amp concentrations as indicated. Cells were induced to express ssTorA-Aβ42-Bla (white bars) or ssTorA-GM6-Bla (gray bars). All data was normalized to the growth rate of the 1:100-diluted cells expressing ssTorA-Aβ42-Bla in the absence of Amp. Data represents the average of six replicate experiments and the error bars represent the standard error of the mean (s.e.m.).

strong blue fluorescence [Fig. 3(b)]. This observation indicated that in these cells the CCF2/AM substrate was activated by periplasmic cutinase and subsequently cleaved by periplasmic Bla, thereby resulting in dominant coumarin donor fluorescence. Thus, heterologous expression of cutinase allowed for facile fluorescent detection of Bla in the periplasm of living *E. coli* cells. Importantly, when periplasmic cutinase was coexpressed with versions of Bla that were not exported (i.e., due to absence of an export signal), cells emitted green fluorescence (see, for instance, Table I) indicating that a positive signal required colocalization of both cutinase and Bla to the periplasm. This is significant because it shows that there is no measurable false-positive signal arising from a reaction between nonlocalized Bla and CCF2 in the cytoplasm. We speculate that the reason for this is that CCF2 becomes negatively charged in the periplasm due to cutinase

activity and thus is incapable of diffusing across the negatively charged inner membrane.

To determine if CCF2/AM hydrolysis was suitable for detecting Aβ42 solubility and transport, we transformed cells with (i) plasmid pBKPC expressing Sec-targeted cutinase (ssPhoA-CutA) from an arabinose-inducible promoter and (ii) an Aβ42 reporter plasmid encoding either ssTorA-Aβ42-Bla or ssTorA-GM6-Bla. After incubation with CCF2/AM, cells were analyzed in a fluorescence microplate reader (excitation 409 nm; emission 447 nm or 520 nm). Cells expressing cutinase alone emitted a weak blue signal that was just above background [Fig. 3(c)]. In contrast, cells coexpressing cutinase and the efficiently exported ssTorA-

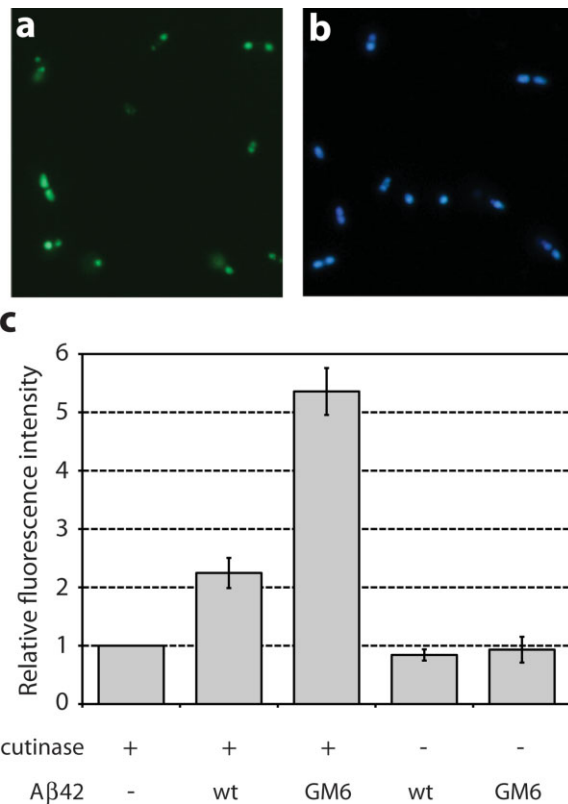


Figure 3. Fluorescence-based detection of Aβ42 aggregation in living *E. coli* cells using CCF2/AM. (a) Negative control cells expressing Sec-targeted cutinase (ssPhoA-CutA) from pBKPC and (b) positive control cells coexpressing ssPhoA-CutA and TEM-1 Bla with its native Sec signal peptide both from pBPC. Cells were incubated with 1 μM CCF2/AM for 1 h with gentle rocking and visualized by fluorescence microscopy (ex: 409 nm). (c) CCF2 fluorescence for cells expressing ssTorA-Aβ42-Bla or ssTorA-GM6-Bla in the presence (+) or absence (-) of cutinase as indicated. CCF2 fluorescence readings were normalized by dividing fluorescence values measured at 447 nm by those measured at 520 nm, and then normalized to OD₆₀₀ of the cells. All data was then normalized to the background fluorescence measured in cells expressing cutinase only. Data represents the average of three replicate experiments and the error bars represent the s.e.m.

Table I. 96-Well Plate Assays of A β 42 Solubility

Reporter construct	FI/OD ^a	SD ^b	CV (%) ^c	S-to-B ^d	SD above mean ^e
ssTorA-A β 42-Bla	1111	55	5.0	1.0	—
ssTorA-GM6-Bla	2566	154	6.0	2.3	9.4
A β 42-Bla	1144	62	5.4	1.0	0.5
GM6-Bla	1198	67	5.6	1.1	1.3

^a Specific fluorescence activity calculated as the fluorescence intensity (FI) divided by the optical density (OD) (average of 96 readings).

^b Standard deviation (SD) from measurements across an entire plate.

^c Coefficient of variance (CV) calculated as the (SD/FI/OD)*100 across an entire plate.

^d Signal-to-background (S-to-B) calculated by normalizing signals to the FI/OD for ssTorA-A β 42-Bla negative control.

^e Number of standard deviations above the average of the ssTorA-A β 42-Bla negative control.

GM6-Bla emitted a high level of blue fluorescence that was >12-fold above that observed for cutinase-only control cells and, more importantly, nearly 3-fold more fluorescent than cells expressing the export-incompetent ssTorA-A β 42-Bla fusion [Fig. 3(c)]. These fluorescent signals were dependent upon cutinase expression as no signal above background was observed for either wt or GM6 versions of A β 42 expressed in the absence of cutinase [Fig. 3(c)]. Likewise, no signal above background was observed when the cutinase and A β 42 constructs were coexpressed in Δ *tatC* cell lacking a functional Tat system (data not shown), indicating that the fluorescent phenotype was entirely dependent on the Tat export system. Collectively, these results indicate that heterologous expression of cutinase allows for facile and quantitative fluorescent discrimination between the soluble ssTorA-GM6-Bla fusion and its aggregation-prone counterpart ssTorA-A β 42-Bla in living *E. coli* cells using the cell membrane-permeable substrate CCF2/AM.

Statistical evaluation of Tat-based HTS screen

Next, we sought to demonstrate the robustness and reproducibility of the fluorescent version of our A β 42 solubility assay in 96-well plate format. For these studies, 96-well plates containing 100 μ L of growth medium (M9 minimal medium supplemented with casamino acids and 0.4% arabinose; chosen due to its extremely low fluorescence background) were inoculated with log-phase *E. coli* cells carrying plasmids for the expression of cutinase and one of the following: (i) ssTorA-A β 42-Bla; (ii) ssTorA-GM6-Bla; (iii) A β 42-Bla or (iv) GM6-Bla (the latter two constructs each lacked a Tat signal peptide and thus served as nonexported negative controls). After 18 h of growth and induction of protein expression at 30°C, CCF2/AM was added to each well and incubated for 1 h. Using a fluorescence microplate reader, the fluorescence intensity (FI; ex: 409 nm; em: 447 nm, 520 nm) and cell density (OD; absorbance at 600 nm) in each well were measured. For each of the four Bla fusions, measurements across an entire 96-well plate were made (Table I). Consistent with data in Figure 3, we observed a near 2.5-fold increase in signal for ssTorA-GM6-Bla versus ssTorA-A β 42-Bla. Importantly, the signal for the positive con-

trol ssTorA-GM6-Bla construct (2566 average, 154 standard deviation) was more than nine standard deviations above the average for the ssTorA-A β 42-Bla negative control (1111 average, 55 standard deviation). Two additional negative controls, A β 42-Bla and GM6-Bla, yielded signals that were virtually indistinguishable from ssTorA-A β 42-Bla. It is noteworthy that the coefficient of variation (CV) for 96 measurements made across an entire plate was \leq 6.0% for each construct tested. Additionally, the Z-factor,²⁰ which measures the quality or power of an HTS assay, was 0.6 (a value between 0.5 and 1.0 is indicative of an excellent assay). Finally, between-plate and day-to-day variations for each construct were determined to be \leq 7.0% and 9.6%, respectively (data not shown).

A search for inhibitors of A β 42 aggregation

To test whether our assay was able to isolate small molecules that inhibit A β 42 aggregation by intermolecular effects, we screened a combinatorial library of \sim 1000 compounds derived from the triazine scaffold.^{21,22} Combinatorial diversity in this library was introduced by varying the substituents at positions R_1 and R_2 on the triazine scaffold [Fig. 4(a)] and the chemical composition of these substituents is described in our earlier work.^{21,22} We chose this triazine library because: (1) previous studies by Hecht and coworkers showed that aggregation inhibitors already exist in this library²³; and (2) compound RS-0466, which was shown by Selkoe and coworkers to block A β oligomerization and rescue long-term potentiation, is a triazine derivative.²⁴

To implement our screen, we added *E. coli* cells coexpressing cutinase and ssTorA-A β 42-Bla to 96-well plates containing growth medium supplemented with inducer and 5 μ M of each candidate molecule from the library of triazine derivatives in dimethyl sulfoxide (DMSO). After 18 h of growth and induction of protein expression, CCF2/AM was added to each well and the fluorescence and cell density of each well was measured with a fluorescence microplate reader. In parallel, each compound was screened in control plates containing *E. coli* cells expressing A β 42-Bla (lacking the Tat signal sequence). The purpose of this secondary screen was to rule out false positives that might arise

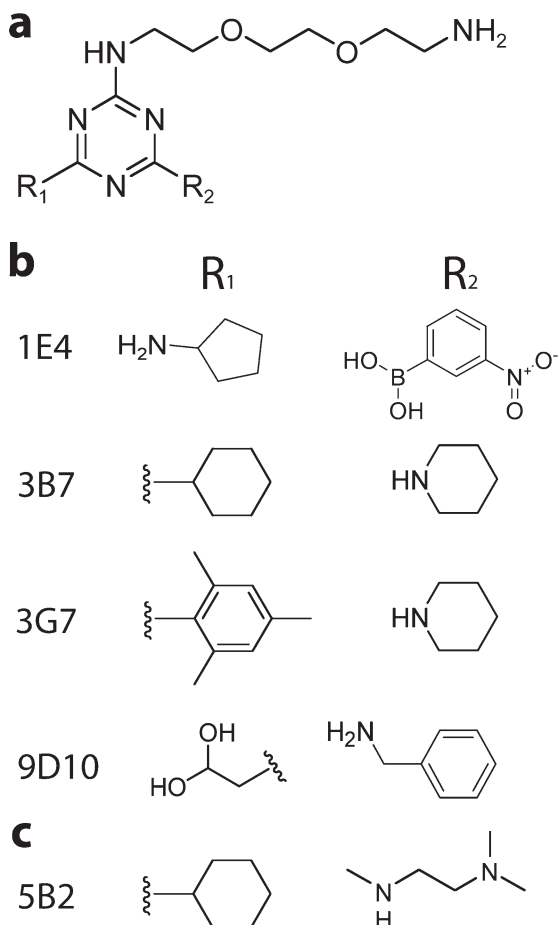


Figure 4. Isolation of triazine derivatives that inhibit A β 42 aggregation in *E. coli*. (a) Triazine scaffold where R_1 and R_2 substituents were varied to generate a combinatorial compound library. (b) R_1 and R_2 substituents for the four compounds identified as hits using the A β 42 HTS CCF2/AM assay. (c) Compound 5B2 is structurally similar to positive hit 3B7 at the R_1 position but scored as an inactive compound in our cell-based screen.

due to factors such as: (i) high intrinsic fluorescence of inactive compounds; (ii) nonspecific Bla localization to the periplasm; and/or (iii) release of Bla into the medium caused by compound-induced cell lysis. Importantly, several wells containing triazine derivatives fluoresced at levels that were significantly above background (>three standard deviations above the average signal measured for the ssTorA-A β 42-Bla negative control with blank DMSO added) for ssTorA-A β 42-Bla expression but not for A β 42-Bla. Each of the ~1000 compounds was tested in quadruplicate and the identification of “hits” was consistent across the four replicates. We consider four of these fluorescent hits, namely compounds 1E4, 3B7, 3G7, and 9D10, to be putative inhibitors of A β 42 aggregation [Fig. 4(b)]. It is noteworthy that compound 3E2, previously identified by Hecht and coworkers using a bacterial screen based on A β 42 fused to the green fluorescent protein,²³ was scored as a positive hit in some of our

plates (three out of four replicates) but was not as strong a hit as the four compounds shown in Figure 4 (each was identified in four out of four replicates). It seems that at the low concentration used here for screening (5 μ M versus 30–100 μ M in,²³ 3E2 is not as effective as the other lead compounds. It should also be noted that all library compounds were diluted into plates from a 10 mM DMSO stock solution. The use of higher concentrations of our hit molecules (and thus higher DMSO levels in each well) did not seem to have any adverse effects on the cell-based assay. Specifically, we observed a dose-dependent increase in signal up to 30 μ M of each compound and the signal remained constant over a concentration range of 30–100 μ M for each compound (data not shown). Also interesting is the observation that our HTS assay was capable of effectively discriminating between hits and inactive candidates, for example 5B2, which is identical to 3B7 at position R_1 and differs only at position R_2 [Fig. 4(c)]. Thus, in the future, it might be possible to screen collections of molecules that only differ at one position as a means to establish the structure/activity relationship between the substituents at each position and the resulting level of aggregation inhibition. Additionally, all of the putative hits were cross-checked for their ability to rescue growth of ssTorA-A β 42-Bla-expressing cells and each was found to confer a 6–8-fold increase in Amp resistance relative to control cells treated with blank DMSO. Recall that the solubility-enhanced ssTorA-GM6-Bla fusion conferred a ~12–16-fold increase in Amp resistance relative to control cells (see Fig. 2 above); thus, it seems that both the fluorescence- and viability-based assays are capable of discriminating active and inactive compounds.

Confirmation of hits as A β 42 aggregation inhibitors

To verify whether our isolated compounds were *bona fide* inhibitors of A β 42 aggregation, we monitored the effect of each compound on the aggregation behavior of synthetic A β 42. It has been established that A β 42 peptide can be rendered soluble and monomeric after filtration, addition of organic solvents and sonication.²⁵ Dilution of these soluble, monomeric A β 42 peptides into aqueous buffer results in peptide aggregation and the formation of amyloid fibrils, which can be readily assayed by the binding and subsequent fluorescence of ThT.²⁶ We tested each of our compounds with shaking where the rate of A β 42 aggregation is faster.²³ Specifically, 20 μ M of synthetic A β 42 was incubated in the presence of varying concentrations of each compound (or blank DMSO) for 2.5 h and the resulting fibril formation was measured by the change in ThT fluorescence that results from fibril binding. All four compounds seemed to inhibit A β 42 in a concentration-dependent manner, similar to the known A β 42 inhibitor tannic acid (see Fig. 5). Compounds 3B7 and

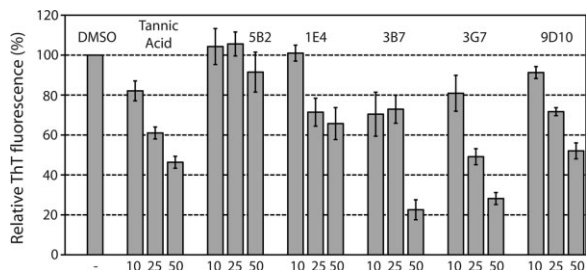


Figure 5. *In vitro* characterization of aggregation inhibition using synthetic A β 42. ThT fluorescence of synthetic A β 42 fibrils after incubation of 20 μ M A β 42 with 10, 25, or 50 μ M compounds as indicated. Tannic acid, a known inhibitor of A β 42 aggregation, served as a positive control. 5B2 was structurally similar to our triazine hits but did not score as a hit in our screen and thus served as a negative control. 1E4, 3B7, 3G7, and 9D10 were compounds identified as A β 42 aggregation inhibitors in the cell-based CCF2/AM screen of \sim 1000 triazine derivatives. ThT fluorescence of A β 42 incubated with triazine derivatives was expressed as a percentage of the DMSO-only negative control (note that the compounds were all dissolved in DMSO). Data represents the average of three replicate experiments and the error bars represent the s.e.m.

3G7 exhibited the most potent inhibition with IC_{50} values between \sim 25 and 50 μ M. These compounds caused a \sim 70–80% reduction in ThT fluorescence at a concentration of 50 μ M (see Fig. 5) and 90% reduction at concentrations of 100 μ M or greater (data not shown). In contrast, control compound 5B2, which was scored as inactive during the HTS process, did not show any significant inhibition activity (see Fig. 5).

Discussion

We performed a high-throughput screen of a library containing \sim 1000 small molecules derived from triazine and identified four lead compounds that promoted efficient Tat-dependent export of ssTorA-A β 42-Bla, a proven reporter of A β 42 aggregation.¹⁶ This is significant because there are currently only four mildly effective drugs for the treatment of Alzheimer's patients and none of these addresses the underlying molecular cause of AD. Our lead compounds 1E4, 3B7, 3G7, and 9D10 were identified as A β 42 aggregation inhibitors using a novel fluorescence-based HTS screen enabled by the substrate CCF2/AM. Each compound was cross-checked with the absorbance-based version of our folding assay, then counter-screened in cells that lacked a functional Tat system or with a A β 42-Bla reporter that lacked an export signal and finally confirmed as *bona fide* inhibitors of A β 42 aggregation using an *in vitro* ThT assay. Compounds 3B7 and 3G7 showed the strongest inhibition (IC_{50} of \sim 25–50 μ M) whereas the compound 5B2, which has the same R_1 group as 3B7, showed no inhibitory effect on A β 42 aggregation. The IC_{50} values found here for 3B7 and 3G7 are comparable to that reported for the triazine derivative 3E2 (\sim 30 μ M) by Hecht and coworkers.²³

Our compounds also compared favorably to previously reported inhibitors. In particular, IC_{50} values for compounds assayed in a manner similar to our analysis (i.e., inhibition of A β 42 fibril formation using the ThT assay) ranged between 0.1 and 50 μ M and included: curcumin (1.1 μ M) and its analog rosmarinic acid (1.1 μ M),^{27,28} ferulic acid (5.5 μ M), rifampicin (9.1 μ M) and tetracycline (10 μ M),²⁹ tannic acid (0.1 μ M), myricetin (0.43 μ M) and nordihydroguaiaretic acid (0.87 μ M),³⁰ bis-styrylpyridine and bis-styrylbenzene derivatives (0.1–2.7 μ M),³¹ nicotine (50 μ M)³² and *N,N'*-bis(3-hydroxyphenyl)pyridazine-3,6-diamine (50–100 μ M).³³ Of all these compounds, tannic acid seems to be one of the best A β 42 inhibitors with a reported IC_{50} of \sim 0.1 μ M.³⁰ It is noteworthy, however, that there appears to be a fair amount of lab-to-lab discrepancy between reported values. For instance, whereas³⁰ report a value of \sim 0.1 μ M for tannic acid,²³ reported an IC_{50} value for tannic acid (\sim 25–50 μ M) that was similar to the value we measured. This is likely caused by differences in experimental conditions such as incubation time, incubation buffer and source of synthetic A β 42 between the different reports. Importantly, Kim et al. reported that at concentrations of 25 and 50 μ M, the inhibitory effect of their best compound, E2, was similar to or better than tannic acid or another known inhibitor dopamine. Likewise, we observed that our best compounds could inhibit A β 42 aggregation on par with tannic acid. Thus, while there may be disagreement over the absolute IC_{50} value for tannic acid, it is clear that the triazine compounds isolated here and by Kim et al. are relatively potent A β 42 aggregation inhibitors.

It is noteworthy that 5B2 and 3B7 each have the same R_1 position side group but differ in their R_2 moiety: in 5B2 it is aliphatic whereas in 3B7 it is cyclic. This finding suggests that, like the known A β 42 aggregation inhibitor tannic acid, steric bulk may be important in blocking A β 42 aggregation. The other compounds identified in this study all have cyclic moieties in their side groups. We note that compound 3E2,²³ shown here to be a slightly less efficient inhibitor in the context of our cell-based screen, contains aliphatic side groups. This would suggest that the binding of A β 42 by these different triazine derivatives may be site-specific, although at present we do not have any mechanistic data regarding the interactions between A β 42 and any of the isolated compounds.

A key advantage of our approach is that isolation of these compounds did not require synthetic A β 42; instead, biosynthesis of A β 42 was carried out by *E. coli* cells thereby making this an extremely cost-effective strategy compared to earlier screening studies that were performed *in vitro* using synthetic A β 42 peptides.^{9,10,12} Another advantage of our cell-based approach is its inherent ability to select for membrane-permeant, noncytotoxic compounds. This seems to be rather important in light of emerging evidence

that intraneuronal formation of soluble, low-molecular-weight oligomers of A β 42 is the pathogenically critical process in AD.^{15,34–39} In fact, one recent report implicates soluble A β 42 dimers as the smallest synaptotoxic species.⁴⁰ Thus, compounds that can efficiently cross cell membranes and inhibit the earliest stages of intracellular A β 42 aggregation may prove to be the most effective therapeutic direction for the treatment of AD. Although we do not currently know the export-incompetent conformation (low-n oligomers (e.g., dimers, trimers, and tetramers), dodecamers, etc.) of A β 42 in our assay, inhibitors that promote export are highly likely to render ssTorA-A β 42-Bla monomeric or, at most dimeric, because assemblies larger than this are likely to be incapable of passage through the Tat translocation pore.⁴¹ Thus, we suspect that our assay is inherently capable of identifying inhibitors of the earliest stages of A β 42 aggregation, a feat that is difficult with HTS assays that are dependent upon assembly of A β into amyloid (e.g., ThT assay).

To date, only a handful of bacterial HTS assays have been used to screen compound libraries against nonbacterial targets but each of these has successfully uncovered interesting lead molecules including estrogen receptor modulators,⁴² HIV protease inhibitors^{43,44} and, akin to our studies here, A β 42 aggregation inhibitors.²³ The use of bacteria for HTS studies is fast, inexpensive and reliable; however, one possible limitation is the potential difficulty of getting diverse compounds into cells. As evidence that permeability is not necessarily a limiting factor, other groups have used *E. coli*-based screening assays to isolate lead compounds from structurally diverse libraries.^{42–44} Along similar lines, Hecht and coworkers successfully employed wt *E. coli* cells expressing an A β 42-GFP reporter to isolate an aggregation inhibitor from a library of triazine derivatives that was nearly identical to the library screened here.²³ It should be noted that their hit compound E2 was also a positive hit in our study, although at the lower screening doses used here it was not as effective as the four inhibitors that we characterized here. Regardless, the ability of their assay and ours to isolate inhibitors would suggest that the triazine scaffold is privileged in its ability to cross biological barriers and/or interfere with intracellular A β 42 aggregation. Indeed, our own sampling of known A β 42 aggregation inhibitors that were structurally unrelated to triazine, such as ferulic acid, curcumin, nordihydroguaiaretic acid, tannic acid, nicotine, proline, and tramiposate revealed that none of these promoted export of ssTorA-A β 42-Bla in *E. coli* (data not shown). While the reason for this is currently unknown, there are several possible explanations: (i) the outer membrane of may be impermeable to these compounds; (ii) these compounds may be metabolized by *E. coli* cells or actively effluxed from cells; and/or (iii) the stage at which the compound inhibits A β 42 aggregation may not be compatible with inhibition of

ssTorA-A β 42-Bla aggregation. To improve screening in the future, we are currently investigating the use of different mutant strains (i.e., with increased outer membrane permeability or decreased efflux activity) or the application of a periplasmic folding assay for A β 42 aggregation (Mansell, Fisher and DeLisa, manuscript in preparation) since penetration of compounds to the periplasm is reportedly less restricted than to the cytoplasm.^{45–47} Finally, with the recent development of two eukaryotic cell-based assays for A β 42 aggregation including an A β 42-GFP assay in yeast⁴⁸ and an amyloid precursor protein expressing CHO cell line that secretes SDS-stable Abeta oligomers,²⁴ it will be of great interest to see whether compounds isolated using *E. coli*-based assays are reliable inhibitors in these other host strains.

Materials and Methods

Bacterial strains and plasmids

E. coli strain BLR(DE3) (Novagen; F⁻ *ompT hsdSB* (r_B⁻ m_B⁻) *gal dcm* (DE3) Δ (*srl-recA*)306::Tn10 (TetR)) was routinely used in these studies, although a BW25113 *tolC*⁻ strain from the Keio collection⁴⁹ was also used. The plasmids pSALect-A β 42 and pSALect-GM6 for expressing ssTorA-A β 42-Bla or ssTorA-GM6-Bla, respectively, have been described previously.¹⁶ The deletion of the ssTorA signal peptide from pSALect-A β 42 and pSALect-GM6 was carried out using QuikChange Site-Directed Mutagenesis Kit (Stratagene), yielding plasmids pSALect(Δ ssTorA)-A β 42 and pSALect(Δ ssTorA)-GM6. For the CCF2 assays described below, plasmid pBKPC was constructed by cloning the *Fusarium solani pisi* cutinase gene (kindly provided by K. Griswold and G. Georgiou) immediately after DNA encoding the Sec-specific signal peptide from *E. coli* alkaline phosphatase (ssPhoA) in pBAD18-Kan.⁵⁰ Plasmid maintenance was with 25 μ g/mL chloramphenicol (Cam) for pSALect derivatives and 50 μ g/mL kanamycin (Kan) for pBKPC. Initial CCF2/AM validation experiments were carried out using pBAD18-ssPhoA-CutA (pBPC) that is Amp^R and expressed both cutinase and Bla via the Sec pathway.

CCF2/AM assay

Cells grown overnight in Luria-Bertani broth (LB) supplemented with Cam were diluted 1:100 and grown for 3 h at 37°C. These exponential phase cultures were diluted 1:100 in 96-well black, clear-bottom plates (Corning Costar) containing M9 minimal medium supplemented with 0.5% casamino acids, 0.4% arabinose as sole carbon source and inducer of cutinase expression, 1 mM IPTG, 25 μ g/mL Cam, and 50 μ g/mL Kan. Cells were incubated at 30°C under quiescent conditions for 18 h. CCF2/AM (Invitrogen) was added to the cultures at 1 μ M final concentration and gently mixed by rocking at room temperature for 1 h. CCF2 was excited at 409 nm and emission recorded at 449

nm and 520 nm on a Gemini EM microplate spectrofluorometer (Molecular Devices). Optical density (OD) of cultures was read at 600 nm using a Bio-Tek Synergy HT microplate reader (Bio-Tek Instruments). Fluorescence readings were normalized by dividing fluorescence values measured at 447 nm by those measured at 520 nm, and then normalized to OD₆₀₀. Five micromolar of each triazine derivative was added when required. Because the compounds were diluted in DMSO, DMSO was added to all cultures at 0.3% final concentration. Cells analyzed by microscopy were cultured identically to the protocol given above for the 96-well plate assay using CCF2/AM. After 1 h incubation with CCF2/AM, 5 μ L of cells were removed from the plate and directly applied to a microscope slide for visualization. Fluorescence microscopy was performed on an Axioskop 40 microscope (Zeiss) with Bla filter set (Chroma Technology).

Growth selection assay

Cells grown overnight in LB supplemented with Cam were diluted 1:100 and grown for 3 h at 37°C. These exponential phase cultures were diluted 1:500 in 96-well plates (Corning Costar) containing LB supplemented with 25 μ g/mL Cam, 1 mM IPTG, 50 μ g/mL Amp selection and, when required, 30 μ M triazine-derived compounds. The plates were incubated at 37°C for 16 h under quiescent conditions. Culture OD was read at 600 nm. All cultures contained 0.3% DMSO.

ThT assay

ThT assays were performed with synthetic A β ₄₂ (Anaspec) as described previously²³ with shaking at 37°C for 2.5 h. Compounds were added at 10, 25, and 50 μ M. ThT fluorescence was measured at 483 nm (450 nm excitation) on a Gemini EM microplate spectrofluorometer. Readings were normalized to the DMSO-only negative control and expressed as percentage relative fluorescence.

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