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Small-molecule aggregates inhibit amyloid polymerization

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Many amyloid inhibitors resemble molecules that form chemical aggregates, which are known to inhibit many proteins. Eight known chemical aggregators inhibited amyloid formation of the yeast and mouse prion proteins Sup35 and recMoPrP in a manner characteristic of colloidal inhibition. Similarly, three known anti-amyloid molecules inhibited β-lactamase in a detergent-dependent manner, which suggests that they too form colloidal aggregates. The colloids localized to preformed fibers and prevented new fiber formation in electron micrographs. They also blocked infection of yeast cells with Sup35 prions, which suggests that colloidal inhibition may be relevant in more biological milieus.

The aggregation of proteins into amyloid fibers is associated with a growing list of diseases, including diabetes, Alzheimer's, Parkinson's, Huntington's and the prion diseases. In these disorders, proteins aggregate into long, unbranched fibers after misfolding into a β-sheet–rich conformation[1]. Though there are no approved therapies targeting amyloid formation directly, many organic molecules inhibit fibrillization *in vitro*[2–7]. Some, such as the chelator clioquinol (**1**), even have activity *in vivo*[4]. These results have inspired the hope of therapeutic applications for some molecules[3–5].

Curiously, many fibrillization inhibitors resemble molecules known to form promiscuous chemical aggregates. These colloidal particles are composed of small organic molecules and range in size from 50 to over 600 nm[8]. Once formed, they physically sequester proteins and inhibit enzymes nonspecifically[8,9]. Like many inhibitors of amyloid polymerization, these colloidal inhibitors are typically highly conjugated, hydrophobic and dye-like (**Supplementary Table 1** online)[8,9]. A good example is the amyloid inhibitor Congo red (**2**), a dye that was one of the first molecules observed to exhibit colloidal inhibition[8]. The flavonoid baicalein (**3**), an inhibitor of α-synuclein polymerization[6], resembles the known chemical aggregator quercetin (**4**), and 4,5-dianilinophthalimide (DAPH, **5**), an inhibitor of Alzheimer's amyloid formation[2], resembles the aggregator bisindoylmaleimide (**6**; **Supplementary Fig. 1** online). Given that chemical aggregates function through enzyme sequestration, we wondered whether they might also sequester protein molecules from each other, thereby preventing amyloid polymerization. Here, we investigate this hypothesis in two classic amyloid-forming proteins: the yeast prion protein Sup35 (ref. 10) and the recombinant mouse prion protein recMoPrP$_{89–230}$ (ref. 11). We ask whether known chemical aggregators can inhibit amyloid fiber formation, whether known fibrillization inhibitors form colloidal aggregates and whether amyloid inhibition by these molecules is in fact mediated via colloidal aggregation.

Eight known chemical aggregators and two known nonaggregators[8,9] were tested for inhibition of Sup35 fibrillization in a thioflavin T (ThT, **7**) fluorescence assay. All eight inhibited Sup35 fibrillization both in seeded and unseeded polymerization reactions, whereas the two nonaggregators were inactive (**Table 1** and **Supplementary Fig. 2a,b** online). Likewise, three amyloid inhibitors (DAPH, baicalein and clioquinol) also inhibited Sup35 polymerization. Among the most potent molecules was the chemical aggregator tetraiodophenolphthalein (TIPT, **8**), which had a half-maximal inhibitory concentration (IC_{50}) of 2.5 μM (**Fig. 1a**). To control for spectroscopic interference, we also tested two chemical aggregate-forming molecules for inhibition by dynamic light scattering (DLS). At 2 μM TIPT the DLS reaction was 30% inhibited, and at 20 μM the reaction was more

Table 1 Inhibition of amyloid polymerization

Compound	Percent inhibition Sup35	Percent inhibition Sup35 + BSA	Percent inhibition recMoPrP
Known colloidal inhibitors			
30 μM TIPT (**8**)	99.3	6.8	0[a]
50 μM clotrimazole (**9**)	62.7	5.6	21.5[b]
50 μM sulconazole (**10**)	75.5	10.1	100
50 μM nicardipine (**11**)	61.5	0	22.7
30 μM rottlerin (**12**)	99.4	40.5	71.1[b]
50 μM S3218 (**13**)	33.7	0	0
10 μM 4BPAP (**14**)	36.4	2.1	100[b]
5 μM Congo red (**2**)	100	100	100
Known nonaggregating molecules			
50 μM fluconazole (**15**)	0	9	5.9
50 μM lidocaine (**16**)	1.1	0	0[b]
Known inhibitors of amyloid formation			
50 μM clioquinol (**1**)	54.7	0	0
50 μM baicalein (**3**)	95.4	17.6	34.3
50 μM DAPH (**5**)	87.1	44.7	73.6[b]

Amyloid polymerization inhibition by 8 known colloidal inhibitors, 2 nonaggregators and 3 known fibrillization inhibitors, ±5 mg ml^{-1} BSA. Compounds were tested at concentrations sufficient to observe a significant effect.
[a]Inhibition of recMoPrP$_{89–230}$ was assayed at 50 μM. [b]Inhibition of recMoPrP was assayed at 25 μM.

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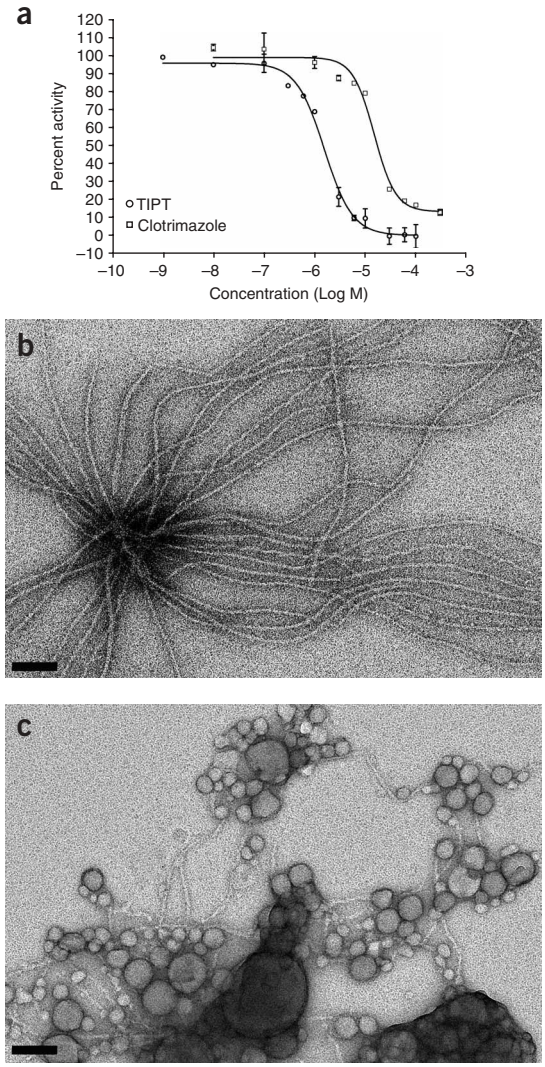

Figure 1 Chemical aggregators inhibit amyloid formation in biochemical assays. (**a**) The known chemical aggregators TIPT and clotrimazole inhibit seeded Sup35 polymerization in a dose-dependent manner. Inhibition of Sup35 polymerization was measured by ThT fluorescence and plotted as a function of time. Percent inhibition was calculated as the ratio of the initial rate of the inhibited reaction compared with control. Error bars represent the s.d. of triplicate measurements. (**b**) Transmission electron micrographs of control Sup35 prion fibers grown in the absence of compound. (**c**) Preformed Sup35 fibers mixed with chemical aggregates of TIPT (concentration of compound 100 μM). Scale bar, 100 nm.

than 99% inhibited. Similarly, the reaction with 25 μM of clotrimazole (9) was 98% inhibited (**Supplementary Fig. 2c,d**).

Given that aggregate-based inhibition is nonspecific, chemical aggregators should also inhibit other amyloidogenic proteins. In a ThT-based assay, six of the eight colloidal inhibitors also inhibited fibrillization of the mouse prion protein (recMoPrP). In most cases potency was lower than that observed against Sup35 (**Table 1**), which is likely due to the use of 3 M urea in the recMoPrP assay, a condition that is known to disrupt the formation of colloidal aggregates[8]. Consistent with this view, one of the chemical aggregators that did not inhibit recMoPrP fiber formation, TIPT, did do so in an electron microscopy assay that lacked urea. TIPT particles not only associated with preformed recMoPrP fibers, but also inhibited fibrillization, resulting in a grid empty of fibers (**Supplementary Fig. 3** online).

If known chemical aggregators inhibit fibrillization, do known fibrillization inhibitors form colloidal aggregates? We tested five published amyloid inhibitors for detergent-sensitive inhibition of β-lactamase (**Supplementary Table 2** online). Detergent disrupts the colloids, and molecules that inhibit β-lactamase in the absence but not the presence of detergent are likely to act via this mechanism[9,12,13]. These five molecules included the α-synuclein fibrillization inhibitor baicalein[6], the chelator clioquinol[4], DAPH[2] and Congo red[3]—all inhibitors of Aβ fiber formation—and the dye Direct Yellow 20 (17), an inhibitor of huntingtin polymerization[7]. The IC_{50} values against β-lactamase for these molecules ranged from 1 to 30 μM in the absence of detergent, but addition of 0.01% Triton X-100 essentially eliminated inhibition, which is consistent with chemical aggregate formation. These IC_{50} values resemble those required to inhibit fibrillization by these same molecules.

To understand whether the inhibition of fibrillization was mediated through colloidal forms of the organic molecules, we evaluated inhibition for characteristic features of this mechanism. These include the sensitivity of inhibition to the presence of a large amount of secondary protein, such as bovine serum albumin (BSA)[12,13], time-dependence of inhibition and sensitivity to the concentration of the target protein[8,9]. Inhibition of Sup35 polymerization displayed all

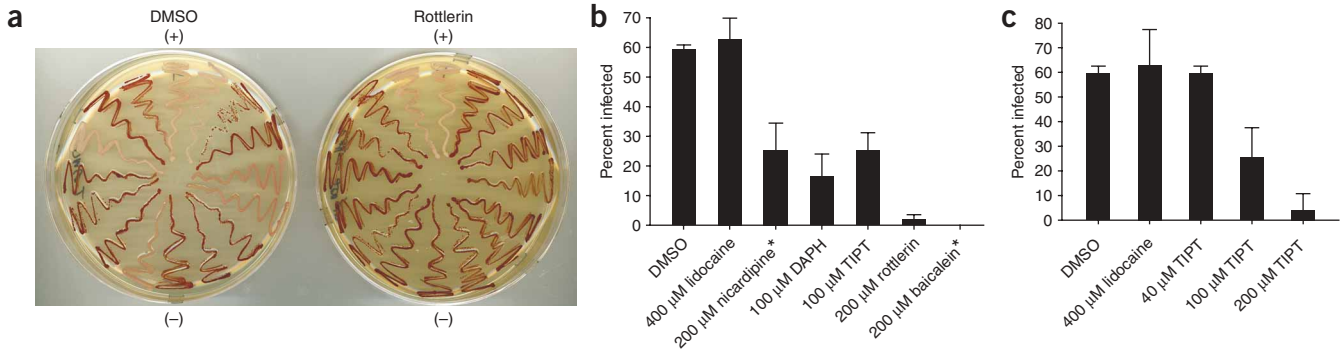

Figure 2 Chemical aggregators inhibit prion infection in yeast culture. (**a**) Representative cultures of infected and noninfected yeast cells. The infected phenotype, PSI^+, appears as white or pink colonies, whereas the uninfected PSI^- colonies are red. Rottlerin, a known chemical aggregator, inhibits infection. (+) and (–) correspond to positive and negative controls for infection. (**b**) The known chemical aggregate-forming molecules nicardipine, DAPH and baicalein block infection to varying degrees. (**c**) Infection is blocked in a dose-dependent manner by TIPT. *Indicates that slightly fewer than 56 viable colonies were observed due to mild cell toxicity. Error bars represent the s.d. in colonies counted.

of these traits. Inhibition was inversely proportional to the amount of seed in polymerization reactions; for a fixed concentration of 50 μM clotrimazole, reactions with 0.5%, 1% and 5% seed were inhibited 99%, 95% and 30%, respectively. Similarly, inhibition was increased by a 5-min preincubation (**Supplementary Table 3** online). For enzymes, nonionic detergents can be used to disrupt colloidal inhibitors, making this a useful assay for aggregation-based inhibitors. Because detergent itself inhibits fiber formation, here we use sensitivity to BSA as a proxy for this effect. BSA seems to attenuate colloidal inhibition by saturating the colloid prophylactically, thus protecting the target protein from sequestration[12]. Addition of BSA typically attenuated inhibition, often substantially (**Table 1** and **Supplementary Fig. 2a**). For instance, whereas 30 μM of TIPT inhibited 99.3% of fibrillization, incubation with 20 mg ml^{-1} BSA (final concentration 5 mg ml^{-1}) reduced inhibition to 6.8%. Similarly, 50 μM of nicardipine inhibited fibrillization by 61.5% in the absence of BSA but did not inhibit in its presence. BSA also prevented inhibition by the fibrillization inhibitors DAPH, baicalein and clioquinol, which is consistent with their own activities due to colloidal formation.

To further characterize this mechanism, fibers of both Sup35 and recMoPrP were examined by transmission electron microscopy. In control experiments, long, unbranched Sup35 fibers were distributed densely on the grid (**Fig. 1b**). However, when mixed with 100 μM TIPT, preformed fibers of Sup35 became coated with colloidal particles (**Fig. 1c**). Similar images were obtained from identical experiments performed with recMoPrP and 50 μM of the colloidal inhibitor rottlerin (**Supplementary Fig. 4a,b** online). When Sup35 and recMoPrP were polymerized in the presence of 100 μM TIPT and 50 μM rottlerin, respectively, hardly any fibers were observed at all (**Supplementary Fig. 4c,d**).

Several known inhibitors of amyloid fiber formation are active in cell culture[4,5]. To establish whether aggregate-based inhibition is relevant in more biological milieus, several chemical aggregators were tested for inhibition of Sup35 prion infectivity in yeast cell culture. Cells were made infection-competent through enzymatic digestion of the cell wall (**Supplementary Methods** online)[14]. The resulting spheroplasts were exposed to sonicated prion fibers with and without compound. Infection was quantified by color: uninfected cells are red due to build-up of a red adenine precursor. Prion-infected colonies are white or pink, as prion-mediated inactivation of Sup35 restores adenine biosynthesis (**Fig. 2a**). Of the eight molecules tested in this assay, 4-bromophenylazophenol (4BPAP) and clioquinol were toxic, and their effects were not considered. The other six chemical aggregators inhibited infection at concentrations between 100 and 200 μM (**Fig. 2b**). For instance, baicalein and rottlerin almost completely ablated infectivity at 200 μM, whereas 100 μM nicardipine inhibited infectivity by about 50%. For TIPT, inhibition of infectivity was measured over a range of concentrations from 40 to 200 μM, resulting in a half-maximal effective concentration (EC_{50}) of approximately 100 μM (**Fig. 2c**). As a control, the non-aggregator lidocaine was also tested, and no inhibition of infectivity was observed.

A cautionary conclusion to emerge from these studies is that chemical aggregators may be common among inhibitors of amyloid fibrillization. Eight of eight known chemical aggregators inhibited Sup35 polymerization, and most inhibited that of recMoPrp. Correspondingly, five of five amyloid inhibitors form chemical aggregates at relevant concentrations. Mechanistically, the inhibition of amyloid formation by these molecules displays all the characteristics of aggregate-based inhibition of enzymes, and presumably operates by a similar sequestration-based mechanism. More encouragingly, colloidal aggregation, of all the confusing mechanisms of inhibition in early drug discovery, is among the easiest to control for and may be rapidly distinguished from the direct, specific binding that might be expected of therapeutic candidates. From the standpoint of the behavior of colloidal aggregates themselves, it is noteworthy that many are active in cell culture, presumably because they remain stable and retain the ability to sequester protein even in a more biological environment.

Note: Supplementary information and chemical compound information is available on the Nature Chemical Biology website.

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AUTHOR CONTRIBUTIONS

B.Y.F. performed the experiments unless otherwise noted, and wrote the manuscript with B.K.S. B.H.T. helped perform the Sup35 infectivity assay. H.W. performed the electron microscopy studies. D.W.C. performed the recMoPrP polymerization experiments. S.R.C. helped perform the Sup35 polymerization experiments. B.C.H.M., S.B.P. and J.W. provided guidance and helped edit the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturechemicalbiology/.

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1. Nelson, R. *et al. Nature* **435**, 773–778 (2005).
2. Blanchard, B.J. *et al. Proc. Natl. Acad. Sci. USA* **101**, 14326–14332 (2004).
3. Lorenzo, A. & Yankner, B.A. *Proc. Natl. Acad. Sci. USA* **91**, 12243–12247 (1994).
4. Ritchie, C.W. *et al. Arch. Neurol.* **60**, 1685–1691 (2003).
5. Yang, F. *et al. J. Biol. Chem.* **280**, 5892–5901 (2005).
6. Zhu, M. *et al. J. Biol. Chem.* **279**, 26846–26857 (2004).
7. Heiser, V. *et al. Proc. Natl. Acad. Sci. USA* **97**, 6739–6744 (2000).
8. McGovern, S.L., Caselli, E., Grigorieff, N. & Shoichet, B.K. *J. Med. Chem.* **45**, 1712–1722 (2002).
9. McGovern, S.L., Helfand, B.T., Feng, B. & Shoichet, B.K. *J. Med. Chem.* **46**, 4265–4272 (2003).
10. True, H.L. & Lindquist, S.L. *Nature* **407**, 477–483 (2000).
11. Abid, K. & Soto, C. *Cell. Mol. Life Sci.* **63**, 2342–2351 (2006).
12. Coan, K.E.D. & Shoichet, B.K. *Mol. Biosyst.* **3**, 208–213 (2007).
13. Feng, B.Y., Shelat, A., Doman, T.N., Guy, R.K. & Shoichet, B.K. *Nat. Chem. Biol.* **1**, 146–148 (2005).
14. Tanaka, M. & Weissman, J.S. *Methods Enzymol.* **412**, 185–200 (2006).