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Emergence and natural selection of drug-resistant prions

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Abstract

Drug resistance is a refractory barrier in the battle against many fatal diseases caused by rapidly evolving agents, including HIV, apicomplexans and specific cancers. Emerging evidence suggests that drug resistance might extend to lethal prion disorders and related neurodegenerative amyloidoses. Prions are self-replicating protein conformers, usually ‘cross- β ’ amyloid polymers, which are naturally transmitted between individuals and promote phenotypic change. Prion conformers are catalytic templates that specifically convert other copies of the same protein to the prion form. Once in motion, this chain reaction of conformational replication can deplete all non-prion copies of a protein. Typically, prions exist as ensembles of multiple structurally distinct, self-replicating forms or ‘strains’. Each strain confers a distinct phenotype and replicates at different rates depending on the environment. As replicators, prions are units of selection. Thus, natural selection inescapably enriches or depletes various prion strains from populations depending on their conformational fitness (ability to self-replicate) in the prevailing environment. The most successful prions confer advantages to their host as with numerous yeast prions. Here, I review recent evidence that drug-like small molecules can antagonize some prion strains but simultaneously select for drug-resistant prions composed of mammalian PrP or the yeast prion protein, Sup35. For Sup35, the drug-resistant strain configures original intermolecular amyloid contacts that are not ordinarily detected. Importantly, a synergistic small-molecule cocktail counters prion diversity by eliminating multiple Sup35 prion strains. Collectively, these advances illuminate the plasticity of prionogenesis and suggest that synergistic combinatorial therapies might circumvent this pathological vicissitude.

1. Introduction to prion phenomena

1.1 Prions are replicators that store and transmit biological information

Life is based upon the replication, transfer and evolution of specific heritable information. The information that encodes life can only proliferate and be edited by natural selection, however, if selection acts more rapidly than mutation, drift or other competing processes.¹ Various physical media can meet this demand and constitute the material replicators that stably store, copy and transmit heritable information.¹ None have succeeded like DNA, the most familiar genomic medium, which can adopt an indefinitely large number of distinct self-replicable structures distinguished by the sequence of bases. DNA is an unlimited hereditary replicator² with unbounded information content and evolutionary potential. Within cells, however, numerous other structures exist with a more limited, but still potentially very large repertoire of self-replicating, heritable states, which can exert rapid and profound effects on phenotype.^{2–4} Extraordinary examples of limited hereditary replicators are provided by prions.

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Prions are proteins that can fold into several alternative, functionally distinct conformations, at least one of which can replicate itself and become infectious.^{5,6} Typically, the self-replicating prion conformation is an amyloid template: a 'cross- β ' fibrous structure in which the β -sheet strands align orthogonal to the fiber axis.^{5–16} This highly stable conformation resists chaotropes, detergents, proteases and mechanical stress,^{17–19} and confers the inherent specificity of the self-replicating process.^{5,6,17} Fibers elongate at both ends by specifically capturing and converting natively folded copies of the same protein to the cross- β form^{17,20,21} (Fig. 1). Conformational replication involves remodeling intramolecular non-covalent bonds of the native state and establishing intermolecular hydrogen bonds and other non-covalent bonds of the amyloid state.^{17,22} Newly incorporated monomers align reproducibly and in register to duplicate the previously established steric adaptations of the catalytic cross- β form^{17,22–24} (Fig. 1). Thus, an active template is regenerated with high fidelity after each round of replication^{17,22–24} (Fig. 1). Steric constraints dictate that generally only polypeptides with the same or very similar primary sequence can be efficiently converted.^{17,24} This self-templating or 'seeding' activity is critical for infectivity. Seeding, however, must be combined with a mechanism to fragment fibers (Fig. 1). Fibers will fragment spontaneously upon reaching a certain length^{18,19} or fragmentation can be catalyzed by cellular factors, such as Hsp104 in yeast.^{14,25–30} Fragmentation ensures that self-templating ends are continuously exposed and disseminated.^{6,9,31} Thus, transmission of specific prion-encoded information between cells or individuals becomes possible.

1.2 Beneficial prions

The vast majority of prions have been uncovered in the yeast, *Saccharomyces cerevisiae*, which may deploy more than twenty proteins as prions to provide a vast reservoir of heritable information.^{6,7,32} In yeast, the self-perpetuating change in protein conformation elicited by prions encodes dominant phenotypic traits, without any underlying change in DNA sequence. These dominant traits are inherited in a non-Mendelian manner.^{6,32} Typically, a loss of function phenotype specific to the prion protein in question arises because steric effects of the amyloid form often diminish functionality.³³ Remarkably, yeast have evolved a proteostasis network^{34,35} able to exploit prions as metastable switches in protein function that impart selective advantages in diverse environments.^{6,7,32,36–38}

Perhaps the best-characterized yeast prion is [*PSI*⁺], which functions as an evolutionary capacitor.³⁹ Following standard nomenclature, the italicized capital letters denote a dominant genetic element and the brackets denote non-Mendelian inheritance. [*PSI*⁺] unleashes usually silent, cryptic genetic variation to generate potentially beneficial phenotypes in response to environmental stress.^{6,32,36–43} [*PSI*⁺] arises when the release factor, Sup35, forms infectious amyloids that transmit heritable and immediate increases in nonsense suppression.^{10,15,44} These modest prion-encoded reductions in translation termination fidelity immediately alter mRNA stability, gene expression and protein function on a system-wide scale.^{6,36,37,44,45} Consequently, complex multigenic phenotypes can develop rapidly in a single step.^{6,36,37,44–46} Typically, these traits are neutral or deleterious, but ~25% of the time they are advantageous.³⁷ [*PSI*⁺] is reversibly gained and lost from yeast at a low spontaneous rate of ~1 in 10⁵ to 10⁷ cells.⁶ Thus, any detrimental effects of [*PSI*⁺] have little effect on global fitness of yeast populations.⁶ Moreover, under specific circumstances even neutral diversity can accelerate adaptation.⁴⁷ Importantly, [*PSI*⁺] induction can increase ~60-fold with increasing environmental stress.³⁸ This increase allows a sub-population of cells to rapidly sample complex multigenic traits that might promote survival in response to stress.^{38,44} The epigenetic nature of [*PSI*⁺] (and other prion-based switches) enables a single genotype to explore a larger phenotypic space without any permanent alteration to the genomic DNA or commitment to immediate fixation.^{6,36} Should a [*PSI*⁺]-dependent trait be favored for extended periods, the fraction of the population with that phenotype will amplify and increase the probability of

genetic assimilation *via* new mutations. Remarkably, traits that are originally [*PSI*⁺]-dependent can become fixed and [*PSI*⁺]-independent.³⁶ Thus, [*PSI*⁺] is a transient adaptation that enhances survival of yeast in variable environments by promoting evolvability.^{6,32,36–43}

A disproportionate number of other yeast prion proteins are positioned in regulatory hubs of high connectivity, which control the expression of large portions of the genome.^{6,7,32} For example, Swi1, Cyc8 and Mot3 are all global regulators of transcription that can switch to inactive prion forms, alter the expression of a panoply of genes and generate potentially advantageous phenotypes.^{7,48,49} Indeed, simulations of complex gene networks and genome-scale expression data from yeast indicate that the reversible loss-of-function conferred by any prion is predicted to release phenotypic variation and potentially accelerate the acquisition of new adaptations and phenotypic optima.^{6,50} With over twenty potential prion-based switches in protein function, individual yeast within an isogenic population can rapidly access distinct complex multigenic phenotypes on a probabilistic basis.^{7,32,40} The ability to plumb such deep reserves of heritable diversity and explore phenotypic space in response to stress likely protects yeast populations against fluctuating and disparate environmental perturbations. Indeed, in yeast, prions serve as bet-hedging adaptations that promote survival in fluctuating environments.^{7,32,40}

This utility of prion-based switches has led to proposals that prions might be relatively common adaptive agents found throughout nature.^{6,51} Indeed, neuronal isoforms of cytoplasmic polyadenylation element binding protein (CPEB) might form prions that promote the formation of stable synapses that contribute to long-term memory formation in *Aplysia*^{52,53} and *Drosophila*.⁵⁴ Curiously, CPEB activity is potentiated in the prion state: CPEB prions stimulate the translation of specific mRNAs more effectively than soluble CPEB.⁵² Although not generally considered infectious,⁵⁵ various amyloid conformers are also found in functional settings.⁵⁶ For example, amyloid forms of Pmel17 promote melanosome biogenesis in mammals^{57–59} and CsgA amyloids promote biofilm formation in *E. coli*.⁶⁰ Specific nucleoporins might even form amyloid-like gels that create the permeability barrier of nuclear pores.⁶¹ In other perhaps more notorious contexts, however, prions and amyloids are inextricably tied to several fatal and untreatable neurodegenerative diseases.

1.3 Pathogenic prions

In yeast and perhaps other organisms, prions can be advantageous.^{6,7,32,53} By contrast, in mammals, conflict can arise between these limited hereditary replicators⁴ and the individual, such that prions or amyloids replicate at the expense of the host. The initiation of such selfish replication launches a microevolutionary process in which the prion or amyloid replicator initially prospers and amplifies but ultimately destroys the host.⁶² The mammalian nervous system is particularly vulnerable to this conflict and can become severely and selectively devastated by proteins undergoing prionogenesis or amyloidogenesis.⁶² The precise neurodegenerative phenotype depends on the specific protein that has morphed into a self-replicating cross- β form.⁶² A major risk factor for these neurodegenerative disorders is aging.⁶³ Indeed, because natural selection acts less powerfully on genetic variation expressed at post-reproductive age, many genes may harbor ‘late-expressing’ harmful mutations.⁶⁴ Some of these mutations may predispose proteins to forming prions or amyloids in the environment of an aging individual. Several examples are found in mammalian prion protein (PrP).⁶⁵ At the other extreme, certain mutations practically guarantee that a protein will enter a self-replicating amyloid state and promote devastating early-onset neurodegeneration.^{65–71}

In mammals, prions are lethal pathogens. They cause several inexorably fatal neurodegenerative disorders, including: bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in deer and elk, scrapie in sheep, and Gerstmann–Sträussler–Scheinker syndrome (GSS), kuru, Creutzfeldt–Jakob Disease (CJD) and fatal familial insomnia

(FFI) in humans.^{5,72} There are no treatments for these transmissible spongiform encephalopathies (TSEs), which result in 100% mortality once the earliest clinical symptoms present. TSEs can be idiopathic, inherited or acquired by infection.^{65,73} All of these disorders originate from the prionogenesis of one specific protein, PrP,^{74,75} a glycosyl-phosphatidylinositol (GPI)-anchored plasma membrane protein of unclear function.⁷⁶ Initially, the identity of the infectious agent was mired in controversy because it is devoid of nucleic acid.^{77,78} It is now widely accepted, however, that infectious amyloid forms of PrP are causative.^{16,79} Perhaps the most compelling evidence comes from the generation of infectious species from purely recombinant PrP that induce transmissible neurodegenerative disease upon inoculation into rodents.^{9,11,80–82}

A propensity to form self-templating amyloid is not, however, unusual to prion proteins. Several fatal neuro-degenerative diseases are intimately connected with the accumulation of self-templating amyloid forms of specific proteins.⁶² For example, β -amyloid (A β) fibers accumulate in Alzheimer's disease^{83,84} (AD), tau forms amyloid in various tauopathies and AD,^{85–88} huntingtin forms amyloid in Huntington's disease^{66,89} (HD), whereas α -synuclein undergoes amyloidogenesis in Parkinson's disease^{90–92} (PD). Several recent advances suggest that the amyloid forms that underpin these distinct disorders are experimentally transmissible in various contexts. For example, intracerebral injection of primates or transgenic mice with dilute A β fiber-containing extracts from AD brains induces widespread A β fibrillization and associated pathology.^{93,94} Amyloid forms of tau, poly-glutamine and α -synuclein are readily transmitted and propagated by various cells in culture.^{95–98} Moreover, neuronal grafts (from fetal donors) can develop amyloid inclusions of α -synuclein in the brains of PD patients.^{99,100} It appears that amyloid conformers can spread from cell to cell in a highly predictable and stepwise manner within the brains of afflicted individuals thereby spreading the specific neurodegenerative phenotypes distinctive to the protein being converted to amyloid.^{62,101–103} These data are consistent with the spread of a self-replicating, transmissible agent that is in conflict with the host.⁶² Unlike prion diseases, these disorders are not infectious. They are not transmitted naturally from individual to individual. To distinguish these experimentally transmissible amyloid templates from prions, the term prionoid has been introduced.^{55,104}

Prions are naturally transmitted between individuals.^{5,105} Indeed, infectious forms of PrP can move from the environment, sometimes *via* the food chain, and travel between tissues to the brain of an individual.^{5,105} Precisely why prion conformers can accomplish this whereas prionoids cannot remains unclear. One possibility is that infectious PrP conformers are exceptionally stable and can endure harsh environments like the gut,¹⁰⁶ saliva,¹⁰⁷ blood¹⁰⁸ and feces.¹⁰⁹ However, amyloid forms of other proteins are extremely stable too.^{18,19} Another possibility is that prions have an optimized balance between seeding activity and fiber fragmentation that facilitates more effective dissemination and transmission of infectious material^{9,31,62,110} (Fig. 2). PrP might also be uniquely suited to transfer between cells because it is a GPI-anchored plasma membrane protein, whereas prionoid proteins connected with neurodegeneration do not bear this modification. This modification may allow dendritic cells to bring PrP into contact with sympathetic nerves, eventually enabling access to the central nervous system.¹¹¹ Indeed, the GPI anchor allows infectious forms of PrP to rapidly spread among cell populations *via* cell-to-cell contact,¹¹² tunneling nanotubes¹¹³ or exosomes,¹¹⁴ and gain access to unconverted substrate. Once infected with prions, transgenic mice that express only PrP lacking the GPI anchor do not succumb to a scrapie-like disease and death.^{115,116} Collectively, these studies suggest that the GPI anchor of PrP plays a crucial role in the distinctive transmissible pathogenesis of prion disease.

1.4 Prion strain phenomena

A universal feature of amyloidogenesis is that the same polypeptide can fold into multiple, structurally distinct amyloid forms or ‘strains’, which confer distinct phenotypes.^{8–10,13,15,17,31,72,117–122} For example, PrP forms different infectious strains, perhaps 15 or more, that elicit different transmissible neurodegenerative diseases characterized by distinct pathology and rate of disease progression.^{5,9,11,122,123} A β and polyglutamine assemble into distinct amyloid strains that vary in inherent neurotoxicity suggesting that strain diversity might contribute to the pathology of other neuro-degenerative amyloidoses.^{119,124,125} Beneficial prions also form distinct strains.⁶ For example, Sup35 assembles into multiple different prion forms, which confer distinct [*PSI*⁺] variants that are differentiated by the strength of their nonsense suppression phenotype.^{10,15,31,126}

Distinct strains confer distinct phenotypes, but how their structures differ at the atomic level remains unclear. All strains adopt a cross- β form. However, multiple precise folds might fit within this generic framework.^{120,121} Thus, distinct strains are likely to differ considerably in local steric detail. Indeed, there is potentially enormous freedom for the main chain within the constraints of cross- β architecture.²⁴ Main chain dihedrals might populate the β -compatible polyproline II basin or the adjacent β -basin, which comprise ~50% of the available Ramachandran space.²⁴ This freedom means that single prion proteins have an intrinsic tendency to spontaneously populate multiple self-templating strains. A key question is then how side chains pack to achieve various energy-minimized cross- β structures. Accumulating evidence suggests that one solution is for side chains to align in register, in parallel intermolecular β -sheets due to potentially favorable interactions between identical side-chains involving hydrophobic, aromatic or amide contacts.^{120,121,127–129} However, several different solutions exist for the same primary sequence. Thus, side chains might be accommodated in subtly different ways to achieve some minimum threshold of net stability that makes spontaneous reversion to the non-amyloid state highly improbable. Indeed, several local energy minima are likely populated as well as the most stable amyloid state (Fig. 2). Once formed, each single solution (strain) then amplifies by high fidelity self-templating. The set of solutions that a single polypeptide might find depend on the environment (*e.g.* temperature, pH, and macromolecular crowding). Thus, different assembly conditions can shift the relative populations of amyloid strains and generate distinct strain ensembles (Fig. 3). For example, Sup35, polyglutamine and PrP assemble into distinct ensembles of amyloid strains at 4 °C *versus* 37 °C, which have very different phenotypic effects.^{9,15,31,119}

Pioneering structural studies on short amyloidogenic peptides (4–12 amino acids) have yielded atomic resolution information on how a single sequence might generate cross- β diversity.^{22,129,130} Depending on the assembly conditions, a single peptide can form intermolecular β -sheets with a different registration of interdigitating residues in the same basic interface (registration polymorphism).²² Under the same assembly conditions, a single peptide can assemble into intermolecular β -sheets with different interfaces (facial polymorphism).²² Moreover, multiple short peptides within an amyloidogenic protein can form intermolecular β -sheets^{22,130,131} and each single peptide can form a different atomic cross- β structure (segmental polymorphism).²² How these findings might translate to full-length proteins remains uncertain, but it seems likely that similar principles will apply to the intermolecular contacts of all amyloids. Indeed, pioneering work on Sup35 suggests that different prion strains are characterized by different intermolecular contacts and by the exact stretch of primary sequence that is sequestered in cross- β structure.^{23,132–135}

How structural polymorphism engenders distinct phenotypes or disease states remains poorly defined. However, different amyloid strains of the same polypeptide can have different biophysical properties. Two parameters are of particular importance: the rate at which individual fiber ends self-template and the rate at which fibers fragment to liberate new fiber

ends. Different strains can have very different properties with regard to these parameters. Moreover, for each strain these parameters can change depending on the environment. For example, Sup35 prions assembled at 4 °C (termed NM4) seed efficiently at 4 °C, but poorly at 23 °C.³¹ Slow seeding can be compensated by increased fragmentation. NM4 prions are more readily fragmented by mechanical shearing than NM37 prions.³¹ Indeed, an emerging principle for both yeast and mammalian prions is that strains that are more readily fragmented, but not so fragile that they are cleared by the proteostasis machinery, tend to induce more severe phenotypes (Fig. 2). Thus, more frangible Sup35 prions confer a stronger $[PSI^+]$ phenotype³¹ and more labile PrP strains amplify and kill the host more rapidly.^{9,11,136} Increased frangibility yields more fiber ends, the active sites of prion replication, per unit mass and, consequently, more rapid conversion of available monomers and contingent phenotypic change (Fig. 2). Thus, for Sup35 and PrP prions, conformational stability is inversely correlated with strength of the prion phenotype^{9,11,31,136} (Fig. 2). If an amyloid form is too stable, it may not fragment frequently enough to become infectious, *i.e.* be a prion¹¹⁰ (Fig. 2). Thus, perhaps the distinction between prions and non-infectious amyloids is that prions occupy local free energy minima rather than the most stable amyloid state, which might be too difficult to fragment and disseminate (Fig. 2).

1.5 Natural selection of prion strains

As replicators, prions are units of selection.⁶ Indeed, prions possess all three qualities of successful replicators.^{2,4} First, the intrinsic chemical stability of the amyloid form confers longevity. Second, optimized seeding together with fragmentation confers fecundity. Third, the steric constraints of each cross- β form ensure that the amyloid template is accurately duplicated after each round of conformational replication and confers fidelity. The natural tendency for single prion proteins to spontaneously populate multiple, structurally distinct self-templating strains provides the natural variation upon which selection can act. Each strain has a distinct conformational fitness, *i.e.* ability to self-replicate conformation. Moreover, conformational fitness can vary depending upon the environment. Thus, natural selection inescapably enriches or depletes various prion strains from populations depending on their conformational fitness in the prevailing environment. Prior to the realization of the extent of cross- β diversity and contingent diversity of conformational fitness, it was suggested that prions could not evolve during an infection.¹³⁷ However, elegant studies have now revealed that prions can evolve during an infection.¹³⁸ For example, upon transfer from brain to cell culture, cell-adapted prion strains gradually amplify at the expense of brain-adapted strains¹³⁸ (Fig. 3). Conversely, if the evolved cell-adapted strain population is returned to brain then the original brain-adapted ensemble of forms is re-established.¹³⁸ Thus, environmental change can alter strain selection pressures and favor previously rare strains (Fig. 3).

Another process, termed strain conversion, might also contribute to strain diversity and subsequent selective amplification when the environmental change involves a switch in the PrP sequence being converted by the prion. That is, when a prion crosses a species barrier.^{139,140} In mouse infection studies, some promiscuous prion strains replicate their structure even when the primary sequence of the PrP being converted is non-identical.^{139,141} By contrast, for other strains a distinct prion conformation emerges.^{78,139,141} This might simply reflect strain selection events (Fig. 3). However, pure protein studies have revealed that strain conversion can be detected within individual amyloid fibers of PrP.^{142,143} That is, distinct portions of a single PrP amyloid fiber have a distinct conformation, which depends on the primary sequence of PrP at that portion of the fiber.^{142,143} How frequently this event happens and how rapidly it can contribute to prion population evolution during infection remains unclear. Regardless, the evolvability of prion populations makes them extremely challenging drug targets.

2. Small molecule antagonists of prions

There are no effective treatments for prion disorders or related neurodegenerative amyloidoses. Yet, hope remains in the vastness of unexplored chemical space,¹⁴⁴ which may harbor potent small molecule (~0.5 kDa or less) antagonists of prionogenesis. The conformational diversity and shifting balance of prion strain populations in response to fluctuating environmental cues and contingent selection pressures severely complicate the development of small-molecule therapies. The problem is accentuated by the likelihood that potential small-molecule regimens would only be applied after substantial accumulation of diverse amyloid forms. Indeed, a key issue that remains relatively unaddressed is whether small molecules can antagonize the entire repertoire of structurally distinct misfolded forms.

Two broad strategies might be considered to antagonize prionogenesis with small molecules. First, small molecules might be developed that interact directly with a prion protein and either prevent or reverse prionogenesis. Second, small molecules might be developed that operate more indirectly by modulating proteostasis to enhance natural cellular pathways that eliminate or detoxify prions.

2.1 Direct antagonists

Small molecules must overcome several daunting challenges to antagonize prionogenesis directly.¹⁴⁵ It is inherently challenging for small molecules of limited steric bulk to prevent or disrupt protein–protein interactions in which the binding energy is distributed among numerous amino acids and thousands of square angstroms of contact area.¹⁴⁶ As well as being large, protein–protein contacts can be relatively flat and exhibit sufficient plasticity to accommodate small molecule binding without being disrupted.¹⁴⁶ Intermolecular amyloid contacts are particularly recalcitrant because they lie at the extremes of protein stability and resist disruption by chaotropes and detergents.¹⁷ Moreover, crystal structures of small amyloidogenic peptides suggest that intermolecular amyloid contacts might be particularly inaccessible because they exclude water to form a dry steric zipper.^{22,129,130} Furthermore, different prion strains can have different intermolecular contacts.^{23,132,134,135} An effective small molecule would need to target all of these. In addition, prior to reaching the final amyloid state, many amyloidogenic proteins populate toxic oligomeric forms that share a conformation that is distinct to fibers.¹⁴⁷ Effective small molecules must prevent or reverse amyloidogenesis in a manner that prevents the accumulation of these toxic oligomeric forms. Finally, another key issue for neurodegenerative amyloidoses is the ability to cross the ‘blood–brain-barrier’ (BBB), which strictly limits the nature and size of the small molecule.¹⁴⁸

Despite these challenges several small molecules have emerged that inhibit^{149–155} and sometimes even reverse amyloidogenesis.^{134,135,156–158} One effective strategy might be to stabilize the soluble native structure by small molecule binding and decrease the probability of amyloidogenic folding trajectories. This approach has proven effective for transthyretin, which has a well-characterized native structure.¹⁴⁹ However, many amyloidogenic or prionogenic proteins are natively unfolded in their soluble states and it is difficult to know how to effectively stabilize this form.¹⁵⁹ Encouragingly, several small molecule antagonists also reduce the intrinsic toxicity of amyloid conformers in cell culture settings.^{150,152,156} Nonetheless, there are still no approved drugs that directly resolve amyloid or prion conformers.

2.2 Proteostasis modulators

Another approach to antagonize prion conformers is to employ small molecules that enhance or modulate the proteostasis network, which controls the conformation, concentration, binding interactions, activity, post-translational modification and locations of each protein in the

proteome.^{35,160} Within the remit of this huge framework there are numerous opportunities for small molecule intervention. It is hypothesized that proteostasis can be precisely manipulated to boost natural system-wide defenses that detoxify, inhibit, refold or eliminate prion or amyloid conformers and their cytotoxic oligomeric antecedents.^{35,160} Typically, these small molecules do not interact with the amyloidogenic protein directly, but affect it indirectly *via* the proteostasis network. A common strategy is to manipulate signaling pathways that result in transcriptional or translational upregulation of proteostasis components, such as molecular chaperones, as in the unfolded protein response (UPR) or heat shock response (HSR).^{35,160}

This approach can be very effective in model systems of protein misfolding disorders. For example, geldanamycin, an inhibitor of Hsp90, induces the HSR, and ameliorates a *Drosophila* model of PD.¹⁶¹ Similarly, celastrol (an HSF1 activator) and MG132 (a proteasome inhibitor) activate the HSR and the UPR, and can correct specific protein folding defects connected with Gaucher's disease.¹⁶² Genetic ablation of the HSR by deletion of HSF1 confers a more rapid progression of prion disease in mice infected with the RML prion strain, although there was no effect on the age of onset compared to wild-type mice.¹⁶³ Thus, eliciting the HSR might have applications to prion disease,¹⁶³ particularly because prion-infected cells appear to have a defective HSR,¹⁶⁴ which can be restored with geldanamycin.¹⁶⁵

Numerous alternative strategies to modulate proteostasis might be devised. Small molecule activators of autophagy might enhance clearance of misfolded proteins.^{166–169} Calcium pump inhibitors that deplete endoplasmic reticulum (ER) Ca^{2+} stores facilitate the release of substrates from Ca^{2+} -dependent ER chaperones, which can allow ER-detained Delta F508-CFTR to reach the plasma membrane and correct the cystic fibrosis defect.¹⁷⁰ Small molecules that inhibit the insulin growth factor signaling pathway might have profound therapeutic benefits for AD.^{171–174} Calorie restriction increases lifespan in a variety of organisms and this effect has been connected with activation of Sir2, an NAD-dependent deacetylase.¹⁷⁵ Resveratrol, a small molecule activator of Sir2, protects against neurodegeneration in several disease models.^{175,176} However, in prion-infected mice, although calorie restriction or Sir2 (SIRT1) deletion delays disease onset, disease progresses more rapidly and life span is ultimately reduced.¹⁷⁷

Intriguingly, a single 1,2,3,4-tetrahydroquinolinone analog can antagonize disparate pathological events connected with PD, including ER–Golgi trafficking defects, mitochondrial dysfunction, pathological transcriptional profiles and α -synuclein aggregation in various model systems ranging from yeast to *Caenorhabditis elegans* to rat midbrain neurons.¹⁷⁸ However, the small molecule did not affect the fibrillization of pure α -synuclein.¹⁷⁸ The target remains unknown, but it is probably deeply rooted and operates upstream or unifies these diverse pathological pathways.¹⁷⁸ The ability of a single small molecule to simultaneously correct multiple defects of a complex disorder such as PD is extremely encouraging.¹⁷⁸

In isolation or in combination the use of direct antagonists and proteostasis modulators holds great promise for treating various neurodegenerative disorders. However, recent evidence suggests that direct small molecule antagonists and proteostasis modulators can also create selection pressures that cause the unexpected emergence of drug-resistant prion strains.^{135, 138,179} Drug resistance, therefore, needs to be taken into consideration as prion and amyloid therapeutics are developed.

3. Drug-resistant mammalian prions

It has been difficult to address the issue of whether small molecules can directly antagonize entire repertoires of mammalian prion strains, owing to the substantial difficulties in generating prions from pure protein *de novo* that infect wild-type animals and cause transmissible disease.^{9,11,80,81} Indeed, this difficult challenge has only just been realized.⁸⁰ Nonetheless, several

promising small molecule antagonists of mammalian prion replication have been uncovered in cell culture models, where crude homogenates from prion-infected animals are added to cells in culture that stably propagate prions.^{180–185} This approach has revealed that small molecules can be effective against one strain but not another.^{181,182,185} Yet, it remains unclear whether the small-molecule effects are direct or reflect alterations in proteostasis. Some of these small molecules antagonize pure PrP amyloidogenesis or conversion of recombinant PrP catalyzed by crude extracts, indicating that they might act directly.^{181,182,184–186} However, because the vast majority of these PrP amyloid species do not cause infectious disease in wild-type mice, lingering doubts remain as to whether they are an accurate proxy.^{9,11,186–188} Thus, the direct effects of small molecules on distinct infectious forms of PrP remain uncertain. Several small molecules that are effective in cell culture have no effect on pure PrP amyloidogenesis *in vitro* or conversion of recombinant PrP catalyzed by crude extracts, suggesting they might act solely through modulating proteostasis.^{181,186,189} However, the possibility remains that they might directly inhibit the formation of infectious forms of PrP that are simply not assembled in these *in vitro* assays. Whatever the case, it is disappointing that while potent in cell culture, many of these small molecules are ineffective against various prion strains in mice^{190–193} and humans.^{194–196} Two recent studies have illuminated the difficulties in finding small-molecule remedies for mammalian prion disorders.

3.1 Quinacrine

Quinacrine (Fig. 4a) induces rapid clearance of protease-resistant forms of PrP from prion-infected neuroblastoma cells,^{183,189} but does not inhibit amyloidogenesis of pure PrP¹⁸⁶ or conversion of recombinant PrP catalyzed by crude extracts,¹⁸⁹ suggesting that it might therapeutically modulate proteostasis. Curiously, quinacrine can repress the HSR.¹⁹⁷ The anti-prion activity generated optimism because oral quinacrine is safe, can cross the BBB to some extent¹⁹⁸ and has been used for many years as an anti-malarial drug.¹⁹⁹ Unfortunately, quinacrine is ineffective against prion disorders in mice.^{192,193} In humans, quinacrine has provided transient benefits but ultimately has proven ineffective.^{194–196} This failure has been attributed to the ABC transporter, Pgp, which promotes quinacrine efflux from the brain.¹⁹⁸ Thus, quinacrine fails to accumulate at sufficient concentrations in the brain to be efficacious.¹⁹⁸

To address this issue, transgenic mice were generated with ABC transporter genes deleted. These mice were infected with prions and treated with various quinacrine regimens post-infection.¹⁷⁹ Quinacrine concentration in the brain was increased ~100-fold in these mice and protease-resistant forms of PrP were initially reduced.¹⁷⁹ Remarkably, however, this effect was ephemeral. Quinacrine-resistant prion forms subsequently emerged and ultimately quinacrine did not prolong disease progression or life span.¹⁷⁹ Intriguingly, compared to the original strain, quinacrine-resistant prions had subtly reduced conformational stability and reacted differently with a panel of PrP antibodies.¹⁷⁹ These strain properties were not maintained upon transfer to mice in the absence of quinacrine.¹⁷⁹ Thus, this prion strain fails to propagate in the absence of quinacrine.¹⁷⁹ These findings in mice were corroborated in division-arrested neuroblastoma cells in culture.¹⁷⁹ Collectively, these data suggest that quinacrine effectively eliminates a large portion of pre-existing prion strains, but a rare quinacrine-resistant strain endures or arises anew upon exposure to the drug, which then amplifies at the expense of other strains.¹⁷⁹ One possibility raised by this study is that an intermittent regimen of quinacrine where the drug is administered, withdrawn and then added again might delay disease progression. Indeed, the incubation period for mice that were given a single transient dose of quinacrine was increased slightly by ~10–15%.¹⁷⁹

3.2 Swainsonine

Swainsonine (Fig. 4b) is a potent inhibitor of lysosomal α -mannosidase as well as Golgi α -mannosidase II.²⁰⁰ The latter activity impairs the removal of two mannose residues from proteins in the *medial* and *trans* Golgi and thereby impedes the subsequent maturation of complex N-glycans.^{200,201} Swainsonine is a strain-selective inhibitor of mammalian prions in cell culture.¹³⁸ Swainsonine initially decreases prion levels in cells infected with swainsonine-sensitive species.¹³⁸ However, after this initial decline swainsonine-resistant prions gradually amplify.¹³⁸ If swainsonine was then withdrawn, swainsonine-sensitive prions then began to dominate the population once again.¹³⁸ Importantly, lack of complex glycosylation of PrP was not responsible for swainsonine resistance and fully glycosylated prions can be resistant or sensitive to the drug.¹³⁸ Swainsonine-resistant and swainsonine-sensitive prions displayed similar chemical stability and protease-sensitivity indicating that the structural differences between the strains were subtle or were not uncovered by these indirect methods.¹³⁸ Swainsonine-resistance might be due to a low level of pre-existing swainsonine-resistant prions or because swainsonine-resistant prions arise *de novo* in response to the small molecule.¹³⁸ However, careful titration studies revealed that ~0.5% of prions were swainsonine-resistant prior to exposure to the drug.¹³⁸ It is therefore likely that this rare strain arises spontaneously with some low probability but then amplifies in the presence of swainsonine.¹³⁸

Both quinacrine and swainsonine likely antagonize prions by affecting proteostasis. However, the precise mechanistic basis for their activity and the exact characteristics of the resistant strains that enable escape from inhibition remains unclear. Nonetheless, these studies help establish that drug-resistant strains can amplify in response to proteostasis modulators and have important implications for drug development.^{138,179} Yet, how small molecules directly affect the folding, formation and integrity of pure mammalian prion strains continues to remain uncertain. Mechanistic insights into how small molecules directly affect different prion strains are beginning to emerge from the study of the yeast prion protein, Sup35.^{134,135}

4. Drug-resistant yeast prions

4.1 Mechanism of Sup35 prionogenesis

Sup35 provides an unparalleled, well-defined system to study the direct effects of small molecules on different prion strains comprised of pure protein. Distinct infectious strains of Sup35 are readily generated using pure protein.^{10,14,15,23} Moreover, Sup35 is one of the best-studied amyloidogenic proteins, with analytical tools to study amyloid structure that are not yet available for other polypeptides. These include position-specific fluorescent probes that report the formation of inter- and intra-molecular prion contacts.^{23,132,134,135} A facile yeast transformation assay enables rapid assessment of which prion strain (if any) has formed.^{10,15} Pure Sup35 fibers are sufficient to transform [*psi*⁻] yeast cells (which lack Sup35 prions) to the corresponding strain of [*PSI*⁺].^{15,23} [*PSI*⁺] strains are distinguished using an *ade1* nonsense reporter, which allows their (weak or strong) translation termination defect to be determined.¹²⁶ Thus, [*psi*⁻] colonies are red and require adenine, whereas [*PSI*⁺] colonies do not require adenine and range from pink (weak) to white (strong) depending on the extent of Sup35 aggregation and contingent inactivation^{31,126} (Fig. 5).

The prion domain of Sup35, termed NM, spontaneously assembles into prions after a lag phase^{15,23} (Fig. 5). During lag phase, NM rapidly partitions between monomeric and oligomeric pools and populates a variety of transiently unfolded states^{26,134,202–205} (Fig. 5, step 1). The specific intermolecular contacts required for prionogenesis ultimately occur in structurally fluid NM oligomers.^{23,26,204} Monomers within these molten oligomers slowly rearrange to form amyloidogenic oligomers (Fig. 5, steps 2 and 3), which have a conformation distinct to fibers.^{14,26} The intermolecular contacts that distinguish fibers form very rapidly

after the appearance of these obligate, transient intermediates^{14,23,26,204} (Fig. 5, step 4). Once formed, fibers then seed their own rapid assembly^{21,204} (Fig. 5, step 5). Short prion recognition elements with the N-terminal domain (N), termed 'Head' and 'Tail' are proposed to make homotypic intermolecular contacts such that fibers are held together by alternating Head-to-Head and Tail-to-Tail contacts that are separated by a central core^{23,120,132} (Fig. 5). Both the Head and Tail regions can nucleate prionogenesis, although the Head nucleates more rapidly.^{23,120,132}

Which Sup35 prion strain forms is also readily manipulated by altering reaction conditions. For example, NM forms distinct ensembles of infectious amyloid strains at 25 °C *versus* 4 °C, termed NM25 and NM4.^{15,23,133} Strain biases can also be controlled by introducing specific missense mutations into NM²⁰⁶ or by specifically crosslinking single cysteine NM mutants in the Head or Tail region, using the flexible 11 Å crosslinker 1,4-bis-maleimidobutane (BMB), to create covalent NM dimers.²³ Strain biases created by missense mutations and crosslinking overcome those created by temperature.^{23,206} Thus, NM that is BMB-crosslinked in the Head forms NM4 at 4 °C and 25 °C, whereas NM that is BMB-crosslinked in the Tail forms NM25 at 4 °C and 25 °C.²³ Infecting [*psi*⁻] cells with NM4 induces predominantly strong [*PSI*⁺] strains and NM25 induces predominantly weak [*PSI*⁺]^{15,23} (Fig. 5).

NM4 and NM25 strains possess different intermolecular contacts and sequester overlapping, but distinct portions of N in their amyloid core.^{23,133} The length of the central core and position of the Tail-to-Tail contact are markedly different between different strains (Fig. 5). Residues N-terminal to the Head are organized differently in NM4 and NM25.^{23,133} The atomic structures of Sup35 prion strains remain unknown and several models have been advanced.^{17,23,120,130,132,133,207,208} Nonetheless, different strains are readily distinguished, even at the resolution of spatial arrangements of individual amino acids.^{23,133} Thus, Sup35 provides a unique opportunity to explore precisely how small molecules directly affect prion strains. Furthermore, findings made with Sup35 are not restricted in relevance to the prionogenesis of this protein. Several small molecules that cure cells of [*PSI*⁺] also inhibit mammalian prion replication in cell culture.^{209,210}

4.2 Epigallocatechin-3-gallate (EGCG)

EGCG (Fig. 4c) is the major polyphenol in green tea. EGCG inhibits the *de novo* amyloidogenesis of several proteins including polyglutamine, α -synuclein, PrP, tau, A β 42 and prostatic acidic phosphatase (PAP) fragments.^{151–153,184,211} Remarkably, EGCG can also remodel preformed amyloid forms of PAP fragments, which are connected with semen-mediated enhancement of HIV infection.^{211,212} Surprisingly, Sup35 folds into a spectrum of infectious conformations with differing sensitivities to EGCG.¹³⁵ Using a variety of methods to control strain bias, it became clear that EGCG selectively inhibited formation of NM25 by preventing the formation of the inter- and intramolecular contacts that distinguish this strain.¹³⁵ Indeed, EGCG prevented the reorganization of molten oligomers that facilitates formation of NM25.¹³⁵ By contrast, under various conditions that ordinarily promote formation of NM4, EGCG failed to inhibit prion assembly.¹³⁵ Remarkably, however, NM4 no longer formed.¹³⁵ Rather, a new prion strain, termed NM4E, assembled which configured original EGCG-resistant intermolecular contacts that do not ordinarily assemble.¹³⁵ In NM4E, both the Head and Tail contact sites were shifted toward the N-terminus¹³⁵ (Fig. 5). This finding provides a first molecular glimpse of how prion folding can adjust and establish new intermolecular amyloid contacts that elude small molecule inhibition.

Curiously, both NM4 and NM4E conferred mostly strong [*PSI*⁺] indicating that the repertoire of prion conformations encoding strong [*PSI*⁺] is more nuanced than previously suspected¹³⁵ (Fig. 5). Moreover, EGCG selects which strong strain is deployed.¹³⁵ Infection of [*psi*⁻] cells with NM4E yielded exclusively strong [*PSI*⁺], whereas NM4 yielded a mixture

of strong (~75%) and weak (~25%) [*PSI*⁺].¹³⁵ Thus, EGCG strongly selected against strains that encode weak [*PSI*⁺]. These findings were corroborated *in vivo*. [*PSI*⁺] induction by overexpression of NM-YFP in the presence of EGCG yielded predominantly strong [*PSI*⁺] rather than the usual mixture of strains.¹³⁵ The ability to form entirely new strains with shifted intermolecular contacts that escape small-molecule inhibition exposes the plasticity of prionogenesis and the difficulty it poses to drug development. It is important to identify small-molecule scaffolds that facilitate the appearance of novel amyloid polymorphs, and either modify the scaffold so this is avoided or avoid this type of scaffold altogether.

EGCG also exerted strain-selective effects on preformed Sup35 prions. EGCG remodeled NM25 by disrupting inter- and intramolecular contacts to generate non-templating, β -sheet-rich oligomeric species.¹³⁵ EGCG partially remodeled NM4 fibers, but this effect was less pronounced, and self-templating amyloid forms persisted.¹³⁵ By contrast, NM4E was resistant to EGCG and was unaffected.¹³⁵ These effects were also observed *in vivo*. Strong [*PSI*⁺] strains encoded by NM4E were refractory to EGCG, whereas EGCG cured [*PSI*⁺] strains encoded by NM4 to some extent, but showed the greatest curing activity against weak [*PSI*⁺] strains encoded by NM25.¹³⁵ Once established [*PSI*⁺] variants do not generally switch between weak and strong or *vice versa*.^{126,213} However, EGCG cured weak [*PSI*⁺] but simultaneously induced switching from weak to strong [*PSI*⁺].¹³⁵ It seems likely that some weak [*PSI*⁺] variants harbored low levels of EGCG-resistant prions, which in the presence of EGCG could then amplify and cause switching to strong [*PSI*⁺].¹³⁵ These results reinforce that a single small molecule may be insufficient to counter prion polymorphism and can even select for drug-resistant forms. Thus, EGCG eradicates some strains, but also facilitates the appearance of new drug-resistant strains.

4.3 DAPH-12

Co-application of another small molecule that antagonizes prionogenesis might counter the appearance of drug-resistant strains. Hence, we considered other small molecules to combine with EGCG. We selected DAPH-12 (Fig. 4d), which directly antagonizes NM25 assembly, remodels NM25 and cures strong [*PSI*⁺].¹³⁴ DAPH-12 also inhibits assembly of A β 42 fibers and remodels preformed A β 42 fibers, but is ineffective against mammalian prions.¹³⁴ DAPH-12 inhibits Sup35 prionogenesis and remodels preformed fibers in a manner distinct to that of EGCG.¹³⁵ First, DAPH-12 prevents the formation of both NM4 and NM25, by disrupting early folding events in molten oligomers, which preclude the recognition events that nucleate prionogenesis.^{134,135} Unlike EGCG, a new prion strain does not form in the presence of DAPH-12.¹³⁵ However, DAPH-12 is a more potent antagonist of NM25 formation than of NM4.¹³⁵ DAPH-12 remodels the intermolecular contacts of preformed NM25, NM4 and NM4E.^{134,135} Here too, DAPH-12 preferentially remodels NM25, which is converted to mostly non-templating species, whereas NM4 and NM4E are less affected.^{134,135} In contrast to EGCG, DAPH-12 does not disrupt the intramolecular contacts of NM25.^{134,135} These various strain-dependent effects were corroborated *in vivo*. DAPH-12 cures weak [*PSI*⁺] variants conferred by NM25 more effectively than strong [*PSI*⁺] variants encoded by NM4 or NM4E.¹³⁵ Thus, although DAPH-12 is also a strain-selective antagonist it is mechanistically distinct to EGCG.¹³⁵ DAPH-12 fails to eradicate some strains, but unlike EGCG, DAPH-12 does not promote the formation of new drug-resistant strains.

4.4 Combination of DAPH-12 and EGCG

These different modes of action indicated that DAPH-12 and EGCG might synergize to counter Sup35 prionogenesis. Indeed, when combined DAPH-12 and EGCG synergized to prevent and reverse formation of NM4 and NM25.¹³⁵ The inter- and intramolecular contacts that distinguish these strains were more effectively disrupted by the combination.¹³⁵ Moreover, the EGCG-resistant strain, NM4E, was unable to form in the presence of EGCG and

DAHP-12.¹³⁵ Once formed, NM4E was susceptible to DAPH-12, but the combination of DAPH-12 and EGCG was no more effective than DAPH-12 alone.¹³⁵ Importantly, the combination of DAPH-12 and EGCG synergized to cure weak and strong [*PSI*⁺] variants.¹³⁵ Moreover, weak [*PSI*⁺] was now cured without the appearance of EGCG-resistant strong [*PSI*⁺] variants.¹³⁵ Thus, DAPH-12 and EGCG synergize to cure multiple [*PSI*⁺] strains.¹³⁵

5. Implications for drug development

These findings have implications for the development of successful small-molecule treatments for prion and amyloid disorders. Like [*PSI*⁺], mammalian prion disorders and other neurodegenerative amyloidoses are likely the result of a continuum of phenotypic outcomes caused by an underlying continuum of amyloid strains and misfolded forms, rather than any single pure form⁶² (Fig. 2 and 3). Treatment with a small molecule that targets only one strain can only be effective if the disease state is caused by a purely susceptible strain. However, if mixtures of susceptible and resistant strains cause other disease states, then the resistant strain, even if present at low levels, might amplify. Analogy may be drawn from the ability of EGCG to effectively cure weak [*PSI*⁺] and simultaneously cause the appearance of strong [*PSI*⁺].¹³⁵ Moreover, quinacrine and swainsonine allow the amplification of drug-resistant mammalian prions.^{138,179} At the other extreme, disease states caused by purely resistant strains would be refractory to a small molecule that might be effective against other strains. Here, analogy may be drawn from EGCG being unable to cure strong [*PSI*⁺] strains conferred by NM4E.¹³⁵ Such a small molecule might, alone, fail in broad clinical trials. Indeed, perhaps the amplification of quinacrine-resistant prions¹⁷⁹ has contributed to the disappointing clinical results with this small molecule.^{194–196} While the foregoing views are likely an oversimplification, a central goal for small-molecule therapeutics must be to cope with the vicissitude of prionogenesis, and to target the diversity of misfolded forms and disease states.

The application of a single direct small molecule antagonist or a single proteostasis modulator can give rise to drug-resistant strains.^{135,138,179} For direct antagonists, pure protein studies with Sup35 have revealed a resistant strain can configure unique intermolecular amyloid contacts that are refractory to disruption by the small molecule.¹³⁵ For proteostasis modulators, it is not clear what changes in the prion or the cell or tissue confer drug resistance.^{138,179} Many mechanisms of drug resistance might arise *in vivo* including alterations in the responsiveness of a tissue or cell such that the drug is removed, inactivated or unable to reach its target. In this way, latent reservoirs of prions might persist that evade treatment. One important new concept is that context-dependent protein–protein interactions can radically alter cellular pharmacology.²¹⁴ Thus, A-kinase activating proteins interact directly with protein kinase C and can protect the kinase from otherwise potent ATP-competitive inhibitors.²¹⁴ By analogy, some direct antagonists of prionogenesis might fail to inhibit certain strains *in vivo* because of strain-specific differences in protein-binding partners that alter small molecule interactions.

The application of a small-molecule antagonist of prions creates a familiar situation in Darwinian dynamics in which a heterogeneous population of replicators (Fig. 3) must overcome a severe negative selection pressure to repopulate a niche.^{215,216} A successful treatment must drive a prion population to extinction before resistant strains accumulate that maintain the infection and facilitate the evolution of escape. The risk of escape depends on several parameters including population size, the efficacy of treatment, the rate at which new strains arise *de novo*, the kinetic and thermodynamic difficulty of adopting a resistant form and the selective pressure against resistant strains prior to treatment. The primary objective of treatment is to maximize the difficulty of acquiring a prion conformation able to escape inhibition.

Ultimately, prion disorders and neurodegenerative amyloidoses may require combination therapies involving small-molecule cocktails that antagonize every strain permutation. The rapid evolution of resistance to a single drug in many settings ranging from cancer to viral, bacterial, fungal or malarial infection has stimulated the development of multi-drug treatments, which combat the amplification of rare drug-resistant forms.^{217–224} Drug combinations can be synergistic, additive or antagonistic depending on whether the combined effect is greater, equal or less than one of the drugs by itself.²¹⁹ Synergistic and additive combinations can be more potent and help prevent the evolution of resistance by rapidly reducing population size to such an extent that the probability of spontaneous acquisition of resistance through mutation is minimized.^{215,216,225,226} Perhaps counter-intuitively, however, antagonistic combinations can also prevent drug resistance by exposing portions of the fitness landscape that reduces or even inverts the selection pressure to acquire resistance.^{225,226} Thus, in the presence of particular antagonistic combinations wild-type forms outcompete resistant forms despite global reductions in fitness.^{225,226} Lessons learned from coping with drug resistance in other systems might have important applications to treating prion disorders and neurodegenerative amyloidoses.

The observation that DAPH-12 and EGCG synergized to block and reverse the formation of multiple Sup35 prion strains *in vitro* and *in vivo* provides proof of principle that small molecule combinations can directly counter prion diversity.¹³⁵ This pairing is the first that directly and synergistically eradicates diverse prion strain structures.¹³⁵ How common such synergistic pairings might be remains unclear, but Sup35 provides a valuable paradigm to begin to identify synergistic pairings able to disrupt entire strain spectra. The significance of this specific pairing to other amyloidogenic proteins remains to be explored, however, I suspect that equivalent combinations will be elucidated for various amyloidogenic proteins. Indeed, recently an oligopyridylamide was found to synergize with insulin to inhibit the spontaneous, lipid-catalyzed amyloido-genesis of amylin, which occurs in type II diabetes.²²⁷

Synergy need not be restricted to direct antagonists and might occur between a direct antagonist and a proteostasis modulator, or between proteostasis modulators. Three recent examples are particularly encouraging. The first comes from cell-based models of Gaucher's disease, a lysosomal storage disorder, in which missense mutations in glucocerebrosidase (GC) cause misfolding of the enzyme in the ER and extensive ER-associated degradation. Consequently, GC fails to reach the lysosome in sufficient amounts. A direct antagonist of GC misfolding and a proteostasis modulator synergized to restore a wild-type phenotype.¹⁶² Thus, coapplication of celastrol or MG-132 to induce the HSR and UPR, with *N*-(*n*-nonyl)-deoxynojirimycin to directly stabilize natively-folded GC, led to synergistic recovery of GC function in the lysosome.¹⁶² Second, combining two proteostasis modulators, quinacrine and LY411575 (a γ -secretase inhibitor), synergistically eradicated mammalian prions from several brain regions of infected mice.²²⁸ Unfortunately, the toxicity of LY411575 precluded an assessment of how incubation times were affected.²²⁸ Finally, intracerebral administration of pentosan polysulfate and Fe(III)*meso*-tetra(4-sulfonatophenyl)porphine 14–28 days after prion inoculation increased survival times of transgenic mice that overexpress PrP.²²⁹ This effect appeared to be synergistic, but in cell culture was found to be additive and the mechanism of action is unclear.²²⁹ Regardless, the combination therapy was considerably more effective than either small molecule alone.²²⁹

The results from these combination therapies are encouraging. However, can a single small molecule antagonist of all prion strains be found? It seems unlikely that prions are as adaptable as RNA viruses. RNA viruses have the highest mutation rates in nature, which along with their short generation time helps facilitate escape from various interventions.²³⁰ Even for these rapidly evolving agents, certain events can be inhibited that are so invariant and stringently required that drug resistance cannot arise. For example, Hsp90 mediates the essential folding

and maturation events of various picornavirus capsid proteins and small molecule Hsp90 inhibitors inhibit poliovirus replication without the emergence of drug-resistant escape mutants.²³¹

Two events in prion replication are essential for the success of all strains. First, all strains must capture and convert non-prion conformers (Fig. 1). Second, prions must fragment and disseminate their self-replicating activity (Fig. 1). Strategies that indiscriminately target either or both of these events would halt all strains. Thus, one approach would be to target the repertoire of non-prion conformers such that they cannot be converted to the prion state. Stabilization of the native state by small molecules has proven extremely effective in inhibiting transthyretin amyloidogenesis.¹⁴⁹ Another strategy might be to deplete the protein being converted to the prion state. Indeed, genetically depleting the substrate of the transmissible conformer, even after the onset of disease symptoms, has proven to be an effective therapy in various transgenic models of prion or amyloid disorders.^{232–238} Finally, it might be possible to eradicate all strains by inhibiting prion fragmentation. This strategy is extremely effective for yeast prions. Here, the AAA + ATPase Hsp104 catalyzes prion fragmentation.^{14,25–30} Uncompetitive inhibition of Hsp104 ATPase activity with low concentrations of guanidium chloride^{239,240} or deletion of Hsp104 cures yeast of virtually all known amyloidogenic prions.^{7,48,49,241–247} Unfortunately, the factors that drive the fragmentation of mammalian prions remain unknown. Identification of these factors could reveal key drug targets. The GPI anchor of PrP is also extremely important for infectivity^{115,248–250} and might represent another potential drug target common to all strains.^{248,249} Overall, several avenues might still be explored to indiscriminately eliminate all prion strains.

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Fig. 1.

Prion replication and fragmentation. Prion conformers replicate their conformation by capturing and converting native conformers to the prion state. Typically, this process requires native conformers to exist in a transiently unfolded state or to harbor an intrinsically unfolded domain, which is captured and converted by both ends of a cross- β amyloid polymer. In the prion state, prion recognition elements (red or green) establish homotypic, intermolecular cross- β contacts, which are separated by a region sequestered by intramolecular cross- β contacts^{23,120,132} (blue). Amplification and dissemination of conformational replication is achieved by fragmentation of prion conformers to expose new fiber ends. Prions can fragment spontaneously upon reaching a critical length^{18,19} or fragmentation may be catalyzed by specific factors, such as Hsp104 in yeast.²⁶

Fig. 2.

The continuum of amyloid and prion strains. The same polypeptide can access numerous distinct self-templating amyloid forms or prion strains. These are distinguished by the length of primary sequence sequestered in cross- β structure and by the specific residues that comprise the intermolecular contacts. These different structures have different physical properties and confer different phenotypes. At one extreme (far left) is an amyloid conformer, which occupies the free energy minimum (see energy landscape below). This form is so stable (perhaps due to longer stretches of intermolecular contact) that it is difficult to fragment and typically is not infectious. Prion strain A (middle) is distinguished by distinct intermolecular contacts and is less stable and can be readily fragmented. At the other extreme (far right) is prion strain B, which sequesters less primary sequence in cross- β structure, has distinct intermolecular contacts and is the least stable prion form. This strain is the most readily fragmented and typically confers the strongest phenotype because it consistently generates the most fiber ends, the active sites of prion replication.^{9,31} Thus, a gradient of forms with increasing frangibility exist. Typically, the most frangible form confers the strongest phenotype.

Fig. 3.

Natural selection of prion strains. A brain-adapted ensemble of prion strains (far left) harbors multiple variants at different frequencies, but is dominated by one form (circled far left). Upon transfer to cells in culture this form replicates less rapidly than a cell-adapted form (circled far right), which then begins to amplify and ultimately dominates the ensemble of cell-adapted prions. If this cell-adapted ensemble is then returned to brain, the brain-adapted form then begins to amplify again and eventually dominates the population. Despite their reduced fitness several strains persist at a low level in both brain-adapted and cell-adapted populations. This strain-selection model has been referred to as the 'cloud' model.^{72,138}

Fig. 4. Strain-selective small molecule antagonists of prionogenesis. Chemical structures (upper panel) and three dimensional view (lower panel) of (a) quinacrine, (b) swainsonine, (c) EGCG, and (d) DAPH-12.

Fig. 5.

Mechanism of Sup35 prionogenesis. Sup35 is composed of a C-terminal GTPase domain (amino acids 254–685, black) that confers translation termination activity, a highly charged middle domain (M, amino acids 124–253, dark grey) and a prionogenic N-terminal domain (N, amino acids 1–123, light grey) enriched in glutamine, asparagine, tyrosine and glycine. Within N, prion recognition elements termed the ‘Head’ (red) and ‘Tail’ (green), which flank a ‘Central Core’ (blue), play important roles in prionogenesis. After a lag phase (steps 1–3), Sup35 prions assemble rapidly (steps 4 and 5). Prion recognition elements within N make homotypic intermolecular contacts such that Sup35 prion fibers are maintained by an alternating sequence of Head-to-Head (red) and Tail-to-Tail (green) contacts. The Central Core (blue) is sequestered by intramolecular contacts. Different prion strains can form depending on the environmental conditions. Thus, NM25 fibers form at 25 °C. NM25 fibers also form at 4 °C when NM is chemically crosslinked, with BMB (a flexible 11 Å crosslinker) in the Tail region (green). NM4 fibers form at 4 °C. NM4 fibers also form at 25 °C when NM is chemically crosslinked with BMB in the Head region (red). NM4E forms in the presence of EGCG at 4 °C. Note that the Central Core (blue) and Tail (green) are comprised of different amino acids in the NM25, NM4 and NM4E fiber conformations. NM4E has a different Head-to-Head contact. Transformation of NM25 fibers into $[psi^-]$ cells yields mostly weak $[PSI^+]$, whereas transformation of NM4 fibers into $[psi^-]$ cells yields mostly strong $[PSI^+]$ and NM4E yields exclusively strong $[PSI^+]$.