Published in final edited form as:

Curr Opin Pharmacol. 2019 February ; 44: 20–27. doi:10.1016/j.coph.2018.12.005.

Identification of anti-prion drugs and targets using toxicity-based assays

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Abstract

Prion diseases are untreatable and invariably fatal, making the discovery of effective therapeutic interventions a priority. Most candidate molecules have been discovered based on their ability to reduce the levels of PrP^{Sc}, the infectious form of the prion protein, in cultured neuroblastoma cells. We have employed an alternative assay, based on an abnormal cellular phenotype associated with a mutant prion protein, to discover a novel class of anti-prion compounds, the phenethyl piperidines. Using an assay that monitors the acute toxic effects of PrP^{Sc} on the synapses of cultured hippocampal neurons, we have identified p38 MAPK as a druggable pharmacological target that is already being pursued for the treatment of other human diseases. Organotypic brain slices, which can propagate prions and mimic several neuropathological features of the disease, have also been used to test inhibitory compounds. An effective anti-prion regimen will involve synergistic combination of drugs acting at multiple steps of the pathogenic process, resulting not only in reduction in prion levels but also suppression of neurotoxic signaling.

Introduction

Prion diseases are fatal, infectious neurodegenerative disorders of humans and animals. The central molecular event underlying these diseases is the conformational conversion of the cellular prion protein (PrP^C), which consists of alpha helical and natively unstructured domains, to a primarily β -sheet-containing conformer (PrP^{Sc}) [1]. This conformational change imparts biochemical differences upon PrP^{Sc} that distinguish it from PrP^C, namely resistance to proteolytic digestion, and detergent insolubility (a reflection of molecular aggregation). PrP^{Sc}, the sole constituent of infectious prions, propagates through the CNS by a self-templating process in which additional molecules of PrP^C are converted to PrP^{Sc}. Accumulation of PrP^{Sc} leads to characteristic neuropathological features, including synaptic loss, neuronal death, astrogliosis, and spongiform change, which lead eventually to clinical symptoms, including dementia, ataxia, and myoclonus.

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Conflict of interest statement

Nothing declared.

There are currently no effective therapies or cures for prion diseases. In contrast to the situation with Alzheimer's disease, the low incidence of prion diseases in the population (~1 case/million people/year) has discouraged pharmaceutical companies from investing in the discovery of new therapeutic modalities and the development of effective drugs.

Nevertheless, a variety of approaches to anti-prion therapy have been pursued over the past 30 years. One major strategy has been to use small molecule inhibitors to prevent the accumulation of PrP^{Sc}, either by blocking its formation from PrP^C, or by enhancing its clearance. Many such inhibitory compounds have been discovered, either by trial-and-error, or by high-throughput screening campaigns using cultured cells capable of propagating prions [2,3]. Although some of these compounds prolong scrapie incubation times in animal models, none of them completely prevents the disease, and their efficacy is usually diminished when administered in the symptomatic phase. There have been very few clinical trials of existing anti-prion agents in humans, and those that have been undertaken have proven disappointing [4,5].

Importantly, the molecular target of many existing antiprion compounds is unknown. In a recent systematic study of anti-prion compounds identified in a high throughput screen, it was reported that most hits were compounds that did not interact with either PrP^C or PrP^{Sc}, and presumably targeted non-PrP molecules [6[●]]. These authors note that, 'proteins that play auxiliary roles in prion propagation may be more effective targets for future drug discovery efforts'. However, there is very little information available on which non-PrP proteins play a role in PrP^{Sc} formation in a cellular setting, although there is evidence that certain molecules (lipids, polyanions) participate in PrP^{Sc} formation in cell-free conversion systems [7]. The identification of such ancillary molecules has enormous therapeutic implications, since these represent prime targets for anti-prion drugs. These molecules also promise to illuminate poorly understood aspects of prion biology, such as the identity of PrP^C-interacting partners, and the mechanisms by which PrP^{Sc} initiates downstream neurotoxic sequelae.

In this review, we will discuss several new approaches to identification of anti-prion compounds. These are based on novel assay systems, developed in our laboratory and those of others, which monitor the downstream neurotoxic effects of PrP^{Sc} or mutant forms of PrP. Some of the compounds to emerge from these screens target cellular signaling pathways initiated by PrP^{Sc}/PrP^C interactions on the cell-surface, which lead to synaptic dysfunction and neuronal loss. These toxicity-based assays and the compounds they identify represent important alternatives to existing anti-prion screening efforts, which are focused primarily on discovering molecules that reduce overall levels of PrP^{Sc}.

Strategies for discovery of anti-prion compounds

Laboratory rodents have been indispensable for understanding prion biology, but the long incubation period of prion diseases in these animals (2–5 months) makes them impractical for drug discovery. To overcome this limitation, most searches for anti-prion compounds over the past 30 years have utilized as an assay a mouse neuroblastoma cell line (N2a), which is capable of sustaining a chronic infection with several strains of scrapie prions [8,9]. Infected N2a cells (designated ScN2a) are exposed to test compounds, after which the

presence of protease-resistant PrP^{Sc} is assessed by digestion of cell lysates with proteinase K (PK) followed by western blot analysis. This system has been adapted to a high-throughput format by growing cells in 96-well plates and detecting PK-resistant PrP by ELISA-based methods [10–12]. This has allowed for the screening of large compound libraries for their anti-prion activity [11,12].

While ScN2a cells are an experimentally convenient system, they suffer from several limitations. First, some forms of PrP^{Sc} (particularly those that may be the most pathogenic) are protease-sensitive, and so are not detected by assays that depend on PK digestion [13–15]. A second limitation is related to the confounding variable of prion strains. Prion strains arise from conformational differences in PrP^{Sc}, which cause characteristic and divergent pathological and clinical outcomes in identical hosts [16,17]. Since ScN2a cells are mouse cells that are typically infected with strains of sheep (scrapie) prions (RML, Me7 or 22L), these cells will not respond to compounds that target human prion strains with conformations that cannot be adopted, or are not represented, by mouse PrP^{Sc}. An immortalized cell line stably infected with human prions is a major goal of the field and the recent report of human prion replication in iPS-derived astrocytes represents a potentially important advance in this direction [18].

Virtually all current therapeutic approaches for the treatment of prion diseases have focused on compounds that prevent accumulation of PrP^{Sc} in the CNS. However, an additional or alternative strategy would be to block the downstream, neurotoxic signaling events that are triggered by PrP^{Sc}, or other pathogenic forms of PrP. There is very little known about the cellular and molecular mechanisms by which PrP^{Sc} causes neuronal dysfunction and pathology, although it is likely that an initial event is the binding of PrP^{Sc} to cell-surface PrP^C. This binding step itself, or the subsequent conversion of PrP^C to PrP^{Sc}, would then trigger signal transduction events within the neuron that lead to toxic consequences, including synaptic dysfunction [19]. One recent example of therapeutically targeting prion-induced signaling pathways is the use of inhibitors of the endoplasmic reticulum kinase PERK, which mediates one branch of the unfolded protein response, to reduce neurodegeneration in prion-infected mice [20].

How might one systematically identify compounds that inhibit PrP^{Sc}-initiated neurotoxic signaling? One essential requirement is a cell-based assay system that registers the relevant toxic signaling events. ScN2a cells are not suitable for this purpose, since they display no overt signs of cytotoxicity as a result of prion infection [8,9]. We will now outline the use of cellular systems, two of which were developed in our laboratory, which provide useful alternatives to the ScN2a system for the discovery of anti-prion molecules. These systems have led to the identification of several novel compounds and molecular targets, and they provide a new conceptual framework for designing therapies for these devastating disorders.

A PrP activity-based cellular assay for drug screening

In principle, one approach to discovering novel anti-prion compounds would be to take advantage of an assayable, physiological activity of PrP^C. Such an approach is based on the assumption that abnormalities in the normal functioning of PrP^C might play a role in the

neurotoxic effects of PrP^{Sc} [19]. If so, then compounds that affect the normal physiological activity of PrP^C might be useful in blocking downstream signaling activities elicited by PrP^{Sc}. However, despite intensive investigation, the physiological role and biological activity of PrP^C have remained enigmatic. Although PrP knockout (*Prnp*^{0/0}) mice are completely resistant to prion infection, they develop normally and show only minor phenotypes in the later stages of life (for a comprehensive reviews see Refs. [21,22]). Thus, a simple loss of PrP^C function is unlikely to account for the pathogenesis of prion disease.

Clues to how alterations in PrP^C function might have neurotoxic effects have emerged from studies of transgenic mice expressing several kinds of PrP deletion mutants. With the aim of elucidating the region(s) of PrP required for prion propagation and/or pathogenesis, PrP molecules with progressively larger deletions of the N-terminus were expressed in transgenic mice on a *Prnp*^{0/0} background. While deletion of residues 31–106 was found to be benign, loss of residues 32–121 or 32–134 caused a severe neurodegenerative phenotype [23]. To define further the boundaries of this critical region, our laboratory created transgenic mice expressing a PrP molecule (designated CR) that harbors a deletion of 21 residues (105–125) in the central region. Astonishingly, these animals displayed a neonatal lethal phenotype that was even more severe than that displayed by mice expressing PrP with longer deletions [24]. A polybasic region of nine amino acids comprising the extreme N-terminus of PrP (residues 23–31) was found to be required for expression of the observed neurodegenerative phenotypes [25]. It was concluded that the neurotoxic action of these three deletion mutants reflected the subversion of an as-of-yet undefined function of PrP^C, as these phenotypes could be rescued in a dose-dependent manner by co-expression of wild type PrP^C [23,24].

Hints to the mechanism of CR toxicity have come from electrophysiological experiments using patch-clamping techniques, which revealed that CR induces spontaneous ionic currents in a variety of cultured cells and neurons [26,27,28●]. Importantly, these currents could be suppressed by co-expression of wild type PrP, paralleling the effect of wild-type PrP in reversing the neurodegenerative phenotypes observed in transgenic mice expressing CR and the other internal deletion mutants. Several point mutations in PrP that cause familial prion diseases had a similar, though less pronounced, effect. The polybasic region is required for these ionic currents, possibly because they act as a ‘protein transduction domain’ that forms transient pores in the plasma membrane (Figure 1a).

Although the ion-channel activity of cells expressing CR could, in principle, be used as a screenable phenotype for discovery of drugs that act on PrP-related biological pathways, the patch-clamping techniques required do not lend themselves to a high-throughput format. Surprisingly, CR PrP-expressing cells display another, more readily screenable property: they are hypersensitive to several common antibiotics, including G418 and Zeocin, which are normally used for selection of transfected cell lines [29●] (Figure 1b). Whereas normal cells are typically killed by these antibiotics with 7–10 days, cells expressing CR PrP are eliminated within 3 days. This phenomenon may reflect increased influx of these antibiotics via CR PrP-induced pores. We have developed a drug-based cellular assay (DBCA) that is based on the antibiotic hypersensitivity phenotype of CR PrP-expressing HEK cells [30].

Identification of a new class of anti-prion compounds

Several, known anti-prion agents are known to reduce accumulation of PrP^{Sc} in ScN2a cells via interaction with endogenous PrP^C [31,32]. We wondered whether some of these compounds might also suppress the antibiotic hypersensitivity of HEK cells expressing CR PrP in the DBCA. Consistent with this prediction, we observed that pentosan polysulfate (PPS), Congo red, and a cationic tetrapyrrole (TMPyP), all of which inhibit PrP^{Sc} accumulation in ScN2a cells, also reversed the G418 hypersensitivity of CR PrP-expressing HEK cells in the DBCA (Figure 1c,d). Given this correlation between the inhibitory activity of compounds in ScN2a cells and in the DBCA, we decided to use the DBCA as the read-out in a high-throughput screen to discover previously unknown anti-prion molecules [33●●] (Figure 1e). A library of 75 000 molecules was screened, resulting in a total of 68 hits (0.1%), each of which reduced both G418-induced and Zeocin-induced cell death in the DBCA by 50% (Figure 1f). Confidence in the validity of this assay was bolstered by the finding that hit compounds represented nine distinct chemotypes, seven of which were represented by multiple, structurally related molecules.

We chose to focus further efforts on one of the lead molecules, LD7 (Figure 1g), which reduced the levels of PK resistant PrP^{Sc} in ScN2a cells infected with two different strains of prions, RML and 22L (Figure 1h). LD7 was refined by testing 34 chemical derivatives in the ScN2a assay to develop a structure-activity relationship. This process led to a more potent molecule, JZ107, with an EC₅₀ of 3.1 μM in both RML-infected and 22L-infected ScN2a cells [33●●] (Figure 1f,h). LD7, JZ107 and other active compounds are phenethyl piperidines, and they share a phenethyl amine group that is required for activity (Figure 1g).

We are currently attempting to identify the molecular target of JZ107 and other related compounds, and are investigating the mechanism by which they inhibit PrP^{Sc} accumulation. We know that the expression level and cellular distribution of PrP^C are unchanged following incubation with JZ107 [33●●]. A direct interaction between the original hit molecule, LD7 and recombinant PrP was not detected using surface plasmon resonance. This result suggests that PrP^C is not molecular target of the phenethyl piperidines, in contrast to the case for PPS, Congo red, and TMPyP. As mentioned, there is evidence that many previously identified anti-prion compounds have non-PrP^C targets [6●]. On the contrary, however, we found that, at concentrations well above their EC₅₀ values in the ScN2a assay, JZ107 and other active compounds displayed inhibitory activity in the real time quaking induced conversion (RT-QuIC) reaction, an *in vitro* assay for protein misfolding that uses purified, recombinant PrP substrate [33●●]. These results keep open the possibility that phenethyl piperidines may interact with PrP^C in a cellular context, perhaps in complex with other currently unidentified membrane components. It is noteworthy that the phenethyl piperidines, as well as PPS, Congo red and TMPyP, are active in two orthogonal, apparently unrelated assays, the DBCA and in the ScN2a assay. This result suggests that there is a mechanistic connection between the cellular processes accessed by these two assays, perhaps related to the folding or conformation of PrP.

A PrP^{Sc} synaptotoxicity assay using cultured neurons

To identify therapeutic agents that block prion neurotoxicity, it would be desirable to employ neuron-based assay systems that reproduce these toxic effects *in vitro*. However, there has been relatively little published literature on prion infection of cultured primary neurons, and it has not been clear how well these cells propagate prions or how they respond to the infectious process [34–37]. Our laboratory has recently developed a neuronal culture system that reproduces one of the earliest and potentially most critical steps in prion neurotoxicity: synaptic damage. Numerous studies pinpoint synapses, in particular dendrites and dendritic spines, as important initial targets of prion neurotoxicity [38,39]. Dendritic spines are protuberances on dendrites at which primarily excitatory synaptic contacts occur [40]. Changes in spine morphology are now thought to regulate the synaptic plasticity associated with learning and memory, in addition to the synaptic disruption that occurs during aging and neurological disease [41,42].

We have found that acute exposure of cultured hippocampal neurons to purified PrP^{Sc} results in rapid retraction of dendritic spines (within 24 hours), as well as decrements in synaptic function as assayed by electrophysiological techniques (Figure 2, left) [43●●,44●●]. These effects are entirely dependent on expression of PrP^C by target neurons, and on the presence of the nine-amino acid, polybasic region at the N-terminus PrP^C, consistent with the idea that binding of PrP^{Sc} to cell-surface PrP^C initiates a synaptotoxic signaling cascade. We have used this neuronal culture system to identify what we believe to be a core synaptotoxic signaling pathway triggered by PrP^{Sc} [44●●]. This pathway includes, sequentially, binding of PrP^{Sc} to cell-surface PrP^C, activation of NMDA receptors, calcium influx, stimulation of p38 MAPK and several downstream kinases, and collapse of the actin cytoskeleton within dendritic spines.

In addition to providing powerful insights into the biology of prion neurotoxicity, these results identify new molecular targets and therapeutic agents for treatment of prion diseases. Pharmacological inhibition of any one of the components in the signaling cascade we have identified, including NMDA receptors and p38 MAPK, as well as expression of a dominant-negative form of p38 MAPK, blocked PrP^{Sc}-induced spine degeneration [44●●]. Of particular significance, p38 MAPK inhibitors actually reversed the degenerative process after it had already begun [44●●]. This result suggests the existence of a therapeutic window for treatment of patients who have already been infected with prions, and who might even have sustained a certain level of synaptic damage. NMDA receptor antagonists, as well as p38 MAPK inhibitors, have already been developed for therapy of CNS disorders and inflammatory diseases [45–47], and it may be feasible to re-purpose these agents for treatment of prion diseases. Interestingly, the phenethyl piperidine compound, JZ107, also causes significant protection from PrP^{Sc}-induced spine retraction in our system, suggesting that blocking prion propagation prevents initiation of downstream synaptotoxic signaling [33●●].

Prion neurotoxicity assays using brain slices

The neuronal culture system described above registers the acute synaptotoxic effects of PrP^{Sc} exposure over a time course of 6–24 hours [43^{●●},44^{●●}]. A complementary system to discover candidate therapeutic compounds that inhibit PrP^{Sc}-induced neurotoxicity would be one that reproduces the neuropathological features of chronic prion infection. Recently, the Aguzzi laboratory has introduced the Prion Organotypic Slice Culture Assay (POSCA) as a model of chronic prion infection and pathology [48] (Figure 2, right). Prion infection has been successfully achieved in cerebellar [49^{●●},50] and hippocampal [51^{●●}] slices, leading to amplification of PrP^{Sc} to levels similar to those observed *in vivo*, but in a significantly shorter time (14–21 days). These slice cultures reproduce certain aspects of prion pathology that are observed *in vivo*, including progressive loss of neurons, spongiform change, development of tubulovesicular structures, and loss of dendritic spines [49^{●●},52]. Interestingly, different prion strains result in distinct patterns of PrP^{Sc} deposition in the slices, supporting the relevance of this assay [49^{●●}].

Although slice cultures have not been used in a drugscreening format, they have proven to be a useful tool for the discrimination of anti-prion compounds that have neuroprotective activity *in vivo*. For example, when assayed using cerebellar slices, compounds that interact with PrP^{Sc} or that directly suppress prion replication such as PPS, Congo red and amphotericin B all suppressed prion-induced toxicity in the slice assay [49^{●●}]. In contrast, compounds with efficacy in ScN2a cells, but which failed to have beneficial effects *in vivo* (quinacrine, curcumin and cannabidiol), were not protective in slices [49^{●●}]. Slice cultures have also been used to evaluate the therapeutic potential of bile acids, as well as metabotropic glutamate receptor antagonists [51^{●●},53]. Of note, mGluR1 and mGluR5 antagonists prevented neuronal loss without altering PrP^{Sc} levels in brain slices [51^{●●}], suggesting that they were affecting downstream neurotoxic pathways independent of PrP^C→PrP^{Sc} conversion.

Conclusions

In this review, we have highlighted three recently developed *in vitro* assays which, because they monitor cellular toxicity as opposed to the traditional readout of PrP^{Sc} levels, provide novel avenues in the search for anti-prion compounds. Two of these assay systems, the hippocampal dendritic spine assay and POSCA, are well suited for secondary validation of new anti-prion compounds, and we recommend their routine use in the search for effective therapeutics. While ScN2a cells will remain valuable tools in primary screens for anti-prion compounds, they are unable to identify molecules with protective effects downstream of prion conversion, for example, the recently identified p38 MAPK inhibitors [44^{●●}] or inhibitors of the endoplasmic reticulum kinase PERK [20]. The labor required for the preparation of brain slice cultures makes it unlikely that they could be used in a high throughput format. However, it may be possible to adapt the hippocampal dendritic spine assay we have developed for use in high-throughput screens. Human pluripotent stem cells have been used in the past to generate large numbers of differentiated neurons for screens of 100+ molecules [54,55]. A similar approach, paired with an automated microscopy, would allow for the screening of large chemical libraries to identify molecules with anti-prion

activity in human neurons. Elucidation of the molecular targets of anti-prion compounds is important avenue of research, as it may lead to the repurposing of existing drugs that recognize these targets, while also illuminating unknown aspects of prion biology.

The results outlined above lead us to argue for a synergistic, two-pronged therapeutic approach for the treatment of prion diseases: inhibition of PrP^{Sc} accumulation, as well as suppression of downstream neurotoxic pathways. This stands in contrast to the monotherapies that have been tested thus far, most of which rely exclusively on reducing PrP^{Sc} levels. It is our hope that the approaches and methodology outlined above will lead to the identification of additional anti-prion compounds that can be used to halt, or even reverse, the progression of these devastating neurodegenerative disorders.

Acknowledgements

Work in the Harris laboratory is supported by grants from the N.I.H. (NS065244, NS101659, NS107755) and the Creutzfeldt-Jakob Disease Foundation.

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PrP, and the isolated N-terminal domain induced currents when expressed in the absence of the C-terminal domain. Moreover, anti-PrP antibodies targeting epitopes in the C-terminal domain induced currents, and caused degeneration of dendrites on cultured hippocampal neurons, effects that entirely dependent on the effector function of the N-terminus. Finally, NMR experiments demonstrated intramolecular docking between N-terminal and C-terminal domains of PrPC, reflecting a novel auto-inhibitory mechanism that regulates the functional activity of PrPC.

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Figure 1.

High throughput screen using the Drug-Based Cellular Assay (DBCA).

(a) WT PrP, represented in blue, is shown on the outer leaflet of the plasma membrane, where it is attached by a glycosyl-phosphatidylinositol anchor (not shown). CR PrP, represented in red, creates spontaneous ionic currents by transient permeabilization of the lipid bilayer. The 21-residue deletion is indicated by a black dashed line. The nine-residue polybasic region, which is essential for current activity, is indicated by a ball with three plus symbols. (b) An MTT viability assay reveals that CR PrP-expressing cells are more sensitive than WT PrP-expressing cells to cationic antibiotics such as Zeocin or G418. This effect is the basis of the DBCA, which can be used to identify compounds that suppress the antibiotic hypersensitivity of CR PrP cells. (c) ScN2a cells (RML) were treated for seven days with DMSO, Congo red (CR), tetrapyrrole (TMPyP), or pentosan polysulfate (PPS). Lysates were treated with proteinase K and analyzed by western blot. Molecular weights are indicated in kDa. (d) Dose response curves for restoration of cell viability in the DBCA by CR, TMPyP, and PPS in the presence of G418. These compounds also block CR PrP-associated current activity (not shown). (e) Percent inhibition of cell death in the DBCA for 17 000 compounds from the Harvard Laboratory for Drug Discovery in Neurodegeneration

small compound library. Hits are circled in red. **(f)** Structures of LD7 and JZ107. **(g)** Dose response curves showing (left axis) the amount of PrP^{Sc} remaining in ScN2a cells infected with RML or 22 L prions following seven days of treatment with LD7; and (right axis) cell viability monitored by MTT assay. Values are expressed relative to untreated cells. **(h)** Same as panel G, but for JZ107. Panels C, D, G, and H are taken from [33^{●●}]. Panel A was produced using Servier Medical Art (<http://smart.servier.com>).

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Figure 2.

In vitro assays of prion neurotoxicity and possible points for pharmacological intervention. **Top:** PrP^C (blue) is converted to PrP^{Sc} (red) through a self-templating mechanism. An anti-prion drug might act by blocking any one of three different steps: the conversion process itself (e.g. PPS, JZ107), acute synaptotoxic signaling (e.g. NMDAR, p38 MAPK inhibitors), or chronic neurotoxic sequelae (e.g. mGluR, PERK inhibitors). The nine-residue polybasic region of PrP is indicated by a ball with three plus symbols. **Left:** The hippocampal neuron synaptotoxicity assay [43^{●●},44^{●●}] monitors dendritic spine morphology and synaptic transmission after acute application of PrP^{Sc}. PrP^{Sc} causes collapse of dendritic spines, which is depicted in the expanded view of the synapse. Only post-synaptic sites of excitatory synapses are affected. Electrophysiological traces, recorded by whole cell patch clamping, show decreased frequency and amplitude of miniature excitatory post synaptic currents (mEPSCs) caused by the application of PrP^{Sc}. Electrophysiological traces are taken from Ref. [44^{●●}]. **Right:** The Prion Organotypic Slice Culture Assay (POSCA) measures neurotoxicity during chronic infection with prions [48,49^{●●},50]. Infected brain slices display PrP^{Sc} accumulation, neuronal loss, and spongiform vacuolation. Elements of this figure were produced using Servier Medical Art (<http://smart.servier.com>).