Insights from Therapeutic Studies for PrP Prion Disease

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Although an effective therapy for prion disease has not yet been established, many advances have been made toward understanding its pathogenesis, which has facilitated research into therapeutics for the disease. Several compounds, including flupirtine, quinacrine, pentosan polysulfate, and doxycycline, have recently been used on a trial basis for patients with prion disease. Concomitantly, several lead antiprion compounds, including compound B (compB), IND series, and anle138b, have been discovered. However, clinical trials are still far from yielding significantly beneficial results, and the findings of lead compound studies in animals have highlighted new challenges. These efforts have highlighted areas that need improvement or further exploration to achieve more effective therapies. In this work, we review recent advances in prion-related therapeutic research and discuss basic scientific issues to be resolved for meaningful medical intervention of prion disease.

Prion diseases, also called transmissible spongiform encephalopathies, in humans and animals, are rare neurodegenerative illnesses that are currently still incurable and fatal. Creutzfeldt–Jakob disease (CJD) (Creutzfeldt 1920; Jakob 1921) is the most representative human prion disease and comprises several clinicopathological subtypes (Parchi et al. 1999; Puoti et al. 2012). Most cases of CJD show a subacute progressive disease process after symptoms appear, making it difficult for patients to receive beneficial effects from therapeutic intervention. In this work, we will first review the history of medical intervention in prion diseases, which includes clinical trials and recent advances in therapeutic development. Next, we will examine the new insights provided by re-cent clinical trials and drug-discovery research. Finally, we will discuss the advances in basic science that need to be made to achieve better medical interventions for prion diseases in the near future.

HISTORICAL OVERVIEW OF MEDICAL INTERVENTIONS FOR CJD

Figure 1 shows the chronology of therapeutic development for CJD. In the early history of experimental CJD intervention (before 1990), preliminary experimental treatments (in addition to symptomatic treatments) were occasionally given to CJD patients in case report studies. The first drugs used to treat CJD patients were antivirals such as acyclovir (David et al. 1984;
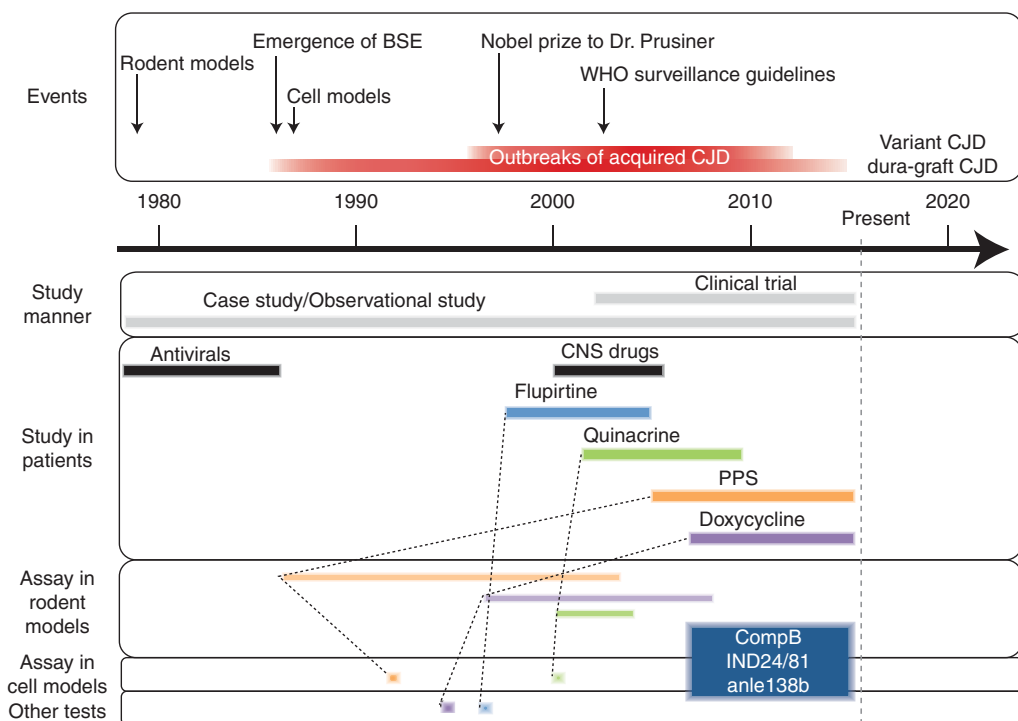
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Figure 1. Chronology of therapeutic development for Creutzfeldt–Jakob disease (CJD). BSE, Bovine spongiform encephalopathy; WHO, World Health Organization; CNS, central nervous system; PPS, pentosane polysulfate.

Newman 1984), amantadine (Braham 1971; Norris 1972; Herishanu 1973; Sanders and Dunn 1973; Ratcliffe et al. 1975; Terzano et al. 1983; Neri et al. 1984), interferon (Kovanen et al. 1980), and vidarabine (Furlow et al. 1982). All of these drugs were used to address the transmissible features of CJD pathogens. The next group of drugs used to treat the disease were those that targeted agents affecting the central nervous system, including antidepressants (Dervaux et al. 2001), analgesics (Otto et al. 2004), and anticonvulsants (Imperiale et al. 2003), which were used to treat the encephalopathic features of the disease.

Following the discovery of prions as CJD pathogens (Prusiner 1998), many studies predominantly investigated compounds or biological materials that inhibited formation of the abnormal prion protein (PrP^{Sc}) or that facilitated PrP^{Sc} degradation to better understand the curious nature of the prion. Concurrently, two tragic events accelerated the search for spe-

cific CJD remedies: the emergence of variant CJD (Will et al. 1996) and the prevalence of iatrogenic CJD (Nozaki et al. 2010). Because CJD and other types of human prion diseases are rare and difficult to diagnose at early stages, most reports regarding experimental intervention until the mid-2000s were from a single or few patients in the form of case reports or observational studies. However, it is very difficult to draw definite conclusions on the effectiveness of therapeutic agents because of the considerable variations in disease duration. Stewart et al. (2008) systematically summarized such reports and emphasized that disease course and treatment of all patients must be evaluated within a structured framework, preferably within randomized controlled trials.

Along with a stream of evidence-based medicine, several clinical trials have recently been performed within a structured framework, including flupirtine trials in Germany (Otto et al. 2004), quinacrine trials in the United

Kingdom (Collinge et al. 2009) and United States (Geschwind et al. 2013), and doxycycline trials in Italy and France (Haïk et al. 2014). Table 1 summarizes these clinical trials and their results, and each trial is described in detail in the following section. It is important to note that recent CJD clinical trials have been multi-institutional or multinational collaborations of clinicians and researchers. Furthermore, outreach by patients and their families in support of nonprofit organizations has also been helpful in pushing therapeutic development forward.

RECENT EXPERIMENTAL AND TRANSLATIONAL STUDIES IN CJD PATIENTS

Researchers have made considerable effort to search for antiprion drugs or compounds using in silico screening, in vitro models, persistently prion-infected cell models, and prion-infected rodent models (Trevitt and Collinge 2006; Sim and Caughey 2009). In this section, we focus on drugs and compounds recently tested in CJD patients.

Flupirtine

Flupirtine (Fig. 2A) is a centrally acting, nonopioid analgesic found to have cytoprotective activity in vitro (Raffa and Pergolizzi 2012). This activity was also shown in a cell-viability assay against toxic PrP 106-126 aggregates, reminiscent of PrP^{Sc} (Perovic et al. 1997). Flupirtine is a well-established drug—a randomized double-blind clinical trial of 28 CJD patients started in Germany in 1997 (Otto et al. 2004). It was concluded that flupirtine has beneficial effects on cognitive function but no significant effects on survival (Otto et al. 2004).

Quinacrine

Quinacrine (Fig. 2B) was discovered to inhibit PrP^{Sc} formation in prion-infected cell models (Doh-ura et al. 2000; Korth et al. 2001). Thereafter, stereochemical (Ryou et al. 2003) and structure–activity relationship studies (Murakami-Kubo et al. 2004; Nguyen et al. 2008, 2011) were conducted. Aliphatic side-chain

bonding to nitrogen at position 9 of the tricyclic scaffold of quinacrine is one of the key structures conferring potency in prion-infected cell models. However, quinacrine treatment was found to show no beneficial effects in prion-infected rodent models (Collins et al. 2002; Barret et al. 2003), even when administered by cerebroventricular infusion (Doh-ura et al. 2004). Kocisko and Caughey (2006) also showed the ineffectiveness of mefloquine, an antimalarial drug approved by the U.S. Food and Drug Administration that crosses the blood–brain barrier (Kocisko and Caughey 2006). It is interesting to note that quinacrine eliminates and/or modifies a specific subset of PrP^{Sc} conformers, resulting in the survival of drug-resistant PrP^{Sc} conformers (Ghaemmaghami et al. 2009) or selective amplification of drug-modified PrP^{Sc} conformers (Bian et al. 2014).

Quinacrine has also been used for CJD patients in several observational studies (Kobayashi et al. 2003; Scoazec et al. 2003; Benito-León 2004; Haïk et al. 2004; Nakajima et al. 2004; Satoh et al. 2004; Bertrand et al. 2005; Martínez-Lage et al. 2005; Wroe et al. 2006) that appeared after widespread media reports about a variant CJD patient who tentatively showed rapid improvement of neurological symptoms after quinacrine administration. However, the results of these studies were controversial. Tentative improvements in mental and neurological symptoms observed in some patients were regarded as part of an adverse reaction in the central nervous system, and the medication was discontinued by many patients because of its noxious effects (e.g., liver toxicity and bone marrow aplasia). A large-scale clinical trial of quinacrine used in an open-labeled, patient-preference manner (PRION-1 study) was launched in the United Kingdom in 2004 and included 107 patients with sporadic, iatrogenic, variant, or familial CJD (Collinge et al. 2009). This study concluded that there was no difference in mortality between the treated and untreated groups. Another clinical trial of quinacrine conducted in the United States from 2005 to 2009 in a double-blind, placebo-controlled, stratified-randomization manner concluded that quinacrine did not increase survival of

Table 1. Recent experimental and translational studies of Creutzfeldt–Jakob disease (CJD) patients

Country	Compound	Study period	Report	Study type	Result	Note
Germany	Flupirtine	1997–2001	Otto et al. 2004	Randomized, double-blind trial; 28 patients of CJD	Effective in cognitive functions but ineffective in survival	No preclinical studies of the drug in prion-infected cells or animals
Japan	PPS	2004–2007	Tsuboi et al. 2009	Open prospective observation; 11 patients of CJD	No apparent improvement of clinical features	Continuous cerebroventricular infusion of the compound through an osmotic pump device
United Kingdom		2003–?	Newman et al. 2014, and others	Case reports (unorganized trial); five patients of variant CJD	Significantly extended survival in four patients	
United Kingdom	Quinacrine	2004–2007	Collinge et al. 2009	Open-labeled, patient-preference trial (PRION-1); 107 patients of CJD	No difference in mortality between treated and nontreated groups	Transient improvement of mental and neurological signs caused by adverse brain reactions to the drug
United States		2005–2009	Geschwind et al. 2013	Double-blind, placebo-controlled, stratified randomization trial; 54 patients of CJD	No significant difference between drug groups and placebo groups in survival	
Italy France	Doxycycline	2007–2010 2009–2012	Haïk et al. 2014	Double-blind, placebo-controlled, randomized trial; 121 patients of CJD	No significant difference between drug groups and placebo groups in survival	Minimal benefits in prion-infected animals even in cerebroventricular liposomal delivery of the drug

PPS, Pentosane polysulfate.

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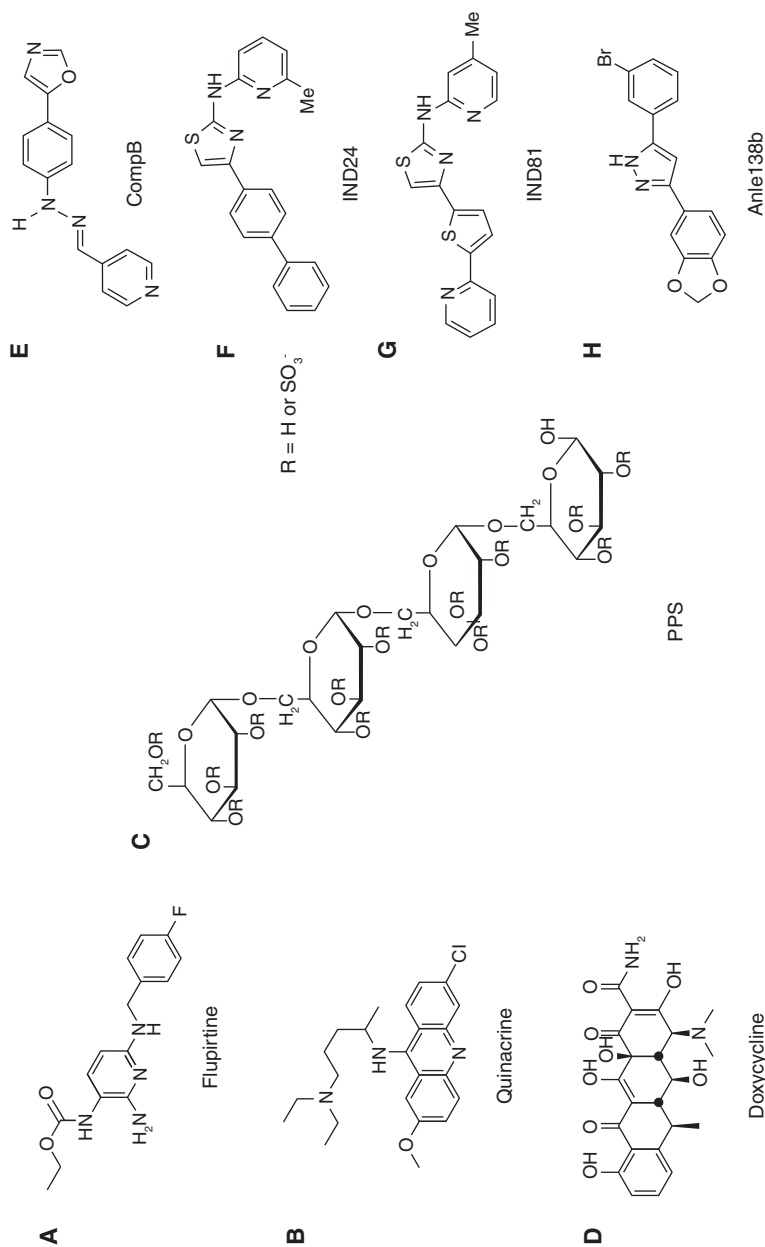
Figure 2. Chemical structures of compounds recently used in clinical trials and recently developed representative lead compounds. Compounds used in clinical trials include (*A*) flupirtine, (*B*) quinacrine, (*C*) pentosan polysulfate (PPS), and (*D*) doxycycline. Representative lead compounds include (*E*) compB, (*F*) IND24, (*G*) IND81, and (*H*) anle138b.

sporadic CJD patients compared with the placebo (Geschwind et al. 2013).

Pentosan Polysulfate (PPS)

Farquhar and Dickinson (1986) injected sulfated glycans, such as dextran sulfate and PPS, intraperitoneally or intravenously in peripherally prion-infected animals and found that these compounds inhibit prion replication in the lymphoreticular system and prolong incubation periods within the animals. Subsequently, Caughey and Raymond (1993) reported that these sulfated glycans inhibit PrP^{Sc} formation in prion-infected cells by interacting with normal (PrP^C) or abnormal (PrP^{Sc}) prion–protein isoforms. As another example of polyanionic macromolecules, dietary seaweed fucoidan, a complex sulfated fucosylated polysaccharide, was also reported to delay disease onset in enterally prion-infected animals when administered orally (Doh-ura et al. 2007a).

PPS (Fig. 2C) is a sulfated semisynthetic polysaccharide with a heparin-like nature. Because of its highly charged polymeric structure, PPS does not penetrate the blood–brain barrier when administered orally or parenterally. To solve this issue, Doh-ura et al. (2004) performed cerebroventricular administration of PPS through an infusion-pump device. Continuous administration directly into the brain suppressed PrP^{Sc} accumulation, neurodegenerative changes, and infectivity, and, consequently, prolonged the life spans of intracerebrally prion-infected animals, even when administered after the appearance of PrP^{Sc} accumulation in the brain. However, PPS administered in this manner persisted around the infusion site and did not diffuse throughout the ventricular system—specifically, into the contralateral side of the brain where pathological changes were not well suppressed.

Based on the results of cerebroventricular PPS administration in animals, long-term cerebroventricular PPS treatment was then given to patients with prion diseases. After High Court hearings in the United Kingdom supported this treatment method for variant CJD, five patients were treated with cerebroventricular PPS (Todd

et al. 2005; Whittle et al. 2006; Parry et al. 2007; Rainov et al. 2007; Bone et al. 2008; Newman et al. 2014). Four of the five PPS-treated patients survived significantly longer than untreated patients. The survival periods of these five PPS-treated patients were 16, 45, 84, 105, and 114 months. However, the mean survival of variant CJD patients was 17 months, and the maximum survival period of untreated patients was 40 months (Newman et al. 2014). From a postmortem study of a long-term CJD survivor, Newman et al. (2014) observed that treatment with cerebroventricular PPS did not reduce overall neuropathological changes in the brain and concluded that the reason for long-term survival of CJD patients treated with cerebroventricular PPS remains unclear; however, the effect of the treatment on disease pathology cannot be excluded.

Conversely, Tsuboi et al. (2009) concluded that cerebroventricular PPS treatment of 11 Japanese patients with sporadic, familial, or iatrogenic CJD produced no apparent improvements in clinical features. These results are apparently discordant with those of variant CJD, suggesting that PPS efficacy might vary with disease subtype. Terada et al. (2010) reported a case of sporadic CJD treated with cerebroventricular PPS in Japan, wherein they observed a reduction in the amount of PrP^{Sc} in the brain, although overall neuropathological changes were not reduced. In addition, Honda et al. (2012) reported neuropathological findings of four cases treated with cerebroventricular PPS in Japan, wherein the results suggest that PPS possibly modifies the accumulation of PrP^{Sc} oligomers and the protein expression profile of astrocytes.

Doxycycline

Discovery of doxycycline (Fig. 2D) for CJD treatment began with the investigation of a structurally similar molecule, 4′-iodo-4′-deoxy-doxorubicin, which binds to amyloid fibrils and induces amyloid resorption in patients with systemic amyloidosis. Tagliavini et al. (1997) found a reduction in both protease resistance and infectivity of PrP^{Sc} when co-incubated with 4′-iodo-4′-deoxy-doxorubicin. Doxycy-

cline and other tetracycline compounds were shown to have similar activity against PrP^{Sc}, but with much less toxicity, by a subsequent series of in vitro and in vivo studies (Tagliavini et al. 2000; Forloni et al. 2002; Barret et al. 2003). These analogs were shown to prolong survival of peripherally or intracerebrally prion-infected animals when administered peripherally or intracerebrally (De Luigi et al. 2008).

Following an observational study of a considerable number of patients with CJD, a multi-institutional, double-blind randomized trial of 121 CJD patients treated with doxycycline versus placebo was conducted in Italy and France (Haïk et al. 2014). The results, however, did not show a significant difference in survival or disease progression in patients from either group. Researchers have speculated that treatment with oral doxycycline after disease symptoms first appear is relatively ineffective. In fact, an animal study by De Luigi et al. (2008) showed that even cerebroventricular liposomal delivery of doxycycline had very limited effect when administered after animals began to show signs of disease. At present, another clinical trial of

doxycycline in Italy is reportedly providing preventive treatment to persons carrying a genetic mutation associated with fatal familial insomnia (FFI), a type of familial prion disease (Forloni et al. 2015). However, no data on doxycycline efficacy in an animal model of FFI have ever been reported.

RECENT ADVANCES IN THERAPEUTIC DEVELOPMENT

Figure 3 is a representation of prion propagation and possible therapeutic targets. Therapeutic targets include inhibition of PrP^C expression, enhancement of PrP^C degradation, inhibition of PrP^C–PrP^{Sc} interaction, inhibition of PrP^{Sc} oligomer formation, and enhancement of PrP^{Sc} degradation. All of these targets have been intensively studied to understand the enigmatic nature of prions. In addition to these targets, some cellular factors have been implicated as possible targets in PrP^C-mediated or PrP^{Sc}-induced neurodegenerative processes (Mouillet-Richard et al. 2000; Moreno et al. 2012; Pietri et al. 2013; Alleaume-Butaux et al. 2015). One
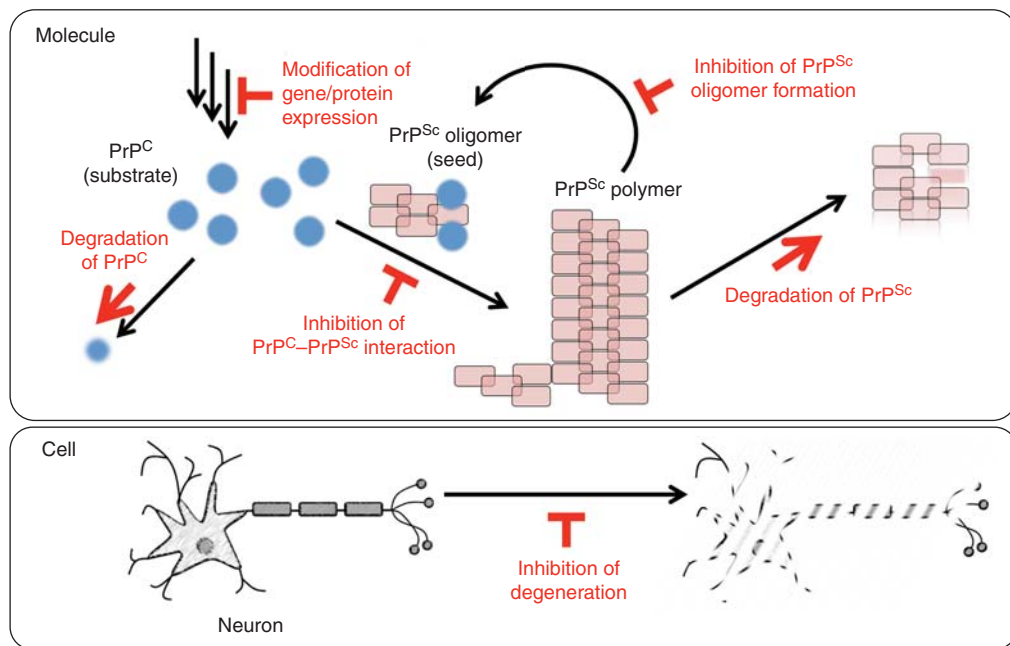
Figure 3. Schematic of possible targets for Creutzfeldt–Jakob disease (CJD) therapeutics.

example was reported in an in vivo study of the growth arrest and DNA-damage-inducible protein GADD34, which promotes dephosphorylation of the α-subunit of eukaryotic translation initiation factor 2 and reverses translational suppression caused by the prion infection–induced unfolded protein response in the endoplasmic reticulum (Moreno et al. 2012).

Thus far, PrP^C has been the most attractive target for therapeutic development since it was reported that *Prnp* knockout mice are apparently healthy and resistant to prion infection (Büeler et al. 1993). In the *Prnp*-less background, residual infectivity disappeared within 4 days after inoculation, indicating efficient prion clearance in vivo (Aguzzi and Zhu 2012). Moreover, conditional knockout of *Prnp* expression halted disease progression and recovered certain brain functions even after disease onset (Mallucci et al. 2002, 2003). Subsequently, gene therapy studies of suppressed *Prnp* expression (Pfeifer et al. 2006; White et al. 2008) or immunotherapeutic targeting of PrP^C (White et al. 2003; Song et al. 2008; Roettger et al. 2013) have been reported. Concurrently, compounds that suppress PrP^C expression have also been investigated (Karapetyan et al. 2013). For example, the compound tacrolimus has been shown to reduce both membrane and intracellular PrP^C levels by a nontranscriptional mechanism (Karapetyan et al. 2013), to suppress neurodegenerative processes in prion-infected animals by acting as a calcineurin inhibitor (Mukherjee et al. 2010), and to activate autophagy in both prion-infected cells and animals (Nakagaki et al. 2013).

Recent advancements in structural chemistry and biology have made it possible to identify important structural features of PrP^C, which has facilitated the investigation of interactions between PrP^C and test compounds and has guided rational drug design (Kuwata 2013; Baral et al. 2014). Accordingly, Venko et al. (2014) computationally analyzed and summarized structure–activity relationships of small organic compounds using prion-infected cell-based assays. Kamatari et al. (2013) compared results of docking studies with the antiprion activity of designed compounds in prion-infected cell-

based assays, and concluded that compounds potently inhibiting PrP^{Sc} formation must not only bind to PrP^C but also change the environment around the binding site to suppress the PrP^C–PrP^{Sc} conversion reaction. Recently, Shirai et al. (2014) proposed a structural model of PrP^{Sc} based on a comprehensive PrP^C mutation study in prion-infected cell-based assays. Interfaces on which to build PrP^C–PrP^{Sc} and PrP^{Sc}–PrP^{Sc} interactions are highlighted in the literature.

Conversely, facilitating PrP^{Sc} degradation is also an attractive target strategy. Autophagy is reported to facilitate PrP^{Sc} clearance (Heiseke et al. 2010), and autophagy-related antiprion compounds, including astemizole (Karapetyan et al. 2013) and tacrolimus (Nakagaki et al. 2013), are reported to have certain effects in prion-infected animals. Conversely, Marzo et al. (2013) have recently reported that 4-hydroxytamoxifen conveys PrP^C and PrP^{Sc} to lysosomes independent of autophagy, suggesting the existence of a lysosomal degradation pathway for PrP^{Sc} clearance.

RECENTLY DEVELOPED REPRESENTATIVE LEAD COMPOUNDS

In this section, we focus on three types of recently developed representative lead compounds that show remarkable inhibition of PrP^{Sc} formation in prion-infected cells, as well as remarkable prolongation of survival periods in cerebrally prion-infected animals when the compounds are administered orally. The compounds include compound B (compB), IND series, and anle138b. All of these compounds are featured as having a planar structure comprising aromatic rings linked with conjugated bonds (Teruya and Doh-ura 2013).

CompB

The phenylhydrazine derivative 4-(oxazol-5-yl)phenyl)-2-((pyridin-4-yl)methylene)hydrazine, called compB (Fig. 2E) (Kawagoe et al. 2004), has been shown to be highly effective in prolonging the incubation of cerebrally prion-infected animals when administered orally (Ka-

wasaki et al. 2007), with a brain-to-plasma concentration ratio of 2.6. Ten minutes after oral intake, 0.03% of the initial dose was found in the brain (Kawasaki et al. 2007; Suzuki N, unpubl.). Additionally, compB has no active efflux system, which hinders the clinical use of quinacrine. Together these characteristics make compB a suitable oral treatment for prion diseases. Orally administered compB prolonged incubation periods from 68.5 ± 5.9 days in untreated control mice to 154.3 ± 19.9 days in mice treated with 0.2% compB in their feed (approximately 300 mg/kg/d). Even at the terminal stage of the disease, orally administered compB maintained PrP^{Sc} levels and infectivity titer of the brain at a remarkably low level. Although these features make compB a promising therapeutic compound, two shortcomings must be overcome: prion strain–dependent efficacy and inhibitory activity against several common P450 isozymes, which potentially cause a drug–drug interaction. Furthermore, high lability of an aryl hydrazine moiety (Hwu et al. 2004) may be a possible reason for the limited potency in vivo compared with that in vitro. Potency and pharmacokinetics in mice have been confirmed by others (Lu et al. 2013). According to Lu et al. (2013), degradation of compB via Schiff base hydrolysis may generate a toxic metabolite and carcinogen. Lu et al. (2013) have shown that compB prolongs the survival of animals infected with the RML prion strain but is ineffective in animals infected with prions from MM1-type sporadic CJD, the most common CJD subtype. These data on prion strain specific efficacy of compB are consistent with those of Kawasaki et al. (2007).

IND Series

Two 2-aminothiazole compounds, IND24 (Fig. 2F, 4-(biphenyl-4-yl)-*N*-(6-methylpyridin-2-yl)thiazol-2-amine) and IND81 (Fig. 2G, 4-methyl-*N*-[4-[5-(2-pyridinyl)-2-thienyl]-2-thiazolyl]-2-amine), were developed via a battery of large cell-based screenings, extensive structure–activity relationship studies, and pharmacokinetic analyses (Ghaemmaghami et al. 2014). One million diverse compounds

were analyzed using an ELISA-based assay (Ghaemmaghami et al. 2010), and it was revealed that the 2-aminothiazole scaffold exerts antiprion activity. Animal studies showed that compounds with a 2-aminothiazole scaffold were orally absorbed when properly formulated in liquid for rodents and achieved steady-state brain concentrations well in excess of their in vitro antiprion potencies (Gallardo-Godoy et al. 2011). Li et al. (2013) improved the potency of 2-aminothiazole compounds to maintain favorable in vivo pharmacokinetic profiles. Mice receiving IND24 or IND81 showed no adverse effects even at the highest dose of 210 mg/kg/d. Subsequently, a full pharmacokinetic investigation of IND24 and IND81 in mice (Silber et al. 2013) showed that IND24 prolonged the survival of mice infected with RML and ME7 prions from scrapie or prions from chronic wasting disease, but was ineffective against prions from MM1- and VV2-type sporadic CJD (Berry et al. 2013).

Anle138b

Anle138b (Fig. 2H, 3-(1,3-benzodioxol-5-yl)-5-(3-bromophenyl)-1*H*-pyrazole) was discovered after screening a primary library comprising two million diverse drug-like compounds and a subsequent focused library of 150 compounds using a combination of methods (Wagner et al. 2013), including "scanning for intensely fluorescent targets," a novel method based on single-particle spectroscopy that allows targeting of oligomer formation (Bieschke et al. 2000; Bertsch et al. 2005). As a lead compound, anle138b has three more favorable features than compB: greater survival effects in mice; better pharmacokinetic properties (i.e., a longer half-life in the brain); and activity against all prion strains tested, including murine prions (RML and ME7 from scrapie and 301C from bovine spongiform encephalopathy) and human prions from sporadic and variant CJD. Therefore, anle138b may be the most promising drug candidate for the treatment of prion diseases at present. However, Berry et al. (2013) imply that the presence of a methylenedioxyphenol group in anle138b is a potential problem be-

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cause methylenedioxyphenol compounds were shown to produce neurotoxic or hepatotoxic effects (Murray 2000).

INSIGHTS FROM THERAPEUTIC STUDIES ON HUMANS AND ANIMALS

Although immeasurable efforts have made it feasible to conduct large-scale clinical trials, few meaningful outcomes have benefited CJD patients, as summarized in Table 1. The rapidly progressive disease process, the mechanism of which remains enigmatic, makes the timing of therapeutic intervention particularly difficult. At this moment, we are still very far from the goal of life-long survival with preserved quality of life, let alone a cure. In this section, we discuss the issues that should be resolved to achieve significantly beneficial intervention against prion disease.

Preemptive Intervention

One of the main reasons clinical trials commonly fail in terms of survival is delayed intervention. In fact, the more delayed the intervention in prion-infected animals, the less effective the antiprion compound is at prolonging survival (Doh-ura et al. 2004; Kawasaki et al. 2007). As shown in Figure 4, the most opportune time for therapeutic intervention of prion diseases is very early in the preclinical stage. At this stage,

the odds of preventing or delaying disease onset are most favorable because exponentially accumulated amounts of prions almost reach a plateau in the brain before symptomatic disease onset (Prusiner 1987; Sandberg et al. 2014). Consequently, suitable preclinical diagnostic measurements are required for preemptive intervention.

Preclinical Diagnosis

Current diagnosis of CJD relies on a combination of results from magnetic resonance imaging, cerebrospinal fluid analysis, and electroencephalography (Zerr 2009; Wang et al. 2013). In addition, new technologies, such as quaking-induced conversion analysis for detecting an ultra-trace amount of PrP^{Sc} in the cerebrospinal fluid (Atarashi et al. 2011; McGuire et al. 2012; Sano et al. 2013; Cramm et al. 2015; Orrú et al. 2015), nasal brushings (Orrú et al. 2014), urine (Moda et al. 2014), or blood (Orrú and Caughey 2011; Orrú et al. 2012), have presented new opportunities for early diagnosis. These recent advances in diagnostic techniques, whose sensitivity and specificity are 89%–97% and 100%, respectively, are remarkable (Masters 2014; Moda et al 2014; Orrú et al. 2014, 2015). However, these techniques still may be insufficient to diagnose very early preclinical stages in healthy prion carriers or individuals predisposed to CJD. Further advances in this field, including
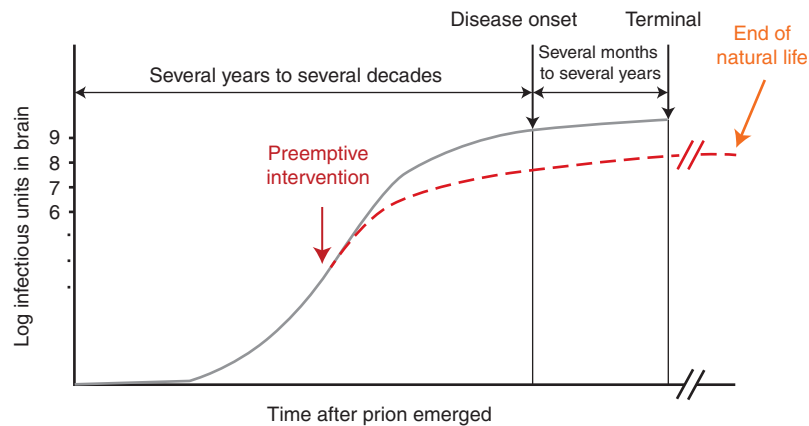
Figure 4. Schematic of the kinetics of prion accumulation in the brain and timing of medical intervention.

discovery of surrogate markers, are necessary to detect healthy individuals at high risk.

Prion diseases have long incubation periods from infection to disease onset. It remains unclear whether the illness invariably occurs in those who have already been infected with prions, as this illness apparently only occurs in a certain portion of people predisposed to prions in any prion disease case. A genome-wide analysis has been performed to evaluate genetic predisposition to prion disease, but thus far no strong genetic factors other than *PRNP* have been disclosed. Many missense or insertional mutations in *PRNP* are linked to familial types of prion disease (Mastrianni 2010). In addition, polymorphic codon 129 of *PRNP* is partly associated with the risks of acquired prion diseases such as kuru, variant CJD, or iatrogenic CJD (Collinge et al. 1991, 1996), whereas polymorphic codon 127 and codon 219 are reportedly resistant to kuru (Mead et al. 2009; Asante et al. 2015) and sporadic CJD (Shibuya et al. 1998), respectively. However, carrying these types of *PRNP* polymorphisms may not suggest whether a person will or will not develop the disease (Kobayashi et al. 2015). Even carrying disease-linked *PRNP* mutations does not necessarily mean that a person will inevitably develop the disease. For instance, no positive family history has ever been reported for patients with a valine-to-isoleucine mutation at codon 180 of *PRNP*, although this mutation is the most common (40%) in patients with genetic prion diseases in Japan (Jin et al. 2004). Consequently, it is presumed that there are other genetic and environmental factors strongly affecting disease susceptibility. Understanding these factors will be useful for identifying those healthy individuals at high risk.

Limitations of Single Compounds

Another issue is the strain dependency of antiprion compounds, which causes the emergence of drug-resistant prion conformers in prion-infected cells or animals (Kawasaki et al. 2007; Ghaemmaghami et al. 2009; Berry et al. 2013; Miller-Vedam and Ghaemmaghami 2013). This issue is predicted based on similar drug-resis-

tant phenomena in chemotherapy with antibiotic, antiviral, or anticancer drugs. However, the molecular mechanisms of strain-dependent activity and subsequent emergence of drug-resistant prion conformers remain to be elucidated. Disclosing antiprion pharmacophores targeting all prion strains is crucially important for the development of therapeutics for prion disease, considering that the most beneficial compound (anle138b) is incapable of halting disease progression even without the emergence of drug-resistant prion conformers. Limitations of single-compound therapy are attributable to the induction of detoxification systems in the body, which is frequently an obstacle to monochemotherapy for cancers or viruses. Consequently, with reference to abundant knowledge of chemotherapy for cancer and infectious diseases, combination therapy using drugs with different structures and targets should also be considered for treating prion diseases. The beneficial effects of such an approach have been shown in principle in prion-infected rodents (Kocisko et al. 2006).

A Combination of Multiple Targets

As already described, many compounds or biological materials have been discovered to have antiprion activity related to the inhibition of PrP^{Sc} formation or the enhancement of PrP^{Sc} degradation through a combination of in silico, in vitro, prion-infected cell models, and prion-infected animal screening (Sim and Caughey 2009; Teruya et al. 2009). Among these models, prion-infected cell models have been the most frequently used since Congo red and polyanionic glycans were found to possess antiprion activity (Caughey and Race 1992; Caughey and Raymond 1993). In fact, our research group has identified dozens of antiprion compounds, including quinacrine and compB, using prion-infected cell models (Doh-ura et al. 2000, 2007b; Ishikawa et al. 2004, 2006; Murakami-Kubo et al. 2004; Kawatake et al. 2006; Kawasaki et al. 2007; Nguyen et al. 2008, 2011; Hamanaka et al. 2011, 2015; Teruya and Doh-ura 2013; Nishizawa et al. 2014). However, these cell models are not fully compatible with in vivo prion-

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infected neuronal cells. All persistently prion-infected cell models are mitotic and invulnerable to accumulated PrP^{Sc}, whereas prion-infected neuronal cells of the brain are postmitotic and presumably vulnerable to accumulated PrP^{Sc}. Therefore, more suitable prion-infected cell models are necessary for assaying not only PrP^{Sc} formation and degradation but also PrP^{Sc}-induced neuronal cell death.

Regarding neurodegeneration, levels of PrP^{Sc} accumulation and/or infectivity are not parallel to those of neurological deterioration (Prusiner 1987; Sandberg et al. 2014). In addition, a study of mice lacking a glycosylphosphatidylinositol anchor for PrP^{C} has indicated that these mice survive for very long periods despite remarkable levels of PrP^{Sc} or infectivity present in the brain (Chesebro et al. 2005). These data suggest that innovations in preemptive treatment strategies against PrP^{Sc}-induced neurodegenerative processes might be as or more important than those for inhibiting PrP^{Sc} formation or facilitating its degradation. Even lifelong survival with a preserved quality of life may be possible irrespective of PrP^{Sc} levels in the brain if the most effective specific treatments for the neurodegenerative processes could be introduced at very early preclinical stages. This wishful expectation, however, requires further evaluation.

CONCLUDING REMARKS

Large-scale clinical trials for prion diseases have been made feasible with a structured framework, and several lead candidates for antiprion therapy have been developed. In addition, efforts have been made to better understand the prion pathogen and its pathogenesis and to establish more sensitive and specific diagnostics, more susceptible experimental disease models, and more convenient drug-screening methods. Thus far, however, obtaining meaningful benefits from medical interventions after disease onset remains elusive. To gain really beneficial results, the timing of intervention should be shifted to an earlier preclinical stage of disease, wherein PrP^{Sc} or infectivity in the brain remains low. Concomitantly, further advances are nec-

essary to elucidate the prion-specific neurodegeneration mechanism, to identify endogenous and environmental factors susceptible or resistant to the disease, and to discover diagnostic surrogate markers for detecting healthy prion carriers. These advances could be made from recent research but will require continued innovative approaches and/or strategies.

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