Therapeutic strategies for identifying small molecules against prion diseases

REVIEW

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Abstract

Prion diseases are fatal neurodegenerative disorders, for which there are no effective therapeutic and diagnostic agents. The main pathological hallmark has been identified as conformational changes of the cellular isoform prion protein (PrP^{C}) to a misfolded isoform of the prion protein (PrP^{Sc}). Targeting PrP^{C} and its conversion to PrP^{Sc} is still the central dogma in prion drug discovery, particularly in *in silico* and *in vitro* screening endeavors, leading to the identification of many small molecules with therapeutic potential. Nonetheless, multiple pathological targets are critically involved in the intricate pathogenesis of prion diseases. In this context, multi-target-directed ligands (MTDLs) emerge as valuable therapeutic approach for their potential to effectively counteract the complex etiopathogenesis by simultaneously modulating multiple targets. In addition, diagnosis occurs late in the disease process, and consequently a successful therapeutic intervention cannot be provided. In this respect, small molecule theranostics, which combine imaging and therapeutic properties, showed tremendous potential to cure and diagnose *in vivo* prion diseases. Herein, we review the major advances in prion drug discovery, from anti-prion small molecules identified by means of *in silico* and *in vitro* screening approaches to two rational strategies, namely MTDLs and theranostics, that have led to the identification of novel compounds with an expanded anti-prion profile.

Keywords prion diseases, *in vitro* screening, *in silico* screening, multi-target-directed ligands, theranostics

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Introduction

Prion diseases, otherwise known as transmissible spongiform encephalopathies (TSE), are a group of fatal and neurodegenerative infectious disorders of humans and animals (Prusiner 1982; 2001). These include sporadic, genetic and acquired forms of Creutzfeldt-Jakob disease (CJD) in humans, scrapie in sheep, and bovine spongiform encephalopathy (BSE) in cattle. Despite being quite rare (the global incidence of CJD is approximately 1 case per million per year) (Uttley et al. 2020), the intrinsic transmissibility of prions results in acquired prion diseases (2), with significant public health consequences.

These diseases are generally characterized by neurodegeneration due to conformational changes of the cellular isoform prion protein (PrP^{C}) and accumulation of a misfolded isoform of the prion protein (PrP^{Sc}). The formation of PrP^{Sc} is accompanied by profound changes in the structure of PrP^{C}. PrP^{C}, rich in α-helical regions, is converted into a misfolded protein with a mainly β-sheet structure, which makes PrP^{Sc} highly insoluble and partially resistant to proteolytic digestion (Pan et al. 1993). Furthermore, PrP^{Sc} acts as a template to promote further conversion of PrP^{C} into nascent PrP^{Sc} molecules. The deposition of PrP^{Sc}, either in the form of aggregates or as diffuse deposit, depends on the host species and prion strain (Soto and Pritzkow 2018), and defines different conformational arrangements of PrP^{Sc}. PrP^{Sc} deposits are characterized by different biochemical properties, such as electrophoretic mobility, glycosylation profile and proteolytic resistance, and *in vivo* biological features, such as different incubation period, clinical signs, and lesion profiles. Generally, PrP^{Sc} causes distinctive histopathological brain lesions associated with spongiform changes, neuronal loss, vacuolation, microglial activation, and astrogliosis. However, the exact mechanism of PrP^{Sc}-induced neurodegeneration and pathology is still an open question. It has been suggested that either loss of function of PrP^{C} or toxic gain-of-function of PrP^{Sc} and/or during prion conversion might trigger progressive neurodegeneration (Westergard et al. 2007).

PrP^{C} is a glycophosphatidylinositol (GPI)-anchored glycoprotein whose highest expression levels are found in nervous tissues within neuronal and glial cells (Bendheim et al. 1992). It is also expressed at lower levels in other cells and tissues, such as blood, digestive tract, lymphocytes, and skin (Bendheim et al. 1992). One of the challenges in prion biology is determining the main physiological functions of the cellular prion protein. This area of investigations has been comprehensively reviewed elsewhere (Castle and Gill 2017; Legname 2017; Watts et al. 2018; Wulf et al. 2017).

Presently, there are no available treatments for prion diseases. In the last decades, several compounds have been found to affect PrP^{Sc} accumulation in *in vitro* models and to prolong survival in TSE-infected animals (Colini Baldeschi et al. 2020). Most of these agents successfully inhibited PrP^{Sc} formation in cell-free system or mouse neuroblastoma cells. However, in humans, the majority of them did not delay the disease onset or alter the disease progression. This situation is exacerbated by the fact that current diagnosis of prion diseases relies on a combination of techniques from magnetic resonance imaging, cerebrospinal fluid analysis, and electroencephalography, that are unable to diagnose very early preclinical stages (Connor et al. 2019; Wang et al. 2013). In fact, prion diseases have long incubation periods from infection to disease onset, which calls for discovery of novel biomarkers to early detect pathological signs and allowing a successful therapeutic intervention.

In this review, we will consider current therapeutic strategies for identifying small molecule-based preclinical candidates against prion diseases. The first section will focus on *in silico* and *in vitro* screening approaches mostly devoted to target prion protein and its pathogenic conversion. In the second part, we will discuss the so-called multi-target drug discovery and theranostic approaches as two rational strategies that have led to the identification of novel compounds with an expanded anti-prion therapeutic profile.

Drug discovery approaches for antiprion compounds

The main strategy for antiprion drug discovery is to target PrP^{C} and prevent its conversion to PrP^{Sc} (Gandini and Bolognesi 2017). Thus, possible strategies act on different stages of prion biogenesis and PrP^{Sc} formation and aggregation. These include: reduction of PrP^{C} expression, prevention of PrP^{C} unfolding, blockade of unfolded PrP binding to PrP^{Sc}, prevention of PrP^{C} recruitment by PrP^{Sc}, inhibition of PrP^{Sc} polymer formation, and induction of PrP^{Sc} degradation (Figure 1). To identify ligands with an anti-prion profile, high-throughput screening (HTS) of large compound libraries has been -and still is- extensively pursued, by employing several cell platforms (Geissen et al. 2011). Marked advancements in the experimental and computational techniques have broaden the discovery of antiprion small molecules, moving from an empirical screening toward a structure-based rational design (Ghaemmaghami et al. 2014). Starting from the year 2000, *in silico* structure-based approaches became possible due to the NMR solution structure of the human prion protein obtained (Zahn et al. 2000). The recent cryo-EM structure of amyloid fibrils formed by prion protein would further enable structure-based drug design approaches (Glynn et al. 2020; Wang et al. 2021; Wang et al. 2020). Additionally, cell-free *in vitro* conversion assays are emerging as major screening techniques (Moda et al. 2019). Real Time Quaking Induced Conversion (RT-QuIC) and Protein Misfolding Cyclic Amplification (PMCA) have opened new horizons for testing compounds in a time- and cost- effective manner (Moda et al. 2019). Furthermore, as in the case of *in vitro* systems, they have clear ethical advantages compared to *in vivo* models (Törnqvist et al. 2014). In addition, the phenotypic readout of such assays allows to

specifically identify drugs able to reduce prion load or its propagation/replication efficiency. By contrast, they do not provide information about the pathways or molecular targets that might be involved in the anti-prion mechanism of action.

Despite the availability of several approaches, the existence of several prion strains, which give rise to different conformational aggregates (Soto and Pritzkow 2018), and drug-resistant pathological conformers has hampered the identification of effective compounds capable of modulating the progression of different forms of the disease. Thus, the search for effective anti-prion candidates remains a hot topic, which has been covered by several comprehensive reviews (Aguzzi et al. 2018; Barreca et al. 2018; Bolognesi et al. 2015; Colini Baldeschi et al. 2020). Therefore, the next sections will focus on the most relevant *in silico* and *in vitro* screening strategies, concentrating on advances in the last ten years.

In silico and *in vitro* screening strategies

Development of screening assays for the discovery of relevant compounds with effective anti-prion activity is of utmost need to combat these rare diseases.

Structural conformation of the cellular prion protein (PrP^C) consists of three α-helices (A, B and C) and two small, antiparallel β-sheets (S1 and S2) (Zahn et al. 2000). In 2002, Kuwata *et al*. characterized a sparsely populated metastable conformer of the PrP^C (PrP*), and defined multiple, unstable residues spanning the B and C helices (Kuwata et al. 2002). The authors hypothesized that by cross-linking distant hotspot PrP residues by a small chemical chaperone could result in a suppression of the PrP^C to PrP^{Sc} conformational rearrangements by stabilizing PrP^C conformation.

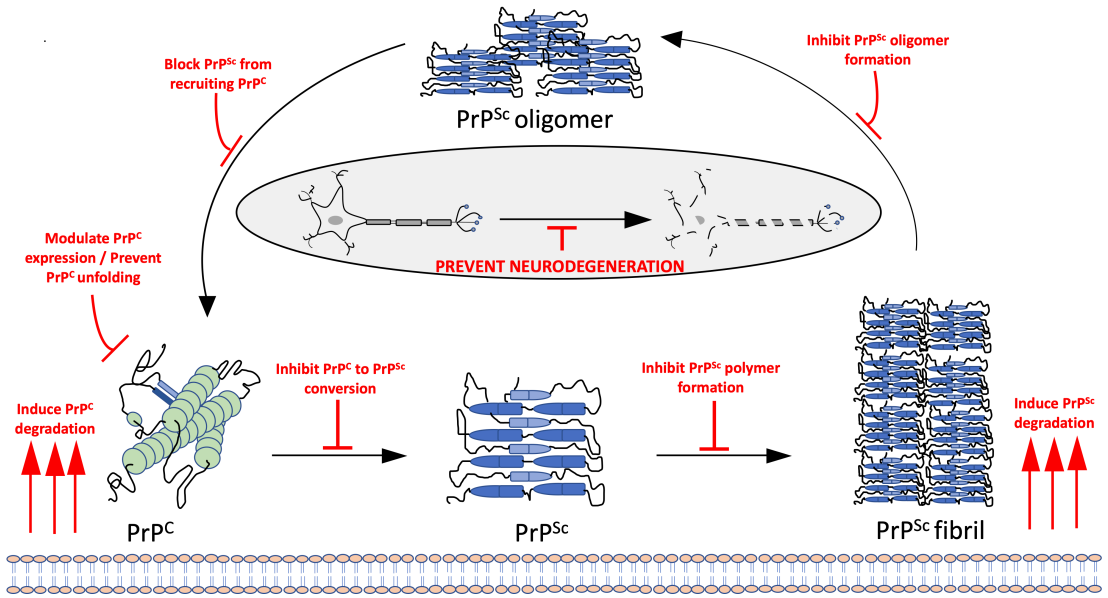

Fig. 1 Therapeutic strategies directed to the different stages of prion biogenesis and PrP^{Sc} formation and aggregation.

To uncover small molecules capable of specifically binding the protein "pocket" created by the hotspot residues, a dynamics-based drug discovery approach that utilizes heteronuclear NMR data combined with computer simulations to conduct *in silico* screening was developed. The hit compounds identified by this strategy were further tested in a cell-based assay and *in vivo*, leading to the discovery of a diphenylmethane derivative, named GN8. Particularly, GN8 treatment significantly reduced PrP^{Sc} levels in a mouse neuronal cell line persistently infected with human GSS agent, and resulted in a prolonged survival of TSE-infected mice. Thermal stability analysis by circular dichroism spectroscopy confirmed that GN8 acts as a chemical chaperone to stabilize PrP^{C} conformation and further validated its potential as a lead compound for prion diseases (Kuwata et al. 2007). GN8 has been the starting point for several further drug discovery endeavors. To investigate the organ distribution and to assess the penetration across the blood–brain barrier (BBB), the same authors incorporated two additional methyl groups to the diphenylmethane structure of GN8, leading to a synthesis of a positron emitting [^{11}C]-labeled GN8 derivative, with an unchanged anti-prion efficacy (Kimura et al. 2013). Furthermore, subsequent positron emission tomography (PET) scan analysis of Sprague-Dawley rats confirmed the ability of the newly synthesized GN8 derivative to cross the BBB and maintain therapeutic concentration in rat brains (Kimura et al. 2013). Additional optimization of GN8 guided the design of a novel PrP^{C} molecular chaperone (MC) with a substantially increased affinity for PrP^{C} (Yamaguchi et al. 2019). Efficacy of its binding and anti-prion activity was accessed via *in vitro* conversion NMR (IVC-NMR) technique that allows the real-time surveillance of chemical shift perturbations within the PrP protein residues following the template-dependent conformational conversion. *In vivo* experiments showed that the MC significantly prolonged the median survival time of TSE-infected mice and, more importantly, postponed the onset of the disease in BSE-infected macaques (Yamaguchi et al. 2019). Another advantage of small molecule chaperones is their potential to act on a broad spectrum of molecular targets in different diseases. Based on a 3D structure of the PrP^{C}, an *in silico* ligand screening using the ICM method led to the discovery of a carbazole derivative with ant-prion properties, named 5Y (Yamashita et al. 2020). Due to chemical resemblance of its binding sites on several different proteins, 5Y not only showed a notable anti-prion activity, but also exhibited anti-cancer and anti-influenza effects (Yamashita et al. 2020).

Another group of small aromatic molecules, termed NPRs, also showed great potential in their anti-prion activities (Ishibashi et al. 2016). These molecules were identified employing a structure-based drug discovery algorithm (named Nagasaki University Docking Engine, NUDE) in conjunction with the DEGIMA supercomputer used for molecular docking simulations of NPRs with hydrophobic "hotspot" pocket of PrP^{C}. Following *in silico* screening, hit molecules were studied for their binding affinities to PrP^{C} via Surface Plasmon Resonance (SPR) analysis and their stabilizing effect on recombinant PrP was examined by Thermal Shift Assay (TSA). Fragment Molecular Orbital (FMO) calculations demonstrated polar interactions as essential for the formation of GN8-PrP^{C} complex (Ishikawa et al. 2009), while Van der Waals forces seemed to drive the NPR-PrP^{C} binding (Ishibashi et al. 2016).

Based on a previously characterized GN8-PrP^{C} NMR structure (Yamamoto and Kuwata 2009), Hyeon et al. generated a pharmacophore model for selection of prospective anti-prion drugs from their in-house chemical database (Hyeon et al. 2015). Candidate compounds generated by their virtual screening were further supported by docking simulations and validated in a novel Multimer Detection System (MSD) *in vitro* assay. Assessment of anti-prion compounds activity with the MSD assay relies on using anti-prion antibodies for precise identification of PrP^{Sc} multimers. Binding affinities of selected compounds to PrP^{C} were evaluated on the SPR biosensor chip and the investigation of their binding mode highlighted reinforced the importance of hydrogen bonding with the critical PrP hotspot residues together with the hydrophobic environment, closely corresponding to the prediction made by their pharmacophore model (Hyeon et al. 2015).

Although bioassays are still irreplaceable tools in drug research, *in vitro* assays offer some advantages in terms of their simplicity, higher speed and tighter control of the experimental environment. *In vitro* drug screening assays based on prion protein aggregation can be used to uncover new anti-prion molecules or as a follow up to conventional bioassays when investigating whether a compound acts directly on PrP or via other cellular targets species (Ladner-Keay et al. 2018). Shaking Induced Conversion (ShIC) followed by Resolution Enhanced Native Acidic Gel Electrophoresis (RENAGE) allows time-dependent monitoring of distinct PrP conformers formed along the PrP conversion and propagation process. Furthermore, regarding the discovery of anti-PrP compounds, this system permits direct molecular target identification among different PrP species (Ladner-Keay et al. 2018). As stated above, other, more commonly used *in vitro* aggregation assays are PMCA and RT-QuIC, utilized in a comparative analysis of stilbene-based compounds on inhibiting prionization of different rodent-derived PrP^{Sc} species (Zhou et al. 2020).

A ligand-based strategy for tackling prion diseases was proposed by our group (Bolognesi et al. 2010). We observed that most of the anti-prion molecules possessed a symmetrical bifunctional structure consisting of two moieties joined via an appropriate spacer (e.g., GN8). Starting from this assumption, we developed bivalent compounds as they might show superior activity profiles compared to their monovalent counterparts (Staderini et al. 2013b). Particularly, the bivalent compounds were designed exploiting the diketopiperazine scaffold as central spacer covalently linked to two identical moieties potentially able to halt the conversion of PrP^{C} and PrP^{Sc} by concurrent interaction with two prion protein binding pockets. A well-established scrapie cell-based screening assay followed by RT-QuIC validated these planar bivalent compounds as conceivable anti-prion therapeutics (Bolognesi et al. 2010).

Another anti-prion approach was recently established by Spagnolli *et al*., aimed at downregulating PrP^C on a post-translational level (Spagnolli et al. 2021). By following the evolution of PrP^C folding trajectories using thermal unfolding and Reactive Molecular Dynamics (rMD) simulations, the authors identified a novel target against prion diseases: a PrP-folding intermediate. Small molecules from a chemical library were docked at the folding intermediate "hotspot" pocket and screened for their capacity to lower the amounts of PrP^C in different cell lines expressing PrP. SM875 was identified as the most potent compound and confirmed to act solely on non-native PrP conformer by Dynamic Mass Redistribution (DMR) technique. The compound was further verified in a Tet-On conditional *PRNP* expression system and by a detergent insolubility assay upon temperature-induced denaturation of PrP (Spagnolli et al. 2021).
Advancements in computational methods have indicated pharmacophore modelling as one of the key components of the rational drug design (Lin and Li 2020). To produce a statistically reliable pharmacophore model, Zaccagnini et al. performed QSAR studies aimed at linking biological activity and physicochemical properties of hitherto known anti-prion compounds (Zaccagnini et al. 2020). Virtual library screening was based on a newly generated model and further selection of molecules was conducted through *in vitro* assays. This study led to the identification of a bivalent compound whose anti-prion activity was attributed to its phenothiazine and 7-chloro-quinoline moieties (Zaccagnini et al. 2020).
However, there is another possibility when searching for anti-prion compounds. Since drug discovery and development is an extremely time- and cost- consuming process, drug repurposing offers a promising alternative to accelerate the "bench to bedside" process, and thus reduce the costs of drug development. In fact, doxycycline, a tetracycline-class antibiotic (Forloni et al. 2002; Haïk et al. 2014), and chlorpromazine (Benito-León 2004; Korth et al. 2001), a phenothiazine-based antipsychotic, have reached clinical phase, albeit with no evident beneficial effects (Forloni et al. 2019). Guanabenz (GA), a drug routinely used for the treatment of hypertension, was also found to act against mammalian and yeast prions (Tribouillard-Tanvier et al. 2008). Varying the positions of chlorine atoms on the benzene ring of GA guided the synthesis of GA derivatives devoid of the initial agonistic activity against α2-adrenergic receptors (Nguyen et al. 2014). The remarkable anti-prion activity of GA analogues was nevertheless retained as confirmed by a phenotypic yeast-based assay as well as by a cell-based assay upon Western blot quantification of the residual PrP^{Sc} (PrP^{res}). Additionally, two of the most potent analogues were further validated as anti-prion agents in an ex vivo experiment employing cultured organotypic cerebellar slices (OCSs) infected by PrP^{Sc} (Nguyen et al. 2014). In a repurposing effort based on a PrP–FRET-enabled high throughput assay, two drugs, the anti-histaminic astemizole (Karapetyan et al. 2013) and tacrolimus, an immunosuppressant widely used in organ transplant, displayed promising effects confirmed in prion-infected animals. Additionally, Marzo et al. have reported that the main metabolite of the anticancer drug tamoxifen, 4-hydroxytamoxifen, induced PrP^C and PrP^{Sc} lysosome-mediated degradation, suggesting the existence of a lysosomal degradation pathway for PrP^{Sc} clearance (Marzo et al. 2013). More recently, Gilch et al have shown that oral administration of efavirenz, a repurposed drug targeting Cyp46A1, increases survival times of prion infected mice (Ali et al. 2021).
HTS platforms are valuable tools when it comes to reducing the time needed for the discovery of novel therapeutics. One such platform, based on yeast growth, was designed by Du et al. (Du et al. 2017; Du et al. 2019). The authors screened thousands of compounds coming from different libraries against the yeast [SWI^+] prion in their formerly created Ura3-based reporter system and associated several new chemical structures with anti-prion potential (Du et al. 2017; Du et al. 2019).
In search for potential anti-prion drugs, scrapie-infected murine cells are commonly used to assay compound libraries in a high-throughput fashion. Apart from the conventional Western blot quantification of the residual proteinase-resistant scrapie prion, reduction of PrP^{Sc}, as a measure of compound efficacy, can also be detected by Enzyme-Linked Immunosorbent Assay (ELISA) (Li et al. 2013a; Li et al. 2013b). Li and colleague reported on a HTS ELISA, which aided the discovery of new anti-prion compounds based on arylamide and arylpiperazine chemical scaffolds (Li et al. 2013a; Li et al. 2013b).
Imberdis et al. developed a surrogate assay employing HEK cells stably transfected with a mutant isoform of PrP (ΔCR PrP). The assay is based on the suppression of antibiotic hypersensitivity mediated by the PrP isoform in HEK cells and adapted for HTS (Imberdis et al. 2016b). With this platform, the authors have identified a novel class of anti-prion molecules, i.e., phenethyl piperidines. These compounds were shown to delay template-seeded PrP^{Sc} aggregation in the RT-QuIC assay and to partially cure scrapie-induced dendritic loss in a neuronal cell culture (Imberdis et al. 2016b).

Pursuing new directions in prion drug discovery

Continuing research in the last decade has shed light on additional layers of complexity that may take many years to explore. Along with strain heterogeneity and drug-resistance mechanisms, developing compounds for prion diseases is clearly highly challenging. To overcome the current lack of effective treatment options for prion diseases, we have explored two drug discovery approaches (Bongarzone et al. 2014): (i) the development of compounds with other therapeutic properties than PrP inhibition (ii) PrP inhibitors endowed with concomitant imaging properties, acting as theranostics, toward a more promising personalized medicine setting.
Similar to other neurodegenerative diseases, there is a general consensus that prion diseases are multifactorial in nature (Booth 2017). Increasing evidence suggests that they arise by an intricate array of pathological events, all occurring

concurrently, which include: (a) PrP misfolding and aggregation (Aguzzi and Calella 2009), (b) oxidative stress and reactive oxygen species (ROS) production (Pamplona et al. 2008); (c) brain inflammation with activation of astrocytes and microglia (Li et al. 2021); and (d) metal ions dyshomeostasis (Toni et al. 2017).

To effectively confront such multifaceted and complex nature, multitarget compounds (often referred to as "multitarget-directed ligands" (MTDLs)), which can hit multiple proteins simultaneously in intertwined pathways, may offer promise (Bongarzone et al. 2014; Cavalli et al. 2008; Gandini and Bolognesi 2017). In terms of PrP inhibitors, this means developing small molecules that can modulate both PrP and other related targets. In principle, this could considerably enhance efficacy and produce more satisfactory clinical outcomes.

Another approach to combat prion diseases is to consider its principal player, PrP, as both therapeutic target and diagnostic biomarker. Hence, the idea to combine into a single molecule both therapeutic and diagnostic properties, the so-called theranostic, has emerged as a promising and innovative therapeutic option. As such, theranostics allow to simultaneously target and label prion protein, thus monitoring disease progression and/or success of treatment (Bolognesi et al. 2016). It is also one of the key strategies for the emergence of personalized medicines, especially important for the high heterogenicity of multiple prion strains (Woerman 2021). Despite far from being applied in clinical practice, theranostics should allow clinicians to precisely assess the benefits of anti-prion protein therapy and to identify the "best patients" for a given treatment (Bolognesi et al. 2016).

In the following, we will summarize our and other efforts in developing MTDLs and theranostics again prion diseases.

Multi-target strategies

In 2006, Klingenstein and co-authors opened the way for the development of MTDLs for prion, by designing the first dual molecule in the field. Quinpramine (Figure 2) was rationally designed by combining the structural features of two drugs showing therapeutic potential in prion diseases, i.e., quinacrine and desipramine with the aim of obtaining a compound retaining the activity of both the starting drugs, with synergistic effects. Remarkably, quinpramine exhibited anti-prion activity in ScN2a cells 5-fold higher than quinacrine and 10-fold than desipramine (Klingenstein et al. 2006).

Encouraged by these results and based on our expertise in the field of MTDLs for neurodegenerative diseases (Bongarzone et al. 2014; Cavalli et al. 2008; Gandini and Bolognesi 2017), we developed different series of MTDLs with *in vitro* anti-prion potential.

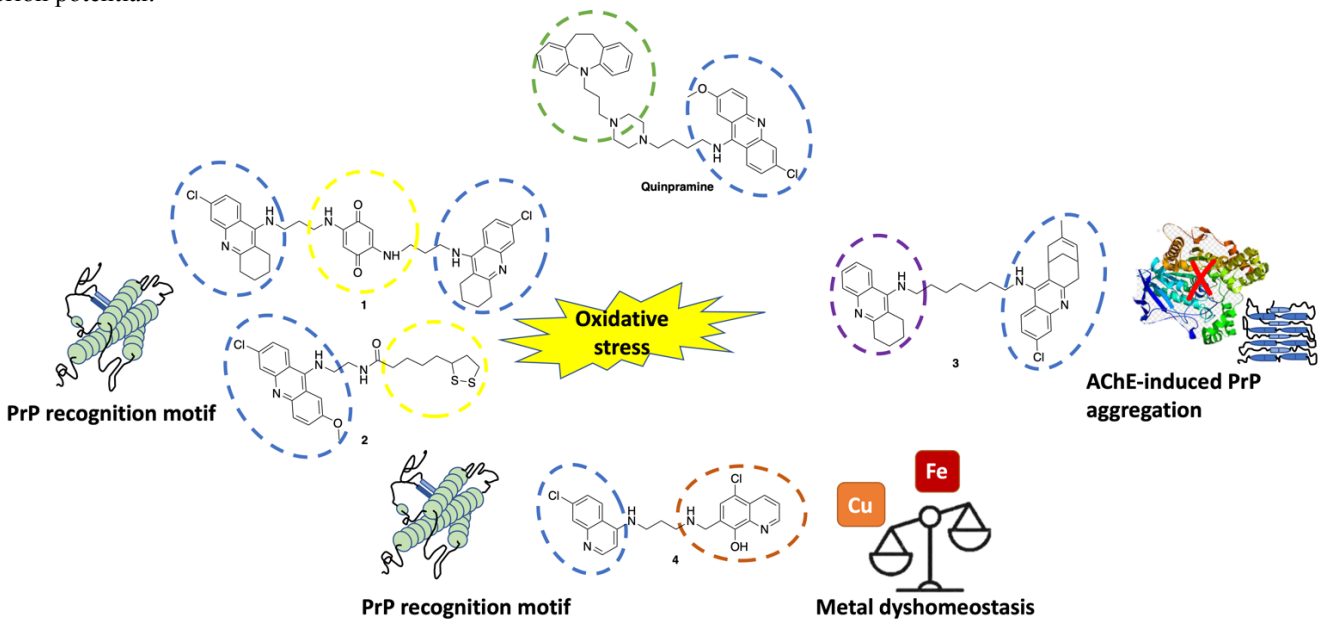

Fig. 2. MTDLs able to modulate multiple targets involved in prion diseases.

We sought to develop compounds directed towards two or multiple pathological targets, recognized as critically involved in prion pathogenesis (Bongarzone et al. 2014; Cavalli et al. 2008; Gandini and Bolognesi 2017). The generation of ROS has been reported to be one of the main downstream effectors of prion-induced neurotoxicity, and administration of antioxidants effectively extended the survival of prion-diseased mice (Mizrahi et al. 2014). Consistently, oxidative damage was observed in the brains of infected mice and patients with sporadic and familial CJD (Van Everbroeck et al. 2004; Yun et al. 2006).

With this in mind, we envisaged that the conjugation of the privileged anti-prion motif of quinacrine with the well-known

antioxidant properties of the benzoquinone framework, might lead to effective anti-prion compounds capable of modulating both prion misfolding and oxidative stress (Bongarzone et al. 2010). We developed a series of MTDLs featuring a 2,5-diamino-1,4-benzoquinone central core linked thorough different spacers to diverse anti-prion privileged motifs. The resulting MTDLs turned out to inhibit PrP^{Sc} aggregation and reduce oxidative stress in ScGT1 cells (Bongarzone et al. 2010). Remarkably, compound **1** (Figure 2) was active against prion replication in the submicromolar range with an EC_{50} of 170 nM, lower than that of quinacrine (EC_{50} = 320 nM). Furthermore, it displayed negligible cytotoxicity in ScGT1 cell line.
In a continuation of our efforts aimed at developing a new series of anti-prion MTDLs, we combined the structural features of the quinacrine with that of lipoic acid (Bongarzone et al. 2011), a well-known antioxidant (Bolognesi et al. 2006), demonstrating potential beneficial effects in prion patients (Drisko 2002).The resulting MTDL **2** (Figure 2) was able to simultaneously block PrP^{Sc} accumulation in ScGT1 cells with an EC_{50} value of 150 nM. It also delayed fibril formation in an amyloid seeding assay, and reduced oxidative stress (Bongarzone et al. 2011).
Galdeano and colleagues developed a series of dual compounds active against both Alzheimer's and prion diseases (Galdeano et al. 2012). The discovery of a pathological chaperoning effect of acetylcholinesterase (AChE) toward prion peptide aggregation supported the idea to inhibit AChE for reducing prion aggregation (Pera et al. 2009; Pera et al. 2006). Binding of AChE to prion protein is suggested to be at the AChE peripheral site (Pera et al. 2006), located above the catalytic one. Hence, compounds able to bind AChE peripheral site or both sites (the so-called dual binding inhibitors) might modulate the pathological chaperoning effect of AChE in prion diseases. The authors developed a series of tacrine-huprine dual binding inhibitors (Galdeano et al. 2012). Derivative **3** turned out to be a potent inhibitor of AChE in the nanomolar range. More importantly, it was effective in inhibiting the chaperoning effect of AChE toward the aggregation of a prion peptide (PrP106–126).
The imbalance of metal ions has been shown to play a functional role in the PrP^{C} to PrP^{Sc} conformational conversion, both *in vitro* and *in vivo* (Toni et al. 2017). In fact, Cu^{2+} induced PrP^{C} misfolding and aggregation, probably causing a β-sheet like conformational change (Giachin et al. 2015). Furthermore, metal ions-PrP^{Sc} complex is associated with a marked increase of ROS production and neurotoxicity (Singh et al. 2010). In addition, calcium binding seems capable of promoting PrP^{C} aggregation propensity (Marrone et al. 2016; Storchi et al. 2015).
Thus, we purposely designed the multi-target compound **4** (Figure 2), by conjugating a heteroaromatic prion motif (i.e., a 7-chloroquinoline) with an 8-hydroxyquinoline (8HQ), as a metal chelator (Bolognesi et al. 2015). 8HQ derivatives have been reported to cross the BBB and to chelate metals from the brain (Prachayasittikul et al. 2013). **4** caused a persistent clearance of proteinase-resistant PrP^{Sc} from scrapie-infected cells, with a remarkable EC_{50} of 40 nM, emerging as a potent small molecule in ScGT1 prion model. It also reduced metal-induced prion aggregation. At 1 μM concentration, it displayed promising antioxidant effects.
While these antiprion MTDLs effectively inhibit PrP^{Sc} accumulation *in vitro*, it should be noted that they have not been tested *in vivo* yet.

Theranostic strategies

Prion diseases can only be confirmed by taking a sample of brain tissue during a biopsy or after death. The only reliable molecular marker for prion diseases is PrP^{Sc}, which is believed to precede clinical symptoms by several years (Kübler et al. 2003). Hence, imaging of fibrillar aggregates may be particularly suitable to diagnose the disease onset in its early stage when there is no clear clinical evidence, as well as to monitor its progression. In the last years, marked advancements have been made in the field of molecular imaging of amyloid deposits *in vivo*. In this context, PET ligands are imaging tools with established clinical applications in Alzheimer's disease (Vandenberghe et al. 2013), in spite of presenting the limitation of short half-lives, which require the on-site synthesis of PET tracers and access to radiochemistry equipment and a cyclotron. In parallel, fluorescence spectroscopy has emerged as noninvasive alternative for studying fibrillar aggregates (Staderini et al. 2015). This is because it is a versatile and sensitive method that provide a rapid, inexpensive and nonradioactive imaging system for neurodegenerative disorders (Staderini et al. 2015). A more promising strategy is the one based on theranostics, i.e., single chemical entities able to deliver therapy (via targeting PrP misfolding) and diagnosis (via PrP staining by fluorescence imaging) simultaneously. In principle, the simultaneous ability of a theranostic to allow assessment in real-time of its delivery to a pathological district and to the desired target, as well as the visualization of molecular changes due to its therapeutic effect, makes it an attractive strategy. Furthermore, at clinical level, such approach might offer the unique opportunity for improving treatment and trials, by enabling patient-risk stratification and patient selection, as well as treatment monitoring (Bolognesi et al. 2016).
Although we are far from the clinical application, a high number of fluorescent dyes has been developed for their dual ability to stain and target PrP misfolding (Mustazza et al. 2020). Of note, heterocyclic structures with a planar conformation, e.g., tetracyclines and phenothiazines, shared the capacity to bind amyloid structures and to possess fluorescence properties (Jameson et al. 2012). Despite not deliberately designed as theranostics, Congo Red derivatives and oligothiophenes have been demonstrated to prevent abnormal PrP formation in cell and animal models of prion diseases, while showing fluorescent

imaging properties (Mustazza et al. 2020). However, no significant survival prolongation was observed in diseases animal models and several imaging limitations, such as low specificity and poor sensitivity, have limited their translation into clinics (Mustazza et al. 2020; Staderini et al. 2013a).

An aminonaphthyl 2-cyanoacrylate (ANCA)-based probe (compound **5** in Figure 3) was capable of fluorescently labeling prion protein aggregates in neuronal tissue with excellent specificity and reproducibility (Cao et al. 2012). Intriguingly, probe **5** exhibited different fluorescence emission properties when bound to aggregates of amyloid-beta and prion. Inspired by these initial findings, a novel set of amino-aryl cyanoacrylates (AACAs) was synthesized and evaluated in an *in vivo* model (Cao et al. 2018). Particularly, in mice inoculated with infectious mouse-adapted chronic wasting disease prions, probe **6** showed promising results (Figure 3).

The first small molecule deliberately designed as a theranostic agent for prion diseases, was the styrylquinoline G8 (Figure 3) (Staderini et al. 2013a). Notably, G8 displayed no sign of neurotoxicity and promising anti-prion activity in two different cell models of prion diseases (ScN2a and ScGT1 cells). G8 exhibited a therapeutic profile comparable to the gold standard quinacrine, showing an EC_{50} of 0.5 μM. As regards to the imaging profile, G8 possessed the ability to fluorescently labeling PrP^{Sc} in treated cells, as demonstrated by fluorescent microscopy. Furthermore, G8 was predicted brain permeable.

Imberdis and colleagues identified a family of thienyl pyrimidine drugs that allow to detect SDS-resistant PrP^{Sc} (rSDS-PrP^{Sc}) dimers and trimers after proteinase K digestion (Ayrolles-Torro et al. 2011). In order to better understand the mechanism of action of previously identified thienyl pyrimidine compounds, as well as to evaluate their diagnostic and therapeutic potentials, novel analogs with improved activity were developed (Imberdis et al. 2016a). Based on the results of a structure-activity study, a quaterthiophene-bis-triazine compound, called MR100 (Figure 3), was identified as potential anti-prion theranostic agent (Imberdis et al. 2016a). In fact, MR100 exhibited promising anti-prion activity at nanomolar concentrations in prion-infected cells. Moreover, fluorescence interaction studies of MR100 with mouse PrP fibrils showed substantial modification of its fluorescence properties, supporting the binding of MR100 to PrP fibrils. Finally, the authors demonstrated that MR100 can also detect rSDS-PrP^{Sc} oligomers in prion-infected brain homogenates of various species (Imberdis et al. 2016a). Thus, MR100 emerged as a powerful tool not only for inhibiting prion aggregation, but also for selectively labeling prion-infected samples.

In a recent report, a virtual screening protocol, based on a three-dimensional quantitative structure-activity relationship

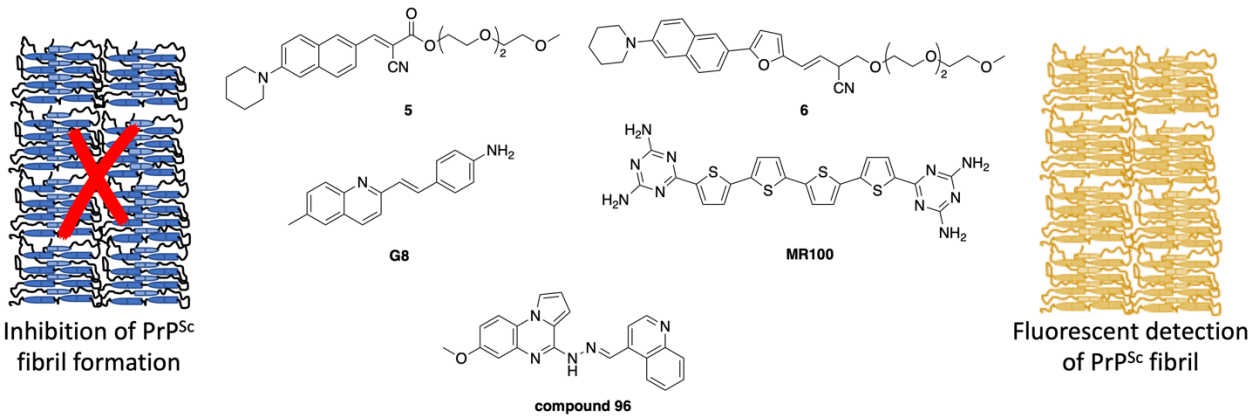

Fig. 3. Theranostics showing both diagnostic and therapeutic properties in prion diseases.

(3D-QSAR) model coupled with molecular docking was used to find novel theranostic tools able to prevent PrP^{C} misfolding (Zaccagnini et al. 2017). Moreover, prediction of physical and chemical properties, including cell membrane and BBB permeability, has been considered to select the hit compounds with potential anti-prion activity. Subsequently, cell toxicity and anti-prion activity were assessed. The employed screening workflow has allowed to identify compounds with low toxicity and remarkable anti-prion activity. In particular, the most promising hit identified, namely compound 96 (Figure 3), was not only able to interfere with PrP^{Sc}, but was also able to stain PrP^{Sc} aggregates in infected ScN2a cells with a fluorescence staining pattern comparable to that found for the amyloid dye Thioflavin-T. This peculiar profile makes the identified hit a suitable diagnostic tool for prion diseases with a concomitant therapeutic potential (Zaccagnini et al. 2017).

Conclusion

Although significant efforts have been made to better understand prion pathogenesis and to identify effective drugs and diagnostics, more sensitive experimental disease models, and more convenient drug screening methods are still necessary. So far, however, no therapeutic treatment after disease onset has reported beneficial effects. To be effective, the timing of

treatment should start at an earlier preclinical stage of disease, when pathological alterations have not occurred yet. Concurrently, further advances are required to elucidate the prion-specific neurodegeneration mechanisms, to identify the network of endogenous targets and factors underlying disease pathogenesis, and to discover diagnostic markers for an early detection.

While specifically targeting PrP^{C} and its conversion to PrP^{Sc} remains a dynamic and evolving research field in prion drug discovery, the rationale for MTDLs design strategy clearly stems from the multifactorial etiological basis of prion diseases. In parallel, fluorescent neuroimaging shows promise as a future clinical diagnostic tool for prion protein. Furthermore, the use of fluorescent compounds as theranostics could represent a promising approach not only to detect, but also to treat prion diseases. This combination in the same molecule enables to monitor real-time biodistribution and target site accumulation. The concomitant delivery and readout of efficacy can be exploited to tailor treatment regimens for specific patients. However, since no in vivo proof of concept has been reported for both MTDLs and theranostics, at this stage it is impossible to predict their clinical translation. This leaves unaddressed the question of whether they offer superior efficacy with respect to more conventional single-target drugs or diagnostics and therapeutics used separately.

Effective treatments are urgently needed. While it is too early to evaluate which strategy will be more successful, we are confident that the marked screening advancement and the diversified drug portfolio including repurposed drugs, MTDLs, and theranostics, will provide powerful agents to combat prion diseases.

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Declarations

Ethical approval This article does not contain any studies with humanparticipants or animals performed by any of the authors.

Informed consent For this type of study, formal consent is not required.

Conflict of interest The authors declare no competing interests.

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