Review

Exploring Anti-Prion Glyco-Based and Aromatic Scaffolds: A Chemical Strategy for the Quality of Life

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Abstract: Prion diseases are fatal neurodegenerative disorders caused by protein misfolding and aggregation, affecting the brain progressively and consequently the quality of life. Alzheimer’s is also a protein misfolding disease, causing dementia in over 40 million people worldwide. There are no therapeutics able to cure these diseases. Cellular prion protein is a high-affinity binding partner of amyloid β (Aβ) oligomers, the most toxic species in Alzheimer’s pathology. These findings motivate the development of new chemicals for a better understanding of the events involved. Disease control is far from being reached by the presently known therapeutics. In this review we describe the synthesis and mode of action of molecular entities with intervention in prion diseases’ biological processes and, if known, their role in Alzheimer’s. A diversity of structures is covered, based on glycans, steroids and terpenes, heterocycles, polyphenols, most of them embodying aromatics and a structural complexity. These molecules may be regarded as chemical tools to foster the understanding of the complex mechanisms involved, and to encourage the scientific community towards further developments for the cure of these devastating diseases.

Keywords: prion; protein aggregation; amyloid; anti-prion compounds; structure optimization

1. Introduction

Prion diseases, also called transmissible spongiform encephalopathies (TSEs), are infectious and fatal neurodegenerative diseases. They affect the brain and the nervous system, causing deterioration of mental and physical abilities, amyloid plaque formation, neuronal death, and production of holes in the cortex that appears, when examined under a microscope, as a sponge. The clinical signs in humans may include memory problems and personality changes, depression, lack of coordination, involuntary movements or insomnia. In the later stages of the disease, dementia and loss of ability to move or speak are common. Prion diseases are the single conformational diseases transmissible, either experimentally or by natural routes, mainly by ingestion. Prions have an extraordinary resistance to conventional sterilization procedures and are capable to bind to metal and plastic surfaces without losing infectivity. They are caused by an abnormal conformational isoform (PrPSc) of the host-encoded cellular prion protein (PrPC) [1]. Full-length PrPC is a 253 amino acid-long glycoprotein ubiquitously expressed in all mammals and anchored to the external cell membrane by a glycosylphosphatidylinositol (GPI) moiety [2]. Although there is evidence on its relevant physiological role as a cellular signalling molecule [3–5], once in the presence of infectious PrPSc seeds, PrPC units are remodelled to reproduce the conformation of the misfolded isoform. Newly formed PrPSc molecules are then incorporated into prion amyloid aggregates that tend to accumulate in neurons, astroglia and microglia, eventually leading to synaptic damage and vacuolar neuropathology characteristic of TSEs [6]. The exact mechanism by which PrPSc aggregates lead to neurotoxicity is, nevertheless, poorly understood.
Human TSEs are rare and include familial Creutzfeld-Jacob disease (CJD) caused by polymorphisms in the PRNP gene giving rise to PrP<sup>Sc</sup>, sporadic CJD, as well as the variant CJD triggered by the consumption of bovine PrP<sup>Sc</sup>-contaminated food [1,7]. Attempts to develop small organic compounds targeting either PrP<sup>C</sup>, PrP<sup>Sc</sup>, or the conversion from one conformer to another have been carried out in the past two decades, but even though a few orally available compounds have been shown to significantly enhance the survival of mice infected with mouse PrP<sup>Sc</sup>, the transposition of such effects to human PrP<sup>Sc</sup> has been challenging and led to rather disappointing results so far. The reasons for this include undesired toxicity and metabolic profile or, in some cases, reduced compound blood brain barrier permeability [8]. Yet, the existence of various phenotypic TSE variants caused by several different PrP<sup>Sc</sup> strains is possibly the major factor contributing to these consecutive failures in the search for effective therapeutic approaches. Each PrP<sup>Sc</sup> strain leads to a distinct disease incubation period, and each one is associated with a particular pattern of prion distribution in the brain [1,9,10] and, indeed, it has been shown that one particular molecule may exert distinct effects against different prion strains [11].

Despite so many difficulties, the urgent need for efficacious treatments against prion diseases keeps the scientific community motivated to pursuing the discovery of new anti-prion molecules up until today. In this perspective, we herein provide an overview of the latest reports focusing on the development of molecules with anti-prion activity and potential against TSEs. From sugar-based compounds to aromatic and heteroaromatic compounds, this review presents also the synthesis and mode of action of the most promising molecular entities described in the literature, ultimately aiming to stimulate the development of innovative therapies against prion-mediated diseases.

2. Synthesis and Mode of Action of Glyco-Based and Aromatic Scaffolds with anti-Prion Effects

2.1. Glyco-Based Molecular Entities

Sulfated polysaccharides, as the family of heparan mimetics (HMs) and pentose polysulfate (PPS) are among the most active drugs tested in experimental models of prion disease (Figure 1, structures A–C). Heparan sulfate (HS) was found on the amyloid plaques in TSEs [12] and reported as an essential part of the cellular receptor used for prion uptake and as a crucial factor for cell infection [13]. It is known that sulfation degree and patterns of these polysaccharide structures govern the HS-protein interactions [14] and are related to their anti-prion activity. Other endogenous polysaccharides including heparin, chondroitin sulfate (CS) and dermatan sulfate (DS), have also shown to bind prions [15,16], and to inhibit the neurotoxicity of amyloid fibrils [17].

![Figure 1. (A): Heparan mimetic representative fragment, abbreviated as HMCSX where n: molecular length, S: degree of sulfate substitution, C: carboxyl substitution and X: hydrophobic group substituent; (B): Pentosan polysulfate representative fragment; (C): Heparan sulfate/heparin representative fragment.](image)

Based on these findings, Ouidja and co-workers [18] reported a library of HMs of different molecular sizes, containing various sulfation and carboxylation levels, and substituted, or not, by different hydrophobic cores with a general formula HMCSX. (Figure 1, structure A). Studies on their capacity to inhibit the accumulation of the abnormal protease-resistant prion conformation (PrP<sub>res</sub>) in chronically infected cells (ScGT1-7) and on their PrP<sub>res</sub> binding ability showed that an optimal
size and sulfation degree were needed for optimum activity. The incorporation of hydrophobic moieties increased compound efficacy while the presence of carboxymethyl moieties decreased it. It is important to add that PrPres is a disease-promoting PrPSc-like prion protein generated in vivo, following PrPSc-induced misfolding of recombinant PrPC [19].

Ouidja et al. [18] also observed that increasing polyanions size increases product efficacy. When considering the same degree of sulfate substitution, a maximum effect was found at 250 g/s (Figure 2), and tested them, as well as the important roles in prion formation and infection [22]. GAG involvement in prion disease pathogenesis has been widely reported [15,23–25]. Yagamuchi et al. [26] described, following PrPSc formation in prion-infected cells.

It is important to add that PrP res is a disease-promoting PrP Sc-like prion protein generated in vivo, rich in hydrophobic residues.

On the other hand heparin analogs may be structurally based on saccharide units whose disaccharide precursor GlcNS6S-IdoA2S 1 is presented in Scheme 1. In an attempt to know whether this disaccharide unit has sufficient anti-prion activity in prion-infected cells, recently, Teruya et al. [20] have characterized the binding profile of the representative structure of unprotected GlcNS6S-IdoA2S 1 to PrP. The synthesis of disaccharide 1 has been described by Saito and co-workers [21] using glucurono-6,3-lactone and glucosamine hydrochloride as starting materials to obtain the disaccharide building block by reaction of the sugar acceptor 2 with the conveniently protected 2-azido glycosyl donor 3 catalysed by tert-butyldimethylsilyl trifluoromethanosulfonate (TBDMSOTf). Acceptor 2 and donor 3 were synthesized over 15 steps from D-glucurono-6,3-lactone, and over 9 steps from glucosamine hydrochloride, respectively (Scheme 1).

![Scheme 1](image)

**Scheme 1.** Synthesis of disaccharide 1. **Reagents and conditions:** (a) TBDMSOTf, DCM, MS 4 Å, −20 °C → r.t., 18 h, 77%.

Teruya et al. described a significant binding of unprotected disaccharide GlcNS6S-IdoA2S 1 with recombinant prion protein (PrP) (K_D = 9.7 μM), that was confirmed by competitive inhibition using heparin or pentosane polysulfate by surface plasmon resonance. However, the disaccharide structure did not exhibit anti-prion activity in prion-infected cells.

Glycosaminoglycans (GAGs) are biomolecules of interest for prion-related diseases playing important roles in prion formation and infection [22]. GAG involvement in prion disease pathogenesis and PrPC conversion into PrPSc has been widely reported [15,23–25]. Yagamuchi et al. [26] described, for the first time, polymers of acrylamide decorated, via an amide bond, with regioselectively sulfated 2-acetamido-2-deoxyglycopyranosyloxyphenyl moieties (Figure 2), and tested them, as well as the p-nitrophenyl sulfated glycoside precursors mimicking GAGs monomers, to screen for their ability to inhibit PrPSc formation in prion-infected cells.

Among the sulfated glycopyranosides and the polymeric compounds examined, the 4-sulfated derivative 6, and two glycopolymers, 10 and 11 (Figure 2), inhibited PrPSc formation with 50% effective doses below 20 μg/mL (ED50 = 10, 4 and 9 μg/mL, respectively). A combination of an N-acetyl group
at C-2 and a sulfate group at either C-4 or C-6 on glucopyranoside might be involved in the inhibition of PrP<sub>Sc</sub> formation. Furthermore, PrP<sub>Sc</sub> formation was inhibited by polymeric compound 11 but not by the glucoside 7, suggesting the importance of a polyvalent structure.

**Figure 2.** Compounds tested for the inhibition of PrP<sub>Sc</sub> formation in prion-infected cells: sulfated p-nitrophenyl glycosides, and glycopolymers composed by a polyacrylamide chain decorated with sulfated 2-acetamido-2-deoxy-β-D-glucopyranosyloxyphenyl moieties via amide bond (adapted from [26]).

The synthesis of the p-nitrophenyl glycosides sulfated at 3, 4 or 6 position is depicted in Scheme 2 [27]. Position 6 of pNP-β-D-GlcNAc was selectively sulfated with a sulfur trioxide complex (Me<sub>3</sub>N·SO<sub>3</sub>) affording compound 7 in 52% yield. In order to generate sulfated glucosides at position 3 and 4, tert-butylidiphenylsilyl chloride (TBDPSCI) was chosen to firstly protect position 6. After sulfation with sulfur trioxide complex both 5 and 6 regioisomers were obtained in 63% overall yield and then separated by column chromatography.

**Scheme 2.** Synthesis of compounds 5–7. Reagents and Conditions: (a) Me<sub>3</sub>N·SO<sub>3</sub>, DMF, 40 °C, 3 h, Dowex (Na<sup>+</sup>), 52%; (b) TBDPSCI, py, DMAP, r.t., 24 h, 95%; (c) Me<sub>3</sub>N·SO<sub>3</sub>, DMF, 40 °C, 10 h, Dowex (Na<sup>+</sup>), 63% (5/6 = 1.4/1); (d) TBAF, THF, r.t., 24 h, 98%.
More recently Nishizawa et al. introduced the p-methoxyphenyl aminoglucoside \( 13 \) (Figure 3) as a new type of anti-prion compound, commercially available, and able to inhibit abnormal prion protein formation in prion-infected neuroblastoma cells in a prion strain-independent manner, when the cells were treated for more than 1 day. The 50% inhibition dose for Pr\( \text{Pr}^{\text{res}} \) formation is 5.36 and 3.33 (\( \mu \text{g/mL} \)) in prion-infected ScN2a cells and N167 cells, respectively [28].

![Figure 3. Molecular structure of the aminoglucoside 13.](image)

In 2009, Charvériat and co-workers presented two different families of prion replication inhibitors [29]. One of them is based on 3-aminosteroid structures and the other on erythromycin A derivatives bearing saccharide residues and an oxime functionality (Figure 4). The eight compounds inhibited Pr\( \text{Pr}^{\text{res}} \) accumulation in two cell cultures (SN56 and GT1 cell lines) infected by the Chandler scrapie strain, and by mouse-adapted scrapie strain 22 L, respectively.

![Figure 4. Chemical structure of inhibitors belonging to distinct chemical families: 3-aminosteroids 14–20 and erythromycin A derivative 21 [29].](image)

Dose–response curves, with the identification of IC\( _{50} \) by densitometry, confirmed the concentration-dependent Pr\( \text{Pr}^{\text{res}} \) inhibitory activity of compounds \( 14–21 \) on Chandler-infected SN56 cells [29]. Compound \( 17 \) and erythromycin derivative \( 21 \) showed the lowest IC\( _{50} \) (0.4 and 0.3 \( \mu \text{M} \)), respectively. Interestingly, they found that compound \( 21 \) interacted directly with Pr\( \text{Pr}^{\text{res}} \) stability observing a change in the shape of the \( T_m \) curve in the fluorescence-based thermal shift assay. These results indicated that the interaction between Pr\( \text{Pr}^{\text{C}} \) and compound \( 21 \) leads to a peculiar conformation of Pr\( \text{Pr}^{\text{C}} \) that could induce alteration of its amyloidogenic properties, and consequently lead to the inhibition of conversion to Pr\( \text{Pr}^{\text{res}} \) [29].

More recently other natural glycosides with anti-prion activity have been described in literature, namely bacoside-A (22, Figure 5). This natural triterpene glycoside bearing a trisaccharide moiety has been recently found to exhibit anti-amyloid properties [30]. It is an active principle of the medicinal plant *Bacopa monniera*, whose components include amphiphilic compounds structurally based on sterol glycosides, and is used in traditional Indian medicine to treat various nervous disorders, and to promote memory enhancement [31,32].
Recent studies have suggested that bacoside-A might exhibit therapeutic effects against amyloid diseases, such as Alzheimer’s disease (AD) [33,34]. More recently Malishev et al. have investigated the interactions between bacoside-A and the 21-residue amyloidogenic determinant of the prion protein PrP106–126 by spectroscopy and microscopy experiments [35,36]. They evaluated the consequence of bacoside-A/PrP(106–126) interactions upon membrane bilayers (Figure 6). The experimental data revealed acceleration of PrP106–126 fibril formation in the presence of lipid bilayers. Importantly, they found that the enhanced fibrillation of the peptide, induced by bacoside-A, went in parallel with significant reduced membrane interactions and bilayer disruption, demonstrating a direct relationship between externally-induced accelerated fibrillation and inhibition of membrane interactions (Figure 6), indicating bacoside-A (22) as a potential therapeutic agent for TSE and amyloid diseases.

Figure 5. Molecular structure of bacoside A (22).

Figure 6. Schematic model for the activity of Bacoside-A; up: PrP106–126 alone forms abundant pre-fibril aggregates which interact with, and disrupt membrane bilayers; down: when PrP106–126 is pre-incubated with bacoside-A, fibrillation is accelerated resulting in lower abundance of membrane-active species (adapted from [30]).

2.2. Aromatic Scaffolds with Nitrogen Containing Six-Membered Ring Heterocycles

2.2.1. Pyridine Dicarbonitrile Derivatives

Pyridine dicarbonitrile derivatives were firstly published in 2000 as a new class of rationally designed leads with activity against PrPSc replication in PrPSc-infected mouse neuroblastoma cells (ScN2a) [37]. In particular compound 23 (Figure 7) was reported to inhibit PrPSc formation in ScN2a...
cells in a dose-dependent manner, with an IC₅₀ of 18.0 µM in ScN2a cells, and low levels of toxicity [37].

It has been proposed that it may bind to a chaperone required itself to bind to PrP for its conversion into PrPSc, consequently inhibiting this conversion [37,38].

![Image of three pyridine dicarbonitrile derivatives](image_url)

**Figure 7.** Three pyridine dicarbonitrile derivatives with in vitro anti-prion activity.

In 2006, Reddy and co-workers focused on improving the activity of compound 23 through the development and evaluation of a small library of pyridine dicarbonitrile derivatives [39]. After performing docking studies with a proposed binding site model for compound selection, 45 structurally diverse compounds were synthesized by a one-pot three-component coupling reaction, herein illustrated in Scheme 3 for compound 24, the most promising compound in this study.

![Scheme 3](image_url)

**Scheme 3.** (A) One-pot synthesis of pyridine dicarbonitrile derivative 24; (B) Proposed reaction mechanism. *Reagents and conditions:* (a) EtOH, reflux piperidine, 3 h, then air-exposed overnight (19%) [39].
Reaction optimization involved the study of the optimum aldehyde/thiol/malononitrile ratio, which was found to be 1:1:2, as well as the best solvent, the best catalytic base and the ideal reaction duration. Mechanistically, as depicted in Scheme 3B, during the first 3 h of reaction in ethanol under reflux, the initial base-catalyzed attack of a malononitrile anion to the aldehyde followed by elimination of water gives an adduct, which subsequently suffers the addition of benzylthiol to give an iminonitrile intermediate. Then, a second molecule of malononitrile is added to promote a cyclization reaction that affords the central dihydropyridine core; from this point, air-derived O₂ is required for the aromatization into the final pyridine ring by chemical oxidation. Even though the yields obtained for this one-pot reaction were up to 52%, compound 24 was achieved in only 19% yield.

Compound 24 successfully interacted with all three PrP<sup>C</sup> forms used in this study: human PrP<sup>C</sup> (huPrP<sup>C</sup>; binding affinity at 40 μM, %RU<sub>max</sub> = 55.5), truncated human PrP<sup>C</sup> (t-huPrP<sup>C</sup>; %RU<sub>max</sub> = 28.8) and murine PrP<sup>C</sup> (moPrP<sup>C</sup>; %RU<sub>max</sub> = 52.6). The analog possessing a 3-chlorophenylthiol at C-4 had a significantly higher affinity towards PrP<sup>C</sup> (huPrP<sup>C</sup> %RU<sub>max</sub> = 123.6; t-huPrP<sup>C</sup> %RU<sub>max</sub> = 91.8; moPrP<sup>C</sup> %RU<sub>max</sub> = 72.2); however, it was found to be toxic. Nevertheless, compound 24 was more potent than the lead compound 23 in PrP<sup>Sc</sup>-infected mouse brain mesodermal (SMB) cells, and presented a high association/dissociation response ratio, which is ideal when looking for a potential new drug candidate.

Overall, most pyridine dicarbonitrile derivatives possessing the benzylsulfanyl moiety displayed some level of affinity towards PrP<sup>C</sup>. Through the evaluation of all synthesized analogs, Reddy and co-workers concluded that the substituent at C-4 of the central pyridine core of pyridine dicarbonitrides does not influence the binding to PrP<sup>C</sup> as much as the substituent at C-6. In fact, this had been predicted in prior docking poses where the thioether moiety was placed deep inside the hypothetical pocket, whereas the substituent at C-4 was generally placed towards the opening of the cavity.

Compound 25 (Figure 7) was published in a subsequent report by May et al. presenting a thorough structure-activity relationship study on pyridine dicarbonitrile derivatives bearing amine substituents at C-6 and furan or halobenzene substituents at C-4 [38]. The synthetic approach towards designed derivatives was quite distinct from the previous one, and is herein illustrated for compound 25 in Scheme 4.

Scheme 4. Synthesis of pyridine dicarbonitrile derivative 25. Reagents and conditions: (a) β-alanine, EtOH, r.t., 72 h; (b) 2-cyanoacetamide, piperidine, EtOH, reflux, 8 h; (c) 10% KOH, DMF, r.t., 1 min; (d) (diethylamino) alkyl halide, r.t., 5 h [38].

Firstly, a β-alanine-catalysed Knoevenagel condensation afforded the arylidene malononitrile 26, which was subsequently converted into intermediate 27 by reacting with a molecule of 2-cyanoacetamide, in the presence of piperidine, under reflux. Then, after treatment with 10% KOH in dimethylformamide (DMF), the haloamine was added to form the desired sulfanyl moiety, thus affording the final compound 25. Although the yields for each reaction step are not specified, final compounds were obtained in 10–70% overall yield.

Most of these pyridine dicarbonitrile derivatives were not toxic up to 25 μM and exhibited EC<sub>50</sub> values below 15 μM in ScNa2 cells. Moreover, they were generally more active than compound 24 (Figure 7) which, in this study, presented no activity up to 25 μM. Compounds with tertiary
alkyl amines at C-6 were generally more active than those bearing primary amine moieties, whereas meta-substituted halobenzenes at C-4 were found to be preferable to furan-substituted compounds or to the corresponding para-substituted analogs. Among all synthesized pyridine dicarbonitrile derivatives, compound 25 was found to be the most promising, with an EC_{50} value of 5.5 μM in ScNa2 cells. Moreover, it presented high water solubility and a good permeability rate in a standard parallel artificial membrane permeability assay (PAMPA) (logPe = −6.0 ± 0.1), indicating a good pharmacokinetic profile. However, no in vivo data are available up to this point.

### 2.2.2. Piperazine Derivatives

Based on the previously described neuroprotective activity and ability of the 2,5-diketopiperazine scaffold to modulate protein-protein interactions [40], Bolognesi and co-workers have generated a small library of compounds bearing different aromatic and heteroaromatic substituents in positions 3 and 6 of a central 3,6-dimethylpiperazine-2,5-dione core [41]. The authors envisioned that a planar, symmetrical molecule with aromatic end groups would be ideal to optimize the desired anti-aggregating properties and, in 2010, presented compound 28 (Figure 8) as a new anti-prion lead for further development.

![Scheme 5](image)

**Figure 8.** Four piperazine derivatives with anti-prion activity.

Compound 28 inhibited PrP^{Sc} accumulation in PrP^{Sc}-mouse hypothalamus (ScGT1) cells with an EC_{50} value of 4.1 μM, while maintaining approximately 75% cell viability at this concentration. Mechanistic studies focusing on aggregation kinetics revealed that this molecule acts by interacting directly with recombinant prion protein (recPrP) to inhibit its conversion into the pathological PrP^{Sc}-like conformer and, interestingly, while the corresponding 3- and 4-pyridyl analogs were roughly ten times less active against PrP^{Sc} accumulation, compounds presenting benzene, furan, thiophene or quinolone moieties did not exhibit any activity.

The straightforward synthesis of compound 28 involved only one reaction step (Scheme 5). In this reaction, two equivalents of picolinaldehyde were mixed with one equivalent of the commercially available 1,4-diacetylpiperazine-2,5-dione dissolved in DMF, in the presence of triethylamine. A double aldol-condensation at room temperature gave compound 28 in 64% yield, affording exclusively the desired (Z,Z)-isomer.
The isopropylpiperazine derivative 29 (Figure 8) was later published by Li et al. as the most promising compound of a small library of optimized arylamides with anti-prion activity, which were developed based on a moderately active piperazine arylamide derivative [42]. Here, the piperazine ring is placed in one of the extremities of the molecule, thus contrasting with Bolognesi’s rationale in which the diketopiperazine was the core moiety acting as the linker between two identical pyridine groups. The synthesis of this amide bearing the N-alkyl piperazine ring is very straightforward and involves only one reaction step, namely the standard coupling between a carboxylic acid and an arylamine carried out in the presence of the uranium salt O-(7-azabenzo triazol-1-yl)-N,N,N′,N′′-tetramethyluronium hexafluorophosphate (HATU) as an acid activating reagent and N,N-diisopropylethylamine (DIPEA), in anhydrous DMF, at room temperature gave compound 29 in 66% yield (Scheme 6).

![Scheme 6. Synthesis of piperazine derivative 29. Reagents and conditions: (a) HATU, DIPEA, anhydrous DMF, r.t., 12 h (66%) [42].](image)

The initially conducted structure-activity relationship studies revealed that the biphenyl group linked to the carbonyl of the amide functionality led to more potent compounds than those bearing only one aromatic ring. The piperazine moiety was regarded as an important feature for the maintenance of compound solubility and thus was kept throughout this series of derivatives. Furthermore, 4-biphenyl analogs were preferable to their 3-biphenyl congeners, and compounds containing large N-alkyl moieties in the piperazine ring were more potent than those containing smaller N-alkyl groups. With an EC₅₀ of only 22 nM against PrPSc accumulation in in PrPSc-infected mouse neuroblastoma (ScN2a-cl3) cells, compound 29 stood out among this series of derivatives and was found to be 100-fold more potent than the original lead compound. It displayed acceptable metabolic stability in mouse liver microsomes (t₁/₂ ≥ 60 min) and good brain exposure upon oral administration in mice, thus coming across as a promising lead for further development. However, the in vivo evaluation of this compound to confirm whether the nanomolar range potency observed in vitro is indeed transposable to TSE in vivo models is still lacking.

Compound 30 (Figure 8), on the other hand, was published by Leidel and colleagues as a piperazine derivative with activity in PrPSc-infected mice [43]. This molecule was identified after high-throughput screening of 10,000 compounds in SMB cells, where four piperazine derivatives emerged as a new class of PrPres conversion/amplification inhibitors. Noteworthy, a significant decrease in PrPres formation was not achieved by compound 30 in a cell-free assay but it was shown to be very potent when tested in mouse neuroblastoma (N2a) cells, displaying an IC₅₀ of 0.4 μM. Moreover, proteasome activity was not affected in its presence, indicating that it does not influence cellular trafficking or processing of PrP.C. Taken together, these results suggest an indirect inhibitory mechanism of action for compound 30, in which it probably targets misfolding or aggregation co-factors present in the cell-based assay, but absent when using only the purified prion protein. Alternatively, it may act by inducing cellular PrPres clearance. Anyhow, this piperazine derivative successfully prolonged the incubation time in PrPSc-infected C57BL/6 mice from 144 to 157 days when administered via an intraperitoneal injection, while a direct analog with a lower IC₅₀ and bearing a 3-chlorophenyl moiety, instead of the 2-pyridyl group, did not.

This study succeeded to illustrate in vivo effects of piperazine derivatives against TSEs, and provided important hints on the potential anti-prion mechanism of action of these types of compounds. It is relevant to note that another piperazine derivative, compound 31 (Figure 8), was found to enhance the clearance of PrPSc in ScN2a cells, with an IC₅₀ of 0.1 μM [44]. Interestingly, this compound is a
well-known tyrosine kinase inhibitor indicated for the treatment of chronic myeloid leukaemia, and was shown to strongly activating the lysosomal degradation of PrPSc, without affecting the biosynthesis or localization of PrPSc. These results are consistent and clarify the findings regarding the mechanism of action of the piperazine derivative 30.

Several possible synthetic routes for compound 31, also known as 5TI571 or Imatinib, were reported since the early 1990s [45–47]. Scheme 7 shows one of the most recently published methodologies involving eight reaction steps with an excellent overall yield of 37% [48]. In this synthesis, the commercially available acetylpyridine was firstly enaminated and subsequently converted into the pyrimidyl amine 33 in the presence of guanidine nitrate (Scheme 7A). In parallel, after bromination of p-nitrotoluene, the so formed intermediate 34 was coupled to 33 using CuI as catalyst in a ligand assisted Ullmann-type N-arylation reaction. Subsequent reduction of the nitro group gave the arylamine 35 in 69% over two reaction steps (Scheme 7B). Finally, after full chlorination of 4-(hydroxymethyl)benzoic acid by SOCl2, compound 35 was coupled to the so formed acid chloride in the presence of triethylamine, affording amide 36 in 93% yield (Scheme 7C). Further N-alkylation of 1-methylpiperazine under reflux gave the final product, compound 31, in 91% yield.

Even though piperazine derivatives did not show evidence of disease-modifying effects against TSEs so far, the above studies highlight some chemical features that may be important in future structure optimization approaches, namely the presence of an amide bond together with at least two aromatic rings and, conceivably, pyridine and pyrimidine moieties. However, additional in vivo studies are required for a better understanding of these requirements.

2.2.3. Acridine Derivatives

Quinacrine is one of the most known compounds presenting anti-prion properties and many analogs have been synthesized and studied over the last two decades. In 2001 Prusiner et al. reported that tricyclic derivatives of acridine and phenothiazine exhibited half-maximal inhibition of PrPSc formation at effective concentrations in scrapie-infected neuroblastoma (ScN2a) cells [49]. The EC50 values for chlorpromazine was 3 μM, whereas quinacrine was 10 times more potent (Figure 9), suggesting that they may be candidates for the treatment of CJD and prion diseases.

Scheme 7. Synthesis of piperazine derivative 31 by the reaction steps presented in (A–C). Reagents and conditions: (a) xylene, (CH3)2NCH(OCH3)2, reflux, 20 h (93%); (b) guanidine nitrate, NaOH, n-butanol, reflux, 12 h (86%); (c) Br2, Fe, 80 °C, 1.5 h (90%); (d) 33, DMEDA, CuI, K2CO3, dioxane, 100 °C, 20 h (82%); (e) N2H4·H2O/FeCl3, MeOH, reflux, 6–8 h (84%); (f) SOCl2, DCM, reflux, 5 h (87%); (g) 35, THF, TEA, 0 °C, 3 h (93%); (h) 1-methylpiperazine, reflux, 3 h (91%) [48].
Copper-catalyzed Ullmann–Jourdan reaction [51] is applied to substituted 2-chlorobenzoic compound by reaction with phenol which acts as a catalyst at high temperature (Scheme 10). The corresponding 9-chloroacridines by reaction with POCl₃ (Scheme 9).

For the synthesis of quinacrine and their analogs, the corresponding anthranilic acids are used as starting materials by applying Ullmann-Jourdan reaction or Buchwald Hartwig amination [50] (Scheme 8). Copper-catalyzed Ullmann–Jourdan reaction [51] is applied to substituted 2-chlorobenzoic acids and the corresponding aniline derivatives yielding the anthranilic acids in moderate yields. However, the scope of the reaction is limited to electron-rich anilines and electron-deficient benzoic acids, leading to yield decrease. Using a palladium-catalyzed Buchwald–Hartwig amination seems to be a more robust method for the synthesis of anthranilic acids. Reaction of methyl 2-iodobenzoates and anilines using Pd(ac)₂, bis-[2-diphenylphosphino)phenyl]ether (DPEPhos) and Cs₂CO₃ as base, and subsequent hydrolysis gave the corresponding acids [50] (Scheme 8).

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In order to obtain the acridine scaffold, the resulting anthranilic acids are easily transformed into the corresponding 9-chloroacridines by reaction with POCl₃ (Scheme 9).

Quinacrine derivatives are obtained from 9-chloroacridines and the corresponding amino spacer compound by reaction with phenol which acts as a catalyst at high temperature (Scheme 10).
In 2003, Vogtherr et al. identified the binding site of quinacrine at the C-terminal helix of human prion protein (hPrP) using NMR spectroscopy [52] Tyr225, Tyr226 and Gln227 residues of hPrP helix α3 turned to be the responsible of the interaction with quinacrine. $K_D$ value was of 4.6 μM, 4 orders of magnitude weaker than the $EC_{50}$ value found for PrP$^{Sc}$ inhibition in the cell culture assay [49], probably due to a specific enrichment of this drug in cell organelles.

In spite of the inhibition effectiveness of quinacrine, the lack of efficacy was attributed to inadequate accumulation of quinacrine in the brain, due to its removal by P-glycoprotein (Pgp) which is an efflux protein found in the blood brain barrier [53]. Indeed, when quinacrine was administered to prion-infected mice deficient in the genes encoding Pgp, brain levels of quinacrine were significantly increased [53,54]. Since quinacrine failed to extend the survival of prion-infected animals, authors speculated that continuous quinacrine treatment promoted the formation of drug resistant prions. This phenomenon could have contributed to the lack of in vivo efficacy of quinacrine. In spite of its limitations, quinacrine structural modification has yielded analogs with more potent in vitro anti-prion activity. Thus, other quinacrine derivatives with different substituents R$_1$ and R$_2$ and different spacers have been synthesized and their biological activity tested in the last two decades. Authors as Nguyen et al. showed that nature of side chain influenced compound cell-based potency, PAMPA permeability and binding affinity to hPrP$_{121-231}$ when compared to quinacrine [55]. Several promising analogs were found with a more favorable anti-prion profile than quinacrine, in terms of potency and activity across different prion-infected murine cell models. Analogs 37–39 depicted in Figure 10 have shown sub-micromolar activities on several prion-infected mouse neuroblastoma cell lines, including the more resistant F3 where host cells were stably infected with a human prion strain. In particular, Nguyen Thi et al. found that piperazin analog 37 had $EC_{50}$ values ranging from 0.1 to 0.7 μM on all cell models, being able to clear PrP$^{Sc}$ at non-toxic concentrations of 1.2–2.5 μM, and presenting more activity than quinacrine in terms of $EC_{50}$ values [56]. Behavior of analog 7 appeared to be unusual, showing $EC_{50}$ values of 0.13 and 0.19 μM on ScN2a and F3 cell models respectively (Figure 10 and Table 1).

**Scheme 10.** Synthesis of quinacrine derivatives. *Reagents and conditions:* (a) phenol, 100 °C.

![Scheme 10](image)

Figure 10. Quinacrine and analogs 37–39 with anti-prion activity against PrP$^{Sc}$ in different cell lines.
In vitro anti-prion activities of quinacrine and compounds 37–39 on murine neuroblastoma cells infected mouse adapted scrapie strains (ScN2a and N167) and a human prion strain (F3), binding responses of test compounds to hPrP$_{121-231}$ by SPR, permeability by PAMPA-BBB assay, log D and efflux ratio across wild type MDCK-MDR1 cell monolayers.

| Compound | EC$_{50}$ (µM) ScN2a | EC$_{50}$ (µM) N167 | EC$_{50}$ (µM) F3 | Binding to hPrP$_{121-231}$ (%RU$_{\text{max}}$) | Permeability PAMPA-BBB Assay (logD$_{7.4}$) | Efflux Ratio * MDCK-MDR1 |
|----------|----------------------|---------------------|------------------|---------------------------------|---------------------------------|---------------------|
| Quinacrine | 0.23 | 0.59 | 1.88 | 87.3 | 1.89 | 5 |
| 37 | 0.10 | 0.42 | 0.68 | 120.3 | 3.36 | 2.4 |
| 38 | 0.42 | 0.49 | 0.80 | 67.5 | 3.75 | - |
| 39 | 0.13 | 0.23 | 0.19 | 169.5 | 3.41 | - |

* Ratio of apparent permeability from the basolateral (B) to apical (A) compartment, to apparent permeability from apical (A) to basolateral (B) compartments. Efflux ratio $\frac{\text{Papp}(B/A)}{\text{Papp}(A/B)}$.

In general, potent and broad ranging anti-prion activities were found in the presence of side chains with aromatic residues substituted with basic functionalities. These structures also have shown a decreased effective permeability in PAMPA-BBB by increasing partition coefficient logD$_{7.4}$, but kept within the permeability range of CNS compounds. Interestingly, compound 37 crossed the permeability P-glycoprotein assay (MDCK-MDR1) barrier with an efflux ratio that is half that of quinacrine (Table 1), indicating that it may be a weaker Pgp substrate.

Other quinacrine analogs with non-aromatic and basic side chains exhibited strong binding affinities for hPrP$_{123-231}$ and good PAMPA permeabilities. Unfortunately, anti-prion activities were limited to ScN2a and did not extend to other cell models [55].

The synthesis of analog 37 was carried out by a sequence of three steps (Scheme 11). Buchwald-Hartwig amination of 1-iodo-4-nitrobenzene with 1-methylpiperazine in the presence of a palladium acetate/2,2′-binaphthalene (BINAP) catalyst generated compound 41 in 95% yield. Catalytic hydrogenation of the nitro group gave the corresponding amine to react with 6,9-dichloro-2-methoxyacridine in refluxing ethanol affording anti-prion compound 37 in 65% yield [56].

![Scheme 11. Synthesis of quinacrine analog 37. Reagents and conditions: (a) amine, Pd(OAc)$_2$, BINAP, Cs$_2$CO$_3$, anhydrous toluene, 120 °C, 95%; (b) i) H$_2$, Pd/C; ii) 6,9-dichloro-2-methoxyacridine, ethanol, reflux, 24 h, 65% over the two steps.](image)

In 2003, Cohen et al. disclosed bis(acridine) analogs as an alternative to the acridine-based compounds [57]. They postulated that covalent dimers of quinacrine could be more potent inhibitors of prion replication due to an increased local concentration of the active moiety. Thus, dimeric acridine compounds were synthesized exhibiting a ten-fold higher activity than the respective monomeric compounds. The best results were obtained with the three bis(acridine) analogs 42–44 (Figure 11) that showed half-maximal inhibition of PrP$_{Sc}$ formation in ScN2a cells at 40, 25, and 30 nM, respectively, and were not cytotoxic to uninfected neuroblastoma cells at concentrations of 500 nM.
Structure–activity analysis revealed that spacer length and structure are determinant for inhibition of prion replication in cultured scrapie cells [57]. Compound 42 showed improved activity which could result from additional hydrogen-bonding interactions between the heteroatoms of the linker and the target receptor. Synthesis of bis(acridines) 42–44 [58,59] was carried out by reacting polyamines with 3 equiv. of 6,9-dichloro-2-methoxyacridine in DMF at reflux, in the presence of potassium carbonate as an inorganic base (Scheme 12A). In the case of compound 44, a side chain central secondary amino group reacted with a carboxylic acid in the presence of bromotripyrrolidinophosphonium hexafluorophosphate (Py-BroP) to give the amide functionality [59] (Scheme 12B).

**Scheme 12.** (A). General synthesis of bis(acridines). Reagents and conditions: (a) K₂CO₃, DMF; (B). Preparation of bis(acridines) bearing a central amide group. Reagents and conditions: (a) RCOOH, PyBrop, DIEA, DMF.

Bongarzone et al. [60] evaluated a small library of bis(acridine) analogs, bearing a 2,5-diamino-1,4-benzoquinone moiety as spacer component, against prion infection. The most active one acting on prion replication in the submicromolecular range was 45 containing the 6-chloro-1,2,3,4-tetrahydroacridine moiety (Figure 12), and showing an EC₅₀ of 0.17 μM on ScGT1 cells, which was lower than that displayed by reference compound 42 (EC₅₀ = 0.32 μM on ScGT1).
Compound 45 was synthesized starting from 6,9-dichloro-1,2,3,4-tetrahydroacridine by reaction with sodium iodide, followed by addition of a large excess of diamine at 120 °C for 5 h to generate the N-substituted diamine 47 in 50% yield (Scheme 13), whose reaction with 2,5-dimethoxy-1,4-benzoquinone at 60 °C for 5 h gave 45 in 87% yield [60].

More recently Galdeano et al. [61] developed a family of huprine-tacrine heterodimers demonstrating a potent inhibition of human acetylcholinesterase (AChE) activity, in vitro neutralization of the pathological chaperoning effect of AChE toward \( \beta \) -amyloid peptide (A\( \beta \)) and prion protein aggregation. These heterodimers were able to cross the blood brain barrier in ex vivo experiments with OF1 mice. The general structure of these huprine-tacrine heterodimers is presented in Figure 13.
Enantiopure (−)-48b and (−)-49b showed a 5-6-fold more potent inhibitory effect (IC₅₀ = 1.33 and 2.04 nM respectively) towards hAChE than their dextrorotatory homologs. All the huprine–tacrine heterodimers, at 100 µM, turned out to be potent inhibitors of the AChE-induced PrP₁₀₆−₁₂₆ aggregation with percentages of inhibition higher than 80% but no significant differences between compounds bearing an unsubstituted or chloro-substituted tacrine unit or between enantiomers were found.

Thus, alkylation of tacrine [62] with 1,9-dibromoheptane 50 in the presence of KOH in DMSO [63] gave bromoalkyltacrine 51b. Alkylation of racemic huprine Y with 51b under similar reaction conditions afforded the heterodimer (−)-48b in 13% isolated yield [64] (Scheme 14).

![Scheme 14](image)

Scheme 14. Synthesis of compound 48b. Reagents and conditions: KOH, DMSO, 4 Å MS. (a) 2 h; (b) 3 days, 13% isolated yield over the two steps.

Quinacrine has been reported to be an important prion inhibitor [65] However this compound did not increase the survival in a murine model of prion disease [49] and in high doses led to liver damage without therapeutic efficacy in humans affected with CJD.

Interestingly, a shared mode of action between acridines and aminoquinolines, whose core structure is embodied in acridines, has been discussed [66]. Aminoquinolines with anti-prion activity were also reported by Macedo and co-workers [67,68]. These aminoquinoline derivatives have been developed ten years earlier as potential antimalarial drugs. The hypothesis that the aminoquinoline scaffold could also act against PrPSc formation was based on a previous publication reporting the PrPres-inhibitory activity of the aminoquinoline derivative mefloquine (52, Figure 14), despite its lack of efficacy in Tg7 mice challenged with 263 K brain homogenates [69]. By studying these compounds, the authors aimed at gaining new insights on the minimum structure required for anti-prion efficacy, while trying to improve the therapeutic potential of both quinacrine and mefloquine. Compounds 53 and 54 (Figure 14) emerged as the most promising molecular entities with the aminoquinoline scaffold, with IC₅₀ values of 0.2 µM and 0.1 µM, respectively, for the inhibition of the Syrian hamster prion protein ShaPrP₁₀₉−₁₄₉ aggregation.
By comparing the activity of all analogs studied by Macedo et al., it becomes clear that the primary amine present in both compounds 53 and 54 is an important feature for activity, particularly in the case of the latter [67]. In fact, while N-methylation of 53 led to an increase in ShaPrP\textsubscript{109-149} aggregation from 8.4% to 38.6% at 1 \( \mu \text{M} \), N-alkylation of compound 54 drastically increased the aggregation of ShaPrP\textsubscript{109-149} to 82%. Moreover, the replacement of the primary amine by a hydroxy group also led to a dramatic decrease in activity, thus excluding the role of the primary amine as a hydrogen bond donor or acceptor in the affinity of these types of compounds towards the target protein.

These compounds definitively need to be further investigated to be considered as anti-prion leads for further development. Indeed, as evidenced by the studies presented and discussed in previous sections, the best inhibitors of PrP aggregation in cell-free assays are not necessarily the best compounds in cell-based assays, demonstrating that in vitro activity does not assure significant in vivo effects.

### 2.3. Aromatic Scaffolds Bearing Five-Membered Ring Heterocycles as Core Structure

#### 2.3.1. Diphenylpyrazole and Analogs

In addition to piperazine derivatives (30, Figure 8), Leidel and co-workers identified diphenylpyrazoles as another class of promising compounds with anti-prion activity in vivo [70]. In a high throughput screening study against SMB and ScN2a cells conducted in 2011, compound 55 (Figure 15) stood out, exhibiting IC\textsubscript{50} values of 0.6 \( \mu \text{M} \) and 1.2 \( \mu \text{M} \), respectively. When compared to similar compounds also evaluated in this report, the methyl group is recognized as a key motif for the enhanced activity in SMB cells, but not in ScN2a; conversely, when the fluorine atom is replaced by a bromine, the activity drops in both cell lines. Compound 55 did not influence PrP\textsuperscript{c} expression or proteasome activity in ScN2a cells, and did not display any effect in a cell-free assay. The exact mechanism of action remains, for now, undisclosed. Yet, this diphenylpyrazole successfully prolonged the incubation time by 42 days in Tga20 mice challenged with the RML prion strain upon oral treatment, and increased survival time by 20 days in PrP\textsuperscript{Sc} infected C57BL/6 mice in a prophylactic approach. The \( p \)-fluoro analog 56 (Figure 15) was later tested on SMB cells and presented an IC\textsubscript{50} of only 0.1 \( \mu \text{M} \), but no in vivo studies have been published so far [43].

![Figure 14. Aminoquinoline derivatives 52–54 with anti-prion activity.](image1)

![Figure 15. Three diphenylpyrazole derivatives (55–57).](image2)

Just two years later, compound 57 was identified by Wagner et al. another diphenylpyrazole with anti-prion activity [71]. After screening 20,000 structurally diverse compounds, the
benzylidene-benzohydrazide unit came across as a potential lead chemical scaffold and, once optimized into the diphenylpyrazole core, a library of 150 diphenylpyrazole derivatives was prepared. The synthetic methodology is herein exemplified for compound 57: briefly, after condensing the adequate ester and acetophenone starting materials in the presence of sodium hydride to afford de diketone intermediate, the pyrazole core is formed following treatment with hydrazine in ethanol, under reflux (Scheme 15). Compound 57, also known as Anle138b, was obtained by this approach in 77% overall yield [71].

Scheme 15. Synthesis of Anle138b (57). 
Reagents and conditions: (a) NaH, DMSO, THF, 10 °C, then r.t., 15 h (87%) (b) H₂N-NH₂-H₂O, EtOH, reflux, 3 h (89%) [71].

In this study published in 2013, Anle138b (57) was reported to be an amyloid oligomer modulator able to inhibit PrPSc formation in a cell-free protein misfolding cyclic amplification (PMCA) in vitro assay in 84%. Moreover, it was found to block the accumulation of PrPSc and neuronal cell death in mice infected with different PrPSc strains (EC₅₀ = 7.3 μM for the murine RML prion strain or 7.1 μM for the vCJD human prion strain), while PrPSc expression was not affected. Importantly, neuronal cell death was found to correlate with the rate of PrPSc amplification and not with the absolute levels of PrPSc. Compound 57 permeates BBB, is orally available and, interestingly, it also inhibits the aggregation of α-synuclein (α-syn), an amyloid protein involved in the pathophysiology of Parkinson’s disease (PD). Indeed, amyloid proteins such as PrPSc, α-syn and Alzheimer’s amyloid β 1–42 (Aβ₁–₄₂) share common structural features underpinning their similar aggregation-prone behavior [72]. This molecule was thus proposed to act transversally against amyloids, being potentially useful against different protein misfolding diseases [71]. Since then, Anle138b (57) has been and continues to be studied due to such promising results against amyloid protein aggregation. In agreement with Wagner’s results, it was found to increase survival in a mouse model of PD by 66 days [73]. Moreover, it showed affinity towards aggregated tau protein, which is implicated in the pathology of both PD and AD, while inhibiting tau aggregation in vitro and in vivo [74]. It prevented synapse and neuronal loss in tau transgenic PS19 mice, effectively increasing survival of these animals [74]. Hence, Anle138b seems to be not only a promising new lead against TSEs, but also against other diseases caused by amyloid proteins. Villa et al. studied the neuroinflammatory role of ciclooxigenase (COX) activity, and its potential targeting for anti-prion therapies by comparing ketoprofen and celecoxib (Figure 16), preferential inhibitors of COX1 and COX2, respectively, on PrP90-231-induced microglial activation. Celecoxib, but not ketoprofen significantly reverted the growth arrest as well as NO and prostaglandine PGE2 secretion induced by PrP90-231, indicating that PrP90-231 pro-inflammatory response in microglia is mainly dependent on COX2 activation [75].
Synthesis of celecoxib is based on Claisen condensation of acetophenone with ethyl trifluoroacetate providing the adduct 59 in good yield. Reaction with (4-sulfamoylphenyl)hydrazine hydrochloride gave exclusively the desired regioisomer celecoxib in 46% yield \[76\] (Scheme 16).

\[
\text{Reagents and conditions: (a) 25% NaOMe/MeOH, MTBE, ethyl trifluoroacetate, 94%, (b) (4-sulfamoylphenyl)hydrazine hydrochloride, EtOH, reflux 46%}.
\]

2.3.2. Thiazolamines and Oxazolamines

Thiazoles and oxazoles are found in the structure of a wide variety of biologically active molecules. In particular thiazol-5-amines are present in antibiotics \[77\] and photosensitisers \[78\]. Heal and co-workers \[79\] reported the first example of anti-prion activity in compounds of this type. Synthetic 2,4-diphenylthiazol-5-amine and 2,4-diphenyloxazol-5-amine derivatives 60–62 were found to bind hPrP\(^C\) and showed potent inhibition of PrP\(^{Sc}\) formation in infected SMB cells with EC\(50\) in the range 1.5–20 \(\mu\)M (Figure 17).

\[
\text{Reagents and conditions: (a) PhCOCl, py, NEM; (b) Lawesson's reagent; (c) TFAA, DCM.}
\]

Preparation of oxazole derivative 60 was carried out starting from 2-phenylglycinonitrile hydrochloride. Reaction with benzoyl chloride afforded the intermediate 63, whose treatment with triphosgene and tert-BuOH permitted to obtain the target molecule in 22% overall yield (Scheme 17).
For the synthesis of thiazole 61, D(-)-phenylglycinamide 64 was reacted with benzoyl chloride in the presence of N-ethylmorpholine (NEM) to give N-acylglycinamide 65. Treatment with Lawesson’s reagent to provide bis(thioamide) intermediate 45, and then with trifluoroacetic anhydride (TFAA) gave 61 via a two-step one-pot procedure in 65% yield [80] (Scheme 18).

Thiazol-2-amines are another class of small molecules with anti-prion activity in prion-infected neuroblastoma cell lines. In Figure 18 the structure of some of the most potent anti-prion thiazol-2-amines is illustrated, and also oxazoles, namely compound 68, used by some authors for comparison purposes because it was reported to produce a ≥50% extension of survival in treated animals. Prusiner and co-workers were pioneer identifying thiazol-2-amines as active compounds in ScN2a cells. The inhibition of PrPSc formation seemed to be the mode of action they postulated for these molecular entities [81]. N-(Methylpyridyl)thiazol-2-amines seemed to confer a more potent effect than analogs with unsubstituted pyridyl groups as suggested by SAR studies. In fact, compound 67 showed the more potent anti-prion activity with an EC<sub>50</sub> of 2.5 μM in prion-infected neuroblastoma cell lines (ScN2a), than other tested analogs bearing m-dihydroxyphenyl and pyridyl groups.

Gallardo-Godoy et al. reported the identification of other thiazol-2-amine lead compounds that are orally absorbed and achieved high brain concentrations in animals [82]. In particular compound 69, embodying also an isoxazole ring, displayed excellent stability to rat liver microsomes in vitro and exhibited an EC<sub>50</sub> of 0.94 μM in prion-infected neuroblastoma cells (ScN2a-cl3), reaching a concentration of ~25 μM in mice brain after three days of oral administration. The replacement of 4-methylpyridyl by 3-/4-methoxypyridyl resulted in more potent analogs, namely 70 (EC<sub>50</sub> = 0.23 μM) and 71 (EC<sub>50</sub> = 0.25 μM). Noteworthy, the analogs with a quinoline ring and a diprotected catechol moiety, namely 72 (EC<sub>50</sub> = 0.11 μM, 10-fold more potent than its monocyclic version) and 73 (EC<sub>50</sub> = 0.081 μM) were the most potent agents, even more than 74 bearing a pyridyl group. Also the presence of the hydrogen bonding donor, NH, was not determinant for anti-prion activity as shown by the activity of 73 when compared to that of compound 72 (Figure 18).
Ghaemmaghami et al. [83] reported that N-acyl substitution with small groups (e.g., acetamide, cyclopropylamide) was tolerated to keep the inhibitory effect, as confirmed with the activity of compound 78. On the other hand aryl, heteroaryl or aliphatic rings appended to the aromatic ring attached to position 4 of the thiazolamine were tolerated, e.g., compounds 79 and 80.

Regarding brain exposure evaluation, among the thiazoles embodying the quinoline moiety, compound 74 exhibited the highest brain AUC value (0.02 µM after 40 mg/Kg/day), while a higher brain exposure (1.31 and 0.88 µM after 40 mg/Kg/day, respectively) was found for 78 containing also the benzofurane ring and for 71 bearing the phenylisoxazole in its structure. However, thiazole 75 exhibited the highest brain AUC amongst the evaluated thiazolamines (8.70 µM after 40 mg/Kg/day). Even at the dose of 210 mg/kg/day, mice receiving 75 or 77 exhibited no adverse clinical or behavioral effects, suggesting that these compounds would be well tolerated on prolonged dosing in an animal model of prion disease. In general thiazolamine analogs exhibited higher exposure in brain than in plasma, suggesting that is not subjected to P-gp mediated efflux.

On the other hand, among in vivo efficacy studies published prior to 2013, arylhydrazone with the oxazole ring, compound 68, was the only agent that produced a ≥50% extension of survival in treated animals compared to controls [84]. Based on this finding, Renslo et al. also determined the full PK profile of 75 and 77 compared to that of hydrazone 68 (Figure 18). They concluded that compound 75 achieved nearly 30-fold higher brain exposure following oral dosing than 68, and also exhibited a much longer half-life, lower clearance, a superior brain/plasma ratio, and greater bioavailability. Compound 77 exhibited the highest volume of distribution of the three compounds. On the other hand 75 and 77 produced no signs of toxicity in mice. In contrast it was found that hydrazone 68 exhibited lethal toxicity at a dose of 150 mg/Kg/day.

Moreover, the replacement of the terminal phenyl ring in 75 with heteroaromatic or heteroaliphatic rings produced analogs with clearly superior potency in the ScN2a-cl3 assay. Thus, the presence of pyridyl in 76, of morpholine in 80 and pyrazole in 81 led to analogs of 75 exhibiting EC50 values below 100 nM, a greater than 10-fold improvement in potency when compared to 75. Compound 76 also exhibited excellent brain exposure in animals, yielding a greater than 20-fold improvement in brain AUC/EC50 ratio for 76 compared to 75. More promising results were found with a series of cyclopropylamide substituted thiazole analogs, including compound 78, the direct analog of 75, that exhibited potency and in vivo brain exposure superior to 75, with a brain AUC/EC50 ratio 10-fold higher than that of 75.

However, thiazolamine-treated mice eventually showed accumulation of PrPSc in their brain and ultimately succumbed to disease. Compound 75 proved to be entirely ineffective against human CJD prions in susceptible transgenic mice expressing human PrPC. Prusiner and co-workers demonstrated that the eventual failure of thiazolamines can be accounted for by treatment-induced selection of thiazolamine-resistant prion strains [85].

To comprehend the mechanism of action of thiazolamines it has been suggested that they might interfere with the formation of new PrPSc in the cell, either directly by interference in the misfolding/assembly process or indirectly by modulating endogenous cellular clearance mechanisms [83]. However recent findings supported by X-Ray structure have shown that flexible, unstructured regions of PrPC contribute to forming a cryptic small molecule binding site revealing direct interactions between thiazolamine and PrPC [86].

The synthesis of thiazol-2-amine analogs was carried out by the Hantzsch-type condensation of bromomethyl ketones with thioureas (Scheme 19). Synthesis of compound 69 started with reaction of the commercially available (5-methylpyridin-2-yl)amine with phenyl isothiocyanate in acetone under reflux, dissolved in MeOH and hydrolyzed by treatment with 1 N NaOH at 80 °C to afford thiourea 82 in 65% yield. Reaction with the commercially available 2-bromo-1-(3-phenylisoxazol-5-yl)ethanone under reflux for 4 h gave 69 isolated in 65% yield.
Villa et al. have demonstrated that PrP\textsubscript{90–231} (Figure 19), found to possess anti-inflammatory activity in animal models [94].

Induced by PrP\textsubscript{90–231} lead to the ability of both iNOS and COX-2 activity, and reducing reactive oxygen species (ROS) release. These properties activation induced by neuroinflammatory stimuli, such as PrP\textsubscript{90–231} COX-1 blocker [75]. Later on, the same authors searched for novel compounds able to inhibit glial cell of COX-2, since this effect was selectively inhibited by celecoxib but not by ketoprofen, a prevalent non-steroidal anti-inflammatory drug (NSAIDs) treatment [92,93].

That the neuronal injury induced by glial activation in AD and prion diseases can be reduced by non-steroidal anti-inflammatory drug (NSAIDs) treatment [92,93].

Recently, to study the possible role of NSAIDs in preventing amyloid-driven neuroinflammation, Villa et al. have demonstrated that PrP\textsubscript{90–231} induces PGE2 release from microglia through the activation of COX-2, since this effect was selectively inhibited by celecoxib but not by ketoprofen, a prevalent COX-1 blocker [75]. Later on, the same authors searched for novel compounds able to inhibit glial cell activation induced by neuroinflammatory stimuli, such as PrP\textsubscript{90–231} and lipopolysaccharide LPS. They tested a small library of 2,3-diaryl-1,3-thiazolidin-4-one derivatives structurally related to celecoxib (Figure 19), found to possess anti-inflammatory activity in animal models [94].

Thiazolidin-4-one 83 (Figure 19) inhibits microglia activation more efficiently than all other tested thiazolines and celecoxib, whose structure embodies a pyrazole (see pyrazole section), lowering both iNOS and COX-2 activity, and reducing reactive oxygen species (ROS) release. These properties lead to the ability of 83 to revert neuroinflammation-like responses in mixed astrocyte and microglia induced by PrP\textsubscript{90–231} and LPS.
Compound 83 was obtained by synthesizing the corresponding Schiff base 88 (Scheme 20) [95]. Reaction of 4-methylbenzaldehyde with 4-aminophenylsulfonamide under reflux followed by addition of α-sulfanylacetic acid maintaining the same conditions gave compound 83 in 36% overall yield (Scheme 20).

![Scheme 20. Synthesis of thiazolidin-4-one 83. Reagents and conditions: (a) benzene, reflux, Dean-Stark apparatus; (b) HSCH$_2$COOH, reflux, 36% (two steps).](image)

### 2.3.4. Polythiophenes

Five-membered heterocycle ring polymers as thiophenes have been investigated as anti-prion compounds. Herrmann et al. [96] administered to the brain of prion-infected mice luminescent conjugated polythiophenes (LCPs) varying in the number of polyphene moieties, the number and distribution pattern of carboxyl groups (e.g., compounds 89–96, Figure 20). They found that anti-prion activity required a minimum of five thiophene rings bearing regularly spaced carboxyl side groups. Solid-state NMR analyses and molecular dynamics simulations revealed that anionic side chains interact with complementary, regularly spaced cationic amyloid residues of model prions.

![Figure 20. Polythiophene structures 89–96 studied in prion-infected mice.](image)

While five thiophene moieties were required for the minimal generic anti-amyloid LCP pharmacophore, as mentioned above, anionic side groups linked to the terminal thiophene rings is determinant for activity. Finally the periodicity of the anionic side groups also controlled the anti-prion properties, with the most effective pattern of regioregular charges being ca. 5 Å–10 Å–5 Å as in compound 89.

Reduced PrP deposition was confirmed by immunohistochemistry in brainstems of mice treated with 89 and was associated to a conspicuous reduction in vacuolation and astrogliosis. In addition, the increased survival of 89-treated mice, even when administered to preterminally scrapie-sick mice, suggested that LCPs may attenuate PrP$^S$c toxicity, but confirmation remains open because it cannot be directly quantified. Compound 89 was synthesized [97] using the route represented in Scheme 21. Intermediate 99 was prepared by Suzuki cross-coupling conditions from brominated thiophene 97 and boronic acid 98. After esterification, an overall yield of 71% over the two steps was obtained. Bromination of 99 with N-bromosuccinimide afforded the key precursor 100 in 73% yield, whose
coupling to 101 yielded methyl ester pentamer 102, giving compound 89 by hydrolysis, quantitatively (Scheme 21).

![Scheme 21. Synthesis of compound 89. Reagents and conditions: (a) 1,4-dioxane/MeOH, PEPPSI\(^\text{TM}\)-IPr, K\(_2\)CO\(_3\), 70 °C, 20 min; (b) MeOH, H\(_2\)SO\(_4\), 70 °C, 16 h; (c) NBS, DMF, 0 °C to r.t., 16 h; (d) NaOH (1M), 1,4-dioxane, 60 °C, 16 h.]

2.3.5. Carbazoles

Compounds 103 and 104 (Figure 21) are clearly distinct in terms of their chemical structure; yet, it is interesting to note the presence of key common elements in both structures: the number of hydrogen bond donors and acceptors, a nitrogen-containing five-membered ring, aromatic rings—one of which substituted with a fluorine atom, as well as a free hydroxy group. Compound 103 was also identified as a promising anti-prion scaffold by Kimura and co-workers in 2011 [98].

![Figure 21. Two carbazole derivatives 103, 104 with anti-prion activity.]

Compound 104 was developed inspired on compound 103 (Figure 21), previously reported for its affinity towards PrP\(^\text{C}\) while displaying an IC\(_{50}\) of 8.54 \(\mu\)M against PrP\(^\text{Res}\) accumulation in GT1-7 cells infected with Fukoka 1 (FK-1) [98]. It was synthesized together with a library of fortyseven dihydrocarbazole analogs and derivatives, which were subsequently tested to allow a comprehensive structure-activity relationship study on the activity of the lead compound.

Among the most relevant conclusions drawn by the authors, the fused three-ring system was found to be crucial for the desired effect. Indeed, when the 1,2,3,4-tetrahydrocarbazole moiety was replaced by either a pyrrole, indole or 4,5,6,7-tetrahydroindole unit, the anti-prion activity was lost. Carbazoles and 2,3,4,9-tetrahydrocarbazoles, however, were able to retain the desired effects to the same extent as compound 103. The hydroxy group was also found to be critical for activity by acting as hydrogen bond donor; what’s more, by replacing the original piperidine by secondary amines such as the one in compound 104, the anti-prion activity was further enhanced. The secondary amine function is indeed thought to interact with the target by acting as a hydrogen bond donor, while the hydrophobicity conferred by the N-linked substituent seems to be an important requirement. O-halobenzyl substituents in this position proved to be the most effective ones.

Compounds were firstly prepared as racemic mixtures containing both (\(R\))- and (\(S\))-enantiomers, following a similar synthetic route here presented for the lead compound 103 (Scheme 22A). Herein, the starting material—in this case the 6-methyl-2,3,4,9-tetrahydro-1\(H\)-carbazol-1-one unit—was treated with sodium hydride followed by addition of epichlorhydrin in DMF at 25 °C. Subsequent epoxide opening by piperidine in ethanol then gave the final product in good overall yield [98].
when administered subcutaneously, and was found to stabilize PrP

2.3.6. GN8 and Derivatives

the levels of PrP

S

mixture tested in this study, 

C

12 h; (b) EtOH, reflux, 12 h (51% over two reaction steps); (c) NaH, DMF, 0 °C, then 25 °C, 1 h; (2) S-epichlorhydrin, DMF, 0 °C, then 25 °C, 12 h; (d) EtOH, reflux, 12 h (60% over two reaction steps) [98].

In order to compare the individual contributions of each enantiomer constituting the most active mixture tested in this study, (S)- or (R)-epichlorhydrin was, accordingly, used (Scheme 22B) [98]. Containing a 2,3,4,9-tetrahydro-1H-carbazole unit as the main core, and a o-fluorobenzyl group linked to the secondary amine, compound 104 then arose as the most promising one of this series, with an IC$_{50}$ of 1.11 μM against PrP$_{Sc}$ in GT1-7 cells infected with FK-1. It was found to be twice as potent as its (R)-enantiomer, eight times more potent than the lead compound 103, and in vivo studies are currently taking place to assess whether it may, or not, deliver more encouraging results than those observed for some of the previously presented molecules. It would be interesting to compare those results, for instance, with the effects exhibited by GN8 (107, Figure 22) in vivo, since both compounds displayed identical IC$_{50}$ values in the same cellular disease model.

2.3.6. GN8 and Derivatives

N,N’-[methylenebis(4,1-phenylene)]bis[2-(pyrrolidin-1-yl)acetamide] (GN8, 107, Figure 22) was discovered in 2007 by Kuwata and co-workers and, with an IC$_{50}$ of 1.35 μM, significantly reduced the levels of PrP$_{Sc}$ in a mouse neuronal cell line (GT1-7) infected with the Fukuoka-1 (FK-1) prion strain [99]. This compound also improved the incubation time of PrP$_{Sc}$-infected mice up to 18 days when administered subcutaneously, and was found to stabilize PrP$^{C}$ mostly by binding to its C-terminal domain and, possibly, by interacting with the C-terminal region of the GPI-anchored PrP$^{C}$ [99]. PrP$^{C}$ is highly conserved in mammals and, therefore, GN8 is therefore strain-independent. This constitutes a relevant advantage in regard to previously described anti-prion compounds that act
by targeting PrPSc. Furthermore, this compound is easily accessed in only two reaction steps, with excellent overall yield (Scheme 23).

![Scheme 23. Synthesis of GN8. Reagents and conditions: (a) Py, DMAP, DCM, 0 °C, then 25 °C, 3 h (93%); (b) K₂CO₃, THF, 25 °C, then 60 °C, 12 h (88%) [100].](image)

First, treatment of 4,4′-methyleneedianiline with two equivalents of 2-bromoacetyl bromide in the presence of pyridine and dimethylaminopyridine (DMAP) gives intermediate 110. Then, subsequent di-N-alkylation by pyrrolidine in THF at 60 °C affords the desired product [100].

In 2011 Kimura et al. developed a series of GN8 analogs ultimately aiming at defining structural requirements for the activity of the lead compound [100]. Sixtyfour analogs were synthesized, two of which—compounds 108 and 109 (Figure 22)—displayed improved activity when compared to the prototype structure, both with an IC₅₀ of 0.51 μM in GT1-7 cells infected with FK-1. The synthesis of these compounds involved an additional reaction step for the formation of the dianiline precursor, which is exemplified for compound 109 in Scheme 24. In this reaction, 2,2,2-trifluoro-1-(4-fluorophenyl)ethan-1-one was dissolved in a solution of aniline hydrochloride in aniline at high temperature to afford intermediate 111 in moderate yield. Then, as previously described in the synthesis of GN8 (Scheme 23), two consecutive N-alkylation reactions gave the final compound in 65% yield.

![Scheme 24. Synthesis of GN8 derivative 109. Reagents and conditions: (a) PhNH₂Cl, 25 °C, then 140 °C, 48 h (41%); (b) Py, DMAP, DCM, 0 °C, then 25 °C, 3 h; (c) K₂CO₃, THF, 25 °C, then 60 °C, 12 h (65% over two reaction steps) [100].](image)

Overall, the conclusions of this study indicate that the structure of GN8 as a whole is essential for activity, as the deletion of either both pyrrolidines, or the full 2-(1-pyrrolidinyl)acetyl moieties conduct to a decrease in anti-prion activity. Moreover, N-methylation of the amide function leads to the same result. Indeed, it is supposed that the amide N-H groups and nitrogen atoms of the pyrrolidine units are crucial in the affinity towards PrPSc, acting as hydrogen bond donor and acceptor groups, respectively. In contrast, the carbonyl groups are seemingly less important. All compounds in which the central methylene bridge in GN8 was replaced by other linkers, including compounds 108 and 109, exhibited improved activity when compared to the lead structure. PrPSc levels were not affected by
these compounds; however, conformational analysis showed that substituents at the benzylic position confer structural rigidity to the molecule, placing both phenyl groups in the right orientation and thus favouring the access to the binding site of PrP<sup>C</sup>. Yet, these substituents do not establish additional interaction points to the PrP<sup>C</sup> structure. Compounds 108 and 109 did not present any toxicity at 2 µM in a cell-based assay and therefore constitute a promising class of anti-prion compounds for further development against TSEs.

2.4. Aryl Scaffolds Linked to Nitrogen Containing Functional Groups

2.4.1. Congo Red Analogs

Congo Red (CR, 113, Figure 23) was found to inhibit the formation of PrP<sup>Sc</sup> in cell-free assays back in 1983, and later to prevent the formation and accumulation of PrP<sup>Sc</sup> in ScN2a cells [101,102]. However, it has a relevant lack of specificity and poor blood brain barrier permeability [103,104]. Importantly, the central benzidine moiety—which is released into the bloodstream after cleavage by gut and intestinal enzymes—has a relevant carcinogenic potential [105]. Since Rudyk and co-workers have shown that this moiety can actually be replaced by less toxic units without losing the desired activity, some research groups have joined efforts to design and develop new CR analogs with improved pharmacological profiles [106]. In 2004, Sellerajah and colleagues defined a strategy for the accomplishment of this task [107]. Previous work had already pointed towards both symmetrical parts of the lead molecule as being necessary for activity; additionally, the sulfonate groups could be replaced by carboxyl groups without causing a significant loss in activity [106,108]. Sellerajah and colleagues then envisioned that by replacing both diazo groups with bioisosteric equivalents such as amide bonds, the molecule could be prevented from being metabolized into the carcinogenic benzidine unit [107]. After synthesis and evaluation of a small library of CR analogs, compound 114 (Figure 23) was elected as the most promising for further development, being able to inhibit the formation of PrP<sup>res</sup> in SMB cells with an EC<sub>50</sub> value between 25 and 50 nM. It was significantly more efficient that the lead 113 and was effective in a PrP polymerization assay, which pointed towards a mode of action targeting PrP<sup>C</sup> or the conversion between PrP<sup>C</sup> into PrP<sup>res</sup>. Moreover, it displayed an IC<sub>50</sub> value between 30 µM and 40 µM in a cytotoxicity assay against mouse cerebellar neuronal cells, which indicates that this compound has a good therapeutic window.

![Figure 23. Congo red (113) and two analogs 114, 115 with anti-prion activity.](image)

The synthesis of compound 114 was accomplished through a coupling reaction between (1,1'-biphenyl)-4,4'-dicarbonyl dichloride and methyl 4-amino-3-hydroxybenzoate in the presence of triethylamine, in anhydrous THF at room temperature (Scheme 25) [107]. Using this method, the target compound was obtained in 14% yield. The authors also described the synthesis starting from the dicarboxylic acid and using O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate.
(TBTU) and DIPEA in dimethylacetamide (DMA) at room temperature for 48 h. However, there was no significant improvement, as the yield was only 10%.

![Scheme 25](image)

Scheme 25. Synthesis of Congo Red analog 114. Reagents and conditions: (a) TEA, anhydrous THF, 0 °C, 10 min, then rt, 18 h (14%) [107].

With an EC50 value of 75 nM, compound 115 (Figure 23) also exhibited tremendous potential in this study, and its synthesis, although involving one additional step, was much more effective than the previous two: after condensation of terephthalaldehyde with methyl 4-amino-3-hydroxybenzoate in ethanol at 55 °C to afford the imine intermediate 116, a NaBH4-promoted reduction in DCM and MeOH at room temperature gave the desired product in 49% yield (Scheme 26) [107].

![Scheme 26](image)

Scheme 26. Synthesis of Congo Red analog 115. Reagents and conditions: (a) EtOH, 55 °C, 5 h (76%); (b) DCM, NaBH4, MeOH, r.t., 12 h (49%) [107].

The ester moieties in both compounds 114 and 115 were initially conceived based on the hypothesis that they would be readily hydrolysed by esterase enzymes in the brain. Thus, the hydroxy groups were placed in the molecules to assure sufficient aqueous solubility [107]. However, the corresponding free carboxylic acid metabolites were not tested regarding their anti-prion activity in this study, and thus it is not known whether this rationale is, indeed, valid. Yet, in 2007, Webb et al. published a study showing the results of a set of mechanistic tests to verify the mode of action of both compounds, which were later named WSP677 and WSP740, respectively [109]. In this report, proteasome activity was shown to be increased by the interaction of these compounds with PrP. After 24 h, with one single treatment of 1 μM of compound SMB cells were “cured” based on the detectable levels of PrPSc. Remarkably, CD-1 mice inoculated with SMB cell extracts containing compound 115 presented no symptoms after 400 days after inoculation. This implies that the agent of disease transmission was eliminated by the action of this compound. However, compound 114 did not exhibit the same effects. These data not only place compound 115 in a lead position for the development of new molecules for the treatment of TSEs, but also reflect the discrepancy between in vitro and in vivo effects when dealing with anti-prion therapies.

2.4.2. Sulfonated Arylamides

Suramin is a polysulfonated aromatic urea (Figure 24), discovered in the 60’s to treat African sleeping sickness [110]. More recently it has been described as modulator of PrPC expression,
with the ability to modify biochemical properties of PrP\textsuperscript{C} including solubility and its half-life [111]. Kiachopoulos and co-workers described how suramin induces misfolding of the cellular prion protein and interferes with the propagation of infectious scrapie prions [112]. Gilch et al. characterised the effects of suramin on prion biogenesis in cell culture and in vivo models. They reported suramin inhibiting de novo formation of PrP\textsuperscript{Sc} by inducing intracellular PrP\textsuperscript{C} aggregates which are targeted from Golgi/Trans Golgi Network (TGN) compartments to a lysosomal degradation pathway [113].

![Figure 24. Suramin-related structures tested for anti-prion activity.](image-url)

All these previous findings have led to the evaluation of new suramin-related compounds developed by Nunziante and co-workers [111] (Figure 24). Among the compounds tested, only those with symmetrical bipolar structure and sulfonic acid groups showed potent inhibition of PrP\textsuperscript{Sc} synthesis. Two half-molecules lacking symmetrical structure had no effect on PrP\textsuperscript{Sc} accumulation. Uncharged compounds did not have activity, despite their symmetrical aromatic structure. Effective compounds induced aggregation of PrP\textsuperscript{C} and reduced its half-life without affecting PrP\textsuperscript{C} cell surface expression, which was crucial for their activity. The effect of these suramin derivatives on PrP\textsuperscript{Sc} was determined in prion-infected neuroblastoma cells (3F4-ScN2a) using a solubility assay and proteinase
K (PK) treatment followed by immunoblot analysis. The accumulation of PK-resistant material was completely eliminated after treatment with suramin and with compounds 117, 118, 120, 122, 123, 124, 125, and 126. Substances with carbonic or phosphonic acid substitutions, 119 and 121 respectively, showed no activity or only mild inhibitory effects on PrPSc. No PrP signal was detected in cells incubated with 128 owing to the strong toxicity of the compound. Asymmetric and uncharged drugs (129, 130 and 131) were ineffective in reducing the amount of PrPSc, and did not show any effect on PrPSc solubility.

On the other hand, when cells were subjected to a solubility assay, the immunoblot analysis revealed aggregation of PrP when cells were exposed to suramin and to compounds 117, 119, 122, 123 and 124.

Nunziante et al. [111] also examined the influence of suramin analogs on the cellular localization of PrP by confocal microscopy. After treatment of wtN2a cells with 117 or 118, the PrP signal at the cell surface was still consistent with that detected in untreated cells. In addition, 117 and 118 also induced increased partitioning of PrP in intracellular compartiments. This intracellular PrP population was mainly present in lysosomes. These results showed that, in contrast to suramin, these derivatives did not interfere with the cell surface expression of PrPSc.

As an example of arylamide synthesis, Scheme 27 shows the synthetic route to obtain compound 118 [114]. 3-Nitrobenzoyl chloride 132 was reacted with compound 133 in a biphasic mixture of toluene and water at acid pH for 6 hours affording the amide 134 in 83% yield. Reduction of nitro group by hydrogenation generated compound 135 in 98% yield. Finally, reaction of 135 with phosgene gave suramin derivative 118 in 85% yield.

![Scheme 27. Synthesis of compound 118. Reagents and conditions: (a) toluene, r.t., 6 h, 83%, (b) Pd (10%) on charcoal, H2O, 4 bar H2, rt, overnight, 98%, (c) H2O, phosgene (20%) in toluene, r.t., 6 h, 85%.]

2.5. Polyphenols

This structurally diverse family of compounds, the polyphenols, are often found as the major plant components responsible for their medicinal properties. Their bioactivity against neurodegeneration is mainly due to their antioxidant [115–119], anti-inflammatory [120,121], and anti-amyloidogenic [122–124] effects. Polyphenols mode of action includes inhibition of intracellular kinases activity [125], binding to cell surface receptors [126], and modifying cell membrane functions [127]. These natural drugs are potent therapeutic agents exhibiting pharmacological properties against Alzheimer’s disease and dementia, multiple sclerosis, ischemic stroke, Parkinson’s and Huntington’s disease [128].

Rauter and co-workers reported the potent acetylcholinesterase (AChE) inhibitory activity of Salvia sclareoides extracts, a spontaneous plant growing in Iberia (Figure 25A) [129]. Plant extracts also delayed prion propagation in a cell-based screening assay [130], and the prion binding properties were then evaluated by NMR measurements [131]. The n-butanol extract turned out to bind to the cellular form of human prion protein in a specific manner, causing conformational perturbation in three different regions of the protein including the highly dynamic N-terminal region (Figure 25B). The authors found out that the major extract component was rosmarinic acid, and NMR measurements have shown its interaction with the Alzheimer’s disease (AD) amyloid oligomers Aβ1–42, with aromatic protons being mostly involved in the binding (Figure 25C) [132]. Interestingly, a new binding site of rosmarinic acid to AChE was also discovered by the authors, namely the binding site B in Figure 25D [133], opening the way to the development of new drugs based on rosmarinic acid as...
scaffold, aiming to innovate the standard therapy to treat AD patients with donepezil, rivastigmine and galantamine. The search for new AChE inhibitors is encouraged as this cholinesterase is able to accelerate amyloid formation [134]. Indeed PrPSc decrease also accelerates Aβ formation and it was reported that its fate in ageing may be related to the mechanisms involved in neurodegeneration, affecting also AD [135], but the role of PrPSc on AD is not yet fully understood. Noteworthy, prions are amyloid forming proteins and the discovery of anti-amyloidogenic compounds against AD may also be an added-value discovery for the combat of prion diseases.

Figure 25. (A) Salvia sclareoides; (B) Structure of huPrP91–231 indicating residues affected by S. sclareoides butanol extract, where the color and thickness of the ribbon indicates increased interaction. Residues affected: 93–112 in the unstructured N-terminal region of PrPSc, 130–187 comprising the two strands of the beta sheet, helix 1 and the beginning of helix 2, and 205–225 in helix 3 at the C-terminus of the structured domain [131]; (C) Rosmarinic acid interacts with Aβ1-42, and the aromatic protons are mostly involved in the binding, as detected by NMR experiments [132]; (D) Depiction of acetylcholinesterase binding site A for donepezil, a drug in the market to treat AD and that of the newly discovered binding site B for rosmarinic acid [133].

Caughey et al. screened 2000 compounds covering drugs and natural products for the inhibition of PrPSc in ScN2a cells infected with scrapie strain RML or 22L [136]. While 40 compounds showed IC50s below 10 μM, 17 of them, depicted in Figure 15, were the most potent ones, having IC50s below 1 μM against both strains. It was also found that many of them were natural without observed toxicity. Quinacrine and lovastatin were previously identified as PrPSc inhibitors [137,138]. Tannic acid 84, a constituent of foods, turned to be the most potent inhibitor, even better than quinacrine, with IC50 ~100 nM.

The compounds were also evaluated in a solid-phase cell-free (SP-CFC) hamster PrP conversion assay. Only polyphenols 136, katacine 137 and 2′′-bisepigallocatechin digallate 138 inhibited the cell-free reaction, and their IC50 values were near 100 nM. Epigallocatechin 139 and epicatechin 140 were not PrPSc inhibitors, with molecular weights of about 300, although their 3-monogallate derivatives were effective, namely 141 and 142. Noteworthy, compound 143, embodying a double bond in the central ring with a higher hydroxylation pattern than 139 and 140, presented inhibitory properties, demonstrating that changing flavan-type structure by a planar highly hydroxylated flavonol analog had important effects on polyphenol inhibitory efficacy. All other compounds 145–156, with
a diversity of highly substituted core structures, namely steroid, glycosylated triterpene, aromatics and heteroaromatics, did not show inhibitory activity in the SP-CFC reaction at concentrations up to 100 µM.

In addition, Rambold et al. [139] identified 142 and its diastereoisomer 144 (Figure 26) as potent drugs to interfere with the formation of PrPSc in ScN2a cells. Noteworthy 141, with the same configuration as 90 but differing in the hydroxylation pattern, interfered with PrPSc accumulation efficiently at 200 µM concentration while only 50 µM were needed to have the same effect with 90. The additional hydroxy group at the trihydroxyphenyl side chain of 142 clearly increases its activity when compared to that of 141. To test for a direct interaction with PrP, isothermal titration calorimetry (ITC) experiments were carried out using rPrP90–232 showing the lack of interaction of 141 with rPrP90–232. While 142 had a K_D of 130 nM and a ΔH of −43 kJ, no changes in enthalpies with 141 were observed, indicating absence of binding. In addition, compounds 139 and 140 both without the gallate side chain, did not interfere with PrPSc propagation, endorsing, as reported by Kocisko et al. [136] the gallate side chain as essential for activity.

![Figure 26. Structure of compounds 136–156 studied against both the RML and 22 L scrapie strains [136] and rPrP90–232 [139].]
Some polyphenols may not be the best candidates to cross the blood brain barrier for their water solubility, but these molecular entities may be useful by preventing TSE disease. However, radiolabeled epigallocatechin gallate (EGCG) has been detected in mouse brains after oral administration [140].

More recently, Fuchigami and co-workers evaluated the potential of radioiodinated flavonoids (flavone 157, chalcone 158 and aurone 159) and styryl chromones against PrPSc (Figure 27) by single photon emission computed tomography (SPECT) imaging of PrPSc [141].

![Figure 27. Radioiodinated flavonoids 157–159 and styrylchromones 160–165 tested by SPECT imaging of PrPSc.](image)

In vitro binding assays using recombinant mouse PrP (rMoPrP) aggregates revealed that 160 had higher in vitro binding affinity (K_D = 24.5 nM and capacity B_max = 36.3 pmol/nmol as total density of protein in a sample of tissue) than the three flavonoids (K_d = 201 nM and capacity B_max = 11.2 pmol/nmol for 157, K_D = 246 nM and capacity B_max = 16.7 pmol/nmol for 158, and K_D = 125 nM and capacity B_max = 34.9 pmol/nmol for 159). On the other hand fluorescent imaging using brain sections from mouse-adapted bovine spongiform encephalopathy (mBSE)-infected mice demonstrated that 160 labelled PrPSc-positive prion deposits in the mice brain. Fuchigami et al. were pioneers describing in vivo imaging of PrPSc deposits in the mBSE-infected mice brain observing high accumulation of the two methoxy derivatives [125]I162 and [125]I163 by in vitro fluorescence and autoradiography experiments. These derivatives showed binding for rMoPrP aggregates with K_i = 20.8 and 26.6 nM respectively.

Radiochemical synthesis of compound 162 was carried out by an iododestannylation reaction of corresponding tributyltin derivative 166 to give compound 162 in 35–44% radiochemical yield and with a radiochemical purity of 98% as depicted in Scheme 28.

![Scheme 28. Synthesis of radiolabeled compound 162. Reagents and conditions: [123]I NaI, H_2O_2, HCl, EtOH, r.t., 10 min.](image)

Other natural polyphenols, structurally based on stilbenes or cinnamic acid derivatives, have shown anti-prion activity, as resveratrol and curcumin. Resveratrol [systematic name: (E)-5-(4-hydroxystyryl)benzene-1,3-diol, Figure 28] is produced by grapes, mulberries, and nuts, when attacked by pathogens [142]. Over the past couple of decades, resveratrol has been extensively investigated showing health benefits in neuroprotection, cardioprotection, hepatoprotection, antiinflammation, and cancer prevention [143]. Its neuroprotective properties are not restricted to AD or Parkinson’s diseases being also demonstrated its anti-prion efficacy. Jeong et al. [144]
The most recent synthesis of resveratrol has been carried out by El-Deeb et al. [155] and is shown protected resveratrol in high yield (95 and 96%, respectively) which was later demethylated with the presence of resveratrol, significant reduced levels of PrPSc were detected.

Several synthetic approaches have been described for the synthesis of resveratrol, carried out by Wittig [149] or Horner-Wadsworth-Emmons reactions [150], Perkin condensations [151], and also through metal-catalysed processes, such as cross-metathesis [152] or cross-coupling reactions [153,154]. The most recent synthesis of resveratrol has been carried out by El-Deeb et al. [155] and is shown in Scheme 29. They synthesized resveratrol in five steps with a 62% overall yield. The key step is the oxidation of 5-methylcyclohexane-1,3-dione 167 to 3,5-dimethoxytoluene 168 by hemiacetal formation and subsequent dehydration using ethylene and Pd/C in MeOH at 120 °C (Scheme 29). Then, bromination with NBS in CH3CN was carried out to generate compound 169 in 91% yield. Compound 169 was converted in the corresponding aldehyde 170 and the phosphonate 171 to follow two different methodologies as depicted in Scheme 29 for the trans double bond formation. They applied the Horner–Wadsworth–Emmons reaction employing on one hand, the reaction of 3,5-dimethoxybenzaldehyde 170 with diethyl 4-methoxybenzylphosphonate and, on the other hand, 4-methoxybenzaldehyde with diethyl 3,5-dimethoxybenzylphosphonate 171. Both routes gave protected resveratrol in high yield (95 and 96%, respectively) which was later demethylated with the combination of AlCl3 and i-Pr2NH giving resveratrol in 85% yield (Scheme 29).

Figure 28. Structure of resveratrol with anti-prion properties.

Wang and co-workers [147] evaluated the treatment of SMB-S15 cells with Sirt1 activators. In the presence of resveratrol, significant reduced levels of PrPSc were detected.

To address the anti-prion activity of resveratrol, Wang et al. [148] tested the inhibitory activity of resveratrol on prion accumulation in vitro and prion infectivity in vivo using scrapie-infected cell line SMB-S15. They demonstrated that the amounts of PrPSc in SMB-S15 cells decreased in a dose-dependent manner in the presence of resveratrol, with EC50 = 0.61 μM. The removal of PrPSc in SMB-S15 cells by resveratrol seemed to be irreversible as no PrPSc signals could be detected in the resveratrol-treated cells after withdrawal of the drug. Moreover, infectivity of SMB-S15 cells on the sensitive rodent CD1 mice was eradicated after exposing to resveratrol for 1 week.

Several synthetic approaches have been described for the synthesis of resveratrol, carried out by Wittig [149] or Horner-Wadsworth-Emmons reactions [150], Perkin condensations [151], and also through metal-catalysed processes, such as cross-metathesis [152] or cross-coupling reactions [153,154]. The most recent synthesis of resveratrol has been carried out by El-Deeb et al. [155] and is shown in Scheme 29. They synthesized resveratrol in five steps with a 62% overall yield. The key step is the oxidation of 5-methylcyclohexane-1,3-dione 167 to 3,5-dimethoxytoluene 168 by hemiacetal formation and subsequent dehydration using ethylene and Pd/C in MeOH at 120 °C (Scheme 29). Then, bromination with NBS in CH3CN was carried out to generate compound 169 in 91% yield. Compound 169 was converted in the corresponding aldehyde 170 and the phosphonate 171 to follow two different methodologies as depicted in Scheme 29 for the trans double bond formation. They applied the Horner–Wadsworth–Emmons reaction employing on one hand, the reaction of 3,5-dimethoxybenzaldehyde 170 with diethyl 4-methoxybenzylphosphonate and, on the other hand, 4-methoxybenzaldehyde with diethyl 3,5-dimethoxybenzylphosphonate 171. Both routes gave protected resveratrol in high yield (95 and 96%, respectively) which was later demethylated with the combination of AlCl3 and i-Pr2NH giving resveratrol in 85% yield (Scheme 29).
with amyloid fibrils. An apoptosis assay confirmed that fibril-induced apoptosis was largely weakened by the ROS level [159] of N2a cells infected with mPrP fibrils. They found out that mPrP fibril-induced ROS could be completely eliminated by treatment with 2.5 μM of curcumin prior to the fibril treatment. On the other hand, since curcumin binds only to non-native forms of PrP, avoiding prion fibril formation without affecting native PrP [157].

Hafner-Bratkovic et al. showed that this polyphenol protected resveratrol in high yield (95 and 96%, respectively) which was later demethylated with the presence of resveratrol, significant reduced levels of PrPSc were detected. Analysis of transmission electron microscopy (TEM) images showed mPrP fibrils growing in the absence of curcumin, while in its presence the length of the fibrils was shorter. To investigate the effect of curcumin in cells, they first confirmed the disruption of cell membranes in erythrocytes from mouse blood. Mouse erythrocytes are typically oval and biconcave but when the erythrocytes were co-incubated with mPrP fibrils, cells of this group were more rounded and biconcave. Under transmission electron microscopy (TEM), the presence of mPrP fibrils was observed in the absence of curcumin, while in its presence, the length of the fibrils was significantly reduced. To investigate the effect of curcumin in cells, they first confirmed that curcumin inhibits the accumulation of PrPSc in scrapie-infected neuroblastoma (scNB) cells [156]. Caughey and co-workers have found that curcumin inhibits the accumulation of PrPSc in a cell-free environment and in murine neuroblastoma (N2a) cells [157]. They evaluated the effect of curcumin on the amyloid formation of mPrP, based on the kinetics of amyloid formation by fluorescence intensity of thioflavin T. ThT-fluorescence increased significantly, but in the presence of curcumin, only a weak increase was detected. Analysis of transmission electron microscopy (TEM) images showed mPrP fibrils growing in the absence of curcumin, while in its presence the length of the fibrils was shorter. To investigate the effect of curcumin in cells, they first confirmed the disruption of cell membranes in erythrocytes from mouse blood. Mouse erythrocytes are typically oval and biconcave but when the erythrocytes were co-incubated with mPrP fibrils, cells of wrinkled morphology were formed. However, treatment of the erythrocytes with curcumin together with or prior to fibril incubation reduced membrane damage.

Viability of N2a cells turned to increase 33% by treatment with curcumin prior to the co-incubation with amyloid fibrils. An apoptosis assay confirmed that fibril-induced apoptosis was largely weakened when N2a cells were treated with curcumin prior to the fibril treatment. On the other hand, since curcumin is widely applied for its antioxidant properties, it has been investigated its effect on the (ROS) level [159] of N2a cells infected with mPrP fibrils. They found out that mPrP fibril-induced ROS could be completely eliminated by treatment with 2.5 μM of curcumin prior to co-incubation with mPrP fibrils, suggesting that curcumin is involved in the ROS-related signal transduction pathways.

Curcumin [systematic name: 1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione], (Figure 18) is the main pigment derived Curcuma longa that has been identified as an inhibitor of prion fibril formation [156,157] with the ability to cross the blood brain barrier [158]. Curcumin, having an unique conjugated structure including two methoxylated phenols and an enol form of diketone shows a typical radical trapping ability as a chain breaking antioxidant (Figure 29). Structurally, curcumin is similar to Congo red, which is toxic and a poor brain penetrant. However, curcumin does not present apparent toxicity resulting in a promising drug as anti-TSE agent.

Scheme 29. Synthesis of resveratrol. Reagents and conditions: (a) MeOH, Pd/C, ethylene, 130 °C, 1 h, 86%, (b) NBS, CH3CN, 25 °C, 1 h, 91%, (c) hexamethylenetramine, H2O, reflux, 12 h, 99%, (d) P(OEt)3, 80 °C, 12 h, 100%, (e-g) NaH, THF, 28 °C, 3 h, 95% and 94% respectively, (f) AlCl3, tPr2NH, toluene, 110 °C, 4 h, 85%.

![Curcumin](https://example.com/curcumin.png)

**Figure 29.** Structure of curcumin with anti-prion properties.
The major processes of practical utility for the preparation of curcumin follow Pabon’s route [160]. This method involves the reaction of vanillin (4-hydroxybenzaldehyde), acetylacetone and B$_2$O$_3$ in the presence of tributyl borate and butylamine. The reaction is carried out in ethyl acetate at r.t., affording curcumin in 80% yield (Scheme 30).

![Scheme 30. Synthesis of curcumin. Reagents and conditions: (a) B$_2$O$_3$, (BuO)$_3$B, n-BuNH$_2$, AcOEt, r.t., 80% yield.](image)

As shown so far, polyphenols are potent therapeutic agents with a broad range of pharmacological effects, however their biological activity is not completely clear. These compounds modulate many biological pathways and alter functions of different proteins including membrane proteins [161–163] pathways. This characteristic of perturbing cell membrane is believed to be the reason for their multiple functions, instead of specific binding to proteins [164] and there is little evidence for direct binding to some of their numerous effector proteins [165].

Amongst the polyphenols mentioned in this section EGCG, resveratrol and curcumin have been described by Ingolfsson et al. [164] to alter bilayer properties and modify membrane protein function. Curcumin displays essentially all known Pan Assay Interference Compounds (PAINS)-type behavior [164,166]. It contains catechol units which are recognizable PAINS motifs interfering in biological events through different mechanisms [167]. Resveratrol, like many phenols, is readily oxidized and can form reactive quinones [168] which are also PAINS motifs.

The promiscuous activity of these entities should lead us to use them cautiously as control compounds and leave open the challenge to continue structure optimization to investigate their specificity of binding to proteins considered key to the control of neurodegeneration.

3. Concluding Remarks

Prion and Alzheimer’s diseases share being protein misfolding disorders. These diseases are fatal, and the latter accounts for most cases of dementia. It is a much more prevalent amyloid disorder than TSEs and, to this point, no effective disease-modifying therapies have been successfully developed. After continuous drug flops in pivotal clinical trials for AD, a new hope has risen since the recent discovery of PrP$^{C}$ as a high-affinity binding partner of amyloid β (Aβ) oligomers, which are the key pathophysiological toxic entities causing neuronal death in AD patients [169]. In fact, post-mortem examinations of brain tissue derived from AD patients have led to the identification of high molecular mass assemblies of Aβ oligomers and PrP$^{C}$ [170]. Moreover, in contrast with AD transgenic mice, PRNP$^{-/-}$ mice with Aβ plaques do not exhibit hippocampal impairment of synaptic plasticity and associated suppression of cognitive function [171]. These data suggest that Aβ oligomer toxicity in AD may in fact be mediated by PrP$^{C}$, and encourage further research focusing on protein-protein interaction inhibitors (PPII) to block the assembly of Aβ-PrP$^{C}$ aggregates in the treatment of AD. By exploring the molecular entities able to intervene with the common mechanisms of prion assembly and prion-promoted neurodegeneration, perhaps in the future new efficacious therapeutic approaches will be achievable and somehow adjustable to all human prions.

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