




Anti-prion Drugs Targeting the Protein Folding Activity of the Ribosome Reduce PABPN1 Aggregation

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Abstract

Prion diseases are caused by the propagation of PrP^{Sc}, the pathological conformation of the PrP^C prion protein. The molecular mechanisms underlying PrP^{Sc} propagation are still unsolved and no therapeutic solution is currently available. We thus sought to identify new anti-prion molecules and found that flunarizine inhibited PrP^{Sc} propagation in cell culture and significantly prolonged survival of prion-infected mice. Using an *in silico* therapeutic repositioning approach based on similarities with flunarizine chemical structure, we tested azelastine, duloxetine, ebastine, loperamide and metixene and showed that they all have an anti-prion activity. Like flunarizine, these marketed drugs reduced PrP^{Sc} propagation in cell culture and in mouse cerebellum organotypic slice culture, and inhibited the protein folding activity of the ribosome (PFAR). Strikingly, some of these drugs were also able to alleviate phenotypes due to PABPN1 nuclear aggregation in cell and *Drosophila* models of oculopharyngeal muscular dystrophy (OPMD). These data emphasize the therapeutic potential of anti-PFAR drugs for neurodegenerative and neuromuscular proteinopathies.

Key Words Prions · PrP^{Sc} · drug repositioning · PFAR · OPMD · PABPN1

Introduction

Although the concept of infectious proteins was first established for the prion protein PrP in mammals suffering from transmissible spongiform encephalopathy [1], prions

are broadly found in other model organisms including the budding yeast *S. cerevisiae*. Over the last years, we have taken advantage of the existence of prions in yeast to identify new anti-prion compounds by screening drugs able to cure [PSI⁺] and [URE3] yeast prions [2–7]. Some of the compounds we

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have identified are active both *in vitro* and *in vivo* against PrP^{Sc} mammalian prions, demonstrating that some of the molecular mechanisms controlling prion onset and propagation are similar in yeast and mammals [2–6, 8, 9]. In particular, we identified two FDA-approved drugs, guanabenz (GA) and imiquimod (IQ), and 6-aminophenanthridine (6AP), with anti-prion activity [4–6, 9, 10]. A reverse screening approach has allowed us to identify one of the cellular targets of these compounds: the domain V of the large ribosomal RNA (rRNA) of the large 60S ribosome subunit [4–6, 9, 10]. The domain V of large rRNA (23S in *E. coli*, 25S in *S. cerevisiae*, 28S in metazoans) is a ribozyme carrying 2 enzymatic activities: (i) the peptidyl transferase activity and (ii) the protein folding activity, PFAR (protein folding activity of the ribosome), which can refold denatured proteins back to their functionally active forms [11, 12]. Ribosomal RNA from all three kingdoms of life exhibit a protein folding activity [12], which is in good agreement with the fact that both sequence and secondary structure of rRNA domain V are highly conserved [13]. 6AP, GA and IQ were shown to be the first described competitive inhibitors of PFAR that do not affect protein translation [5, 6, 14]. The fact that these three anti-prion compounds are also PFAR inhibitors led us to explore the link between PFAR and prion propagation. We found that PFAR is involved in the *de novo* formation and propagation of [PSI⁺] prion in yeast [15], strongly suggesting that PFAR is a promising cellular target for the treatment of prion diseases.

There is growing evidence that other proteinopathies share key biophysical and biochemical characteristics with prionopathies [16]. In this type of disease, affected proteins are for example A β and Tau for Alzheimer's disease, and α -synuclein for Parkinson's disease. Oculopharyngeal muscular dystrophy (OPMD) is also classified as a proteinopathy, in which poly(A) binding protein nuclear 1 protein (PABPN1) forms aggregates within the nuclei of patients' muscle cells [17, 18]. OPMD is a late-onset, autosomal dominant genetic neuromuscular disease caused by a short expansion of a GCG repeat encoding a polyalanine tract located at the N-terminus of PABPN1 [19]. Ten alanine residues are present in the normal protein and are expanded from 11 to 18 alanine residues in the mutant forms of the protein [20–22]. Similarly to polyglutamine expansion diseases, the triplet-expanded mutant PABPN1 protein aggregates and this is the pathological hallmark of OPMD. More important, we have previously shown that the anti-PFAR drug GA was active in OPMD cells as well as *Drosophila* and mouse models [23, 24], suggesting that PFAR plays a role in PABPN1 aggregation in OPMD. These data highlight the fact that PFAR inhibitors may be beneficial for various proteinopathies, including prion diseases and OPMD.

As the prion field desperately lacks therapeutic solutions for affected patients, and since GA (hypotensive drug) as well as IQ (immune system activator used in epicutaneous administration) are not compatible with a chronic treatment targeting the CNS, we sought to identify other anti-prion compounds which are also approved drugs. Here, we show that flunarizine, an approved drug used to treat migraine, is also a potent anti-prion drug in cell culture. Flunarizine also shows a significant effect on the survival of intracerebrally PrP^{Sc}-infected mice. Following this observation, we employed an *in silico* approach based on similarities with the flunarizine chemical structure to identify other FDA-approved drugs that may also possess anti-prion activity and allow their therapeutic repositioning according to SOSA (selective optimization of side activities of drug molecules [25, 26]) approach. This strategy is based on the screening of drugs that are already in the clinic or in clinical trials to determine if they could have biological activities—in our case, the inhibition of prion propagation—other than those initially described during their therapeutic development. This approach is particularly popular for rare or neglected diseases, as their therapeutic targets are not present in the safety or selectivity profiles in pharmaceutical companies. As their toxicity, safety and bioavailability in humans have already been tested, the drugs identified in these SOSA-based libraries may reach patients faster and may also be directly administered as potential compassionate therapeutics to people presenting symptoms of prion diseases. Using this approach, we have been able to identify 17 drugs approved by the FDA and/or other drug agencies, which are, to our knowledge, newly described anti-PrP^{Sc} drugs, and provide a new perspective for their therapeutic repositioning as anti-prion compounds in the clinic. Among a selection of the most potent anti-prion drugs identified, we found that, like flunarizine, azelastine, duloxetine, ebastine, loperamide and metixene can inhibit PrP^{Sc} propagation in organotypic mouse cerebellum slice cultures. From a mechanistic point of view, flunarizine and its six structural analogs were also found to be potent PFAR inhibitors, confirming our previous findings that PFAR is a relevant therapeutic target to curb prion propagation. As PFAR has also been shown to play a role in OPMD [23] and since there is no pharmacological treatment currently available for this disease, flunarizine and some of its structural analogs were also challenged for their capacity to reduce the phenotypes of OPMD in both cellular and *Drosophila* models. These new anti-PFAR molecules were able to reduce not only PABPN1 aggregation in cell culture but also muscular defects in OPMD *Drosophila*.

Altogether, we report here the identification of six new anti-PFAR FDA-approved drugs, namely azelastine, duloxetine, ebastine, flunarizine, loperamide and metixene as potent drug candidates for prion diseases as well as for OPMD. In addition, these data confirm our previous observations that PFAR plays a major role in prion propagation and toxicity of PABPN1 aggregation [23].

Methods

Selection of Anti-prion Drug Candidates

ROCS Shape-Based Virtual Screening The latest release of DrugBank (version 5.1.0, released 2018-04-02) was downloaded directly from <https://www.drugbank.ca> and filtered to only conserve FDA-approved or withdrawn drugs. Multiconformer files were generated by OMEGA v2.5.1.4 from OpenEye Scientific Software (<http://www.eyesopen.com/>), leading to 1887 approved or withdrawn drugs saved in oeb.gz format. These generated multiconformational files were used as the input database for performing similarity search with Rapid Overlay of Chemical Structures (vROCS 3.2.2.2 from OpenEye Scientific Software (<http://www.eyesopen.com/>)). ROCS is designed to carry out large-scale 3D database searches. It performs similarity searches by using a shape- and/or pharmacophore-based superposition method that finds the similar but non-intuitive compounds. It uses only the heavy atoms of a ligand ignoring the hydrogens. The output files of the similarity search were then ranked according to their ComboScore, which is based on a combination of shape and pharmacophore similarity.

The structure of flunarizine used as query was extracted from experimentally crystallized structure [27] obtained from the Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/structures (CCDC identifier: JOBSIE).

Azelastine, biperiden, cinnarizine, diphenhydramine, epinastine, ethosuximide, flunarizine, imipramine, loperamide, orphenadrine, paramethadione, thioridazine, triflupromazine, zonisamide and GuHCl were purchased from Sigma-Aldrich. Astemizole, clemastine, duloxetine, ebastine and metixene were purchased from CarboSynth. Benzydamine and nefopam were purchased from LGC. Antazoline, atomoxetine, cetirizine, citalopram, diazepam, ketotifen and zimeclidine were purchased from Tocris. Alimemazine, chloropyramine, dicyclomine, diphenidol, mirtazapine and prenylamine were purchased from Prestwick.

PrP^{Sc} Clearance Assay in Cultured MovS6 Cells

These experiments were performed as previously described [4, 5, 28]. Briefly, MovS6 cells chronically infected with ovine 127S prion strain were plated in 6-well plates (Starlab, France), treated for 6 days with the indicated concentrations of candidate drugs and then lysed in a buffer containing 0.5% sodium deoxycholate, 0.5% Triton-X100 and 5 mM Tris-HCl pH 7.5. Cell lysates (250 µg) were digested by 20 µg/mL of proteinase K (PK, Thermo Scientific, Waltham, MA) to identify PrP^{Sc} proteins which are resistant to proteolytic degradation. PK-treated homogenates were loaded on 10% Bis-Tris SDS-polyacrylamide gels (NuPAGE, Invitrogen/Thermo Scientific), and subjected to western blotting onto

nitrocellulose membranes (GE Healthcare, Barrington, Illinois). For normalization purposes, 25 µg of non-PK-treated homogenates was also loaded on 10% Bis-Tris SDS-polyacrylamide gels, and subjected to western blotting onto nitrocellulose membranes. PrP proteins were immunolabelled on PK-treated and non-PK-treated samples using an anti-PrP antibody (Sha31, 1/40,000, Bertin pharma, [29]). Loading accuracy was controlled by tubulin detection on non-PK-treated samples (YOL1/34, 1/3000, Abcam, Cambridge, UK).

Mouse Lines and Ethical Statement

We used mice overexpressing ovine PrP (tg338 line previously described [30]). All animal experiments were carried out in strict accordance with EU directive 2010/63 and were approved by the author's institution local ethics committee (Comethea, INRA Agroparitech ethics committee, permit number 12/034) and by the French Ministry of Education, higher education and research (authorization no. 4292). All efforts were made to minimize suffering.

Drug Treatment of a Mouse Model for Prion Disease

Experiments were performed as previously described [5, 31]. Briefly, thirty 8-week-old female mice overexpressing ovine PrP (tg338 line [30]) were infected intracerebrally with 20 µL of the 127S scrapie strain at the 0.01% (*w/v*) dose, corresponding to $2 \times 10^{3.2}$ LD₅₀ (the infectious titre of 127S in tg338 mouse brain is $10^{9.2}$ LD₅₀/g of brain, [32]). Forty days post-infection, 9 mice were intraperitoneally injected 3 times per week with a dose of 100 µL at 4 mg/mL (20 mg/kg) of flunarizine solubilized in 8% DMSO. As negative controls, 11 mice were intraperitoneally injected 3 times per week with 100 µL of 8% DMSO. To avoid unethical suffering of prion-infected mice, the treatment was stopped around day 70 post-infection, when the first symptoms appeared in the control group of mice. Mice were euthanized at the terminal stage according to ethics rules. Spleens and brains were collected from 4 mice euthanized at the terminal stage of the disease and analysed for their PrP^{Sc} content, as previously described [5, 6, 32].

Luciferase Refolding Assay

[*psi-*] *Itv1Δ/hsp104Δ* 74-D694 *S. cerevisiae* yeast strain (ΔLΔH, [15]) was transformed with pDCM90 plasmid [33, 34], allowing constitutive expression of temperature-sensitive luciferase polypeptide (LuxAB). Transformants were exponentially grown at 29 °C. Cells were treated with the indicated concentrations of drugs or DMSO 2 h before the heat shock. The 100% reference corresponds to luciferase activity measured just before the heat shock. Luciferase was then heat-inactivated by incubation at 43.5 °C for 60 min.

Cycloheximide (10 µg/mL; Sigma-Aldrich, St. Louis, MO) was added after 45 min at 43.5 °C to prevent luciferase synthesis during recovery. Luciferase activity was immediately measured after 60-min post-heat shock (0 min) and cells were then left to recover at 29 °C for indicated times, luciferase activity being assessed at 90 and 150 min by adding 10 µL n-decylaldehyde (Decanal, Sigma-Aldrich) to 120 µL yeast culture. Luminescence was quantified using a Varioscan microplate reader (ThermoFisher). Luciferase activity at 0-, 90-, and 150-min recovery was then expressed as a percentage of the activity before the heat treatment for each strain (100%).

Drug Treatment of Prion-Infected Cultured Organotypic Cerebellar Slices

The anti-prion activity of drugs was evaluated using cultured organotypic cerebellar slices (OCSs), as described in [4] using tg338 transgenic mice overexpressing the VRQ allele of the ovine prion protein [31]. The preparation and culture of slices were performed as described in [4, 35] using insert (Millipore), except for prion infection which was performed the day of cerebellum slicing for 1 h on ice in 24-well plates (Starlab) with 50 µg/mL of a brain stock prepared from terminally ill tg338 mice experimentally infected with 127S prion strain. Seven days after infection, the drugs were added to the cerebellum slices at indicated concentrations. As a negative control, vehicle only (DMSO) was added. The slices were then cultured for an additional 21 days before harvesting. Fresh drugs were added at each medium change, 3 times per week, at indicated concentrations.

Pools of slices (at least seven slices/condition) were harvested by scrapping and homogenized in 350 µL of lysis buffer (0.5% sodium deoxycholate, 0.5% Triton-X100, 5 mM Tris-HCl pH 7.5) with the Beadbug homogenizer (Benchmark Scientific, Sayreville, NJ) as previously described [4]. Slice lysates (100 µg) were treated by 20 µg/mL proteinase K (PK, Thermo Scientific) for 30 min at 37 °C. For the analysis, PK-treated slice homogenates and 25 µg non-PK-treated slice homogenates were denatured for 10 min at 95 °C and then loaded on 10% SDS-polyacrylamide gels (NuPAGE, Invitrogen) and electrotransferred onto nitrocellulose membranes (GE Healthcare). PK-treated and non-PK-treated samples were immunoblotted with anti-PrP antibody (Sha31, 1/40,000, Bertin Pharma, Montigny-le-Bretonneux, France). Loading accuracy was controlled by immunoblotting 25 µg of non-PK-treated homogenates with anti-tubulin antibody (YOL1/34, 1/3000, Abcam).

Assessment of the Action of Drugs on Nuclear Aggregate Number in an OPMD Cell Line

The OPMD mutated PABPN1 (Ala17) stable cell line [36] was cultivated on a matrigel-coated surface (µ-slide 8 wells

ibidis, Biovalley) in DMEM medium supplemented with 20% of fetal calf serum (Invitrogen), 0.5% of chick embryo extract (MP Biomedicals, Irvine, CA), 100 U/mL penicillin-streptomycin antibiotic (ThermoFisher), 500 µg/mL geneticin (G418, Life Technologies, Carlsbad, CA) and 10 U/mL interferon gamma (Millipore/Merck, Darmstadt, Germany) at 33 °C in a humidified 5% CO₂ air atmosphere. At 80% confluence, Ala17 cells were differentiated in DMEM supplemented with 10% horse serum and 1% penicillin-streptomycin at 37 °C in a 5% CO₂, humidified atmosphere.

At 2 days of differentiation, half of the differentiation medium was replaced with fresh differentiation medium containing the drugs solubilized in DMSO at indicated concentrations. Two days later, cells were washed once in phosphate-buffered saline (PBS) and fixed for 15 min with 4% paraformaldehyde for PABPN1 immunostaining. Cells were then incubated for 15 min in PBS-0.1 M glycine and permeabilized in blocking buffer containing 3% BSA, and 5% Goat serum in PBS-0.2% Triton X-100. Anti-PABPN1 primary antibody (1/200, ab75855, Abcam) was incubated 1 h at room temperature in blocking buffer. Cells were washed five times with PBS-0.1% Triton X-100 prior incubation with goat anti-rabbit secondary antibody conjugated to Alexa Fluor 488 (Life Technologies) together with Phalloidin 555 (Interchim, Montluçon, France). Cells were then washed five times with PBS-0.1% Triton X-100, and stained with Hoechst for 10 min at room temperature. Pictures were taken with the ×40 objective and five to seven hundred nuclei were counted for each condition.

OPMD *Drosophila* Treatment

The *Drosophila* OPMD model contains an alanine-expanded mammalian PABPN1 cDNA (PABPN1-17ala) that is specifically and constitutively expressed in adult indirect flight muscles from the *Act88F-PABPN1-17ala* transgene [37]. Drug-supplemented food was prepared as follows: instant *Drosophila* medium (Carolina Biological Supply Company, Whitsett, NC) was reconstituted in each vial with a solution of 1% yeast in water, supplemented with indicated concentrations of either drug solubilized in DMSO or DMSO alone. Each vial contained 2 mL of reconstituted medium for twenty flies per vial. The flies were put on freshly prepared medium every day. Abnormal wing posture was quantified every day by pooling males by batches of five in an empty vial and scoring abnormal wing position by direct observation of the flies through the vials, without anaesthesia.

Results

Flunarizine Is a Potent Anti-prion Drug

Flunarizine (Fig. 1a) was identified in our laboratory through a yeast-based screening strategy and was shown to reduce the

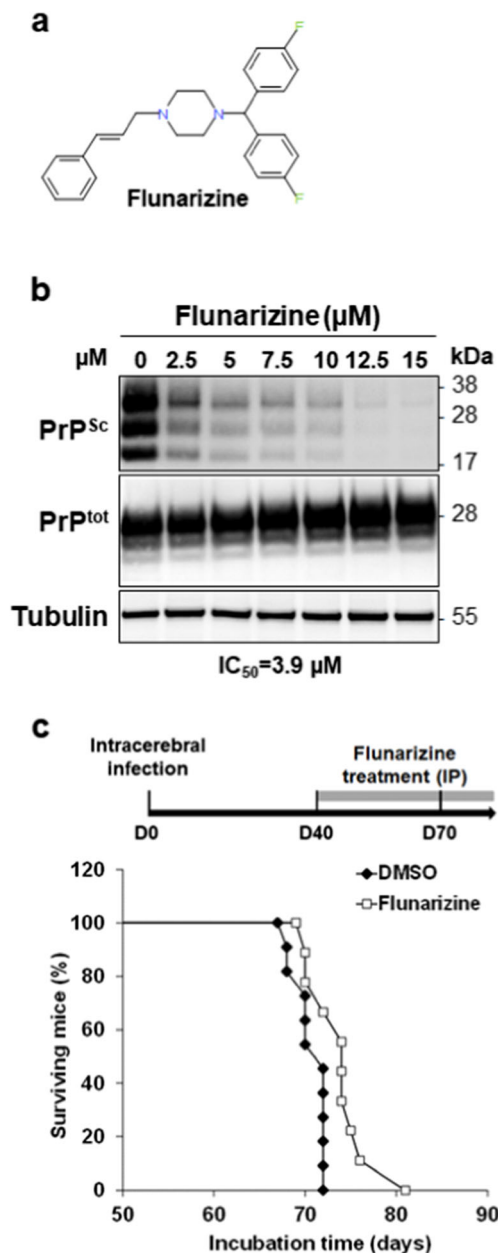


Fig. 1 Flunarizine is a potent anti-prion drug. Flunarizine was identified through a yeast-based screening strategy [7]. (a) Structure of flunarizine. (b) Flunarizine activity against PrP^{Sc} in chronically prion-infected MovS6 cells. Scrapie-infected MovS6 cells (127S prion strain, [28]) were treated for 6 days with a range of flunarizine concentrations. Cell lysates were then subjected to PK digestion to specifically reveal PrP^{Sc} by immunoblot. The effect of flunarizine on the steady state level of PrP (PrP^{tot}) was determined on the same MovS6-treated cell lysates in the absence of PK treatment (lower panel). The ratios of western blot PrP^{Sc}/PrP^{tot} signals are presented in the form of histograms below the blot. The same blot was used to check the loading homogeneity using an anti-tubulin antibody (bottom panel). The blot shown is representative of at least two independent experiments which all produced similar results. Flunarizine IC₅₀ is 3.9 μM. (c) Survival of intracerebrally infected mice treated with flunarizine. Tg338 transgenic mice overexpressing ovine PrP were intracerebrally infected with scrapie (strain 127S). Forty days post-infection, eleven mice were treated with DMSO as control, and nine mice were treated three times a week with 20 mg/kg of flunarizine solubilized in DMSO, until the appearance of the first clinical signs in mice from the control group at day 70, as illustrated by the top scheme. $p < 0.0146$, Kruskal-Wallis test

amount of PrP^{Sc} in chronically prion-infected MovS6 cells, with an IC₅₀ of 3.9 μM (Fig. 1b). Flunarizine is a selective calcium entry blocker as well as an antagonist of histamine H1 receptor that readily crosses the blood-brain barrier [38]. This drug is used in many developed countries (except USA, UK and Japan) to treat migraine headaches, occlusive peripheral vascular disease and vertigo of central and peripheral origin, and as an adjuvant in the therapy of epilepsy [38–42].

We next challenged the effect of flunarizine using an *in vivo* model of prion infection. We monitored the survival of tg338 mice intracerebrally infected with a relatively high dose of 127S prion at day 0, and treated by 20 mg/kg flunarizine three times per week from 40 days post-infection onward. Mice treated by flunarizine survived significantly longer than DMSO-treated mice ($p < 0.0146$, Kruskal-Wallis test) with a mean survival time of the flunarizine group mice increased by approximately 6% compared to the control group ($n = 9$ for flunarizine and $n = 11$ for DMSO, Fig. 1c). These results confirm that flunarizine is a potential anti-prion drug.

Identification of New Approved Anti-prion Drugs Based on Flunarizine Structure

In order to broaden the range of new anti-prion drugs that could potentially be used to treat patients with prion diseases, and considering that flunarizine might not be the most optimized anti-prion drug, we performed an *in silico* screening, ranking the 1887 FDA-approved or withdrawn drugs present in the Drugbank database (<https://www.drugbank.ca/>) for their structural similarities with flunarizine. In terms of pharmacophoric pattern, flunarizine is composed of three aromatic moieties, as well as two protonable nitrogen atoms, which can interact with specific biological targets through electrostatic or H-bond acceptor interactions (Fig. 2a). We chose to use the ROCS method from OpenEye Scientific, using a shape- and/or pharmacophore-based superposition method that finds similar but non-intuitive compounds. FDA-approved drugs exhibiting similarities with flunarizine were then ranked according to the scoring function ComboScore, which is based on a combination of shape and pharmacophore similarities. The resulting ranking was a manner of classifying the FDA-approved drugs according to their similarities with flunarizine (Table S1). As flunarizine is known to be a non-selective calcium channel blocker with antihistamine activity as well as other actions including serotonin receptor and dopamine D2 blocking activities, it was not surprising to find in the top 100 several compounds targeting histamine, muscarinic and dopamine, as well as both serotonin receptors and transporters.

From the ranking shown in Table S1, a series of thirty molecules was selected throughout the classification. According their ROCS ranks, we chose compounds exhibiting a high or a medium level of similarity with flunarizine, short-

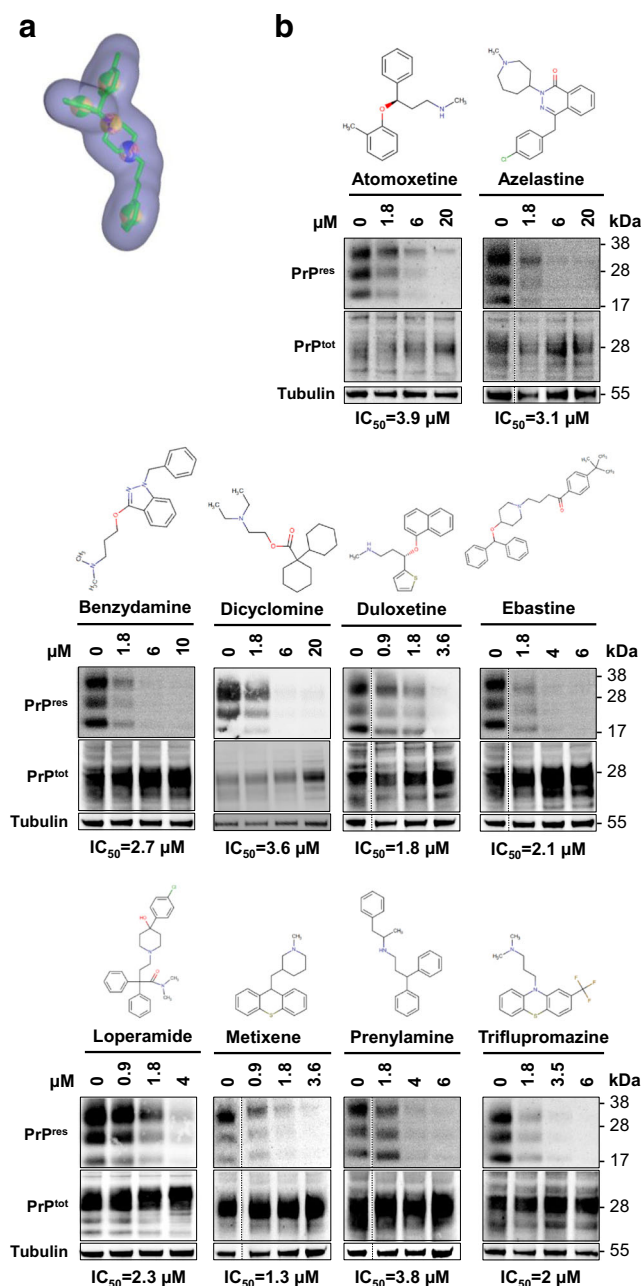


Fig. 2 Anti-prion activity of seventeen structural analogs of flunarizine. (a) Five-site pharmacophore model of flunarizine containing two nitrogen atoms (pink/yellow and blue/pink), and three aromatic rings (green/yellow). (b) Anti-prion activity of the ten most active ($\text{IC}_{50} \leq 4 \mu\text{M}$) flunarizine structural analogs using scrapie-infected MovS6 cells as described in Fig. 1. Cells were treated for 6 days with indicated drug concentrations, lysed and then subjected to PK digestion to specifically reveal PK-resistant PrP^{Sc} (PrP^{Pres}) by immunoblot. Structures of molecules and PrP^{Sc} immunoblots of MovS6-treated cells are presented on the top of each western blot. Drug-treated samples and their corresponding DMSO control were processed on the same immunoblot. The blots shown are representative of at least two independent experiments which produced similar results. The effect of compounds on the steady-state level of PrP (PrP^{Tot}) was determined on the same non-PK-treated cell lysates (middle panel). The same blot was used to check the loading homogeneity using an anti-tubulin antibody (bottom panel). The IC_{50} of each drug is indicated below the blot

listing compounds sharing targets with flunarizine, such as H1 receptor, as well as compounds targeting cellular pathways common or not with flunarizine (Table 1 and Table S2). In other words, we consider the ROCS ranking as a guideline to classify approved drugs exhibiting more or less structural similarities with flunarizine, and combine that with a diversity of addressed biological targets in order to select these thirty compounds. To evaluate the potential involvement of calcium channels in PrP^{Sc} propagation, three calcium antagonists were selected, although they are not closely related to the calcium antagonist flunarizine (ranking > 500) (ethosuximide, paramethadione, zonisamide, Table S2). Altogether, the thirty selected FDA-approved drugs represent thirteen different cellular targets (Table S2). In addition to the thirty selected drugs resulting from our similarity screening approach, we also selected three reference compounds, namely astemizole [43–45], clemastine [44] and thioridazine [44, 46] which have previously been shown to inhibit prion propagation (labelled # in Table 1; Table S1).

We first evaluated the anti-PrP^{Sc} activity of the thirty selected structural analogs of flunarizine using the MovS6 cell line infected by the 127S scrapie prion strain [30]. We showed that seventeen out of the thirty tested drugs showed anti-PrP^{Sc} activity, ten of which with an $\text{IC}_{50} \leq 4 \mu\text{M}$ (Fig. 2b) and 7 with an IC_{50} between 4 and 20 μM (Fig. S2), without affecting PrP^{Tot} level (Fig. 2b and Fig. S2). Thirteen out of the thirty selected drugs were inactive ($\text{IC}_{50} \geq 20 \mu\text{M}$; Fig. S3). Overall, this approach allowed us to identify seventeen new potential anti-prion FDA-approved drugs.

Flunarizine and Some of Its Structural Analogs Are Active Against PrP^{Sc} in Organotypic Cerebellar Slice Culture

We next evaluated the anti-prion activity of the 7 most active newly identified drugs (azelastine, duloxetine, ebastine, flunarizine, loperamide, metixene and triflupromazine) on organotypic cerebellum slice culture (OSC), a relevant alternative to animal experimentation which has been shown to closely mimic prion replication and propagation on a structured organ in which cohesion between cells and tissues is maintained [35]. The selected FDA-approved drugs are marketed to target different biological targets (GPCRs: H1, μ -opioid, 5-HT, D2, M1; transporters: SERT and NET; channels: VSCC). Cerebellum slices derived from ovine PrP transgenic mice (tg338 line) and infected with 127S prions [47] were treated for 21 days by the indicated concentrations of the drugs, 7 days post-infection. Tested at doses ranging from 20 to 35 μM , azelastine, duloxetine, ebastine, flunarizine, loperamide, metixene and the reference drug astemizole showed anti-PrP^{Sc} activity in OSC (Fig. 3), whereas triflupromazine and the reference drugs clemastine and thioridazine showed no activity. None of the active drugs except

Table 1 Anti-PrP^{Sc} IC₅₀ and ROCS ranks of flunarizine, the 30 selected flunarizine structural analogs and the three anti-prion references. For each drug, IC_{50s} against PrP^{Sc} in cell culture are indicated, together with ROCS ranks. # indicates drugs previously described as anti-PrP^{Sc}, * indicates withdrawn drugs

	Drugs	IC ₅₀	ROCS ranks	Drugs	IC ₅₀	ROCS ranks	
1	Alimemazine	11.4	197	18	Ethosuximide	Neg	1471
2	Antazoline	Neg	129	19	Flunarizine	3.9	1
3	Atomoxetine	3.9	41	20	Isradipine	Neg	327
4	Azelastine	3.1	140	21	Ketotifen	Neg	78
5	Benzylamine	2.7	368	22	Loperamide	2.3	25
6	Biperiden	9.2	33	23	Metixene	1.3	101
7	Cetirizine	Neg	10	24	Mirtazapine	Neg	225
8	Chloropyramine	18.5	133	25	Nefopam	Neg	471
9	Cinnarizine	Neg	2	26	Orphenadrine	9.1	16
10	Citalopram	10.4	23	27	Paramethadione	Neg	1475
11	Diazepam	Neg	595	28	Prenylamine*	3.8	6
12	Dicyclomine	3.6	112	29	Triflupromazine	2	256
13	Diphenhydramine	14.8	21	30	Zimelidine*	14.3	149
14	Diphenidol	Neg	24	31	Zonisamide	Neg	1216
15	Duloxetine	1.8	69	32	Astemizole*#	0.5	36
16	Ebastine	2.1	14	33	Clemastine#	2	27
17	Epinastine	Neg	683	34	Thioridazine#	1.7	159

loperamide affected the level of PrP^{tot}. Altogether, these data indicate the potency of azelastine, duloxetine, ebastine, flunarizine, loperamide and metixene to reduce PrP^{Sc} burden and highlight their therapeutic potential.

Flunarizine and Its Structural Analogs Are PFAR Inhibitors

We next wondered if the known targets of our selected active FDA-approved drugs could be involved in their anti-PrP^{Sc} activity. Despite the fact that some of them are not used for their H1 receptor antagonist property, seven out of the seventeen active drugs identified were able to antagonize histamine H1 receptor, suggesting that histamine H1 receptor could be involved in PrP^{Sc} clearance. We challenged this hypothesis by comparing the binding to H1 (H1 binding IC₅₀ from the literature) with the anti-PrP^{Sc} IC₅₀ of flunarizine and its 30 selected structural analogs. The three reference drugs astemizole, clemastine and thioridazine were also included. We found no correlation between H1 affinity and anti-PrP^{Sc} activity (Fig. S4). The fact that flunarizine was identified in an assay based on *S. cerevisiae* yeast cells that lack known homologues of histamine receptors [48] is an additional argument indicating that the H1 receptor is not involved in the anti-prion effect of the drug tested. Finally, since 5 out of the 13 inactive compounds tested also bind to H1 receptor, we concluded that H1 receptor is unlikely to be involved in PrP^{Sc} clearance. Altogether, this set of thirty-three anti-prion compounds binds to sixteen different cellular targets (Fig. S5), suggesting that no common mechanisms can explain their capacity to clear PrP^{Sc}.

As we recently identified PFAR as being involved in [PSI⁺] prion propagation in yeast [15], we sought to determine whether flunarizine and its structural analogs could also inhibit PFAR. To this aim, we monitored PFAR inhibition using a luciferase refolding assay in yeast, which measures renaturation of a heat-denatured luciferase over time [33]. We performed this assay in PFAR-enriched yeast cells that were deleted for the protein chaperon Hsp104, and that have previously been shown to be particularly suitable to monitor PFAR inhibition [15]. The PFAR assay is based on *S. cerevisiae* cells which are much less permeable to drugs than mammalian cells. However, as yeast cell permeability is different for each drug, the tested concentrations had to be individually adapted to each drug [4, 5, 14]. Yeast cells expressing luciferase were heat-shocked to unfold luciferase, and cycloheximide was added to inhibit translation and avoid *de novo* luciferase synthesis, in order to only monitor luciferase refolding by PFAR over time (Fig. S6). For each drug, a range of concentrations was tested. Luciferase activity remained basal in the presence of 45 μM ebastine, 100 μM metixene, 150 μM flunarizine, loperamide, duloxetine and azelastine, as well as 50 μM of the reference compound astemizole, indicating that these molecules are potent PFAR inhibitors (Fig. 4). On the contrary, antazoline and diazepam, which showed no anti-prion activity in cultured MovS6-infected cells (Fig. S3), were unable to inhibit PFAR (Fig. 4). Altogether, these data indicate that flunarizine and six of its anti-prion structural analogs astemizole, azelastine, duloxetine, ebastine, loperamide and metixene are PFAR inhibitors and share a common mechanism explaining their anti-prion activity.

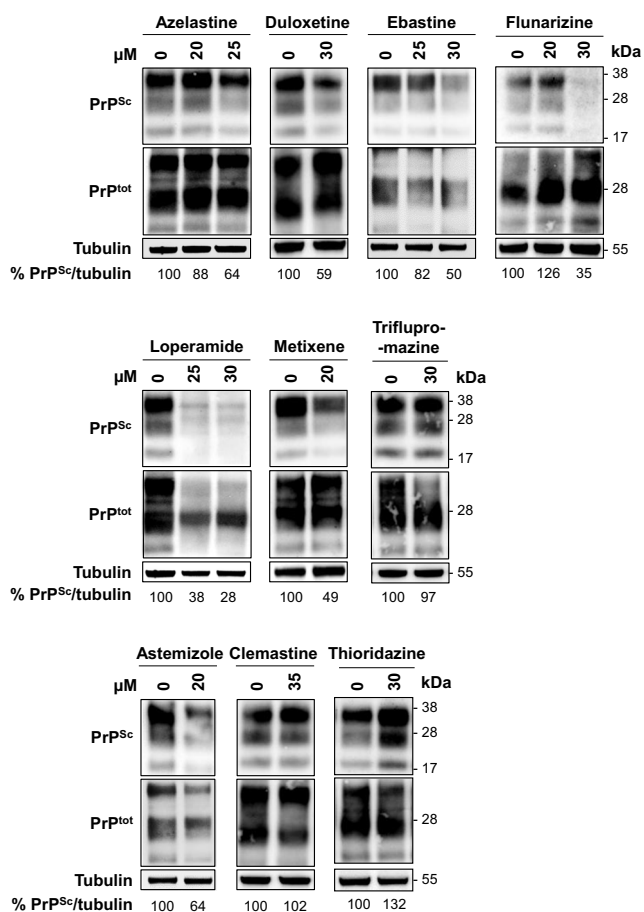


Fig. 3 Activity of flunarizine and 9 of its structural analogs on prion-infected organotypic cerebellar slice culture. Cerebellar slices from P9 to P12 tg338 transgenic mice were infected with 127S ovine prion strain. Seven days after infection, slices were treated with indicated concentrations of drugs for 21 days. Infected cerebellar slice lysates were subjected to PK digestion to specifically reveal PrP^{Sc} by western blot. The effect of compounds on the steady-state level of PrP (PrP^{tot}) was determined on the same slice lysates in the absence of PK treatment (middle panel). The same blot was used to check the loading homogeneity using an anti-tubulin antibody (bottom panel). The blots shown are representative of two to four independent experiments which all produced similar results. Ratios of western blot PrP^{Sc}/tubulin signals are indicated below each lane

Flunarizine Is Active in OPMD Cellular and *Drosophila* In Vivo Models

We previously showed that anti-PFAR and anti-prion drugs 6AP and GA efficiently decreased muscle degeneration in an OPMD *Drosophila* model, and had a synergistic effect with partial deletions of the DNA locus encoding rRNA that bear PFAR [23]. In addition, GA was also shown to reduce the size and number of PABPN1 nuclear aggregates in an OPMD Ala17 muscle cell model and improve muscle force *in vivo* in an OPMD mouse model [24]. Together, these data suggest that PFAR might also play a role in PABPN1 aggregation in OPMD. We therefore tested the efficiency of 10 of the newly identified anti-PFAR drugs in the OPMD Ala17 muscle cell

model. When differentiated, this myogenic cellular model expresses PABPN1-Ala17, resulting in the formation of a large number of nuclear aggregates [36, 49]. Drugs were added to the cells at day 2 of differentiation and maintained in the medium for 48 h. As 10 μM of GA was previously shown to significantly reduce the number and size of PABPN1-Ala17 aggregates in Ala17 differentiated cells [24], GA was used as a positive control. Similarly, anti-PFAR drugs were tested at 10 μM. In these conditions, treatment with 10 μM of flunarizine, metixene, thioridazine, astemizole, loperamide, duloxetine, azelastine and ebastine led to a significant reduction in the number of PABPN1-Ala17 aggregates (Fig. 5a), without altering cell differentiation. Moreover, flunarizine, metixene, thioridazine, loperamide and ebastine showed a better efficiency than GA (Fig. 5a) [24]. However, clemastine and triflupromazine, which showed no anti-PrP^{Sc} activity in OSC, also showed no significant reduction in the number of PABPN1-Ala17 aggregates, similarly to the negative control diazepam which showed neither anti-PFAR nor anti-prion activity (Fig. 5a).

As flunarizine, metixene and ebastine induced a strong reduction in PABPN1 aggregates in OPMD cells and were more efficient than GA (Fig. 5a), we next tested their effect in the OPMD *Drosophila* model. This model recapitulates the disease characteristics, namely progressive muscle weakness and degeneration, and formation of PABPN1 nuclear aggregates [37, 50]. Specifically, PABPN1-17ala expression in muscles leads to abnormal wing posture (Fig. S7a) resulting from affected indirect flight muscle function and muscle degeneration [23, 37]. The relevance of the *Drosophila* model has been validated by the fact that the molecular mechanisms identified as participating in OPMD in *Drosophila* have been confirmed in OPMD patient biopsies [37, 51]. As 2 mM GA was previously shown to significantly reduce the wing position defects of OPMD *Drosophila* when provided in the food from day 2 of adulthood [23], anti-PFAR drugs were also tested at 2 mM. However, at this concentration, flunarizine and ebastine showed toxicity. These two drugs were thus further used at 1.5 mM, concentration at which both drugs showed no toxicity (Fig. S7b). Drugs were administered to adult flies orally in the food at the indicated concentrations from day 2 to day 5, and fresh food with drug was provided every day. Wing posture defects were recorded from day 3 to day 6 of adulthood. The effect of drugs was quantified by recording the number of PABPN1-17ala-expressing flies with abnormal wing posture. Flunarizine, metixene and ebastine showed a beneficial effect with a significant decrease in the number of flies with abnormal wing posture compared to DMSO alone (Fig. 5b), without affecting *Drosophila* survival (Fig. S7b). Altogether, these data demonstrate that these new anti-PFAR drugs are active in alleviating defects in OPMD cell and *Drosophila* models. This indicates that these drugs might have a therapeutic potential for OPMD patients.

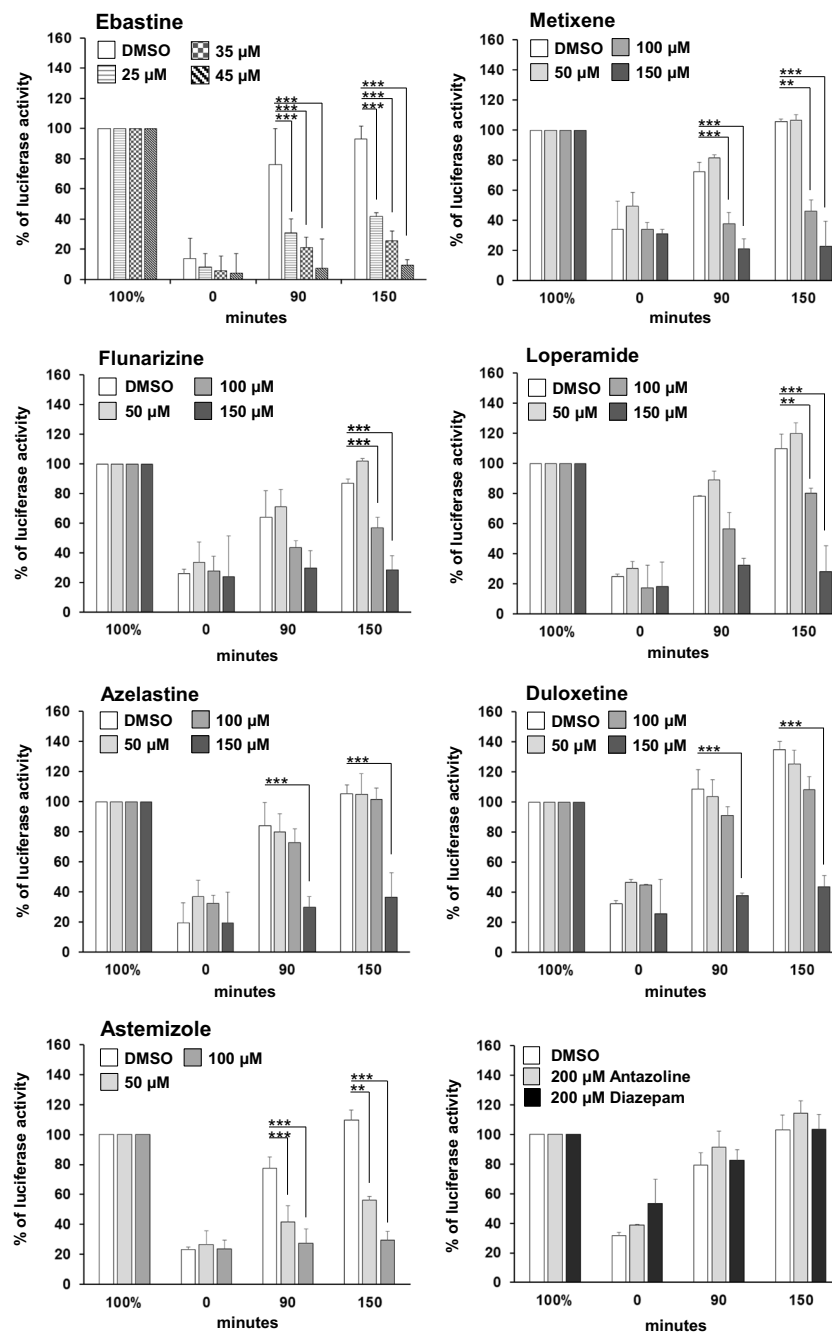


Fig. 4 PFAR activity monitoring in the presence of flunarizine and six of its most active structural analogs. The effect of astemizole, azelastine, duloxetine, ebastine, flunarizine, loperamide and metixene was tested on PFAR using a luciferase refolding assay in yeast cells (Fig. 4 Fig. S6). The heat shock reduced luciferase activity down to 20 to 30% of its initial level. In the presence of DMSO, luciferase activity increased over time (white bars), which indicates that it recovers its active conformation thanks to PFAR. In the presence of increasing concentrations of anti-PFAR drugs, luciferase activity recovery is less and less efficient. Ebastine showed a strong anti-PFAR activity from 25 μ M, astemizole

Discussion

We started this study by identifying flunarizine as an anti-prion drug in yeast and mammalian cells. We next established a proof

from 50 μ M, metixene and flunarizine from 100 μ M, and loperamide, azelastine, and duloxetine from 150 μ M. In the presence of antazoline and diazepam, luciferase activity increased over time as in the presence of DMSO, which indicates these two drugs do not inhibit PFAR, which is in good correlation with the fact they are inactive against PrP^{Sc}. Experiments were repeated two to three times. A representative assay including three technical repeats is shown with error bars. Bar height represents the mean relative to DMSO-treated cells at each time point; ** p value < 0.01 or *** p < 0.001, ANOVA one way

of principle that flunarizine exerts an anti-prion activity *ex vivo* and *in vivo*. The mice survival was significantly improved, despite the use of a very stringent transgenic mouse model, which overexpresses 8-fold ovine PrP and was inoculated by

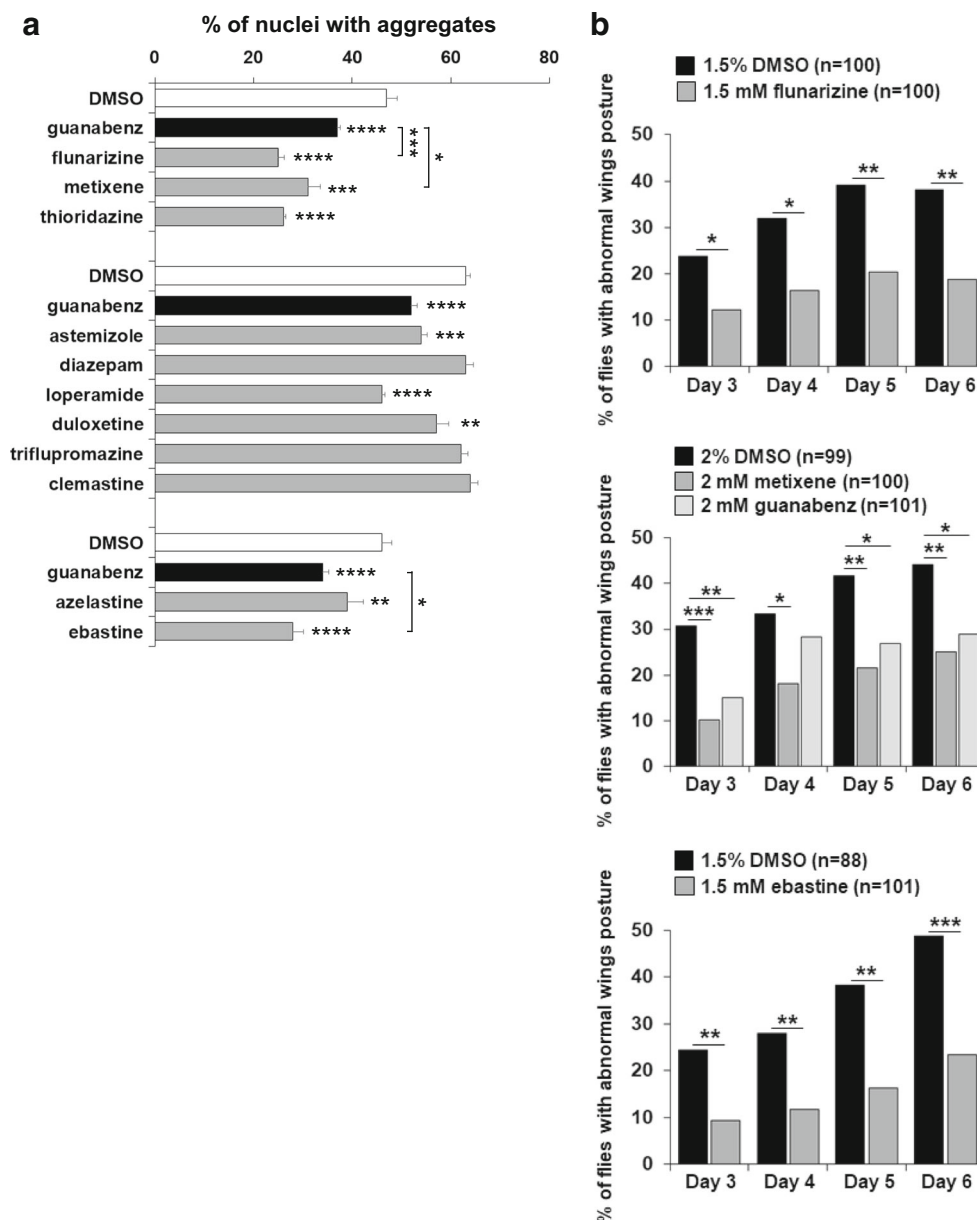


Fig. 5 Anti-PFAR drugs are active in OPMD cellular and *Drosophila* models. (a) Effect of flunarizine and nine of its structural analogs on PABPN1 nuclear aggregates in OPMD muscle cells during differentiation. OPMD cells were plated and left to differentiate. At 2 days of differentiation, 10 μ M drugs were added to the medium at indicated concentrations. At 4 days of differentiation, the myotubes were fixed, permeabilized and stained with PABPN1. Since the percentage of nuclei containing aggregates in the OPMD Ala17 cells is linked to the differentiation status of the cells [36, 49], the values obtained with DMSO differ between experiments. Therefore, for each independent experiment, DMSO and GA (10 μ M, positive control) were always added as internal controls to compare from one experiment to another the percentage of nuclei containing aggregates. For each condition, 500 to 700 nuclei were

counted. Diazepam (10 μ M) was used as a negative control. Values are expressed as means \pm SD compared to DMSO (** p value < 0.01, *** p < 0.001, **** p < 0.0001, unpaired t test) or GA (* p value < 0.05, *** p < 0.001, Turkey's multiple comparisons test). (b) Effect of flunarizine, metixene and ebastine on the wing posture defect in the *Drosophila* OPMD model (Fig. 5-Figure Supplement 1). Quantification of PABPN1-17ala-expressing flies (*Act88F-PABPN1-17ala/+*) showing wing position defects. Flies were fed either with DMSO or with the indicated concentration of flunarizine, metixene or ebastine at 25 $^{\circ}$ C. GA was used as a positive control. Feeding with GA or the three tested compounds led to a decreased number of flies with abnormal wing posture. * p value < 0.05, ** p < 0.01 and *** p < 0.001 using the chi-square test

the intracerebral route with a relatively high dose of prion. Furthermore, the treatment was initiated at the late stage of the disease and was administered by the intraperitoneal route.

Based on this promising result, we decided to look for other FDA-approved drugs and used a strategy mixing structural similarities with flunarizine (ROCS ranking) and diversity in the

addressed biological targets, leading to the selection of thirty drugs. The anti-prion activity of the selected molecules was mainly correlated with their ROCS ranking, as 76% of the selected drugs showing an anti-prion activity have a rank ≤ 200 (Fig. S8). These observations indicate that our screening strategy was relevant to select molecules active against PrP^{Sc} and it is possible that there are still some undescribed anti-prion drugs present in the top two hundred drugs shown in Table S1.

Thanks to this approach, we report here the identification of seventeen new anti-prion drugs that are FDA-approved for other pathologies and that could be readily usable to treat patients with prion diseases for whom no treatment is currently available. Notably, flunarizine and ten of these newly identified anti-prion drugs, namely atomoxetine, azelastine, benzydamine, dicyclomine, duloxetine, ebastine, loperamide, metixene, prenylamine and triflupromazine, have an $IC_{50} \leq 4 \mu\text{M}$ in mammalian cell culture. We further showed that azelastine, duloxetine, ebastine, flunarizine, loperamide and metixene are active against PrP^{Sc} in organotypic mouse cerebellum slice culture, thus emphasizing the therapeutic potential of these drugs for the treatment of prion diseases.

While we have identified anti-prion drugs exhibiting structural similarities, we may also have found a common mechanism explaining their anti-prion activity. Indeed, flunarizine and six of its most potent anti-prion structural analogs (astemizole, azelastine, duloxetine, ebastine, loperamide and metixene) are potent inhibitors of PFAR, a protein folding activity born by rRNA which has previously been shown to be involved in [PSI⁺] prion propagation [15]. PFAR has been described for the first time *in vitro* by the group of C. Das Gupta who showed that any denatured protein can recover its functional conformation thanks to domain V of 23S, 25S or 28S rRNA [12]. Ribosome-assisted folding was then described for ribosomes from all living kingdoms and for all classes and protein sources tested [12, 23], which is consistent with the high conservation of the sequence and the secondary structure of rRNA domain V [13]. We previously identified anti-prion drugs 6AP and GA which also target PFAR *in vitro* [9] as well as in living bacteria [9] and yeast [15]. Although the main role of protein chaperones is to prevent protein misfolding and aggregation, the implication of a chaperone such as PFAR in propagating protein prion conformation is intuitive, since amyloid proteins exist in several conformations and their replication corresponds to the propagation of these particular conformations. This is illustrated by the role of Hsp104, a protozoan-specific protein chaperone that, together with Hsp70 and Hsp40, is the only cellular factor known to date to be essential for prion propagation [PSI⁺] in yeast [52]. Using [PSI⁺] as a prion model, we showed that, similarly to Hsp104, PFAR is involved in prion propagation [15]. Therefore, since PFAR fosters the propagation of pathologic protein conformation, inhibiting PFAR will allow ones to reduce the burden of aggregation of these pathologic proteins. Our present data confirm the therapeutic potential of anti-PFAR

drugs and highlight this second enzymatic activity of the ribosome as an innovative therapeutic target for prion diseases.

OPMD is a dominant hereditary neuromuscular disease caused by the extension of a polyalanine repeat in the PABPN1 protein. Wild-type PABPN1 is localized in nuclear speckles, whereas polyalanine-expanded PABPN1 forms nuclear aggregates in skeletal muscle fibres, which is the pathological hallmark of OPMD. We thus evaluated the potential of anti-prion drugs on OPMD cellular and animal models. We previously showed that oral treatment with the anti-PFAR drugs 6AP and GA reduces OPMD-associated phenotypes, including defective wing posture due to thoracic muscle defects and the size of PABPN1 nuclear aggregates [23]. It should be noted that the OPMD *Drosophila* model previously allowed us to demonstrate a direct link between PFAR and the aggregation of mutated PABPN1 protein responsible for OPMD phenotypes [23]. Eight out of the ten new anti-PFAR drugs tested were also able to reduce the number of PABPN1 aggregates in a cell model of OPMD. Moreover, flunarizine, metixene and ebastine, which were more active than GA, were also able to alleviate muscle degeneration in the *Drosophila* OPMD model. Together, these data strengthen the role of PFAR in PABPN1 aggregation and highlight PFAR as a therapeutic target to treat OPMD. It is interesting to note that the activity spectrum of the drugs is remarkably similar for both diseases. Loperamide, which poorly crosses the blood-brain barrier, is a better candidate for OPMD than for prion diseases, but might be considered in prion diseases, to limit the peripheral propagation of PrP^{Sc} in acquired forms of the diseases.

Flunarizine and some of its anti-prion structural analogs identified in this study have been previously assessed in other neurodegenerative disorders, some of which were also proteinopathies. Furthermore, flunarizine has recently been shown to increase impedance in learning and memory in a murine pharmacological model of Alzheimer's disease [53]. Flunarizine has also been shown to have a beneficial effect in spinal muscular atrophy (SMA) models [54], a hereditary neurodegenerative disease characterized by the death of spinal cord motor neurons and skeletal muscle atrophy. Survival motor neuron (SMN) protein forms nuclear aggregates associated to Cajal bodies. Altered Cajal body localization of mutant SMN protein is a hallmark of SMA. Interestingly, flunarizine restores SMN localisation into the Cajal body of SMA-derived fibroblasts and spinal cord motor neurons in SMA mice, reduces the synaptic alterations of spinal cord motor neurons, improves motor function and expands survival of SMA mice [54]. Finally, flunarizine has also been shown to rescue the short lifespan of a *C. elegans* model of CLN3 disease (Spielmeier-Vogt-Sjogren-Batten disease), a paediatric-onset progressive neurodegenerative disease caused by recessive mutations in *CLN3*, in which the C subunit of mitochondrial ATP synthase is known to abnormally accumulate and which is characterized by progressive vision loss, seizures, loss of cognitive and motor function

and early death [55]. Loperamide has been shown to be able to clear A30P α -synuclein aggregates in a Parkinson's disease cellular model [56], and reduce the accumulation of polyQ misfolded proteins in a Huntington's disease cellular model [57] by promoting autophagic degradation. We cannot exclude that the anti-prion activity of some drugs like loperamide, which is known to induce cell autophagy, could also be due to their capacity to activate autophagy.

Altogether, these data reinforce the concept that prion diseases share common mechanisms of pathogenic protein conformation propagation with other proteinopathies. They also suggest that PFAR may be a pertinent therapeutic target common to several of these diseases. It seems increasingly obvious that the success of the fight against these pathologies will come from complementary and synergistic therapies. Together, these data show that, despite being fundamentally different diseases, the common protein aggregation hallmark of protein misfolding diseases is a potent therapeutic target that could be used in combination with drugs targeting specific hallmarks of each disease. In this respect, anti-PFAR drugs like astemizole, azelastine, duloxetine, ebastine, flunarizine, loperamide and metixene appear to be of particular interest.

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Compliance with Ethical Standards

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Competing Interests The authors declare that they have no competing interests.

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