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Structure–Activity Relationship Study around Guanabenz Identifies Two Derivatives Retaining Antiprion Activity but Having Lost α2-Adrenergic Receptor Agonistic Activity

Phu hai Nguyen,[†,#] Hassan Hammoud,[‡,#] Sophie Halliez,[§,#] Yanhong Pang,[∥] Justine Evrard,[†] Martine Schmitt,[‡] Nassima Oumata,[⊥] Jean-Jacques Bourguignon,[‡] Suparna Sanyal,[∥] Vincent Beringue,[§] Marc Blondel,[†] Frédéric Bihel,*[,‡,#] and Cécile Voisset*[,†,#]

[†]Inserm UMR 1078, Université de Bretagne Occidentale, Faculté de Médecine et des Sciences de la Santé; Etablissement Français du Sang (EFS) Bretagne; CHRU Brest, Hôpital Morvan, Laboratoire de Génétique Moléculaire, 29200 Brest, France

[‡]Laboratoire d'Innovation Thérapeutique, UMR7200, CNRS, Université de Strasbourg, Faculté de pharmacie, 74, route du Rhin, 67400 Illkirch, France

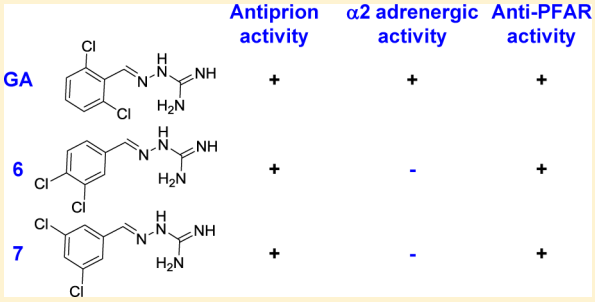
[§]Virologie Immunologie Moléculaires, UR892, Institut National de la Recherche Agronomique (INRA), 78352 Jouy-en-Josas, France

[∥]Department of Cell and Molecular Biology, Box-596, BMC, Uppsala University, 751 05 Uppsala, Sweden

[⊥]Laboratoire de Chimie Organique 2, Inserm U1022, Université Paris Descartes, 75006 Paris, France

Supporting Information

ABSTRACT: Guanabenz (GA) is an orally active α2-adrenergic agonist that has been used for many years for the treatment of hypertension. We recently described that GA is also active against both yeast and mammalian prions in an α2-adrenergic receptor-independent manner. These data suggest that this side-activity of GA could be explored for the treatment of prion-based diseases and other amyloid-based disorders. In this perspective, the potent antihypertensive activity of GA happens to be an annoying side-effect that could limit its use. In order to get rid of GA agonist activity at α2-adrenergic receptors, we performed a structure–activity relationship study around GA based on changes of the chlorine positions on the benzene moiety and then on the modifications of the guanidine group. Hence, we identified the two derivatives **6** and **7** that still possess a potent antiprion activity but were totally devoid of any agonist activity at α2-adrenergic receptors. Similarly to GA, **6** and **7** were also able to inhibit the protein folding activity of the ribosome (PFAR) which has been suggested to be involved in prion appearance/maintenance. Therefore, these two GA derivatives are worth being considered as drug candidates.



KEYWORDS: *Antiprion compounds, guanabenz, yeast model for prion diseases, PrP^{Sc} prion protein, structure–activity relationship study, α2-adrenergic agonist*

Since the outbreak of bovine spongiform encephalopathy and the emergence of its human counterpart, the new variant of Creutzfeldt–Jakob disease, a growing interest has been raised for these mammalian unconventional infectious prion diseases caused by the autocatalytic conversion of the host-encoded cellular prion protein PrP^{C} into an amyloidogenic and pathogenic isoform called PrP^{Sc}. Thousands of molecules have been screened for their antiprion capacities, and various chemical classes of compounds have been shown to present some antiprion activity when tested in vitro, ex vivo, and in vivo.[1−5] These compounds target either the expression or the stability of PrP, the conversion of PrP^{C} to PrP^{Sc}, or PrP^{Sc} propagation. Neuroprotecting compounds that either increase protein chaperone levels or reduce ER stress have also been identified,[4,6] but none of them can prevent or protect against prion diseases. It is now clear that therapies based on combinations of compounds will be necessary to restrain prion spreading and subsequent neurological damage.

Proteins behaving like prions have also been identified in the budding yeast *Saccharomyces cerevisiae*.[7] Among the yeast prions described so far, the most well-known are [PSI^{+}] and [URE3]. We set up a yeast-based assay whose aim was to identify compounds acting in trans, that is, targeting cellular pathways involved in the prion propagation and conserved from yeast to mammals,[8,9] instead of compounds acting in cis directly on PrP^{Sc} or PrP^{C}. Using this systematic approach to screen large chemical libraries, we determined that guanabenz (GA), an aminoguanidine derivative already in use for the treatment of

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hypertension under the trade name Wytensine, was active against both yeast and mammalian prions.[5] GA was further shown to inhibit the protein folding activity of the ribosome (PFAR), a second enzymatic activity borne by the large rRNA of the large subunit of the ribosome at the level of domain V, as peptidyl transferase activity.[10−13] These data suggest that GA-mediated inhibition of PFAR may be the cause of its antiprion activity and that PFAR may be involved in the prion propagation. Therefore, the treatment of prion-based diseases constitutes a potential new therapeutic indication for GA in addition to its current use as an antihypertensive agent.

Afterward, the effect of GA has also been studied in the context of endoplasmic reticulum stress. Treatment with a massive dose of GA over a short period was shown to rescue cells artificially stressed by the deleterious accumulation of misfolded proteins that yield to cellular proteostasis disruption.[14] In this study, the authors suggested that this protective effect of GA was due to its specific binding to PPP1R15A subunit which led to a transient increase of the phosphorylated eIF2α level. However, a later report from the same authors showed that a sustained increase of the phosphorylated eIF2α level was detrimental for the survival of prion-infected mice and that the decrease of the phosphorylated eIF2α level was neuroprotective.[15] Altogether with the fact that GA has been shown to have an antiprion effect at a much lower dose and through a prolonged treatment,[5,16] these data cast doubt on eIF2α phosphorylation as a bona fide therapeutic target for prion-based diseases. They rather suggest that GA mode of action as an antiprion drug is due to the inhibition of PFAR rather than PPP1R15A.

Recent evidence suggests that other neurodegenerative diseases linked to amyloid-forming proteins (e.g., tau and Aβ for Alzheimer's disease, αSyn for Parkinson's disease, FUS and TDP43 for amyotrophic lateral sclerosis, or Htt for Huntington's disease) may also involve conformational prion-like autocatalytic changes of their native cellular conformers into amyloid, thereby allowing their spread from cell to cell and thus the progression of the disease from one tissue to another.[17−21] In accordance with these studies, we recently showed that GA was able to suppress, in a drosophila model, all the typical features of OPMD (oculopharyngeal muscular dystrophy). This inherited dominant myodegenerative disease is caused by the extension of a polyalanine tract in PABPN1 protein, which causes its accumulation as amyloids within the nuclei of muscular cells, thereby leading to a muscular cell death and eventually to a muscle disorganization and degeneration.[16] GA has also been shown to reduce paralysis, neurodegeneration, and oxidative stress in *Caenorhabditis elegans* and *Danio rerio* models for the amyotrophic lateral sclerosis expressing mutant TDP-43.[22] The increasing understanding of the prionization process and the broadening list of antiprion compounds may thus benefit all proteinopathies. The quest for antiprion drugs with new cellular targets or original modes of action is thus an important issue which goes far beyond the rare prion-based diseases.

Guanabenz (GA) is an orally active α2-adrenergic agonist, an activity that is responsible for its anti-hypertensive effect. GA is able to cross the blood brain barrier.[23−25] GA has been safely used in clinic for more than 30 years on a daily basis, and pharmacological and toxicological analyses showed that GA treatment has no major side effects. However, in the perspective of using GA for the treatment of amyloid-based diseases, its potent antihypertensive activity becomes an annoying side-effect that could limit its use. We therefore sought to design chemical derivatives of GA that have retained potent antiprion activity but have lost their α2-adrenergic agonist activity for future use as therapy against prion and other amyloid-based diseases.

We first performed a structure−activity relationship (SAR) study around GA based on the changes of the chlorine positions on the benzene moiety and then on the modifications of the guanidine group. All GA derivatives were tested for their antiprion activity against PrP^{Sc}. We focused on two derivatives, **6** and **7**, which were more active against PrP^{Sc} than GA. In addition, our results show that compounds **6** and **7** appeared to be devoid of any agonist activity at α2-adrenergic receptors A, B, and C. They also show that, similarly to GA, their antiprion activity is linked to their ability to inhibit PFAR. GA derivatives **6** and **7** are thus worth being considered as drug candidates for the treatment of amyloid-based diseases and can also be valuable tools to decipher the biological role of PFAR, a protein chaperon activity which is still poorly understood.

RESULTS AND DISCUSSION

Structure−Activity Relationship (SAR) Study around GA. In order to identify some potent antiprion compounds devoid of α2-adrenergic agonistic activity, we synthesized a set of GA analogues that were tested for their activity against the mammalian prion PrP^{Sc} ex vivo. This assay is based on the use of the MovS6 murine peripheral neuroglial cell line. MovS6 cells express the ovine PrP encoding gene under the control of its endogenous promoter and are chronically infected by 127S sheep scrapie prion.[26,27] Prion infection is classically monitored in these cells by quantifying, by immunoblot or immunostaining, the levels of proteinase K (PK) resistant PrP^{Sc}, named PrP^{res}.[26] Indeed, while PrP^{C} is PK-sensitive, PrP^{Sc} is partially resistant due to conformational changes. MovS6 cells were treated for 6 days with a 0−20 μM concentration range of the various GA derivatives, and the amount of PrP^{res} was determined by western blot. The data are presented as histograms (Figures 1 and 2) depicting PrP^{res} levels in the culture at each drug concentration (Figure S1, Supporting Information).

As shown in Figure 1, the absence of chlorine (derivative **1**) led to a substantial loss of activity. The presence of one chlorine atom at position 2 (derivative **2**), 3 (derivative **3**), or 4 (derivative **4**) of the benzene ring increased the antiprion activity, but only at the concentration of 20 μM. 3-chlorobenzene and 4-chlorobenzene derivatives showed a similar or superior antiprion activity compared to that of GA. The insertion of a second chlorine atom led to more potent compounds. 2,4-dichloro (derivative **5**) and 3,4-dichlorobenzene (derivative **6**) derivatives showed a significant antiprion activity at 6 μM, but both compounds were toxic at 20 μM. In contrast, 3,5-dichlorobenzene derivative (derivative **7**) appeared very efficient even at 1.8 μM and was not toxic at the maximal tested concentration (20 μM). Next, we evaluated a varied fonctionalization of the guanidine moiety (Figure 2). We observed that, depending on the position of the chlorine atoms on the benzene ring, the modifications of the guanidine moiety had variable impacts on their antiprion activity. Consequently, the guanidine derivatives were synthesized using three different aromatic rings (2,6-dichlorobenzene, 3-chlorobenzene, and 3,4-chlorobenzene). The insertion of a methyl group at the terminal nitrogen ω did not affect the antiprion activity (derivatives **11** and **15**). In contrast, the introduction of a

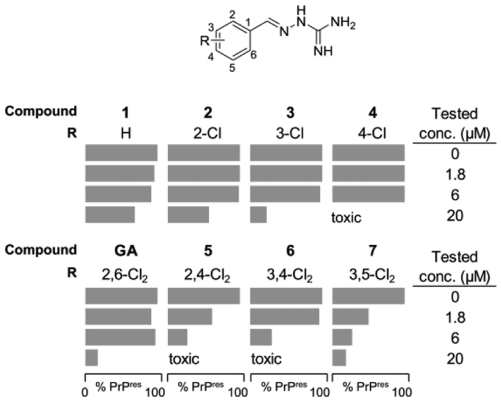

Figure 1. Molecular structure and anti-PrP^{Sc} activity of compounds from SAR around GA based on its benzene ring. This first set of SAR around GA was performed by modifying the position and the number of chlorine atoms on the benzene ring, without modification of the guanidine moiety. The antiprion activity of these seven GA derivatives was evaluated using scrapie-infected MovS6 cells. The cells were treated for 6 days with increasing amounts of compounds, lysed, and then subjected to PK digestion to specifically reveal PK-resistant PrP^{Sc} (PrP^{res}) by immunoblot. The effect of compounds on the steady state level of PrP (PrP^{tot}) was determined on the same MovS6-treated cell lysates in the absence of PK treatment (Figure S1). If the basal level of PrP^{tot} (PrP^{C} + PrP^{Sc}), which is determined by using cell lysates not treated by PK, remained unchanged upon a drug treatment, it indicates that the drug does not act by decreasing the level of PrP. The ratios of western blot PrP^{res}/PrP^{tot} signals are presented in the form of histograms for each compound.

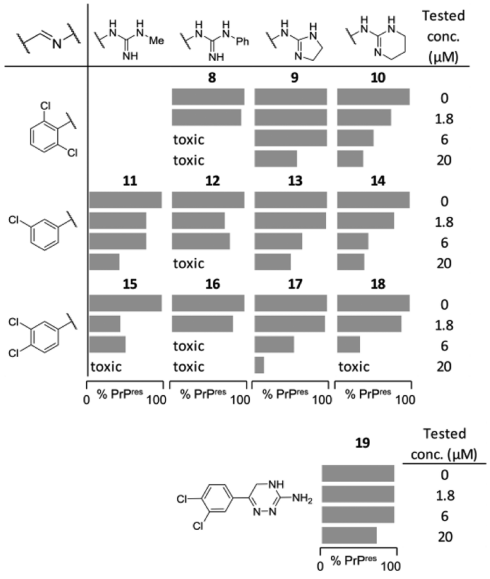

Figure 2. Molecular structure and anti-PrP^{Sc} activity of SAR around GA based on its guanidine moiety. A second set of SAR around GA was performed by modifying GA guanidine moiety using three different aromatic rings (2,6-dichlorobenzene, 3-chlorobenzene, and 3,4-chlorobenzene). The antiprion activity of these 12 GA derivatives was evaluated as described in Figure 1. The ratios of western blot PrP^{res}/PrP^{tot} signals are presented in the form of histograms for each compound.

phenyl group at the same position led to toxicity at 6 μM (derivatives **8** and **16**) or 20 μM (derivative **12**), without any improvement of the antiprion activity at 1.8 μM. The incorporation of guanidine into cyclic systems, such as dihydroimidazoylhydrazone (derivatives **9**, **13**, and **17**) and tetrahydropyrimidylhydrazone (derivatives **10**, **14**, and **18**), did not lead to any significant improvement of the antiprion activity. Finally, the scaffold rigidification led to constrained aminotriazine **19**, but this compound appeared to be inactive. In terms of physicochemical properties, most of the compounds exhibit similar properties to those of GA. For example, the log *P* of GA analogues **5**−**7** is equal to 1.35 (calculated at pH 7.4) and is exactly the same as that of GA. Consequently, the difference in the activity observed is unlikely due to a difference in cell permeation. Globally, the whole set of compounds show log *P* values in accordance with the Lipinski rules. In conclusion of this structure−activity relationship study, we showed that the positions of the chlorine atoms on the benzene ring appeared critical for the antiprion activity, highlighting 3,5-dichlorobenzene as the best combination. On the contrary, modifications of the guanidine moiety did not lead to any significant gain or loss of activity, except for the introduction of the phenyl moiety which induced toxicity.

GA derivatives depicted in Figures 1 and 2 were assayed for their activity against [*PSI*$^+$] and [URE3] yeast prions to assess whether they targeted some prionization mechanisms conserved from yeast to mammals. Briefly, [*PSI*$^+$] and [URE3] yeast cells were spread on a rich solid (agar-based) medium, small filters were placed on the agar surface, and then 50 nmol of each compound solubilized in DMSO were loaded on each filter. This yeast-based assay uses a colorimetric detection system in which the prion-containing yeast cells form pink or white colonies, depending on the strength of the prion strain used.[8,9] Yeast cells from which the prion has been eliminated (cured) form red colonies. An antiprion compound would thus lead to the formation of a halo of red colonies around the filter onto which it has been loaded. Among the 20 compounds tested, only 7 were active against PrP^{Sc}, [*PSI*$^+$] (STRg6 strain) and [URE3] (Figure S2A and Table S1). STRg6 yeast strain contains a strong [*PSI*$^+$] strain that is harder to cure than the weak [*PSI*$^+$] strain. We thus evaluated the antiprion activity of GA derivatives on a weak variant of [*PSI*$^+$] prion. As shown in Figure S2B (left panel), compounds **7** and **15** showed some activity against the weak [*PSI*$^+$] prion and the activity of **19** was confirmed. Compounds **12**, **16**, **17**, and **18** that were toxic on STRg6 strain were active against the weak [*PSI*$^+$] prion. Their toxicity was reduced probably because we used a WT strain whose permeability is lower than the one of STRg6 yeast strain in which *ERG6* gene has been deleted, thereby increasing yeast cell permeability. In order to enhance weak [*PSI*$^+$] strain permeability, the yeast medium was supplemented with a small quantity of SDS, a detergent that has recently been shown to increase yeast permeability without disturbing cell growth.[28] The presence of SDS at 0.003% allowed the detection of the antiprion activity of compounds **5** and **6** and confirmed the activity of **15** (Figure S2B, right panel). The antiprion activity of the 19 compounds analysed is summarized in Table S1. Despite the use of a weaker prion strain and the increase of yeast cells' permeability, some discrepancies between the activity of the compounds against the mammalian and yeast prions were observed. Dissimilarities for compounds **1** and **2** may be due to slight differences between species in the conserved targeted mechanisms. For compound **8**, this disparity is probably due to the high toxicity of the compound for MovS6 cells. These discrepancies between the activity of the compounds against the mammalian prion and yeast prions may also be due to a lower stability of some compounds on the yeast

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acidic solid medium, an enhanced metabolic degradation by yeast cells, or an increased rejection by multidrug resistance (MDR) pumps which exist in yeast as well as in mammals. In light of this SAR study around GA showing that the modification of the position of the chlorine residues was associated with an antiprion activity change (Figure 1), which was not the case for the modification of the guanidine moiety (Figure 2), we chose to focus our analysis on the two most promising compounds, **6** and **7**. Tighter concentration ranges of **6** and **7** were tested on MovS6 cells to define their IC_{50}. Compared to GA for which the IC_{50} is 12.5 ± 2.7 μM, the IC_{50} for **6** is 2.8 ± 1.3 μM, whereas the one for **7** is 1.1 ± 0.5 μM (Figure S3).

6 and 7 Are Not Agonists of α2-Adrenergic Receptors. GA is a potent nonselective α2-adrenergic agonist, clinically used for treating hypertension. In the absence of hypertension, GA administration led to side effects such as dizziness or weakness. To our knowledge, no structure−activity relationship study has ever been reported for GA activity toward α2-adrenergic receptors. The agonist activity of the two most promising compounds **6** and **7** at the human α2-adrenergic receptors was thus evaluated using CHO cells overexpressing one of the three human α2-adrenergic receptor isoforms (α2A-AR, α2B-AR, or α2C-AR). The activity of **6** and **7** was compared to the activity of epinephrine (α2A-AR and α2C-AR) or dexmedetomidine (α2C-AR), which are potent α2-adrenergic receptor agonists. The data presented in Figure 3 clearly show that neither **6** nor **7** displays any agonist activity at the human α2-adrenergic receptors up to 3 μM, indicating that these two potent antiprion compounds are deprived of the agonist activity that is responsible for GA antihypertensive action. In contrast, GA is known to exhibit agonist activities in the nanomolar range for the three α2-adrenergic receptor subtypes (K_i of α2A-AR = 2.0 nM; K_i of α2B-AR = 2.9 nM; K_i of α2C-AR = 0.9 nM[29]). Moreover, these data confirm our previously published data suggesting that GA antiprion activity is not due to its α2-adrenergic agonist activity,[5] which is confirmed by its activity in yeast that does not contain any kind of α2-adrenergic receptors. Compounds **6** and **7** are thus worth being considered as promising antiprion drug candidates as they have lost their ability to activate α2-adrenergic receptors.

6 and 7 are Inhibitors of the Protein Folding Activity of the Ribosome (PFAR). In previous studies, we showed that GA is a specific inhibitor of PFAR. Apart from the peptidyl transferase activity involved in translation, PFAR is a second rRNA-borne function of the ribosome that is still unsung but which may play a central role in the propagation of prions.[13,30−32] As for the peptidyl transferase activity, rRNA nucleotides involved in PFAR are located in domain V of the large rRNA component of the ribosomal large subunit.[10−13,33,34] Because compounds **6** and **7** are close derivatives of GA, their anti-PFAR capacity was tested. For that purpose, we used an in vitro assisted folding assay based on the refolding of a denaturated enzyme, human carbonic anhydrase-1 (hCA-1), by purified ribosomes. hCA-1 correct refolding was assessed by following the reappearance of its enzymatic activity in comparison to native hCA-1. Self-folding, that is, hCA-1 capacity to recover an active conformation without any assistance, was about 25%. PFAR displayed by purified *Escherichia coli* ribosomes restored about 65% of hCA-1 activity (Figure 4). Compounds **6** and **7** had no effect on hCA-1 self-folding but strongly inhibited the protein folding activity of 70S ribosomes (Figure 4), in the same range as that of GA.[31]

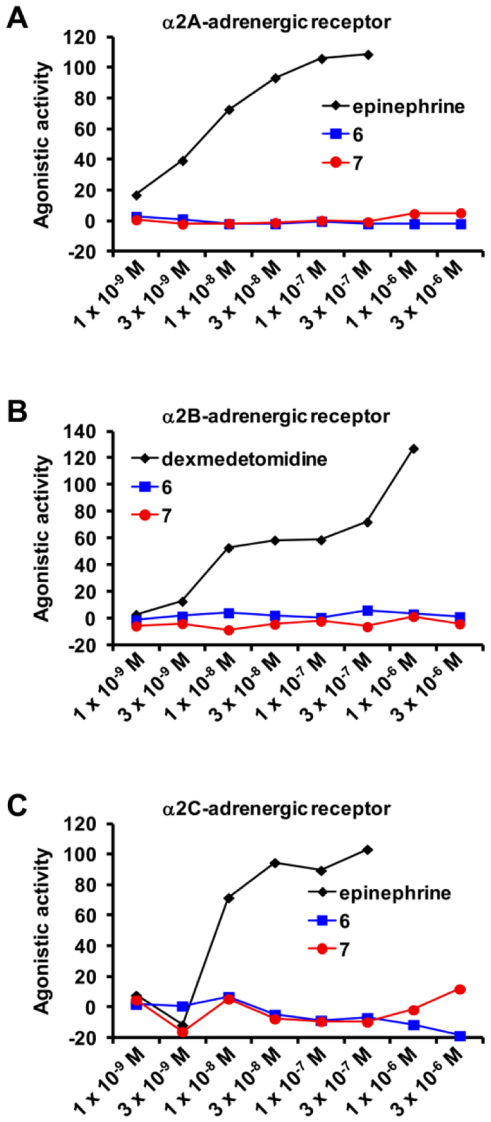

Figure 3. Agonistic activity of **6** and **7** at the human α2-adrenergic receptors. The agonistic activity of compounds **6** and **7** was evaluated using cells overexpressing one of the three human α2-adrenergic receptor isoforms α2A (A), α2B (B), or α2C (C). The results are depicted as percentages of the agonist activity of compounds **6** and **7** at α2-adrenergic receptors compared to DMSO. Epinephrine was used as an agonistic control for α2A-AR (EC_{50} of 5.5 × 10^{-9} M) and α2C-AR (EC_{50} of 9.3 × 10^{-9} M), and dexmedetomidine was used as an agonistic control for α2B-AR (EC_{50} of 2.8 × 10^{-8} M).

Altogether, these data indicate that **6** and **7** conserved the anti-PFAR properties of GA and also reinforce that PFAR might be involved in prion propagation both in yeast and mammals, as confirmed by our recent in vivo data (C.V., J.E., P.h.N., and M.B. unpublished data).

6 and 7 are Able to Reduce PrP^{Sc} Propagation in Cultured Organotypic Cerebellar Slices (OCSs). The antiprion activity of **6** and **7** was finally evaluated using prion infected cultured organotypic cerebellar slices (OCSs). OCSs correspond to slices of the cerebellum of transgenic mouse pups overexpressing ovine PrP that are kept in culture. This assay allows ex vivo prion replication under conditions closely resembling an intracerebral infection and thus enables the evaluation of the antiprion drug effect in a cerebral context. OCSs have been shown to retain the cerebellar architecture

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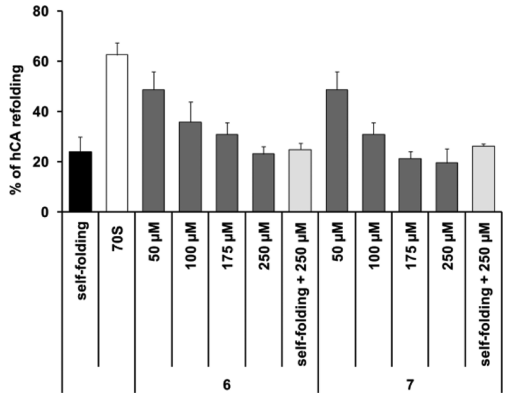

Figure 4. In vitro anti-PFAR activity of GA derivatives **6** and **7**. The effect of compounds **6** and **7** on the ribosome-assisted folding of denatured hCA-1 was evaluated. The correct refolding was assessed by measuring the recovery of hCA-1 enzymatic activity as a function of time in comparison to that of the native enzyme stored undiluted on ice (normalized to 100%). hCA-1 self-refolding was about 25% (black bar). In the presence of 70S ribosome, refolding increased up to 65% due to PFAR (white bar). In the presence of 50−250 μM of compounds **6** and **7**, PFAR was inhibited in a concentration dependent manner (dark gray bars), but **6** and **7** had no effect on the self-folding of denatured hCA-1 at 250 μM (light gray bars).

and, when infected by scrapie PrP^{Sc}, display classical hallmarks of prion diseases such as prion replication, spongiform changes, and neurodegeneration, although neuronal loss is only a late effect that can be observed from 42 days of culture.[35] OCSs also present the significant advantage of minimizing the number of sacrificed animals[36] and of considerably shortening the time-scale of the experiment as PrP^{Sc} can be detected only 2 weeks after the prion inoculation from OCS overexpressing ovine PrP (*tg*338 mouse strain) without any amplification step (S.H., V.B. unpublished data).

Compound **6**, **7** and GA intrinsic toxicity on WT OCSs was first evaluated. In the absence of treatment (Figure S4, panel A) or in the presence of any of the drugs tested up to 6 μM (Figure S4, panels B−D) or DMSO control (Figure S4, panel E), Purkinje cells (GABAergic neurons located in the cerebellum, green staining) were abundant in cerebellar slice cultures and presented highly branched dendrites, indicating that the tested drugs were not toxic for this cell type. In the presence of 20 μM GA, very few Purkinje cells could be observed (Figure S4, panel F). In the presence of 20 μM of compound **6** (Figure S4, panel G) or compound **7** (Figure S4, panel H), Purkinje cells were less abundant than those in the control slices, and the few Purkinje cells present were dying, as indicated by the absence or scarce presence of dendrites (Figure S4, panels A and E) and the presence of fragmented dendritic trees (arrowheads Figure S4, panels G and H). When tested at 20 μM (Figure S4, panels J−L), none of the drugs tested showed toxicity for astrocytes (anti-GFAP, red staining) that presented the same aspect as in the presence of DMSO control alone (Figure S4, panel I). None of the drugs (Figure S4, panels N−P) were toxic at 20 μM for granular cells (cerebellar neurons, which mainly correspond to the layers of highly dense nuclei stained by DAPI), as thick layers of nuclei were preserved in almost all the slices cultivated as in DMSO control alone (Figure S4, panel M). At 40 μM of any of the drugs tested, massive cell loss (astrocytes and neurons) was observed (data not shown).

OCSs were prion-infected after 1 week of culture, cultivated during 1 additional week to allow the prion replication, and then treated during 2 weeks with various concentrations of **6**, **7**, and GA as the reference compound. The treatment was not carried out concomitantly with the infection in order to prevent any side effects on the initial uptake of the inoculum by the slices and also to match medical treatment conditions. OCSs were then either lysed to determine the presence of PrP^{res} (Figure 5) or immunostained to control the integrity of OCSs

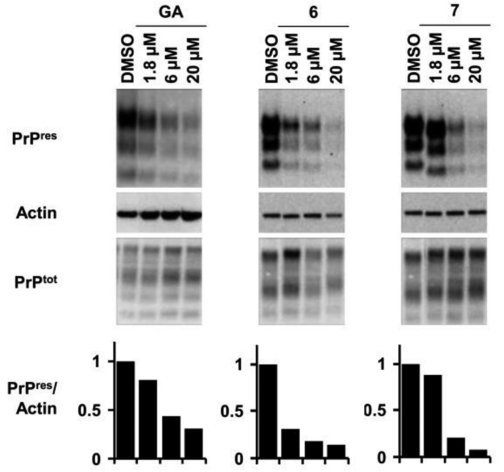

Figure 5. Antiprion activity of GA derivatives 6 and 7 using cultured organotypic cerebellar slices. The cerebellar slices from *tg*338 transgenic mice were prion infected after 7 days and were treated 7 days after infection with increasing concentrations of GA, **6** or **7**. Three weeks after infection, among which was 2 weeks of treatment, the cerebellar slice cultures infected by 127S prion strain were harvested for protein analyses by western blot. OCS lysates were subjected to PK digestion to specifically reveal PrP^{res} by immunoblot. The effect of compounds on the steady state level of PrP (PrP^{tot}) was determined on the same OCS lysates in the absence of PK treatment (lower panel). The same blot was used to check the loading homogeneity using anti-actin antibodies (middle panel). Ratios of western blot PrP^{res}/PrP^{tot} signals are indicated below each lane. The blots shown are representative of three independent experiments which all produced similar results.

(Figure S5). Similarly to what was observed when using prion-infected cells (MovS6, Figures 1, 2, and S1), the quantity of PrP^{res} was reduced on the slices treated by 1.8, 6, and 20 μM of compounds **6** and **7** despite a slight toxicity at 20 μM (Figure S4). Compounds **6** and **7** showed a better antiprion activity than GA at 6 μM.

As 127S prion strain mainly replicates in astrocytes but not in neurons (unpublished observations), we determined whether the antiprion effect of compounds **6**, **7**, and GA did not rely on the astrocyte death. In the presence of any of the drugs tested or DS500 positive control (Figure S5, panels B−D and F−L), astrocytes of OCSs infected by 127S prion strain presented the same aspect as in the presence of DMSO control alone (Figure S5, panels A and E), suggesting that the tested drugs were not more toxic for astrocytes infected by 127S prion than for noninfected astrocytes, and that their antiprion activity did not occur through the death of astrocytes. The antiprion effect of compounds **6** and **7** did not rely either on a decrease of PrP^{C} expression level as PrP^{tot} was not affected in the presence of any of the drugs tested (Figures 5 and S5). Thick layers of nuclei mainly corresponding to granular cells were largely preserved in

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the presence of any of the drugs tested or DS500 (Figure S5, panels N–P and R–X) as in the presence of DMSO control alone (Figure S5, panels M and Q), suggesting that the tested drugs are not toxic for granular cells in the context of a prion infection. Nevertheless, some areas devoid of granular cells can be found in slices independently of the drug applied and its concentration and even in the presence of DMSO control alone (arrowheads, Figure S5), suggesting that granular cells are particularly sensitive in the context of the prion infection, independently of the applied treatment. Altogether, these OCS data suggest that compounds **6** and **7** display a potent antiprion activity in vivo.

This structure–activity relationship study around GA thus allowed the identification of compounds **6** and **7**, two potent antiprion and anti-PFAR compounds devoid of any agonist activity at α2-adrenergic receptors. **6** and **7** are thus worth to be considered as drug candidates to treat amyloid-based diseases.

METHODS

Synthesis of GA Derivatives. See the Supporting Information.

PrP^{Sc} Clearance Assay in MovS6 Cells. Experiments were performed as previously described.[3,26] See the Supporting Information for further details.

Yeast-Based Assay. The yeast strains used in this study were STRg6 (74-D694, Mata, *ERG6::TRP1*, *ade1–14*, *trp1–289*, *his3Δ200*, *ura3–52*, *leu2–3,112*) containing the strong [PSI^+] prion strain, WT (74-D694 Mata, *ade1–14*, *trp1–289*, *his3Δ200*, *ura3–52*, *leu2–3,112*) containing the weak [PSI^+] prion strain, and SB34 (Mata, *ERG6::TRP1*, *dal5::ADE2*, *ade2–1*, *trp1–1*, *leu2–3,112*, *his3–11,15*, *ura2::HIS*) containing [URE3] prion. Yeast cells were grown and used as previously described.[8] Experiments were performed as previously described.[3,8,37] See the Supporting Information for further details.

α2-Adrenergic Agonistic Activity Assays. The agonistic activity of compounds at α2-adrenergic receptors was performed using CHO cells endogenously expressing human α2A-AR (CEREP, #G120-2558, according to ref 38), α2B-AR (CEREP, # G011-1813, according to ref 39), and α2C-AR receptors (CEREP, # G006-1736, according to ref 40). See the Supporting Information for further details.

In Vitro Ribosome Assisted Protein Folding Assay. 70S *E. coli* ribosomes were prepared using sucrose gradient zonal ultracentrifugation as previously described,[41] and refolding experiments in vitro were performed as previously described.[31] See the Supporting Information for further details.

Ethics Statement. The animal experiments were carried out in strict accordance with EU directive 2010/63 and were approved by the local ethics committee of the authors' institution (Comethea, INRA Agroparitech ethics committee, permit number 12/034). All efforts were made to minimize suffering.

Organotypic Slice Cultures (OCSs). The antiprion activity of drugs was evaluated using cultured organotypic cerebellar slices (OCSs). Cerebella were dissected from 9–11 day old *tg*338 transgenic mice overexpressing VRQ allele of the ovine prion protein.[42] The preparation and culture of slices were performed as described in the protocol published for the Prion Organotypic Slice Culture Assay[36] except for prion infection. Briefly, cerebella were embedded in 2.5% low melting point agarose (Invitrogen) dissolved in Gey's balanced salt solution (Eurobio) supplemented with 1 mM of glutamate receptor antagonist kynurenic acid (Sigma) and 33 mM of glucose (Sigma). Slices of 350 mm thickness were cut on a vibratome (HM650 V, Microm), recovered from agarose, and then placed on 6-well Millicell culture inserts (Millipore) in groups of six to eight slices. The inserts were transferred to a cell culture plate and cultured in a sterile slice culture medium (SCM) composed of 50% minimum essential medium (Gibco), 25% Basal Medium Eagle (Gibco), and 25% horse serum (Gibco) and supplemented with glucose, penicillin/streptomycin, and stable glutamine (PAA). The slices were cultured at 37 °C in a humidified atmosphere with 5% CO_2, and the culture medium was replaced three times a week. A 10^{-3} dilution of brain stock prepared from terminally ill *tg*338 mice experimentally infected with 127S prion strain was prepared in SCM. The brain dilution (2 μL/slice, i.e., 2 μg of brain tissue per slice) was applied on the slices after 7 days in vitro (DIV). After 14 DIV (i.e., 7 DIV postinoculation), the drugs were added to SCM at 1.8, 6, and 20 μM. As a negative control, vehicle only (DMSO) was added to SCM. DS500 (dextran sulfate 500, 5 μg/mL in SCM) was used as a positive antiprion control. The slices were then cultured for an additional 14 DIV before harvesting. Fresh drug was added at every medium change at indicated concentrations. The slices were harvested for protein analyses or immunochemical analyses after 28 DIV, i.e., 3 weeks after infection among which was 2 weeks of drug treatment.

Toxicity Assay of Antiprion Drugs. The cellular toxicity of antiprion drugs was tested on cerebellar slice cultures established from 9–11 day old C57BL/6 mice prepared in the conditions previously described. After 1 DIV, the drugs to test were added to SCM at 6, 20, and 40 μM. As negative controls, SCM with and without DMSO only (vehicle) were used. The slices were then cultured for an additional 20 DIV, which corresponds to a treatment 6 days longer than the one applied to prion-infected OCSs. The immunofluorescence staining of Purkinje cells, astrocytes and nuclei was performed as described below.

PrP^{res} Immunoblot on Slice Cultures. Pools of slices (at least 4 slices/condition) were harvested by scrapping and homogenized at 20 μg/μL in 5% sterile glucose solution with a Rybolyser (Hybaid, Middlesex, U.K.). PrP^{res} was extracted following the Bio-Rad test protocol[43] by using 20 μg/mL proteinase K (PK) for 10 min at 37 °C. For analysis, samples corresponding to 25 μL of homogenate (~1 slice equivalent) were processed and then loaded on a SDS-polyacrylamide gel. For normalization purposes, 5 μL of non-PK-treated homogenate was also loaded on a SDS-polyacrylamide gel. After denaturation, the samples were run on 12% Criterion XT Bis-Tris gels (Bio-Rad), electrotransferred onto nitrocellulose membranes (Bio-Rad), and immunoblotted with anti-PrP antibody Sha31b[44] for PK-treated samples and anti-β-actin antibody (Sigma) for non-PK-treated samples. The immunoreactivity was revealed by chemiluminescence (reagents by Amersham Pharmacia Biosciences), imaged via a GeneGnome machine (Syngene) and GeneSnap software (Syngene), and quantified via GeneTools software (Syngene).

Immunofluorescence Staining on Slice Cultures. The slices not harvested for the immunoblot analysis were fixed in 4% paraformaldehyde for 45 min. Purkinje cells (anti-Calbindin, Chemicon, 1:1000) and astrocytes (antiglial fibrillary acidic protein GFAP, DAKO, 1:500) were specifically labeled overnight. The slices were then incubated for 3 h with appropriate 1:500 Alexa 488 (anti-Calbindin green staining) or Alexa 555 (anti-GFAP red staining) conjugated secondary antibodies (Invitrogen), and nuclei were stained using DAPI nuclear marker (4′,6-diamidino-2-phenylindole, Sigma) for 30 min and finally mounted in Fluoromount (Sigma). Fluorescence was imaged via a Zeiss microscope and AxioVision software (Zeiss).

ASSOCIATED CONTENT

Supporting Information

PrP^{res} levels in the culture at each drug concentration (Figure S1), compounds' activity against [PSI^+] and [URE3] prions (Figure S2), tighter concentration ranges of GA, **6**, and **7** tested on MovS6 cells to define their IC_{50} (Figure S3), and compound **6**, **7**, and GA intrinsic toxicity on WT OCS evaluation (Figure S4) and immunostaining to control the integrity of OCSs (Figure S5). This material is available free of charge via the Internet at http://pubs.acs.org/.

AUTHOR INFORMATION

Corresponding Authors

*E-mail: fbihel@unistra.fr.
*E-mail: cecile.voisset@univ-brest.fr.

Author Contributions

[#]P.h.N., H.H., and S.H. contributed equally to this work. F.B. and C.V. contributed equally.

Author Contributions

H.H. and M.S. did compound synthesis; P.h.N and J.E. did prion-based experiments; C.V., M.B., P.h.N., and J.E. analysed prion-based data; F.B., C.V., H.H., N.O., and J.J.B. analysed RSA data; CEREP performed agonistic activity assays; C.V. and F.B. analysed agonistic activity data; Y.P. did PFAR assays; Y.P., S.S., C.V. and M.B. analysed PFAR data; S.H. did OSC-based assays; S.H., V.B. and C.V. analysed OSC-based data; C.V., F.B., M.B., and S.H. wrote the manuscript.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Kamatari, Y. O., Hayano, Y., Yamaguchi, K., Hosokawa-Muto, J., and Kuwata, K. (2013) Characterizing antiprion compounds based on their binding properties to prion proteins: implications as medical chaperones. *Protein Sci. 22*, 22−34.

(2) Karapetyan, Y. E., Sferrazza, G. F., Zhou, M., Ottenberg, G., Spicer, T., Chase, P., Fallahi, M., Hodder, P., Weissmann, C., and Lasmezas, C. I. (2013) Unique drug screening approach for prion diseases identifies tacrolimus and astemizole as antiprion agents. *Proc. Natl. Acad. Sci. U.S.A. 110*, 7044−7049.

(3) Oumata, N., Nguyen, P. H., Beringue, V., Soubigou, F., Pang, Y., Desban, N., Massacrier, C., Morel, Y., Paturel, C., Contesse, M. A., Bouaziz, S., Sanyal, S., Galons, H., Blondel, M., and Voisset, C. (2013) The toll-like receptor agonist imiquimod is active against prions. *PLoS One 8*, e72112.

(4) Sim, V. L. (2012) Prion disease: chemotherapeutic strategies. *Infect. Disord.: Drug Targets 12*, 144−160.

(5) Tribouillard-Tanvier, D., Beringue, V., Desban, N., Gug, F., Bach, S., Voisset, C., Galons, H., Laude, H., Vilette, D., and Blondel, M. (2008) Antihypertensive drug guanabenz is active in vivo against both yeast and mammalian prions. *PLoS One 3*, e1981.

(6) Boyce, M., Bryant, K. F., Jousse, C., Long, K., Harding, H. P., Scheuner, D., Kaufman, R. J., Ma, D., Coen, D. M., Ron, D., and Yuan, J. (2005) A selective inhibitor of eIF2alpha dephosphorylation protects cells from ER stress. *Science 307*, 935−939.

(7) Liebman, S. W., and Chernoff, Y. O. (2012) Prions in yeast. *Genetics 191*, 1041−1072.

(8) Bach, S., Talarek, N., Andrieu, T., Vierfond, J. M., Mettey, Y., Galons, H., Dormont, D., Meijer, L., Cullin, C., and Blondel, M. (2003) Isolation of drugs active against mammalian prions using a yeast-based screening assay. *Nat. Biotechnol. 21*, 1075−1081.

(9) Bach, S., Tribouillard, D., Talarek, N., Desban, N., Gug, F., Galons, H., and Blondel, M. (2006) A yeast-based assay to isolate drugs active against mammalian prions. *Methods 39*, 72−77.

(10) Chattopadhyay, S., Das, B., and Dasgupta, C. (1996) Reactivation of denatured proteins by 23S ribosomal RNA: role of domain V. *Proc. Natl. Acad. Sci. U.S.A. 93*, 8284−8287.

(11) Das, B., Chattopadhyay, S., Bera, A. K., and Dasgupta, C. (1996) In vitro protein folding by ribosomes from Escherichia coli, wheat germ and rat liver: the role of the 50S particle and its 23S rRNA. *Eur. J. Biochem. 235*, 613−621.

(12) Das, D., Das, A., Samanta, D., Ghosh, J., Dasgupta, S., Bhattacharya, A., Basu, A., Sanyal, S., and Das Gupta, C. (2008) Role of the ribosome in protein folding. *Biotechnol. J. 3*, 999−1009.

(13) Voisset, C., Thuret, J. Y., Tribouillard-Tanvier, D., Saupe, S. J., and Blondel, M. (2008) Tools for the study of ribosome-borne protein folding activity. *Biotechnol. J. 3*, 1033−1040.

(14) Tsaytler, P., Harding, H. P., Ron, D., and Bertolotti, A. (2011) Selective inhibition of a regulatory subunit of protein phosphatase 1 restores proteostasis. *Science 332*, 91−94.

(15) Moreno, J. A., Radford, H., Peretti, D., Steinert, J. R., Verity, N., Martin, M. G., Halliday, M., Morgan, J., Dinsdale, D., Ortori, C. A., Barrett, D. A., Tsaytler, P., Bertolotti, A., Willis, A. E., Bushell, M., and Mallucci, G. R. (2012) Sustained translational repression by eIF2alpha-P mediates prion neurodegeneration. *Nature 485*, 507−511.

(16) Barbezier, N., Chartier, A., Bidet, Y., Buttstedt, A., Voisset, C., Galons, H., Blondel, M., Schwarz, E., and Simonelig, M. (2011) Antiprion drugs 6-aminophenanthridine and guanabenz reduce PABPN1 toxicity and aggregation in oculopharyngeal muscular dystrophy. *EMBO Mol. Med. 3*, 35−49.

(17) Brundin, P., Melki, R., and Kopito, R. (2010) Prion-like transmission of protein aggregates in neurodegenerative diseases. *Nat. Rev. Mol. Cell Biol. 11*, 301−307.

(18) Costanzo, M., and Zurzolo, C. (2013) The cell biology of prion-like spread of protein aggregates: mechanisms and implication in neurodegeneration. *Biochem. J. 452*, 1−17.

(19) Frost, B., and Diamond, M. I. (2010) Prion-like mechanisms in neurodegenerative diseases. *Nat. Rev. Neurosci. 11*, 155−159.

(20) Goedert, M., Clavaguera, F., and Tolnay, M. (2010) The propagation of prion-like protein inclusions in neurodegenerative diseases. *Trends Neurosci. 33*, 317−325.

(21) Jucker, M., and Walker, L. C. (2013) Self-propagation of pathogenic protein aggregates in neurodegenerative diseases. *Nature 501*, 45−51.

(22) Vaccaro, A., Patten, S. A., Aggad, D., Julien, C., Maios, C., Kabashi, E., Drapeau, P., and Parker, J. A. (2013) Pharmacological reduction of ER stress protects against TDP-43 neuronal toxicity in vivo. *Neurobiol. Dis. 55*, 64−75.

(23) Holmes, B., Brogden, R. N., Heel, R. C., Speight, T. M., and Avery, G. S. (1983) Guanabenz. A review of its pharmacodynamic properties and therapeutic efficacy in hypertension. *Drugs 26*, 212−229.

(24) Schafer, S. G., Kaan, E. C., Christen, M. O., Low-Kroger, A., Mest, H. J., and Molderings, G. J. (1995) Why imidazoline receptor modulator in the treatment of hypertension? *Ann. N.Y. Acad. Sci. 763*, 659−672.

(25) Sica, D. A. (2007) Centrally acting antihypertensive agents: an update. *J. Clin. Hypertens. 9*, 399−405.

(26) Archer, F., Bachelin, C., Andreoletti, O., Besnard, N., Perrot, G., Langevin, C., Le Dur, A., Vilette, D., Baron-Van Evercooren, A., Vilotte, J. L., and Laude, H. (2004) Cultured peripheral neuroglial cells are highly permissive to sheep prion infection. *J. Virol. 78*, 482−490.

(27) Vilotte, J. L., Soulier, S., Essalmani, R., Stinnakre, M. G., Vaiman, D., Lepourry, L., Da Silva, J. C., Besnard, N., Dawson, M., Buschmann, A., Groschup, M., Petit, S., Madelaine, M. F., Rakatobe, S., Le Dur, A., Vilette, D., and Laude, H. (2001) Markedly increased susceptibility to natural sheep scrapie of transgenic mice expressing ovine prp. *J. Virol. 75*, 5977−5984.

(28) Fernandez-Acero, T., Rodriguez-Escudero, I., Vicente, F., Monteiro, M. C., Tormo, J. R., Cantizani, J., Molina, M., and Cid, V. J. (2012) A yeast-based in vivo bioassay to screen for class I phosphatidylinositol 3-kinase specific inhibitors. *J. Biomol Screening 17*, 1018−1029.

(29) Piletz, J. E., Zhu, H., and Chikkala, D. N. (1996) Comparison of ligand binding affinities at human I1-imidazoline binding sites and the

high affinity state of alpha-2 adrenoceptor subtypes. *J. Pharmacol. Exp. Ther. 279*, 694−702.
(30) Pang, Y., Kurella, S., Voisset, C., Samanta, D., Banerjee, D., Schabe, A., Das Gupta, C., Galons, H., Blondel, M., and Sanyal, S. (2013) The antiprion compound 6-Aminophenanthridine inhibits protein folding activity of the ribosome by direct competition. *J. Biol. Chem. 288*, 19081−19089.
(31) Reis, S. D., Pang, Y., Vishnu, N., Voisset, C., Galons, H., Blondel, M., and Sanyal, S. (2011) Mode of action of the antiprion drugs 6AP and GA on ribosome assisted protein folding. *Biochimie 93*, 1047−1054.
(32) Tribouillard-Tanvier, D., Dos Reis, S., Gug, F., Voisset, C., Beringue, V., Sabate, R., Kikovska, E., Talarek, N., Bach, S., Huang, C., Desban, N., Saupe, S. J., Supattapone, S., Thuret, J. Y., Chedin, S., Vilette, D., Galons, H., Sanyal, S., and Blondel, M. (2008) Protein folding activity of ribosomal RNA is a selective target of two unrelated antiprion drugs. *PLoS One 3*, e2174.
(33) Kudlicki, W., Coffman, A., Kramer, G., and Hardesty, B. (1997) Ribosomes and ribosomal RNA as chaperones for folding of proteins. *Folding Des. 2*, 101−108.
(34) Sanyal, S. C., Bhattacharyya, D., and Das Gupta, C. (2002) The folding of dimeric cytoplasmic malate dehydrogenase. Equilibrium and kinetic studies. *Eur. J. Biochem. 269*, 3856−3866.
(35) Falsig, J., Sonati, T., Herrmann, U. S., Saban, D., Li, B., Arroyo, K., Ballmer, B., Liberski, P. P., and Aguzzi, A. (2012) Prion pathogenesis is faithfully reproduced in cerebellar organotypic slice cultures. *PLoS Pathog. 8*, e1002985.
(36) Falsig, J., and Aguzzi, A. (2008) The prion organotypic slice culture assay—POSCA. *Nat. Protoc 3*, 555−562.
(37) Tribouillard, D., Bach, S., Gug, F., Desban, N., Beringue, V., Andrieu, T., Dormont, D., Galons, H., Laude, H., Vilette, D., and Blondel, M. (2006) Using budding yeast to screen for anti-prion drugs. *Biotechnol. J. 1*, 58−67.
(38) Bavadekar, S. A., Ma, G., Mustafa, S. M., Moore, B. M., Miller, D. D., and Feller, D. R. (2006) Tethered yohimbine analogs as selective human alpha2C-adrenergic receptor ligands. *J. Pharmacol. Exp. Ther. 319*, 739−748.
(39) Eason, M. G., Kurose, H., Holt, B. D., Raymond, J. R., and Liggett, S. B. (1992) Simultaneous coupling of alpha 2-adrenergic receptors to two G-proteins with opposing effects. Subtype-selective coupling of alpha 2C10, alpha 2C4, and alpha 2C2 adrenergic receptors to Gi and Gs. *J. Biol. Chem. 267*, 15795−15801.
(40) Regan, J. W., Kobilka, T. S., Yang-Feng, T. L., Caron, M. G., Lefkowitz, R. J., and Kobilka, B. K. (1988) Cloning and expression of a human kidney cDNA for an alpha 2-adrenergic receptor subtype. *Proc. Natl. Acad. Sci. U.S.A. 85*, 6301−6305.
(41) Ederth, J., Mandava, C. S., Dasgupta, S., and Sanyal, S. (2009) A single-step method for purification of active His-tagged ribosomes from a genetically engineered Escherichia coli. *Nucleic Acids Res. 37*, e15.
(42) Le Dur, A., Beringue, V., Andreoletti, O., Reine, F., Lai, T. L., Baron, T., Bratberg, B., Vilotte, J. L., Sarradin, P., Benestad, S. L., and Laude, H. (2005) A newly identified type of scrapie agent can naturally infect sheep with resistant PrP genotypes. *Proc. Natl. Acad. Sci. U.S.A. 102*, 16031−16036.
(43) Deslys, J. P., Comoy, E., Hawkins, S., Simon, S., Schimmel, H., Wells, G., Grassi, J., and Moynagh, J. (2001) Screening slaughtered cattle for BSE. *Nature 409*, 476−478.
(44) Feraudet, C., Morel, N., Simon, S., Volland, H., Frobert, Y., Creminon, C., Vilette, D., Lehmann, S., and Grassi, J. (2005) Screening of 145 anti-PrP monoclonal antibodies for their capacity to inhibit PrPSc replication in infected cells. *J. Biol. Chem. 280*, 11247−11258.