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Treatment with a Non-toxic, Self-replicating Anti-prion Delays or Prevents Prion Disease *In vivo*

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SUMMARY

Transmissible Spongiform Encephalopathies (TSEs) are fatal neurological disorders caused by prions, which are composed of a misfolded protein (PrP^{Sc}) that self-propagates in the brain of infected individuals by converting the normal prion protein (PrP^C) into the pathological isoform. Here, we report a novel experimental strategy for preventing prion disease based on producing a self-replicating, but innocuous PrP^{Sc}-like form, termed anti-prion, which can compete with the replication of pathogenic prions. Our results show that a prophylactic inoculation of prion-infected animals with an anti-prion delays the onset of the disease and in some animals completely prevents the development of clinical symptoms and brain damage. The data indicate that a single injection of the anti-prion eliminated ~99% of the infectivity associated to pathogenic prions. Furthermore, this treatment caused significant changes in the profile of regional PrP^{Sc} deposition in the brains of animals that were treated, but still succumbed to the disease. Our findings provide new insights for a mechanistic understanding of prion replication and support the concept that prion replication can be separated from toxicity, providing a novel target for therapeutic intervention.

INTRODUCTION

Prions are proteinaceous particles that are able to self-propagate within hosts in a similar way as classic infectious agents¹. Prions are composed mainly or solely by a misfolded

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Supplemental Data

Additional figures are available as supplementary information.

AUTHORS CONTRIBUTIONS

R.D.E. performed most of the *in vitro* experiments, designed the *in vivo* and *in vitro* experiments and prepared most of the figures. R.M. performed all the i.c. inoculations, designed the *in vivo* experiments and participated in lesion profile assessments. L.C.M. purified recombinant PrP, performed part of the biochemical analysis and carried out the experiments of *in vitro* competition by PMCA. I.M.G and F.M. did the histological work and analysis and I.M.G prepared the histology figures. C.S. conceived, supervised the work and prepared the final version of the manuscript.

Conflict of Interest

The authors declare no conflict of interest with any of the studies reported in this paper

protein and they rely on the presence of their normally folded counterpart protein as substrate to perpetuate their abnormal conformations. In mammals, prions are mainly known for their involvement as causative agents of TSEs². These are fatal neurological disorders characterized by brain dysfunction, neuronal death and widespread motor impairment. In humans, Creutzfeldt-Jakob disease (CJD) is the most common type of TSE.

The TSE infectious agent is composed of aggregates of variable size consisting of a misfolded version of the prion protein, termed PrP^{Sc}. PrP^{Sc} is able to self-propagate its abnormal conformation in the brain and several peripheral tissues of affected individuals³. The substrate for PrP^{Sc} propagation is a membrane-attached protein (termed PrP^C) of identical sequence that is constitutively expressed in different tissues including the brain and spleen⁴. During conversion, PrP^{Sc} aggregates are believed to directly interact with PrP^C monomers, introducing them into the polymers and forcing a template-guided conformational change that leads to growing PrP^{Sc} particles^{5,6}. Large particles are later fragmented by a yet unknown mechanism, releasing more seeds for further prion replication in an autocatalytic manner^{5,6}. The exclusively proteinaceous nature of prions was proposed as part of the protein-only hypothesis⁷. However, a structural and functional involvement of accessory molecules such as lipids and RNA has emerged as an essential requirement for the generation of *bona fide* infectious prions^{8,9}. *In vitro* reconstitution experiments have shown that both brain-derived and recombinant PrP can be converted into PrP^{Sc}-like aggregates that produce a TSE disease upon inoculation on animal models¹⁰⁻¹².

The relationship between PrP^{Sc} self-propagation and brain toxicity is not well understood. PrP-knockout mice do not show clear neurological disorder, suggesting that PrP^C depletion during prion propagation is not a likely source of toxicity¹³. Moreover, the development of clinical symptoms appears to be temporally disengaged from prion accumulation¹⁴⁻¹⁷. Considerable amounts of PrP^{Sc} in the brain of affected animals can be observed well before the onset of the clinical stage, which suggests that widespread PrP^{Sc} self-propagation can occur without immediate neuronal damage¹⁶. Furthermore, transgenic mice expressing a secreted form of PrP, that lacks the GPI-anchoring motif, accumulate large quantities of PrP^{Sc} but have only minimal clinical alterations¹⁸.

As part of our studies to generate infectious prions in the test tube from recombinant prion protein, we tried many conditions to induce misfolding, including addition of salts, denaturant agents, detergents, etc. Although for various of these conditions we were able to generate protease-resistant PrP with similar biochemical properties to PrP^{Sc} (ref¹⁹), including the ability to self-propagate *in vitro*, the majority of the preparations were not infectious in animals. A similar experience has been reported by others²⁰⁻²³. Interestingly, although inoculation of some of these preparations in wild type animals did not lead to disease in the first passage, sometimes disease was observed after successive passages in animals^{24,25}. It was also reported that some of these preparations were able to induce the accumulation of PrP^{Sc}-like aggregates in the animal, despite the absence of disease or any indication of neurodegeneration²⁴⁻²⁶. Considering that pathogenic and non-pathogenic forms of PrP^{Sc} replicate at expenses of the same substrate (PrP^C), we hypothesized that it might be possible to generate innocuous variants of PrP^{Sc} (here referred as “anti-prions”) which upon replication may interfere with disease-causing prions and delay the onset of the

disease. The process of prion interference has been illustrated before by injecting animals with two different strains of pathogenic prions with distinct incubation periods in specific hosts^{27–32}. Detailed *in vitro* and *in vivo* experiments indicated that strain interference was due to competition for the PrP^C substrate or a cellular factor implicated in prion conversion³³.

The main goal of this study was to test the idea that artificially produced, non-pathogenic PrP^{Sc}-like forms can self-propagate and outcompete the replication of pathogenic prion strains, delaying or even suppressing neurodegenerative and clinical abnormalities produced by pathological prions. This strategy may be expanded in the future to develop potential treatments for more prevalent protein misfolding diseases that have been shown to spread using the prion principle, such as Alzheimer's or Parkinson's disease^{1,34,35}. The most interesting aspect of this approach is that a single injection of the molecule is enough to produce the desired outcome, suggesting that the anti-prion self-replicates in the body producing more of the interfering molecule. The findings generated in this study may also contribute to understand the relationship between prion replication and pathogenicity.

MATERIALS AND METHODS

Purification of recombinant PrP

Purification of recombinant PrP (rPrP) was conducted as reported earlier¹⁹. An expression plasmid vector containing the sequence of PrP was commercially obtained (DNA 2.0) and transformed into DH10B *E. coli* cells (InVivoGen®). The cells were IPTG-induced at an optical density of 0.6–0.9 and then grown for several hours at 37°C with proper agitation and aeration. After centrifugation, the cell pellets were lysed by lysozyme treatment followed by sonication. The resulting inclusion bodies were then centrifuged at 22,000 x g and the pellet was washed several times with washing buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 100 mM NaCl, 0.05% Triton X-100) to remove attached contaminants. The solubilization of the inclusion bodies was performed in binding buffer (10 mM Tris-HCl, 100 mM Na₂HPO₄, pH 8.0, 100 mM NaCl, 10 mM β-mercaptoethanol, 6 M GdnCl) for 2 hrs at room temperature under mild agitation. Non-solubilized debris was ultracentrifuged at 100,000 x g and the recombinant PrP contained in the supernatant was purified by IMAC chromatography. Briefly, the supernatant was batch-incubated in a Nickel-charged sepharose resin previously equilibrated with binding buffer. Recombinant PrP bound to the column was on-column-refolded through gradient-exchange from binding buffer to refolding buffer (10 mM Tris-HCl, 100 mM Na₂HPO₄, pH 8.0, 100 mM NaCl) followed by elution on refolding buffer supplemented with 500 mM imidazole. The eluted recombinant PrP solution was then desalted by using Zeba-desalting columns according to manufacture instructions (Pierce®) and then flash-frozen and stored at –80°C. Purity and protein structure was checked by SDS-PAGE and Circular Dichroism, respectively.

Preparation of AP1

To produce AP1, full-length hamster rPrP was aggregated following a previously established protocol with minor modifications^{24,36}. Following lyophilization, recombinant PrP was resuspended in phosphate buffered saline (PBS) containing 6 M GdmCl and incubated for

several minutes. The sample was then supplemented to achieve the final composition of the solution reaction (PBS pH 7.0, 1 M GdmCl, 3M Urea and 150 mM NaCl). The reaction was incubated for several days at 37°C under 600 RPM agitation in a Thermomixer (Eppendorf®) until fibrils were visible to the naked eye. Formation of fibrils was confirmed by adding 10 µM Thioflavin-T and checked by fluorescence using excitation at 435 nm and recording the emission spectra from 450 to 600 nm. Recombinant PrP aggregates were dialyzed against PBS, pH 7.0 and then subjected to an annealing procedure as described before^{24,36}. Briefly, a 1:1 mixture of recombinant PrP aggregates and 10% hamster normal brain homogenate was submitted to cycles of heat and cooling using a PCR machine (5 cycles of 1 min incubation at 80°C followed by 1 min at 37°C). This sample was called AP1. As negative control (buffer), we used the buffer sample from the dialysis that was also submitted to the same annealing procedure. The annealing procedure was performed just before starting the infectivity experiments.

Infectivity assays

AP1, buffer and 263K were injected intra-cerebrally (5 µl) in 3–4 weeks old female Golden Syrian hamsters at different times as indicated in the figures. Animals were visually inspected in a weekly manner for the appearance of clinical symptoms. The onset of clinical disease was measured by scoring the animals twice a week using our previously described scale³⁷: Stage 1: normal animal; stage 2: mild behavioral abnormalities, including hyperactivity and hypersensitivity to noise; stage 3: moderate behavioral problems, including tremor of the head, ataxia, wobbling gait, head bobbing, irritability, and aggressiveness; stage 4: severe behavioral abnormalities, including all of the above plus jerks of the head and body and spontaneous backrolls. Animals scoring level 4 during 2 consecutive weeks were considered sick and were humanely sacrificed by exposure to carbon dioxide followed by decapitation. Half of the brain was collected in Carnoy solution (60% ethanol, 30% chloroform, 10% acetic acid) for histological analysis and the other half was recovered and frozen at –80°C for biochemical studies. Animals that did not exhibit clinical symptoms were kept alive until they started showing signs of excessive aging or were affected by non-prion related health issues such as tumors. The euthanasia procedure and collection of brain tissue for these animals was performed in the same manner as that for prion-diseased animals. All animal experiments were carried out in accordance with the NIH regulations and approved by the committee of animal use for research at the University of Texas Health Science Center at Houston.

In vitro replication of Prions by PMCA

Protein misfolding cyclic amplification (PMCA) was performed as previously reported³⁸. Brain tissues were manually homogenized in conversion buffer (PBS supplemented with 150 mM NaCl, 1% Triton X-100 and protease inhibitor cocktail) at 10% w/v, centrifuged at 800 x g and supernatant was kept frozen at –80°C until used. Brains from young healthy hamsters (3–4 weeks old) were used as PMCA substrate. Different prion or control inoculums previously homogenized in PBS buffer were used as seeds to initiate the reaction as specified in the figures. PMCA was run using 48-hr rounds at 37–40°C with cycles of 29.5 min incubation and 30 sec sonication on a Q700 sonication system connected to a microplate horn assembly (QSonica®) and temperature controlled by an incubator. Samples

were submitted to proteolysis analysis by adding proteinase K (PK) at a 50 µg/mL final protein concentration and incubating the reactions for 1 hr at 37°C under constant shaking (450 rpm) in a thermomixer (Eppendorf®). For analysis, the reactions were diluted with Nu-PAGE loading buffer (Invitrogen®) and then heat-denatured at 95°C for 10 min followed by electrophoresis (SDS-PAGE) and immunoblotting. Anti-PrP specific monoclonal antibody 6D11 was used for identification of PrP. The presence of protease-resistance PrP in brains was studied by treating the 10% brain homogenates under the same proteolytic conditions and subsequent analysis by Western blots.

For the experiments of inhibition of PMCA amplification in the presence of the anti-prion, various concentrations of AP1 (containing PBS and 5% brain homogenate, subjected to annealing) were either used directly or incubated with 10% healthy hamster brain homogenate and used as substrate for the PMCA reaction. Samples were pre-incubated at 37°C for 6 hours before addition of serial dilutions of 263K PrP^{Sc} and the initiation of PMCA cycles. After amplification, the amount of PK-resistant PrP^{Sc} formed was evaluated by Western blot, as indicated above.

Neuropathological analyses

Brain samples were fixed in Carnoy solution (60% ethanol, 30% chloroform and 10% acetic acid), dehydrated and included in paraffin. Ten µm tissue slices were stained with Hematoxylin-eosin (H&E) or immuno-stained with a monoclonal antibody to PrP (6H4, 1:1000; Prionics) and a polyclonal antibody against reactive astrocytes (GFAP, 1:2000; Dako). For PrP^{res} staining, slides were subjected to 10 µg/mL PK for 5 min and 3M guanidinium isothiocyanate treatment for 20 min. Slides were incubated overnight using the 6H4 antibody (Prionics®) and non-specific binding blocked using the Dako ARK® (Animal Research Kit) following manufacturer's recommendations. Immunostaining was developed using HRP-conjugated streptavidin and visualized with DAB as chromogen. Tissues were later counterstained with Hematoxylin for 30 sec and rinsed in tap water for 10 minutes. For GFAP staining, slides were post-fixed in 10% formalin and subjected to treatment with 3% H₂O₂ for 20 minutes. Samples were finally incubated with GFAP antibody overnight. Immunostaining was developed using anti-rabbit HRP secondary antibody and visualized with DAB as chromogen and counterstained with Hematoxylin. Later, slides were dehydrated in ethanol, cleared with xylene, and mounted with resinous mounting medium. Sections were examined under a bright field DMI6000B Leica® microscope. Spongiform profiles were determined on H&E-stained sections, by scoring the vacuolar changes in nine standard grey matter area as described³⁹.

RESULTS

Treatment with an anti-prion significantly delays the onset of prion disease

Recent studies have shown that under various conditions recombinant PrP can be induced to misfold and aggregate forming PrP^{Sc}-like structures that, despite having many of the biochemical characteristics of brain-derived PrP^{Sc}, do not cause clinical disease when directly injected into animals^{19–25}. Strikingly, some of these preparations were able to induce the accumulation of PrP^{Sc}-like in the animal, despite the absence of disease or any

indication of neurodegeneration^{24–26}. In order to assess whether these non-toxic PrP aggregates can interfere with propagation of *bona fide* prions *in vivo*, we studied an anti-prion candidate produced by incubation of purified recombinant PrP in the presence of denaturing agents followed by annealing in normal brain homogenate, using a protocol previously described by Baskakov and colleagues⁴⁰. This procedure led to the formation of amyloid-like aggregates that were positive for thioflavin T and resistant to proteolytic degradation (Figure S1). The resulting structure, termed anti-prion 1 (AP1), was intracerebrally injected in three groups of 263K infected hamsters divided according to the time of AP1 treatment with respect to 263K injection: (i) post-infection, (ii) pre-infection and (iii) simultaneous challenge (Figure 1). We observed that simultaneous injection of 263K prions and AP1 produced a clear and statistically significant ($P=0.035$) increase on the incubation period for prion infection (Figure 1A). When AP1 was injected weeks after 263K inoculation, we observed a trend for a delay in the appearance of clinical symptoms for AP1 treated animals, but did not yield statistically significant differences ($P=0.056$) (Figure 1B). According to the hypothesis for the anti-prion action, a treatment with AP1 before administration of the pathogenic prion should have a greater degree of interference, because the anti-prion would be able to self-replicate and accumulate before the competition with the pathogenic PrP^{Sc}. Confirming this prediction, we observed that prophylactic inoculation of AP1 produced significant ($P=0.019$) increases in the survival of the 263K-treated animals compared to the control group (Figure 1C). Furthermore, the bigger increases in survival respect to the control group were obtained upon earlier prophylactic treatment with AP1, and the most dramatic result was observed when AP1 was inoculated 63 days before 263K challenge. Indeed, in this group the average incubation periods were increased by $>50\%$ and the maximum survival by $>100\%$ (Figure 1C). Considering that all the treatments were performed as a one-time inoculation of material, the results suggest that AP1 is producing a lasting effect in the brain of the animals that allows efficient interference with 263K replication when inoculated in a prophylactic manner.

AP1 can completely prevent the onset of clinical symptoms in prion infected animals

Next we studied the anti-prion effect on animals exposed to lower quantities of infectious prions, which may correspond more closely to the natural situation during prion infection. For this purpose, we repeated the same prophylactic treatment with AP1 at the most efficient time observed earlier (63 days before challenge), but now inoculating animals with different dilutions of the 263K agent ranging from 10^{-3} to 10^{-5} with respect to the brain. Higher dilutions were not considered as these can produce incomplete attack rates and high variability among the groups⁴¹. The lowest 263K prion dilution (10^{-3}) showed a substantial increase in survival time for the experimental group, but as before all the animals succumbed to prion disease (Table 1). At this dilution, the average survival of the control group is within the expected values for 263K strain⁴¹. The intermediate prion dilution (10^{-4}) showed a more dramatic effect influencing both the survival time and attack rate (Table 1). One of the animals from this group did not show any clinical symptoms so it was sacrificed at the end of the experiment (500 days post challenge with 263K prions). The highest tested dilution (10^{-5}) exhibited a striking decrease in the attack rate compared to the control group (Table 1). Three out of five animals did not show any clinical sign during their lifespan when treated with AP1, whereas all animals infected with the same dilution of 263K developed

typical signs of prion disease. Overall, the significant changes on survival and especially the reduced attack rate compared to the respective control groups are consistent with the idea of a long term “protection” effect against prion disease upon a one-time prophylactic inoculation of AP1.

The PrP^{Sc} deposition pattern is altered in prion-infected animals treated with AP1

Since we observed significant changes in the incubation period and attack rates in the animals treated with AP1, we then evaluated whether treatment with this anti-prion molecule may affect some of the clinical and neuropathological features of the 263K prion strain. All of the animals that developed prion disease presented classical scrapie-like clinical signs at the final stages of the disease that are consistent with those typically observed for 263K, including severe ataxia, hyperactivity, hypersensitivity to touch and some weight loss^{10,41}. We did not observe gross behavioral differences between animals affected in the experimental and the control groups, but more sophisticated test would be necessary to appreciate subtle differences. We then analyzed the deposition pattern of protease-resistance PrP (PrP^{res}) in the brains of diseased animals by immunohistochemistry using an anti-PrP antibody. Surprisingly, we observed that the animals treated with AP1 exhibited considerably less deposition of PrP^{res} in some brain areas compared to the classical 263K deposition pattern (Figure 2A). The differences were most clear in the group of animals inoculated with a 10⁻⁵ dilution of 263K brain homogenate with or without prophylactic injection of AP1. This is likely because at this low dilution of pathogenic prion there are more chances for the anti-prion to effectively compete. The most significant changes were observed in the supragranular layers of the frontal cortex, septal nuclei, dentate gyrus and fimbria of the hippocampus (Figure 2B). The molecular layer of the dentate gyrus was completely spared from PrP^{Sc} deposition in prion-infected animals treated with AP1. The type of PrP deposits (mostly synaptic and diffuse) was not affected by the treatment with AP1 and biochemical analyses revealed no differences in the electrophoretical mobility of PrP (Figure S2). All the samples showed the typical diglycosylated-dominant pattern that is characteristic of 263K. Taken together, these results suggest that treatment with AP1 is mainly affecting the extent and/or location of PrP^{Sc} accumulation.

The brain of asymptomatic animals treated with AP1 showed reduced or complete absence of PrP^{Sc} accumulation

We analyzed the brains of 263K-treated animals that did not develop clinical symptoms upon prophylactic treatment with AP1, as illustrated in Table 1. We found much less PrP^{Sc} deposition in these brains compared to that of the animals belonging to the same group that conversely did succumb to the disease (Figure 3A). However, we were intrigued by the fact that some faint deposition was indeed clearly detectable in the brain from several of these animals. The type of PrP deposition observed in those brains was rather diffuse. To further confirm the reduction of PrP^{Sc} in the brain of these animals, we performed a biochemical analysis of these brains and looked for the presence of protease-resistant PrP^{Sc}. Upon treatment with proteinase K, we did not observe the presence of protease-resistant PrP by Western blot in any of the brains from the asymptomatic animals (Figure 3B). Since minute amounts of PrP^{Sc} may still be present, we analyzed the homogenates by Protein Misfolding Cyclic Amplification (PMCA) that is able to detect classical PrP^{Sc} in the attomolar range³⁸.

After three consecutive PMCA rounds, we observed the typical signal associated to 263K-PrP^{Sc} in two of the four asymptomatic animals (Figure 3C). PMCA of an aged control brain (550 days) did not show any signal suggesting that the PrP^{Sc} detected in the asymptomatic animals was not due to age. Considering that PMCA is a highly sensitive technique for detection of prions and under our current conditions three 48-hour rounds permit the maximum amplification rate, equivalent to a few particles of PrP^{Sc} (ref⁴²), we can safely conclude that extremely low or no PrP^{Sc} was present in the brains from hamsters rescued from clinical disease by AP1 treatment.

Animals inoculated with AP1 alone do not develop neurodegenerative alterations, but show accumulation of protease-resistant PrP

Injection of AP1 alone in hamsters did not produce disease during the entire lifespan of the animals in agreement with previous reports from Baskakov and colleagues²⁴. Considering the subtle but clear PrP^{res} deposition obtained in the asymptomatic prion-infected animals treated with AP1 (Figure 3A), we tested whether AP1 alone would also produce some protease-resistant PrP deposition. We therefore inoculated two independent groups of animals with AP1 and checked their lifespan and putative accumulation of PrP^{res}. Some of the animals were sacrificed at different time points in order to assess any time-dependent PrP deposition. As expected, we did not observe any neurological clinical sign in any of the AP1-treated animals, corroborating that AP1 does not produce TSE disease. When the brains from these animals were analyzed by PrP immunohistochemistry, we observed a diffuse pattern of protease-resistant PrP deposition similar to that of asymptomatic prion-infected animals (Figure 4). Furthermore, we observed that PrP^{res} accumulation increased with age. In order to rule out that this deposition could be age-related, we performed immunohistochemical staining of brains from age-matched untreated hamsters. We observed that the diffuse PrP^{res} deposition observed in brains of AP1-treated animals is not present in the untreated animal (Figure 4) which suggests that these PrP deposits are associated with AP1 injection. Strikingly, the diffuse deposits of PrP^{res} were located in the same regions as those observed for the asymptomatic animals (compare Figure 3A and 4). The extent of PrP^{res} deposition appears to be equivalent for both groups, although these levels are considerably lower than those observed in diseased brains of animals treated with 263K alone. In order to check whether these deposits are associated to the presence of classical PrP^{Sc}, we homogenized all the brains and ran Western blots as well as three rounds of PMCA. Using these methods, we were unable to detect a signal of PrP^{Sc} in any of the analyzed samples (data not shown) which strongly suggests that the diffuse PrP^{res} deposition observed on these brains is not associated to classical PrP^{Sc}. Treatment with lower concentrations of proteinase K or development of the blots with C-terminal antibodies R20 and SAF-84, that may be able to pick up smaller fragments, did not enable to see any clear difference between AP1 inoculated animals and age-matched controls (Figure S3). Although we saw a signal on the expected molecular weight, this was not different from that seen with age-matched untreated animals, so it likely reflects undigested PrP^C or some other non-specific band. These results suggest that the PrP^{Sc}-like form of AP1 is not resistant to proteolytic degradation. This is not entirely surprising, since it has been shown that different prions strains have a differential susceptibility to proteases and in some forms the large majority of the material is protease-sensitive⁴³.

Prion-infected animals responsive to AP1 treatment do not show significant brain damage

The main neuropathological features of TSE, other than PrP^{Sc} deposition, include extensive spongiform degeneration and brain inflammation⁴⁴. As expected, animals injected with 263K alone showed a typical lesion profile characterized by significant accumulation of damage spread over many regions across the brain including the medulla, midbrain, thalamus and the hippocampus (Figure 5). The thalamus exhibited the most pronounced degree of spongiosis. Interestingly, despite the changes on PrP^{res} deposition, hamsters treated with AP1 that did succumb to disease showed also extensive spongiosis which was not significantly different from animals inoculated with 263K alone. This is not entirely surprising as the animals from both groups exhibited typical clinical symptoms and 263K-associated behavioral features with comparable duration of the clinical phase. Considering that we did not observe clinical signs in some of the prion-infected animals treated with AP1, we assessed these samples for the presence of vacuoles. Prion-infected animals in which the treatment with AP1 precluded onset of clinical disease, exhibited a completely different lesion profile characterized by reduced or non-existent neuropathological alterations in the same brain areas (Figure 5). Interestingly, the overall profile of spongiosis in these animals was very similar to that obtained for hamsters injected with AP1 alone or to age-matched controls, suggesting that brain damage associated to 263K strain was effectively avoided by prophylactic treatment with AP1 on these animals.

AP1 inhibits 263K prion replication *in vitro*

Our data so far support the hypothesis that the anti-prion interferes with prion pathogenesis by inhibiting prion replication. To analyze more directly the effect of AP1 on 263K prion propagation, we utilized the PMCA technology that mimics *in vitro* the process of prion replication. For this purpose, the PMCA substrate (10% healthy hamster brain homogenate) was incubated for 6h with different concentrations of AP1. Thereafter, 10-fold dilutions of 263K brain homogenate were added and samples were subjected to 2 rounds of PMCA amplification. As controls the same brain homogenate substrate was supplemented with the same volume of PBS. In the absence of AP1, PrP^{Sc} signal was observed at 263K dilutions of 10⁻⁴ and 10⁻⁶/10⁻⁷ after 1 or 2 rounds of PMCA, respectively (Fig. 6D and E). When the substrate was pre-incubated with the highest concentration of AP1, a complete inhibition of PrP^{Sc} replication was observed, since no PrP^{Sc} signal was obtained after 1 or 2 rounds of PMCA (Fig. 6A). Conversely, a lower degree of inhibition was observed when the brain homogenate substrate was pre-incubated with 50% of the original AP1 concentration (Fig. 6B). A less drastic inhibitory effect was observed when samples were incubated with 25% AP1 (Fig. 6C). These results indicate that AP1 inhibits in a concentration-dependent manner prion replication *in vitro*.

DISCUSSION

Prion disease are 100% fatal disorders for which no cure or even palliative treatment is available. Many different strategies have been attempted in the past⁴⁵⁻⁴⁷, including small molecules inhibiting prion replication⁴⁸⁻⁵², rationally designed peptides⁵³, vaccines and antibodies^{54,55} as well as downstream targets at the level of the unfolded protein response⁵⁶ or compounds that inhibit the signaling pathways implicated in prion-induced

neurodegeneration⁵⁷. Prions are a unique class of infectious agents that have several unprecedented features and can produce dramatic diseases that are 100% fatal. The phenomenon of prion strains has been one of the most puzzling observations in the field, difficult to reconcile with an infectious agent composed exclusively by a protein⁵⁸. In some experimental rodents, more than 20 different prion strains have been recognized, differing in the clinical signs, incubation periods, attack rate, profile of neuropathological damage, or biochemical properties of PrP^{Sc} (refs ^{59–61}). New prion strains are often generated upon infection of an animal with prions from a distinct species. Interestingly, prion strains have been shown to progressively mature by adaptation to the new host and can also exhibit peculiar features such as strain memory and strain interference⁶⁰. With the introduction of modern *in vitro* prion replication techniques, several new prion strains have been generated^{11,37,62,63}, some of which exhibit the capacity to self-replicate but do not produce disease in a first passage^{24,25}. These results support the current view that self-propagation of PrP^{Sc} can be temporally or biochemically disconnected from toxicity both *in vitro* and *in vivo*^{14–18,64}. Combining these findings with the well-established concept that prion strains can interfere between themselves *in vivo*^{28–32}, we hypothesized that it should be possible to generate particular prion strains that are not pathogenic, but can outcompete the replication of disease-causing prions. These innocuous prion strains (termed here as anti-prions) may use the prion principles of self-propagation to generate more and more of the non-pathogenic structures and successfully compete with pathological prions.

In the current study, we demonstrated that treating animals with a non-toxic, self-propagating anti-prion greatly interfered with the pathological progression of the disease induced by inoculation with the 263K prion strain and even completely prevented several animals from succumbing to disease. The magnitude of the anti-prion effect can be estimated by comparing survival curves of animals injected with different dilutions of 263K prions. As shown in Figure S4A, the survival curve of hamsters inoculated with a 10⁻⁵ dilution of 263K brain homogenate was very similar to that obtained when animals pre-injected with AP1 were inoculated with a 10⁻³ dilution of the same preparation. A similar conclusion was obtained by comparing the survival curves of hamsters inoculated with a 10⁻⁶ dilution of prion-containing brain or a 10⁻⁴ dilution of this material when previously injected with AP1 (Figure S4B). These data indicate that a single prophylactic treatment with AP1 reduced prion infectivity by 2 logs, i.e. ~99% of efficacy. Considering that 263K is one of the most aggressive prion strains available in hamsters in terms of infectivity titer, duration of incubation period and disease progression⁴¹, we consider that delaying or preventing the clinical symptoms associated to this strain with a one-time prophylactic treatment with an anti-prion is a significant achievement. An interesting observation in several of our groups of animals treated with AP1 was that the effect appears to be binary, i.e. some animals treated with the anti-prion get a high level of protection and others do not show much of an effect (see for example Fig 1C and Table 1). We are currently investigating the possible mechanisms for this behavior, but we suspect that it is related to the intrinsic properties of prions. Indeed, decades of research in the field have demonstrated that infectivity experiments done with limiting dilutions of prions (without any treatment) lead to disease in only a proportion of the animals and others in the cohort treated in the same manner remain healthy throughout their lifespan. A possible explanation for this binary

behavior of prions at limiting conditions (e.g in animals exposed to low concentrations of prions or to a competing agent like the anti-prion) is that during inoculation animals may receive different amount of prions, due to the fact that prion solutions are actually a suspension of relatively large aggregates. It is also possible that small differences due to animal-to-animal variability lead to substantial changes in the disease outcome owing to the fact that prions replicate in an exponential fashion. Another potential source of variability is that inoculation of 263K prions and AP1 were not done following specific stereotaxic coordinates. In future experiments, it would be interesting to test the effect of AP1 by administration in distinct areas of the brain. Also interesting would be to compare in more detail the clinical characteristics of the disease in the groups treated and untreated with AP1, considering the observation that the anti-prion effect appears higher in some areas of the brain than others.

Although the exact mechanism for anti-prion activity is unknown, we hypothesize that self-propagation of AP1 may decrease replication of 263K prions by reducing the availability of PrP^C or a cellular conversion factor, in a similar way as proposed in the prion interference experiments³³. This hypothesis is supported by the following observations: (i) Animals inoculated with AP1 alone progressively accumulate a protease-resistance form of PrP in their brains, suggesting that the anti-prion is self-replicating in the animal body. (ii) The *in vitro* studies using PMCA showed that AP1 can inhibit 263K prion replication in an extent that depends on the concentration of anti-prion added to the reaction. (iii) The fact that prophylactic inoculation of AP1 had the greatest effect on the onset of 263K-induced TSE suggests that AP1 replication in the brain is necessary to accumulate a quantity of the anti-prion that can effectively compete with the pathogenic prion. This conclusion is also supported by the results obtained in the experiments in which lower doses of 263K prions were given to animals prophylactically treated with AP1. As shown in Table 1, several animals treated in this manner were completely protected from prion disease. The results obtained in this experiment are important because it probably reflects better the natural conditions of infection in which individuals are likely exposed to lower concentrations of the infectious agent. The clear differences in the histological pattern of PrP^{Sc} deposition in prion-infected animals treated with AP1 also support the idea of a competition between 263K and AP1. Prion strains are known to have different regional distribution of PrP^{Sc} deposition^{60,65}; hence, our interpretation of these results is that the brain areas where there is lower accumulation of typical PrP^{Sc} corresponds to regions in which AP1 replicates better and competes more efficiently with 263K prions. This interpretation is supported by the data showing that the regions having more profuse protease-resistant PrP staining in the AP1 group were also the areas least affected in both the amount and intensity of PrP^{Sc} deposition in the brains of prion-infected animals treated with AP1. Interestingly, considering that AP1-treated and untreated animals develop the same clinical symptoms and profile of spongiform damage, our data suggest that PrP^{Sc} deposition is not directly responsible for the disease onset. Alternatively, it is possible that small protease-resistant PrP oligomers that are not detected by immunohistology are responsible for brain damage and disease.

Other putative mechanisms to explain the anti-prion activity, instead of the self-replication of the anti-prion at expenses of PrP^C thus reducing the amount of substrate for prion conversion, include: (i) a direct interaction between PrP^{Sc} and AP1, leading to the capture of

the infectious protein resulting in inhibition of prion replication; (ii) a neuroprotective activity of the anti-prion; (iii) a boosting by AP1 of the biological clearance pathways, such as the unfolded protein response or autophagy; (iv) an activation of an inflammatory or immune response against PrP^{Sc}. Although we cannot completely rule out these alternative models, the available evidence suggests they are unlikely to explain the results. For example, if the target of AP1 was PrP^{Sc} itself, we should have expected the best effect when these materials were administered at the same time. Since prophylactic intervention occurs at a stage well before any brain damage is expected, the AP1-mediated protection is most likely unrelated to a direct neuroprotective effect of the anti-prion. In addition, we did not observe changes on the length and/or evolution of the disease from the onset of clinical symptoms suggesting that AP1 is not altering the toxicity associated to 263K. With respect to the possible effect of AP1 in boosting neuro-inflammatory and/or clearance pathways that can prime the brain to be prepared for an infection with a pathogenic prion, we consider these scenarios unlikely because of several reasons. Firstly, no significant brain inflammation was observed in animals treated only with AP1 when compared to an age-matched animal (Figure S5). In addition, PrP aggregates have typically low immunogenicity and the generation of anti-PrP^{Sc} specific antibodies is very challenging requiring multiple boosts and addition of potent co-adjuvants or even deletion of PrP^C expression to overcome endogenous PrP tolerance^{66–69}. A specific activation of the unfolded protein response (UPR) pathway upon prion infection has been indeed demonstrated previously⁷⁰. However, this response has been shown to be tightly related with neurodegeneration due to an overall blockade of protein synthesis caused by phosphorylation of the eIF2a protein involved in translation in the affected brains that leads to synaptic failure, neuronal loss and spongiosis⁷¹. These alterations typically underlie the clinical symptomatology of TSE. Considering we did not observe any significant disease-associated symptom or spongiosis in animals treated only with AP1, we do not think this mechanism is a significant contributor to the anti-prion protective effect. Furthermore, we reason that a significant acute activation of these indirect mechanisms leading to protection against TSE would have caused a more robust effect if done at the moment in which pathogenic prions start to replicate.

A potential drawback of using anti-prions as a TSE treatment might be the possibility that these innocuous prion strains may mutate or progressively mature *in vivo* and convert into pathogenic prions. Indeed, it was shown that aggregated recombinant PrP produced with the same protocol used for generation of AP1 leads to a new form of prion disease upon successive passages in hamsters²⁴. The goal of the current study was to provide proof-of-concept data that anti-prions may indeed be used to interfere with the replication of pathogenic prions. One of the important tasks for the future would be to generate new, more efficient and more stable anti-prion candidates that overcome the strain conversion process. This could lead to a new “self-replicating” therapy utilizing the prion principles to generate therapeutic molecules.

Recent exciting reports have shown that some of the most common forms of neurodegenerative diseases, such as Alzheimer’s and Parkinson’s diseases as well as various systemic amyloidosis, may utilize the prion principle to spread the process of protein misfolding and, in this manner, progressively increase the pathogenesis to the point of irreversible damage and disease^{1,34,35}. Since the mechanism of spreading and accumulation

of misfolded protein aggregates implicated in these diseases are very similar to the prion replication process, it is possible that the anti-prion concept may provide a new strategy for intervention in many diseases associated to the accumulation of misfolded protein aggregates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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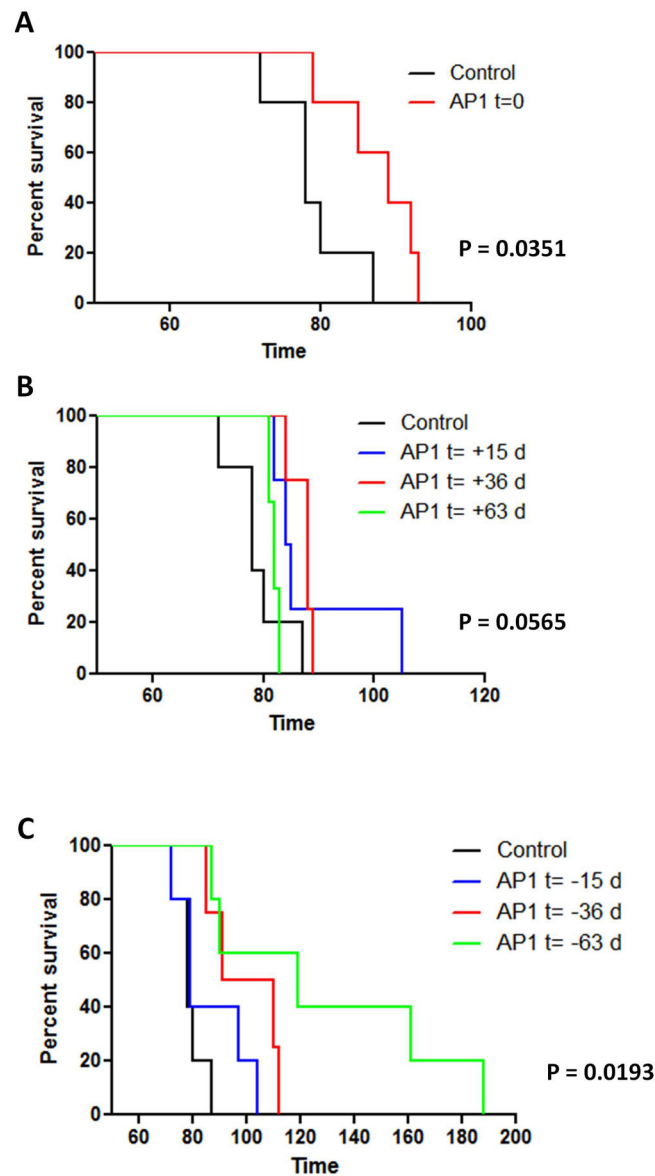


Figure 1. Effect of AP1 on the onset of prion disease triggered by injection of 263K prions
 (A) 263K and AP1 injected at the same time into wild type hamsters. Two groups of hamsters were intra-cerebrally inoculated with AP1 and 263K (1% brain homogenate) at the same time, but through two separate injections. Control animals were treated using the same strategy but using buffer instead of AP1.

(B) Treatment with AP1 after prion exposure. Three groups of hamsters were inoculated intra-cerebrally with the 263K prion strain and then inoculated with AP1 at three different times (15, 36 and 63 days after 263K injection). Control hamsters were inoculated with a second injection of buffer sample at the time in which AP1 was injected in the experimental groups.

(C) Treatment with AP1 before prion infection. Three groups of hamsters were inoculated intra-cerebrally with AP1 at different times before exposure to 263K (15, 36 or 63 days prior

263K injection), as indicated in the figure. Control hamsters were treated with buffer instead of AP1.

For all the groups, 5 animals per treatment condition were used and the onset of clinical disease was measured as previously described³⁷. Animals with definitive signs of prion disease, scoring level 4 in our scale during 2 consecutive weeks were considered sick and were humanely sacrificed. Time in the X-axis refers to days after inoculation with the 263K prion agent. The “t” in the legends refers to the time of AP1 inoculation relative to the time of 263K challenge. Differences on the survival curves were evaluated by the Log-rank (Mantel-Cox) test using the Graph Pad software and P values are indicated in each panel.

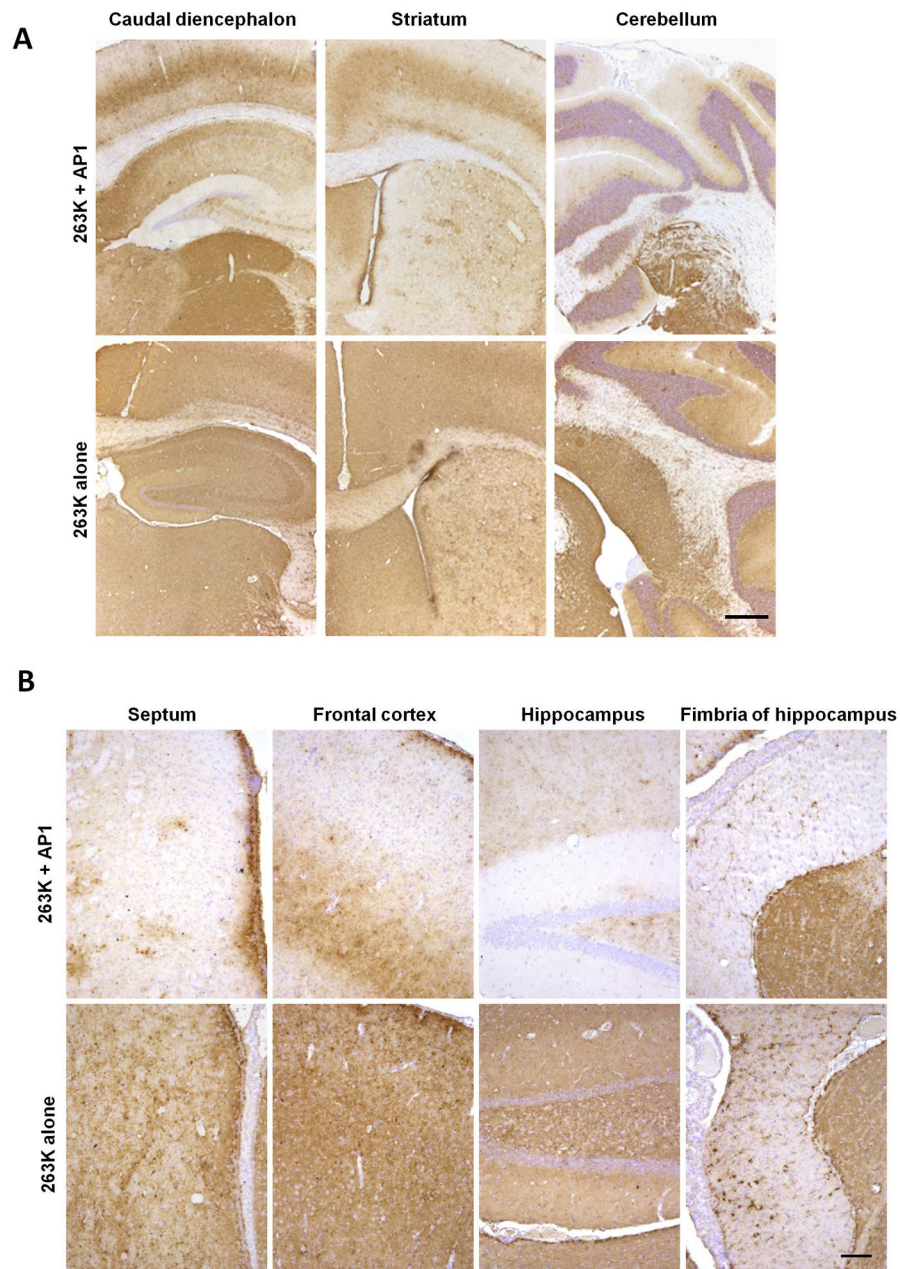


Figure 2. Brain deposition of PrP^{res} in the brains of animals treated with AP1 that succumbed to prion disease

(A) The brains of the animals described in Table 1 that went through the clinical phase of TSE were histologically analyzed by anti-PrP immunostaining after protease treatment (see Experimental Procedures). Three main brain areas (hippocampus/cortex, striatum and cerebellum) that exhibited the greatest differences in PrP deposition between the experimental (treated with AP1) and control group (inoculated with buffer) are shown. Scale bar 500 μ m. Pictures correspond to representative pictures of many slides analyzed for all the animals in each group.

(B) A closer view of PrP deposition on specific zones (septum, frontal cortex, hippocampus, and fimbria) within the brain regions shown in (A). Scale bar 100 μm .

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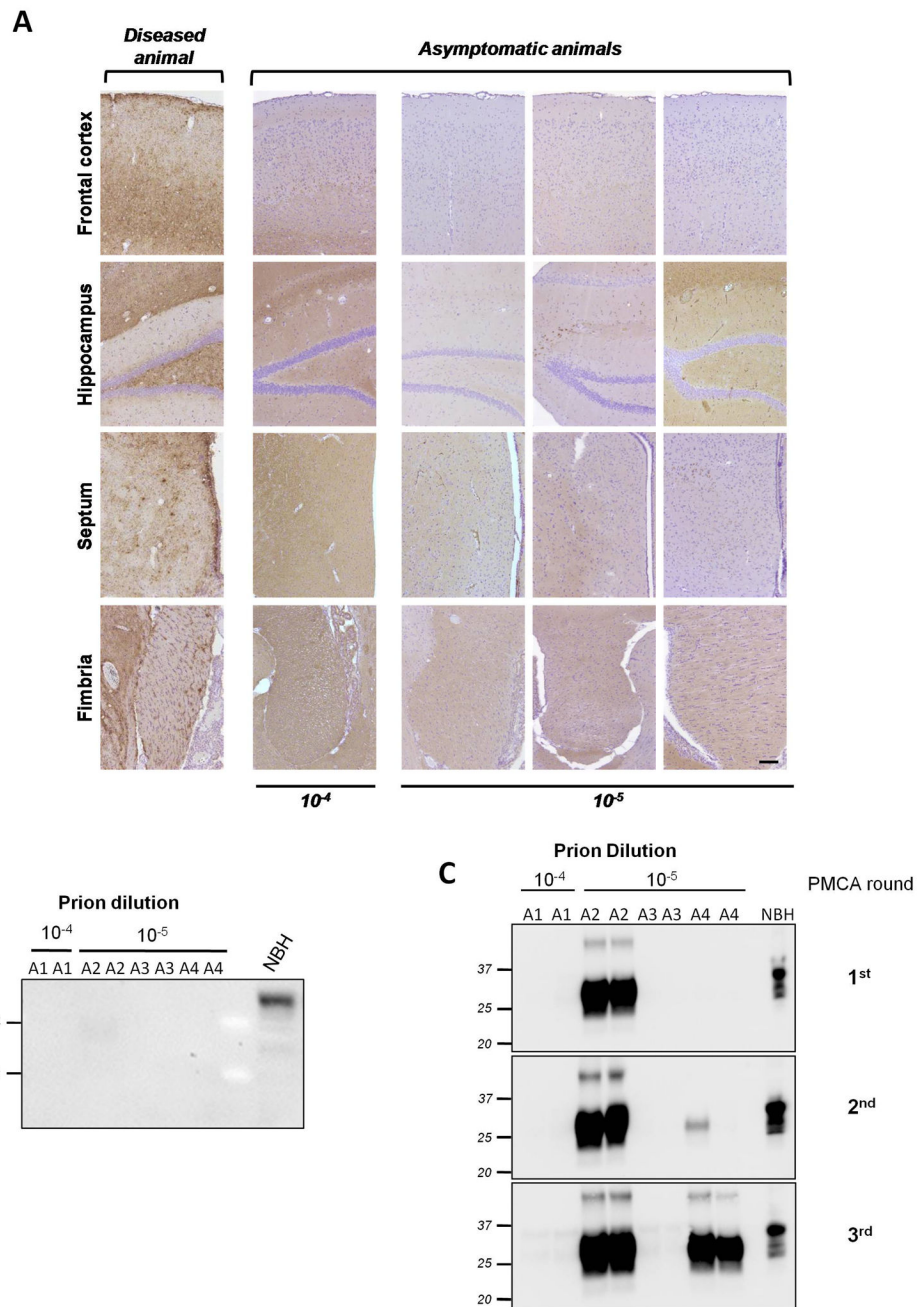


Figure 3. Histological and biochemical detection of PrP^{Sc} in the brains of asymptomatic prion-infected animals treated with AP1

(A) Histological comparison of PrP deposition between the brains of animals treated with AP1 that did not develop clinical symptoms (asymptomatic animals) versus a representative animal that succumbed to prion disease (diseased animal), as described in Table 1. For comparison, the same zones shown in Figure 2B are depicted. Scale bar 100 μ m. Pictures correspond to representative pictures of many slides analyzed for all the animals in each group.

(B) Detection of PrP^{Sc} by direct Western blot in 4 asymptomatic animals treated with a prophylactic injection of API 63 days before inoculation with a 10⁻⁴ (animal A1) or 10⁻⁵ (animals A2, A3 and A4) dilution of 263K brain homogenate. Samples were run in duplicates. All samples were treated with proteinase K (PK) (50 µg/ml), except for the normal brain homogenate (NBH), used as electrophoretic migration marker.

(C) Detection of PrP^{Sc} by PMCA. 10% (w/v) homogenates from the brains of asymptomatic animals described in (A) were analyzed in duplicate for the presence of PrP^{Sc} by the PMCA technique. Three consecutive PMCA rounds were performed and the samples were analyzed by immunoblotting with 6D11 anti-PrP antibody (see Experimental Procedures). A brain homogenate from a healthy hamster not treated with protease (NBH) is shown on the last lane to show the molecular weight shift of PrP^{Sc} after protease treatment.

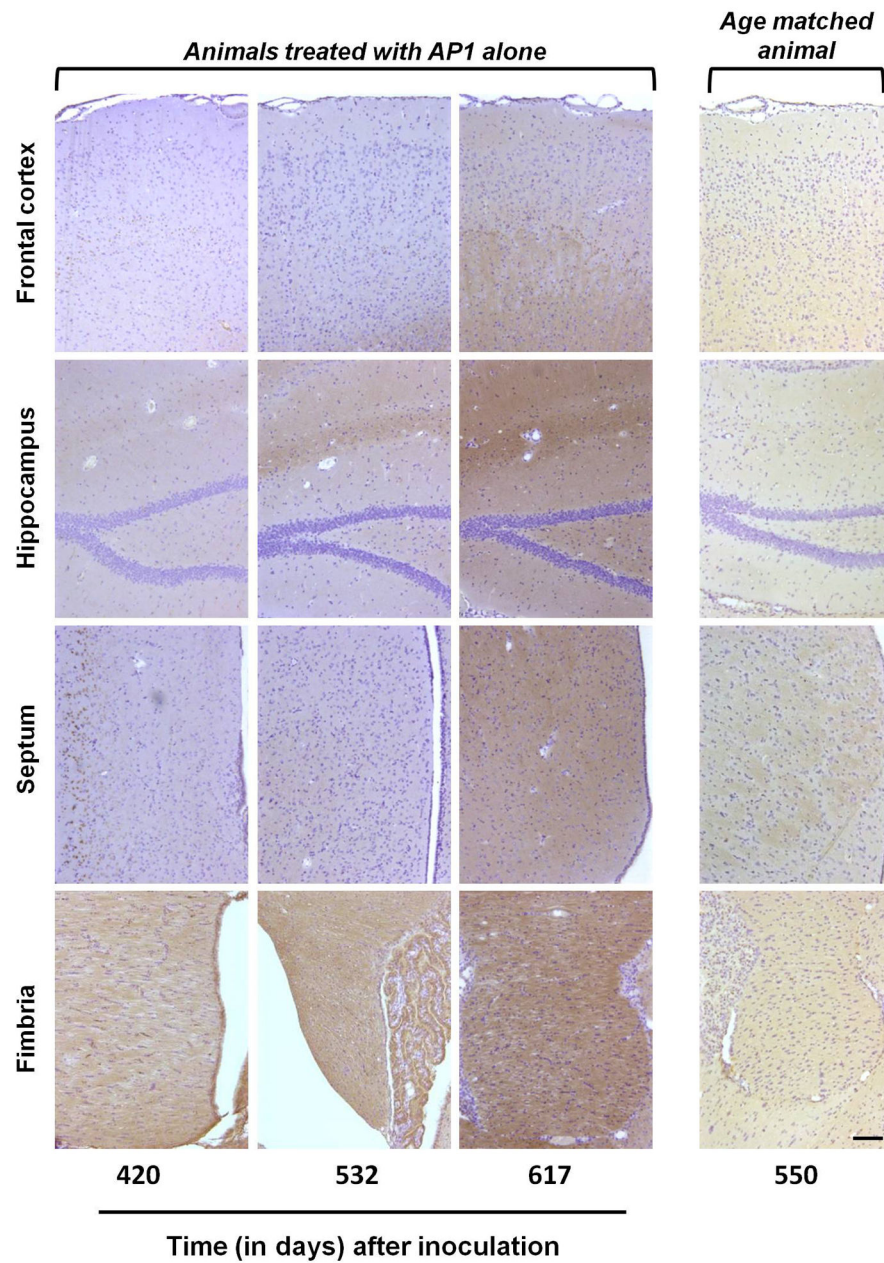
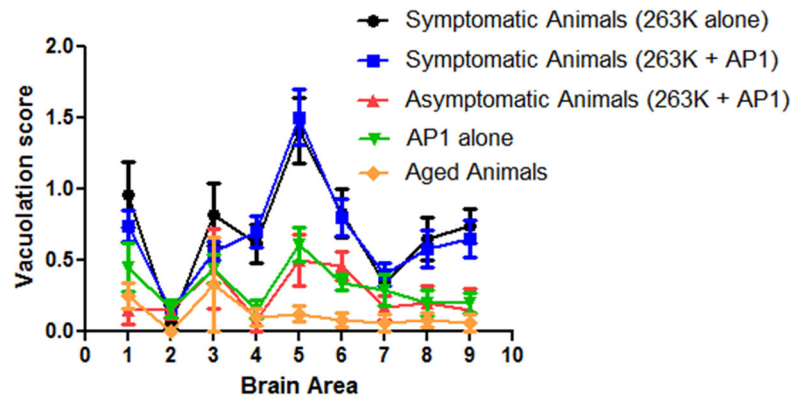


Figure 4. Histological analysis of protease-resistant PrP deposition in brains treated with AP1 alone

The brains of animals intra-cerebrally inoculated with AP1 alone were analyzed by anti-PrP immunohistochemistry after treatment with PK (see Experimental Procedure). The same brain zones (frontal cortex, hippocampus, septum and fimbria) shown in previous figures are depicted here for comparison. The samples were arranged according to the age in which animals were sacrificed (420, 532 and 617 days post anti-prion inoculation). The brain of an untreated aged hamster (550 days old) is shown on the right as control for basal PrP deposition due to age in the same brain areas. Pictures correspond to representative pictures of many slides analyzed for all the animals in each group. Scale bar 100 μ m.



Symptomatic animals (263K alone) vs symptomatic animals (263K + AP1)	ns
Symptomatic animals (263K alone) vs asymptomatic animals (263K + AP1)	**
Symptomatic animals (263K alone) vs AP1 alone	*
Symptomatic animals (263K alone) vs aged animals	***
Symptomatic animals (263K + AP1) vs asymptomatic animals (263K + AP1)	*
Symptomatic animals (263K + AP1) vs AP1 alone	*
Symptomatic animals (263K + AP1) vs aged animals	***
Asymptomatic animals (263K + AP1) vs AP1 alone	ns
Asymptomatic animals (263K + AP1) vs aged animals	ns
AP1 alone vs aged animals	ns

Figure 5. Profile of spongiform degeneration in the brains of experimental and control animals
Brain damage associated to vacuolation was assessed as described in Experimental Procedures. The vacuolation profile for each animal and brain region was performed independently by two different investigators blinded to the samples being analyzed. The brain areas analyzed were the typical regions in which TSE pathology has been previously evaluated to profile different prion strains, namely: dorsal medulla (1), cerebellar cortex (2), superior culliculus (3), hypothalamus (4), thalamus (5), hippocampus (6), septum (7), retrosplenial and adjacent motor cortex (8) and cingulate and adjacent motor cortex (9). Each point represents the average vacuolation score and standard error for all the animals in a group (n=5). Data was analyzed by one-way ANOVA followed by the Tukey's multiple comparison post-test. Differences between curves were highly significant with $P < 0.0001$. Significance of differences among the different groups are shown below the graph. ns, no significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

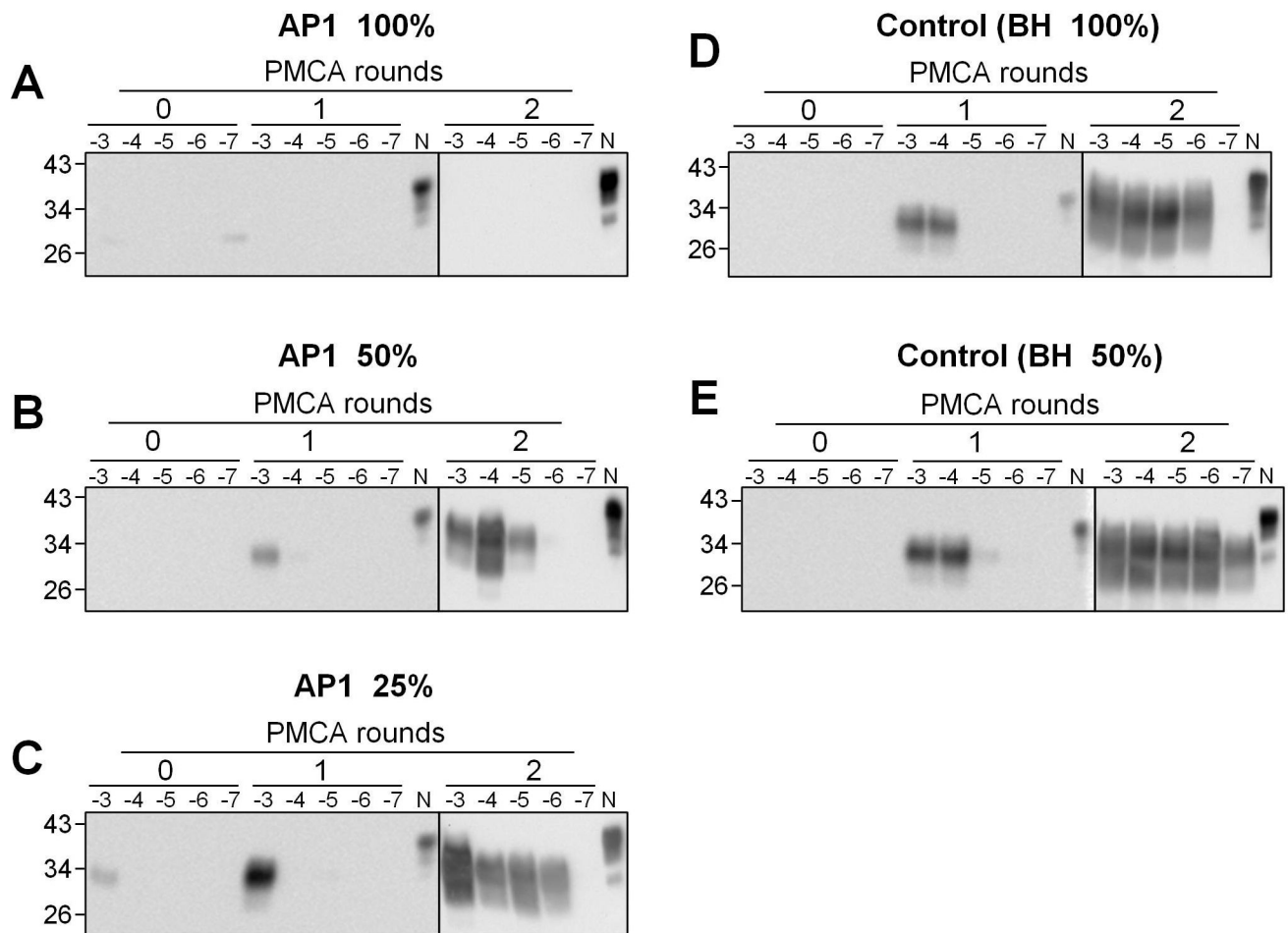


Figure 6. AP1 inhibits 263K PrP^{Sc} replication *in vitro* in a concentration-dependent manner
 Aliquots of AP1 at different concentrations were added to the substrate for PMCA consisting of 10% healthy hamster brain homogenate (BH). Samples were incubated at 37°C for 6h. Thereafter, 10-fold dilutions of 263K sick brain homogenate were added and samples subjected to 2 rounds of 96 PMCA cycles. The presence of PK-resistant PrP^{Sc} was evaluated by Western blot. In all panels, all samples were treated with PK (50 µg/ml), except for the normal brain homogenate (N), used as electrophoretic migration marker. Numbers on the left of each blot depict the molecular weight markers (kDa). Numbers at the top of each blot indicate the log₁₀ dilutions of 263K sick brain homogenate, e.g -3 refers to 10⁻³, -4 to 10⁻⁴, etc. These experiments were repeated at least 3 times and data shown corresponds to representative results of all experiments performed.

(A) AP1 100%: For this reaction, AP1 was used as PMCA substrate. As described in Experimental Procedures, AP1 contains the equivalent of 5% healthy hamster BH. The control of this experiment is BH 100%, as described in panel D below.

(B) AP1 50%: For this reaction, 1 volume of AP1 was incubated with 1 volume of 10% healthy hamster BH. Since AP1 contains 5% hamster BH, the final BH concentration in this reaction was 7.5%. The exact control for this experiment is shown in BH 50%, panel E, below.

(C) AP1 25%: For this experiment, 1 volume of AP1 was incubated with 3 volume of 10% healthy hamster BH. The final concentration of BH in this reaction was 8.75%.

(D) Control BH 100%: This is the control of panel A. For this, 1 volume of PBS was mixed with 1 volume of 10% hamster BH and subjected to 5 min annealing, as indicated in Methods. This material was used for PMCA substrate after 6h of incubation at 37°C.

(E) Control BH 50%: This is the control of panel B. For this, 1 volume of PBS was mixed with 1 volume of 10% hamster BH and subjected to 5 min annealing, as indicated in Methods. This sample was mixed with 1 volume of 10% BH and incubated for 6h at 37°C.

Table 1

Effect of AP1 on the survival times and attack rates in animals treated with different doses of 263K prions

<i>Prion dilution</i>	<i>Treatment</i>	<i>Survival time</i>	<i>Attack Rate</i>
10 ⁻³	<i>API-treated</i>	171 ± 66	5/5
	<i>Buffer-Treated</i>	100 ± 7	5/5
10 ⁻⁴	<i>API-treated</i>	105, 148, 158, 116	4/5
	<i>Buffer-treated</i>	109 ± 1	5/5
10 ⁻⁵	<i>API-treated</i>	119, 120	2/5
	<i>Buffer-treated</i>	173 ± 47	5/5

Each group of hamsters was i.c. inoculated with either AP1 or buffer 64 days before i.c. injection of 263K. Three different dilutions of 263K diseased brain were used (10⁻³, 10⁻⁴ and 10⁻⁵). Animals were euthanized at the final stage of the clinical phase or due to aging or to prion-unrelated health problems. Survival times are expressed in days after 263K inoculation. For the groups with complete attack rates, the average survival ± the standard error (SEM) is shown.

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