Comparison of CR36, a new heparan mimetic, and pentosan polysulfate in the treatment of prion diseases

Claire Larramendy-Gozalo,1 Agnès Barret,1 Estelle Daudigeos,1 Emilie Mathieu,1 Lucie Antonangeli,1 Cécile Riffet,2 Emmanuel Petit,3 Dulce Papy-Garcia,2 Denis Barritault,3 Paul Brown1 and Jean-Philippe Deslys1

Correspondence
Jean-Philippe Deslys
jpdeslys@cea.fr

1CEA, IMETI/SEPIA, 18 route du Panorama, BP6, 92265 Fontenay-aux-Roses cedex, France
2Laboratoire CRRET, CNRS FRE24-12, Université Paris XII-Val de Marne, Avenue du Général de Gaulle, 94010 Créteil, France
3OTR3 Sarl, 4 Rue Française, 75001 Paris, France

Sulfated polyanions, including pentosan polysulfate (PPS) and heparan mimetics, number among the most effective drugs that have been used in experimental models of prion disease and are presumed to act in competition with endogenous heparan sulfate proteoglycans as co-receptors for prion protein (PrP) on the cell surface. PPS has been shown to prolong the survival of animals after intracerebral perfusion and is in limited use for the experimental treatment of human transmissible spongiform encephalopathies (TSEs). Here, PPS is compared with CR36, a new heparan mimetic. Ex vivo, CR36 was more efficient than PPS in reducing PrP res in scrapie-infected cell cultures and showed long-lasting activity. In vivo, CR36 showed none of the acute toxicity observed with PPS and reduced PrP res accumulation in spleens, but had only a marginal effect on the survival time of mice infected with bovine spongiform encephalopathy. In contrast, mice treated with PPS that survived the initial toxic mortality had no detectable PrP res in the spleens and lived 185 days longer than controls (+55%). These results show, once again, that anti-TSE drugs cannot be encouraged for human therapeutic trials solely on the basis of in vitro or ex vivo observations, but must first be subjected to in vivo animal studies.

INTRODUCTION

Prion diseases, or transmissible spongiform encephalopathies (TSEs), are a group of diseases that can occur in sporadic, genetic or environmentally acquired forms. They affect humans as Creutzfeldt–Jakob disease (CJD), kuru, Gerstmann–Straussler–Scheinker syndrome and fatal familial insomnia. In animals, they occur as bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep and goats and chronic wasting disease in cervids. They evolve in two stages: first, a long-lasting incubation period (approx. 10–20 years after environmental exposure), during which the prion agent replicates in lymphoid tissues before invading the central nervous system (probably at some point in the middle of this period), followed by a shorter period of weeks to months when clinical signs appear, corresponding to central nervous system degradation.

TSEs are characterized by the accumulation in the lymphoreticular system and ultimately in the central nervous system of a misfolded, proteinase-resistant conformation (PrP res) of a normal host protein (PrPC) with two sites of N-glycosylation, which is bound to the plasma membrane by a glycosylphosphatidylinositol anchor. The normal function of PrPC is unknown, although it has been postulated to play a role in various cellular processes, such as signal transduction (Kuwahara et al., 1999; Spielhaupter & Schatzl, 2001), differentiation (Graner et al., 2000) and adhesion (Schmitt-Ulms et al., 2001). Recognition domains that interact with heparan sulfate proteoglycans (Hundt et al., 2001) and the LRP/LR laminin receptor (Graner et al., 2000) have been described.

Although concern about BSE and variant CJD has decreased in parallel with the currently waning epidemics, a new concern has been raised by the appearance of secondary transmission of infection associated with blood transfusion (Llewelyn et al., 2004; Peden et al., 2004). The problem is compounded by the fact that no preclinical diagnostic test or preventive therapy has been achieved.

A growing list of compounds has been screened for therapeutic efficacy in prion diseases (Brown, 2002; Cashman & Caughey, 2004; Weissmann & Aguzzi, 2005).
Among these, a large number of molecules have been reported to inhibit PrP\textsuperscript{Sc} accumulation in chronically infected cells, but only a few were able to prolong incubation time and survival in animal models, and usually only when administered early in the course of the disease, before the appearance of clinical signs.

To date, only two treatments have been proposed for use in humans: quinacrine and pentosan polysulfate (PPS). Quinacrine was shown to be efficient in cellular models of infection (Korth et al., 2001) and, due to its previous use in human therapeutics as an antimalarial drug, was advocated for immediate use in humans with TSE (Korth et al., 2001). However, subsequent studies showed it to have no therapeutic effect in either experimental animals (Collins et al., 2002; Barret et al., 2003) or humans (Furukawa et al., 2002; Kobayashi et al., 2003; Nakajima et al., 2004). PPS is a polyanion that was also reported to be efficient in cellular models (Caughey & Raymond, 1993; Priola et al., 1994) and to delay the appearance of prion disease in hamsters (Ladogana et al., 1992) and mice (Diringer & Ehlers, 1991; Farquhar et al., 1999). PPS has been administered directly into the CNS via intraventricular shunts in experimental animals, with significant prolongation of the incubation period (Doh-ura et al., 2004), and in a small number of symptomatic human patients, with questionable improvement in neurological condition and continuing progression in brain atrophy (Todd et al., 2005).

None of these treatments thus appears to be either practical or efficient.

Among the most effective \textit{ex vivo} anti-TSE drugs is a family of molecules known as heparan mimetics (HMs). HMs are substituted polysaccharides obtained by controlled chemical substitution of dextran with precise amounts of carboxymethyl, sulfate and hydrophobic groups (Schonberger et al., 2003). HMs were initially synthesized for their tissue-regeneration properties (Meddahi et al., 1994; Blanquaert et al., 1995; Desgranges et al., 1999) and later tested for their anti-prion properties. These drugs are presumed to act in competition with endogenous heparan sulfate proteoglycans that can bind to PrP (Gabizon et al., 1993; Brimcombe et al., 1999; Gonzalez-Iglesias et al., 2002; Warner et al., 2002), play an active role in the PrP endocytic pathway (Shyang et al., 1995), act as co-receptors for the binding of PrP to the cellular receptor LRP/LR (Hundt et al., 2001) and influence PrP\textsuperscript{Sc} synthesis (Ben-Zaken et al., 2003; Horonchik et al., 2005) and amplification (Deleault et al., 2005).

Adjou et al. (2003) described one HM derivative, HM2602, that abolished prion propagation in scrapie-infected GT1 cells, hampered PrP\textsuperscript{Sc} accumulation in scrapie- and BSE-infected mice and prolonged the survival time of 263K scrapie-infected hamsters. However, therapy with this molecule was compromised by the presence of potentially mutagenic benzylamide groups. We describe here a novel HM derivative (CR36) that is devoid of these chemical structures.

### METHODS

**Cells.** The cells used throughout the experiments were GT1 cells (hypothalamic neuronal cells) and ScGT1 cells (GT1 cells chronically infected with the Chandler scrapie strain), kindly provided by S. Lehmann, Montpellier, France. They were cultured as described previously (Mange et al., 2000), except that Dulbecco’s modified Eagle’s medium was replaced by OPTI-MEM (Invitrogen) as culture medium.

**Chemicals.** CR36 and HM2602 were obtained by the CRRET laboratory, Créteil, France. These products were prepared by controlled chemical substitution of dextran T40 (Pharmacia) with defined amounts of carboxymethyl, sulphate and benzylamide (HM2602) or phenylalanine methyl ester (CR36) groups as described previously (Papy-Garcia et al., 2002). Prior to use, the products were solubilized in sterile 0.9 % NaCl.

PPS was kindly provided by Sanofi-Synthelabo, Chilly-Mazarin, France, and quinacrine was purchased from Sigma-Aldrich. These products were solubilized in sterile 0.9 % NaCl and used under the same conditions as CR36.

**Characterization of the efficacy of the drugs on a cellular model.** GT1 and ScGT1 cells were seeded in 75 cm\(^2\) flasks at 900 000 cells per flask and treated for 4 days with the desired drug concentration. On day 5, cells were washed with PBS, lysed at 4 °C in lysis buffer [0.5 % sodium deoxycholate, 0.5 % Triton X-100, 50 mM Tris/HCl (pH 7.4)] and the nuclear fraction was removed. The protein concentration was measured by using a bicinchoninic assay (microBC assay; Interchim) and all samples were normalized to equal protein concentration. ScGT1 samples were then separated into two fractions: one was treated with proteinate K (PK) at 15 μg (mg protein)\(^{-1}\) for 30 min at 37 °C, then Pefabloc was added at a 4 mM final concentration; the other sample was treated immediately with Pefabloc, as were the GT1 samples. All samples were then precipitated with 2.5 vols cold acetone (1 h at −20 °C), centrifuged at 8000 g for 10 min and the pellet was resuspended in deposition buffer and analysed by an ELISA test. Semiquantification of PrP was performed by comparison with serial dilutions of 20 % uninfected mouse-brain homogenate (positive control) in a matrix of 20 % PrP-KO mouse-brain homogenate (negative control), purified as described previously but without PK treatment. The experiments were performed in duplicate and the mean ± SD was calculated.

Long-term efficacy of treatment on PrP\textsuperscript{Sc} accumulation was evaluated by Western blot analysis of passaged cells that had been initially subjected to two consecutive drug exposures. ScGT1 cells were seeded on six-well plates on day 0, treated with medium supplemented with CR36 at 10 or 1 μg ml\(^{-1}\) for two consecutive periods of 4 days, then passaged approximately twice a week. At each passage, a fraction of the cells was lysed at 4 °C with lysis buffer and the nuclear fraction was removed after a 2 min 9000 g centrifugation. The protein concentration was measured by using a bicinchoninic assay. All samples were normalized to equal protein concentration, treated with PK at 15 μg (mg total protein)\(^{-1}\) for 30 min at 37 °C, then centrifuged at 4 °C for 90 min at 21 000 g. The pellets were resuspended in deposition buffer and analysed by Western blot with a PrP-specific monoclonal antibody, Saf83.

**PrP\textsuperscript{Sc} accumulation in the spleens of mice.** C57Bl/6 female, 8-week-old mice were inoculated intraperitoneally on day 0 with 100 μl of a BSE (6PB1)- or scrapie (C506M3)-infected 2 % (w/v) brain homogenate or of uninfected brain homogenate for control mice. Groups of five animals were treated intraperitoneally twice a week from day 0 to day 35 with CR36 at 1, 10 or 25 mg kg\(^{-1}\), HM2602 at 25 mg kg\(^{-1}\), PPS at 25 or 75 mg kg\(^{-1}\), quinacrine at 60 mg kg\(^{-1}\) or normal saline for ‘mock-treated’ mice. They were
sacrificed at day 35, the spleens were collected and 10 % (w/v) spleen homogenates were prepared in 5 % sterile glucose by using a RiboLyser (Bio-Rad). PrP<sub>res</sub> was purified by centrifugation in the presence of detergents after PK digestion, according to a previously described scrapie-associated fibril (SAF) protocol (Lasmezas et al., 1997). PrP<sup>res</sup> accumulation in the spleens was quantified by ELISA, according to a previously described protocol (Grassi et al., 2001), modified to detect murine PrP. To compensate for mouse-to-mouse spleen-weight differences, PrP<sup>res</sup> measurements were normalized to individual spleen weights.

**Evaluation of the efficacy on survival time.** C57Bl/6 female, 8-week-old mice were inoculated intraperitoneally on day 0 with 100 µl of the same 2 % 6PB1 brain homogenate as was used in the splenic PrP<sup>res</sup> study and dosed intraperitoneally twice a week with CR36 or PPS at 50 mg kg<sup>−1</sup> or normal saline for control mice either from day 1 to day 35 or from day 1 to day 110. Survival times were measured and PrP<sup>res</sup> levels in the brains and spleens at death were quantified by using the ELISA test described above.

**Statistical analyses.** Statistical analysis used to characterize the efficacy of the drugs on cellular models and to test variations in PK resistance was ANOVA followed by a Newman–Keuls multiple-comparison test. For all in vivo experiments, analysis was performed by using a t-test for independent samples.

**RESULTS**

CR36 inhibits ex vivo PrP<sub>res</sub> accumulation in the ScGT1 cellular model

After a 4 day treatment with CR36 at 100, 10, 1 or 0.1 µg ml<sup>−1</sup>, PPS at 100, 10, 1 or 0.1 µg ml<sup>−1</sup> or HM2602 at 10 µg ml<sup>−1</sup>, no significant difference was detected in the amount of PrP<sup>c</sup> in GT1 uninfected cells or in ScGT1 cells not treated with PK (data not shown). In contrast, the amount of PrP<sup>res</sup> in ScGT1 cells treated with PK was reduced significantly after treatment (<i>P</i> < 0.001, except for CR36 at 0.1 µg ml<sup>−1</sup>; <i>P</i> < 0.01) (Fig. 1). These results were confirmed by Western blotting analysis (data not shown); PrP<sup>res</sup> disappeared after a 4 day treatment with CR36 or HM2602 at 1 or 10 µg ml<sup>−1</sup>, but complete disappearance was not obtained with PPS, even after a 1000 µg ml<sup>−1</sup> treatment. No cytopathic effect was observed with any of the treatments, and a WST-1 cell-viability assay (Roche) confirmed the absence of drug toxicity (data not shown).

To assess whether the effect of CR36 treatment could persist even after treatment was stopped, we studied the reappearance of the PrP<sup>res</sup> after two consecutive 1 or 10 µg ml<sup>−1</sup> CR36 treatments, the second treatment aiming at clearing any potential residual inoculum. PrP<sub>res</sub> was cleared after both treatments at 1 and 10 µg ml<sup>−1</sup> and did not reappear until day 64 (fifteenth passage) after the 1 µg ml<sup>−1</sup> treatment and at least until day 90 (twenty-first passage) in the cells treated with 10 µg CR36 ml<sup>−1</sup> (data not shown). Thus, treatment of the cells with CR36 resulted in long-term inhibition of PrP<sub>res</sub> formation.

**Reduction of PrP<sub>res</sub> accumulation in the spleens of mice treated with CR36**

All treatments reduced PrP<sub>res</sub> in mice infected with the BSE strain significantly compared with untreated animals (Fig. 2; <i>P</i> < 0.05). At 25 mg kg<sup>−1</sup>, CR36 was as efficient as HM2602 at 25 mg kg<sup>−1</sup> and more efficient than quinacrine at 60 mg kg<sup>−1</sup> or PPS at 25 or 75 mg kg<sup>−1</sup> (which produced 60 % immediate mortality). Comparable results were obtained in scrapie-infected mice (data not shown).

**Effect of CR36 treatment on survival time and brain PrP<sub>res</sub> accumulation**

In the first experiment, mice were treated intraperitoneally from day 1 to day 35 post-infection, corresponding to the time needed for the level of PrP<sub>res</sub> to reach a plateau in the spleen. CR36 at 50 mg kg<sup>−1</sup> twice a week delayed the median appearance of clinical signs and death by only about 10 % (Fig. 3) and mean PrP<sub>res</sub> content of the positive brains was four times lower than for controls (data not shown). Forty per cent of animals with clinical signs had no detectable PrP<sub>res</sub> in the spleen. Longer treatment (days 1–110) was followed by a shortened survival time (−30 %), with no PrP<sub>res</sub> detected in the brain (data not shown).

PPS at 50 mg kg<sup>−1</sup> (days 1–35) produced significant mortality (40 %) during the treatment period, but surviving mice lived 185 days longer (+55 %) than controls and CR36-treated mice. These animals all accumulated PrP<sub>res</sub> in the brain at levels 40 % lower than those of controls (Fig. 3; data not shown), but none of them had detectable PrP<sub>res</sub> in the spleen (Fig. 3).

**DISCUSSION**

HMs have been studied as potential therapeutic candidates for prion diseases because of their homology with
endogenous heparan sulfates that are thought to play a role in prion infection. Studies of dextran sulfate and PPS, the prototype compounds, were followed by work with a compound named HM2602, which was a more potent inhibitor of PrP\textsuperscript{res} accumulation than the prototype compounds (Adjou et al., 2003), but its benzylamide groups had potential carcinogenic activity. A new derivative (CR36), with phenylalanine methyl ester substituted for benzylamide, eliminated this activity and was shown to be as potent as HM2602 and more potent than PPS in our scrapie-infected culture model of ScGT1 cells. The potency was shown to be dose-dependent and, after 1 week treatment at 10 \( \mu \text{g ml}^{-1} \), a complete disappearance of PrP\textsuperscript{res} was observed, with no reappearance in following passages (up to 90 days) (Fig. 1; data not shown). In contrast, PPS, even at doses up to 1000 \( \mu \text{g ml}^{-1} \) (Fig. 1; data not shown), could not suppress the PrP\textsuperscript{res} signal totally, suggesting possible differences in the mechanism of action of these molecules.

The high hopes engendered by these cell-culture studies for comparably improved in vivo activity of this new HM seemed to be supported by the initial studies of PrP\textsuperscript{res} accumulation in the spleens of infected animals: no PrP\textsuperscript{res} could be detected after 1 month treatment at a dose of 25 mg kg\(^{-1}\) in the spleens of CR36-treated animals (Fig. 2). However, this blockage of prion replication in lymphoid tissues was not absolute, as was shown in the long-term experiments on BSE-infected mice: five of nine examined animals showed PrP\textsuperscript{res} in the spleen and all animals dying comparatively late also had PrP\textsuperscript{res} in the brains. In comparison, none of four animals treated with PPS had PrP\textsuperscript{res} in their spleens (even when PrP\textsuperscript{res} was detected in their brains) (Fig. 3).

With respect to survival time, the results were even more disappointing. Although CR36 showed none of the toxicity of PPS (40–60\% mortality following intraperitoneal injection), the drug produced only a marginal (not statistically significant) prolongation of survival time compared with untreated animals, and substantially shorter than that in mice treated with PPS which survived the initial toxic mortality (Fig. 3). The acute toxicity of PPS has been described previously (Farquhar et al., 1999) and is thought to be at least partially due to its anticoagulant properties at the high doses used. The long-term survival of animals resisting acute toxicity has also been observed previously, but here we show for the first time that it is coincident with what appears to be a permanent blockage of PrP\textsuperscript{res} accumulation in lymphoid tissues. It is also the first time that PPS has been shown to be efficient in a BSE experimental model.

In other experiments in which treatments were begun later in the course of the disease (> 120 days post-infection), we observed no effect whatsoever, consistent with the supposition that CR36, like PPS, does not pass through the blood–brain barrier and thus cannot interfere with prion replication after neuroinvasion has occurred unless infused directly within the subdural space.

Further studies are needed to understand the different actions of these molecules. A paradoxical increase of PrP\textsuperscript{res} formation with heparan sulfates has been observed in vitro.

---

**Fig. 2. In vivo:** relative PrP\textsuperscript{res} accumulation in the spleen normalized to PrP\textsuperscript{res} level in untreated mice, after 35 days treatment consecutive with intraperitoneal infection with 2% brain homogenate. UT, Untreated animals; MT, mock-treated animals; HM, HM2602; Q, quinacrine. The asterisk indicates 60% mortality during the treatment period.

**Fig. 3. In vivo:** survival and presence or absence of PrP\textsuperscript{res} in the brains and spleens of BSE-infected mice treated twice a week with CR36 or PPS (50 mg kg\(^{-1}\) given intraperitoneally) for 35 days, beginning 24 h after intraperitoneal infection (2% brain homogenate). Pos control: mock-treated animals (0.9% NaCl).
in cell-free conversion systems (Wong et al., 2001), suggesting that ex vivo and in vivo mechanisms of action may not be identical. Moreover, the persistent effect of PPS on peripheral prion replication compared with the reversible effect of CR36, as well as the difference in toxicity, suggests that the potency of PPS may in part result from the destruction of peripheral target cells. Models based on lymphoid tissues (Beringue et al., 2000) should take into account such delayed effects for the evaluation of future therapeutic molecules.

Finally, our studies on these drugs emphasize, once again, the risk of recommending human trials based solely on efficacy in chronically infected cell cultures and the absolute necessity of first conducting treatment trials in experimental animals. There should be no exceptions to the ‘first rule’ of necessity of first conducting treatment trials in experimental animals. Treatment trials should be conducted in experimental models.

ACKNOWLEDGEMENTS

This work was supported in part by the European Community (QLK2-CT-2001-0285 and QLK3-CT-2001-00283) and the network of excellence NeuroPrion. C. L.-G. is the recipient of a fellowship from Region Ile-de-France. We are grateful to J. Grassi and his colleagues for the anti-PrP antibodies.

REFERENCES


