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A novel generation of heparan sulfate mimetics for the treatment of prion diseases

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The accumulation of PrP^{res}, the protease-resistant abnormal form of the host-encoded cellular prion protein, PrP^{C}, plays a central role in transmissible spongiform encephalopathies. Human contamination by bovine spongiform encephalopathy (BSE) has propelled many scientific teams on a highway for anti-prion drug development. This study reports that heparan sulfate mimetics (HMs), developed originally for their effect on tissue regeneration, abolish prion propagation in scrapie-infected GT1 cells. PrP^{res} does not reappear for up to 50 days post-treatment. When tested *in* vivo, one of these compounds, HM2602, hampered PrP^{res} accumulation in scrapie- and BSE-infected mice and prolonged significantly the survival time of 263K scrapie-infected hamsters. Interestingly, HM2602 is an apparently less toxic and more potent inhibitor of PrP^{res} accumulation than dextran sulfate 500, a molecule known to exhibit anti-prion properties *in vivo*. Kinetics of PrP^{res} disappearance *in vitro* and unaffected PrP^{C} levels during treatment suggest that HMs are able to block the conversion of PrP^{C} into PrP^{res}. It is speculated that HMs act as competitors of endogenous heparan sulfates known to act as co-receptors for the prion protein. Since these molecules are particularly amenable to drug design, their anti-prion potential could be developed further and optimized for the treatment of prion diseases.

INTRODUCTION

Transmissible spongiform encephalopathies (TSEs) or prion diseases are a group of fatal neurodegenerative disorders with long incubation periods and an invariably fatal outcome. They include

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Creutzfeldt–Jakob disease (CJD) in humans, scrapie in sheep and bovine spongiform encephalopathy (BSE) in cattle. The neuropathological changes observed in these diseases are restricted to the CNS and classically consist of spongiosis, neuronal loss and astrogliosis (Fraser, 1976). From a biochemical point of view, TSEs are characterized by the accumulation in the brain of an abnormally folded isoform (PrP^{res}) of the host-encoded prion protein (PrP^{C}), partially resistant to proteolysis (Bolton *et al.*, 1982; Prusiner, 1982). The conversion of the prion protein into its abnormal counterpart is a crucial event in the pathogenesis of TSEs and, therefore, constitutes the hitherto preferred target for therapeutic intervention.

The appearance of variant CJD (vCJD) in Europe has been linked to the contamination of the human food chain by BSE-infected cows. Because the extent of the vCJD epidemic in forthcoming years is still uncertain (Ghani *et al.*, 2000, 2002), and given that no effective treatments are presently available for TSEs, the development of new therapeutic strategies is of crucial importance. One class of molecules that has shown significant efficiency in the treatment of prion diseases is the class of sulfated polyanions, such as dextran sulfate 500 (DS500), pentosane polysulfate and suramin (Farquhar & Dickinson, 1986; Kimberlin & Walker, 1986; Ladogana *et al.*, 1992; Caughey & Raymond, 1993; Beringue *et al.*, 2000; Gilch *et al.*, 2001). However, their use is limited by their toxicity, restricted efficiency and narrow window of intervention following infection (Diringer & Ehlers, 1991; Ladogana *et al.*, 1992).

Sulfated polyanions found in biological systems are heparan sulfate proteoglycans. These molecules are either secreted or membrane-bound and consist of a protein core to which variably sulfated glycosaminoglycan (GAG) chains are attached. During their biosynthesis, specific modifications of the GAG chains can generate a vast diversity of molecules, each with specific functions, ranging from mechanical support to regulation of adhesion, motility and proliferation (reviewed by Bernfield *et al.*, 1999; Turnbull *et al.*, 2001). In addition to their diverse biological functions, heparan sulfates may be good candidates for therapeutic intervention in prion diseases, since they can bind to PrP (Gabizon *et al.*, 1993; Brimacombe *et al.*, 1999; Gonzalez-Iglesias *et al.*, 2002; Warner *et al.*, 2002), play an active role in the PrP endocytic pathway (Shyng *et al.*, 1995) and act as co-receptors for the binding of PrP to the cellular receptor LRP/LR (Hundt *et al.*, 2001). For these reasons, various heparan sulfate mimetics (HMs) (see Fig. 1 for structural information) containing specific structure modifications in their GAG domains, developed initially for their interesting properties in wound healing (Desgranges *et al.*, 1999), were tested *in vitro* for their activity as anti-prion drugs. Two molecules designated HM2602 and HM5004 were chosen and analysed *in vivo*. We report in this study that HM2602 can (i) inhibit PrP^{res} accumulation in scrapie-infected GT1 (ScGT1) cells in a dose-dependent manner, (ii) exert a long-term effect (PrP^{res} does not reappear up to 50 days following removal of the drug), (iii) hamper PrP^{res} formation in the spleens of mice infected intraperitoneally with either scrapie or BSE and (iv) prolong significantly the survival of 263K scrapie-infected hamsters. In contrast, and although it was efficient *in vitro*, HM5004 displayed no

anti-prion activity *in vivo* in any of the experimental models used. Structure analysis provides some clues that the hydrophobicity of the molecule may be important for its anti-prion activity. Since HMs are amenable to various chemical modifications, this study paves the way for the development of this family of compounds as anti-TSE drugs.

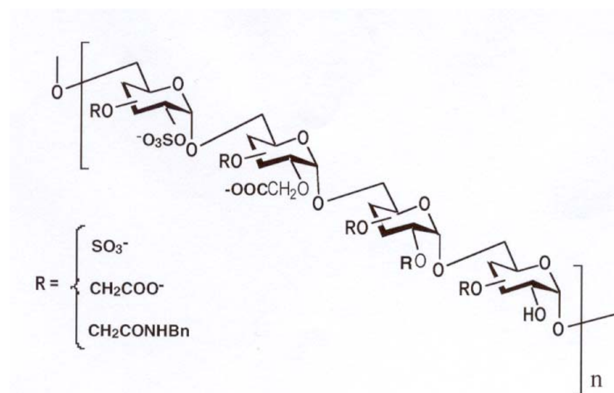

Fig. 1. General chemical formula of HMs, which are produced by chemically substituting hydroxyl groups of a dextran polymer by defined amounts of carboxymethyl (CM), hydrophobic benzylamide (Bn) and sulfate (S) groups. The molecules designated HM2602 and HM5004 contain the following degree of substitution per sugar unit: 0.8 CM, 0.2 Bn and 0.5 S, and 0.5 CM and 1.3 S, respectively.

METHODS

Preparation of HMs. The HMs (Fig. 1) described in this study were obtained by controlled chemical substitutions of T40 dextran (Pharmacia) with defined amounts of carboxymethyl (CM), hydrophobic benzylamide (Bn) and sulfate (S) groups (Desgranges *et al.*, 1999), with modifications as described in Petit *et al.* (2002). The molecules designated as HM2602 and HM5004 contain the following degree of substitution per sugar unit: 0.8 CM, 0.2 Bn and 0.5 S, and 0.5 CM and 1.3 S, respectively.

Cell culture. ScGT1-7 cells (GT1 hypothalamic neuronal cells chronically infected with the Chandler isolate of scrapie), generously provided by S. Lehmann (Montpellier, France), were cultured as described previously (Mange *et al.*, 2000), except that DMEM was replaced by Opti-MEM.

Treatment of cells. HMs and control molecules were added at various concentrations to the medium of cells seeded at a density of 10 % and cultures were incubated with the molecules for 4 days.

Analysis of cellular PrP^{res} levels. ScGT1-7 cells from a 75 cm^2 flask were collected and lysed in a buffer containing 50 mM Tris/HCl (pH 7.4), 0.5 % sodium deoxycholate and 0.5 % Triton X-100 at 4 °C for 10 min. Samples were centrifuged at 10 000 ***g*** for 1 min and the supernatant was collected. Following this step, protein concentrations were measured using a BCA Protein Assay kit (Pierce) and all samples were normalized to equal protein concentrations, treated with proteinase K (PK) (10 µg mg^{-1} total protein) for 60 min at 37 °C and centrifuged at 20 000 ***g*** for 45 min at 4 °C. The pellet was resuspended in 50 µl Laemmli's sample buffer and boiled for 5 min. Proteins were separated by SDS-PAGE on a 12 %

polyacrylamide gel, immunoblotted with a PrP-specific monoclonal antibody, SAF84 (kindly provided by J. Grassi, CEA, Saclay, France), and revealed using enhanced chemiluminescence (Amersham).

Kinetics of PrP^{res} elimination. For time-course experiments, ScGT1-7 cells were seeded to 10 % confluency in five 75 cm^2 flasks. One flask was left untreated, while the other flasks were treated with 10 μg HM2602 ml^{-1}, each flask at intervals of 24 h to obtain different durations of treatment when harvesting all flasks on the same day at the end of the experiment. The cells in each flask were lysed 96 h after the start of the treatment of the first flask, normalized for equal protein concentration and subjected to the PrP^{res} purification protocol described above.

Analysis of spleens. Female, 8-week-old, C57BL/6 mice (Centre d'élevage René Janvier, Le Genest-St-Isle, France) were injected intraperitoneally with 100 μl 2 % (wt/vol) brain homogenates of scrapie-(C506M3) or BSE-infected (6PB1) mice (Lasmézas *et al.*, 1996) diluted in 5 % glucose. Following inoculation, mice were treated intraperitoneally twice a week with 25 mg HMs kg^{-1}, diluted in 5 % glucose, for a total period of 30 days, the first treatment being given 24 h post-inoculation. Treatment with DS500, diluted in 5 % glucose, was performed with the same time-schedule and at the same dose. Control mice were treated with a 5 % glucose solution. To determine the levels of PrP^{res} in the spleens of these mice, animals were sacrificed at 30 days post-inoculation and spleens were collected and homogenized at 10 % (w/v) using a ribolyser (Bio-Rad) in 5 % sterile glucose. PrP^{res} was purified by centrifugation in the presence of detergents, after PK digestion, according to the scrapie-associated fibril (SAF) protocol reported previously (Lasmézas *et al.*, 1997). The level of PrP^{res} in the spleen of each animal was revealed by Western blot using the PrP-specific antibodies JB007 (see Fig. 5) and SAF60 (see Fig. 6) (kindly provided by J. Grassi) and quantified by ELISA, according to a protocol described previously (Grassi *et al.*, 2001), but modified to detect murine PrP, in which the antibodies SAF53 and 11C6 were used as the capture and detection antibodies, respectively.

Survival-time experiments. Outbred, weanling, female golden Syrian hamsters (9 to 10 weeks of age) were obtained from the Centre d'élevage René Janvier. Hamsters were injected intraperitoneally with the 263K scrapie agent, a gift from H. Fraser, Edinburgh, UK (titre of stock suspension, 2.2×10^{11} LD_{50} g^{-1} brain). A 100 μl sample of 2 % (wt/vol) brain homogenate was injected into the right abdomen of each hamster; thus, each animal received 4.4×10^{8} IC infectious doses. Hamsters were treated intraperitoneally with 25 mg HM2602 or HM5004 kg^{-1} body weight (diluted in 5 % glucose), once a week from the day of inoculation until death.

RESULTS

Inhibition of PrP^{res} accumulation in ScGT1 cells

We wanted first to investigate whether HM2602 or HM5004 could inhibit PrP^{res} propagation in ScGT1 cells, which harbour a robust PrP^{res} phenotype [ScGT1 cells stably propagate PrP^{res} and, in our hands, drugs like tricyclic compounds are less potent in these cells than in scrapie-infected neuroblastoma cells (Barret *et al.*, 2003)]. Cells were treated for 4 days with different concentrations of HMs (HM2602 and HM5004), ranging from 0.1 to 10 μg ml^{-1}, and levels of PrP^{res} were analysed. An amphotericin B derivative, MS-8209, known to delay BSE and scrapie replication *in vivo* (Adjou *et al.*, 1995, 1996), was included as a positive control. No apparent cytotoxicity was observed after 4 days of treatment with HM2602 or HM5004 at the concentrations used. HM2602 inhibited PrP^{res} accumulation in ScGT1 cells in a dose-dependent manner (Fig. 2a). At a concentration of 0.1 μg ml^{-1}, HM2602 reduced only partially the levels of PrP^{res} (two of three samples show a moderate decrease, Fig. 2a). At 1 μg ml^{-1}, the reduction in the PrP^{res} signal was always more drastic than that observed at 0.1 μg ml^{-1}, but some residual PrP^{res} was observed in most experiments (Fig. 2a, c), suggesting that these doses were limiting under our cell culture conditions. At the highest concentration, we observed consistently a disappearance of PrP^{res} in the infected cells (Figs 2, 3 and 4). HM5004 showed a comparable effect (Fig. 2b). In contrast, a complete suppression of the PrP^{res} signal could not be obtained with MS-8209, since this molecule was toxic at doses higher than 1 μg ml^{-1} (Fig. 2b). Comparison of the effect of HMs with DS500, another sulfated polyanion proven to be effective *in vivo* (Farquhar & Dickinson, 1986; Kimberlin & Walker, 1986; Beringue *et al.*, 2000), showed a similar reduction in PrP^{res} levels in our *in vitro* model at 1 and 10 μg ml^{-1} (Fig. 2c).

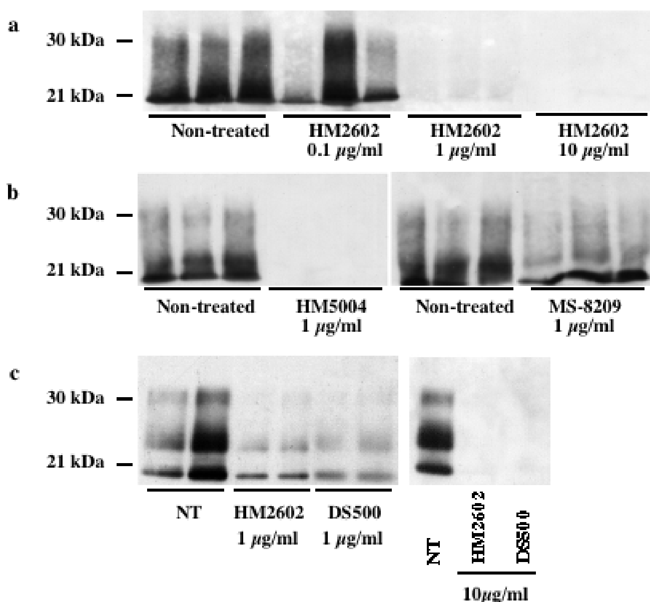

Fig. 2. HM2602 and HM5004 inhibit PrP^{res} accumulation in ScGT1-7 cells. ScGT1-7 cells were incubated for 4 days in medium containing the designated concentrations of HM2602 and HM5004. An amphotericin B derivative, MS-8209, was used as a positive control for anti-prion activity. PrP^{res} was purified from cells and analysed by Western blot using the PrP-specific monoclonal antibody SAF84. (a) Effects seen after treatment with HM2602 (in triplicate). (b) Effects obtained after treatment with HM5004 and MS-8209 (in triplicate). (c) Comparison of *in vitro* effects observed after treatment with HM2602 and DS500 (in duplicate).

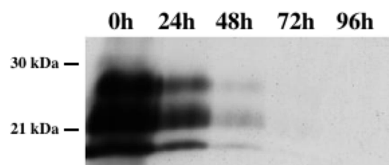

Fig. 3. Kinetics of PrP^{res} elimination in ScGT1-7 cells during treatment with HM2602. Cells were treated with HM2602 for 24, 48, 72 and 96 h. Cell lysates were then collected and protein content normalized. PrP^{res} was purified as described in Methods and visualized by Western blot using the PrP-specific monoclonal antibody SAF84.

To determine the kinetics of PrP^{res} elimination during treatment with HMs, cells were exposed to HM2602 at a dose of 10 µg ml^{-1} for 24, 48, 72 or 96 h. Cells treated with HM2602 showed a significant reduction in the PrP^{res} signal after only 24 h of exposure and a progressive fading of the PrP^{res} signal thereafter, leading to complete disappearance at 96 h (Fig. 3).

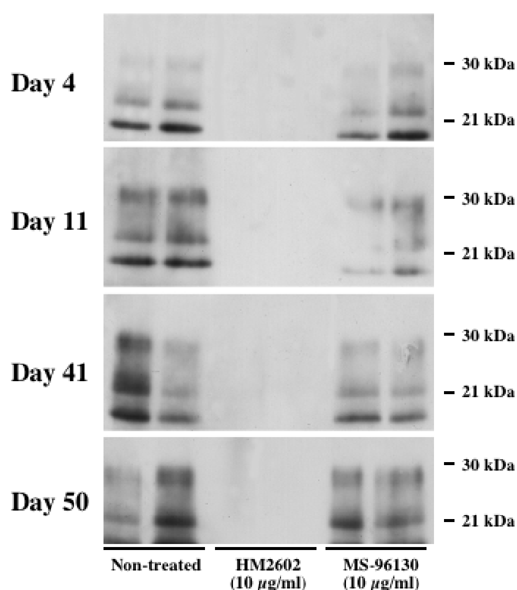

Fig. 4. HM2602 abolishes PrP^{res} accumulation in ScGT1-7 cells up to 50 days post-treatment. ScGT1-7 cells were treated in duplicate with 10 µg ml^{-1} of either HM2602 or MS-96130 for 4 days and then transferred to a new flask and the same treatment repeated the next day. Cell samples were collected at various time-points post-treatment (days 4, 11, 41 and 50) and PrP^{res} levels were revealed by Western blotting using the anti-PrP monoclonal antibody SAF84. The PrP^{res} signal disappeared completely and was not restored after removal of the drug, even after 50 days of culture. The negative control MS-96130 did not exert an effect on PrP^{res} propagation, demonstrating the specificity of the assay.

Long-term inhibition of PrP^{res} formation by HMs

To assess further the extent of the *in vitro* effect of HM2602 and HM5004, we verified whether the ‘PrP^{res}-free’ status of the cells achieved after 96 h of treatment could be maintained. For this purpose, duplicates of ScGT1-7 cells were treated with HM2602 at a concentration of 10 µg ml^{-1} for 4 days, then transferred into a new flask and the same treatment was repeated for an additional 4 days. This second exposure of the cells to HM2602 was performed to eliminate any contamination of the new flask with residual PrP^{res} from the transferred cells. Cell lysates were then collected at various time-points up to day 50, day 1 corresponding to the first day after removal of HM2602. The PrP^{res} signal did not reappear for the whole 50 day period post-treatment (corresponding approximately to 12 passages, Fig. 4). Thus, two consecutive 4 day treatments with HM2602 result in a long-term inhibition of PrP^{res} formation. Exposure of the cells to the polyene antibiotic derivative MS-96130, chosen as a negative control as it exhibited no

anti-prion activity *in vivo* (data not shown), did not cause a significant reduction in the formation of PrP^{res} (Fig. 4), demonstrating the specificity of the treatment by HM2602.

HMs hamper the development and progression of scrapie and BSE infection in animals

These encouraging results obtained *in vitro* prompted us to investigate whether HMs were also effective in animals. For this purpose, we used a rapid animal model developed recently for the screening of anti-prion molecules (Deslys *et al.*, 1998). This model is based on the fact that after peripheral infection, TSE agents are directed to the spleen where they replicate with a concomitant accumulation of PrP^{res}. Thus, to assess whether a drug interferes with prion replication, the level of PrP^{res} in the spleens of mice treated with the compound is measured at the time that a plateau would be reached normally in untreated mice. Based on this principle, HM2602 and HM5004 were administered twice a week at a dose of 25 mg kg^{-1} to a group of 10 scrapie-infected mice for a period of 30 days. Following treatment, the spleen of each animal was collected and the levels of PrP^{res} in this organ were determined (Fig. 5). Our results showed that compared to non-treated animals, biweekly injections of HM2602 reduced significantly the accumulation of PrP^{res} in the spleen of infected mice (Fig. 5b), suggesting that HM2602 can slow the pace of infection. Intriguingly, although HM2602 and HM5004 were both efficient in our *in vitro* model, HM5004 did not exhibit any effect when used at the same dose in scrapie-infected mice (Fig. 5c). The response of the mice to treatment was quantified using ELISA (Fig. 5a), which showed a reduction in PrP^{res} levels of about 70 % after exposure to HM2602 ($P<0.005$, Student's *t*-test).

As it has been shown that BSE is sometimes more resistant than scrapie to therapeutic intervention (Adjou *et al.*, 1996), we next tested the therapeutic potential of HM2602 in a mouse BSE infection model. Moreover, because the effects of DS500 and HM2602 were indistinguishable in ScGT1-7 cells (Fig. 2c), DS500 was tested also in this model to determine whether it had the same therapeutic potential *in vivo*. HM2602 exhibited potent anti-prion properties in BSE-infected mice; only faint PrP^{res} signals were detected in the spleens of animals treated with HM2602 compared to non-treated controls (Fig. 6). Quantification of the Western blot signals showed a 95 % reduction in the PrP^{res} signal with HM2602 as compared to 68 % with DS500. Furthermore, we observed 10 % mortality in mice treated with DS500, demonstrating that the threshold of toxicity was attained with this molecule but not with HM2602.

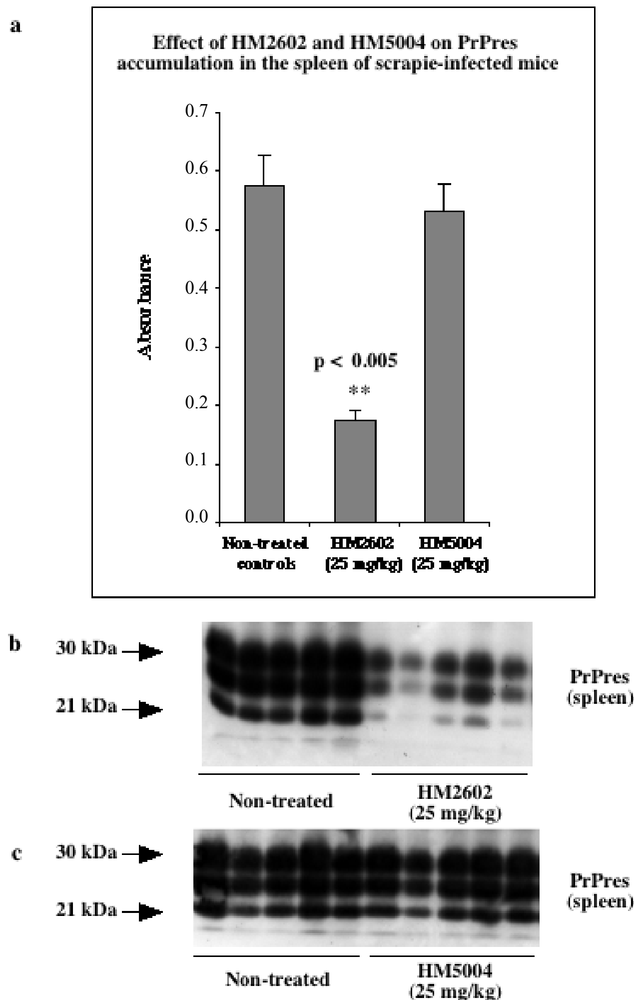

Fig. 5. PrP^{res} levels in the spleens of scrapie-infected mice (C506M3) after treatment with HM2602 and HM5004 at 30 days post-infection. Groups of 10 C57BL/6 female mice were inoculated intraperitoneally with the C506M3 strain of scrapie and treated twice weekly with either HM2602, HM5004 or 5 % glucose (25 mg kg^{-1} body weight) for 30 days. (a) ELISA quantification of PrP^{res} in the spleens of scrapie-infected mice treated with HM2602 ($P<0.005$, unpaired Student *t*-test). (b) Immunoblot of PrP^{res} levels in the spleens of mice treated with 25 mg HM2602 kg^{-1} body weight. (c) Immunoblot of PrP^{res} level in spleens of mice treated with 25 mg HM5004 kg^{-1} body weight.

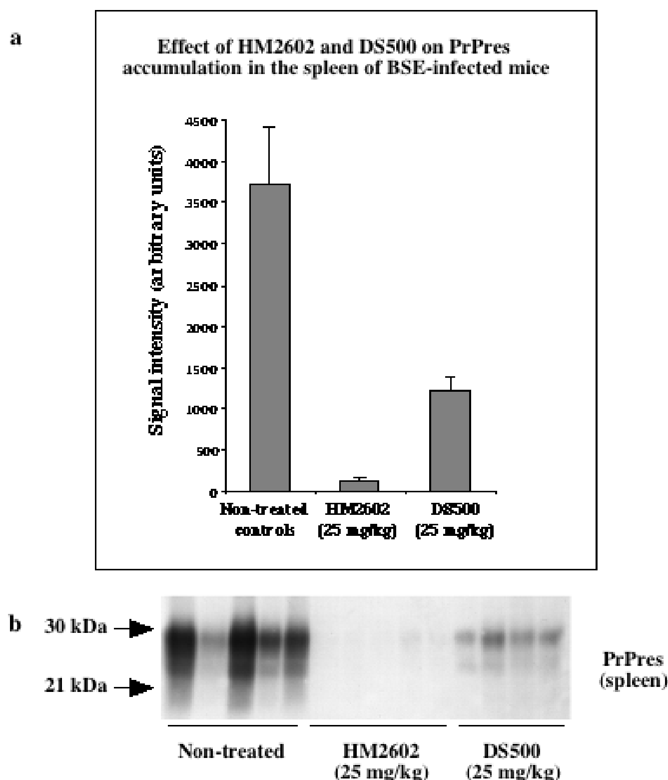

Fig. 6. Effects of treatment of HM2602 and DS500 on the accumulation of PrP^{res} in the spleens of BSE-infected mice. Groups of eight C57BL/6 female mice were inoculated intraperitoneally with the 6PB1 strain of BSE and treated twice weekly with either HM2602, DS500 or 5 % glucose (25 mg kg^{-1} body weight) for 30 days. (a) Quantification of PrP^{res} signals obtained by Western blot. (b) Electrophoresis analysis of PrP^{res} levels in the spleens of treated mice.

We next set out to determine whether the hindrance of PrP^{res} accumulation in the lymphoreticular system would affect the CNS and translate into a delay in the progression of disease and occurrence of clinical signs. Thus, hamsters were inoculated with the neuroinvasive 263K strain of scrapie and treated with 25 mg HM2602 or HM5004 kg^{-1} body weight. Treatments were performed once a week until time of death. We observed that the survival time in animals treated with HM2602 was prolonged by 24 days (196±4 days for treated animals compared to 172±2 days for control animals) (Fig. 7). Interestingly, HM5004 did not prolong significantly the survival time of infected hamsters (mean survival time 174±2 days), which is in agreement with the results obtained in the spleens of infected mice.

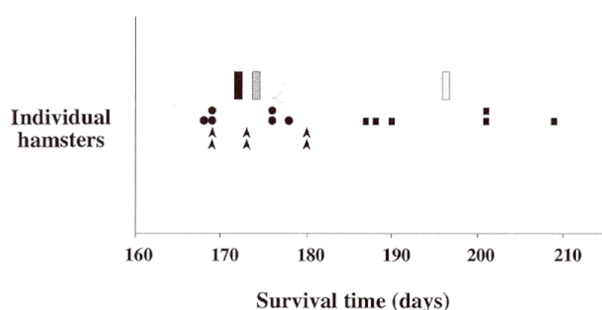

Fig. 7. Survival times of 263K scrapie-infected hamsters treated with HM2602 or HM5004. Groups of six female golden Syrian hamsters were inoculated with the neuroinvasive 263K strain of scrapie and treated intraperitoneally with HM2602 (squares) and HM5004 (arrowheads) diluted in 5 % glucose (25 mg kg^{-1} body weight) once a week from the day of inoculation until death. HM2602 prolonged the mean time of survival of scrapie-infected hamsters by 24 days [mean survival times: HM2602-treated hamsters, 196±4 days (white bar); non-treated hamsters, 172±2 days (black bar); HM5004-treated animals, 174±2 days (grey bar)].

DISCUSSION

It has been shown that PrP can bind specifically to heparan sulfate-like molecules (Gabizon *et al.*, 1993; Brimacombe *et al.*, 1999; Gonzalez-Iglesias *et al.*, 2002; Warner *et al.*, 2002) and that some of them exhibit anti-prion properties (Ehlers & Diringer, 1984; Farquhar & Dickinson, 1986; Kimberlin & Walker, 1986; Ladogana *et al.*, 1992; Caughey & Raymond, 1993; Farquhar *et al.*, 1999; Beringue *et al.*, 2000). For these reasons, various HMs developed initially for their properties to enhance tissue repair were screened to identify inhibitors of PrP^{res} accumulation. The advantage of working with HMs is that they can be modified as desired by varying group substitutions and, thus, the anti-prion activity of lead compounds can be optimized subsequently by structure–function studies.

Two molecules, HM2602 and HM5004, were examined for their capacity to interfere with PrP^{res} formation in ScGT1-7 cells. This type of assay, where cells chronically infected with prions are used as a screening method to identify potential therapeutic molecules, has been exploited on several occasions (Caughey & Raymond, 1993; Mange *et al.*, 2000; Korth *et al.*, 2001; Peretz *et al.*, 2001; May *et al.*, 2003). It is widely accepted that a decrease in the levels of PrP^{res} in cultures is indicative of a hindrance to prion replication (Supattapone *et al.*, 2001). In ScGT1-7 cells, we found that both HMs were efficient at

blocking prion replication (Fig. 2). The effect was dose dependent and at the higher dose the PrP^{res} signal was abolished. The more efficient dose of 10 μg HMs ml^{-1} was, therefore, used in subsequent experiments (Figs 3 and 4). The effect of this new class of compounds was clearly superior to the one observed using the highest non-toxic dose of the amphotericin B derivative MS-8209 (Fig. 2), a drug known to delay significantly the incubation time of experimental scrapie *in vivo* (Adjou *et al.*, 1995).

To assess whether HMs could durably eliminate PrP^{res} from ScGT1 cultures, we treated the cells for 4 days twice consecutively and then monitored PrP^{res} levels over 50 days. During this whole period, PrP^{res} was undetectable (Fig. 4). This result suggested that PrP^{res} was eliminated from the cultures or decreased to trace amounts insufficient to act as a seed for PrP^{res} formation. These cells were then sub-passaged further in our laboratory and, to our surprise, we observed the reappearance of PrP^{res} at day 120. While we have no explanation for this observation, this finding suggests that minute amounts of the abnormal prion protein present in a few cells are sufficient to reinitiate the growth of infection. Furthermore, our findings underline the need, in the future, for longer monitoring periods when assessing the therapeutic potential of anti-prion molecules *in vitro*.

The kinetics experiment showed that the molecule decreases the PrP^{res} level early after its application (Fig. 3, 24 h); we estimated the time of treatment needed for PrP^{res} to become undetectable by Western blot to be around 96 h. Compared to the kinetics of action of Congo red and polyene antibiotics tested previously in the same ScGT1 cells, where a PrP^{res} signal is still present 5 and 15 days after drug application, respectively (data not shown; Mange *et al.*, 2000), HM2602 appears to act faster. Moreover, the time of disappearance of PrP^{res} after HM2602 treatment (96 h) corresponds to that of PrP^{res} catabolism described previously (Caughey *et al.*, 1989), suggesting that the drug does not contribute to PrP^{res} degradation but acts by inhibiting the *de novo* production of PrP^{res} by the cells. Moreover, HMs do not alter the level of expression of PrP^C in cell cultures nor in the spleens of mice (data not shown), demonstrating that the block in PrP^{res} formation does not occur by inhibiting PrP^C synthesis.

Given the kinetics of elimination of PrP^{res} in our infected cells, the heparan-binding properties of PrP (Gabizon *et al.*, 1993; Brimacombe *et al.*, 1999; Warner *et al.*, 2002) and the role of heparan sulfates in the binding of PrP to the cellular receptor LRP/LR and in PrP endocytosis (Shyng *et al.*, 1995; Hundt *et al.*, 2001), two mechanisms of action can be proposed at this stage. HMs might interact with PrP^C and hamper its conversion into PrP^{res} through steric inhibition. Alternatively, HMs might compete with the natural cellular heparan sulfates and block the PrP–LRP/LR interaction. We speculate that this latter interaction is relevant to prion propagation because (i) LRP/LR is essential to the endocytosis of PrP^C (Gauczynski *et al.*, 2001) and/or PrP^{res}, which, in turn, is necessary for the accumulation of PrP^{res} in the cells and (ii) LRP/LR may be a direct co-factor for PrP conversion. These hypotheses have been

underpinned recently by our demonstration that antibody blockage or expression inhibition of LRP/LR inhibits PrP^{res} formation in cultured cells (Leucht *et al.*, 2003). Therefore, a blockage of the interaction of PrP with LRP/LR through HMs would prevent cell-to-cell prion propagation.

Turning to *in vivo* experiments, we show by two different methods that HM2602, unlike HM5004, can interfere with the progression of TSEs. The first depends on a 'rapid' spleen assay consisting of measurement of PrP^{res} formation in the spleen of mice 30 days after intraperitoneal inoculation. Although when a species barrier is crossed, PrP^{res} and infectivity do not always correlate (Lasmézas *et al.*, 1997; Hill *et al.*, 2000; Race *et al.*, 2001, 2002), measuring PrP^{res} levels is now generally accepted as a screening method for drugs or inactivation procedures using host-adapted TSE models (Lee *et al.*, 2000). By this method, we have observed a substantial decrease in PrP^{res} levels in the spleen of HM2602-treated mice compared to controls; HM5004, on the other hand, did not exert an effect (Fig. 5). Secondly, a survival experiment was set up in the 263K golden Syrian hamster model (Kimberlin & Walker, 1977). The animals treated with HM2602 (injection by intraperitoneal route with 25 mg HMs kg^{-1} body weight, once weekly) lived, on average, 24 days longer than untreated animals, corresponding to a 14 % increase in survival time. Interestingly, HM5004 did not have any effect on the survival times of these hamsters (Fig. 7). Given the short plasma retention time of this family of compounds (Meddahi *et al.*, 2002), it is probable that increasing the frequency of treatment would improve substantially the observed therapeutic effect of HM2602. This is substantiated by our *in vitro* data showing a positive correlation between the treatment regimen and the hindrance of PrP^{res} formation (Fig. 2) and by a separate experiment in which scrapie-infected mice were treated with HM2602 every day for 30 days (instead of twice weekly as in Fig. 5), resulting in a reduction in spleen PrP^{res} below detectable levels (data not shown). This argues that in the hamster 263K model, a higher frequency of treatment would result in a further increase in animal survival time. Overall, both the survival data and the results obtained in the rapid spleen assay demonstrate the therapeutic potential of HM2602. Therefore, to assess if this drug might be useful to treat vCJD, we also tested its efficacy in a BSE-infection model. This was important since it was shown for other drugs like amphotericin B that BSE is often more resistant than scrapie to therapeutic intervention (Adjou *et al.*, 1996). HM2602 inhibited PrP^{res} accumulation in the spleens of BSE-infected mice such that the protein was barely detectable at 30 days post-inoculation. HM2602 was more efficient than the reference molecule DS500 (Fig. 6) and also less toxic, thus allowing a much larger therapeutic window.

Surprisingly, although both HMs successfully reduced the accumulation of PrP^{res} in ScGT1-7 cells, only HM2602 impeded PrP^{res} formation *in vivo* (Fig. 5). The mechanisms responsible for this discrepancy are not known. However, since the activity of anti-prion drugs has been shown to vary depending on the strain of prions (Adjou *et al.*, 1996), it is conceivable that the variation seen here might be due to the different strains of scrapie used in the experimental models (Chandler for the ScGT1-7 cells and

C506M3/263K for the animal infections). Analysis of the chemical composition of the compounds provides clues to an alternative explanation: HM2602, due to the presence of a Bn group, exhibits a higher hydrophobicity profile than HM5004. This difference probably affects the tissue distribution of the molecules in such a way that the concentration of the less hydrophobic HM5004 in target organs is too low to exert an effect. Moreover, the conversion of PrP^{C} into PrP^{res} is characterized by the exposure to the surface of hydrophobic residues that are normally buried in PrP^{C} (Safar *et al.*, 1998). This phenomenon, which explains partly the high propensity of PrP^{res} to aggregate, also suggests the possibility of a stronger interaction with a more hydrophobic molecule like HM2602 and hence a better inhibition of the template-assisted conversion by PrP^{res}. This effect might act in conjunction with the higher tissue concentration of the drug. Such a hydrophobic interaction between PrP^{res} and anti-prion drugs like Congo red and iododoxorubicin has been demonstrated previously (Prusiner *et al.*, 1983; Merlini *et al.*, 1995; Tagliavini *et al.*, 1997).

In this study, we have demonstrated that HM2602 and HM5004 block PrP^{res} accumulation in ScGT1 cells. *In vivo*, HM2602 was shown as a potential therapeutic agent against prion diseases. The inhibition of PrP^{res} accumulation in the spleen observed with HM2602 was more effective than we had experienced previously with other drugs such as amphotericin B derivatives. Combined with our survival data, these results constitute a strong incentive towards the development of long-term trials using these molecules, with increased frequency of administration. Importantly, the effect was not strain-restricted, as it could be observed with both scrapie- and BSE-infected mice, suggesting a general mechanism of action. Notwithstanding the need for further drug characterization and development, our study defines HMs as a new class of drugs for the treatment of TSEs. These molecules are particularly amenable to drug design and may prove efficient even in later stages of the disease due to their properties to enhance tissue repair (Desgranges *et al.*, 1999).

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