MS-8209, a water-soluble amphotericin B derivative, affects both scrapie agent replication and PrPres accumulation in Syrian hamster scrapie

Karim T. Adjou,1 Remi Demaimay,1 Jean-Philippe Deslys,1 Corinne I. Lasmézas,1 Vincent Beringue,1 Severine Demart,1 Francois Lamoury,1 Michel Seman2 and Dominique Dormont1

1 CEA, Service de Neurovirologie, DSV/DRM/CRSSA, B. P. 6, 60-68, Avenue du Général Leclerc, 92265 Fontenay aux Roses Cedex, France
2 Laboratoire d’Immunodifférenciation, Institut Jacques Monod, Université Paris 7, 2 place Jussieu, 75251 Paris Cedex 05, France

Amphotericin B (AmB) has been shown to delay hamster scrapie. Infectivity studies have been performed previously using AmB in order to understand the relationship between the accumulation of an abnormal isoform (PrPres) of the prion protein and 263K scrapie agent replication in the brain. The first study reported that AmB had no effect upon agent replication, although it delayed the development of both clinical signs and PrPres accumulation. However, subsequent experiments using the same model showed a significant effect both on agent replication and PrPres accumulation early in infection. This fundamental discrepancy was assumed to be linked to differences in experimental protocols. In order to unravel the issue, a new experiment has been performed encompassing different protocols and using an AmB derivative, MS-8209, that can be used at higher doses because of its lower toxicity. The findings of this study exclude the suspected differences in the protocols as the reason for previous conflicting results, and suggest strongly that these discrepancies were due to a low dose of AmB causing a ‘threshold effect’. Overall, this study indicates that, in this model, PrPres cannot be dissociated from infectivity by polyene antibiotics.

Introduction

Experimental hamster scrapie is a useful model for the transmissible spongiform encephalopathies (TSEs), which are neurodegenerative diseases of many mammalian species. TSEs include Creutzfeldt–Jakob disease, Gerstmann–Sträussler Scheinker syndrome, kuru and fatal familial insomnia in man and, in animals, transmissible mink encephalopathy, chronic wasting disease of mule deer and elk, feline spongiform encephalopathy and bovine spongiform encephalopathy (BSE).

These fatal diseases are characterized by a long incubation period. The histopathological changes they induce in the central nervous system are a progressive neuronal vacuolization in the dendritic and axonal processes and neuron cell bodies and gliosis with hypertrophy and the possible proliferation of astrocytes. TSEs are caused by transmissible infectious agents named TSE agents or prions.

At the molecular level, these disorders are characterized by the accumulation of an abnormal isoform (PrPres) of the host-encoded prion protein (PrP) (Bolton et al., 1982). PrPres is resistant to limited proteolysis and is derived from PrP by post-translational modification (Bolton et al., 1982). PrPres copurifies with the scrapie agent and is considered as the only component, or the major part, of the agent (Prusiner, 1982, 1992; Weissmann, 1991). The transcriptional accumulation of glial fibrillary acidic protein (GFAP), a specific marker for astrocytes, has also been observed. GFAP and its mRNAs are overexpressed in both natural and experimental scrapie (Dormont et al., 1981; Georgsson et al., 1993; Lazarini et al., 1994; Mackenzie, 1983).

At present, no treatment is available for these diseases. Many unsuccessful therapeutic strategies have been evaluated in experimental animal models: they include the use of antiviral drugs, hormones, antibiotics and antifungal agents (Brown, 1990). Three groups of drugs exhibited relative efficacy against scrapie: polyanions (Diringer & Ehlers, 1991; Ehlers & Diringer, 1984; Kimberlin & Walker, 1983, 1986; Ladogana et
Amphotericin B :  \( R = \text{NH}_2 \)  
MS-8209 :  \( R = \text{NH}_2 \) 

**Fig. 1.** Structures of AmB and MS-8209. AmB contains a hydrophilic polyhydroxyl chain along one axis and a lipophilic polyene hydrocarbon chain along the other. Although these features confer very low solubility in both aqueous and organic solvents, they allow AmB to intercalate into cell membranes.

with the duration of the treatment. AmB prolongs the incubation time but the duration of the clinical phase and the clinical symptoms remain identical. The anti-scrapie effect of AmB depends upon the period of treatment; treatment during the early stages of infection appears to be the best regimen (Pocchiari et al., 1989b) and no significant effect has been observed when hamsters are treated after the appearance of clinical symptoms (Pocchiari et al., 1989b).

At the molecular level, AmB treatment significantly delays the accumulation of PrPres and GFAP in the brain. Infectivity studies have been performed to understand the relationship between PrPres accumulation, agent replication and the onset of the disease. The first study, using Syrian hamsters infected with the 263K scrapie strain, suggested that AmB had no effect upon agent replication though it delayed the development of both clinical signs and PrPres accumulation (Pocchiari et al., 1989b; Xi et al., 1992). However, other experiments using the same model showed an effect both on scrapie agent replication and on PrPres accumulation early in infection (McKenzie et al., 1994). This discrepancy may be due to different experimental conditions, such as the composition of the inoculum; purified fractions were used in the former, whereas crude brain homogenates, theoretically containing AmB, were used in the latter.

MS-8209 is an AmB derivative that exhibits antifungal properties identical to those of AmB but has the advantage of being more soluble and at least five times less toxic (Saint Julien et al., 1992). The development of new AmB derivatives with lower toxicity such as MS-8209 has resulted in an improvement in the therapeutic benefit of polyene antibiotics in scrapie (Adjou et al., 1995, 1996; Demainay et al., 1994, 1997). Thus, we used high doses of MS-8209 as a new tool to investigate the link between 263K scrapie agent replication and PrPres accumulation in the central nervous system of scrapie-infected hamsters.

**Methods**

- **Infection of animals.** Outbred, weanling female golden Syrian hamsters (age 9–10 weeks; weight 100 g) were obtained from the Centre d’élevage René Janvier (Le Genest-St-Ise, France). The animals received water and food *ad libitum.* Hamsters were injected intracerebrally with the 263K scrapie agent, a gift from H. Fraser (Institute for Animal Health, Edinburgh, UK) (title of the stock suspension was \( 2.2 \times 10^{11} \text{LD}_{50}/\text{g} \) brain tissue). Fifty µl of a 1% (w/v) brain homogenate was injected into the right cerebral hemisphere.

- **Drugs.** Parenteral desoxycorticosterone AmB (Fungizone) was obtained from Squibb. MS-8209 is the N-methylglucamine (NMG) salt of 1-deoxy-1-aminoo-4,6-O-benzylidene-o-fructosyl-AmB (Mayoly-Spindler Laboratories) (see Fig. 1). Drugs were diluted in sterile 5% (w/v) glucose solution. Animals were treated by the intraperitoneal route for 6 days a week, from the day of inoculation throughout the duration of the disease until death. They were monitored regularly for the onset of clinical symptoms. Control groups were either untreated or injected intraperitoneally with the same volume of NMG.
Protein analysis

Protein preparation and immunoblotting. Three hamsters from each group were killed by cervical column disruption when the first clinical signs were observed in infected, untreated animals, in order to estimate PrPres and GFAP accumulation in the brain. The central nervous system (including the cerebellum) was then removed, frozen immediately in liquid nitrogen and kept at −80 °C until use. Brain homogenates were suspended at 20% (w/v) in a 5% glucose solution. Aliquots (50 µl) were digested with a final concentration of 50 µg/ml proteinase K for 1 h at 37 °C. Denaturation buffer containing 1× Tris–glycine, 2% SDS, 2% β-mercaptoethanol and 5% sucrose was added, and the samples were heated at 100 °C for 5 min. The samples were then sonicated and centrifuged for 5 min at 15,000 r.p.m. at 20 °C, and subsequently separated by SDS–PAGE at 100 V for 2 h. Proteins were transferred onto nitrocellulose membranes by electrotransfer. The membranes were saturated with a milk buffer (5%, w/v, milk powder, 0.1% Tween 20 and 0.1% NaN₃ in PBS), immunostained for 263K PrPres with 3F4 monoclonal antibody (1:50,000), washed three times in PBS containing 0.1% Tween 20 and incubated for 30 min at room temperature with monoclonal antibodies to peroxidase-conjugated mouse immunoglobulin (1:2500). Immunodetection was carried out with an enhanced chemiluminescence kit (Amersham). Signals were quantified by laser densitometry.

Purified fractions of PrPres (PrP27–30). For PrPres purification, the whole brain hemisphere was homogenized at 20% (w/v). Briefly, proteinase K was used at 10 µg/ml for 1 h at 37 °C and digestion was blocked with PMSF (5 mM). After addition of Sarcosyl to 10% and Tris–HCl (pH 7.4) to 10 mM, samples were incubated for 15 min at room temperature. Samples were then centrifuged at 245,000 g for 4 h at 20 °C on a 10% sucrose cushion (Beckman TL100). Pellets were resuspended in Laemmli buffer and run on a 12% polyacrylamide gel. Protein immunoblotting procedures using chemiluminescence were as described above.

Infectivity studies. Brain infectivity was measured at 40 days post-infection (p.i.) by titration in golden Syrian hamsters. Titres were estimated by the end-point dilution method (Reed & Muench, 1938) or by measuring relative incubation periods and estimating the titre from a standard dose-period incubation curve. The inocula used in these experiments consisted either of crude brain homogenates or of purified brain fractions, also named ‘scrapie-associated fibrils’ (SAF) (Merz et al., 1983). Aliquots for titration were diluted in sterile 5% glucose (w/v) solution.

Results

Effects of long-term treatment on survival time and PrPres accumulation

The experiment was designed to determine the optimum pharmacological effect of a long-term treatment with MS-8209 and Amb. Animals were treated from the day of experimental infection, throughout the course of the disease until death. As shown in Fig. 2(a), the pharmacological action of MS-8209 was dose dependent and all doses tested were effective. Both Amb and its derivative increased the incubation period of the disease significantly. Hamsters treated with 1 or 2.5 mg Amb or 2.5 mg MS-8209 per kg body weight had mean incubation periods prolonged by +46, +66 and +75%, respectively (Fig. 2a). Doses of 10 and 25 mg MS-8209 per kg induced delays in scrapie onset of +100%.

To examine the effects of MS-8209 and Amb on PrPres and GFAP accumulation in the central nervous system, three hamsters from each group of animals were killed at 72 days p.i., which is when clinical signs appeared in untreated, infected hamsters.

The levels of PrPres (Fig. 2b) and GFAP (Fig. 2c) were markedly lower in the treated groups than in the controls.
PrP27-30

30 kDa

21 kDa

Dilutions

Fig. 3. Measurement of PrP27-30 by Western blotting in purified fractions of the brains from untreated and AmB- and MS-8209-treated hamsters after 40 days p.i. Lanes: 1, uninfected control; 2, infected, untreated control at 40 days p.i. (dilution 1/4); 3, NMG at 40 days p.i. (dilution 1/4); 4, AmB (1 mg/kg); 5, AmB (2.5 mg/kg); 6, MS-8209 (2.5 mg/kg); 7, MS-8209 (10 mg/kg); 8, MS-8209 (25 mg/kg); 9–15, infected, untreated controls at the terminal stage of the disease (dilutions 1/2000, 1/1000, 1/500, 1/200, 1/100, 1/50 and 1/10).

Table 1. Infectivity of 263K scrapie agent at 40 days p.i., calculated in hamsters infected intracerebrally with brain homogenates and treated with AmB and MS-8209

The increase in the incubation period for MS-8209- or AmB-treated groups was calculated from the mean of the solvent-treated and untreated groups. The increases were significant (P < 0.005, Mann–Whitney U test) unless indicated otherwise. ns, Not significant; –, not applicable. The infectivity titres were measured at 40 days p.i., based on the end-point dilution method (Reed & Muench, 1938).

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Incubation period (days) (mean ± SEM)</th>
<th>Increase in incubation period (days)</th>
<th>Infectivity of 263K scrapie agent (log10 LD50)</th>
<th>Difference in infectivity titre (logs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>87.7 ± 1.1</td>
<td>0</td>
<td>7.4</td>
<td>–</td>
</tr>
<tr>
<td>NMG solvent</td>
<td>85.5 ± 0.7</td>
<td>0</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>AmB (1)</td>
<td>120 ± 4.0</td>
<td>0</td>
<td>6.5</td>
<td>0–9</td>
</tr>
<tr>
<td>AmB (2.5)</td>
<td>144 ± 1.7</td>
<td>0</td>
<td>5.7</td>
<td>2.4*</td>
</tr>
<tr>
<td>MS-8209 (2.5)</td>
<td>151 ± 0.7</td>
<td>0</td>
<td>5.3</td>
<td>2.1*</td>
</tr>
<tr>
<td>MS-8209 (10)</td>
<td>171 ± 1.0</td>
<td>0</td>
<td>4.4</td>
<td>3.0*</td>
</tr>
<tr>
<td>MS-8209 (25)</td>
<td>171 ± 1.2</td>
<td>0</td>
<td>4.5</td>
<td>3.4*</td>
</tr>
</tbody>
</table>

*Significantly different from controls (P < 0.05, Mann–Whitney U test).

However, at the terminal stage of the disease, all brain samples from all groups contained similar levels of PrPres and GFAP (data not shown).

Effects on scrapie agent replication

Crude brain homogenates. PrPres and scrapie infectivity were measured in the same fraction of brains removed from non-treated and AmB- or MS-8209-treated hamsters at 40 days p.i. (Fig. 3), which is the time when the greatest difference in the PrPres levels was observed between AmB-treated and non-treated hamsters by Xi et al. (1992). As shown in Table 1, AmB (2.5 mg/kg) and MS-8209 (2.5, 10 and 25 mg/kg) reduced 263K scrapie agent replication markedly. Indeed, infectivity levels in 2.5 mg/kg AmB- or MS-8209-treated animals were more than 2 logs lower than those of non-treated animals (Table 1). Moreover, the difference between the infectivity titres calculated for brains of non-treated and 10 and 25 mg/kg MS-8209-treated animals was 3 and 3.4 logs, respectively.

In order to estimate the effects of polyene antibiotics on PrPres accumulation in the brain at 40 days p.i., we measured PrP27-30 by Western blotting in purified, infectious fractions of brains from non-treated and AmB- or MS-8209-treated hamsters used for infectivity studies. As shown in Fig. 3, comparison of the staining intensities on the blots demonstrated that PrPres accumulation was lower in treated animals than in controls (non-treated and solvent-treated). In fact, the estimated amounts of PrPres in brains of treated animals killed at 40 days p.i. were 10- to 1000-fold lower than in non-treated control hamsters: 1 mg/kg AmB reduced the accumulation of PrP27-30 at least 10-fold, and 2.5 mg/kg AmB and 10 and 25 mg/kg MS-8209 reduced accumulation 100- and 1000-fold, respectively (Fig. 3). The reductions of
PrP27-30 levels correlated well with the differences observed in the estimated infectivity titres (Fig. 4).

Purified brain fractions (SAF). This experiment was done in order to determine whether the composition of the inoculum used in the bioassay studies was responsible for the discrepancy reported in the two previous studies (McKenzie et al., 1994; Xi et al., 1992). We therefore also infected recipient hamsters intracerebrally with SAF obtained from brains of AmB- or MS-8209-treated and non-treated animals. Infectivity was then titrated by measuring relative incubation periods and estimating the titre from the standard dose-incubation period curve (Fig. 5). As shown in Table 2, infectivity titres at 40 days p.i. were reduced significantly compared to those of controls. The differences in infectivity titres obtained in this experiment were similar to those obtained with crude brain homogenates, especially for animals treated with high doses of MS-8209 (10 and 25 mg/kg) (Tables 1 and 2). These findings confirm that treatment with AmB and derivatives interferes with the replication of the scrapie agent.

**Discussion**

In spite of the large accumulation of evidence supporting the role of PrP in the pathogenesis of prion diseases, the relationship between PrP and infectivity remains controversial (Caughey & Chesebro, 1997). Recent data suggest that only a fraction of the PrPres is associated strongly with infectivity (Somerville & Dunn, 1996). On the other hand, first-passage rats with Creutzfeldt–Jakob disease agent showed obvious clinical signs, activated microglia and spongiform changes in the brain, but PrPres was not detected in histological sections from clinically ill animals (Manuelidis et al., 1997). Hill et al. (1997) reported that they were not able to detect PrPres by Western blotting on primary passage in BSE-inoculated, HuPrP+/+ Prnp0/0 transgenic mice. The BSE agent could also be transmitted to wild-type C57BL mice without detectable cerebral PrPres accumulation, as determined by immunoblotting (Lasmézas et al., 1997).

In order to understand the relationship between abnormal PrP accumulation and scrapie agent replication in the brain, infectivity studies have been performed previously using pharmacological tools such as AmB, giving conflicting results. The results of one study suggested that AmB had no effect upon agent replication, although it delayed both clinical signs and accumulation of PrPres (Xi et al., 1992), while the results of another study showed an effect on both scrapie agent replication and PrPres accumulation early in infection (McKenzie et al., 1994). A major difference between these two studies lies in the experimental design. Xi and colleagues used purified brain fractions (SAF) whereas McKenzie and colleagues used crude brain homogenates in the bioassay studies. This difference between the two inocula was perceived as possibly important, since crude brain homogenates might contain residual AmB that had accumulated in the brain during treatment and may have an effect on the survival time during the bioassays. In contrast, this effect would be abolished by the use of purified brain fractions (SAF) (M. Pocchiari, personal communication).

This hypothesis has been taken into account in our experimental design. In our study, however, the use of the two types of inocula gave similar results in terms of infectivity (Tables 1 and 2). According to these data, in this experimental model, potentially residual AmB in the inoculum cannot explain the discrepancies reported.

On the other hand, our experiments indicate that the effects of polyene antibiotics on infectivity are clearly detectable only...
at doses of at least 2.5 mg/kg body weight. The maximum dose used in previous studies was 2.5-fold lower, which seems to constitute the key to the discrepancies observed. Indeed, our findings point out that 1 mg/kg AmB seems to be a critical dose where replication of the agent begins to be affected, with a significance that probably depends on slight differences in the experimental procedures.

However, by using MS-8209 at higher doses, we observed clear-cut effects in terms of a marked delay in scrapie agent replication (Tables 1 and 2). The maximum differences in infectivity obtained with MS-8209 therapy were more than 3 logs compared with that of controls. This strong decrease of TSE-associated infectivity confirms that at present polyene antibiotics constitute one of the best candidates as a basis for future therapeutic strategies, which will most probably require a multi-drug approach (Adjou et al., 1998).

Our findings suggest that, in the Syrian hamster model, there is a close association between PrPres accumulation and 263K scrapie agent replication in the central nervous system. Previous observations suggested strongly that the anti-scrapie effects of AmB and derivatives are directed towards the PrPres accumulation process (Adjou et al., 1997). The results presented in this study are consistent with the idea that the drugs act on the PrP to PrPres conversion step, the central event of scrapie agent replication, but also with the hypothesis that the action on PrPres accumulation hampers replication of the agent more indirectly, for example by limiting a co-factor of replication or interfering with uptake into cells.

We thank Dr Kamel Cherifi and Mayoly-Spindler Laboratories for providing the MS-8209 derivative. We are also grateful to René Rioux and Jean-Claude Mascaro for the excellent animal care. This work has been supported by a grant from DRET and ACCSV no. 10 of the French Ministry of Research. This paper has been presented in part at the International Symposium of Prion and Lentiviral Diseases in Reykjavik, Iceland (20–22 August, 1998).

Table 2. Estimated infectivity of 263K scrapie agent at 40 days p.i. in the brains of recipient hamsters infected intracerebrally with purified brain fractions (SAF)

Infectivity titres of the 263K scrapie agent were estimated based on the dose-incubation curve shown in Fig. 5. – Not applicable.

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Infectivity of 263K scrapie agent (log_{10} LD_{50})</th>
<th>Difference in infectivity titre (logs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>7.2</td>
<td>–</td>
</tr>
<tr>
<td>AmB (1)</td>
<td>6.0</td>
<td>1.2</td>
</tr>
<tr>
<td>AmB (2:5)</td>
<td>4.1</td>
<td>3.1*</td>
</tr>
<tr>
<td>MS-8209 (2:5)</td>
<td>3.9</td>
<td>3.3*</td>
</tr>
<tr>
<td>MS-8209 (10)</td>
<td>3.8</td>
<td>3.4*</td>
</tr>
<tr>
<td>MS-8209 (25)</td>
<td>3.6</td>
<td>3.6*</td>
</tr>
</tbody>
</table>

*Significantly different from controls (P < 0.05, Mann–Whitney U test).

References


