Pharmacological studies of a new derivative of amphotericin B, MS-8209, in mouse and hamster scrapie

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Transmissible subacute spongiform encephalopathies (TSSE) are neurodegenerative diseases characterized by the presence of a modified, partially proteinase-resistant host protein, PrP^{Sc}, which accumulates in the brains of infected individuals. Recently it has been reported that amphotericin B (AmB) treatment of hamsters infected with scrapie strain 263K prolongs the incubation period of the disease, and dissociates *in vivo* replication of the scrapie agent from PrP^{Sc} accumulation. We report here on data obtained after treatment with AmB and one of its derivatives, MS-8209, in experimental scrapie of mouse and hamster. Treatment was carried out by the intraperitoneal route 6 days per week, at three different dosages initiated at the time of infection. Two regimens were used: during the early time of infection or throughout the experimental infection. Results indicate that MS-8209 was as efficient as AmB in prolonging the incubation time and decreasing PrP^{Sc} accumulation in the hamster scrapie model. A dose-dependent response was observed in mice treated early after experimental infection. At a dose of 2·5 mg/kg, MS-8209 significantly prolonged the incubation period (by 11·9%). In long-term treatment of mice, MS-8209 and AmB markedly reduced PrP^{Sc} levels in the preclinical stage of the disease. These data demonstrate that the effect of AmB is not restricted to one model (hamster–263K). This regimen leads to an inversion of the PrP^{Sc} to proteinase-sensitive protein (PrP^{Sens}) ratio, suggesting PrP^{Sens} (presumably cellular PrP^{C}) accumulation occurs before its conversion into PrP^{Sc}. As it has been shown that AmB does not modify the infectivity titre, we conclude that the drugs could act by inhibiting either the interaction of the scrapie agent with PrP^{Sens} during the early times of infection or the conversion of PrP^{Sens} into PrP^{Sc}.

Unconventional transmissible agents, also named prions, are responsible for diseases in both humans and animals. Their nature and mechanism of infection are still elusive. This group of diseases is characterized by a long incubation period and a fatal outcome after the onset of clinical signs. Neuropathological characteristics (spongiosis and gliosis) are found in both natural diseases and experimental models in rodents and primates. A post-translational accumulation of a pathological isoform (PrP^{Sc}) of a host-coded protein (PrP^{C}) (Bolton *et al.*, 1982; Prusiner, 1982) is detectable. This isoform is characterized by its partial resistance to proteolytic cleavage (Oesch *et al.*, 1985). Furthermore, an accumulation of glial fibrillary acidic protein (specific to astrocytes) and its mRNA is observed (Dormont *et al.*, 1981; Lazarini *et al.*, 1992; Mackenzie, 1983). Several unsuccessful treatments have been investigated in the past, including hormones, anti-neoplastics, drugs against parasites, bacteria, viruses and fungi, and so on (for a review see Brown, 1990). Two groups of drugs proved their efficacy against experimental scrapie: polyanions (Dormont *et al.*, 1986; Ehlers & Diringer, 1984; Farquhar & Dickinson, 1986; Kimberlin, 1978) and amphotericin B (AmB) (Amyx *et al.*, 1984; Pocchiari *et al.*, 1986). In experimental scrapie, treatment with the polyene macrolide antibiotic AmB prolongs the incubation period in hamsters infected with the 263K scrapie strain either intracerebrally or intraperitoneally. The anti-scrapie effect of AmB is limited to the period of treatment and treatment during the early stages after infection appears to be the best regimen. The observed dose-dependent effect of AmB seems to be mediated by inhibition of PrP^{Sc} accumulation in brain (Xi *et al.*, 1992). Yet the maximum dose used in chronic disease treatment is 5 mg/kg. At this dose, efficient inhibition has been shown in the hamster model. A new derivative of AmB, MS-8209 (Cefai *et al.*, 1991), has been developed that exhibits antifungal properties identical to AmB, but with less toxicity (St Julien *et al.*, 1992). Furthermore, MS-8209 inhibits infection by human immunodeficiency

virus types 1 (Cefai *et al.*, 1991) and 2 (P. Clayette, personal communication) *in vitro*.

Experiments reported here were designed (i) to assess the efficiency of MS-8209 compared to AmB in the hamster model, (ii) to test the drug in mice infected by the C506M3 scrapie strain (similar to the ME7 strain), which represents a model closer to the natural disease than the strain 263K–hamster model (Kimberlin & Walker, 1977, 1979) and (iii) to explore the accumulation of PrP^{Sc} in order to determine whether the anti-scrapie effect is similar to that proposed by XI *et al.* (1992) for AmB.

Scrapie agent C506M3 was obtained from brain homogenate of C57BL/6 mice (a gift from Dr D. C. Gajdusek, NIH, Bethesda, Md., U.S.A.), after the seventh passage of infection, at the terminal stage of disease (5×10^7 LD$_{50}$/g of brain). 263K scrapie agent was obtained from the brain homogenate of hamster (a gift from Dr D. C. Gajdusek) at the terminal stage of disease (4.4×10^8 LD$_{50}$/g of brain). AmB was obtained from the deoxycholate salt (Fungizone; Squibb). MS-8209 is the *N*-methyl glucamine (NMG) salt of 1-deoxy-1-amino-4,6-*O*-benzylidine-D-fructosyl-AmB. Drugs were suspended in a sterile 5% (w/v) glucose solution. All animals were treated 6 days per week by the intraperitoneal route. Controls consisted of one untreated group and one group treated with NMG.

Animals were sacrificed by cervical column disruption. The central nervous system (CNS) including the cerebellum was dissected, rapidly frozen in liquid nitrogen, and kept at -80 °C until use. Brain homogenates were suspended at 20% (w/v) in a 5% (w/v) glucose solution. Some aliquots (50 µl) were digested by Proteinase K (20 µg/ml for 1 hour at 37 °C). Denaturation buffer (150 µl; Tris–glycine, 2% SDS, 2% 2-mercaptoethanol, 5% sucrose) were added to samples and kept at 100 °C for 5 min. They were sonicated and centrifuged at 15000 r.p.m. for 5 min at 20 °C before being separated by 12% SDS–PAGE at 100 V for 2 h. Proteins were then transferred onto nitrocellulose membranes by electrotransfer. After saturation with a milk buffer (PBS, 5% w/v, milk, 0·1% Tween 20, 0·1% NaN$_3$), membranes were incubated with an anti-mouse PrP polyclonal antibody or with an anti-hamster PrP monoclonal antibody (both were kindly provided by Dr R. J. Kascsak, Institute for Basic Research in Developmental Disabilities, New York, N.Y., U.S.A.) for 2 h at room temperature. After three washes in PBS containing 0·1% Tween 20, membranes were incubated with monoclonal antibodies to rabbit or mouse Ig conjugated with peroxidase for 30 min at room temperature. Immunodetection was carried out with an ECL kit (Amersham). Signals were quantified by laser densitometry.

In a first experiment, hamsters were intracerebrally

Table 1. *Survival time of hamsters, infected intracerebrally with scrapie, after various treatments*

Treatment* (number)	Survival (days p.i. ±s.e.m.)	Delay† (days)	Mann-Whitney test
Untreated (3)	81·3±3·3		
NMG (2)	88±0	7·7	NS‡
AmB (3)	98·7±0·7	14·7	$P < 0.03$
MS-8209 (3)	100·7±0·3	16·7	$P < 0.03$

* Treatment from 0 to 7 days p.i. with 2·5 mg/kg of the indicated drug.

† Delay in time of death for NMG-treated group was in comparison with untreated group and delay for AmB- and MS-8209-treated groups was calculated from the average of untreated and NMG-treated groups.

‡ NS, Not significant.

infected (50 µl of 1% w/v CNS homogenate) and were treated during the first week of infection with 2·5 mg/kg of AmB or MS-8209. No significant difference in survival was observed between the two control groups (untreated infected animals and infected animals treated with NMG). Death occurred at 83 days post-inoculation (p.i.) (Table 1). After treatment with MS-8209 or AmB at 2·5 mg/kg, survival was significantly different from that observed in the untreated group ($P < 0.03$ and $P < 0.03$, respectively; Mann-Whitney test). The survival times of animals treated with AmB and MS-8209 were prolonged by 14·7 and 16·7 days respectively (Table 1). This delay was related to an increase in the incubation time, as the duration of the clinical phase and the clinical symptoms were identical in all groups. For PrP analysis, hamsters were sacrificed at 72 days p.i., which is the time of appearance of clinical signs in untreated, infected hamsters. In both treated groups the level of PrP^{Sc} was markedly reduced compared to control groups (Fig. 1). These PrP levels were similar to those found in untreated, infected hamsters at 50 days p.i. (data not shown). Yet, at the end of the experimental disease all animal brains contained PrP^{Sc} at a similar level in all groups (data not shown).

In a second experiment, three groups of intraperitoneally infected mice (80 µl or 1% w/v CNS homogenate) were treated from day -7 to day 40 p.i. at different dosages of MS-8209 (0·25, 2·5 or 25 mg/kg). The onset of clinical signs was delayed. In the first experiment no effect was observed at dose below 2·5 mg/kg (Table 2). Statistical analysis (using both the non-parametric Mann-Whitney and parametric ANOVA tests on StatView II software) did not reveal any difference in survival time between the untreated group, the placebo group and the group treated with 0·25 mg/kg. The survival of mice treated with 2·5 mg/kg was significantly different from the other groups ($P < 0.05$) and death occurred, on average, 39 days later than for the untreated group (Table 2). We also tested,
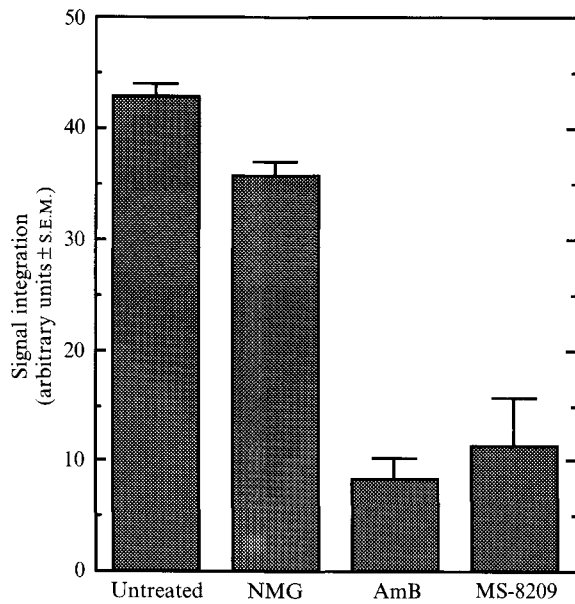
Fig. 1. Accumulation of proteinase K-resistant PrP^{Sc}, in hamsters treated during the 6 days following inoculation with 2·5 mg/kg of scrapie-infected brain. Brain homogenates were digested with 50 μg/ml of proteinase K for 1 h at 37 °C.

Table 2. *Survival time of mice, infected intraperitoneally with scrapie, after various treatments*

Treatment* (number)	Survival time (days p.i. ± s.e.m.)	Delay† (days)	Mann-Whitney test
Untreated (6)	332·7 ± 6·7		
NMG (6)	326·5 ± 8·3		NS‡
MS-8209			
0·25 mg/kg (6)	330 ± 3·9	2·2	NS
2·5 mg/kg (5)	349·3 ± 9·5	39	P < 0·03
NMG§ (12)	296·2 ± 6·1		
MS-8209§			
25 mg/kg (12)	357·1 ± 7·5	60	P < 0·001

* Treatment was as indicated, from −7 to +40 days p.i.
† Delay for MS-8209-treated groups was calculated from the average of NMG-treated and untreated groups.
‡ NS, Not significant.
§ These experiments were carried out using the same inoculum as the previous experiment.

under the same conditions, a third dose of 25 mg/kg. This treated group was significantly different from the others (*P* < 0·001) and animal survival was prolonged for 60 days (Fig. 2). It is noteworthy that the maximum dose used in this experiment was fivefold higher than the dose used for AmB (Pocchiari *et al.*, 1989), and no apparent toxicity was observed.

In a third experiment, intracerebrally infected mice (20 μl of 1 % w/v CNS homogenate) were treated on the day of inoculation and throughout the course of the disease with 2·5 mg/kg of AmB or MS-8209. Untreated, infected animals died at 170 days p.i. (Table 3), in
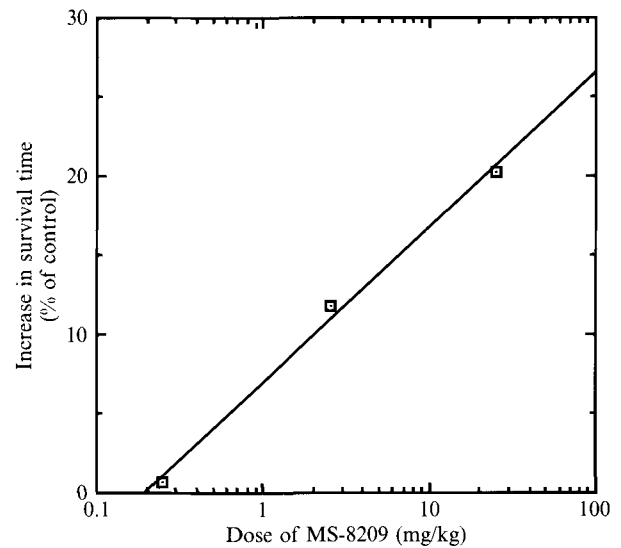

Fig. 2. The effect of dose on survival time in intraperitoneally infected mice, treated with MS-8209 early after infection (−7 to +40 days p.i.). Correlation coefficient $R^2 = 0·994$; $y = 7 + 9·8 \log(x)$.

Table 3. *Survival time of mice intracerebrally infected with scrapie and subjected to long term drug treatment*

Treatment	Survival time (days p.i. ± s.e.m.)	Delay (days)	Mann-Whitney test
Untreated (3)	170·7 ± 2·3*		
AmB			
2·5 mg/kg (6)	222·7 ± 7·1	52	P < 0·03
MS-8209			
2·5 mg/kg (6)	214·8 ± 16·6	44·2	P < 0·03

* This result is identical to that of a duplicate experiment performed using 24 mice (data not shown).

accordance with previous data obtained in our laboratory (Lazarini *et al.*, 1992). Mice treated with 2·5 mg/kg of either AmB or MS-8209 died significantly later than the untreated, infected group (*P* < 0·02 and *P* < 0·025 respectively; Mann-Whitney test). The delay observed in survival time was 52 days for mice treated with AmB and 44·2 days for mice treated with MS-8209 (Table 3). Analysis of PrP accumulation was performed at the time of death of untreated infected mice (170 days p.i.; Fig. 3). For untreated mice, the difference between the undigested PrP (PrP^{Tot}) and PrP^{Sc} integration signals was identical to the level of PrP routinely detected in the uninfected control animals. Therefore, under our experimental conditions, this difference might be representative of the cellular protease-sensitive PrP (PrP^{Sens}). The level of PrP^{Tot} and PrP^{Sc} was reduced in the two treated groups. AmB seems to be more efficient at blocking the accumulation of PrP^{Sc}. Again, in AmB-treated mice, the PrP^{Sens} level was similar to that in the negative control group and untreated, infected mice.
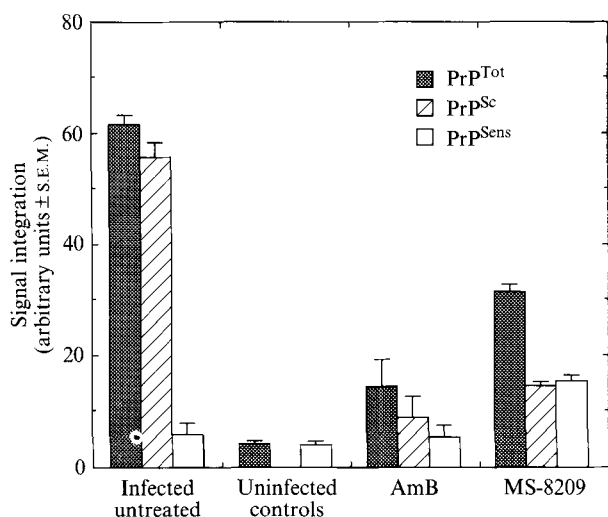
Fig. 3. Detection of undigested PrP (PrP^{Tot}), proteinase K-digested PrP (PrP^{Sc}) and estimated PrP^{Sens} (PrP^{Tot} — PrP^{Sc}) in intracerebrally infected mice, treated at the day of inoculation and throughout the experimental disease. Mice were sacrificed at 170 days p.i.

In MS-8209-treated mice, the amount of PrP^{Sc} was proportionally lower than in AmB-treated or untreated, infected animals. Thus, the estimated PrP^{Sens} level seems to be greater in this group. As for hamsters, the levels of PrP^{Tot} and PrP^{Sc} were identical in all groups at the end of the experimental disease (data not shown).

Until now, the 263K-infected hamster was the only model in which AmB has shown efficacy, despite attempts to reproduce this effect in other experimental scrapie models (Xi *et al.*, 1992). This led several authors to suggest that the action of AmB is restricted to the 263K scrapie strain or to the recipient species (Caughey & Raymond, 1993; Prusiner *et al.*, 1993). Our data demonstrate that the effect of AmB can be extended to other models. Although both scrapie strains used in our studies were established from naturally infected sheep, the passage histories of C506M3 and 263K are totally different (Gibbs *et al.*, 1964; Kimberlin & Walker, 1977). Thus, we may hypothesize that interaction between AmB and the scrapie agent might be non-specific, probably mediated by interaction with membranes in accordance with the previously described mechanism (Medoff *et al.*, 1983). Failure in prolonging the incubation period in other experimental models might be due to the dose of AmB used: only 1 mg/kg for hamsters infected with the 139H strain and 3 mg/kg for mice infected with the 139A strain (Xi *et al.*, 1992). Despite this non-specific mechanism, the response to AmB and its derivative might be specific for the two strains used.

MS-8209 increases the length of the experimental disease in the two animal models. Since apparent toxicity of MS-8209 is at least fivefold lower than that of AmB

(Cefai *et al.*, 1991), an improved action of the drug might be anticipated by use at higher doses. Unlike mepartricin, another derivative of amphotericin that prolongs incubation time only in intraperitoneally infected hamsters (Pocchiari *et al.*, 1989), no difference was observed with MS-8209 between intracerebrally and intraperitoneally infected mice. Thus, we may also have to determine whether MS-8209 prolongs the incubation time in intraperitoneally infected hamsters. Moreover, at the preclinical stage, accumulation of PrP^{Sc} was reduced in treated animals, in agreement with previous results (Xi *et al.*, 1992). Since it has been shown that AmB and MS-8209 do not modify the infectivity titre in infected brains, it might be concluded that the effects of this drug on clinical disease could be related to inhibition of PrP^{Sc} accumulation.

In MS-8209-treated mice, the PrP^{Sc} level is proportionally lower than for groups of untreated animals and AmB-treated, infected animals. The estimated PrP^{Sens} level seems to be higher in this group. This might support the hypothesis of the pre-existence of PrP^{Sens} accumulation before PrP^{Sc} conversion. Nevertheless, the increase in immunoreactivity observed after Proteinase K digestion (Serban *et al.*, 1990) should be considered; we might have underestimated the level of PrP^{Sens} in our samples. Some characteristics of PrP^{Sc}, compared to PrP^C, are (i) a partial resistance to Proteinase K digestion (Oesch *et al.*, 1985), (ii) insolubility in detergent (Meyer *et al.*, 1986) and (iii) non releasability by phosphatidyl-inositol-specific phospholipase C digestion (Stahl *et al.*, 1990). In accordance with the first characteristic, it is possible, theoretically, that the calculated level of PrP^{Sens} in our samples is representative of PrP^C. This accumulation is in agreement with the finding that the level of PrP^C is a predominant factor for susceptibility to scrapie infection (Büeler *et al.*, 1993).

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