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Short Communication

Oral pravastatin prolongs survival time of scrapie-infected mice

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Statins are potent inhibitors of HMG–CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase in the cholesterol-biosynthesis pathway. They are either lipophilic (e.g. simvastatin) or hydrophilic [e.g. pravastatin (PRV)] compounds, considered mainly for long-term treatment of hypercholesterolaemic individuals. Beneficial effects of statins are not related exclusively to their lipid-lowering action; they also possess cholesterol-independent, pleiotropic effects (e.g. anti-inflammatory and antioxidant). Recent studies revealed that simvastatin treatment increased survival significantly in scrapie-infected mice. Although PRV treatment results in measurable drug levels in the mouse brain, the anti-prion effect of this compound has not been investigated. Therefore, we aimed to test the potential therapeutic action of PRV in a murine scrapie model. Our study showed that high-dose and long-term oral PRV treatment prolonged survival times of strain 139A scrapie-infected mice significantly (194 versus 177 days) in the absence of any obvious toxicity, suggesting that protective effects of statins may be independent of absolute solvent or water solubility of the drug.

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Prion diseases or transmissible spongiform encephalopathies (TSEs) are lethal neurodegenerative disorders affecting animals, e.g. scrapie in sheep and goats and Creutzfeldt–Jakob disease (CJD) in humans. The conversion of cell-surface glycosylphosphatidylinositol (GPI)-anchored prion protein, referred to as PrP^{C}, into the pathological scrapie isoform, PrP^{Sc}, is the key event in the pathogenesis of TSEs (Aguzzi *et al.*, 2008). Cholesterol, a necessary component of lipid rafts, was demonstrated to be essential for the cell-surface localization of PrP^{C} (Bate *et al.*, 2004; Gilch *et al.*, 2006). Cholesterol depletion by high doses of statins disrupts lipid rafts, altering protein localization and function on the cell membranes (Simons & Ehehalt, 2002; Michel & Bakovic, 2007). Statins act as reversible competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG–CoA) reductase, which, as the key rate-controlling enzyme in cholesterol biosynthesis, catalyses the conversion of HMG–CoA to mevalonic acid. Statins are categorized pharmacologically as lipophilic (e.g. lovastatin and simvastatin) or hydrophilic [e.g. pravastatin (PRV)] compounds, depending upon their solubility in lipid solvents or water, and are considered to be first-line therapeutics for the prevention of coronary heart disease and atherosclerosis (Schachter, 2005). However, the beneficial effects of statins are not related exclusively to their lipid-lowering activity. They also possess a cholesterol-independent action, the so-called pleiotropic effects (e.g. anti-inflammatory, antioxidant and vasoprotective), which are probably attributable to the cellular consequences of depletion of intermediates (isoprenoids) in the cholesterol-biosynthetic pathway (Liao & Laufs, 2005). Growing research supports the hypothesis that lipid rafts harbour the pathogenic scene both for the conformational conversion of PrP^{C} into the infectious form, PrP^{Sc} (Taraboulos *et al.*, 1995; Vey *et al.*, 1996), and for the proteolytic processing of the β-amyloid (Aβ) peptide in Alzheimer's disease (AD) (Reid *et al.*, 2007), linking these two events to each other (Parkin *et al.*, 2007; Taylor & Hooper, 2007; Debatin *et al.*, 2008).

It was shown in mouse brain that statins, although in different patterns, may disturb *trans*-bilayer cholesterol distribution and domain sorting, rather than cellular bulk cholesterol levels (Kirsch *et al.*, 2003; Burns *et al.*, 2006). Recent studies showed that simvastatin treatment, at 1–100 mg (kg body weight)$^{-1}$ day^{-1}, was able to delay disease onset and to increase survival significantly of experimentally scrapie-infected mice (Mok *et al.*, 2006; Kempster *et al.*, 2007; Haviv *et al.*, 2008). Lipophilic statins, such as simvastatin, are commonly considered to be able to cross the blood–brain barrier (BBB) promptly by passive

diffusion. However, it was shown in rodents that hydrophilic statins, such as PRV, may enter hepatic cells (Nezasa *et al.*, 2003; Evers & Chu, 2008), as well as brain capillary endothelial cells at the BBB (Kikuchi *et al.*, 2004; Cheng *et al.*, 2005), via an ATP-dependent anion-transport polypeptidic system, referred to as Oatps in rodents and OATPs in humans. These transporters, expressed in a variety of different tissues including gut, kidney and brain, play important roles in drug absorption, distribution and excretion (Kivisto & Niemi, 2007; Seithel *et al.*, 2008). PRV was not previously thought to cross the BBB, although it was shown that PRV (0.5–10 mg kg^{-1} day^{-1} for 4 weeks) reduced Aβ deposition as much as the lipophilic drug lovastatin in the brains of mice in a transgenic-mouse model of AD (Chauhan *et al.*, 2004). Moreover, it was recently demonstrated in C57BL/6 mice that high-dose oral PRV treatment (100 mg kg^{-1} day^{-1}) for 21 days resulted in measurable drug levels in the brain (as much as with simvastatin and lovastatin treatment), at levels above the IC_{50} (i.e. 50 % inhibitory concentration) for inhibition of HMG–CoA reductase activity (Johnson-Anuna *et al.*, 2005). Statin levels in the brain declined quickly between 1 and 6 h following acute administration, probably due to active transport and/or metabolism (Thelen *et al.*, 2006). Moreover, in guinea pigs, a species considered to be a suitable model of humans with respect to lipoprotein and lipid metabolism, oral administration of high doses of simvastatin or PRV lowered levels of the cholesterol precursor lathosterol and its ratio to cholesterol significantly in the brains of treated animals (Lütjohann *et al.*, 2004).

Thus, the main goal of the present study was to test the effect of the hydrophilic statin PRV as a potential therapeutic anti-prion agent in a murine scrapie model.

One-month-old female C57BL/6 mice (Charles River) weighing 18–20 g, identified individually by a passive integrated transponder, were inoculated intracerebrally (i.c.) in the left hemisphere with 1 % (w/v) brain homogenate prepared from terminally ill, strain 139A scrapie-infected mice as described previously (Vetrugno *et al.*, 2005) and assigned randomly to the control or PRV-treated groups. PRV sodium salt (mouse oral LD_{50}, 8939 mg kg^{-1}), kindly provided by Bristol–Myers Squibb, was administered in the drinking water at a dose of 200 mg (kg body weight)$^{-1}$ day^{-1} from the time of scrapie inoculation. Water consumption was monitored twice weekly and drug concentration was adjusted as required. Control animals received water without PRV. Mice were examined twice weekly until the appearance of scrapie clinical signs and then observed daily until they reached the terminal stage of the disease, when they were euthanized by carbon dioxide. Brain, heart, liver and muscles (i.e. quadriceps) were collected as scheduled. Each mouse brain was divided into the two hemispheres; one was frozen (at -80 °C) for immunoblot analysis and the other was formalin-fixed. The other tissues were formalin-fixed for histopathological examination. Biochemical, histopathological and immunohistochemical studies were performed as described previously (Nonno *et al.*, 2006; Di Bari *et al.*, 2008). Statistical analyses were finally performed on 13 PRV-treated and 10 control scrapie-infected mice and results are expressed as the mean $\pm$ SD. Mean values of survival times were compared by using a Mann–Whitney test. Bonferroni's correction was adopted to control for type I error in multiple comparisons carried out by *t*-test on body weights at different times.

Compared with the control group, oral PRV treatment delayed disease symptoms and prolonged survival times of strain 139A scrapie-infected mice by a mean of 17 days (194.3 ± 7.5 versus 177.4 ± 4.4 days; Mann–Whitney test, $P=0.0001$) (Table 1). Kaplan–Meier survival curves showed higher proportions of survivors in the PRV-treated group during the observation period (log-rank test, $P<0.0001$) (Fig. 1). In order to study the effect of PRV treatment on scrapie-associated pathology in terminally sick scrapie-affected mice, we investigated both PrP^{Sc} accumulation in the brain by Western blotting, and astrocytosis in the brain [glial fibrillary acidic protein (GFAP)] by immunohistochemistry. No significant difference in terms of deposition, abundance and glycotype pattern of PrP^{Sc} was observed between treated and control brains (data not shown). Hence, in agreement with other authors (Mok *et al.*, 2006; Kempster *et al.*, 2007), we found no direct *in vivo* correlation to the reported abrogation of PrP^{Sc} deposition by cholesterol-lowering drugs in scrapie-infected cell cultures. GFAP staining of brain sections revealed no significant difference between treated and untreated scrapie-infected mice, in accordance with the results of Mok *et al.* (2006) (data not shown).

Mok *et al.* (2006) reported that C57/B6 mice treated with simvastatin [100 mg (kg body weight)$^{-1}$ day^{-1}], administered as an ingredient of the mouse chow pellets since 100 days post-infection (p.i.), survived on average from 16 days (mean $\pm$ SD: 194 ± 6 days, $n=10$, versus 178 ± 7 days,

Table 1. Survival times of PRV-treated and control scrapie-infected mice

Group	Survival times (days p.i.)	Mean $\pm$ SD*
PRV-treated ($n=13$)	181, 183, 188, 189, 191, 195, 196, 196, 199, 199, 202, 202, 205	194.3 ± 7.5
Control ($n=10$†)	171, 171, 175, 175, 176, 180, 181, 181, 182, 182	177.4 ± 4.4

*PRV-treated group versus control group: Mann–Whitney test, $P=0.0001$.
†Three mice in this group died prematurely from causes unrelated to scrapie.

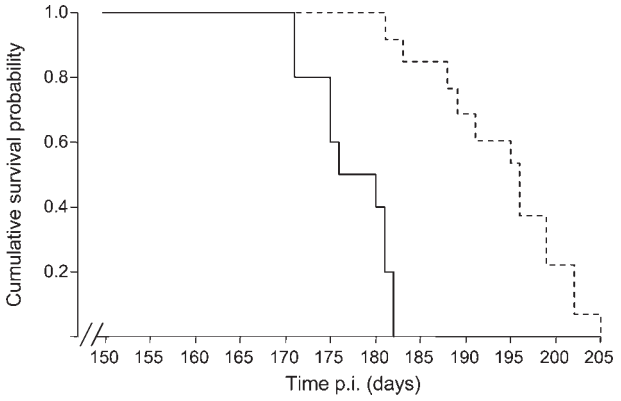

Fig. 1. Kaplan–Meier survival curves of control (solid line) and PRV-treated (dashed line) scrapie-infected groups of mice (comparison was carried out by log-rank test, $P<0.0001$).

$n=10$; *t*-test, $P=0.00003$) to 20 days (213 ± 9 days, $n=10$, versus 193 ± 11 days, $n=9$; *t*-test, $P=0.0003$) longer when challenged i.c. with, respectively, 10^{-3}- or 10^{-4}-diluted strain 139A scrapie-infected brain homogenate.

Kempster *et al.* (2007) demonstrated that low-dosage simvastatin [i.e. 1 mg (kg body weight)$^{-1}$ day^{-1}], administered in the drinking water, was still effective at prolonging survival times significantly, on average by 10 days (mean ± SEM: 193 ± 1.1 versus 183 ± 2.2 days, $n=8$; *t*-test, $P<0.05$) of C57BL/6J female mice infected i.c. with 20 µl of a 10 % strain ME7 scrapie-infected brain homogenate. However, no significant difference was found when mice were treated only at the onset of scrapie-induced behavioural changes.

Haviv *et al.* (2008) reported that simvastatin dosages of 2–20 mg (kg body weight)$^{-1}$ day^{-1}, administered starting at 41 and 72 days p.i. through the drinking water, increased survival of FVB/N female mice infected i.c. with 30 µl of a 1 % RML scrapie brain homogenate by approximately 21 days ($n=20$–24 by cumulating four independent experiments; Breslow test, $P<0.001$). Surprisingly, the authors measured, for the first time, increased PrP^{Sc} levels in simvastatin-treated mouse brains in comparison to untreated scrapie-infected controls. Thus, they proposed the original hypothesis that increased PrP^{Sc} deposition might result from the neuroprotective effect of simvastatin, i.e. the surviving neurons continuously generate and accumulate PrP^{Sc} in a mechanism independent of PrP^{Sc} accumulation. This last finding is in contrast to results produced both by us and also by Mok *et al.* (2006) and Kempster *et al.* (2007). To solve this evident discrepancy, further comparative experimental studies are critically needed. Haviv *et al.* (2008) also reported in their study, in accordance with Mok *et al.* (2006) and Kempster *et al.* (2007), no significant effects of simvastatin treatment on cholesterol levels and, accordingly, attributed increased animal survival to simvastatin pleiotropic effects. Noteworthily, they demonstrated that the pharmacological effect in their model was mediated through the L-mevalonate pathway, i.e. probably by preventing isoprenylation of signalling molecules, as the beneficial effect of statin on survival was reversed completely by the administration of mevalonate to the mouse diet. Thus, further studies are indeed needed to clarify the precise relationship among cholesterol metabolism, PrP^{C}/PrP^{Sc} conversion and statin pharmacological action in the brain.

In summary, the beneficial effects of statins (to date, regarding solely the lipophilic simvastatin and the hydrophilic PRV) on survival time of experimentally scrapie-infected mice seem to be independent of absolute solvent or water solubility of the drug. This observation is not unprecedented, as also in AD, the protective effect of statins was shown to be independent of their lipophilicity, as reported in a large, recently published clinical prospective Rotterdam study (Haag *et al.*, 2009). Furthermore, cumulative evidence suggests that both long-term and high-dosage statin treatment may affect the survival of scrapie-infected mice favourably. However, further dose-escalation and efficacy/safety preclinical studies with statins of higher potency and half-life are highly needed to confirm and extend these observations.

We employed a high PRV dosage (i.e. 200 mg kg^{-1} day^{-1}) without producing apparent adverse effects in C57BL/6 female mice. This dosing regimen was based on preliminary toxicity studies in mice (data not shown) and on data reported in the literature (Smith *et al.*, 1991; Kirsch *et al.*, 2003; Thelen *et al.*, 2006). Body weights of mice, measured monthly for PRV therapy adjustment, were recorded for statistical analysis at baseline (0 days p.i.) and at 150 days p.i. At that time, strain 139A scrapie-infected mice were still in the preclinical phase. It is known that the scrapie infectious agent can produce highly specific effects on body weight that depend upon the mouse strain being infected. However, for most combinations of scrapie agent (e.g. 139A) and mouse strain (e.g. C57/BL), weights during the preclinical phase were similar to or lower than the average weight of mock-infected controls (Carp *et al.*, 1984). At baseline, the body weights of PRV-treated and untreated scrapie-infected mouse groups were similar (18.5 ± 0.9 versus 18.7 ± 0.7 g, respectively). At 150 days p.i., body weights of PRV-treated mice were significantly lower than those of controls (21.9 ± 1.0 vs 23.6 ± 1.4 g; *t*-test, $P=0.0031$). Noteworthily, the difference between the two groups in body-weight gain (measured as a percentage) was also statistically significant (26.3 ± 5.6 versus 18.2 ± 4.1 %; *t*-test, $P=0.0006$). This effect could probably be attributed to any depletion of visceral fat tissue and/or skeletal muscle glycogen, as it has been reported that PRV treatment (100 mg kg^{-1} day^{-1}) may prevent the development of obesity and diabetes in diet-induced obese mice (Araki *et al.*, 2008; Takagi *et al.*, 2008). Moreover, it was reported that simvastatin [10–100 mg (kg body weight)$^{-1}$ day^{-1}], administered for 6 weeks, decreased the body weight of treated mice versus untreated controls significantly in a

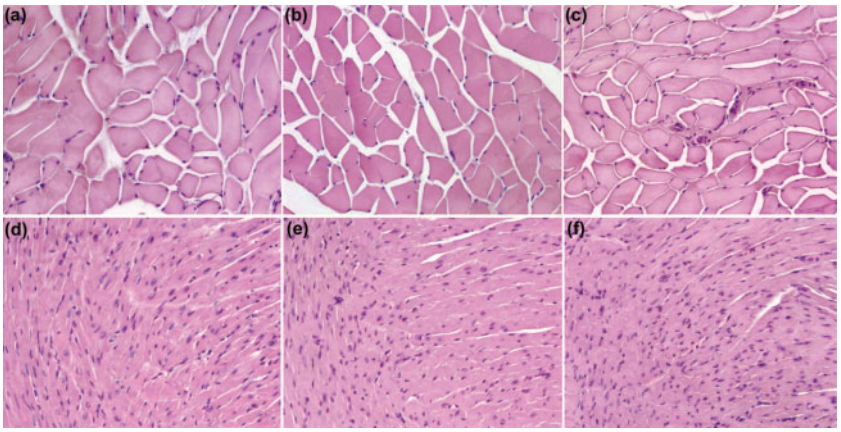

Fig. 2. Histopathological studies (haematoxylin and eosin staining) of quadriceps and heart of healthy, age-matched mouse [(a) and (d), respectively] and of untreated-control (b, e) and PRV-treated (c, f) scrapie-affected mice. The architecture of skeletal and myocardial fibres is conserved; no abnormal findings, such as muscle remodelling, vacuolation, degeneration or signs of necrosis, can be seen in any section.

dose-dependent manner (Sparrow *et al.*, 2001). Although we cannot exclude the possibility that the observed collateral effect of PRV treatment on mouse body weight contributed to prolonging the survival of scrapie-infected mice, we find it very unlikely, as it has also been reported recently that scrapie-infected C57BL/6 mice, subjected to a 30 % calorie restriction daily dietary regimen, displayed instead a shorter life span (on average 10 days) than mice fed *ad libitum* (Chen *et al.*, 2008). It is well-known that statins in some circumstances, e.g. at high doses and mainly in association with compounds such as fibrates, may produce adverse reactions such as myotoxicity, ranging from myalgias to rhabdomyolysis (i.e. massive and acute destruction of muscle fibres, resulting in the release of myoglobin into the bloodstream), probably related to impaired mitochondrial function (Sirvent *et al.*, 2008). To investigate the eventual toxic effects associated with PRV treatment, we performed histopathological examinations of a few tissues and/or organs, such as skeletal muscle (quadriceps), heart and liver. No degeneration or signs of necrosis were observed in either cardiac or skeletal muscle of PRV-treated mice (Fig. 2). Likewise, liver sections from PRV-treated and untreated, scrapie-affected mice did not show any evident difference (data not shown).

To date, prion diseases do not have reliable prophylactic or therapeutic treatment in humans (Stewart *et al.*, 2008), despite the fact that several drugs and/or therapeutic approaches were demonstrated to be able to prolong survival in TSE-infected animal models (Trevitt & Collinge, 2006). A growing scientific literature reports that PRV and other statins might have potential therapeutic implications in various neurological disorders, such as AD (White *et al.*, 2000; Haag *et al.*, 2009), stroke (Switzer & Hess, 2006; Tseng *et al.*, 2007), Parkinson's disease and multiple sclerosis, although the precise molecular mechanisms underlying these wide beneficial effects remain poorly understood (Rajanikant *et al.*, 2007; Reiss & Wirkowski, 2007). The mild but significant effect of statins, to date restricted to simvastatin and PRV, for the treatment of established central nervous system prion infections in mice is encouraging. Doses of PRV used in our animal study may appear higher, by about 2 orders of magnitude, when compared with 40 mg day^{-1}, the highest licensed dose in humans for the treatment of hypercholesterolaemia. However, it is known that, in rodents, higher doses of drugs than in humans are required to achieve a similarly effective concentration, due to their higher rate of liver metabolism (Trinkl *et al.*, 2006). Notably, PRV is also markedly different from other statins. It is eliminated by both the kidney and the liver, mostly as unchanged drug, in urine and bile, whereas lipophilic statins are bound extensively to plasma proteins and metabolized predominantly by cytochrome P450 (Neuvonen *et al.*, 2008). PRV, at the usual clinical dosage for humans and in comparison to other available statins, is considered to have a good safety profile (Simes *et al.*, 2002). Thus, PRV might offer a wider dose range of safety, for high-dose and long-term treatments, than lipophilic statins in the protection and/or treatment of TSE-incubating/affected individuals, e.g. mostly genetic and iatrogenic CJD cases.

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