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The tyrosine kinase inhibitor imatinib mesylate delays prion neuroinvasion by inhibiting prion propagation in the periphery

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Prion diseases are fatal neurodegenerative disorders with no effective therapy. A hallmark of prion disease is the conversion of the normal cellular form of prion protein PrP^{C} into a disease-associated isoform PrP^{Sc}. The authors recently have shown that a tyrosine kinase inhibitor, imatinib mesylate, induces clearance of PrP^{Sc} via specific inhibition of c-Abl in prion-infected cell culture models. In this study, the authors assessed the *in vivo* effects of imatinib mesylate on prion disease using a scrapie-infected mouse model and further investigated prion infectivity of the drug-treated scrapie-infected neuroblastoma (ScN2a) cells. The authors found that imatinib mesylate abolished prion infectivity to almost undetectable level in ScN2a cells and the level of PrP^{Sc} was significantly decreased by the drug in scrapie-infected mouse spleens as well as in ScN2a cells. Moreover, the drug treatment at an early phase of peripheral scrapie infection delayed the appearance of PrP^{Sc} in the central nervous system (CNS) and onset of clinical disease in mice. However, neither intraperitoneal nor intracerebroventricular delivery of the drug exerted any PrP^{Sc} clearance effect in the CNS. *Journal of NeuroVirology* (2007) **13,** 328–337.

Keywords: Gleevec; imatinib mesylate; prion; spleen; STI571

Introduction

Prion diseases are transmissible fatal neurodegenerative diseases characterized by microvacuolation, reactive gliosis, neuronal cell death, and accumulation of a misfolded disease-associated prion protein PrP^{Sc} in the central nervous system (CNS) (Budka, 2003). In addition to the conventional genetic and sporadic forms of human prion diseases, the occurrence of newly diagnosed variant form of Creutzfeldt-Jakob disease during the last decade has been posing a potential risk on public health. There is no effective treatment once the clinical symptoms have developed, and most clinical trails have failed (Cashman and Caughey, 2004; Weissmann and Aguzzi, 2005). The prion is mainly composed of PrP^{Sc}, a detergent-insoluble and partially protease-resistant isoform of the cellular prion protein PrP^{C}. It has been proposed that PrP^{Sc} replicates by autocatalytic conversion of α-helical PrP^{C} to β-sheet–rich PrP^{Sc} (Prusiner, 1998). Although the protective roles of PrP^{C} against harmful conditions, including oxidative stress, have been demonstrated in diverse experimental settings (Li and Harris, 2005; Roucou *et al*, 2004), the mechanism

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of its involvement in prion diseases progression remains largely unknown.

PrP-deficient mice show behavioral abnormality in response to stress (Nico *et al*, 2005) and in cognition (Criado *et al*, 2005). But they have normal life span, do not suffer from typical signs of prion disease, and are resistant to cytotoxic effect of exogenously introduced PrP^{Sc} (Bueler *et al*, 1993). Those findings suggest that the characteristic clinical conditions of prion diseases are elicited by gained abnormal function of PrP rather than the loss of its normal function. PrP is a membrane protein attached via a glycosylphosphatidylinositol (GPI) anchor. In the brains of transgenic mice that secretes anchorless PrP, prion can replicate but the mice do not succumb to the disease (Chesebro *et al*, 2005), implying that membrane-attached PrP mediates the cytotoxicity of prions, probably through transducing noxious signals. Several lines of evidence suggest the involvement of PrP in cell signal transduction. Ligation of PrP^{C} with specific antibodies activates Fyn tyrosine kinase through a caveolin in fully differentiated serotonergic or noradrenergic 1C11 neuroblastoma cells (Mouillet-Richard *et al*, 2000). Yeast two-hybrid screening and immuno-coprecipitation revealed that PrP^{C} directly binds to Grb2, which is an adaptor protein that recruits proline-rich effecter molecules to propagate intracellular signal transduction (Buday, 1999; Spielhaupter and Schatzl, 2001). Based on those findings, we previously screened signal transduction inhibitors in an attempt to search for therapeutic molecules against prion disease and found that a specific tyrosine kinase inhibitor, imatinib mesylate, can effectively diminish the accumulation of PrP^{Sc} in prion-infected cell lines (Ertmer *et al*, 2004). Imatinib mesylate, also known as Gleevec or STI571, is a derivative of 2-phenylaminopyridine, which was developed to inhibit specifically Bcr-Abl tyrosine kinase, which causes chronic myeloid leukemia (Capdeville *et al*, 2002). It competes with ATP on specific binding sites, resulting in the inhibition of several tyrosine kinases, such as c-Abl, c-Kit, and platelet-derived growth factor (PDGF) receptor. Using specific kinase inhibitors, a dominant-negative mutant of c-Abl, and an inhibitor of proteolytic degradation, we have shown that imatinib mesylate induces PrP^{Sc} clearance in prion-infected cells primarily by inhibiting c-Abl–mediated signal transduction, resulting in activation of lysosomal degradation of PrP^{Sc}.

As were used in our previous study, chronically prion-infected cell lines are very useful models for initial drug screening. In fact, tens of thousand compounds have been tested on these cell lines, and more than a hundred synthesized or natural compounds have been shown to reduce PrP^{Sc} level in cell culture system. However, when those compounds were applied to animal models, only a few of them, for example, congo red, amphotericin B, suramin, and pentosan polysulfate, exerted clinical effects by prolonging incubation time after prion infection (Weissmann and Aguzzi, 2005). The discrepancy between the results derived from cell culture and animal experiments and the recently emerged ethical issues on the treatment of human prion disease patients with compounds of which efficacy and safety have not been fully proven emphasize the crucial importance of preclinical studies. Here we report that imatinib mesylate treatment reduces PrP^{Sc} accumulation in scrapie-infected mouse spleens as well as neuroblastoma cells. Furthermore, the drug treatment at an early phase of peripheral scrapie infection delays prion neuroinvasion, consequently prolonging survival time of infected mice.

Results

Reduction of PrP^{Sc} and infectivity by imatinib mesylate in ScN2a cells

The clearance of PrP^{Sc} was determined by the disappearance of the protease-resistant core of PrP^{Sc} using immunoblot analysis. As shown in Figure 1A, 10 days treatment with 10 μM imatinib mesylate induced complete clearance of PrP^{Sc} from 22L-infected

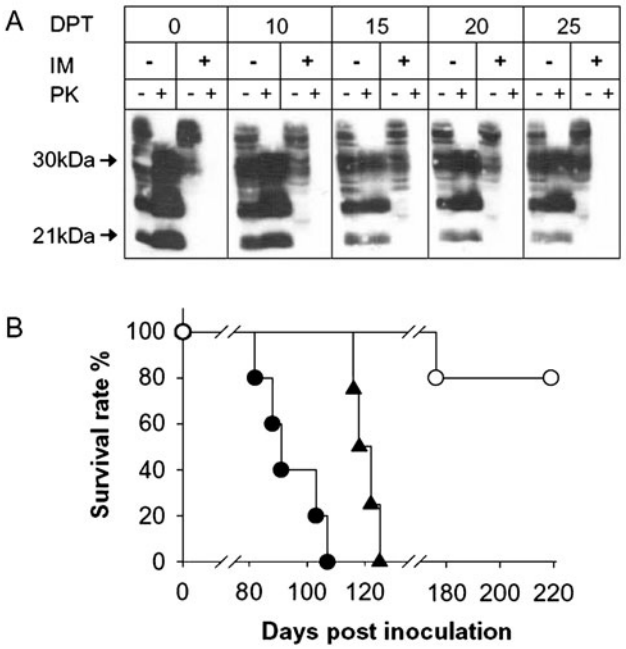

Figure 1 (**A**) Reduction of PrP^{Sc} in 22L-infected N2a cells (ScN2a) by imatinib mesylate. ScN2a cells were treated with 10 μM imatinib mesylate (IM+) or mock-treated (IM−) for 10 days. After that the cells were further cultivated in a drug-free medium for 10, 15, 20, or 25 days. The cells were passaged every 5 days during and after the treatment. Cell lysates were left undigested (PK−) or digested with proteinase K (PK+) and analyzed by immunoblotting using a mAb 4H11. (**B**) Survival curves of tga20 mice after intracerebral inoculation with mock-treated (*closed circle*, 1×10^6 cells, $n = 5$; *closed triangle*, 1×10^4 cells, $n = 4$) or imatinib mesylate-treated (*open circle*, 1×10^6 cells, $n = 5$) 22L-ScN2a cell lysate. DPT, days post treatment.

ScN2a cells. Even after 25 days of further cultivation without the drug, PrP^{Sc} was not detectable by immunoblot. To determine whether the clearance of PrP^{Sc} by imatinib mesylate is also accompanied with infectivity reduction, we performed bioassay with the drug-treated cells. As expected, all Tga20 mice inoculated with 1×10^6 or 1×10^4 mock-treated ScN2a cells developed scrapie after 92 ± 10 ($n = 5$) or 120 ± 4 ($n = 4$) days, respectively. However, only one of five mice inoculated with 1×10^6 imatinib mesylate–treated ScN2a cells developed scrapie at 178 days post inoculation (dpi), whereas all the other mice remained healthy for over 220 days, indicating that less than 1 infectious unit of infectivity was retained in the drug-treated cells (Figure 1B). This result shows imatinib mesylate abolishes not only PrP^{Sc} accumulation but also prion infectivity to almost undetectable level in cell culture system.

Reduction of PrP^{Sc} by imatinib mesylate in the spleen

After prion infection via peripheral routes, the accumulation of infectivity and PrP^{Sc} was detected at first in the lymphoreticular system before the CNS in most cases of prion infection (Kimberlin and Walker, 1988). When a wild-type C57BL mouse was intraperitoneally (ip) infected by RML (see Materials and methods for strain definition), PrP^{Sc} accumulation with high titer of infectivity reached its maximum plateau level in the spleen within 2 months (Heikenwalder *et al*, 2005; Klein *et al*, 2001), and the data obtained from immune deficient transgenic mice have shown that the distance between splenic follicular dendritic cells (FDCs) and sympathetic nerve innervated into the spleen is the key rate-limiting factor in prion neuroinvasion (Prinz *et al*, 2003). Therefore, we first investigated whether imatinib mesylate could reduce the levels of PrP^{Sc} in the spleen of C57BL/6 mice after peripheral challenge with a high dose of RML. Starting the treatment at 2 months post infection (57 dpi), imatinib mesylate was administrated for a period of either 27 or 48 days. Western blots of spleen samples taken from two mice of each group after 27 or 48 days imatinib mesylate treatment showed very weak but detectable signals of PrP^{Sc} by a long time exposure, whereas high levels of PrP^{Sc} were found in the vehicle-treated controls of each group (Figure 2). This PrP^{Sc} clearance effect of imatinib mesylate in spleen was confirmed by a duplicate experiment and an independent experiment as shown in Figure 3A. These results indicate that imatinib mesylate dramatically reduces PrP^{Sc} accumulation in the periphery, but certain levels of PrP^{Sc} remain even after prolonged drug treatment.

No effect of imatinib mesylate on the accumulation of PrP^{Sc} in the CNS

To determine if the imatinib mesylate treatment started at 57 dpi exerted effects also on the CNS by delaying prion spread or reducing PrP^{Sc} level, the infected-mice were treated with the drug for a total period of 30 or 60 days. The mice that received 30 days treatment were kept for additional 30 days without drug treatment. At 117 dpi the mice were sacrificed, and the cervical spinal cords and spleens were taken for PrP^{Sc} detection. This time point of 117 dpi was chosen based on our previous time-course analysis by which we detected PrP^{Sc} in the cervical spinal cord at 105 dpi and in the brainstem at 117dpi in this model (Yun *et al*, 2006). In the spleen, significant reduction of PrP^{Sc} was observed in the 60 days drug-treated mice, while high levels of PrP^{Sc}

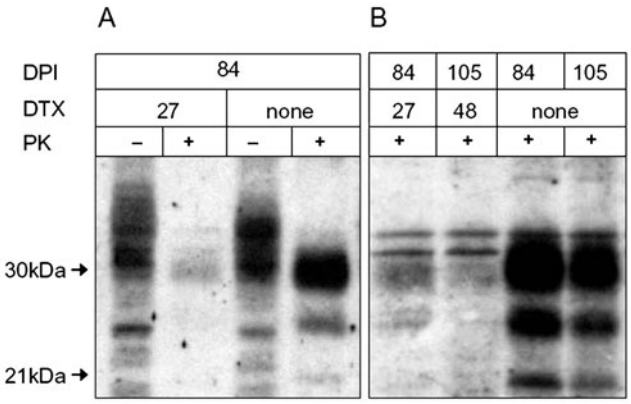

Figure 2 Reduction of PrP^{Sc} in the RML-infected mouse spleens by intraperitoneal imatinib mesylate injection. Spleen samples from RML-infected mice were prepared for PrP detection at the indicated time. (**A**) Normal level of proteinase K–sensitive (PK–) PrP^{C} expression was observed after 27 days of imatinib mesylate treatment at 84 days post inoculation (DPI), whereas PK-resistant (PK+) PrP^{Sc} level was significantly reduced. (**B**) Spleen samples of 27 days and 48 days imatinib mesylate treated mice were digested with PK. Significantly reduced but weak PrP^{Sc} bands were detectable in imatinib mesylate treated mouse spleens by a long-time film exposure. DTX, days of drug treatment.

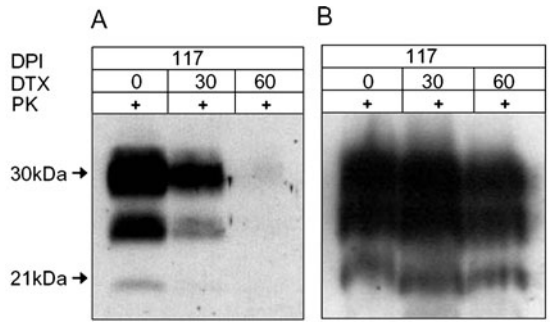

Figure 3 The effect of imatinib mesylate on PrP^{Sc} accumulation in the spleen and the CNS. Intraperitoneal injection of imatinib mesylate was ceased after 30 days treatment for some mice. After additional 30 days treatment for other mice, spleen and cervical spinal cord samples were prepared at 117 days post inoculation. (**A**) Significantly reduced PrP^{Sc} level was detected in the 60-day treated mouse spleens (*right lane*) compare to that of untreated mouse (*left lane*). In the spleens of the drug-treated mice that did not receive treatment during the last 30 days, appreciable amount of PrP^{Sc} was detected in the spleen (*middle lane*). (**B**) In contrast to the spleen, imatinib mesylate treatment exerted no effect on the PrP^{Sc} accumulation in the spinal cord. DPI, days post inoculation. DTX, days of drug treatment. PK, proteinase K.

were found in untreated controls (Figure 3A). PrP^{Sc} was also found in spleens of mice treated only for 30 days and analyzed 30 days later, suggesting that PrP^{Sc} reappeared in the spleen after cessation of imatinib mesylate because treatment for 27 days was sufficient to almost abolish splenic PrP^{Sc} as shown in Figure 2. However, in the spinal cords of mice treated either for 30 or 60 days, PrP^{Sc} was present at comparable levels as found in untreated controls (Figure 3B). The time point of 57 dpi at which we started drug treatment is already the time when low prion titer starts to be detected in the thoracic spinal cord in the same scrapie model we used (Glatzel *et al*, 2001). One important factor that determines drug efficacy in the CNS is the permeability across the blood-brain barrier (BBB). Because of its hydrophobicity and small size, imatinib mesylate had initially been thought to cross the BBB but pharmacokinetic analysis showed that less than 1% of plasma drug level was found in the cerebrospinal fluid (CSF) (Takayama *et al*, 2002; Wolff *et al*, 2003). Because this poor penetrance of the drug into the CNS might be a reason for the discrepancy in PrP^{Sc} clearance effect between spleen and brain, as was suggested in some clinical cases of CNS relapse among the leukemia patients who responded to imatinib mesylate with complete cytogenic remission (Bujassoum *et al*, 2004; Leis *et al*, 2004), we infused imatinib mesylate directly into the cerebral ventricle, bypassing the BBB throughout the whole incubation time, starting 128 days after peripheral infection with a high dose of RML. This treatment did not yield any statistically significant effect in the incubation time. The mean incubation time was 204 ± 6 ($n = 4$) and 200 ± 5 ($n = 5$) days for imatinib mesylate-treated and untreated mice, respectively. In addition, samples of the cerebral cortex at the site where the drug was administered were analyzed for the presence of PrP^{Sc} by immunoblotting, but similar levels of PrP^{Sc} were found in all mice regardless of the treatment (Figure 4). These data together indicate that the imatinib mesylate treatment did not exert therapeutic effects in the CNS.

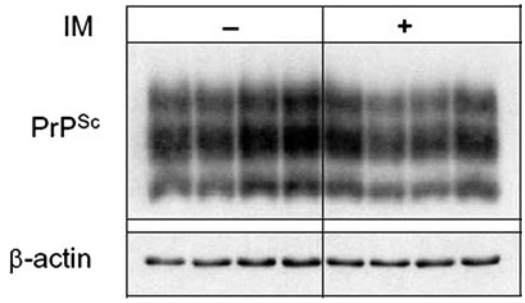

Figure 4 PrP^{Sc} accumulation in the brains of RML-infected mice in spite of imatinib mesylate infusion into the cerebral ventricle. Proteinase K–digested PrP^{Sc} level in the cerebral cortex at the site of the drug infusion was examined by immunoblotting, but no significant difference between the drug-treated ($n = 4$) and untreated controls ($n = 4$) was observed. β-actin was detected from the proteinase K–nontreated samples as a loading control. IM, imatinib mesylate.

Imatinib mesylate treatment at an early phase of peripheral infection delays prion neuroinvasion

It has been known that some complement component–deficient mice show delayed incubation time or even never develop clinical prion disease if prion is challenged peripherally with low titer (1×10^3 LD_{50}) (Klein *et al*, 2001). However, this resistance is completely overwhelmed by a high titer (1×10^6 LD_{50}) prion infection. Because the titer of inoculum we used in the first experimental setting was as high as $1 \times 10^{6.6}$ LD_{50},which might override drug effect, and prion may have already spread into the CNS at 57 dpi, the time point when the drug treatment started, we designed the second experiment to investigate the effect of early drug treatment against peripheral infection with a low-titer of prion. In this experiment, the mice were ip inoculated with 1000-fold further diluted RML inoculum, which had titer of $1 \times 10^{3.6}$ LD_{50}, and then imatinib mesylate treatment was started at 7 dpi and continued for 1 month. We hypothesized that early drug treatment would delay prion neuroinvasion and PrP^{Sc} accumulation in the CNS, otherwise PrP^{Sc} would start to be detectable in the brainstem from around 150 dpi in such an experimental condition. As expected, PrP^{Sc} was detected in all untreated control mouse brainstem tissues at 150 dpi, whereas it was not detected in the drug-treated mouse samples (Figure 5A). We further analyzed cervical spinal cord samples taken at every 20 days between 100 and 160 dpi for a time-course analysis of PrP^{Sc} appearance in the CNS. In untreated controls, PrP^{Sc} was first detectable at 120 dpi and reached its maximal level at 140 dpi, whereas in drug-treated mouse samples, PrP^{Sc} was undetectable at 140 dpi but high level of PrP^{Sc} was detected at 160 dpi (Figure 5B). These results imply that imatinib mesylate treatment at early phase of infection delayed prion neuroinvasion approximately 20 days. Moreover, the drug treatment significantly prolonged the survival time of prion-infected mice compared to that of untreated controls (263 ± 9 versus 246 ± 13 days, $P = .0054$ by log-rank test) (Figure 6).

Normal splenic microarchitecture and PrP^C expression pattern in the spleen of imatinib mesylate–treated mice

The integrity of germinal centers in the lymphoid organs is crucial for peripheral prion propagation (Aguzzi and Heikenwalder, 2005). Because the expression of c-Abl is ubiquitous, and homozygous mutation on its gene causes severe developmental problems including high neonatal mortality and atropy of organs involved in immune system (Schwartzberg *et al*, 1991; Tybulewicz *et al*, 1991), we examined whether our treatment with imatinib mesylate affected normal follicle formation and FDC

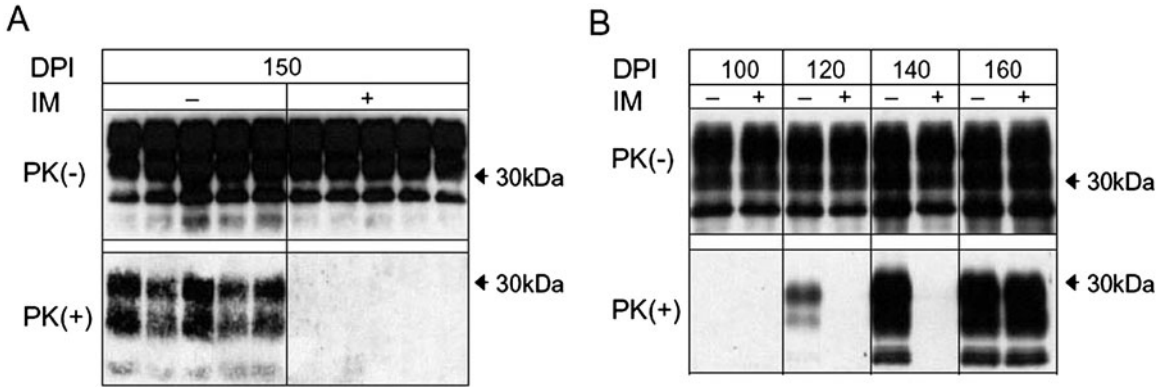

Figure 5 Delayed PrP^{Sc} accumulation in the brain by imatinb mesylate treatment at an early phase of infection. Mice, infected with $1 \times 10^{3.6}$ LD_{50}titer of RML, received intraperitoneal injection of imatinb mesylate for 30 days from 7 days postinoculation. (**A**) Proteinase K–resistant (PK+) PrP^{Sc} was detected in the brainstem of all untreated mice at 150 dpi, whereas it was not detected in any of the drug-treated mice. (**B**) Time-course analysis of PrP^{Sc} accumulation in the cervical spinal cord. IM, imatinib mesylate.

development in the spleen. One week after infection with a high dose of RML, the mice were treated with imatinib mesylate for a period of 30 days, and the spleens of RML-infected drug-treated and untreated mice were compared with uninfected controls (Figure 7). The general morphology of germinal centers of drug-treated mice was similar as untreated controls regardless of prion infection as judged by hematoxylin-eosin staining. Immunostaining of complement receptor 1 (CD35), which is expressed in marginal-zone B cells and in FDCs, showed no alterations of the germinal centers in the drug-treated mice. Because PrP^{C}-expressing FDCs within the germinal centers are required for PrP^{Sc} accumulation after peripheral infection with RML (Kaeser *et al*, 2001), we immunostained the sections of the spleen with an antibody for PrP^{C}. Normal shape of the follicles and segregation of distinct zones of PrP^{C}-expressing cells were apparent on spleen sections of the drug-treated mice. These results demonstrate that our treatment with imatinib mesylate did not disrupt the splenic architecture nor alter the PrP^{C} expression in the spleen despite its antiprion effect. The findings that PrP^{Sc} reappeared after cessation of imatinib mesylate treatment (Figure 3A) and PrP^{C} level remained unaltered (Figure 1A) are also in line with the suggested mechanism of the antiprion effect of imatinib mesylate, that the drug would facilitate the degradation of PrP^{Sc} specifically.

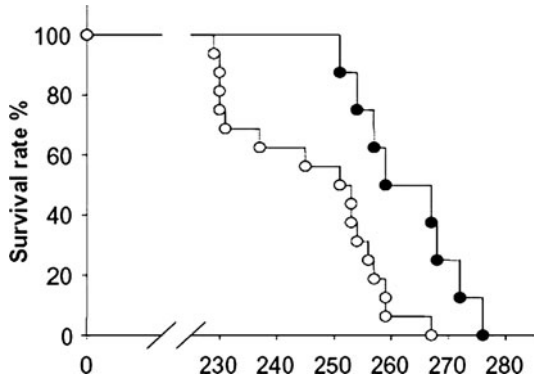

Figure 6 Prolonged survival time of RML-infected mice by imatinib mesylate treatment at an early phase of infection. Mice, infected with $1 \times 10^{3.6}$ LD_{50}titer of RML, received intraperitoneal injection of imatinb mesylate for 30 days from 7 days postinoculation. *Closed circle*, drug-treated mice; *open circle*, untreated controls. $P = .0054$ by log-rank test.

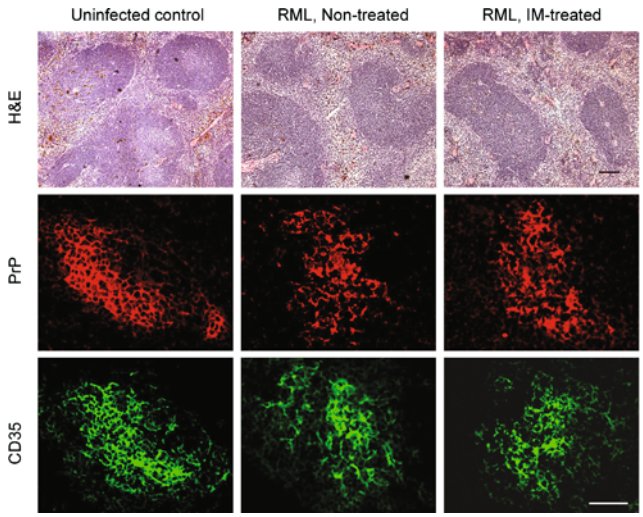

Figure 7 Normal spleen morphology of imatinb mesylate–treated mice. RML-infected mice received intraperitoneal injection of imatinb mesylate from 7 days post inoculation. After 30 days treatment, the spleens of RML-infected drug-treated and nontreated mice were compared with uninfected controls. The splenic architecture of drug-treated mice analyzed by hematoxylin/eosin staining (H&E) was similar in comparison to untreated controls (scale bar, 100 μm). Immunofluorescence staining for PrP and complement receptor 1 (CD35) expressed on follicular dendritic cells showed normal expression levels of those proteins within the germinal centers of drug-treated mice (scale bar, 50 μm). IM, imatinib mesylate.

Discussion

The distinguishing character of PrP^{Sc} is a resistance to protease, and it probably facilitates the protein to accumulate in tissues. However, it has been shown that PrP^{Sc} is continuously degraded in the scrapie-infected mouse brain as well as in neuroblastoma cell culture, with a half-life of about 36 h (Peretz *et al*, 2001; Safar *et al*, 2005). As reported in our previous study, the PrP^{Sc} clearing effect of imatinib mesylate in cell cultures is strongly inhibited in the presence of ammonium chloride, which inhibits lysosomal degradation by raising the lysosomal pH (Ertmer *et al*, 2004). Branched polyamines, which can cure scrapie-infected neuroblastoma cells by reducing the half-life of PrP^{Sc} to 8 h, require the acidic environment of intact endosomes and lysomes (Supattapone *et al*, 1999, 2001). Although the PrP^{Sc} degradation mechanism in live cell is still unknown, the data described above suggest that PrP^{Sc} hydrolysis in endosomes and lysosomes is a critical step in PrP^{Sc} clearance. The inhibitory effect on the exogenous PrP^{Sc} degradation is observed in dendritic cell by cysteine protease inhibitors treatment (Luhr *et al*, 2004). Lysosomal cysteine proteases play a key role in antigen presentation of antigen-presenting cells (Honey and Rudensky, 2003), and imatinib mesylate treatment significantly enhances antigen-presenting function of cultured dendritic cell at the concentration of 1 μM (Wang *et al*, 2005), which is similar to the concentration at which we observed more than 50% PrP^{Sc} clearance effect in cell culture. These findings together suggest that the PrP^{Sc} clearance effect of imatinib mesylate in spleen, demonstrated in Figures 2 and 3A, might be due to accelerated PrP^{Sc} hydrolysis in lysosome triggered by the drug treatment. We showed previously that the PrP^{Sc} clearance effect of imatinib mesylate is mediated primarily by c-Abl inhibition (Ertmer *et al*, 2004). How c-Abl inhibition promotes PrP^{Sc} degradation in lysosome remains to be investigated.

In cell culture system, imatinib mesylate reduces PrP^{Sc} level in a time-dependent manner, finally to an undetectable level, and PrP^{Sc} does not reappear during subsequent cultivation in a drug-free medium (Ertmer *et al*, 2004). This curing effect was further confirmed in this study by a significantly decreased infectivity, as demonstrated in Figure 1B. However, in the mouse spleens, although our drug treatment dramatically reduced PrP^{Sc}, we could detect traceable amount of PrP^{Sc}, which were not further reduced during the prolonged drug treatment period. Furthermore, when the drug treatment was stopped, PrP^{Sc} reaccumulated in the spleen. The presumed reason for this incomplete clearance could be the different response of various spleen cell types, including innervated nerves, to the drug. Cell type–specific difference in PrP^{Sc} clearance by imatinib mesylate was clearly demonstrated in our previous study. PrP^{Sc} of ScN2a and ScGT1 cells was reduced to undetectable level within 3 days after the drug treatment, whereas SMBs.15 cells needed 10 days to show the same effect (Ertmer *et al*, 2004). The spleen has been proven to be an important organ for the establishment of prion infection, especially in peripheral infection; however, it is still not clearly known in which splenic cells prion proliferates and accumulates. Reconstitution of the hematopoietic system with PrP-expressing cells is not sufficient to transfer the prion infectivity from the periphery to the brain in the PrP–knockout mouse (Blattler *et al*, 1997), but prion infectivity accumulates in the spleen of the mouse chronically (Kaeser *et al*, 2001). Ablation of PrP either in stroma or splenocytes prevent prion accumulation in the spleen when the mice are challenged with low-dose prion (Kaeser*et al*, 2001). Depletion of FDCs abolishes prion accumulation in the spleen (Montrasio *et al*, 2000). These data together imply that the splenic lymphocytes and FDCs can serve as a reservoir for prion. Another possible source for the remaining prion in the spleen is the sympathetic nerves innervating splenic tissue. Sympathectomized mice are more resistant to peripheral prion infection than normal mice, whereas transgenic mice, whose lymphoid organs are hyperinnervated by sympathetic nerves, are more susceptible, and higher titer and enhanced PrP^{Sc} accumulation are detected in the hyperinnervated spleen (Glatzel *et al*, 2001). Further studies are necessary to explore which cells in the spleen are responding to imatinib mesylate and whether the drug exerts its antiprion effect in spleen cells the same way as in cell culture models.

Doh-ura *et al* (2004) reported that direct infusion of pentosan polysulfate at a dose of 230 μg/kg/day into the cerebral ventricle not only reduced PrP^{Sc} level in the brain but also significantly prolonged scrapie incubation time in mice, but the authors could not see any effect when quinacrine was delivered by the same way. In our study, direct injection of imatinib mesylate into the brain at a dose of 400 μg/kg/injection did not have any effect on PrP^{Sc} accumulation in the brains as well as survival time of the scrapie-infected mice. The failure of chemotherapy is often attributed to the imbalance between cellular uptake and efflux of drugs that results in inadequate intracellular drug concentration. Imatinib mesylate is a substrate for P-glycoprotein encoded by the multidrug resistance gene MDR1 (Dai *et al*, 2003) and actively transported into the cell by a human organic cation transporter, hOCT1 (Thomas *et al*, 2004). The expression levels of those transporters determine intracellular concentration of imatinib mesylate and confer resistance to the drug when MDR1 is overexpressed or hOCT1 is underexpressed in human leukemia cells (Crossman *et al*, 2005; Mahon *et al*, 2003). In mouse brain, mouse organic cation transporter 1 (mOCT1) expression is not detected by immunohistochemistry or Northern blotting, whereas high level of mOCT3 is expressed in most brain regions (Schmitt *et al*, 2003). Amino acid sequences of OCT1s from human, rat, and mouse

share high homologies; however, OCT1 function, tissue distribution, and substrate specificity are very different between species (Green *et al*, 1999; Jonker and Schinkel, 2004). Although the involvement of P-glycoprotein in imatinib mesylate transporting has been demonstrated both in human and mouse cells, the role of mOCT1 in imatinib mesylate transport has not been known. However, studies have shown that c-Abl is inhibited in primary rat neuronal cell culture by 3 μM imatinib mesylate, thereby affecting normal outgrowth of axon and dendrite (Jones *et al*, 2004; Woodring *et al*, 2003). β-Amyloid production in N2a cells and rat hippocampal primary cell culture are inhibited at 5- to 10-μM ranges of imatinib mesylate, and 7-day intrathecal drug infusion at a dose of 220 μg/kg/day significantly reduces the brain level of β-amyloid in guinea pig (Netzer *et al*, 2003). Those drug concentrations are comparable to the dose we applied for curing ScN2a cells and intracerebroventricular (icv) drug infusion. Although the effect of imatinib mesylate on β-amyloid production in the brain turned out to be not mediated by c-Abl inhibition, the data taken together imply that the imatinib mesylate we infused into the cerebral ventricle could have been transported to inhibit c-Abl in brain cells; however, the role of c-Abl in the intracellular mechanism of PrP^{Sc} degradation in brain cells might be different from that in spleen.

Given the normal expression of PrP^{C} in the spleen and spleen morphology of the imatinib mesylate–treated mouse, it appears that the PrP^{Sc} reduction by the drug is specific. Although imatinib mesylate treatment could not completely protect the mouse from scrapie, we have shown here that the drug treatment at early phase of infection can delay prion neuroinvasion, consequently prolonging the survival time of scrapie-infected mice. Imatinib mesylate has been studied intensively in the field of cancer research since its approval for chronic myeloid leukemia in 2001. Further investigations on the underlying mechanisms of PrP^{Sc} clearance by imatinib mesylate may provide insight into understanding mechanisms of prion proliferation as well as the development of new therapeutic strategies for prion diseases.

Materials and methods

Animals and cell line

Six- to eight-week-old female C57BL/6J mice were purchased from Charles River, Germany. PrP-overexpressing Tga20 mice have been bred in the animal breeding facility of the Institute of Virology and Immunobiology, University of Wurzburg. The prion-infected mice and controls were housed in a specially designated facility for infected animals and provided with water and standard mouse chow (ssniff GmbH) *ad libitum*. All animal experiments were performed according to Governmental and Institutional guideline for animal experiment. N2a cells (ATCC CCL131) were subcloned, and individual clones were seeded on 24-well plates at a density of 0.5×10^5 cells per well for infection.

Scrapie infection

The mice were infected with mouse-adapted Rocky Mountain Laboratory (RML) scrapie strain passaged in Swiss CD-1 mice (passage number 5). The inoculum stock was a 10% (*w*/*v*) homogenate of RML-infected, terminally sick CD1 mouse brains prepared in 0.32 M sucrose, with a titer of 8.6 log LD_{50} units/ml. Mice were challenged by ip injection with 0.1 ml of 1% (high dose) or 0.001% (low dose) of the stock diluted with phosphate-buffered saline (PBS) containing 5% bovine serum albumin and in 0.32 M sucrose. For N2a cell infection, brain homogenate of 22L scrapie-infected C57/BL6 mice was added to each well of cell culture plate for 24 h, to a final concentration of 1%. After removal of inoculum, the cells were rinsed with PBS and allowed to grow in Opti-MEM medium containing 10% fetal calfserum, antibiotics, and glutamine. After three passages, PrP^{Sc}-expressing clone was determined by immunoblotting for subsequent experiments. Diagnosis of scrapie was made by the appearance of typical clinical signs of mouse scrapie such as gait disturbance, kyphosis, rigid tail, and severe weight loss. Terminal disease was confirmed by another independent researcher before the mice were sacrificed by CO_2 overdose.

Imatinib mesylate administration

Gleevec, purchased from Norvatis Pharmaceutical (Basel, Switzerland), was dissolved in distilled water at a final concentration of 5 or 2 mg/ml for ip or icv injection into the mouse, respectively. Insoluble carriers were removed by centrifugation at $1000 \times g$ for 15 min, and the solution was sterilized before injection by filtering through 0.45-μm pore size filter. The mice received ip injection of imatinib mesylate at a dose of 50 mg/kg/day for 7 consecutive days, followed by injection every 2nd day until the designated time point. This dose of the drug has been used for cancer mouse models (Brain *et al*, 2002; Decaudin *et al*, 2005). After the 7-day consecutive treatment, we observed 13% body weight loss, but it was recovered to normal level during the every 2nd day treatment period. For icv administration, 5 μl of the drug solution was infused into the cerebral ventricle via an implanted cannula system (Plastic One) at a dose of 400 μg/kg every 3rd day. The drug preparation and treatment for ScN2a cells were previously described (Ertmer *et al*, 2004). In brief, 1 day after seeding, the cells were cultured either in the absence or presence of imatinib mesylate at a final concentration of 10 μM for 10 days and then the drug-treated and mock-treated cells were further cultured for a period of 25 days in a normal cell culture medium. The

cells were passaged every 5 days during and after the treatments.

Prion infectivity bioassay
Prion infectivity was assayed by a bioassay using the incubation time methods. ScN2a cell lysate of 1×10^6 or 1×10^4 cells per 30 μl were prepared in PBS to be used as inoculums. Tga20 mice received single intracerebral (ic) inoculation of 30 μl of inoculum. The inoculated mice were monitored every 2nd day, and scrapie diagnosis was made by the appearance of typical clinical signs of mouse scrapie.

PrP^{Sc} detection by Western blot
The frozen brain and spleen tissues were weighed and homogenized in 9 volumes of PBS containing 0.5% deoxycholic acid, 0.5% NP-40, and 5 U/ml benzonase using micropestles. After centrifugation at $1000 \times g$ for 5 min, the supernatants were obtained. ConfluentScN2a cell cultures were lysed in cold tris-buffered saline (TBS) containing 10 mM EDTA, 0.5% deoxycholic acid, and 0.5% Triton X-100 for 10 min, and the proteins were ethanol precipitated. Where indicated, aliquots were digested with proteinase K (20 μg/ml) at 37°C for 30 min for PrP^{Sc} detection. The samples were denatured at 95°C for 10 min, resolved in a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel and then transferred to polyvinylidene diflouride (PVDF) membrane (Millipore). The membrane was blocked in 5% nonfat skim milk and incubated overnight at 4°C with an anti-PrP polyclonal antibody SA2125 or monoclonal antibody 4H11. Immunoreactivity was detected by incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) (1:20,000; Zymed) for 1 h, followed by incubation with enhance chemiluminescence (ECL) substrate (Pierce). The chemiluminescence was visualized on a Hyperfilm (Amersham Biosciences).

Histological staining of the spleen
Spleens were quick-frozen in Hanks' balanced salt solution using liquid nitrogen and later cut on a Leica CM1900 cryostat at –20°C. The 10-μm-thick sections were collected on glass slides, fixed in acetone for 10 min and then stored at –20°C in OCT embedding medium until further processing for hematoxylin/eosin staining and immunofluorescence histochemistry. For immunofluorescent staining, the sections were blocked in 10% goat serum for 30 min and incubated with rat anti-mouse CD35 monoclonal antibody (1:200; BD Bioscience) or anti-PrP rabbit antiserum XN (1:500) overnight at 4°C. The sections were subsequently incubated with fluorescein isothiocyanate (FITC)-conjugated goat $F(ab')_2$ anti-rat Igs (1:200; Biosource) or Alexa Fluor 546 goat anti-rabbit IgG (H+L) (1:500; Invitrogen) and mounted in a fluorescence mounting medium (DAKO).

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