Amyloid imaging probes are useful for detection of prion plaques and treatment of transmissible spongiform encephalopathies

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INTRODUCTION

The transmissible spongiform encephalopathies (TSEs or prion diseases) form a group of fatal neurodegenerative diseases including bovine spongiform encephalopathy, Creutzfeldt–Jakob disease (CJD) and Gerstmann–Sträussler–Scheinker syndrome (GSS). These diseases are characterized by the accumulation in the brain of abnormal protease-resistant isoforms of prion protein (PrP), termed PrPSc (Prusiner, 1991). The diseases are rare, but outbreaks of acquired forms of CJD, such as variant CJD (Will et al., 1996) and iatrogenic CJD with cadaveric growth hormone or dura grafts (Hamad et al., 2001), have prompted the development of therapeutic interventions and new diagnostic methods.

There are several drug candidates currently under clinical trial for TSE patients, but unfortunately their potential usefulness remains limited. The problem is that these agents can only be given after the onset of the disease, often in the advanced stage, because there is no reliable means of detecting presymptomatic infection by either neuroimaging or laboratory examination. Some studies have reported that imaging assessments such as positron emission tomography (PET) with [18F]FDG and diffusion-weighted magnetic resonance imaging are useful for some types of human TSE (Demaerel et al., 1997; Murata et al., 2002), but are not always conclusive. At present, TSEs can be diagnosed with certainty only through pathological examination or immunoblotting of the diseased brain. Recently, PET and single photon emission CT (SPECT) using radiolabelled imaging probes, which provide information on neuropathological changes as well as brain metabolism, have been reported to be helpful for the early diagnosis of neurodegenerative disorders. A variety of chemicals have been evaluated for imaging β-amyloid (Aβ) aggregation, which is the major diagnostic marker for Alzheimer’s disease.
hallmark of Alzheimer’s disease. Candidate probes have primarily been derived from amyloid dyes such as thioflavin and Congo red (Bacskai et al., 2002).

Here, we focused on two candidate probes: a thioflavin derivative, 2-[4’-(methylamino)phenyl] benzothiazole (BTA-1) and a Congo red derivative, (trans, trans)-1-bromo-2,5-bis-(3-hydroxycarbonyl-4-hydroxy)styrylbenzene (BSB) (Fig. 1). Both chemicals have been reported to detect amyloid or amyloid-like plaques in either post-mortem human brains or living mouse brains (Mathis et al., 2002; Skovronsky et al., 2000). Since PrPSc tends to exist as amyloid-like fibrils, we used either BTA-1 or BSB to label PrP deposition in TSE brains. We also examined these chemicals for their application as therapeutics for TSE, since some amyloid-binding chemicals have the potential to inhibit PrPSc propagation in in vitro and/or in vivo models of TSE (Supattapone et al., 2002).

**METHODS**

**Chemicals and experimental models.** BTA-1 was synthesized at Tanabe R & D (Saitama, Japan), and BSB was kindly provided by Dojindo Laboratories (Kumamoto, Japan). The chemicals were dissolved in 100 % dimethylsulfoxide (DMSO) and stored at 4 °C until use.

Three kinds of TSE-infected mouse neuroblastoma (N2a) cell lines were used in this study: N2a cells infected with the RML strain (ScN2a, Race et al., 1988), N2a#58 cells infected with the 22L strain (L-1, Nishida et al., 2000) and N2a#58 cells infected with the Fukuoka-1 strain (F-3). N2a#58 cells are known to express five times more normal PrP than N2a cells. All cell lines were individually cultured in OptiMEM (Invitrogen; supplemented with 10 % fetal calf serum).

For in vivo studies, transgenic mice models Tg7, over-expressing hamster PrP (Race et al., 1995; Priola et al., 2000), and Tga20, expressing five- to eightfold higher levels of murine PrP (Fischer et al., 1996), were used. These models showed substantially shorter incubation periods following intracranial infection with 20 µl of 1 % (w/v) 263K scrapie strain homogenate and RML strain, respectively. By 6 weeks post-inoculation, the Tg7 mouse model consistently showed plaque-type PrP deposition in the cerebral white matter between the cortex and hippocampus, and at the disease terminal stage showed synaptic-type deposition in the thalamus, hypothalamus and pons. Similarly, the Tga20 mouse model showed plaque-type deposition in the same brain areas, but not as consistently. Permission for the animal study was obtained from the Animal Experiment Committee of Kyushu University. Each mouse weighed approximately 30 g, and was maintained under deep ether anaesthesia during all surgical procedures.

**PrP imaging in pathological sections.** Brain samples of autopsy-diagnosed sporadic CJD cases (n = 3), GSS cases (n = 2) and non-TSE control cases with amyloid lesions (Alzheimer’s disease, n = 2) or without them (cerebral infarction, n = 1; pancreatic cancer, n = 1) were obtained from the Department of Neuropathology, Kyushu University. Tissue samples of TSE material were immersed in 98 % formic acid for 1 h to reduce infectivity. Each tissue sample was embedded in paraffin, and then cut into 7 µm thick sections. Sections of variant CJD brain were kindly provided by James W. Ironside of the CJD Surveillance Unit, Edinburgh, UK. For neuropathological staining, sections were deparaffinized in xylene and hydrated in ethanol. They were then incubated for 30 min in a solution of 1 µM BTA-1 in 50 % ethanol, rinsed and examined under a fluorescent microscope (DMRXA, Leica Instruments) with a UV filter set. The sections were washed overnight in 50 % ethanol. After verifying clearance of the BTA-1 signal, they were incubated for 30 min in a solution of 1 µM BSB and re-examined under the fluorescent microscope. For comparison, each section was subsequently immunostained as described previously (Doh-ura et al., 2000). Briefly, sections were incubated in 0.3 % H2O2 in absolute methanol for 30 min and then treated by a hydrolitic autoclave procedure (1 mM HCl, 121°C, 10 min). After rinsing with 50 mM Tris/HCl, pH 7-6, the sections were incubated at 4 °C overnight with a rabbit primary antibody c-PrP, which was raised against a mouse PrP fragment, amino acids 214–228 (1:200; Immuno-Biological Laboratories, Gunma, Japan) (Yokoyama et al., 2001), followed by incubation with a horseradish-peroxidase-conjugated secondary antibody (1:200; Vector Laboratories) at room temperature for 1 h. The coloured reaction product was developed with 3,3’-diaminobenzidine tetrahydrochloride solution. In addition, formalin-fixed brains of diseased Tg7 mice were investigated using the same procedure. To ensure specificity of the anti-PrP antibody, brain sections with other amyloid lesions such as senile plaques were also immunostained. No positive reactions were obtained.

**PrP imaging in presymptomatic mice.** BTA-1 or BSB 10–30 mg (kg body weight)−1 in 10 % DMSO/saline, or vehicle alone, was administered intravenously into Tg7 mice intracerebrally infected with the 263K strain at 6 weeks post-infection, or into Tga20 mice intracerebrally infected with the RML strain at 8 weeks post-infection. At this point the mice showed no apparent clinical signs of disease. As another control, either chemical was similarly injected into uninfeeted transgenic mice. The animals were sacrificed at different time-points, and the brains were removed, frozen in powdered dry ice and cut coronally into 10 µm thick sections using a cryostat. The sections were examined under a fluorescent microscope, and then analysed immunohistochemically for PrP as described above.

**PrPSc inhibition in scrape-infected cells.** PrPSc inhibition assays using a cellular model of TSE were performed as described previously (Caughey & Raymond, 1993). Either BTA-1 or BSB in 100 % DMSO was added at the designated concentrations to each of the cell lines in 6-well plates when they reached 5 % confluency. The final concentration of DMSO in the medium was kept to less than 0.2 %. Two controls, one for untreated cells and the other for cells treated with vehicle alone (0.2 % DMSO), were prepared. The cultures were allowed to grow to confluence, and then harvested and analysed for PrPSc content by immunoblotting. Briefly, the cells were lysed with lysis buffer (0.5 % sodium deoxycholate, 0.5 % Nonidet P-40, PBS) and digested with 20 µg proteinase K ml−1 for 30 min at 37°C. The digestion was terminated with 0.5 mM phenylmethylsulfonyl fluoride, and the samples were centrifuged at 100 000 g for 30 min at 4°C. Pellets were resuspended in 30 µl of sample loading buffer and boiled for 5 min. The samples were separated on a 15 %
polyacrylamide Tris/glycine SDS gel and transferred to a PVDF filter (Millipore). PrP Sc was detected using a rabbit polyclonal antibody PrP-2B, which was raised against a mouse/hamster PrP fragment, amino acids 89–103 (1:5000) (Murakami-Kubo et al., 2004), followed by an alkaline phosphatase-conjugated goat anti-rabbit antibody (1:20 000; Promega). Immunoreactive signals were visualized using the CDP-Star detection reagent (Amersham) and analysed densitometrically using image analysis software. More than two independent assays were performed for each experiment.

**Therapeutic treatment in model animals.** BSB dissolved in 10 % DMSO/saline at 1 mg per injection was given intravenously to infected Tg7 mice (n = 5 in each group) or infected Tga20 mice (n = 7 in each group). The treatment was performed for Tg7 mice at 35 days post-infection (p.i.) and 50 days p.i., and for Tga20 mice at 45 days p.i. and 60 days p.i. Two control groups were prepared for both experimental models: untreated mice and mice treated with vehicle alone. The animals were monitored 5 days a week until the obvious clinical stage was reached, which was the day before or the day of death in Tg7 mice and 4–5 days before death in Tga20 mice. The statistical significance was analysed by one-way ANOVA followed by Scheffé’s method for multiple comparisons.

**RESULTS**

**Imaging of PrP deposition in vitro and in vivo**

Imaging of PrP deposition in the brain by BTA-1 or BSB was first examined using histopathological specimens from human TSE cases. Both chemicals fluorescently labelled most of the compact PrP plaques in the cerebellar cortices of GSS cases (Fig. 2a and c). No residual fluorescence of BTA-1 was seen after thorough washing (Fig. 2b). The labelling intensity was stronger in the sections labelled with BSB compared with those labelled with BTA-1, but the size of each plaque detected by BTA-1 was on average larger than that detected by BSB. The plaques were counterstained with an antibody against the C terminus of PrP (Fig. 2d). In the sections from a variant CJD case, dense PrP plaques were detectable by both chemicals, whereas most of the immunopositive PrP deposits, such as fine granular deposits and perivacuolar deposits, were not labelled (Fig. 2e–h). In the

![Fig. 2. Imaging of PrP aggregates in brains with TSE. PrP plaques were labelled with BTA-1 (a, e and i); no residual signal was seen after washing to remove BTA-1 (b, f and j). The plaques were then labelled with BSB (c, g and k), and subsequently immunostained for PrP (d, h and l). The first row shows a cerebellar section from a GSS case (a–d), the second shows a cerebral cortical section from a variant CJD case (e–h) and the third shows cerebral white matter section from a terminal Tg7 mouse (i–l). Only dense plaques are identified in the variant CJD section; the arrowheads point to the same PrP plaque. In the Tg7 section, PrP plaques in the cerebral white matter between the cortex and hippocampus were labelled. Bars, 100 (a–d) and 50 (e–l) μm.](http://vir.sgmjournals.org)
sections from sporadic CJD cases, neither of the chemicals labelled synaptic-type PrP deposition (data not shown). Non-specific labelling was barely observed after rinsing off the excess chemicals. As reported in previous studies (Mathis et al., 2002; Skovronsky et al., 2000), both chemicals stained senile plaques in Alzheimer’s brain, and neither displayed signals in control brain sections without amyloid (data not shown). Similar results to those observed in the human TSE brain sections were obtained from post-mortem brains of Tg7 mice infected with the 263K strain; both chemicals stained the plaque-type PrP deposition in the cerebral white matter between the cortex and hippocampus (Fig. 2i–l). There was no PrP immunohistochemical signal or fluorescent signal in the brains of uninfected Tg7 mice (data not shown).

Since both BTA-1 and BSB have been reported to cross the blood–brain barrier, we performed in vivo experiments using Tg7 mice in a later stage of 263K scrapie infection. A bolus injection of BTA-1 labelled PrP plaques in the white matter between the cortex and hippocampus of the affected brains (Fig. 3a and b). Faint cerebrovascular labelling was occasionally observed at 4 h after the injection, but not at 18 h or later. PrP imaging of BSB in the brain in vivo was almost as effective as that of BTA-1 (Fig. 3c), but non-specific cerebrovascular labelling was more evident. Images of PrP deposition labelled by BSB were not clearly distinguishable in the cerebrovascular images until 24 h post-administration, but background staining was not seen thereafter. The stability of the signals of PrP deposition was examined at various time-points, and both chemicals remained stably visible at 42 h post-injection. In particular, the BSB labelling signals were relatively stable and visible even at 54 h post-injection. There was no significant labelling after an injection of either chemical to uninfected transgenic mice upon examination after sacrifice 24 h later, or after an injection of vehicle alone to infected mice (data not shown). Similar results were obtained for Tga20 mice infected with the RML strain, although labelled PrP plaques were less frequently observed (data not shown).

Anti-prion activities in vitro and in vivo

The anti-prion activities of these chemicals were examined using three cell lines infected with different strains. Both BTA-1 and BSB inhibited PrPSc formation in ScN2a cells in a dose-dependent manner (Fig. 4a). The concentrations giving 50 % inhibition of PrPSc formation in ScN2a cells relative to the untreated control (IC50) were 4 nM for BTA-1 and 1-4 μM for BSB. However, neither chemical was effective in the other cell lines (Fig. 4b and c). Treatment with vehicle (DMSO) alone showed no significant effects when compared with the untreated control. No apparent cell toxicity of the chemicals was observed up to 10 μM for BTA-1 and 100 μM for BSB. To examine the possibility of interference by the chemicals with immunodetection, BTA-1 or BSB at a concentration 10-fold higher than the IC50s were added to lysates of untreated ScN2a cells for 1 h prior to proteinase K digestion. After these treatments, the PrP signals were not affected (data not shown).

Since BSB was potent without significant toxicity at a high concentration of 100 μM in the cell cultures and remained stably bound to PrP aggregates in the affected brains for more than 2 days in vivo, we examined whether BSB could be an effective treatment for TSE in two different experimental animal models. As shown in Fig. 5, treatment with BSB at 1 mg prolonged the incubation period of Tga20 mice.

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**Fig. 3.** In vivo imaging of PrP deposition in the brains of presymptomatic TSE-infected mice. An intravenous bolus injection of BTA-1 was given to Tg7 mice at 6 weeks post-infection, and they were sacrificed 24 h later. PrP plaques in the cerebral white matter between the cortex and hippocampus were detected under a fluorescent microscope (a), and then the brain section was immunostained for PrP (b). Similar results were observed with BSB (c, 24 h post-injection) and immunostaining for PrP (data not shown). Low magnification demonstrates PrP plaques in the cerebral white matter between the cortex and hippocampus were labelled with high specificity. All images are from coronal sections sited around one-third of the distance from the interaural line to the bregma line. Bars, 25 (a, b) and 100 (c) μm.
infected with the RML strain by 13·6% (77·6 ± 3·6 days in the BSB-treated group versus 68·3 ± 1·9 days in the vehicle control), whereas no significant prolongation was observed in the same treatment for Tg7 mice infected with the 263K strain (57·4 ± 1·9 days in the BSB-treated group versus 55·8 ± 1·3 days in the vehicle control). The dosage of BSB examined here corresponds to the concentration sufficient to detect PrP plaques in vivo as described above, and there were no apparent adverse effects of BSB. No significant differences in incubation times were observed between the untreated controls and the controls treated with vehicle (DMSO) alone (68 ± 2·1 days in the untreated control versus 68·3 ± 1·9 days in the vehicle control in Tga20 mice; 57·0 ± 2·4 days in the untreated control versus 55·8 ± 1·3 days in the vehicle control in Tg7 mice).

**DISCUSSION**

Both BTA-1 and BSB have been reported to be candidates for PET/SPECT tracers for the evaluation of Alzheimer’s disease, and the results of this study have shown that they might also be useful for the evaluation of TSE with certain strains. However, the discrepancy in imaging between the plaque-type and the synaptic-type PrP deposition remains. A previous study demonstrated successful labelling of intracellular Aβ(1–42) accumulation in living cells by BSB (Skovronsky et al., 2000), but the same chemical showed no labelling of PrPSc deposits in ScN2a cells (data not shown). These observations suggest that differences in the structures and/or the microenvironments of these PrP aggregates might account for the discrepancy. Further studies using more sensitive detection methods, such as the use of radiolabelling, might be helpful for evaluation.

Together with previous studies (Mathis et al., 2002; Skovronsky et al., 2000), the current study suggests that both BTA-1 and BSB label various amyloids including Aβ aggregates and PrP aggregates, and are not disease specific. However, these chemicals can be still useful to evaluate amyloid aggregates because anatomical distributions of pathological deposition are quite different between different diseases. For example, Aβ plaques are not, or seldom, observed in the cerebellum, while PrP amyloid plaques are predominantly observed there.

We also demonstrated therapeutic efficacies of these two chemicals. Congo red is well known to inhibit new formation of PrPSc in ScN2a cells and prolongs the incubation period of infected animals when administered prophylactically (Caughey et al., 1993; Ingrosso et al., 1995). However, Congo red cannot be used as a therapeutic drug because of its inability to cross the blood–brain barrier and its carcinogenicity due to its benzidine structure. BSB, a Congo red analogue, can enter the brain and lacks the benzidine structure. Here BSB showed a low toxicity and was as potent as Congo red in a cellular model, and furthermore, BSB-treatment prolonged the incubation period of the Tga20-RML infected mouse model despite being introduced at a late stage of TSE infection.

**Fig. 4.** Inhibition of PrPSc formation in TSE-infected cells by BTA-1 or BSB. Various concentrations of each chemical were added to freshly passaged ScN2a cells in (a), L-1 cells in (b) and F-3 cells in (c), and the PrPSc levels were analysed by Western blotting. Lanes: N, untreated cells; 0, cells treated with vehicle (DMSO) alone. Bars on the left indicate molecular mass markers at 35 and 21 kDa.

**Fig. 5.** Effects of BSB treatment on TSE-infected mice. BSB was administered to Tg7 mice infected with the 263K strain (a) and Tga20 mice infected with the RML strain (b). The treatment protocol is described in the text. Each closed circle represents an individual animal. Bars represent the mean and standard deviation of the incubation periods of each group. *P < 0·0001 versus the other groups.
There was a discrepancy in the efficacy of BSB between Tg7 mice infected with the 263K strain and Tga20 mice infected with the RML strain. This discrepancy in vivo is consistent with that found in vitro, since BSB was only effective in ScN2a cells, which are infected with the RML strain. There is a possibility that the differences in susceptibility to these chemicals among the three cell lines might be caused by the differences in the expression levels of normal PrP, because the expression levels of normal PrP in L-1 cells or F-3 cells are five times higher than that of ScN2a cells. However, the data showed that the two chemicals had no effect in either L-1 cells or F-3 cells, even at doses five times greater than the IC50 in ScN2a cells. The findings suggest that the therapeutic efficacies of these chemicals are dependent on the TSE strain. In this study, we observed that the chemicals bound tightly to some kinds of PrP aggregates in the pathological sections of TSE, implying that a direct interaction with abnormal PrP molecules may play a role in the inhibition of PrPSc formation. However, the mechanism of the strain-specific efficacies of these chemicals remains to be elucidated.

Together with previous reports (Caughey et al., 1993; Ingrosso et al., 1995; Supattapone et al., 2002), the current study demonstrated that chemicals with a high affinity for amyloid could be candidates for inhibiting PrPSc formation and increasing the life-span of TSE-infected animals. We tested this further by examining another chemical, 6-OH-BTA-1, which has recently been reported to facilitate PET studies of Alzheimer’s disease (Engler et al., 2002). We observed that this chemical inhibited PrPSc formation in ScN2a cells with an IC50 in the nanomolar order (data not shown), but in vivo studies remain to be performed.

In conclusion, BTA-1 and BSB, known as amyloid imaging probes, detected PrP deposition in the TSE brains both in vitro and in vivo and had anti-prion activities both in vitro and in vivo, although the efficacy depended upon the strain of TSE. These observations suggest that both could be lead chemicals not only for imaging probes, but also for therapeutic drugs for TSEs caused by certain strains.

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