In vitro cell-free conversion of bacterial recombinant PrP to PrP^{res} as a model for conversion

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Prion diseases are associated with the conversion of the normal cellular prion protein, PrP^{C}, to the abnormal disease-associated protein, PrP^{Sc}. This conversion can be mimicked *in vitro* using PrP^{Sc} isolated from the brains of scrapie-infected animals to induce conversion of recombinant PrP^{C} into a proteinase K-resistant isoform, PrP^{res}. Traditionally, the 'cell-free' conversion assay has used, as substrate, recombinant PrP^{C} purified from mammalian tissue culture cells or, more recently, from baculovirus-infected insect cells. The cell-free conversion assay has been modified by replacing the tissue culture-derived PrP^{C} with recombinant PrP purified from bacteria. Bacterial expression and chromatographic purification give high yields of recombinant radiolabelled untagged protein, eliminates artefacts that may be due to cellular factors or antibody fragments normally present in labelled PrP preparations and allows accurate and rapid variation of protein sequence using standard molecular biological techniques. In addition, these cell-free conversion assays were carried out under more physiological conditions, giving more relevance to the assay as a model for conversion. To validate its use in this assay, this bacterial recombinant PrP has been shown to have the conversion properties of mammalian PrP^{C}: (i) it converts to a proteinase K-resistant isoform in the presence of PrP^{Sc}; (ii) the efficiency of this conversion by PrP^{Sc} of different strains and species parallels that found *in vivo*; and (iii) its cell-free conversion is inhibited by Congo Red analogues in a structure-dependent manner similar to that seen in *in vivo* and *in vitro* cell assays.

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INTRODUCTION

Transmissible spongiform encephalopathies (TSEs) are fatal, neurodegenerative diseases of humans and animals and include Creutzfeldt–Jakob disease (CJD), Gerstmann–Straussler–Scheinker syndrome, Kuru, bovine spongiform encephalopathy (BSE), scrapie, transmissible mink encephalopathy and chronic wasting disease. At present, there is no treatment or cure.

The causative agent responsible for TSEs or prion diseases has yet to be fully defined. A fundamental event in disease is the conversion of the normal, detergent-soluble, proteinase K-sensitive isoform of the prion protein, PrP^{C}, to an abnormal, detergent-insoluble, partially proteinase K-resistant isoform, PrP^{Sc}, and the accumulation of this abnormal isoform in the central nervous system of infected animals (Hope *et al.*, 1986; Meyer *et al.*, 1986; Oesch *et al.*, 1985). The protein-only hypothesis suggested that no nucleic acid is needed for replication (Griffith, 1967); this has been refined by defining PrP^{Sc} as the infectious agent and replication as the conversion by PrP^{Sc} of host PrP^{C} to more PrP^{Sc} (Prusiner *et al.*, 1982). Although the mechanism of conversion is unknown, interaction between PrP^{C} and PrP^{Sc} is critically implicated by *in vivo* studies (Caughey & Chesebro, 1997) and features in the current models of replication such as template-assisted (Prusiner, 1991) and nucleation-dependent (Jarrett & Lansbury, 1993) conversion.

The *in vitro* cell-free conversion assay has provided a quick, simple and well-defined system in which to study the molecular factors that influence the transition of PrP^{C} to PrP^{Sc} (Kocisko *et al.*, 1994). In this system, PrP^{Sc}, isolated from the brains of infected animals, induces the conversion of radiolabelled PrP^{C} to a proteinase K-resistant isoform, PrP^{res}. Newly formed PrP^{res} is distinguished from the PrP^{Sc}

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used to seed the conversion by the fact that it is radiolabelled. The assay has been shown to replicate *in vivo* species specificity, strain properties and polymorphism barriers (Bessen *et al.*, 1995; Bossers *et al.*, 1997, 2000; Iniguez *et al.*, 2000; Kocisko *et al.*, 1995; Zhang *et al.*, 2002; Raymond *et al.*, 1997; Horiuchi *et al.*, 2000) but as yet no *in vitro*-generated PrP^{res} has been shown to be infectious (Hill *et al.*, 1999).

Traditionally, cell-free conversion assays have used, as substrate, PrP^{C} purified from mammalian tissue culture cells (Kocisko *et al.*, 1994; Bossers *et al.*, 2000; DebBurman *et al.*, 1997; Saborio *et al.*, 1999; Hill *et al.*, 1999) or, more recently, from baculovirus-infected insect cells (Iniguez *et al.*, 2000; Zhang *et al.*, 2002). In this study, we have modified the cell-free conversion assay by replacing the tissue culture-derived PrP^{C} with PrP purified from bacteria and refolded *in vitro*. In addition, the guanidine conversion buffer, usually required for efficient conversion *in vitro*, has been replaced with a conversion buffer approximating physiological conditions, similar to that used by Horiuchi *et al.* (1999).

We found that PrP derived from bacteria converts to a proteinase K-resistant isoform in the cell-free conversion assay and the assay was shown to mimic the *in vivo* species barriers of transmission of 263K hamster and 87V mouse scrapie between hamster and mouse.

With the emergence of BSE (Wells *et al.*, 1987) and variant CJD (Will *et al.*, 1996), the search for prophylactic and therapeutic compounds is under way. We investigated the use of our modified cell-free conversion assay for identifying compounds that inhibit this conversion. Ten compounds with known anti-TSE activity in cell culture and/or *in vivo* were tested for their ability to inhibit this conversion. Eight of these compounds inhibited cell-free conversion to some extent, suggesting part of their *in vivo* effect could be modulated by binding directly to PrP. Two compounds did not inhibit *in vitro* conversion; these compounds may inhibit *in vivo* conversion indirectly by acting on some other part of cell metabolism.

These studies reinforce the specificity of PrP^{C} and PrP^{Sc} interactions and the utility of the cell-free conversion assay using bacterial recombinant PrP to investigate TSE transmission barriers and identify potential prophylactic compounds.

METHODS

Expression, radiolabelling and purification of recombinant PrP. Full-length mouse PrP of the *Prn-p*^{a} genotype (Locht *et al.*, 1986) with the N-terminal signal sequence replaced with methionine and the C-terminal signal sequence removed (corresponding to aa 23–230) was amplified from genomic DNA using the 5′ and 3′ primers 5′-GGGATCCATCATGAAAAAGCGGCCAAAGCCTGGAG-3′ and 5′-CGAATTCTTAGGATCTTCTCCCGTCGTAATAG-3′, respectively. Full-length hamster PrP (Basler *et al.*, 1986) with the N-terminal signal sequence replaced with methionine and the C-terminal signal sequence removed (corresponding to aa 23–231) was amplified from genomic DNA using the 5′ and 3′ primers 5′-GGGATCCATCATGAAGAAGCGGCCAAAGCCT-3′ and 5′-CGAATTCTCAGGACCTTCTTCC-3′, respectively. Plasmid pTrcHis (Invitrogen) was digested with the restriction enzymes *Nco*I/*Eco*RI to remove the 6-histidine tag. PCR fragments were digested with restriction enzymes *Eco*RI/*Rca*I and ligated into the modified pTrcHis vector. Therefore, the recombinant vector encodes full-length, untagged PrP.

Calcium chloride-competent *Escherichia coli* strain 1B392 were transformed with the recombinant vectors. Cells were grown in 50 ml volumes in methionine-deficient M63 media containing ampicillin to an OD_{600} of approximately 0·2, induced with 1 mM IPTG and proteins labelled with 18·5 MBq of [^{35}S]methionine for 90 min. Cells were harvested by centrifugation, lysed in buffer [50 mM Tris/HCl (pH 8), 100 mM sodium chloride and 1 mM EDTA] containing PMSF (0·1 mM final) and lysozyme (20 μg ml^{-1} final) and then treated with sodium deoxycholate (1 mg ml^{-1} final) and DNase (5 μg ml^{-1} final). Inclusion bodies were isolated by centrifugation and solubilized in 8 M urea. Insoluble material was removed by centrifugation and PrP in the supernatant purified in a two-stage chromatographic procedure of Ni-NTA affinity chromatography (Qiagen), binding in 0·1 M sodium phosphate, 10 mM Tris (pH 8), 8 M urea and 10 mM β-mercaptoethanol and eluting in 0·1 M sodium phosphate, 10 mM Tris (pH 4·5), 8 M urea and 10 mM *β*-mercaptoethanol, followed by cation-exchange chromatography using SP–Sepharose (Pharmacia), binding in 8 M urea and 50 mM HEPES (pH 8) and eluting with a sodium chloride gradient. Purification of PrP was followed by Western blotting using the monoclonal antibody (mAb) 6H4 (Prionics).

Following purification, ^{35}S-labelled PrP was refolded by copper oxidation of the disulphide bond (Jackson *et al.*, 1999) and dialysis into 50 mM sodium acetate (pH 5·5). Estimates of PrP purity were made by Coomassie staining and estimates of concentration were made using dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA, Perkin-Elmer) using unlabelled recombinant PrP as a standard, the mAb FH11 (TSE Resource Centre, I.A.H., Compton, UK) for capture of PrP onto a 96-well plate and europium-labelled mAb 6H4 for subsequent detection.

PrP^{Sc} preparation. PrP^{Sc} was prepared from the brains of terminally ill 87V-infected VM mice and 263K-infected hamsters, based on a method described by Hope *et al.* (1986). Briefly, a 5 % (w/v) brain homogenate was prepared in 10 mM sodium phosphate (pH 7·4) and 10 % (w/v) *N*-lauryl sarcosinate. The suspension was centrifuged at 22 000 ***g*** for 30 min at 10 °C. The supernatant was centrifuged at 215 000 ***g*** for 150 min at 10 °C and the pellet resuspended in H_2O. The volume of the solution was adjusted to 9 ml g^{-1} of brain and its ionic composition to 0·6 M potassium iodide, 6 mM sodium thiosulphate, 1 % (w/v) *N*-lauryl sarcosinate and 10 mM sodium phosphate (pH 8·5) and centrifuged at 285 000 ***g*** for 90 min at 10 °C through a sucrose cushion of 20 % (w/v) sucrose in 0·6 M potassium iodide, 6 mM sodium thiosulphate, 1 % (w/v) *N*-lauryl sarcosinate and 10 mM sodium phosphate (pH 8·5). The pellet was washed in H_2O and centrifuged in a microfuge. The final PrP^{Sc} pellet was resuspended in H_2O by sonication to approximately 1 μg μl^{-1}.

Cell-free conversion assay. Two cell-free conversion protocols were used in this study, either with or without guanidine. The cell-free conversion assay without guanidine was based on that used by Horiuchi *et al.* (1999). Briefly, PrP^{Sc} was sonicated and approximately 1 μg incubated with 200 ng of [^{35}S]PrP in conversion buffer [50 mM citrate (pH 6·5), 50 mM potassium chloride, 10 mM magnesium chloride, 100 mM sodium chloride and 0·1 % (v/v) Nonidet P-40], either with or without compounds to be tested for inhibition, for 24 h at 37 °C in a 20 μl volume reaction. Following incubation,

20 µl H_2O was added. One-twentieth of the reaction mixture was removed for analysis without proteinase K treatment and the rest was treated with 60 µg proteinase K ml^{-1} for 1 h at 37 °C. Proteinase K digestion was stopped by the addition of Pefabloc to 1 mM. All samples were precipitated with 20 µg BSA and 4 vols ice-cold methanol at −20 °C. The resulting pellet was boiled for 10 min in SDS-PAGE sample buffer and analysed by SDS-PAGE in 15 % polyacrylamide gels. Gels were then fixed, dried and exposed to film. Autoradiographs were quantified using Phoretix Gel Analysis software. For cell-free conversion assays in guanidine buffers, we based our method on that described in Kocisko *et al.* (1994). PrP^{Sc} was sonicated briefly and approximately 1 µg pre-incubated in 2 M guanidine, 0·25 % (w/v) zwittergent 3-14 and 50 mM citrate (pH 6·5) in a reaction volume of 10 µl for 2 h at 37 °C. Following pre-incubation, 200 ng [^{35}S]PrP was added to give a final guanidine concentration of 1 M. The reaction was incubated for 24 h at 37 °C and proteinase K-treated as described above.

Compounds. Compounds were obtained from Sigma-Aldrich or Lancaster or synthesized directly. Congo Red (CR) and its analogues were stored as 10× stock solutions in 2 % (v/v) DMSO at −20 °C. Chlorpromazine and quinacrine were freshly prepared as 10× stock solutions in H_2O for each experiment. For compound structures, see Fig. 4.

RESULTS

Production of ^{35}S-labelled PrP in recombinant bacteria

Recombinant mouse PrP (aa 23–230) was expressed in bacteria from the modified pTrcHis expression vector in the presence of [^{35}S]methionine. Following purification and refolding *in vitro*, the recombinant protein was analysed by Western blotting with anti-PrP mAb (Fig. 1, lane 1) and Coomassie staining (Fig. 1, lane 2). The protein had a molecular mass of approximately 26 kDa, as expected for unglycosylated PrP lacking the glycosylphosphatidylinositol (GPI)-anchor addition peptide. The yield was approximately 100 µg [^{35}S]MoPrP (recombinant mouse PrP) per 50 ml of culture medium and purity was estimated by Coomassie staining to be approximately 95 %. Mass spectrometry and circular dichroism spectroscopic analysis of unlabelled protein confirmed the correct expression, formation of the disulphide bond and folding into a normal α-helical formation (data not shown).

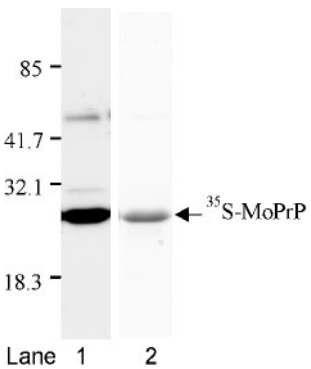

Fig. 1. Western blot and Coomassie-stained gel analysis of [^{35}S]MoPrP. Recombinant MoPrP was radiolabelled during expression from bacteria, purified and refolded *in vitro*. Western blot using mAb 6H4 (lane 1) and Coomassie-stained gel (lane 2) of purified refolded [^{35}S]MoPrP. Molecular mass markers (kDa) are indicated on the left.

Conversion of bacterial PrP to PrP^{res}

PrP^{C} purified from mammalian tissue culture cells (Kocisko *et al.*, 1994; Bossers *et al.*, 2000; DebBurman *et al.*, 1997; Saborio *et al.*, 1999; Hill *et al.*, 1999) and from baculovirus-infected insect cells (Zhang *et al.*, 2002; Iniguez *et al.*, 2000) has been used as substrate in the cell-free conversion assay. In this study, recombinant PrP, purified from bacterial cells and refolded *in vitro*, could also be converted into a proteinase K-resistant form in the cell-free conversion assay. Recombinant mouse PrP, MoPrP, was radiolabelled during expression, purified from bacteria and refolded *in vitro*. [^{35}S]MoPrP was incubated with and without unlabelled PrP^{Sc}, isolated from 87V scrapie-infected VM mouse brains in a guanidine-free conversion buffer at 37 °C for 24 h. Fig. 2 (lanes 1 and 3) show undigested [^{35}S]MoPrP. Following proteinase K digestion, [^{35}S]MoPrP became more proteinase K resistant in the presence (Fig. 2, lane 4) but not in the absence (Fig. 2, lane 2) of PrP^{Sc}. The proteinase K-resistant core of [^{35}S]$MoPrP^{res}$ was approximately 17 kDa, 6–7 kDa smaller than [^{35}S]MoPrP, characteristic of the proteinase K-resistant core of PrP^{Sc}, $PrP^{27\text{-}30}$ (Oesch *et al.*, 1985; Hope *et al.*, 1986). The efficiency of the conversion was approximately 30 %, according to densitometric analysis of labelled PrP before and after proteinase K treatment. This result indicates that recombinant PrP

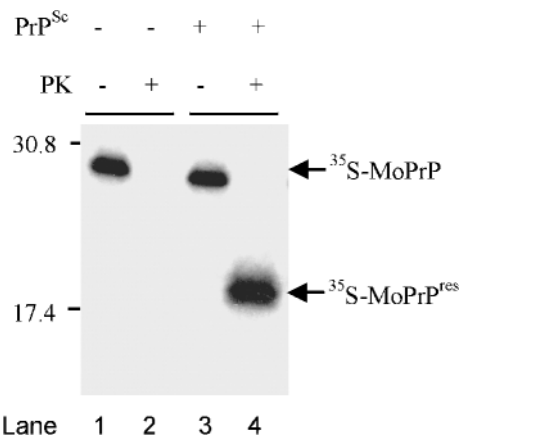

Fig. 2. Cell-free conversion of recombinant bacterially expressed PrP. [^{35}S]MoPrP was incubated with and without unlabelled 87V fibrils in non-guanidine conversion buffer at 37°C for 24 h followed by proteinase K (PK) digestion. [^{35}S]$MoPrP^{res}$ was detected by autoradiography. Lanes 1 and 3 show undigested [^{35}S]MoPrP. [^{35}S]$MoPrP^{res}$ was detected in the presence (lane 4) but not in the absence (lane 2) of PrP^{Sc}. Molecular mass markers (kDa) are indicated on the left.

expressed and purified from bacteria and refolded *in vitro* is a substrate in the cell-free conversion assay under physiologically compatible conditions and conversion to PrP^{res} is PrP^{Sc} dependent.

[^{35}S]MoPrP of the *Prn-p*^{a} allotype was converted to PrP^{res} by 87V (VM)-PrP^{Sc} of the *Prn-p*^{b} allotype (Fig. 2), although, *in vivo*, mice with the *Prn-p*^{a} genotype rarely if ever develop neurological disease following exposure to the 87V (VM) strain of mouse scrapie (M. E. Bruce, IAH, Edinburgh, UK, personal communication). However, this is only an apparent breakdown in the correlation between the cell-free conversion efficiency and *in vivo* transmissibility, since, while *Prn-p*^{a} allotype mice rarely develop clinical signs, 87V scrapie readily replicates in their brains and spleens. Furthermore, pathology typical of brain scrapie has been found in some older mice dying of 'natural causes', suggesting that 87V-infected *Prn-p*^{a} mice have prolonged survival times rather than a resistance to disease (M. E. Bruce, IAH, Edinburgh, UK, personal communication).

Species specificity in the conversion reaction

87V mouse scrapie has a prolonged incubation time in hamsters and mice are highly resistant to infection with 263K hamster scrapie. To determine whether our cell-free conversion assay mimics this *in vivo* species specificity, hamster and mouse PrP were radiolabelled and purified from the bacterial expression system, refolded *in vitro* and incubated in a non-guanidine conversion buffer, with PrP^{Sc} isolated from 263K-infected hamster brains and 87V-infected mouse brains in homologous and heterologous conversion assays. Hamster ^{35}S-labelled PrP, [^{35}S]HaPrP, was converted by hamster PrP^{Sc} into [^{35}S]HaPrP^{res} (Fig. 3, lane 2) and [^{35}S]MoPrP was converted by mouse PrP^{Sc} into [^{35}S]MoPrP^{res} (Fig. 3, lane 6). Very little or no 17 kDa [^{35}S]PrP^{res} was produced when [^{35}S]HaPrP was incubated with mouse PrP^{Sc} (Fig. 3, lane 4) or when [^{35}S]MoPrP was incubated with hamster PrP^{Sc} (Fig. 3, lane 8). These data indicate that the species specificity observed *in vivo* can be mimicked in our cell-free conversion assay using recombinant bacterial PrP.

Inhibition of the cell-free conversion assay

There are several lead anti-TSE compounds, including CR, chlorpromazine and quinacrine (reviewed by Gilbert & Rudyk, 1999). CR inhibits PrP^{res} formation in scrapie-infected mouse brain cells (SMB) (Rudyk *et al.*, 2000), in the cell-free conversion assay and in scrapie-infected neuroblastoma cells (ScN2a) (Demaimay *et al.*, 1998, 2000; Caughey & Race, 1992; Caughey *et al.*, 1993) and can cure these cells of infectivity. CR also prolongs the survival time of scrapie-infected hamsters if administered around the time of experimental infection (Ingrosso *et al.*, 1995). Although CR has anti-TSE activity, it has poor blood–brain barrier permeability and is toxic when broken down in the gut by microbes (Boss *et al.*, 1987); therefore, its use as a drug is limited and structure activity studies have been carried out

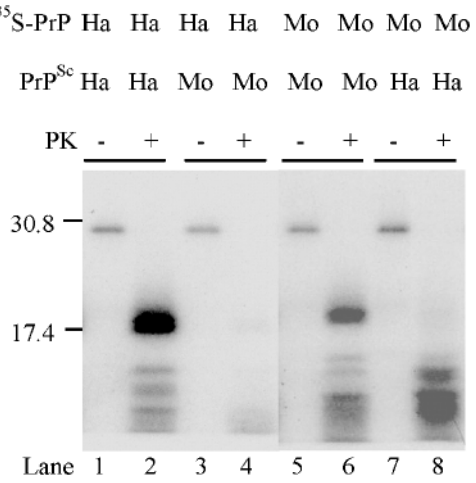

Fig. 3. Species specificity in the cell-free conversion assay. [^{35}S]PrP and PrP^{Sc} preparations were mixed and incubated in non-guanidine conversion buffer at 37 °C for 24 h. Following proteinase K (PK) digestion, [^{35}S]PrP^{res} was detected by autoradiography in the homologous conversion reactions (lane 2 and 6) but very little or no [^{35}S]PrP^{res} was observed in the heterologous conversion reactions (lane 4 and 8). These data are representative of three independent experiments. Molecular mass markers (kDa) are shown on the left. Ha, Hamster; Mo, mouse.

to improve its therapeutic properties (Rudyk *et al.*, 2000; Demaimay *et al.*, 1998). Chlorpromazine and quinacrine have been used in humans for many years as anti-psychotic and anti-malarial drugs, respectively, and have been shown to inhibit PrP^{res} formation in ScN2a cells (Korth *et al.*, 2001; Doh-Ura *et al.*, 2000). Although these drugs have anti-TSE activity, their mechanisms of action are unknown.

We investigated the use of our cell-free conversion assay, using bacterial PrP, to study the inhibitory effect of such anti-TSE compounds. CR and its analogues and chlorpromazine and quinacrine (Fig. 4) were tested for their ability to inhibit formation of PrP^{res} in our non-guanidine cell-free conversion assay using recombinant PrP purified from bacteria and refolded *in vitro*. [^{35}S]MoPrP was incubated with 87V PrP^{Sc} with varying amounts of inhibitor compounds in the cell-free conversion assay. Fig. 5 shows a typical autoradiograph of inhibition of conversion of PrP to PrP^{res} by CR and compound XXIV, Sirius Red (SR). CR and its analogues all inhibited conversion of [^{35}S]MoPrP to PrP^{res} to some extent (Fig. 5). SR was the most effective inhibitor, with an average IC_{50} of $6{\cdot}2 \pm 0{\cdot}26$ μM. This was followed by CR, with an average IC_{50} of $8{\cdot}3 \pm 0{\cdot}35$ μM, then by compounds XVIII, VI, IX, XVI and XII, with average IC_{50} titres ranging from 25 to 70 μM (Fig. 6). Compound III was the least effective inhibitor, with an average IC_{50} of 400 μM. As has been reported elsewhere (Demaimay *et al.*, 1998, 2000; Rudyk *et al.*, 2000) that at low concentrations, CR and its analogues enhanced conversion above the control level. Chlorpromazine and quinacrine did not inhibit conversion

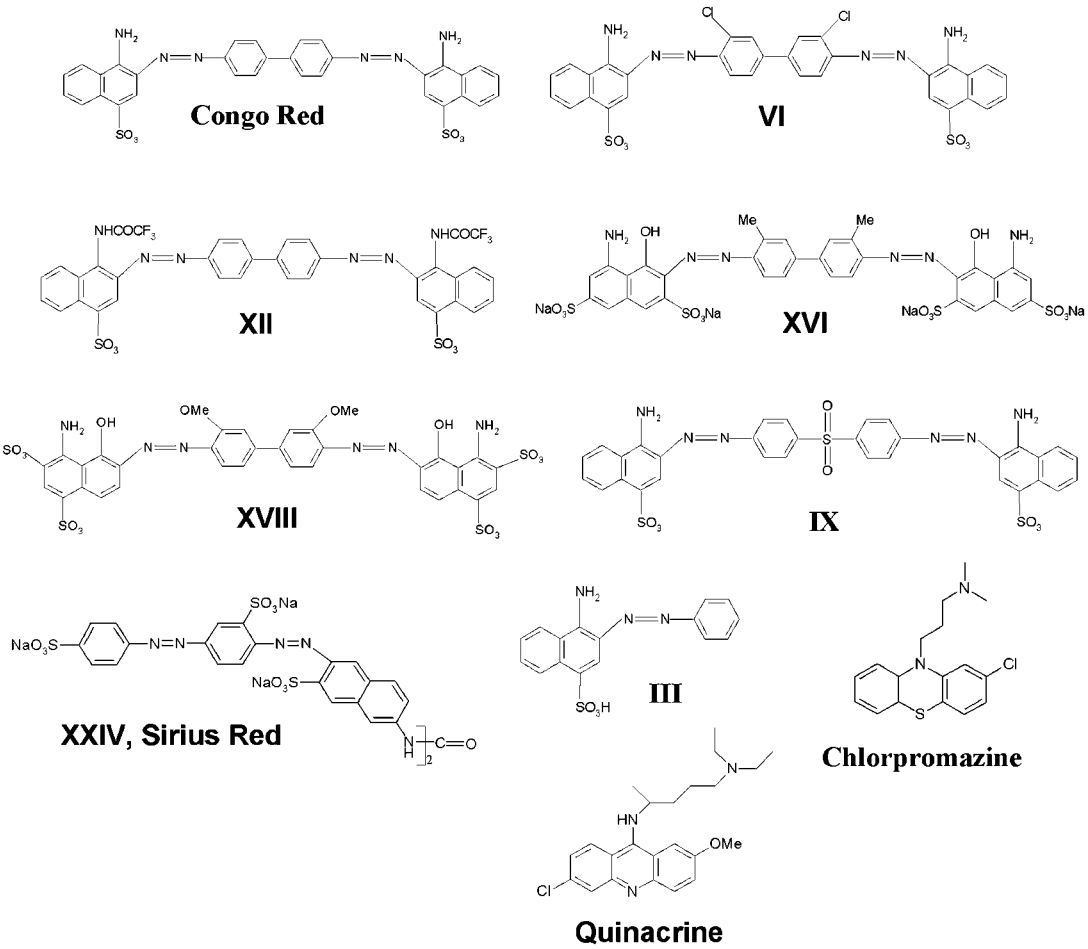

Fig. 4. Chemical structures of inhibitor compounds. CR and related compounds (roman numerals) and chlorpromazine and quinacrine were screened for their ability to inhibit conversion of [^{35}S]MoPrP to [^{35}S]MoPrP^{res} in the cell-free conversion assay.

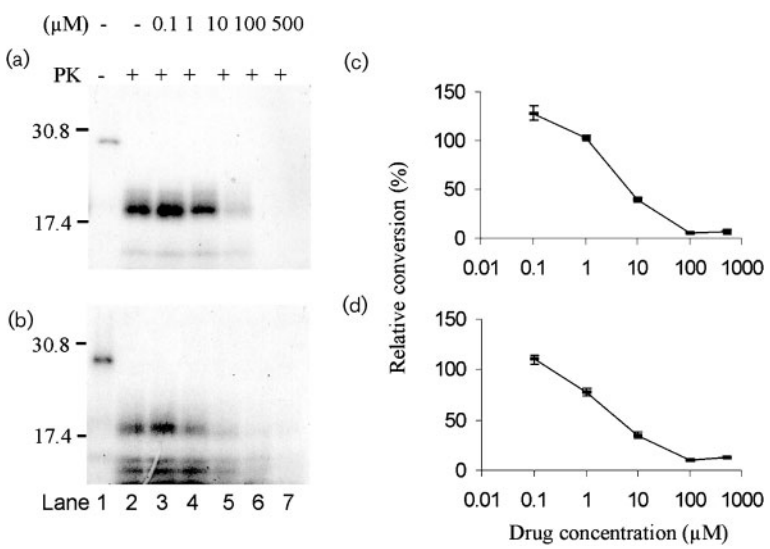

Fig. 5. Dose response relationships for (a) CR and (b) compound XXIV, SR, in the cell-free conversion assay. [^{35}S]MoPrP was incubated with 87V fibrils in non-guanidine conversion buffer at 37°C for 24 h with various concentrations of inhibitor. Amount of [^{35}S]MoPrP^{res} in the absence of inhibitor is shown in lane 2. Lanes 3–7 indicate the amount of [^{35}S]MoPrP^{res} in the presence of increasing concentrations of inhibitor. Molecular mass markers (kDa) are shown on the left. PK, Proteinase K. (c, d) Representation of three independent experiments. Results are plotted as the percentage of [^{35}S]MoPrP^{res} generated in the cell-free conversion assay in the presence of compounds relative to the amount of PrP^{res} generated in the absence of compounds, as determined by densitometry.

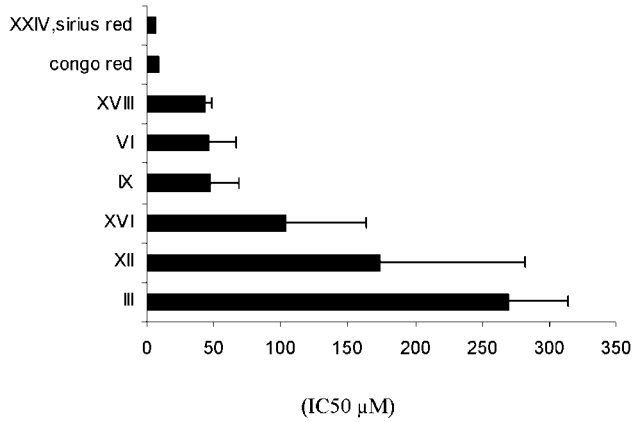

Fig. 6. Average IC_{50} titres for eight compounds that inhibited the formation of [^{35}S]MoPrP^{res} in the cell-free conversion assay. [^{35}S]MoPrP was incubated with 87V fibrils in non-guanidine conversion buffer for 24 h at 37°C with various concentrations of compounds. Following proteinase K treatment and SDS-PAGE, [^{35}S]MoPrP^{res} was detected by autoradiography. Average IC_{50} titres ±SE were calculated from dose response curves from three independent experiments.

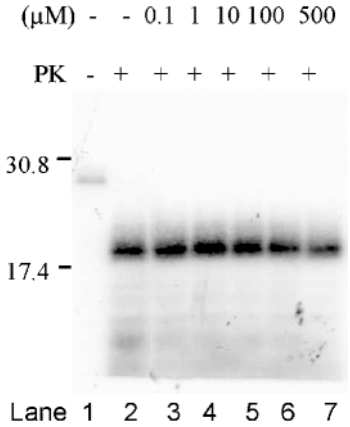

Fig. 7. Dose response relationship for CR in a cell-free conversion assay containing guanidine. 87V fibrils were pre-incubated in 2 M guanidine for 1 h at 37°C followed by dilution to 1 M guanidine by the addition of [^{35}S]MoPrP and various concentrations of inhibitor and incubated at 37°C for 24 h. Amount of [^{35}S]MoPrP^{res} in the absence of inhibitor is shown in lane 2. Lanes 3–7 indicate the amount of [^{35}S]MoPrP^{res} in the presence of increasing concentrations of CR. Molecular mass markers (kDa) are shown on the left. PK, Proteinase K.

at concentrations of 0·1–100 μM in our cell-free conversion assay (data not shown).

These data indicate that CR and its analogues may inhibit *in vivo* conversion by binding directly to PrP, whereas chlorpromazine and quinacrine may inhibit *in vivo* conversion not by binding to PrP but by influencing some other event in the cell.

Guanidine prevents the inhibitory effect of CR

Previously, the inhibitory effects of CR and its analogues on the cell-free conversion of recombinant PrP^{C} were investigated using protocols that incorporated guanidine (Demaimay *et al.*, 1998, 2000). Although CR inhibits conversion of PrP^{C} to PrP^{res} under such conditions, we wanted to measure the effect of CR on conversion without contributions from such non-physiological salts. The efficiency of conversion of [^{35}S]PrP was similar in both the presence and the absence of guanidine (Fig. 5a, lane 2, and Fig. 7, lane 2, respectively). However, when our more physiologically compatible conversion buffer was substituted with conversion buffer containing guanidine, CR no longer inhibited cell-free conversion at similar concentrations (Fig. 7).

DISCUSSION

Kocisko *et al.* (1994) were the first to demonstrate that PrP^{Sc} could induce the conversion of PrP^{C} into a proteinase K-resistant isoform, PrP^{res}, a hallmark of the disease-specific prion protein, in an *in vitro* cell-free assay and that this PrP^{res} was capable of limited self propagation. Their study used recombinant PrP^{C} immunoprecipitated from mammalian cell culture as the substrate for conversion (Kocisko *et al.*, 1994) and although PrP^{C} and PrP^{Sc} are the main constituents of the cell-free conversion assay, it is possible that cellular factors or antibody fragments introduced during the immunoprecipitation may co-purify with PrP^{C} and influence the cell-free conversion assay; chaperones (DebBurman *et al.*, 1997), metal ions (McKenzie *et al.*, 1998) and cell lysates (Saborio *et al.*, 1999) have all been shown to enhance conversion. In addition, the production of eukaryotic protein is time consuming and requires large amounts of radioactivity for low yields of radiolabelled protein.

Recently, recombinant PrP isolated either by immunoprecipitation (Iniguez *et al.*, 2000) or using an histidine tag (Zhang *et al.*, 2002) from baculovirus-infected insect cells has been used as a substrate in the cell-free conversion assay. These studies indicate that a non-mammalian source of PrP can be converted into a proteinase K-resistant species. In this study, we have used recombinant PrP expressed and purified from bacteria and refolded *in vitro* as a substrate in the cell-free conversion assay. This system uses relatively low amounts of radioactivity to produce high yields of recombinant protein, devoid of either an epitope tag or an histidine tag that may influence conversion, and because the protein is purified biochemically, complications such as the potential co-purification of cellular factors or antibody fragments is reduced. In addition, it is a quick and simple procedure and can allow rapid variation of protein sequence using standard cloning and mutagenesis techniques. Full-length mouse PrP (aa 23-230) was expressed, radiolabelled and purified from bacteria and refolded *in vitro*. The

expressed protein had a molecular mass of approximately 26 kDa, characteristic of full-length, aglycosyl PrP lacking the GPI anchor. Kocisko *et al.* (1994) have demonstrated that PrP does not require glycosylation or a GPI anchor to be converted in their cell-free conversion assays. We were able to show that bacterial recombinant PrP can be converted into PrP^{res} in the cell-free conversion assay with efficiencies of conversion similar to those obtained with mammalian and baculovirus recombinant PrP. The most abundant [^{35}S]PrP^{res} product of the cell-free conversion assay is 17 kDa, 6–7 kDa smaller than the precursor [^{35}S]PrP. This is similar to the 6–7 kDa reduction in mass seen upon digestion of brain-derived PrP^{Sc}. Proteinase K-resistant species smaller than 17 kDa were frequently observed in cell-free conversion assays. These smaller species do not correlate with *in vivo* transmissibility and may represent by-products of a non-pathogenic folding pathway.

To validate this bacterial PrP conversion assay, we investigated if the *in vivo* transmissibilities of scrapie between hamster and mouse could be replicated in this assay. The barriers of transmission of TSEs from one species to another generally involve a prolonged incubation period. The species specificity observed *in vivo* has been reproduced in the cell-free conversion assay using mammalian (Kocisko *et al.*, 1995; Raymond *et al.*, 1997; Horiuchi *et al.*, 2000) and baculovirus (Iniguez *et al.*, 2000; Zhang *et al.*, 2002) recombinant PrP. In this study, the results of conversion reactions between recombinant hamster and mouse PrP, and PrP^{Sc} of 263K hamster and 87V mouse, correlates with the relative transmissibility of scrapie between those species *in vivo*. Although molecular compatibility between PrP^{C} and PrP^{Sc} is important in the transmission of TSEs, other factors such as dose, route of infection and strain of agent may influence conversion *in vivo*.

Chlorpromazine, quinacrine and CR (and its analogues) are known inhibitors of PrP^{res} formation in tissue culture cells (Korth *et al.*, 2001; Demaimay *et al.*, 1998, 2000; Rudyk *et al.*, 2000; Caughey & Race, 1992; Caughey *et al.*, 1993) and have shown limited success *in vivo* (Ingrosso *et al.*, 1995). We observed similarities and differences in the ability of these compounds to inhibit conversion in the bacterial PrP cell-free assay. Chlorpromazine and quinacrine, contrary to their inhibitory effects on PrP^{res} formation in ScN2a cells (Korth *et al.*, 2001), did not inhibit PrP^{res} formation in the cell-free conversion assay. This suggests that *in vivo* chlorpromazine and quinacrine inhibit conversion not by binding directly to PrP but in some less direct effect on the cell. A recent study investigating the efficacy of quinacrine in an *in vivo* model of mouse-adapted scrapie failed to show a significant increase in survival time of scrapie-infected mice following quinacrine administration (Collins *et al.*, 2002). Alternatively, this lack of effect in the cell-free conversion assay may be due to host and TSE strain variables that are known to influence the effectiveness of anti-TSE drugs *in vivo*. The CR analogues used in our cell-free conversion assay were screened previously for their ability to inhibit PrP^{res} formation in SMB cells (Rudyk *et al.*, 2000). Although the IC_{50} titres reported in the cellular assay were not identical to the titres generated in the cell-free system, the order of effectiveness as inhibitors was the same. In summary, SR was the most potent inhibitor in both assays. The half molecule of CR had some activity but only at high concentrations. Compounds with an increased level of sulphation, substitution of the naphthelene amino with trifluoroacetamide, 3,3′-modification of the biphenyl or replacement of the biphenyl with bisulphone, all retained some activity. This suggests that it is possible to modify the structure of CR without dramatically affecting its activity as an inhibitor and therefore, it should be possible to design a compound with improved pharmacokinetic properties. The correlation between the cell-free and the cellular conversion assays suggests that *in vivo* CR may prevent conversion by binding directly to PrP. The enhancement of PrP^{res} formation observed at low concentrations of CR requires further investigation due to the obvious therapeutic implications.

The presence of guanidine in the conversion buffer prevented the inhibition of conversion by CR. This contradicts data that demonstrate inhibition of conversion by CR in the cell-free conversion assay in guanidine buffer (Demaimay *et al.*, 1998, 2000). Reasons for the observed difference may be the source of recombinant PrP, the species of PrP or other experimental conditions. However, the physiological conditions of our conversion assay give more relevance to the assay as a model of conversion and the similarity between our *in vitro* data and those from cell culture experiments (Rudyk *et al.*, 2000) support the use of more physiologically compatible buffers.

In summary, we have demonstrated that recombinant PrP expressed in a bacterial system and refolded *in vitro* can be converted into PrP^{res}. In addition, the assay was shown to mimic the *in vivo* species specificity of transmission of 263K hamster and 87V mouse scrapie between hamster and mouse and will allow the study of TSE transmission barriers. Together, the correlation between the cell-free and the cellular conversion assay in the inhibitory effects of anti-TSE compounds, and the use of a physiologically compatible conversion buffer, make the conversion assay using bacterial recombinant PrP a promising model for discovering and investigating potential prophylactics.

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