A systematic review of prion therapeutics in experimental models

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Prion diseases are transmissible, invariably fatal, neurodegenerative diseases which include Creutzfeldt–Jakob disease (CJD) in humans and bovine spongiform encephalopathy and scrapie in animals. A large number of putative treatments have been studied in experimental models over the past 30 years, with at best modest disease-modifying effects. The arrival of variant CJD in the UK in the 1990s has intensified the search for effective therapeutic agents, using an increasing number of animal, cellular and in vitro models with some recent promising proof of principle studies. Here, for the first time, we present a comprehensive systematic, rather than selective, review of published data on experimental approaches to prion therapeutics to provide a scientific resource for informing future therapeutics research, both in laboratory models and in clinical studies.

Keywords: prion; Creutzfeldt–Jakob disease; transmissible spongiform encephalopathy; experimental models; therapeutics

Introduction

Since the 1960s, there have been many attempts to treat prion diseases and prevent prion replication in a wide variety of experimental models. Here we present a systematic collation of these data from the published literature. Although the diversity of both the data and methodology precludes formal meta-analysis, this comprehensive overview allows a degree of qualitative analysis, which is particularly timely at the advent of controlled clinical trials in human prion diseases.

Prion diseases, or transmissible spongiform encephalopathies, include Creutzfeldt–Jakob disease (CJD) in humans and scrapie and bovine spongiform encephalopathy (BSE) in animals. They are incurable, fatal neurodegenerative diseases associated with neuronal cell death, characteristic 'spongiform' vacuolation of the brain tissue and accumulation of a disease-associated isoform of the endogenously expressed prion protein (for review see Collinge, 2001). The human prion diseases have three distinct aetiologies: they may arise sporadically; be autosomal dominantly inherited conditions; or be acquired from exposure to environmental prions (via dietary exposure to human or bovine prions or accidental exposure to human prions during medical and surgical procedures) (Collinge, 2001). The recognition of a novel human prion disease, variant CJD (Will et al., 1996), and the experimental confirmation that it is caused by BSE-like prions (Collinge et al., 1996; Bruce et al., 1997; Hill et al., 1997), have led to fears of a vCJD epidemic in the UK. Fortunately, the number of recognized cases of vCJD has been relatively small to date (~160), but the number of asymptomatic infected individuals incubating or carrying the infection is unknown; human prion incubation periods may span decades (Collinge, 1999) and the recognition...
of secondary transmission by blood transfusion has raised new concerns (Llewelyn et al., 2004; Peden et al., 2004).

The central feature of prion diseases is the accumulation in the brain and some other tissues of the disease-associated PrPSc, which is derived from the host-encoded cellular PrPC. Although its function is unknown, PrPC is implicated in prion pathogenesis as coding mutations of the human prion protein gene (PRNP) result in inherited forms of prion disease (Collinge, 2001), and the presence of PrPC is required for prion propagation and development of prion pathology (Büeler et al., 1993). PrPSc is derived from PrPC by post-translational conformational change (Borchelt et al., 1990; Caughey and Raymond, 1991) and is extracted from diseased brain tissue as aggregated material, which is distinguishable from PrPC by its partial protease resistance and detergent insolubility. A wealth of evidence now supports the ‘protein-only’ hypothesis (Griffith, 1967; Prusiner, 1982), which states that PrPSc is the principal, and possibly the sole, constituent of the transmissible agent or prion (Bolton et al., 1982) and that it serves as a conformational template promoting the conversion of endogenous PrPC to PrPSc (for review see Prusiner, 2001). In vitro methods to produce protease-resistant material from PrPC have been developed (see Supplementary Material), but to date there has been no proof of the ‘protein-only’ hypothesis.

There is also increasing evidence to suggest that PrPSc in vivo is itself not directly neurotoxic and there is a lack of correlation between PrPSc deposition and disease severity (Hsiao et al., 1990; Medori et al., 1992; Büeler et al., 1994; Collinge et al., 1995; Lasmézas et al., 1997; Hill et al., 2000; Mallucci et al., 2003), suggesting that it is the process of conversion of PrPC to PrPSc that is the key event in prion pathogenesis, rather than the accumulation of PrPSc. The mechanism for the conversion and the structure of the infectious agent remain unclear.

On a molecular level, prion disease therapeutics can be targeted to PrPC, PrPSc or to the process of conversion between the two prion protein isoforms. Targeting PrPSc, the disease-associated isoform, may appear to be the most logical approach, but such targeting may have no effect of disease progression, or even enhance or prolong disease if PrPSc is a non-pathological end-point of a pathogenic conversion process, or if the rate of PrPSc deposition is critical to disease progression. Alternatively, targeting PrPSc has the potential to remove the substrate for the pathogenesis and is applicable regardless of the disease aetiology.

The existence of distinct isolates or strains of prion has been recognized for many years. Strains were originally isolated by serial passage of natural scrapie samples in rodents and are distinguished by the production of different phenotypes in inbred mice (for review see Bruce et al., 1992 and Collinge, 2001) and by biochemical differences in the disease-associated prion protein isoform PrPSc (Bessen and Marsh, 1994; Collinge et al., 1996; Telling et al., 1996). It is important to consider prion strain type as well as experimental host with respect to model systems used to evaluate therapeutics; some therapeutic approaches may have different efficacy against different host/strain combinations. The pathogenesis may additionally be affected by the route of prion inoculation; following peripheral infection in some experimental animal models, prion replication is first detectable in the lymphoreticular tissues and spleen where levels plateau before detectable neuroinvasion, which occurs much later in the incubation period (for review see Aguzzi et al., 2003). Experimental models of prion propagation are reviewed in detail in the Supplementary Material.

Methods
This review was carried out using methodology adapted from that for compiling systematic reviews of clinical studies of healthcare interventions (Egger et al., 2001; Alderson et al., 2005). Bibliographic searches of the PubMed database were carried out using the criteria and search strategy detailed in the Supplementary Material, with the most recent search performed on March 10, 2006.

Titles, abstracts or full references from the search results were screened by one individual (C.T.) to assess the eligibility for inclusion in the review. Essentially, experimental data describing the effect of therapeutic agents on prion propagation in animal, cell or cell-free models were eligible for inclusion. Further descriptions of the experimental models and data, both included and excluded from the collection, are given in the Supplementary Material. No unpublished studies were included.

Data from the selected references were extracted directly into the tables by one individual (C.T.). Separate tables were used for each experimental model (see Table 1 for summary of types of experimental model) with therapeutic agents organized as far as possible by structural class and/or pharmacological action (see Table 2 for summary of therapeutic approaches). For each individual agent, experimental data are arranged chronologically. The tables are presented in the Supplementary Material and are supported by descriptive text introducing the specific therapeutic agents and summarizing their effect in the experimental systems, with commentary kept to a minimum. No meta-analysis was performed owing to the heterogeneity of the methodology and data readout.

Results
The PubMed search, as detailed in the Supplementary Material, returned 1648 citations. Of these, 143 articles were selected according to the criteria outlined in the Supplementary Material. The data from these references are tabulated primarily by experimental model and secondarily by therapeutic agent type (as summarized in Tables 1 and 2), and they are presented in the Supplementary Material. Supplementary Table S1 lists experimental data from animal models of disease; Supplementary Table S2 lists the data from cell culture models; Supplementary Table S3 lists the data from cell-free propagation systems and Supplementary Tables S1a and S2a list the secondary infectivity bioassays performed on samples from primary experiments in either animal or cell models, respectively. The structures of many of the therapeutic agents are given in Supplementary Fig. S1. The accompanying text summarizing the data is arranged by therapeutic agent type.
**Polyanionic compounds**

Glycosaminoglycans (GAGs) are the polysaccharide side-chains of proteoglycans (PGs) and include heparan sulphate, heparin, dermatan sulphate, keratan sulphate, chondroitin sulphate and carrageenans. PGs are components of the extracellular matrix and are involved in cell adhesion, migration and proliferation. Some cellular functions are also mediated by the free GAG chains. GAGs share a common architecture, being linear polymers of repeating disaccharide units, including an amino sugar and at least one negatively charged group (sulphate or carboxylate). This basic framework is also shared by the polyanionic glycans pentosan polysulphate.
(PPS) and dextran sulphate (DS), which are simplified semi-synthetic analogues of the endogenous GAGs (see Supplementary Fig. S1).

The endogenous heparan sulphate proteoglycan is associated with PrPSc deposits in prion disease (Snow et al., 1989; McBride et al., 1998) and also with the protein components of other amyloid deposits (for review see Diaz-Nido et al., 2002). GAGs can also associate with PrP (Gabizon et al., 1993; Caughey et al., 1994), with the GAG analogues PPS and congo red (CR) showing higher affinity interactions than the endogenous heparin and chondroitin sulphate (Caughey et al., 1994). As described below, these polyanionic compounds are effective at decreasing detectable PrPSc and increasing prion disease incubation times in cellular and animal models, respectively, and their therapeutic efficacy is thought to be mediated by a competitive inhibition of the interaction between endogenous GAG molecules and PrPSc and/or PrPSc.

**Heteropolyanion-23**

The antiviral compound heteropolyanion-23 (HPA-23, ammonium 5-tungsto-2-antimoniate) was initially tested against scrapie when the prevailing view was that the transmissible spongiform encephalopathies were caused by ‘slow viruses’. It was one of the first agents shown to have a beneficial effect on experimental prion disease, and the only effective one of a selection of antiviral agents tested (Kimberlin and Walker, 1979). HPA-23 delays the onset of scrapie following administration for over 9–12 days from the time of peripheral infection in mice and hamsters (Kimberlin and Walker, 1979, 1983, 1986), but it has no effect on the titre of the inoculum if pre-incubated with the inoculum (Kimberlin and Walker, 1986). The effects of HPA-23 treatment are prion strain dependent, and are less significant if administered after 12 days post infection, or if administered following intra-peritoneal rather than intravenous or subcutaneous inoculation with scrapie prions (Kimberlin and Walker, 1983, 1986).

Two other inorganic polyanions containing alternative or additional metal ions were ineffective against experimental rodent scrapie, whereas other organic polyanions, such as DS, tested alongside HPA-23 were found to be effective (Kimberlin and Walker, 1986). It is suggested that the efficacy of HPA-23 as an anti-prion agent is related to its polyanionic structure rather than its broad-range antiviral activity.

**Dextran Sulphate (DS)**

DS causes short-term impairment of the lymphoreticular system (LRS), and it was investigated as an anti-prion agent following demonstration of the involvement of the LRS in prion pathogenesis (Ehlers et al., 1984) in addition to the polyanion link (Kimberlin and Walker, 1986).

DS of molecular weight 500 kDa (DS500) causes a decrease in PrPSc levels in prion-infected cells (Caughey and Raymond, 1993; Barret et al., 2003) as well as a decrease in cell surface PrPSc in uninfected cells (Shyng et al., 1995). DS500 delays the disease onset following administration of a single dose around the time of i.p. infection in mice (Ehlers and Diringer, 1984; Ehlers et al., 1984; Farquhar and Dickinson, 1986), and both i.p. and i.c. infection in hamsters (Ladogana et al., 1992). There is a significant reduction in the prion titre in spleen following DS treatment (Ehlers and Diringer, 1984), but the effect of pre-incubation of the inoculum with DS500 is ambiguous, with no effect (Ehlers and Diringer, 1984) or a three log reduction in inoculum titre reported (Kimberlin and Walker, 1986) for mouse studies. Analyses of splenic PrPSc accumulation following DS500 treatment indicate a reduction in accumulation during the treatment period (Beringue et al., 2000a; Adjou et al., 2003). DS is most effective when administered at around the time of peripheral infection (Ehlers and Diringer, 1984), and in lymphotropic prion strains. DS500 treatment in mice is effective over a few weeks after peripheral inoculation in mice, during the period of replication in the LRS, but is ineffective if administered after the spleen accumulation plateau (Ehlers and Diringer, 1984; Kimberlin and Walker, 1986). In contrast to many mouse scrapie models, agent replication in the LRS prior to neuroinvasion is not necessary in hamsters peripherally infected with 263K scrapie. In hamsters, DS500 treatment is effective after both i.c. and i.p. inoculation but only if administered within 2 h of infection (Ladogana et al., 1992). Silica and trypan blue were tested along side DS as other agents causing impairment of the LRS, but neither shows any effect on scrapie incubation times (Ehlers et al., 1984).

DS500 is, however, toxic in mice [a 10% mortality is reported for animals treated with multiple doses of over a 4 week period (Adjou et al., 2003)], and other polyanions including PPS have been investigated as less toxic alternatives.

**Pentosan Polysulphate (PPS)**

PPS has anti-coagulant and anti-inflammatory activity, and has a veterinary license in the UK for treatment of osteoarthritis in the dog.

PPS has been reported to decrease PrPSc levels in prion-infected cells (Caughey and Raymond, 1993; Caughey et al., 1994; Birkett et al., 2001), but stimulates PrPSc formation in cell-free conversion system (Wong et al., 2001).

PPS causes a delay to the onset of disease following a single dose administered around the time of i.p. infection in mice (Ehlers and Diringer, 1984; Diringer and Ehlers, 1991; Farquhar et al., 1999), and both i.c. and i.p. infection in hamsters (Ladogana et al., 1992). PPS treatment by intraventricular infusion has also been shown to increase the incubation period of i.c. infected tg7 mice, even if the onset of the 4 week treatment period coincides with the onset of clinical symptoms (42 d.p.i.) (Doh-ura et al., 2004). Treatment was performed using an AZLET osmotic
pump and intra-ventricular cannula inserted into the contralateral hemisphere from that used for scrapie prion inoculation. There are less severe pathological changes in the treated hemisphere, including reduced PrPSc deposition relative to the hemisphere inoculated. In animals treated late in the incubation period, pre-existing PrPSc deposition is not cleared by the treatment, but further PrPSc accumulation is inhibited. There is some evidence of dose response, and an inverse correlation between time of treatment and prolongation of incubation period, though the variability of the incubation time increases with increased PPS dosage. A control peripheral (subcutaneous) infusion experiment was ineffective in prolonging incubation times, and high doses (20 mg/kg/day) were found to cause haemorrhaging around the area of the pump insertion in 80% of animals (Doh-ura et al., 2004). The safety of intraventricular PPS treatment was assessed by continuous i.c.v. infusion of PPS at 110–460 µg/kg/day for 2 months into both rats and dogs. There were no adverse effects reported in rats, but doses >230 µg/kg/day caused severe adverse effects in dogs; the majority of animals suffered fatal seizures within 24 h of initiation of treatment and on pathological examination haematomas in the cerebral white matter surrounding the cannula insertion point was evident in many animals (Doh-ura et al., 2004).

PPS treatment was also found to inhibit PrPres accumulation in a CWD-infected deer-cell model of prion disease, with an IC50 of 10 ng/ml, equivalent to the IC50 for PPS treatment in RML-infected N2a cells (Raymond et al., 2006).

**Endogenous GAGs**

Heparan sulphate and heparin of various molecular weights decrease the accumulation of PrPSc in infected cell cultures (Gabizon et al., 1993; Caughey and Raymond, 1993; Caughey et al., 1994). Conversely, both heparan sulphate and PPS have been shown to stimulate the cell-free conversion of PrPSc to protease-resistant forms (Wong et al., 2001). Other GAGs, including keratan sulphate and chondroitin sulphate, are less effective than heparan sulphate or DS at preventing PrPres accumulation in infected cells but also less effective at stimulating the *in vitro* conversion of PrPSc to PrPres. These results suggest that sulphated glycans have a direct effect on the conversion of PrP, and that depending on the circumstances, they may be either cofactors or inhibitors of conversion. A model to explain the observed effects of GAGs in prion propagation systems proposes that sulphated glycans act as competitive inhibitors of endogenous GAGs in cells, whereas in the cell-free system the sulphated glycans are themselves cofactors for conversion (Wong et al., 2001).

Non-sulphated dextrans are not able to prevent PrPSc accumulation in cells (Caughey and Raymond, 1993), and inhibition of sulphation of GAGs by sodium chlorate also decreases the amount of PrPSc in scrapie-infected cell culture (Gabizon et al., 1993) indicating that the sulphation of these molecules is crucial to their involvement with prion conversion, whether as inhibitors or facilitators.

**Heparan sulphate mimetics**

Heparan mimicking (HM) molecules, developed initially for properties in wound healing (Desgranges et al., 1999), were investigated for activity as anti-prion drugs following observations of the efficacy of DS and other sulphated glycans. HMs are synthesized from dextran polymers by chemical modification of the hydroxyl groups with varying amounts of sulphate, carboxymethyl and benzylamide groups (Adjou et al., 2003; Schonberger et al., 2003); a representative structure is shown in Supplementary Fig. S1. There is a similar response of both HM2602 and HM5004 to DS500 in reducing PrPSc accumulation in chronically infected cells (Adjou et al., 2003; Schonberger et al., 2003), but only HM2606 is effective *in vivo*, offering a 14% increase in incubation time in scrapie prion-infected hamsters and a significant reduction in splenic PrPSc levels in scrapie-prion-infected mice treated from the time of infection (Adjou et al., 2003).

Just as the non-sulphated dextrans are unable to prevent PrPSc accumulation in cells (Caughey and Raymond, 1993), the non-sulphated variants of the synthetic heparan mimetics are also ineffective (Adjou et al., 2003; Schonberger et al., 2003). An increased degree of both sulphate substitution and hydrophobic group substitution on the HM correlates with increased anti-prion efficacy in cells (Schonberger et al., 2003).

**Phosphorothioate oligonucleotides**

Four series of degenerate single-stranded oligonucleotides (DNA and analogues of natural nucleotides, see Supplementary Fig. S1) were investigated in a variety of prion propagation models (Kocisko et al., 2006b) following observation of the interaction of nucleic acids with PrP conversion and aggregation *in vitro* (Cordeiro et al., 2001; Deleault et al., 2005). Only the phosphorothioated oligonucleotides (randomers 1 and 2) are active in mouse-scrapie cell models, with IC50 values of ~20 nM compared with 15–90 µM for related non-phosphorothioated nucleotides (DNA and randomer 3). The ability of the randomers to prevent PrPSc accumulation is strongly size-dependent (oligomers of 17 or more bases are much more effective than smaller oligomers) but largely independent of base-composition. The randomers active in preventing PrPSc accumulation in the cell system were found to bind recombinant mouse or hamster PrP competitively with DS5000 and PPS, and co-localized with internalized PrP in both infected and uninfected cells, suggesting that the effect is not mediated by interaction with PrPSc.

Randomer treatment of peripherally infected Tg7 mice leads to a dramatic increase in survival time; treatment with randomer 1 at 10 mg/kg more than doubles or triples the survival time for s.c. or i.p. inoculated mice, respectively. Pre-treatment of scrapie inocula with randomer also significantly decreases the effective titre of the inocula. Randomers
showed a significantly lower anti-coagulation activity compared with PPS at equivalent molar doses, and may therefore have a clinical advantage over PPS.

Compounds related to GAGs/polysulphate polyanions

Congo Red (CR) and analogues

CR is a widely used histopathological stain for amyloid deposits including PrP. Since the earliest demonstration of CR efficacy in preventing prion accumulation in scrapie infected cells (Caughey and Race, 1992), there have been numerous studies of the effects of CR and its analogues as potential therapeutics for prion diseases.

Treatment of chronically infected cells with CR results in a decrease in PrPSc with IC50s reported between 1 nM and 1 μM (Caughey and Race, 1992; Caughey and Raymond, 1993; Caughey et al., 1993; Caspi et al., 1998; Demainay et al., 1998; Demainay et al., 2000; Milhavet et al., 2000; Mangé et al., 2000b; Rudyk et al., 2000; Poli et al., 2003). It is reported that both neuroblastoma and GT1 cells can be cured of prion infection by treatment with 1 μg/ml CR (Mangé et al., 2000b).

In cell-free conversion systems, CR causes a decrease in PrPres formation with IC50 of ~8 μM (Demainay et al., 1998, 2000; Kirby et al., 2003; Lucassen et al., 2003), although it has been observed that low concentrations of CR (<0.1 μM) actually stimulate PrPres formation under otherwise equivalent conditions (Demainay et al., 1998, 2000; Kirby et al., 2003).

Two in vivo studies report that treatment with CR delays the disease onset in hamsters following ongoing administration starting around the time of i.p. or i.c. infection (Ingrosso et al., 1995; Poli et al., 2004), although the effect was not significant after i.c. infection with low titre scrapie (Poli et al., 2004) and it was noted that there was no effect on the progression of clinical disease (Ingrosso et al., 1995). A further study reports a transient increase in splenic PrPSc levels on CR treatment of peripherally infected mice (Beringue et al., 2000a).

Since CR is toxic, non-specific and does not cross the blood–brain barrier (Klunk et al., 1994, 1998), many analogues and derivatives of CR have been studied in both cell and cell-free systems in order to identify compounds with improved properties. There are three studies of small selections of compounds, either commercially available CR analogues and amyloid dyes (Demainay et al., 1998, 2000) or synthetic naphthalene derivatives (Poli et al., 2003), which correspond to one of the terminal naphthalene groups on the symmetrical CR molecule (see Supplementary Fig. S1). Two further studies investigated large families of structurally related compounds for full structure–activity relationships (Rudyk et al., 2000; Sellarajah et al., 2004).

A general observation from these in vitro studies is that truncated molecules corresponding to one of the terminal moieties of CR are not effective at preventing PrPres propagation (Demainay et al., 1998, 2000). One exception to this is the molecule CR-A (Poli et al., 2003), which in spite of the increased cytotoxicity compared with CR, was taken forward to tests in vivo (Poli et al., 2004) and was found to have similar efficacy to that of CR (see below).

Rudyk et al. (2000) observe that for some of the compounds, treatment with low concentrations of compound actually increases PrPres in SMB cells, whereas high concentrations lead to a decrease in PrPres. This is in accordance with the observed stimulation of cell-free conversion reactions at low concentrations of CR (Demainay et al., 1998, 2000; Kirby et al., 2003). Many of the compounds tested show some activity, although only sirius red (see Supplementary Fig. S1) is as effective as CR in the ScN2a cell model (Demainay et al., 2000). Conversely, in the SMB cells, sirius red is a better inhibitor than CR (Rudyk et al., 2000), and an in depth study of structure–activity relationships in SMB cells identified 10 compounds (from 54 tested) with improved activity compared with CR (Sellarajah et al., 2004). Although these 10 compounds represent 5 of the 7 different structural families tested, some structure–activity relationships have been described (Sellarajah et al., 2004).

A comparison of the cell-free conversion and cell culture experiments shows that there is some correlation between the results with SMB cells and the non-denaturing conversion system (Rudyk et al., 2000; Kirby et al., 2003), but otherwise no clear correlation between compound efficacy in cell culture and in cell-free systems (Demainay et al., 1998, 2000). The amyloid specific dyes (evans red, trypan blue, sirius red, thioflavin S and primuline) are strongly inhibitory in the cell-free system but not in cells (Demainay et al., 2000). Kirby et al. (2003) observe that CR is a less effective inhibitor of conversion under denaturing conversion conditions, which may account for some of the discrepancies between the different experimental systems.

In animals, CR-A (equivalent to the terminal moiety of CR) is more effective than CR after i.c. scrapie infection, but less effective after peripheral infection. CR-B (equivalent to the linker moiety of CR) is not effective in animals. In contrast to the equivalent concentration of CR, a combined treatment of two compounds corresponding to the terminal and linker moieties of CR in ratio 2:1 (CR-A:CR-B) did not increase the incubation time of infected hamsters (Poli et al., 2004).

Although CR is a small molecule (see Supplementary Fig. S1), it is able to stack extensively and mimic larger sulphated polyanions (Woody et al., 1981; Priola and Caughey, 1994) and hence may have some mechanistic similarity to the sulphated polyanionic compounds discussed above. Both CR and other GAg-type compounds cause a decrease in surface PrPSc in uninfected cells (Shyng et al., 1995). CR competes with heparin binding to PrPsen (Caughey et al., 1994) and binds to and causes hyper-stabilization of PrPres, thereby preventing further amyloidogenesis both in cell culture systems and ex vivo brain homogenates (Caspi et al., 1998). The increase in splenic PrPSc after CR treatment (Beringue et al., 2000a) and the stimulation of in vitro conversion at low CR concentrations are consistent
with the proposal that inhibition of PrPSc accumulation by CR is mediated by stabilization of PrPSc (Caspi et al., 1998).

**Suramin**

Suramin (polysulphonated naphthyl urea, see Supplementary Fig. S1) was initially developed to treat trypanosomiasis. It causes downregulation of surface proteins and interferes with the oligomerization state of proteins (Gilch et al., 2001) and has some structural homology to CR. A modest increase in incubation time is observed in hamsters treated with two or three doses of suramin at or around the time of i.p. inoculation (Ladogana et al., 1992), and in mice treated with a single dose around the time of i.p. infection (Gilch et al., 2001). Suramin has been shown to decrease PrPSc levels in infected cells (Gabizon et al., 1993; Doh-ura et al., 2000; Gilch et al., 2001), to decrease surface PrPSc levels and cause intracellular PrP aggregation in uninfected cells and to cause aggregation of recombinant PrP (Gilch et al., 2001).

A variety of suramin derivatives and analogues were also tested in the ScN2a system; compounds effective at decreasing PrPSc accumulation are symmetrical aromatic structures with naphthalene- or benzene-sulphonic acid substitutions, whereas asymmetric or uncharged molecules and those with phosphonic or carbonic substitutions were less active (Nunziante et al., 2005). Unlike suramin, the analogues do not affect the cell surface expression of PrPSc, but like suramin they do induce formation of detergent-insoluble PrP aggregates at the cell surface (Nunziante et al., 2005).

**Polycationic compounds**

In addition to the polyanionic compounds described above, various classes of cationic polyamine compounds, including components of lipid transfection media, have been identified as potential anti-prion agents (Supattapone et al., 1999, 2001; Winklhofer and Tatzelt, 2000; Solassol et al., 2004; Yudovin-Farber et al., 2005).

**Dendritic polyamines**

An incidental discovery showed that exposure of ScN2a cells to SuperFect decreased both pre-existing PrPSc and prevented the formation of de novo PrPSc (Supattapone et al., 1999). The component polyamine compounds of SuperFect that effected the decrease in PrPSc were identified as the dendritic polymers polypolypropyleneimine (PPI) generation 4.0, polyethyleneimine and polyamidoamine generation 4.0 (see Supplementary Fig. S1), which have IC50 for PrPSc decrease in the nanomolar range (Supattapone et al., 1999). Structure–activity relationships indicate that increased anti-prion efficacy correlates with increased branching and an increase in the surface density of primary amine groups. ScN2a cells treated with PPI generation 4.0 are cleared of PrPSc and are no longer infectious to mice (Supattapone et al., 2001). There is a good correlation between compound efficacy in cell culture and in vitro experiments in which purified mouse scrapie or scrapie-infected mouse brain homogenates are incubated with polyamines prior to detection of PrPSc. The in vitro exposure of RML prions to PPI at pH 4 caused disaggregation and a decrease in β-sheet content of the preparation and an increase in proteolytic susceptibility of the protein that is both strain and sequence dependent (Supattapone et al., 1999, 2001). The proposed site of action of the polyamines is in lysosomal compartments, as demonstrated by their localization to lysosomes (Supattapone et al., 2001), their maximal efficacy in vitro at acidic pH (Supattapone et al., 1999), and the fact that the anti-prion effect of the polyamines can be blocked by the lysosome-rupturing agent chloroquine (Supattapone et al., 1999).

Phosphorus-containing dendrimers (pd) are a novel class of dendritic polyamines with improved bioavailability and decreased toxicity compared to the polyamines reported by Supattapone (Solassol et al., 2004). The phosphorus substituents of these dendrimers provide increased stability and tertiary amine groups render the molecule amphipathic. The efficacy of these compounds in the ScN2a cell culture system was found to increase with increasing generation (see Supplementary Fig. S1), but only up to generation 4 dendrimers (molecular weight 33 kDa), and cells cleared of PrPSc by pd-G4 treatment were no longer infectious to susceptible cells. Pd-G4 treatment of scrapie brain homogenate results in decreased PrPSc to varying degrees depending on the scrapie strain. In wild-type mice treated with pd-G4 after i.p. infection there was significant reduction in splenic PrPSc at 30 d.p.i. (Solassol et al., 2004), and although the effect on incubation time is not reported, there is a precedent for a correlation between depletion of splenic PrPSc in the early stages of i.p. infection and a prolongation of incubation time (Beringue et al., 2000b; Heppner et al., 2001; Barret et al., 2003; Mabbott et al., 2003). In addition to their anti-prion properties, these phosphorus dendrimers have the potential for targeting specific tissues and for use as soluble drug carriers (Solassol et al., 2004).

**Other cationic polyamines**

Following various lines of evidence that suggest an involvement of the cell membrane raft domains in the conversion of PrPSc to PrPSc (Taraboulos et al., 1995; Kaneko et al., 1997a; Klein et al., 1998b), Winklhofer and Tatzelt (2000) screened a variety of lipid transfection reagents with membrane-association properties for their effect on PrPSc formation in ScN2a cells. Treatment with the polycationic lipopolyamine DOSPA (see Supplementary Fig. S1) decreased the PrPSc levels in the cells by both clearance of pre-existing PrPSc and blocking de novo formation of PrPSc (Winklhofer and Tatzelt, 2000). The lipopolyamines tested are smaller than the dendritic molecules described above, and most were ineffective in clearing PrPSc from infected cells, including neutral and mono-cationic polyamines, as well as other polycationic lipids related to DOSPA with a spermicide
head-group (Winklhofer and Tatzelt, 2000). Similarly, Supattapone et al. (1999) reported the inability of a mono-cationic lipopolyamine (DOTAP) to decrease PrPSc levels in ScN2a cells. DOSPA treatment of cells showed no adverse effects on the integrity of the sphingolipid-cholesterol-rich membrane microdomains (rafts) (Winklhofer and Tatzelt, 2000). The membrane association of DOSPA is believed to be essential for its anti-prion activity.

Further polycationic compounds in which oligo-amines are conjugated to oxidized dextran or other sugar polymers have been tested for efficacy in the ScN2a cell model (Yudovin-Farber et al., 2005). These compounds are related to cationic polyamines and heparin mimetics and have been investigated for use as wound dressings and as gene transfection agents. Various combinations of polysaccharides and oligoamines were investigated, the most active components being dextran and spermine, respectively. Dextran-spermine was effective at completely clearing ScN2a cells of detectable PrPSc after 4 days of treatment at 31 ng/ml (3 nM); other combinations of saccharides and amines were effective at roughly one order of magnitude greater molarity. Dextran-spermine molecules were derivatized with the addition of methoxypoly-(ethylene glycol) (MPEG) or oleic acid substituents in order to produce compounds with improved bioavailability, but the derivatized compounds were less effective than the parent compound, with an inverse relationship between efficacy and degree of substitution.

Neither the branched (dendritic) polyamines nor the cationic polyamines affect PrPSc production; it is suggested that their mechanism of action may be in part via stimulation of the normal cellular pathways of protein degradation to destroy PrPSc (Supattapone et al., 1999).

Tetrapyrrolic compounds

Tetrapyrrolic compounds are known effectors of protein conformational change, with structural similarities to CR (being both aromatic and sulphated) but with improved toxicological and solubility profiles. They also have the potential for extensive substitution and derivation. A variety of tetrapyrroles, including porphyrins and phthalocyanins, were investigated by Caughey and co-workers as potential anti-prion compounds using the ScN2a cell culture system and were found to decrease PrPRes levels (Caughey et al., 1998), as well as to prevent propagation of PrPRes in the cell-free conversion system with sub-micromolar IC50 (Caughey et al., 1998). Of those tested in the ScN2a model, the three most potent anti-prion compounds (shown in Supplementary Fig. S1) are phthalocyanin tetrasulphonate (PcTS), deuteroporphyrin IX 2,4-bis-(ethylene glycol) iron(III) (DPG2-Fe3+) and meso-tetra(2-N-methylpyridyl)porphine iron(III) (TMPP-Fe3+). In addition, the porphine complex indium (III) meso-tetra (4-sulphonatophenyl)porphine chloride (In-TSP) gives an IC50 of 0.3 μM in the CWD-infected deer-cell model (Raymond et al., 2006).

The most efficient of the cell culture compounds were examined using in vivo prion disease models—transgenic mice expressing hamster PrP and infected with hamster prion strains (tg7) (Priola et al., 2000), and subsequently in wild-type mice (Priola et al., 2003). A delay in the onset of disease follows multiple administrations over the first month after i.p. infection, but no significant effect is seen after i.c. infection, nor after treatment at a later stage in the disease progression after i.p. infection (Priola et al., 2000, 2003). Pre-treatment of the prion inocula with PcTS caused a significant increase in incubation period, but a greater increase in incubation time is observed following multiple treatments post inoculation. The suggested mode of action for the anti-prion efficacy of tetrapyrroles is in peripheral tissues at the initial stage of infection.

Further complexes of various divalent metals with two porphine tetrapyroles, tetra (4-sulphonatophenyl)porphine (TSP) and tetra (4-N,N,N-trimethylammonium)porphine (TAP), were investigated in the tg7 mouse model (Kocisko et al., 2006a). Prophylactic i.p. treatment with Fe-TAP in i.p. infected tg7 mice results in a 4-fold increase in survival time, much greater than that reported in previous tetrapyrrole studies. Significant increases in survival time of a lower magnitude are also seen for Fe-TSP and metal-free TSP and TAP treatments. Even Fe-TAP is ineffective, however, if administration is commenced more than 50 days after scrapie infection. Pre-incubation of the scrapie inoculum with metal–porphine complexes prior to i.c. inoculation results in modest increases in survival time for iron and nickel complexes, correlating to 3–4 log reduction in the effective titre of the inoculum. For i.c. porphine treatments commencing 2 weeks into the incubation period of i.c. infected mice, the most efficacious complex is Fe-TSP rather than Fe-TAP (the most effective in the i.p. treatment and i.p. infection model). Intracerebral Fe-TSP treatment is deemed by the authors to be as effective as similar regime of PPS, at 2-fold lower dose (mass per animal).

Polynye antibiotics

In the 1980s, Amyx reported on the treatment of experimental scrapie in hamsters and CJD in mice with 35 drugs encompassing antiviral, antibacterial, anti-parasitic, anti-fungal and anti-neoplastic drugs, hormonal agents and interferon. Only methotrexate and amphotericihin B were found to significantly prolong the incubation time, following treatment throughout the incubation period, although none of the agents prevented disease. Amphotericihin B treatment was also reported to significantly increase the incubation time of CJD-infected African green monkeys (Amyx et al., 1984).

Amphotericin B (AmB, see Supplementary Fig. S1) is a fungal antibiotic derived from Streptomyces nodosus, which acts by intercalation into and disruption of the cell membrane. AmB shows preferential binding for ergosterol over cholesterol, but in mammalian cells interacts with cholesterol...
and alters the membrane lipid composition by peroxidation and endocytic processes, resulting in modification of raft domain properties. Following the initial report from Amyx, there were many subsequent reports of treatment with amphotericin B and its derivatives in animal and cell culture models of prion propagation.

Amphotericin B treatment delays disease onset in hamsters infected both i.p. and i.c. with 263K scrapie (Pocchiari et al., 1987; Pocchiari et al., 1989; Xi et al., 1992; McKenzie et al., 1994; Adjou et al., 1995, 1999, 2000) and in C57BL/6 mice infected with C506M3 scrapie (Demaimay et al., 1994). In mice, treatment was effective even if administered at a late stage following i.c. infection, when there is already significant infectivity and PrPres accumulation in the brain (Demaimay et al., 1997), suggesting that AmB is an effective inhibitor of prion propagation within brain tissue. Effective late stage treatment in a hamster model has also been demonstrated (Adjou et al., 2000).

MS-8209 is a less toxic derivative of amphotericin B (Adjou et al., 1995), which can therefore be administered at higher doses and is generally more effective than amphotericin B. Treatment with AmB or MS-8209 shows strain-dependent efficacy; hamster scrapie strains DY and 139H show no effect or a less marked increase in incubation time compared with 263K scrapie (Xi et al., 1992; McKenzie et al., 1994), and treatment of mice with C506M3 scrapie is more successful than treatment of mice of the same strain with BSE infection (Adjou et al., 1996). In a transgenic mouse expressing hamster PrP under a neuron-specific enolase promoter (tg52NSE) infected with 263K hamster scrapie (Demaimay et al., 1999), both AmB and MS-8209 treatment was more effective in the absence of endogenous mouse PrP expression. Neither drug showed an equivalent efficacy in tg52NSE mice infected with the DY scrapie prion strain. MS-8209 treatment had no effect on the disease progression in SCID mice but decreased the proportion of mice succumbing to clinical disease (clinical attack rate), and conversely delayed disease onset without altering clinical attack rate in reconstituted SCID (R-SCID) mice (Beringue et al., 1999).

The amphotericin B analogue mepartricin is effective only against i.p. scrapie prion infection (Pocchiari et al., 1989) unlike AmB, which is effective against both i.c. and i.p. infection.

There are discrepancies in the findings from infectivity bioassays of AmB treated 263K-scrapie infected hamsters, which are ascribed to differences in experimental protocols; Xi et al. (1992) report no spleen infectivity and reduced brain infectivity to +50 d.p.i. in partially purified samples, whereas McKenzie et al. (1994) report merely a delay in accumulation of infectivity which reaches control levels at +70 d.p.i. Despite equivalent infectivity between treated and control hamsters, McKenzie et al. (1994) also report that at +70 p.d.i., the treated animals show no clinical sickness and 10-fold lower PrPres in brain tissue, compared to the clinically-sick untreated animals, again highlighting the lack of direct correlation between infectivity in the brain and clinical illness.

In cell culture systems, AmB decreases the PK-resistance of mutant PrP (Mangé et al., 2000a) and causes a decrease in level of PrPSc in infected neuronal cells which is not maintained on cessation of treatment (Mangé et al., 2000b). Filipin is a polyene antibiotic related to amphotericin B which is able to reduce PrPSc accumulation in Scn2a cells with an IC50 of 2 μM (Marella et al., 2002).

**Tetracyclic compounds**

The anthracycline 4′-iodo-4′-deoxy-doxorubicin (IDOX) is an anticancer drug which was found to possess anti-amyloidogenic properties during a trial of cancer patients with immunoglobulin light-chain amyloidosis complications (Gianni et al., 1995). It was shown to encourage resorption of fibrils, and is also able to bind in vitro to a variety of natural amyloid fibres (Merlini et al., 1995). IDOX was tested in experimental hamster scrapie by i.c. administration of the scrapie inocula and drug together due to its high cytotoxicity and poor penetration of the blood–brain barrier; this treatment is effective in delaying the onset of clinical disease and there is an absence of key histopathological features of disease at onset (Tagliavini et al., 1997). Although not applicable as a therapeutic agent, IDOX is proposed as a prototype anti-prion compound (Tagliavini et al., 1997).

The tetracyclic antibiotics were investigated by the same group following the IDOX finding. In common with IDOX, tetracyrrolic compounds and CR, the tetracyclic antibiotics tetracycline and doxycycline contain a hydrophobic core with hydrophilic substituents (see Supplementary Figure S1) and have improved cytotoxicity and pharmacokinetic properties compared with IDOX. The efficacy of tetracycline and doxycycline against experimental scrapie was demonstrated after incubation of the scrapie inoculum with drug prior to i.c. infection (Forloni et al., 2002), which results in a delayed onset of disease by reducing the titre of the initial inoculum. These results are presented as having potential for decontamination rather than for therapeutics (Forloni et al., 2002).

Tetracycline reduces protease-resistant PrP formation in the PMCA replication assay (Barret et al., 2003) and reduces detectable PrPres in treated brain homogenate (Tagliavini et al., 2000; Forloni et al., 2002). It is reported to bind to fibrillogenic synthetic PrP peptides, preventing acquisition of protease resistance (Tagliavini et al., 2000; Barret et al., 2003) and also to prevent PrP106-126 peptide-mediated cytotoxicity in primary cell culture (Tagliavini et al., 2000).

**Tricyclic and related compounds**

Given that the lysosome is a potential site of conversion of PrPSc to PrPSc, Doh-ura et al. (2000) investigated the potential anti-prion effects of various lysosomotropic factors, including the anti-malarial drugs quinacrine and chloroquine (see Supplementary Figure S1), in the scrapie-infected cell
system. Quinacrine was found to be a very efficient inhibitor or \( \text{PrP}^{\text{Sc}} \) propagation in infected cells, with an \( IC_{50} \) of 0.4 \( \mu M \). Prusiner and colleagues later reported a cell culture system in which the efficacy of licensed tricyclic compounds were investigated (Korth et al., 2001). Compounds found to be effective in the ScN2a system included a variety of compounds in the acridine and phenothiazine classes, of which the most effective were quinacrine (IC\( \text{ScN2a} \) 0.3 \( \mu M \)) and chlorpromazine (IC\( \text{ScN2a} \) 3 \( \mu M \)), respectively. Ryou et al. (2003) also tested the relative efficacy of the different enantiomers of quinacrine in the cell system and report a 2- to 6-fold greater activity of (S)-quinacrine compared with (R)-quinacrine.

There are mixed reports of the efficacy of quinacrine in cell-free conversion systems; three groups reports that quinacrine has no effect on Pr\( \text{P}^{\text{Sc}} \) propagation (Do-h-ura et al., 2000; Kirby et al., 2003; Lucasser et al., 2003), whereas Barret et al. (2003) report that quinacrine decreases formation of protease-resistant PrP in the PMCA assay.

Despite its efficacy in cell culture, quinacrine treatment showed no effect on the incubation time of ic-infected animals, in either wild-type mice treated orally (Collins et al., 2002) or in tg7 mice treated by intra-ventricular infusion (Do-h-ura et al., 2004). In fact a high dose of intra-ventricular quinacrine causes a decrease in the scrapie incubation time (Do-h-ura et al., 2004), and a further study reports that ip quinacrine treatment of BSE-infected mice results in an increase in splenic Pr\( \text{P}^{\text{Sc}} \) deposition at 30 days after infection (Barret et al., 2003).

Chlorpromazine is less effective than quinacrine in cell culture (Korth et al., 2001), but was reported to increase incubation time in mice after intracerebral but not intraperitoneal infection (Roikhel et al., 1984). These results were published using the compound name aminasine and have not been substantiated to date.

Following the success of acridine compounds in cells (Korth et al., 2001), a variety of bis-acridines were developed and investigated by the same group (May et al., 2003). Supplementary Figure S1 shows a representative bis-acridine compound. A total of 20 compounds of various linker types and lengths were investigated for bioactivity and cytotoxicity; three compounds were shown to have ~10-fold increased efficacy compared with the parent quinacrine (i.e. IC\( \text{ScN2a} \) ~ 0.03 \( \mu M \)). There are no data reported for in vivo experiments with these compounds.

A study of further quinacrine-related (but not tri-cyclic) compounds has been reported by the same group of investigators who first observed the efficacy of quinacrine in cell systems (Murakami-Kubo et al., 2004). Two families of compounds containing a quinoline ring, typified by quinine and biquinoline (see Supplementary Fig. S1) were investigated. In the scrapie-infected N2a cell model, the effective compounds inhibited Pr\( \text{P}^{\text{Sc}} \) accumulation with IC\( \text{ScN2a} \) in the range 3 nM to 38 \( \mu M \), without any effect on biosynthesis or turnover of Pr\( \text{P}^{\text{C}} \). Other compounds were ineffective, and the authors describe structure--activity relationships for the compound families. In vitro biore analyses show both quinine and biquinoline bind to recombinant Pr\( \text{P}^{\text{C}} \). In vivo studies with transgenic mice expressing hamster PrP or overexpressing mouse PrP were treated for 1 month with an intra-ventricular infusion of quinine or biquinoline using the protocol established for intra-ventricular treatment with PPS. Treatment commenced either shortly after or 1 month after intracerebral scrapie inoculation, and in all cases the variance of the incubation times in the treatment groups was increased compared to the control groups. In spite of increases in the incubation times in many treatment groups, only some of these increases are statistically significant. There is no classical dose response to either compound. Administration of biquinoline at +10 d.p.i. by either intraperitoneal or intra-ventricular route gave a significant increase in incubation time, but neither route of biquinoline administration was significant when treatment was commenced late into the infection process (+35 d.p.i.). Pathological examination of the animals with prolonged incubation periods shows reduced Pr\( \text{P}^{\text{Sc}} \) deposition on the brain hemisphere ipsilateral to the intra-ventricular cannula, but no difference in deposition and pathology on the contralateral side, the side of the scrapie inoculation.

Mefloquine is a quinoline anti-malarial effective against both RML and 22L infected N2a cells, but not effective as prophylaxis against peripherally inoculated scrapie in tg7 mice (Kocisko and Caughey, 2006). Other classes of anti-malarial compounds tested (i.e. not quinoline or acridine compounds) were generally toxic at the treatment concentrations (1–10 \( \mu M \)).

**Beta-sheet breaker peptides**

PrP residues 106–126 appear to be important in the conversion between prion protein isoforms (de Gioia et al., 1994), and an excess of peptides PrP106-136 or PrP109-141 (especially residues 119 and 120 from the hydrophobic sequence \( \text{AGAGAGA}^{270} \)) prevented the recruitment of Pr\( \text{P}^{\text{C}} \) in a cell-free conversion experiment (Chabry et al., 1998). Using prion protein peptides of both mouse and hamster sequences (residues 109–141, of which 119–136 are identical in mouse and hamster protein), Chabry et al. (1999) investigated the species specificity of conversion and report that both mouse and hamster peptides can inhibit conversion of proteins from either species. PrP119-136 is also effective at decreasing Pr\( \text{P}^{\text{Sc}} \) in chronically infected MNB cells with an IC\( \text{ScN2a} \) of 11 \( \mu M \), whereas other peptides, including PrP119-128, did not show this effect (Chabry et al., 1999).

This result, and the successful employment of \( \beta \)-sheet breaker peptides in models of Alzheimer’s disease (Soto et al., 1998), spurred the design of a \( \beta \)-sheet breaker peptide to specifically interact with prion protein conversion (Soto et al., 2000). A \( \beta \)-sheet breaker peptide consists of a sequence from the target protein into which extra proline residues are inserted. Proline is an amino acid unable to take the conformation required by an ordered \( \beta \)-sheet structure and its
presence in a sequence of amino acid residues with otherwise high β-propensity prevents the formation of β-sheet by that peptide. The sequence of the prion protein β-sheet peptide iPrP13 designed by Soto et al. is given in Supplementary Fig. S1. iPrP13 causes some reduction in the incubation time of scrapie infected mice if used to treat the inoculum prior to i.c. infection and decreases protease-resistant PrP in the CHO cell model expressing mutant PrP (Soto et al., 2000). A 1000-fold molar excess of iPrP13 over PrPSc is required for 90% reduction PrPSc in an in vitro experiment with scrapie infected brain homogenate (Soto et al., 2000). There was no decrease in PrPSc levels in chronically infected ScN2a cells treated with either monomeric or polymeric iPrP13 (Oishi et al., 2003).

Compounds identified by screening approaches

**Cell culture screen**

Chronically infected N2a cells were used to screen a library of 2000 commercially available drugs and natural products (The Spectrum Collection, MicroSource Discovery Inc.) for molecules capable of decreasing PrPres propagation (Kocisko et al., 2003). The initial screen conditions were 10 μM compound in RML-infected ScN2a cells for 5 days; compounds positive under these conditions were then screened at 1 μM in both 22L- and RML-infected ScN2a cells, and IC50 values were estimated from these two sets of treatment conditions. A total of 17 compounds were positive against both scrapie strains with sub-micromolar IC50s and without cytotoxicity (Supplementary Table S2). These compounds fall into five distinct classes: anti-histamines (astemizole, terfenadine) and polyphenolic compounds (tannic acid, katochicine, bisepigallocatechin digallate), which define novel classes of anti-scrapie compounds, and anti-malarials (quinacrine, beceberrine, tetradrine, amodiaquine), phenothiazines (thiothixene, prochloroperazine, thioridazine, trifluoperazine) and steroid-type compounds (budesonide, dexamethasone, lovastatin, chrysanthellinA). Compounds from these classes identified with anti-prion activity by other investigators include quinacrine (Doh-ura et al., 2000; Korth et al., 2001), chlorpromazine (Korth et al., 2001), prednisone (Outram et al., 1974) and lovastatin (Taraboulos et al., 1995; Bate et al., 2004b).

Some of the most promising compounds identified in this screen (Kocisko et al., 2003) were tested in the tg7 model of prion disease as both treatment for intracerebral scrapie infection and prophylaxis against peripheral scrapie infection. None showed any significant effect on prion disease incubation time following treatment throughout the incubation period (Kocisko et al., 2004).

Compounds from the initial screen in N2a cells (Kocisko et al., 2003) were subsequently tested in a Rov9 cell model of ovine scrapie, along with 32 novel inhibitors of PrPSc propagation identified by screening of both RML and 22L scrapie infected N2a cells (Kocisko et al., 2005). None of those tested in the Rov9 cells were as effective in the ovine model as in the murine models, with only 3 out of 32 showing IC50s ~40 μM compared with submicromolar IC50s for RML-infected N2a and 1–10 μM for 22L-infected N2a.

**Yeast-based screen**

Further compounds were identified using an assay for prevention of yeast prion formation (Bach et al., 2003). A total of 2500 compounds, synthetic and natural products, were screened in a yeast assay. Two compounds were additionally shown to be effective in preventing prion propagation in the ScN2a model with IC50s of ~5 μM. The kastellpaolitines constitute a new class of anti-prion compounds, and both these and the phenanthridines have some structural similarity to the tricyclic compounds.

**Fluorescence screen**

An assay for identification of potential anti-prion drugs from a library of 10 000 compounds was developed based on the scanning for intensely fluorescent targets (SIFT) technique (Bertsch et al., 2005). The assay involves the formation of a ternary complex between PrPSc, recombinant PrP and mAb which has both red and green fluorescence; in the presence of a compound that interferes with the binding of PrPSc to rPrP, a reduction in the green fluorescence of the complex is observed. Activities of test compounds were compared to that of positive control compound DOSPA (17 μM). A total of 80 promising compounds were tested in a secondary assay in ScN2a cells; 4 showed reproducible decreases in PrPSc signal in the absence of toxicity, with IC50s of 2 and 6 μM reported for two compounds.

**Immunomodulation and immunotherapeutics**

Although there is no obvious humoral immune response stimulated in prion disease (Porter et al., 1973), various elements of the immune system have been manipulated to probe prion pathogenesis, and these experiments in turn have suggested potential strategies for prion therapeutics; for a review of the involvement of the immune system in scrapie pathogenesis see Aguzzi et al. (2003). Early experiments assessed the efficacy of both immunostimulation and immunosuppression (see below), but since the experimental demonstration that antibodies can be raised to the prion protein, efforts have been focused on the therapeutic application of antibodies or of stimulating an antibody response. Initial attempts to produce anti-prion antibodies included immunization with purified prions (Bendheim et al., 1984) or scrapie-associated fibrils (SAFs) (Kascak et al., 1987), with a greater response observed in prion knockout mice (Prusiner et al., 1993) compared to wild-type mice. With appropriate adjuvants, it is also possible to use recombinant
cellular prion protein as an immunogen in both knockout (Kraemmann et al., 1996; Williamson et al., 1996; Korth et al., 1997; Beringue et al., 2003; White et al., 2003; Khalili-Shirazi et al., 2005) and wild-type mice (Souan et al., 2001; Sigurdsson et al., 2002; Gilch et al., 2003; Schwarz et al., 2003).

**Antibodies in in vitro systems**

Early indications of the potential of antibody therapy for prion diseases came from in vitro studies showing a two log reduction of infectivity of a prion inoculum after incubation with an anti-PrP antibody (Gabizon et al., 1988) and the inhibition of a PrPres formation in a cell-free conversion system by a polyclonal serum raised to the prion protein peptide 219–232 (Horiuchi and Caughey, 1999). It was also shown that the polyclonal anti-PrP antibody R073 inhibits the PrPSc-induced neurotoxic effect on primary neuronal cultures (Muller et al., 1993). The monoclonal antibody 3F4 (Kascak et al., 1987) and polyclonal sera raised to peptides 23–37, 90–104, 143–156 and 219–232 were each pre-incubated with PrPsen prior to the in vitro conversion reaction and only the antibody recognizing residues 219–232 was able to prevent the formation of PrPres (Horiuchi and Caughey, 1999). Pre-incubation of the antibody with PrPsen, but not the PrPres seed, resulted in inhibition of de novo PrPres formation, and competition of the antibody with the peptide 219–232 after pre-incubation with PrPsen abrogates the inhibition of PrPres formation, indicating that the effect of the antibody is mediated by a direct interaction with PrPsen.

Many subsequent articles have reported on treatments of cell culture models of prion infection with both monoclonal and polyclonal antibodies, as described below.

The monoclonal antibody 6H4 recognizes residues 144–152 of PrP (Korth et al., 1997), and shows a profound effect in scrapie-infected cells, inhibiting the accumulation of PrPSc with sub-micromolar IC50 (Enari et al., 2001). An equivalent dose is used to prevent infection in uninfected cells pre-treated with antibody and to cause the clearance of PrPSc from infected cells over a 2 week treatment period (Enari et al., 2001). Inhibition of PrPSc propagation is also achieved by removal of PrPSc from cell surface using PIPLC, which cleaves the GPI anchor (Enari et al., 2001); the mechanism proposed by the authors for the inhibition of PrPSc by antibodies and PIPLC is sequestration of PrPSc from the cell surface.

Recombinant Fab fragments of various monoclonal antibodies were tested for their effect on PrPSc formation in Scn2a cells (Peretz et al., 2001). D18 and D13 (recognizing PrP epitopes 132–156 and 95–103, respectively) were the most efficient Fabs, with IC50 of 9 nM and 12 nM, respectively. Other Fabs recognizing C-terminal epitopes inhibited PrPSc formation with IC50 of ~50 nM, whereas Fabs recognizing N-terminal epitopes or an epitope immediately C-terminal to the D18 epitope were unable to inhibit PrPSc formation.

Two antibodies to SAF (Demart et al., 1999) have been investigated in neuroblastoma cells overexpressing PrPC with and without prion infection (Perrier et al., 2004). SAF34 recognizes an epitope in the octa-repeat region of PrP, and does not interact with N-terminally cleaved PrPC (which represents the majority of PrPSc in cells), and SAF61 (epitope 144–152) recognizes both PrP isoforms. These antibodies decrease the levels of both PrPSc in infected cells and PrPSc in uninfected cells, with SAF61 showing the more marked effect, and an enhanced effect when both antibodies are applied together. The proposed mechanism of action for SAF61 is the increased rate of PrPSc clearance which removes the substrate for PrPSc formation, and possibly also enhanced clearance of PrPSc. Since SAF34 does not increase the clearance of PrPSc and is unable to recognize the N-terminally cleaved PrPSc isoform, an alternative mechanism is proposed whereby the antibody prevents the interaction between PrPSc and PrPSc, as also suggested by earlier studies with mAbs (Enari et al., 2001; Peretz et al., 2001). It is also noted that the epitopes for SAF34 and SAF61 correspond to the two proposed binding sites for PrP to the laminin receptor (Hundt et al., 2001), so the anti-prion mechanism of the SAF antibodies could be by disruption of PrP binding to its receptor (Perrier et al., 2004); see also LRP/LR-mediated therapeutics (Leucht et al., 2003).

Beringue et al. (2004) studied a panel of monoclonal antibodies in scrapie-infected Rov cells expressing ovine PrP. The antibodies were raised to recombinant mouse PrP in either α or β conformation (Jackson et al., 1999). Antibodies ICSM 35, ICSM 37 and ICSM 42 were the most efficient inhibitors of PrPSc accumulation, decreasing levels by 500- to 1000-fold after 3 weeks, and an IC50 in the sub-nanomolar range (ng/ml) calculated for ICSM 35. ICSM 18 and ICSM 19 inhibited PrPSc accumulation ~100-fold less efficiently than ICSM 35, and other antibodies had no effect on prion propagation. The determinant for successful prevention of PrPSc accumulation is reported to be the ability of the antibody to recognize PrPSc in addition to PrPC, as demonstrated by immunoprecipitation experiments; the most successful antibodies in this system were those raised to β PrP which recognize the epitope 96–109 (Beringue et al., 2004).

Polyclonal antibodies raised to a dimeric recombinant murine PrP in mice and rabbits were shown to inhibit the de novo accumulation of PrPSc in Scn2a cells (Gilch et al., 2003). The inhibition of PrPSc formation by polyclonal antibodies can be prevented by pre-absorption of Ab with recombinant dimer, indicating the effect of the antibody is directly mediated by its interaction with PrP. Unlike the recombinant Fab fragments, the Fab fragments of these polyclonal antibodies have no effect on the PrPSc levels in Scn2a cells, even after prolonged exposure (Gilch et al., 2003).

Kim et al. generated a large panel of antibodies raised in Pnpp10/0 mice to either purified mouse PrPSc or recombinant mouse PrP (Kim et al., 2004b), and tested a selection of these in the Scn2a model (Kim et al., 2004a). They report that, regardless of the recognition epitope, the antibodies which
are effective at reducing PrPres accumulation are those which are able to bind to cell surface PrP, as determined by flow cytometry (Kim et al., 2004a). Only those antibodies effective in cell culture are listed in Supplementary Table S2; for the full list of antibodies see Kim et al. (2004a, b). The mechanism of inhibition is suggested to be blockade of cell-surface PrPSc, since the flow cytometry shows that the antibodies are not internalized.

Feraudet et al. (2004) studied a separate panel of 145 antibodies, raised in Prnp0/0 mice to a variety of immunogens (PrP peptides, human, ovine or murine recombinant PrP or SAFs), for their efficacy in prevention of PrPSc propagation in two different cell models. An initial treatment of cells with 10 μg/ml Ab for 3 days identified 37 antibodies which were able to decrease PrPSc by more than 20%. IC50s were estimated for 16 of these antibodies from dose-dependent treatments of ScN2a cells (see Supplementary Table S2). The results were corroborated using Rov9 cells expressing ovine PrP, with only slight differences in rank order of efficacy which are probably due to species specificity of the cellular PrP and the immunogen to which the antibodies were raised. Again, the ability of antibodies to prevent PrPSc propagation in ScN2a cells correlates with efficient binding of the antibodies to cell-surface PrP on both N2a and ScN2a cells, as shown by flow cytometry, but not with the PrP recognition epitope.

Antibodies raised in Prnp0/0 mice to recombinant human or sheep protein or to scrapie infected N2a cells (Nakamura et al., 2003) were tested for efficacy in N2a cells infected with three different prion strains: 22L, Chandler (RML) and Fukuoaka (Miyamoto et al., 2005). The effective antibodies were 3S9 (raised to recombinant ovine PrP) and 2H9 (raised to scrapie-infected N2a cells), which recognize epitopes in the C-terminal region of the protein (residues 141–161 and 151–221, respectively) (Miyamoto et al., 2005).

scFv antibodies are mini-antibodies comprising the variable regions of the light and heavy chains (V\textsubscript{L}, V\textsubscript{H}) expressed as a single chain, which maintain antigen specificity and can be engineered for intracellular expression or secretion; two groups have investigated the use of scFvs in prion-infected cell culture systems. A system of paracrine inhibition was developed in which RD-4 cells expressing and secreting scFv(8H4) in HEK-293 cells and PC12 cells also causes retention of PrPSc within the ER. PC12 cells can be infected with scrapie after differentiation with NGF and accumulate PrPres like other prion propagating cell lines. On expression of scFv(8H4) prior to differentiation and infection, PC12 cells showed no PrPres up to 21 days after infection. The expression of scFv(8F9) did not alter the cellular location of PrPSc and was not investigated in infected NGF-differentiated cells.

scFv(8H4)-expressing PC12 cells were further characterized and bioassayed for infectivity in wild-type mice (Vetrugno et al., 2005). A lysate of wild-type PC12 cells prepared at 35 d.p.i. was highly infectious to the host C57BL mouse, whereas only 2 out of 10 animals treated with lysate of infected PC12 cells expressing scFv(8H4) succumbed to prion disease, and it is believed that PrPres in cells rather than de novo scrapie; Lysates of PC12 cells or PC12 cells expressing scFv(8H4) taken at 6 h post scFv exposure were as infectious as the positive control (0.1% 139A scrapie). All 8 of 10 animals with no clinical symptoms at 300 d.p.i. were clear on histopathology and WB (Vetrugno et al., 2005) demonstrating effective treatment.

Active immunizations with PrP or prion peptide prior to infection

Several groups have reported moderate beneficial effects of active immunization of mice with prion protein or peptides prior to infection with scrapie isolates. A prolongation of incubation time by ~10% is observed for mice immunized with recombinant mouse prion protein (residues 23–230) from 14 weeks prior to prion infection, but not for those immunized from the time of infection (Sigurdsson et al., 2002). A higher antibody titre correlated with longer incubation times, but no difference in histopathology and PrPSc levels is observed between treated and control animals at terminal stages of disease (Sigurdsson et al., 2002).

Active immunization with the prion protein peptide 105–125 increases the incubation time of mice subsequently infected orally with scrapie, whereas immunization with a protein corresponding to the structured region of the protein (residues 90–230) is not effective at preventing or delaying disease (Schwarz et al., 2003). The antibodies raised after inoculation with the 90–230 protein responded to an epitope in the region 159–188. The authors propose that antibodies recognizing only prion protein regions 105–125 or 144–152 are active in abrogating prion disease (Schwarz et al., 2003).

An oral vaccination of Salmonella typhimurium expressing moPrP administered to mice prior to oral scrapie exposure significantly increases incubation times and decreases the disease incidence to 70% (Goni et al., 2005). There were no histopathological signs of disease in animals culled at 500 d.p.i. without clinical signs, and no difference in histology between the scrapie-sick treated and control animals. Across all animals (both scrapie-sick and survivors), there are correlations between both IgA and IgG levels and survival time.

Hamsters were immunized with synthetic hamster peptides comprising the N-terminal and central regions of the protein three times prior to and five times after scrapie inoculation (Magri et al., 2005); treatment resulted in a
moderate but not statistically significant increase in survival time, and the treated animals showed decreased spongiosis, PrPSc deposition and pro-inflammatory cytokines (IL-1β and TNF-α) compared to controls.

**Passive immunizations with monoclonal antibodies**

Two groups have reported increased disease incubation times following passive immunization of mice. Peripheral administration of either of the anti-prion antibodies ICSM 18 or ICSM 35 is effective in preventing the appearance of scrapie for up to 500 d.p.i. in mice infected peripherally with scrapie (White et al., 2003). The effect is seen if a high dose of the antibody is administered biweekly from either 1 week or 30 days after infection, during the splenic PrPSc accumulation phase; treatment results in a decrease in splenic PrPSc at 60 d.p.i. and absence of detectable PrPSc in brain at 250 d.p.i. Peripheral antibody treatment is not effective following intracerebral scrapie infection or if applied at onset of clinical symptoms after intraperitoneal scrapie infection (White et al., 2003). The antibodies ICSM 18 and ICSM 35 were raised to recombinant mouse prion protein folded in α or β conformation (Jackson et al., 1999), and recognize the prion protein epitopes 144–152 and 94–105, respectively (Khalili-Shirazi et al., 2005).

Treatment of mice with the monoclonal antibodies 8B4 (epitope 34–52) or 8H4 (epitope 175–185) immediately after peripheral prion inoculation caused only a 10% increase in incubation time with a high prion titre and no difference in histopathology and PrPSc levels between treated and control animals at terminal stage (Sigurdsson et al., 2003b). No significant effect was observed after treatment with mAb 8F9 (epitope 205–233) (Sigurdsson et al., 2003b). The contrast of these results to those of White et al. (2003) may be due to a dose effect of the antibody, which is ~100-fold lower in the Sigurdsson study.

**Transgenic expression of 6H4μ**

The anti-prion antibody 6H4 (epitope 144–152) has been shown to prevent accumulation of PrPSc in scrapie infected cells (Enari et al., 2001). Transgenic expression of the μ-chain of 6H4 results in a high titre of anti-PrP antibodies and complete prevention of scrapie pathogenesis following i.p. infection in hemizygous mice (Heppner et al., 2001). PrPSc levels in brain and spleen both before and after scrapie inoculation are the same as in control mice, and expression of the transgenic μ chains did not cause suppression of B-cell responses. The authors therefore state that the prion resistance of these mice is not mediated by downregulation of PrPSc or by immunosuppression.

**Lymphotoxin-β-receptor mediated approaches**

The LRS is crucial in peripheral prion infection and impairment of the LRS can prolong incubation times or significantly decrease scrapie susceptibility; genetically asplenic mice (Dickinson and Fraser, 1972) and splenectomized mice (Fraser and Dickinson, 1970) are not susceptible to peripheral scrapie prion infection. Depletion of B-lymphocytes prevents pathogenesis after i.p. infection (Klein et al., 1997, 1998a), but depletion of T-cells has no effect on scrapie infection and neuroinvasin (McFarlin et al., 1971; Fraser and Dickinson, 1978). B-cell derived lymphotoxin-β and TNF-α are involved in the differentiation and maturation of follicular dendritic cells (FDCs), which are required for splenic PrPSc accumulation and subsequent neuroinvasin (Brown et al., 1999; Mabbott et al., 2000b). Knockout of TNFα (Mabbott et al., 2002) or complement components involved in antigen recognition by FDCs (Klein et al., 2001; Mabbott et al., 2001), causes an increase in prion disease incubation period, indicating that temporary depletion of FDCs may have therapeutic potential in the early stages of peripheral scrapie infection.

Two groups have reported that neutralization of the LTβ-R pathway with administration of the soluble LTβ-R-Ig fusion protein blocks the maturation of FDCs and prevents scrapie neuroinvasion if administered soon very after i.p. scrapie infection (Mabbott et al., 2000a, 2003; Montrasio et al., 2000). Treatment results in depletion of FDCs in spleen for up to 28 days, and clearance of PrPSc from spleen (Mabbott et al., 2000a, 2003; Montrasio et al., 2000). Treatment prior to i.p. scrapie challenge both increases the incubation time and decreases the disease incidence and surviving animals show no scrapie histopathology and a lack of infectivity in spleen at 70 d.p.i. Scrapie-sick animals show classic histopathology and variable PrPSc at terminal stage. A single treatment with LTβR-Ig followed by oral scrapie challenge resulted in a complete absence of prion disease and PrPSc deposition in brain up to 500 d.p.i. (Mabbott et al., 2003). LTβ-R-Ig treatment 14 days after oral scrapie challenge has no effect, neither does treatment after intracerebral scrapie challenge (Mabbott et al., 2003), which is consistent with a transient depletion of FDCs causing inhibition of neuroinvasion in this model. The effect of LTβR-Ig administration before or after scrapie infection via skin scarification was also investigated (Mohan et al., 2005); treatment was most effective if administered prior to scrapie infection, as observed for LTβR-Ig treatment with peripheral scrapie inoculation (Mabbott et al., 2003).

**Immunostimulation**

Interferons are cytokines of the innate immune system with antiviral and antiproliferative actions known to inhibit the evolution of viral diseases in mice. Treatment of scrapie-infected mice with either interferon (Gresser and Pattison, 1968; Gresser et al., 1983) or interferon stimulators (Field et al., 1969; Worthington, 1972; Allen and Cochran, 1977) has no effect on the disease progression. It is also reported that treatment with interferon or interferon stimulators had no benefit on monkeys clinically ill with CJD, scrapie or kuru (Amyx et al., 1984).
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There are additional reports that general immunostimulants can increase the scrapie incubation period in mice. Administration of the cytokine-guanyl oligodeoxynucleotide 1826 (CpG 1826), which stimulates an innate immune response in mammalian hosts (for review see Lipford et al., 1998), has been reported to have a beneficial effect on mice peripherally infected with scrapie. A modest increase in incubation time is observed with four daily doses of CpG administered directly after infection, and a complete reduction of disease incidence is observed with administration over a 3 week period (Sethi et al., 2002). There are, however, questions about the safety of long-term administration of CpGs; it has been reported that repeated administration leads to destruction of lymphoid follicles and immunosuppression, which may in fact explain the protective effect of CpG treatment on prion infection (Heikenwalder et al., 2004). A further group conducting active immunization experiments observed that treatment with complete Freund’s adjuvant (CFA) alone was sufficient to provide a modest increase in the incubation time of mice infected both i.p. and i.c. with scrapie. The inclusion of prion peptides for active immunization offered no improvement on the response generated by CFA alone (Tal et al., 2003).

Immunosuppression/anti-inflammatory agents

There are varying reports of the efficiency of immunosuppression of experimental scrapie, with no effect observed for treatment with cyclophosphamide (Worthington and Clark, 1971), but a delay in disease onset following treatment with anti-inflammatory agents such as prednisone (Outram et al., 1974) and arachis oil (Outram et al., 1975). Prednisone and arachis oil are able to delay disease onset following administration around the time of i.p. infection, but not following administration late in the disease course, or after i.c. scrapie infection (Outram et al., 1974); younger animals are more responsive to the prednisone treatment, which is likely to be related to the lower susceptibility of neonatal animals to scrapie (Outram et al., 1973).

Non-steroidal anti-inflammatory drugs (NSAIDs) block the activity of the cyclo-oxygenase enzymes (COXs, or prostaglandin synthases). Investigation of the efficacy of the COX-inhibitor indomethacine in vivo reports a modest delay in the onset of clinical disease onset but not death of rats treated after i.c. scrapie infection (Manuelidis et al., 1998). In both neuroblastoma cell lines and primary neuronal cultures, NSAIDs are protective against cytocytotoxicity caused specifically by a prion peptide or by partially purified PrPSc preparations from scrapie sick mouse brains (Bate et al., 2002).

Dapsone is an anti-parasitic and anti-inflammatory drug that shares structural features (amine and sulphonate groups) with CR and suramin (see Supplementary Fig. S1). There are conflicting reports of its efficacy in rodent models of prion disease. It is reported to increase incubation time in ic infected rats (but with no effect on rate of formation of PrPSc deposits) (Manuelidis et al., 1998), and to have no effect on the incubation time of i.c. infected mice (Guenther et al., 2001).

Therapeutic approaches targeted at PrPSc

Transgenic mice in which the Prnp gene has been knocked out do not propagate prions and do not develop pathology following inoculation with scrapie prions (Büeler et al., 1993; Sailer et al., 1994). The removal of PrPSc, the substrate for prion propagation, is a potential therapeutic target, provided it can be achieved without detrimental effects on the host organism. Two different approaches investigated the feasibility of conditional rather than constitutive removal of PrPSc by turning off expression of the prion protein gene Prnp or by post-translational gene silencing mediated by RNA interference.

Transgenic knockout of neuronal PrPSc in adult mice

Conditional knockout of neuronal expression of the prion protein gene in mature mice has been shown to be possible without major deleterious effects (Mallucci et al., 2002). These mice are resistant to prion inoculation administered both before and after the PrP knockout (Mallucci et al., 2003). Knockout of neuronal expression of PrP ~8 weeks after i.p. scrapie infection not only prevents disease but also reverses early spongiform change, and the mice remain asymptomatic despite continued gliosis, extensive extraneural PrPSc deposition, and accumulation of infectivity (Mallucci et al., 2003). These experiments validate removal of the substrate for prion conversion as a therapeutic strategy in vivo.

RNA interference

RNA interference (RNAi) is the tool by which post-transcriptional gene silencing mechanisms in the cell can be initiated. The introduction of small double-stranded RNA to a cell leads to the specific degradation of mRNA homologous to the introduced RNA molecules. RNAi is thus a means by which specific genes can be turned off without the requirement for genomic DNA manipulation. dsRNA encoding mouse Prnp codons 392–410 is effective in reducing the levels of PrPSc in scrapie-infected cell cultures with an IC50 of ~100 nM. The effect is transient, with PrPSc levels returning to normal on subsequent culture following the RNA transfection, but is sequence specific; a scrambled version of the same RNA sequence used as control has no effect in the cell culture system (Daude et al., 2003). Using uninfected cells to monitor the effect of RNA interference on PrPSc expression, a different group of investigators observed that exogenously introduced RNAi-vectors can suppress both exogenous and endogenous Prnp expression (Tilly et al., 2003).
Other therapeutic approaches

Curcumin

Curcumin is the major component of the spice turmeric, it is a planar aromatic small molecule analogous to CR (see Supplementary Fig. S1). It is a non-toxic antioxidant, which has improved blood–brain barrier permeability compared with CR, though this is still limited. It has been reported to decrease β-peptide accumulation in a mouse model of Alzheimer’s disease (Frautschy et al., 2001; Lim et al., 2001). Curcumin is an efficient inhibitor of PrPSc propagation in RML-infected N2a cells (IC50 10 nM), and causes a decrease in detectable protease-resistant PrP in cell-free conversion studies (40% decrease with 10 nM curcumin), but it has no effect on disease progression after i.c. prion infection in hamsters regardless of the treatment regime (Caughey et al., 2003). Curcumin has no effect on N2a cells infected with 22L scrapie nor on an ovine scrapie cell model (Kocisko et al., 2005).

DMSO

Treatment of human amyloidotic patients with the solvent dimethyl sulphoxide (DMSO) resulted in the urinary output of congo-philic amyloid (Ravid et al., 1977), and there has been a trial of DMSO for use in systemic amyloidosis (Ravid et al., 1982). In experimental prion propagation systems, DMSO treatment decreases the amount of detectable PrPSc in ScN2a culture (Tatzelt et al., 1996) and reduces the infectivity titre of scrapie-infected brain material (Shaked et al., 1999). Following these observations, Shaked and co-workers treated scrapie-infected hamsters with DMSO (Shaked et al., 2003). DMSO was administered ad libitum in drinking water to hamsters inoculated both i.p. and i.c. with scrapie, giving an estimated dose of 2.5 g/kg/day. Administration over the course of disease from around the time of i.c. infection increased incubation time by ~15% and delayed PrPSc accumulation in brain, though PrPSc levels in terminal animals were the same as controls. Shorter periods of administration to i.c.-infected hamsters, during the first 2 weeks of infection or from approximately half way through the (control) incubation period, showed no effect on the time of disease onset. Administration to i.p.-infected hamsters from early stages of infection was not possible due to DMSO toxicity over a long treatment period (treatment for up to ~80 days only is possible).

Dominant-negative inhibition and protein X mimetics

Protein X is a putative yet undefined co-factor for prion conversion (Telling et al., 1995). The proposed protein X binding site on PrP includes residues Q168, Q172, T215 and Q219 (residue numbering and type according to human PrP) (Kaneko et al., 1997b). This site corresponds to the ‘dominant negative’ epitope, a group of polymorphisms conferring resistance to prion infection that occur both in sheep (Bossers et al., 1996) and in the Japanese population (Shibuya et al., 1998). Mutant PrPs containing dominant negative polymorphic residues have been expressed both in cell models (Kaneko et al., 1997b) and in vivo (Perrier et al., 2002) and are unable to support prion propagation in addition to preventing prion propagation from endogenous PrP.

In order to mimic the dominant negative inhibition of prion infection mediated by these residues, Perrier and co-workers performed an in silico selection of compounds to bind the dominant negative epitope/protein X binding site of PrPSc (Perrier et al., 2000). Sixty such compounds were screened using the ScN2a cell culture model of prion propagation for prevention of PrPSc formation. These ‘protein X mimetics’ are effective in preventing PrPSc formation in newly infected cells and also in rendering chronically infects cells free from detectable PrPSc. Most compounds inhibits PrPSc formation with IC50s >20 μM, but one, Cp-60 (see Supplementary Fig. S1) has an IC50 of 18 μM.

In silico docking to PrPSc of an extended collection of pyridine dicarbamitrole compounds based on Cp-60 was investigated (Reddy et al., 2006). The best-fit compounds from the in silico analysis were investigated by surface plasmon resonance for binding to recombinant protein, and in the SMB cell culture model of prion propagation. Compound 1 (Cp-60) did not show inhibition of PrPSc accumulation in SMB cells, but one compound, number 42, showed some inhibition at 24 μM.

A complementary approach to utilizing the dominant negative effect for prion therapeutics involves the exogenous administration of the dominant negative mutant PrPs (Crozet et al., 2004; Kishida et al., 2004). ScN2a cells treated with recombinant PrP containing the Q218K mutation show a decrease in PrPres with an IC50 of 0.2 μM (4.5 μg/ml), whereas treatment with wild-type recombinant PrP does not affect PrPres levels (Kishida et al., 2004). Co-treatment of cells with recPrP-Q218K and quinacrine results in enhanced PrPres inhibition compared with either of the two treatments alone.

In a separate study, the expression of two dominant negative PrP mutants in ScN2a cells from a lenti-viral expression vector has been shown to suppress the formation of endogenous PrPres (Crozet et al., 2001). The dominant negative mutation Q218K shows a stronger inhibitory effect than mutation Q167R. The expression of a lenti-virus product in vivo was also demonstrated using a beta-galactosidase reporter gene in wild-type mice.

Cysteine-protease inhibitors

The selective membrane-permeable cysteine-protease inhibitor E64d [L-trans-epoxysuccinyl-leucylamido (4-guanidino) butane] causes a decrease in PrPSc in infected cells (IC50 0.5 μM), with no effect on PrPSc synthesis, but has no effect on the cell-free conversion reaction (Doh-ura et al., 2000; Barret et al., 2003).
RNA aptamers

Aptamers are DNA or RNA molecules that are selected from random libraries of nucleic acid sequences by their ability to bind to other molecules. They may be selected to bind nucleic acid, proteins or small organic compounds. Nucleic acid-resistant 2'-amino(deoxy)pyrimidine-modified RNA aptamers were selected from a library of 10^13 RNA sequences using human PrP peptide residues 90–129 (Prosko et al., 2002). Of those selected, the aptamer DP7 was investigated in cellular and in *in vitro* models of prion propagation: DP7 binds to recombinant hamster PrP with a dissociation constant of 100 nM, recognizing mouse and human PrP with lower affinity, and it reduces de novo PrP<sup>SC</sup> production in 3F4-ScN2a cells without altering total PrP production (Prosko et al., 2002). The *in vitro* conversion of PrP<sup>C</sup> to PrP<sup>SC</sup> is also blocked by aptamer DP7 (Rhie et al., 2003). It is suggested that the aptamers may exert their anti-prion effects via competition with endogenous PrP-binding GAGs due to some structural similarity with the GAGs.

Statins

The statins lovastatin and squalestatin are inhibitors of two enzymes of the cholesterol synthetic pathway (near the beginning and end of the pathway, respectively), and cause a decrease in cellular levels of cholesterol. Squalestatin was developed as an alternative cholesterol-depleting agent to statins such as lovastatin, which also affect the production of non-sterol products (Baxter et al., 1992). The accumulation of PrP<sup>SC</sup> in infected cell lines is prevented by treatment of the cells with lovastatin (Taraboulos et al., 1995) or squalestatin (Bate et al., 2004b). In both cases, this effect is abrogated by the addition of cholesterol, suggesting an important role for cholesterol-sensitive processes in PrP<sup>SC</sup> formation. A redistribution of PrP<sup>SC</sup> in squalestatin-treated cells (Bate et al., 2004b), and an increase in PrP<sup>SC</sup> levels in lovastatin treated cells (Taraboulos et al., 1995) are observed, and it is postulated that the effect of the statins is mediated by reducing the amount of PrP<sup>SC</sup> available for conversion to PrP<sup>SC</sup> by either inaccessibility on the surface, reduced export to plasma membrane or increased partitioning to internal compartments (Taraboulos et al., 1995; Bate et al., 2004b).

Copper

PrP<sup>SC</sup> binds to divalent metal ions such as copper, zinc and manganese with high affinity (Jackson et al., 2001), and there is much speculation about the role of copper binding both physiologically and pathologically; for recent reviews of the role of the interaction between the prion protein and copper see Millhauser (2004) and Harris (2003).

Chelation of copper was investigated to assess the feasibility of copper-targeted therapy for prion disease. Treatment of scrapie-infected mice with the copper chelator D-penicillamine causes a decrease in Cu<sup>2+</sup> levels in the brain by ~30% and gives a small increase in incubation time after peripheral infection (Sigurdsson et al., 2003a).

Clioquinol is a blood–brain barrier permeable, ion-chelating antibiotic which is also structurally related to a number of the quinacrine analogues investigated *in vivo* (Murakami-Kubo et al., 2004). Clioquinol treatment of hamsters infected with scrapie by i.c. or i.p. routes results in a modest prolongation of incubation time, with marginal statistical significance for the i.p. infected group only (Pollera et al., 2005).

Conversely, the work of Hijazi et al. (2003) reports significant delay in incubation time for animals treated with copper in spite of increased intracellular PrP accumulation in the cerebellum. A decrease in PrPres is also observed on copper treatment of ScN2a cells (Hijazi et al., 2003). Since the binding and internalization of PrPres by uninfected N2a cells is also inhibited by pre-incubation with copper, the authors propose that copper causes internalization of PrP<sup>SC</sup>, thereby removing it from the site of interaction with and subsequent conversion to PrP<sup>SC</sup>.

The presence of copper in the sonication-free variation of the PMCA method inhibits PrPres accumulation, but copper itself has no effect on either of the components alone (Orem et al., 2006).

Antiviral agents

Early studies of prion treatment focused on antiviral compounds. It was from a study of the selection of antiviral compounds that HPA-23 was identified as a useful anti-prion agent (Kimberlin and Walker, 1979) (see section above). Other compounds tested *in vivo* include rifampicin, cytosine arabinoside, adenosine arabinoside, isoprinosine, amantadine, methisazone, phosphonoacetic acid, virazole, methisoprinol, β-propiolactone and the antibiotic thiamphenicol; none shows any significant effect on the incubation time of mice infected with CJD or scrapie prions (Haig and Clark, 1968; Cochran, 1971; Kimberlin and Walker, 1979; Tateishi, 1981; Pocchiari et al., 1987).

Transgenic expression of PrP-Fc<sub>2</sub>

PrP-Fc<sub>2</sub> is a fusion protein consisting of full-length murine PrP fused to the Fcγ tail human IgG<sub>1</sub>, expressed as a disulphide dimer (Meier et al., 2003). Transgenic animals expressing PrP-Fc<sub>2</sub> on a wild-type mouse *Prnp* background show prolonged disease onset following either i.p. or i.c. scrapie infection, with a correlation between incubation time and level of PrP-Fc<sub>2</sub> expression. Expression of PrP-Fc<sub>2</sub> alone on a knockout *Prnp* background does not support prion replication, indicating that PrP-Fc<sub>2</sub> is unable to form a disease-associated isoform, although it does become associated with PrP<sup>SC</sup> *in vivo* in the transgenic mice with wild-type *Prnp* background (Meier et al., 2003). The mechanism of action of PrP-Fc<sub>2</sub> is postulated to be binding
to PrPSc and thereby blocking the recruitment of conversion-competent PrPSc for generation of PrPSc.

**Therapeutic approaches directed at LRP/LR**

The laminin receptor (LRP/LR) has been identified as a putative cell surface ligand for PrP (Gauczynski *et al*., 2001). There are two proposed sites of interaction between LRP/LR and PrP; a direct interaction involving residues 144–179 of PrP, and an indirect interaction via endogenous GAGs and the N-terminus of PrP (Hundt *et al*., 2001). Three different strategies for intervention of prion conversion via LRP/LR have been described; antisense RNA and small interfering RNAs directed to the LRP gene, and anti-LRP antibodies have all been shown to decrease the amount of detectable PrPSc in infected cell cultures (Leucht *et al*., 2003).

**Inhibitors of intracellular signalling pathways**

Various studies of the cellular mechanisms involved in PrPres formation have identified inhibitors of PrPres formation, as detailed below.

A total of 50 prototype inhibitors of specific signalling pathways were screened in ScN2a cells in order to determine whether intracellular signalling events have any effect on PrP conversion (Ertmer *et al*., 2004). Only one of these, the tyrosine kinase inhibitor STI571 (Gleevec), was able to decrease PrPres without affecting PrPSc levels and has been reported in detail (Ertmer *et al*., 2004). STI571 decreases PrPres to sub-detectable levels in three cell-lines (ScN2a, ScGT1 and SMB) in a time and dose-dependent manner. Treatment with a high concentration for 10 days also results in the irreversible clearance of PrPres from infected N2a cells. Metabolic labelling studies indicate that STI571 does not prevent de novo PrPres formation, but enhances the clearance of pre-existing PrPres. This clearance is probably mediated by lysosomal degradation pathways, since the lysosomal inhibitor ammonium chloride abrogates the effect of STI571. Suppression of the tyrosine kinase c-Abl was able to reduce PrPres levels, whereas specific inhibition of two other tyrosine kinases had no effect, suggesting that c-Abl is the target of STI571 inhibition involved in PrPres formation.

Inhibitors of various phospholipases and their downstream effectors were tested in the three scrapie-infected cell lines (ScN2a, ScGT1, SMB) to elucidate intracellular signalling pathways involved in PrPres formation (Bate *et al*., 2004a). Phospholipase A2 (PLA2) inhibitors and glucocorticoids (which act to reduce PLA2 activity) decrease PrPres formation in all three cell lines, whereas inhibitors of phospholipase C and prednisone (an inactive precursor of the active glucocorticoid prednisolone) are not able to reduce cellular PrPres levels. Inhibitors of enzymes which act downstream of PLA2 [cyclo-oxygenase (COX) and lipoxygenase (LOX)], were not able to reduce PrPres levels. Platelet activating factor (PAF) is also produced in response to PLA2 activation and it was found that agonists of PAF caused an increase in cellular levels of both PrPres and PrPSc, whereas antagonists caused a decrease in both PrPSc isoforms. These data indicate that the activation of PLA2 and production of PAF are required for PrPres formation in chronically infected cell lines (Bate *et al*., 2004a).

The p53 inhibitor pifitrin-α (PFT), which reduces β-amyloid induced neurotoxicity in cell culture (Culmsee *et al*., 2001), was applied to prion infection models in cell culture and hamsters (Engelstein *et al*., 2005). Treatment in the ScN2a cell model resulted in a transient dose-dependent decrease in PrPSc. In addition to monitoring PrP levels and pathology in vivo, pifitrin-α activity as an anti-apoptotic agent was established by comparison of caspase-3 levels in treated and control groups. There was no change in incubation time, spongiosis or astroglialis in the treated animals, but reduced caspase-3 immunoreactivity was noted, compared with controls. In contrast to previous findings of the same group (Shaked *et al*., 2003), this study reports that a 7.5% dose of the DMSO carrier alone was ineffective.

Following the observation that supplementation of cell growth media with brain-derived neurotrophic factor (BDNF) or serum can enhance the accumulation of PrPSc in ScGT1 cells, Nordstrom *et al*., 2005 investigated inhibitors of various enzymes in the signalling pathways that are stimulated by BDNF. Inhibitors of mitogen-activated protein kinase kinase 1/2 (MEK1/2) decrease the level of PrPSc in treated cells, whereas inhibitors of PI3 kinase have no effect, suggesting that MEK1/2 is involved with PrPSc formation in ScGT1 cells. Encouragingly, a MEK1/2 inhibitor which is also blood–brain barrier permeable (SL327) is also active in decreasing PrPSc levels after a 17 day treatment period (Nordstrom *et al*., 2005).

**Carnitine**

L-carnitine and acetyl-L-carnitine were investigated in a hamster scrapie model alongside the polycation antibiotic amphoterin B (Pocchiari *et al*., 1987), but were not effective. Subsequent to its trial in scrapie infected hamsters (for which no rationale was given), carnitine and other molecules which may have beneficial effects on cellular metabolism have been investigated for use with various neurodegenerative diseases, as reviewed by Tarnopolsky and Beal (2001).

**Neuroprotective therapy and non-specific enhancement of scrapie infectivity**

See Supplementary material.

**Discussion**

Here we present the first comprehensive collection of all published data on therapeutic approaches to the treatment
of prion diseases in experimental models of prion propagation. According to systematic review protocol, we include available negative results which are likely to represent only a fraction of the total negative data since such results often remain unpublished, but which are nevertheless crucial to our developing understanding of prion biology and therapeutics. This review thus also charts the changing treatment rationale as insight into the mechanisms of prion disease evolves. The collation of data from the 1960s through to 2005 provides a key resource which allows some qualitative analysis of the data despite the fact that a formal meta-analysis is not possible because of disparities in both methodology and readout.

In summary, the data presented here of experimental treatments of prion disease indicate that extrapolation of results from in vitro to in vivo systems is unpredictable; there are many promising therapeutic agents identified using cell culture models of disease which have proved disappointing when subsequently tested in vivo. Many of the agents tested in vivo have limited efficacy, producing at best small increases in incubation time, but not affecting the clinical phase of disease or overall survival. A few agents or therapeutic strategies do significantly decrease the clinical attack rate of prion disease, including some in which all treated animals remain healthy throughout their expected normal lifespan (Heppner et al., 2001; Sethi et al., 2002; Mabbott et al., 2003; Mallucci et al., 2003; Meier et al., 2003; White et al., 2003). As transgenic approaches are not directly applicable to the treatment of humans, perhaps the most promising of these for treating human prion disease is passive immunization with anti-PrP antibodies, which has been shown to prevent progression of peripheral prion infection to neurological disease in mice, if the immunization is performed in the early stages of infection (White et al., 2003). Many reports indicate that for effective treatment, therapeutic intervention is necessary in the early stages of experimental prion infection. In practice the use of such post-exposure prophylaxis for treating human prion disease is limited by the fact that it is generally not possible to identify affected individuals, apart from specific cases of iatrogenic prion contamination and asymptomatic carriers of pathogenic PRNP mutations. Advances in diagnostics, specifically the identification of pre- or subclinical disease cases, would allow therapies such as humanized versions of the effective antibodies identified in vivo to be targeted to at-risk groups with known significant prion exposure—e.g. recipients of vCJD prion-contaminated blood transfusions.

To date, the only approach which is effective for the prevention of symptomatic neurological disease in animals with established prion neuropathology is the knockout of neuronal PrPSc in transgenic animals (Mallucci et al., 2003). As described above (‘transgenic knockout of neuronal PrPSc in adult mice’), this model shows that knockout of neuronal PrPSc approximately half way through the control incubation period (a point at which neuropathological changes are seen) completely prevents the development of disease in the mice (Mallucci et al., 2003). The targeting of PrPSc for treatment of prion disease has thus been validated by this and other transgenic experiments which indicate that the disease can be prevented by ablation or knockdown of the substrate for prion conversion (Büeler et al., 1993; Sailer et al., 1994; Mallucci et al., 2003). Although transgenic techniques to ablate the prion gene have no direct clinical application at present, RNA interference offers a corresponding strategy for PrPSc knockdown in vivo (Mallucci and Collinge, 2005).

In humans, prion diseases are generally rapidly progressive terminal diseases which have a high profile in spite of their low incidence. Reports of putative prion treatments in experimental models, such as those reviewed here, are frequently followed by optimistic media coverage. This understandably leads to patient requests for immediate access to highly experimental treatments and unrealistic expectations of efficacy. In such circumstances, experimental treatments are offered on compassionate grounds despite limited understanding of how therapeutic testing in animals can be projected to humans, and the inability to predict in vivo efficacy from in vitro data. Among such treatments given to individual or small numbers of patients with prion disease are quinacrine and PPS. A systematic review of attempts at human prion disease therapy, performed in parallel with this review, gives details of these and other treatments (Stewart LA, Rydzewska LHMT, Keogh GF, Knight RSG, submitted for publication). Controlled clinical trials are urgently needed to properly assess the effects of these and future potential treatments. Such trials for prion diseases are challenging for a number of reasons (for a review see Mallucci and Collinge, 2005). In the UK, at the request of the Government’s Chief Medical Officer, a clinical trial protocol (http://www.controlled-trials.com/isrctn/trial/PRION/0/06722585.html) and infrastructure have been developed in consultation with patients’ representatives (http://www.mrc.ac.uk/prn/pdf-cjd_workshop.pdf) to assess the drug quinacrine and to provide a framework for assessment of novel therapeutics as these become available. Clearly, advances in early diagnosis of prion infection are urgently needed to allow any experimental therapy to be tested before extensive neuronal loss has occurred.

Systematic reviews and meta-analyses, such as those published by the Cochrane Library, are routinely used to assess clinical treatment trials but are currently underutilized in assessment of preclinical studies, despite urgent need for such assessment given limited understanding of how therapeutic testing in animals can be projected to humans. Standardization of methods for conducting and reporting experimental preclinical trials would facilitate formal statistical analysis of the data and an understanding of the significance of the results, in addition to informing the process of prediction between experimental models and from models to patients.
Supplementary material

Supplementary material is available online at http://prion.mrc.ac.uk.

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