Discriminating between cellular and misfolded prion protein by using affinity to 9-aminoacridine compounds

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Quinacrine and related 9-aminoacridine compounds are effective in eliminating the alternatively folded prion protein, termed PrPSc, from scrapie-infected cultured cells. Clinical evaluations of quinacrine for the treatment of human prion diseases are progressing in the absence of a clear understanding of the molecular mechanism by which prion replication is blocked. Here, insight into the mode of action of 9-aminoacridine compounds was sought by using a chemical proteomics approach to target identification. Cellular macromolecules that bind 9-aminoacridine ligands were affinity-purified from tissue lysates by using a 9-aminoacridine-functionalized solid-phase matrix. Although the 9-aminoacridine matrix was conformationally selective for PrPSc, it was inefficient: approximately 5% of PrPSc was bound under conditions that did not support binding of the cellular isoform, PrPC. Our findings suggest that 9-aminoacridine compounds may reduce the PrPSc burden either by occluding epitopes necessary for templating on the surface of PrPSc or by altering the stability of PrPSc oligomers, where a one-to-one stoichiometry is not necessary.

INTRODUCTION

Folding of the cellular prion protein (PrPC) into an alternative conformation (PrPSc) is the central event underlying the prion diseases, a group of uniformly fatal illnesses that affect humans and animals (Prusiner, 2004). Whilst PrPC is soluble, largely α-helical and monomeric, PrPSc is insoluble, β-rich and oligomeric. Prion diseases can arise through (i) transmission of infectious PrPSc, (ii) inheritance of some mutations in the prion protein gene (Prnp) or (iii) sporadic, spontaneous molecular events. Drug development for prion disease has been hampered by a lack of structural resolution of PrPSc and an understanding of the cellular mechanisms involved in the misfolding pathway. However, drug-discovery efforts have moved forward by using biochemical, cellular and animal models of prion replication. Several classes of compound have been identified that reduce the level of PrPSc in cultured cells (Trevitt & Collinge, 2006). The majority of prototypic anti-prion compounds described to date have come from phenotypic-screening efforts and, in some cases, the mode of action of these compounds is poorly understood.

Given the current understanding of the mechanisms involved in prion replication, several approaches to intervention could be imagined and exploited for drug discovery (Fig. 1). Strategies include identifying compounds that: (1.) increase the stability of PrPC or reduce the availability of the substrate, either at the level of PrP gene expression or through changes in cellular localization and/or trafficking; (2.) reduce the availability or involvement of cellular factors that chaperone conversion of PrPC; (3.) act on PrPSc to occlude available sites for templating PrPC; (4.) stabilize higher-order PrPSc oligomers and thus inhibit the necessary generation of lower-order oligomeric templates; and (5.) accelerate PrPSc clearance by decreasing the stability of the infectious isoform or stimulating natural clearance pathways.

The 9-aminoacridine compound quinacrine (Fig. 2a) was described previously as being effective at reducing PrPSc in a cell-based model of prion replication. The concentration
of quinacrine required to reduce the amount of PrP<sup>Sc</sup> by 50 % (EC<sub>50</sub>), relative to untreated control ScN2a cells, was 300 nM (Doh-Ura et al., 2000). Structure–activity relationship (SAR) studies helped to define quinacrine as a prototype for a family of 9-aminoacridine anti-prion compounds (Korth et al., 2001; Ryou et al., 2003; May et al., 2006a). Subsequently, dimeric bis-acridine compounds (Fig. 2a) were identified as a potent class of 9-aminoacridine compound (May et al., 2003). The protective effect of quinacrine administration to animals inoculated experimentally with prions has yet to be clearly demonstrated (Collins et al., 2002; Barret et al., 2003; Doh-Ura et al., 2004; Ryou et al., 2004). The clinical use of quinacrine for over half a century for the treatment of malaria and giardiasis prompted an examination of its effectiveness in the treatment of human prion disease.

As part of our efforts to improve the efficacy of 9-aminoacridine-based therapeutics against prion disease, we sought to understand the phenotypic changes that prion-infected cultured cells undergo upon exposure to quinacrine and related 9-aminoacridine compounds. We have aimed to identify 9-aminoacridine receptors that might be involved in prion replication by using a chemical proteomics approach. To identify key interacting partners, we modified a solid-phase matrix with a 9-aminoacridine ligand, then selectively precipitated proteins from cell extracts. We found that a 9-aminoacridine-functionalized matrix selectively precipitates PrP<sup>Sc</sup>, but not PrP<sup>C</sup>. PrP<sup>Sc</sup> could be captured from lysates prepared from scrapie-infected neuroblastoma cells (ScN2a) and brain homogenates from prion-infected animals, including wild-type (wt) and transgenic (Tg) mice and Syrian hamsters. Our findings suggest a possible mechanism by which 9-aminoacridine compounds might reduce PrP<sup>Sc</sup> levels.

**METHODS**

**Materials and reagents.** All synthetic starting materials, reagents and solvents were obtained from Sigma-Aldrich in the highest purity and used without further purification. Proton nuclear magnetic resonance (<sup>1</sup>H NMR) was recorded on a Varian 400 MHz spectrometer in deuterated DMSO (DMSO-d<sub>6</sub>). Liquid chromatography–mass spectrometry (LCMS) was conducted on a Waters Micromass ZQ in ESI<sup>+</sup> mode, equipped with a Waters 2487 dual-wavelength absorbance detector and Waters Alliance 2695 separations module, eluting through an analytical Xterra C-18 column. A<sub>405</sub> was determined by using a SpectraMax Plus microplate reader running SoftMaxPro. Epoxy-activated Sepharose 6B and aprotinin were obtained from Amersham Biosciences. Sodium phosphotungstic acid (PTA), ethanalamine, PMSF, EDTA, sodium deoxycholate, sodium azide, BSA and Nonidet P-40 were obtained from Sigma-Aldrich. Minimal essential medium (MEM) with Earle’s salts, PBS without Ca<sup>2+</sup> and Mg<sup>2+</sup>, trypsin/EDTA and GlutaMAX were purchased from Gibco. Fetal bovine serum (FBS) was obtained from HyClone and proteinase K (PK) from Invitrogen. Leupeptin was obtained from Roche Applied Science. Anti-PrP Fab antibody D13 (Williamson et al., 1998) was
prepared in house and goat anti-human IgG Fab horseradish peroxidase conjugate was purchased from Fisher Scientific.

**Compound synthesis.** The required spermidine-linked bis-acridine compound 1,4-butanediamine-N-(6-chloro-2-methoxy-9-acridinyl)-N’-[3-{[(6-chloro-2-methoxy-9-acridinyl)amino][propyl]-(9-chloro)-propyl}-(9-chloro)-dichloro-3-methoxyacridine (Girault et al., 2000). The purified compound was characterized by ¹H NMR and LCMS prior to use.

**Affinity-matrix synthesis.** Freeze-dried, epoxy-activated Sepharose 6B beads (100 mg, 10 μmol reactive epoxide) were suspended in deionized H₂O for 30 min to swell the matrix to a bead-bed volume of approximately 350 μl. The resulting slurry was washed for 30 min with deionized H₂O (3 × 200 ml) and filtered. The bis-acridine (40 mg, 6.3 μmol) was dissolved in 50 % N,N-dimethylformamide/0.1 M Na₂CO₃ (40 ml) and then added to the swollen beads (350 μl). Coupling proceeded overnight at 50 °C with vigorous shaking. The bead bed was drained and washed with H₂O (3 × 50 ml) to yield the bis-acridine functionalized bead BA (Fig. 2b) that was stored in 0.1 M NaCl buffer at 4 °C.

To generate the control matrix, Co, (Fig. 2b), epoxy-activated Sepharose 6B beads (100 mg, 10 μmol) were swollen, rinsed, then incubated with 1 M aqueous ethanolamine (10 ml, pH 8.0) for 16 h at 45 °C, washed with H₂O (3 × 50 ml) and stored in 0.1 M NaCl at 4 °C until use.

**Preparation of scrapie-infected cell lysate.** Neuroblastoma (N2a) cells were infected with the Rocky Mountain Laboratory (RML) strain of mouse-adapted scrapie prions and subcloned as described previously (Butler et al., 1988). Both scrapie-infected (ScN2a) and non-infected (N2a) cells were maintained at 37 °C in MEM supplemented with 10 % FBS and 1 % Glutamax. A 100 mM plate of ScN2a or N2a cells was grown to 95 % confluence and 1 ml cold lysis buffer (10 mM Tris/HCl, pH 8.0; 150 mM NaCl; 0.5 % Nonidet P-40; 0.5 % sodium deoxycholate) was added. After 5 min, the nuclear pellet was removed and the cell lysate was transferred. The protein concentration was determined by using a Bio-Rad BCA protein assay kit prior to use. Cell lysates were adjusted to 5 mM dithiothreitol (DTT) and a 1 : 500 dilution of protease inhibitors was added [leupeptin (1 mg ml⁻¹), aprotinin (1 mg ml⁻¹), PMSF (100 mM)]. The final NaCl concentration was adjusted to 1 M by the addition of solid NaCl (50 mg (ml cell lysate)⁻¹).

**Preparation of prion-infected brain homogenate.** Animals (n=4 or more) were inoculated with prions as described by Chandler (1961). Animals were monitored for clinical signs of neurological dysfunction as described previously (Carlson et al., 1986). Terminal animals were sacrificed and whole brains were collected. Brain homogenates (10 %, w/v) were prepared at 4 °C in lysis buffer (10 mM Tris/HCl, pH 8.0; 150 mM NaCl; 0.5 % Nonidet P-40; 0.5 % sodium deoxycholate) in a FastPrep FP120 (BIO 101) tissue disruptor (3 × 45 s). The suspension was clarified by centrifugation at 3000 g for 10 min at 4 °C. The supernatant was removed and adjusted to 5 mM DTT, and a 1 : 500 dilution of protease inhibitors was added [leupeptin (1 mg ml⁻¹), aprotinin (1 mg ml⁻¹), PMSF (100 mM)]. The final NaCl concentration was adjusted to 1 M by the addition of solid NaCl (50 mg (ml brain homog enate)⁻¹).

**Preparation of PTA-precipitated Syrian hamster brain homogenate.** wt Syrian hamsters were inoculated with Sc237 hamster prions and sacrificed at the onset of disease. Whole brains were collected (approx. 1 g) and homogenized in PBS (9 ml) in a FastPrep FP120 (BIO 101) tissue disruptor (3 × 45 s) to give 10 % (w/v) brain homogenates. The sample was diluted with 4 % Sarkosyl in 10 ml PBS to give a 5 % (w/v) brain homogenate and then treated with 25 mg PK ml⁻¹ at 37 °C for 1 h with shaking (350 r.p.m.). PK was inhibited by the addition of 100 mM PMSF (1 : 500 dilution). An aliquot (1.8 ml) of the PK-digested sample was transferred and clarified (500 g, 5 min, room temperature). The supernatant (1.2 ml) was transferred and sodium PTA (38 ml of a 10 % aqueous PTA solution, pH 7.4; 85 mM MgCl₂) was added. The sample was incubated at 37 °C for 16 h with shaking (350 r.p.m.). PK-digested PrpK⁺ was pelleted from the sample (14 000 g, 30 min, room temperature), the supernatant was discarded and the pellet was resuspended in 0.2 % Sarkosyl in PBS (40 ml). Several aliquots were prepared and pooled prior to dialysis to provide sufficient material (approx. 400 μl). The samples were dialysed against 500 ml dialysis buffer (10 mM sodium EDTA, pH 7.4; 0.1 % Sarkosyl; 1 mM NaCl) for 48 h, with a change of dialysis buffer after 24 h. Dialysis cartriides (Pierce) with a 3500 Da molecular mass cut-off were used. Following dialysis, the sample was diluted to 1 ml with 0.2 % Sarkosyl in PBS. Protease inhibitors were added [1 μl; 500, leupeptin (1 mg ml⁻¹), aprotinin (1 mg ml⁻¹), PMSF (100 mM)] and the final NaCl concentration was adjusted to 1 M by the addition of solid NaCl.
Affinity-purification assay. In a typical experiment, ligated matrices (20 μl bead-bed volume) were incubated with either cell lysate or 10% brain homogenate (1 ml) and mixed at 4 °C for 16 h. The sample was transferred onto a mini-spin column (Pierce Handee Mini-Spin Column) and beads were collected by centrifugation. The beads were washed successively with lysis buffer (1 × 400 μl), 1 M aqueous NaCl (3 × 400 μl) and lysis buffer (1 × 400 μl). Washing was aided by the use of a mini-spin column, whereby wash buffer was added, the sample was vortexed for 5 s, and then beads were collected by centrifugation.

Immunoblotting. To the matrices (BA or Co, 20 μl), 20 μl SDS loading buffer (0.125 M Tris/HCl, pH 6.8; 10% 2-mercaptoethanol; 20% glycerol; 4% SDS) was added and the mixture was heated at 100 °C for 5 min. Matrix beads and loading buffer were loaded on a precast Bio-Rad Criterion gel (12.5% acrylamide) and proteins were resolved and transferred to an Immobilon-P membrane (Millipore) as described previously (May et al., 2003). Proteins were visualized by using the recombinant Fab fragments D13, secondary horseradish peroxidase-labeled antibody and an enhanced chemiluminescence (ECL) development system (Amersham Biosciences). Densitometry was performed with the National Institutes of Health ImageJ software, computing at least three independent experiments. Total proteins were visualized by silver staining the intact gel using a Bio-Rad Silver Stain Plus kit according to the manufacturer’s instructions.

RESULTS

9-Aminoacridine compounds bind misfolded PrP from ScN2a cells

An acridine-functionalized matrix, BA (Fig. 2b), was prepared by reacting a spermidine-linked bis-acridine compound with epoxy-activated Sepharose 6B solid-phase beads under basic conditions. The bis-acridine parent compound, 1,4-butanediamine-3-[6-chloro-2-methoxy-9-acridinyl]-N,N’-[3-[[6-chloro-2-methoxy-9-acridinyl]amino]propyl]-9-(chloro), was bioactive at nanomolar concentrations in the ScN2a cell model of prion replication (May et al., 2003). A second matrix was prepared similarly by reacting ethanolamine with epoxide-activated Sepharose 6B beads to yield a ‘non-reactive’ control matrix, Co (Fig. 2b).

Cell lysates were prepared from ScN2a cells and incubated with either the BA or Co matrix. Matrices were washed extensively and captured proteins were resolved by gel electrophoresis. Probing a Western blot of proteins bound by the BA matrix with the anti-prion antibody D13 (Williamson et al., 1998) revealed PrP-immunoreactive proteins of the appropriate molecular mass (27–30 kDa) (Fig. 2c, lane 3). The Co matrix failed to bind PrP-immunoreactive material under the conditions employed (Fig. 2c, lane 2). Whilst protease resistance is not required for prion disease (Nazor et al., 2005), it is a hallmark of the infectious isoform, PrPSc, derived from ScN2a cells (Butler et al., 1988). Limited PK digestion of an ScN2a lysate truncates PrPSc to yield a protease-resistant core, denoted PrP 27–30 (Oesch et al., 1985). Proteins bound by the 9-aminoacridine ligands were digested with PK under standard conditions while remaining bound to the BA matrix. Following the ‘on-bead’ PK digestion and extensive washing of the BA matrix, PK-resistant PrP 27–30 was detected (Fig. 2c, lane 6). To investigate the isoform selectivity of 9-aminoacridine binding, BA and Co matrices were incubated with a cell lysate derived from non-infected neuroblastoma cells (N2a). PrPSc was not detected bound to the BA matrix (Fig. 2c, lane 9), even when a large excess of total cellular proteins was used (Fig. 2c, lane 10).

We correlated the bioactivity of various structurally related compounds in ScN2a cells with the efficiency of affinity purification of PrPSc using these same compounds. Quinacrine and the related compounds azacrine, chlorpromazine and chloroquine were assayed for bioactivity in ScN2a cells by using a procedure established by one of us (May et al., 2006b). The bioactivity of the 9-aminoacridine compound quinacrine (EC50 = 0.9 ± 0.1 μM) was approximately four- to eightfold higher than that of the structurally related compounds (EC50 = 3.5–7.8 μM) (see Supplementary Table S1, available in JGV Online). Separately, we prepared Sepharose matrices coupled to synthetic analogues of the parent compounds quinacrine, azacrine, chlorpromazine and chloroquine (see Supplementary Scheme S1, available in JGV Online). The matrices (QC, AC, CP and CQ; Supplementary Table S1) were incubated with ScN2a lysates, and bound PrPSc was quantified by Western blotting and densitometry. The relative amount of PrPSc captured by the quinacrine-ligated matrix was approximately fivefold greater than for the other ligated matrices (see Supplementary Table S1, available in JGV Online). Thus, the affinity of the various related heterocyclic ligands for PrPSc correlates well with bioactivity in ScN2a cells. If the mechanism of action of this class of related compounds is via binding to PrPSc, it would be expected that ScN2a cell bioactivity and ligand affinity for PrPSc would correlate.

9-Aminoacridine compounds bind misfolded PrP from prion-diseased animals

We sought to determine whether the acridine-functionalized matrix could selectively bind PrPSc from prion-infected animals. wt CD1 mice were inoculated with the mouse-adapted prion strain RML and sacrificed at the onset of prion disease. Brain homogenates were prepared from these ill animals (Fig. 3a, b, lanes 4–6), whilst brain homogenates from uninoculated animals served as controls (Fig. 3a, b, lanes 1–3). Both Co and BA matrices failed to precipitate PrPSc from non-infected animals (Fig. 3a, lanes 2 and 3). In contrast, the BA matrix precipitated PrPSc from prion-infected brain homogenates at approximately fourfold the level found in the Co matrix (Fig. 3a, lane 5), as determined by densitometry. Silver staining of SDS-PAGE-resolved proteins revealed that the BA matrix also enriches for other cellular proteins compared with the Co matrix (Fig. 3b, compare lanes 2 and 3), demonstrating that 9-aminoacridine binding is not wholly selective for PrPSc. The Co matrix was essentially free of non-specifically bound proteins, as judged by silver stain (Fig. 3b, lanes 2 and 5).
The apparent selectivity of the BA matrix for PrP<sub>Sc</sub> was verified further by using a brain homogenate prepared from a Tg mouse overexpressing PrP<sub>C</sub>. The Tg line Tg4053 carries more than 30 copies of the Prnp gene, resulting in approximately eightfold overexpression of PrP<sub>C</sub> relative to that in wt mice (Carlson et al., 1994). Affinity purification of a Tg4053 homogenate using either the Co or the BA matrix did not isolate substantial amounts of PrP<sub>C</sub> (Fig. 4a, lanes 2 and 3, respectively), even in the presence of a large excess of PrP<sub>C</sub> (approx. 900 µg PrP<sub>C</sub> per 20 µl BA matrix; data not shown) derived from the Tg overexpression.

We investigated whether the selectivity of the BA matrix was limited to a particular PrP sequence or prion strain. We prepared brain homogenates from two lines of Tg mice expressing truncated PrP<sub>C</sub> [MoPrP(Δ23–88,Δ141–176)] (Supattapone et al., 1999) or MoPrP(Δ23–88) (Supattapone et al., 2001) after RML inoculation (Fig. 4b, lanes 3 and 9). We also prepared brain homogenates from B6.I mice (Carlson et al., 1994) inoculated with mouse-passaged bovine spongiform encephalopathy prions (301V) (Farquhar et al., 1996) (Fig. 4b, lane 6), spontaneously ill Tg mice bearing the Gerstmann–Sträussler–Scheinker mutation [Tg(MoPrP,P101L)2866] (Telling et al., 1996) (Fig. 4b, lane 15) and Syrian hamsters inoculated with Sc237 hamster prions (Marsh & Kimberlin, 1975) (Fig. 4b, lane 12). The BA matrix readily precipitated PrP<sub>Sc</sub> from all samples. The PrP-immunoreactive fraction precipitated from Syrian hamster brain homogenate included higher-molecular-mass and presumably oligomeric PrP<sub>Sc</sub> (Fig. 4b, lane 12).

9-Aminoacridine–PrP binding is unlikely to be mediated by additional proteins or polynucleic acids

We sought to investigate the involvement of other proteins in the observed 9-aminoacridine–PrP<sub>Sc</sub> interaction. If additional proteins mediate the observed acridine–PrP<sub>Sc</sub> binding, then the BA matrix might bind PrP<sub>Sc</sub> from a brain homogenate prepared from a Tg mouse overexpressing PrP<sub>C</sub> (Fig. 4a, lane 3). However, we found that the BA matrix failed to bind PrP<sub>Sc</sub> from a brain homogenate prepared from the Tg4053 mice overexpressing PrP<sub>C</sub> (Fig. 4a, lane 3). This suggests that the observed 9-aminoacridine–PrP<sub>Sc</sub> interaction is not mediated by additional proteins or polynucleic acids.

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**Fig. 3.** Acidine-ligated matrix binds PrP from RML-infected mouse brain homogenate, but not from non-infected mouse brains. (a) Brain homogenates were prepared from RML-infected (lanes 4–6) and non-infected (lanes 1–3) CD1 mice. Control (Co, lanes 2 and 5) and acidine-ligated (BA, lanes 3 and 6) matrices were incubated with brain homogenates. Non-infected CD1 (lane 1) and RML-infected CD1 (lane 4) mouse brain homogenates (BH) were used as controls. (b) An identical gel was prepared and silver stained to reveal total proteins bound to the BA (lanes 3 and 6) and Co (lanes 2 and 5) matrices. Markers are shown in kDa.

**Fig. 4.** 9-Aminoacridine compounds bind misfolded PrP from various prion-diseased animals. (a) The 9-aminoacridine-ligated matrix (BA) (lane 3) fails to affinity-purify PrP<sub>C</sub> from a brain homogenate prepared from the Tg4053 mice overexpressing PrP<sub>C</sub>. A Tg4053 brain homogenate (BH) was used as a control (lanes 1). (b) The bis-acridine-ligated matrix binds PrP<sub>Sc</sub> in brain homogenates from Tg mice expressing either truncated MoPrP(Δ23–88,Δ141–176) (lanes 1–3) or MoPrP(Δ23–88) (lanes 7–9) and inoculated with RML prions; B6.I mice inoculated with 301V prions (lanes 4–6); Syrian hamsters inoculated with Sc237 hamster prions (lanes 10–12); and spontaneously ill Tg(MoPrP,P101L)2866 mice (lanes 13–15). Brain homogenates (BH) from each animal model were used as controls (lanes 1, 4, 7, 10 and 13). Markers are shown in kDa.
binding, it would be expected that protease digestion of a scrapie-infected lysate would result in loss of the observed binding between the 9-aminoacridine matrix and PrP\textsuperscript{Sc}. Thus, a brain homogenate prepared from an ill Syrian hamster was digested with PK under standard conditions and incubated with sodium PTA to selectively precipitate PrP 27–30 (Lee et al., 2005). The sample was dialysed to remove PTA and peptide fragments, and incubated with BA and Co matrices. Only the BA matrix precipitated PrP 27–30 (Fig. 5a, lane 3). This result suggests that additional proteins are not involved in the 9-aminoacridine–PrP\textsuperscript{Sc} interaction. However, we cannot rule out the involvement of low-abundance proteins that are protected from protease digestion by their close association with PrP\textsuperscript{Sc}.

To rule out the involvement of oligonucleotides in 9-aminoacridine–PrP\textsuperscript{Sc} binding, conditions were established whereby nucleic acids were removed from an ScN2a cell lysate by using benzonase digestion. Benzonase characteristically digests DNA and RNA to 5’-monophosphate-terminated oligonucleotides 2–5 bases in length, and effectively removed all high-molecular-mass polynucleotides from an ScN2a lysate (Fig. 5b, compare lanes 2 and 3). Subsequently, benzonase-treated ScN2a cell lysates were incubated with BA and Co matrices. In the absence of polynucleic acids, the BA matrix was able to precipitate PrP (Fig. 5c, lane 5). It was also possible to perform an ‘on-bead’ digestion by treating BA matrix-bound proteins with benzonase at 20 U ml\textsuperscript{-1}. No loss in PrP immunoreactivity was observed by Western blot of the precipitated proteins (Fig. 5c, lane 6).

**DISCUSSION**

A 9-aminoacridine-functionalized solid-phase matrix was synthesized to provide a multivalent reagent for affinity purification of cellular 9-aminoacridine receptors with relevance to prion replication and/or clearance. We targeted a functionalized matrix where an appropriately substituted bis-acridine compound was linked to a Sepharose matrix (Fig. 2b). Given the existing SAR of this class (Korth et al., 2001; May et al., 2003, 2006a), we believed that this attachment strategy would permit binding to one or more cellular 9-aminoacridine receptors and subsequent purification of the receptor(s).

A major hurdle to successful target identification by affinity purification is the need to overcome non-specific binding of cellular macromolecules to the solid-phase matrix (Burdine & Kodadek, 2004). By using the control matrix (Co), we identified conditions that gave negligible non-specific binding of proteins to the Co matrix, as judged by silver staining after SDS-PAGE (Fig. 3b, lanes 2 and 5). The isotonic strengths of the cell lysate and lysis detergent were found to be important determinants of non-specific binding to the Co matrix (see Supplementary Fig. S1, available in JGV Online).

Given the amenability of the ScN2a cell model to the study of prion replication and the fact that 9-aminoacridine compounds are effective at reducing PrP\textsuperscript{Sc} in ScN2a cells, we chose to use this system initially for our mechanistic studies. Additionally, given their central involvement with prion disease, we initially focused on the affinity purification of the PrP isoforms by the BA matrix. Our results suggested that 9-aminoacridine compounds presented on a multivalent scaffold have a higher affinity for PrP\textsuperscript{Sc} than for PrP\textsuperscript{C}. These findings were extended to the affinity purification of PrP\textsuperscript{Sc} from brain homogenates prepared from prion-diseased animals. Affinity purification of PrP\textsuperscript{Sc}

![Figure 5](http://vir.sgmjournals.org)

**Fig. 5.** 9-Aminoacridine binding does not appear to be mediated by additional proteins or polynucleic acids. (a) The acridine matrix (BA) binds PrP 27–30 that was precipitated selectively from prion-inoculated Syrian hamsters following PK digestion by using PTA. Following dialysis of the sample, the proteins were concentrated, resuspended and incubated with the BA and control (Co) matrices. BA binds PrP 27–30 (lane 3), whereas Co fails to bind PrP 27–30 (lane 2). A sample of PK-digested, PTA-precipitated brain homogenate (BH) was used as a control (lane 1). Markers are shown in kDa. (b) Binding to PrP by a 9-aminoacridine-ligated matrix does not require polynucleotides. Conditions were established to remove high-molecular-mass polynucleotides from an ScN2a cell lysate. ScN2a cell lysates were treated with 20 U benzonase ml\textsuperscript{-1} (lane 3) and the nucleic acids were resolved by 1.0% agarose gel electrophoresis. A 1 kb DNA ladder (lane 1) and untreated ScN2a cell lysate (lane 2) were used as controls. (c) ScN2a cell lysates were either untreated (lanes 1–3) or treated (lanes 4–6) with 20 U benzonase ml\textsuperscript{-1} prior to incubation with either the 9-aminoacridine matrix (BA) or the control matrix (Co). ScN2a cell lysate (CL) was used as a control (lane 1). An untreated ScN2a cell lysate was also incubated with the BA matrix, washed, then treated with benzonase (BA*, lane 6). Markers are shown in kDa.
from brain homogenates prepared from Tg(MoPrP,Δ23–88,Δ141–176) and Tg(MoPrP,Δ23–88) mice expressing truncated PrP ruled out the existence of relevant acridine-binding epitopes at the N terminus (residues 23–88) and between residues 141 and 176 (Fig. 4b, lanes 3 and 9).

PrP is known to interact with a number of cellular factors, making it possible for 9-aminoacridine–PrP binding to be either direct (9-aminoacridine–PrP) or indirect (9-aminoacridine–X–PrP), where binding is mediated by a cellular component (X). Identifying additional binding components is of importance, as these molecules would be of therapeutic and biological relevance to prion disease. Macromolecules are known to bind PrP, including neural cell-adhesion molecules, glucosaminoglycans, nucleic acids, plasminogen and the laminin receptor (Lee et al., 2003).

Whilst the role of these molecules in prion replication and disease is not clearly defined, the possibility exists that one or more of these molecules mediates the observed binding interaction between 9-aminoacridine ligands and PrPSc (9-aminoacridine–X–PrPSc). Additionally, 9-aminoacridine compounds are relatively promiscuous ligands. Receptors of quinacrine or related 9-aminoacridine compounds include nicotinic acetylcholine receptor (Spitzmaul et al., 2001), DNA (Gaugain et al., 1981), diamine oxidase (Ma & Sourkes, 1980) and phospholipase A2 (Mustonen et al., 1998). Other proteins, in addition to PrP, were shown to bind the 9-aminoacridine matrix (Fig. 3b). Future studies aim to identify these proteins by using suitable mass-spectroscopy techniques.

To investigate the possible involvement of proteins in the observed 9-aminoacridine–PrPSc binding, we sought to eliminate additional proteins during affinity purification of PrPSc. An ‘on-bead’ digestion of 9-aminoacridine-captured proteins failed to reduce the amount of bound PrPSc significantly (Fig. 2c). However, because it is possible that the solid-phase affinity matrix, or bound PrPSc, could provide protection to coordinated proteins from protease digestion, we attempted to digest a scrapie-infected cell lysate with PK prior to affinity purification. Protease pretreatment of the lysate prior to affinity purification resulted in substantial non-specific binding to the Co matrix, presumably resulting from the presence of low-molecular-mass peptide fragments (data not shown). To circumvent the non-specific binding, we selectively precipitated PrP 27–30 following PK digestion (Fig. 5a).

Binding of the 9-aminoacridine matrix to PrPSc in the absence of additional cellular proteins was not reduced, suggesting that additional proteins did not mediate 9-aminoacridine–PrPSc binding.

Although not required for prion conversion or disease (Safar et al., 2005a), nucleic acids are known to associate with PrP (Deleault et al., 2003). Anti-DNA antibodies have been used to immunoprecipitate PrPSc from prion-infected brain homogenates, presumably as they immunoreact with nucleic acids present in PrP complexes (Zou et al., 2004). The binding of 9-aminoacridine and bis-acridine compounds to polynucleotides is well characterized and responsible for the observed cytotoxicity of certain acridine compounds (Le Pecq et al., 1975). Thus, we sought to address whether the observed binding of the BA matrix to PrPSc was mediated by polynucleotides. As benzonase digestion did not reduce the amount of PrPSc precipitated by the acridine-ligated matrix, we conclude that nucleic acids do not participate in the 9-aminoacridine–PrPSc complex.

PrPs are anchored to the cell membrane by a glycosylphosphatidylinositol (GPI) moiety (Stahl et al., 1987) and reside in caveolae-like domains of lipid rafts (Prusiner, 2004). Quinacrine and related acridine compounds are lipidophilic and are known to associate with lipid membranes (Dise et al., 1982; Pajeva et al., 1996). This complementary lipid binding allows for the possibility that the observed binding of PrPSc to the BA matrix was lipid-mediated. The use of NP-40-based cell lysates reduces the likelihood that intact lipid membranes mediate the observed 9-aminoacridine–PrPSc binding. In addition, the failure of the BA matrix to capture the GPI-anchored protein Thy-1 (Madore et al., 1999) provides further evidence against lipid-mediated 9-aminoacridine–PrPSc binding (see Supplementary Fig. S2, available in JGV Online).

Considerable effort has been devoted to developing diagnostic reagents for the detection of PrPSc isoforms (Castilla et al., 2005; Safar et al., 2005b). These reagents have immediate application in protecting the blood supply from prion contamination and for early diagnosis of prion diseases. Whilst 9-aminoacridine compounds precipitate PrPSc selectively, the low efficiency of this process precludes their current application in prion detection. We found that our current bis-acridine-ligated matrix recovers only approximately 5% of the PrPSc from scrapie-infected cell lysates (Fig. 6). This low recovery of PrPSc by the BA matrix might be attributed either to low PrPSc–BA matrix binding efficiency or to the fact that only a small fraction of the total PrPSc possesses conformations that are compatible with binding to the BA matrix. It is possible that, after further structural optimization of the acridine class or optimization of acridine-ligated matrices, increased binding efficiency may result.

Recognizing the importance of advancing lead compounds as therapeutic candidates, several groups have undertaken studies to deconvolute the mechanism of action of 9-aminoacridine compounds in the context of prion disease. Quinacrine interacts directly with DNA and downregulates expression of certain proteins by blocking transcription activation (Stuhlmeier, 2003). However, 9-aminoacridine compounds do not reduce PrPSc expression levels, thus ruling out PrPSc depletion as a possible mechanism of action (Korth et al., 2001). It has been suggested that quinacrine acts on PrPSc formation by destabilizing cellular lipid rafts (Klingenstaet et al., 2006) that are important for prion conversion (Taraboulos et al., 1995). Others have suggested that 9-aminoacridine compounds may modify lysine residues of PrP, leading to inhibition of prion
PrPSc discrimination using 9-aminoacridine compounds

Determination of the percentage recovery of PrPSc by the acridine-ligated matrix (BA). (a) BA (20 μl) was incubated with 1 ml brain homogenate prepared from RML-inoculated mice at concentrations between 10 and 0.5 % (as indicated in lanes 1–5). Bound PrPSc was visualized with anti-PrP antibody D13. The analysis revealed that binding of 20 μl BA matrix to PrPSc reached saturation at a brain homogenate concentration between 2.5 and 5 %. (b) BA (20 μl) was incubated with a 3.75 % RML-infected brain homogenate (1 ml) and bound PrPSc was visualized by Western blot with anti-PrP antibody D13 (lane 2). A separate reference sample was prepared in which a 3.75 % RML-infected brain homogenate (1 ml) was digested with PK under standard conditions and centrifuged (30 min, 3500 r.p.m.) to precipitate the PK-resistant material. The pellet was resuspended in 1 ml SDS loading buffer and 20 μl of the sample was resolved by gel electrophoresis and Western blotting (lane 1). A 4.8 % recovery was calculated according to the following equation: percentage recovery = [density of signal from BA matrix/density of signal from BH control matrix] × (1000 μl/20 μl)] × 100. Markers are shown in kDa.

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