Charged bipolar suramin derivatives induce aggregation of the prion protein at the cell surface and inhibit PrP^{Sc} replication

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Summary

The conversion of the cellular prion protein (PrP^{c}) into a pathogenic isoform (PrP^{Sc}) is one of the underlying events in the pathogenesis of the fatal transmissible spongiform encephalopathies (TSEs). Numerous compounds have been described to inhibit prion replication and PrP^{Sc} accumulation in cell culture. Among these, the drug suramin induces aggregation and re-targeting of PrP^{c} to endocytic compartments. Plasma membrane and sites of conversion into PrP^{Sc} are thereby bypassed. In the present study, a library of suramin analogues was tested as a potential class of new anti-prion compounds and the molecular mechanisms underlying these effects were analysed. Treatment of prion-infected neuroblastoma cells with compounds containing symmetrical aromatic sulfonic acid substitutions inhibited de novo synthesis of PrP^{Sc} and induced aggregation and reduction of the half-life of PrP^{c} without downregulating PrP^{c} cell surface expression. Half-molecule compounds lacking the symmetrical bipolar structure or the anionic groups had no effect on PrP^{Sc} synthesis or PrP^{c} solubility. Cell surface expression of PrP^{c} was necessary for the activity of effective compounds. Suramin derivatives did not induce aggregation of PrP^{c} when transport along the secretory pathway was compromised, suggesting that their effects occur at a post trans-Golgi network (TGN) site, possibly close to the compartment of conversion into PrP^{Sc}. In vitro studies with recombinant PrP demonstrated that the inhibitory effect correlated with direct binding to PrP and induction of insoluble PrP aggregates. Our data reveal an anti-prion effect that differs from those characterising other sulphated polyanions and is dependent on the presence of the symmetrical anionic structure of these molecules.

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Key words: Prion, PrP^{c}, PrP^{Sc}, Suramin, Bipolar structure, Anti-prion compound

Introduction

For humans and animals, transmissible spongiform encephalopathies (TSEs) represent lethal diseases commonly characterised by massive degeneration of the central nervous system (CNS), neuronal loss, astrocytic proliferation and sometimes the presence of amyloid plaques. Scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) and Creutzfeldt-Jakob disease (CJD) in humans belong to this group of transmissible pathological conditions. The direct link between the BSE epidemic and the appearance of the new variant CJD (vCJD) in Europe caused by dietary exposure (Will et al., 1996; Scott et al., 1999) has heightened the urgency for development of therapeutic and prophylactic approaches against these fatal neurodegenerative disorders. So far, the common infectious agent causing these diseases appears to be a β-sheet-rich and partially protease-resistant protein denoted PrP^{Sc}, derived from post-translational conversion of the α-helical, protease-sensitive cellular prion protein (PrP^{c}) (Cohen et al., 1994; Prusiner et al., 1998; Collinge, 2001; Aguzzi and Polymenidou, 2004; Legname et al., 2004; Castilla et al., 2005). Correctly processed PrP^{c} localises at the outer layer of the plasma membrane via a glycolipid anchor (Borchelt et al., 1990; Taraboulos et al., 1990; Caughey and Raymond, 1991). Conversion of PrP^{c} into the pathogenic prion protein probably occurs after PrP^{c} has reached the plasma membrane or along the endocytic pathway (Taraboulos et al., 1995). Direct interaction of the two PrP isoforms is thought to be required for this conversion (Prusiner and Scott, 1997; Prusiner, 1998; Horiuchi and Caughey, 1999) in a process involving changes in the secondary structure of the protein (Prusiner, 1991; Caughey and Raymond, 1991; Pan et al., 1993).

Numerous experimental approaches have been undertaken for the prophylaxis and therapy of prion diseases (Gilch and Schatzl, 2003; Nunziante et al., 2003). As the nervous system suffers severe damage during the course of TSEs even in the early clinical phase of the disease, it is difficult to imagine that neuronal loss and degeneration can be reversed by pharmacological treatment. Surprisingly, however, it has

recently been shown that depleting endogenous neuronal PrP^c in mice with established neuroinvasive prion infection reversed early spongiform changes and progression to clinical disease (Mallucci et al., 2003). Experimental anti-prion treatments are useful when infection occurs from peripheral sites of the body, when accumulation and replication of the infectious agent are also seen before neuroinvasion, particularly in the lymphoreticular system. Recent data on the role of the immune system in prion diseases (Klein et al., 1997; Klein et al., 2001; Mabbott et al., 2001) have led to promising vaccination strategies against prion infection (Heppner et al., 2001; Peretz et al., 2001; Weissmann et al., 2001; Gilch et al., 2003; White et al., 2003). A different approach is the use of substances that might prolong the incubation time and decelerate the pace of the disease by inhibiting the accumulation of PrP^{Sc} or de novo conversion of PrP^c into its pathogenic isoform. Among these, Congo Red (Caughey and Race, 1992; Caughey et al., 1993), polyene antibiotics (Adjou et al., 1996; Adjou et al., 1999), anthracycline derivatives (Tagliavini et al., 1997), sulphated polyanions (Caughey and Raymond, 1993; Shyng et al., 1995), porphyrins (Priola et al., 2000) and branched polyamines (Supattapone et al., 2001) have shown to prolong the survival time of scrapie-infected animals, to transiently reduce brain infectivity or to inhibit PrP^{Sc} accumulation in cell culture models. The effects exerted by some of these agents result primarily from their ability to interfere with trafficking of PrP^c (Shyng et al., 1995). We recently characterised the effects of the compound suramin on prion biogenesis in cell culture and in vivo models (Gilch et al., 2001; Gilch et al., 2004). This polysulfonated aromatic urea derivative inhibited formation of PrP^{Sc} and delayed the onset of disease in infected animals by inducing misfolding of PrP^c in a post-ER/Golgi compartment and re-targeting the protein to acidic compartments, thereby preventing PrP^c from reaching the cell surface and the cellular compartment(s) of conversion.

The experiments described in the present work were aimed at screening the capacity of a class of suramin derivatives to inhibit PrP^{Sc} accumulation in cell culture. The molecular mechanisms of these agents on the metabolism and cellular trafficking of PrP^c were examined in different cell lines. Of the compounds tested, only those with symmetrical bipolar structure and sulfonic acid groups showed potent inhibition of PrP^{Sc} synthesis. Two half-molecules lacking symmetrical structure had no effect on PrP^{Sc} accumulation, nor had an uncharged compound despite its symmetrical aromatic structure. Effective compounds analysed here induced aggregation of PrP^c and reduced its half-life without affecting PrP^c cell surface expression. Furthermore, cell surface expression of PrP^c was crucial for their activity. These findings outline the fact that suramin analogues interfere with PrP^{Sc} propagation either by a crosslinking mechanism or by inducing misfolding of the protein, thereby deterring the essential substrate for prion propagation.

Materials and Methods

Reagents and antibodies

Suramin (Bayer) was dissolved in NaCl (0.9%) at a stock dilution of 200 mg/ml. Of the suramin analogues, NF449, NF023 and NF007 were purchased from Calbiochem and ANTS from Molecular Probes. All other substances were synthesised in-house and their structures were confirmed by NMR according to methods previously published (Hohenegger and Klinger, 1998; Hulsmann et al., 2003; Kassack et al., 2004). N,N′-bis(3,6-di-tert-butylphenyl)-3,4,9,10-perylendicarboximide (NN) was purchased from Sigma. Stock dilutions of 200 mg/ml were made in H_2O. All compounds were protected from light and kept at 4°C. Cell culture media and trypsin-EDTA were from Invitrogen. Protein A-Sepharose was from Amersham Biosciences. PNGase F and Pefabloc proteinase inhibitor were from Roche Molecular Biochemicals. Sulfo-X-NHS biotin and horseradish peroxidase-conjugated streptavidin were obtained from Pierce and trypsin inhibitor and Brefeldin-A (BFA) from Sigma. FuGene transfection reagent and leupeptin were from Roche Molecular Biochemicals. Monoclonal PrP-specific antibody 3F4 (Signet Pathology) recognises the sequence encompassing amino acids 109 and 112 in hamster and human PrP. Monoclonal mouse anti-PrP antibody 4H11 was generated using a dimeric murine PrP as an immunogen (E. Kremmer, National Research Center for Environment and Health, Munich, Germany, personal communication). Polyclonal anti-PrP specific antibody A7 was obtained in our laboratories after immunisation of rabbits with recombinant dimeric mouse PrP. Monoclonal anti-HA was from Santa Cruz Biotechnology and anti-actin antibodies from Sigma.

Cell culture and expression of PrP constructs

The mouse neuroblastoma cell line N2a (ATCC CCL 131) has been described (Butler et al., 1988; Schatzl et al., 1997). 3F4-N2a and 3F4-ScN2a cells represent stably transfected clones that overexpress 3F4-epitope-tagged murine PrP. 3F4-ScN2a cells were persistently infected with RML prion strain. WtN2a cells represent stably transfected N2a cells overexpressing wild-type murine PrP^c. Cells were maintained in Dulbecco's modified Eagle's (DMEM) or Opti-MEM medium containing 10% foetal calf serum (FCS), penicillin/streptomycin and glutamine in a 5% CO_2 atmosphere. The medium was changed every 48 hours. For stable and transient transfections, PrP constructs were cloned into the pcDNA3.1/Zeo expression vector (Invitrogen). Lipofection of cells with recombinant plasmids was carried out according to manufacturer's directions. For stable transfection, recombinant clones were selected by addition of 300 μg/ml of Zeocin (Invitrogen). N2a cells were lysed 72 hours post transient transfection. Construction of PrP chimeric constructs was done by PCR-based standard techniques using 3F4-tagged mouse PrP as a template. Insertion of appropriate restriction sites allowed cloning of PCR fragments into the multiple cloning site of the vector pcDNA3.1/Zeo using standard cloning techniques. A PrP-CD4 encompassing the C-terminal 62 amino acids of the murine CD4 protein in the vector pSPOX was courteously provided by S. B. Prusiner (Taraboulos et al., 1995). A fragment of this construct encompassing PrP residues 94-231 and the CD4 fragment was subcloned into the vector pcDNA3.1 using *Kpn*I and *Xho*I sites. A PrP fragment (amino acids 1-93) containing a Flag epitope between residues 22 and 23 was inserted into this vector. Thy-1 protein without the N-terminal signal peptide was obtained by RT-PCR using RNA prepared from murine NIH 3T3 cells as a template (amino acids 20-162). This sequence was inserted into the pcDNA 3. 1. vector in which the PrP signal peptide followed by HA epitope had been cloned. For cloning of PrP-LAMP, a PCR fragment encompassing the transmembrane and cytoplasmic region of the murine LAMP-1 protein (amino acids 348-382) was generated from murine genomic DNA and was fused to a truncated 3F4-PrP comprising residues 1-231. All constructs were verified by nucleic acid sequencing.

Immunoblot analysis and detergent solubility assay

Confluent transfected cells were lysed in lysis buffer (100 mM NaCl, 10 mM Tris-HCl pH 7.5, 10 mM EDTA, 0.5% Triton X-100, 0.5% DOC). Post-nuclear cell lysates were supplemented with 0.5 mM Pefabloc protease inhibitor and precipitated with methanol. Samples

were centrifuged for 30 minutes at 2500 ***g*** and the pellets were resuspended in TNE buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA) with gel loading buffer. After boiling for 10 minutes, an aliquot was analysed by 12.5% SDS-PAGE. Proteins were transferred to polyvinylidene difluoride (PVDF) membrane. Membranes were blocked with non-fat dry milk (5%) in TBST (0.05% Tween 20, 100 mM NaCl, 10 mM Tris-HCl pH 7.8), incubated overnight with the appropriate antibody at 4°C, as indicated and stained using enhanced chemiluminescence blotting kit from Amersham Bioscience. For the solubility assay, post-nuclear cell lysates were supplemented with 0.5 mM Pefabloc protease inhibitor and N-lauryl sarcosine to 1%, and centrifuged for 1 hour at 100,000 ***g*** and 4°C in a Beckman TL-100 centrifuge. Soluble fractions (supernatant) were precipitated with methanol. Insoluble fractions (pellet) were resuspended in 50 μl TNE and analysed in immunoblot or RIPA assays.

Proteinase K treatment

Aliquots of post-nuclear lysates were incubated for 30 minutes at 37°C with 20 μg/ml proteinase K (PK); the proteolysis was stopped by addition of protease inhibitor Pefabloc. Samples were precipitated with methanol and analysed by immunoblot assay.

Metabolic labelling and immunoprecipitation

80% confluent 3F4-ScN2a cells were rinsed twice with phosphate-buffered saline (PBS) and starved for 1 hour in RPMI medium without methionine/cysteine containing 1% FCS. Labelling was carried out by adding 400 μCi/ml [^{35}S]Met/Cys (Amersham) to the medium overnight, in the presence of 200 μg/ml of the analysed compound or left untreated as a control. After incubation, cells were rinsed twice with cold PBS and lysed in 1 ml lysis buffer on ice for 10 minutes. Cell debris was removed by centrifugation for 40 seconds at 18,000 ***g***. Postnuclear lysates were then digested with 20 μg/ml PK for 30 minutes at 37°C and digestion was stopped with Pefabloc. Lysates were subjected to solubility assay as described above and pellets were resuspended in 100 μl RIPA buffer (0.5% Triton-X100; 0.5% DOC in PBS) supplemented with 1% SDS before boiling at 95°C for 10 minutes. Samples were placed on ice and Pefabloc added together with 900 μl lysis buffer and N-lauryl sarcosine to 1%. Samples were then incubated with anti-PrP antibody, overnight at 4°C (1:300). Protein A-Sepharose beads were added for 90 minutes at 4°C. Immunoadsorbed proteins were washed in cold RIPA buffer supplemented with 1% SDS at 4°C. All samples were deglycosylated with 0.1 U/μl PNGase F at 37°C overnight and analysed by 12.5% SDS-PAGE. Gels were exposed to an X-ray film (Kodak) or the autoradiographic signals were quantified by Phosphor Imager analysis of the gel (Molecular Dynamics) or ImageQuant TL (Amersham). The amount of total PrP present at each time point after the chase was expressed as a percentage of nascent PrP rescued 1 hour after the labelling period. For evaluation of PrP^{c} half-life, lysates of treated or mock-treated cells were incubated with anti-PrP antibody immediately after lysis (and boiling at 95°C) and processed as described above.

Fluorescence-activated cell sorting (FACS) analysis

For detection of PrP surface expression in compound treated or transfected cells, 4 ml EDTA (1 mM) were applied to detach cells from culture dishes. Cells were centrifuged, resuspended in FACS buffer (2.5% FCS, 0.05% sodium azide in PBS) and incubated on ice. Cells were incubated with primary antibodies in FACS buffer for 45 minutes on ice and washed three times with FACS buffer. Secondary antibodies (FITC-labelled, Dianova) were incubated for 45 minutes. After washing, propidium iodide (2 μg/ml) was added for staining of dead cells. For detection of intracellular PrP, cells were fixed in 10% formalin (37% formaldehyde, Merck) at room temperature and then permeabilised using saponin buffer (0.1% saponin in FACS buffer). Flow cytometry was performed in a FACS Calibur (Becton and Dickinson) cell sorter. As a negative control, cells incubated with the secondary antibody alone were used and the resulting signals were subtracted from the values obtained using both antisera.

Surface biotinylation assay

The effect of suramin or its analogues on the solubility of PrP^{c} localised at the cell surface was assessed by surface biotinylation assay. Briefly, confluent transfected N2a cells were rinsed twice with cold PBS and incubated on ice for 15 minutes with 1 ml PBS containing 250 μg/ml membrane-impermeable sulfo-biotin-X-NHS (Pierce). Cells were washed again three times with cold PBS to remove unbound biotin and culture medium containing 200 μg/ml suramin or one of the analysed analogues was added before incubation at 37°C for 4 hours. Cells were washed with PBS, harvested with lysis buffer on ice for 10 minutes and subjected to solubility assay. Supernatants and pellets were immunoprecipitated as described above, using the indicated antibodies. Immunoadsorbed proteins were subjected to 12.5% SDS-PAGE and transferred to PVDF membranes (Amersham, Pharmacia). Blots were developed with horseradish peroxidase-conjugated streptavidin and visualised using enhanced chemiluminescence.

Confocal laser microscopy

N2a cells transiently transfected with PrP-LAMP or wtN2a cells were rinsed twice in PBS and fixed with 3% paraformaldehyde (PFA) for 30 minutes at room temperature. After sequential treatment for 10 minutes each with 50 mM NH_4Cl/20 mM glycine, 0.3% Triton X-100 and 0.2% gelatine, monoclonal antibody 3F4 or anti-PrP antibody A7 were added at a concentration of 1:100 in PBS for 30 minutes at room temperature. Cells were washed with PBS and incubated with FITC- or Rhodamine-conjugated secondary antisera. Lysosomes were either stained with anti-LAMP monoclonal antibody (BD Pharmingen) or 30 μM acidotropic amine 3-(2,4-dinitroanilino) 3′-amino-*N*-methyl dipropylamine (DAMP) were added to the culture medium for 30 minutes, followed by PBS washing and re-incubation with medium for 30 minutes at 37°C. Staining was completed by adding a FITC-conjugated anti-DAMP antibody (Molecular Probes) at a dilution of 1:50 in PBS. Rabbit anti-Rab6 antibody (Santa Cruz Biotechnology) was used for detection of Golgi compartments. Slides were mounted in anti-fading solution (Histogel) and kept at 4°C. Confocal laser scanning was carried out using a LSM 510 laser-scanning microscope (Zeiss).

In vitro aggregation assay

Purification of solubilised recombinant mouse PrP (23-231) were carried out as described (Proske et al., 2002). Eluted protein was refolded by dialysis against 10 mM sodium acetate buffer, pH 4.5, before raising the pH to 7. The solubility measurements of recombinant PrP were performed as described above in the presence of 10 or 100 μg/ml of the indicated compounds overnight at room temperature. The preparations contained 50 μg/ml rec mouse PrP in 10 mM sodium acetate buffer at pH 7.0.

MTT viability assay

Cytotoxicity to suramin derivatives was determined using the MTT assay (Sigma). 3F4-ScN2a cells were cultured in 96-well microtitre plates for 4 days in the presence of 200 μg/ml of each suramin analogue. Culture medium was changed daily and the compounds applied freshly. Cells incubated for 16 hours with 2 μM staurosporin were used as a positive control. Mock-treated cells were incubated in culture medium in the absence of compounds. Treatment with each

substance was performed in triplicate. After treatment, 20 μl 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide (MTT, 5 mg/ml) was added and the plates were incubated for an additional 3 hours under the same conditions. During this incubation, the yellow MTT tetrazolium salt is reduced to purple-blue formazan crystals by mitochondrial dehydrogenase of viable cells only. Formazan crystals were dissolved by adding 150 μl acidified isopropanol for 2 hours and the optical density, which is linearly related to the number of viable cells, was measured at 570 nm on a spectrophotometer.

Results

Symmetrical bipolar suramin analogues reduce the amount of PrP^{Sc} in prion-infected cells and inhibit de novo prion conversion

Our previously performed studies revealed abrogation of PrP^{Sc} accumulation in different cell lines by suramin. These data led to the analysis of the activity exerted by a larger number of polyanionic compounds with an aromatic structure closely related to suramin (Fig. 1). Compound N,N′-bis(3,6-di-tert-butylphenyl)-3,4,9,10-perylendicarboximide (NN) represents an example of a large uncharged aromatic molecule with a symmetrical structure. Characterisation of the activity of these compounds on PrP^{Sc} was performed in prion-infected neuroblastoma cells overexpressing 3F4-tagged PrP (3F4-ScN2A). Each substance was tested by addition to the culture medium for 3 or 4 days. The concentrations applied were 50 and 200 μg/ml (for each compound). Suramin was used as a control. Cell viability upon incubation with 200 μg/ml substance for 4 days was measured by MTT-assay (supplementary material Fig. S1). Despite the high doses added to the culture medium, only one compound (NF710) exerted strong cytotoxicity. NF307 and NF542 had mild toxic effects on cultured cells (between 50 and 60% viability), although no visible change in cell morphology or growth rate was macroscopically detected (data not shown). The different proteinase K (PK) resistance and solubility in non-ionic detergent of PrP^{c} and PrP^{Sc} allowed separation of the two PrP isoforms by standard biochemical assays. Postnuclear lysates of compound-treated cells were either digested with PK or subjected to ultracentrifugation in the presence of 1% sarcosyl. All samples were analysed by SDS-PAGE followed by immunoblotting. After incubation of cells for 4 days at a concentration of 200 μg/ml, mock-treated control cells contained high amounts of PK-resistant and detergent-

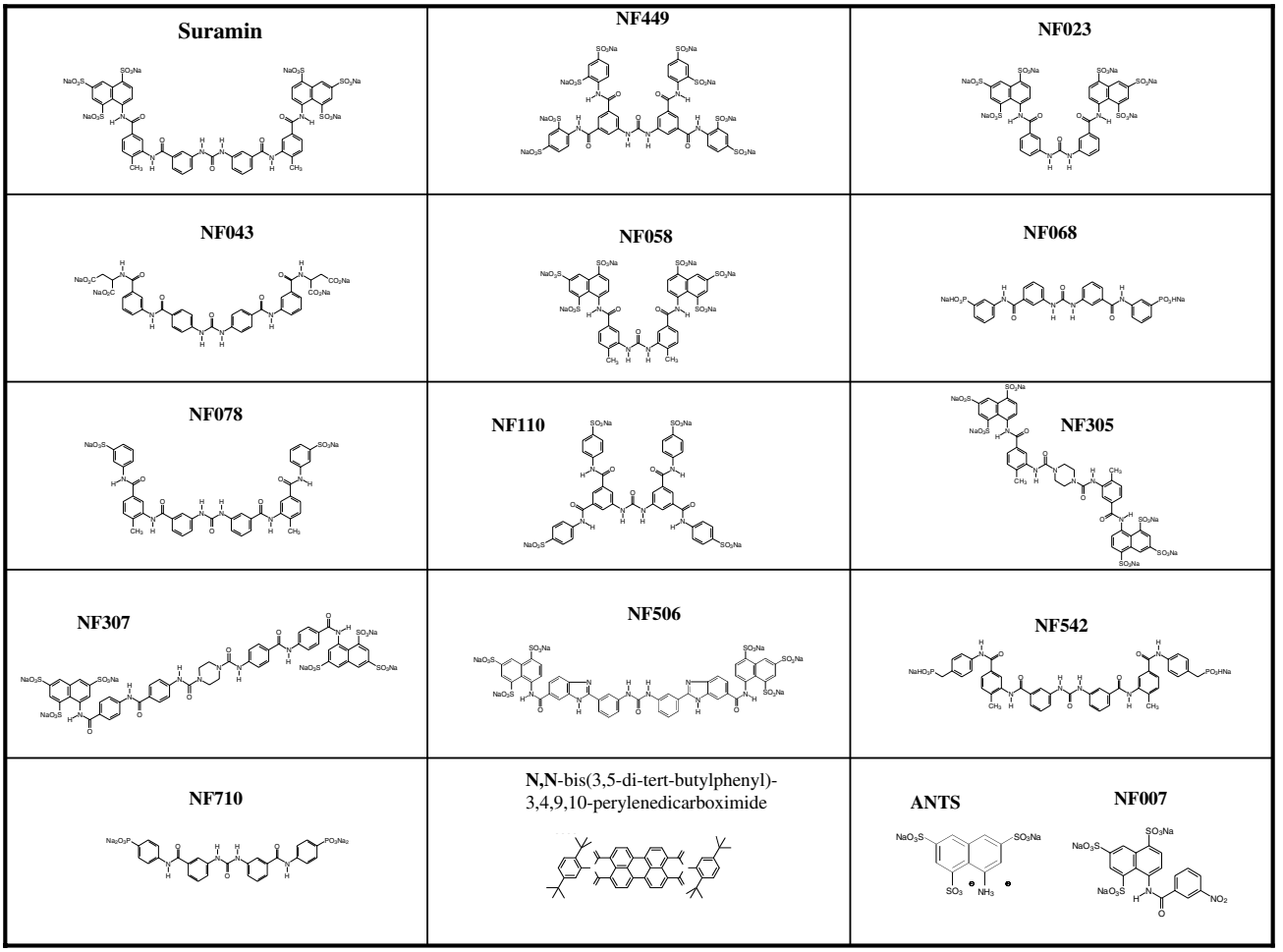

Fig. 1. Chemical structures of suramin and its naphthalene and benzene derivatives with sulfonic, carbonic or phosphonic acid substitutions. Suramin, NF449, NF023, NF058, NF078, NF110, NF305, NF307, NF506 are characterised by symmetrical aromatic structure with sulfonic acid substitutions. Symmetrical compounds NF068, NF542, NF710 present phosphonic and NF043, carbonic acid groups. ANTS and NF007 have sulfonic acid substitutions but lack bipolar structure. NN is an uncharged aromatic compound with symmetrical structure.

insoluble PrP^{Sc} (Fig. 2A,C,D, lanes 1 and 2), as revealed with the monoclonal antibody 4H11. The accumulation of PK-resistant material was completely abolished after treatment with suramin, NF058, NF078, NF110, NF305, NF307, NF506, NF023 and NF449 (Fig. 2A, lanes 4,10,16; Fig. 2B, lanes 1,4,7,10; Fig. 2C, lanes 4,7,10). In prion-infected 3F4-ScN2A cells, insoluble PrP is normally constituted of N-terminally truncated molecules (residues ~90-231) owing to the action of endogenous proteases without addition of exogenous proteases (Fig. 2A, lane 2). In the described experiments, the insoluble moiety found in most drug-treated cells after ultracentrifugation consisted of full-length PrP-molecules (Fig. 2A, lanes 5,14,17; Fig. 2B, lanes 2,5,8,11; Fig. 2C, lanes 8,11). All of the effective compounds are characterised by symmetrical aromatic structure with naphthalenesulfonic or benzenesulfonic acid substitutions. Substances with phosphonic (NF068) or carbonic (NF043) acid substitutions showed no or only mild inhibitory effects on PrP^{Sc} (Fig. 2A, lanes 7,13). No PrP signal was detected in cells incubated with NF710 (Fig. 2B, lanes 16-18) owing to the strong toxicity of the compound (Fig. 2B). Neither of the asymmetric or uncharged drugs (NF007, ANTS and NN) was effective in reducing the amount of PrP^{Sc}, or showed an effect on PrP^{c} solubility (Fig. 2C, lanes 13,14 and 16,17; Fig. 2D, lanes 4,5). 3F4ScN2a cells treated with lower doses (50 or 100 μg/ml) or for shorter time (3 days) still harboured fractions of PK-resistant material (supplementary material Figs S2 and S3). Actin loading controls below each blot show the specificity of the results.

Several mechanisms can account for the anti-prion activity shown by the tested compounds. PrP^{Sc} reduction can be explained by the increased degradation rate of PrP^{Sc} or by interference with its de novo biogenesis. We therefore metabolically labelled prion-infected 3F4-N2A cells for 16 hours in the presence of suramin or its analogues (Fig. 3A shows four representative examples). Cells were subsequently lysed, subjected to PK digestion (20 μg/ml) and ultracentrifuged in the presence of sarcosyl. Precipitation of insoluble material with the anti-PrP polyclonal antibody A7 allowed detection of PrP^{Sc} molecules converted during the labelling time. Deglycosylation of the immunoprecipitated proteins with PNGase F followed by SDS-PAGE revealed a specific signal at 19 kDa in untreated cells, representing PK-resistant, detergent-insoluble PrP^{Sc} (Fig. 3A, lane 1). In prion-

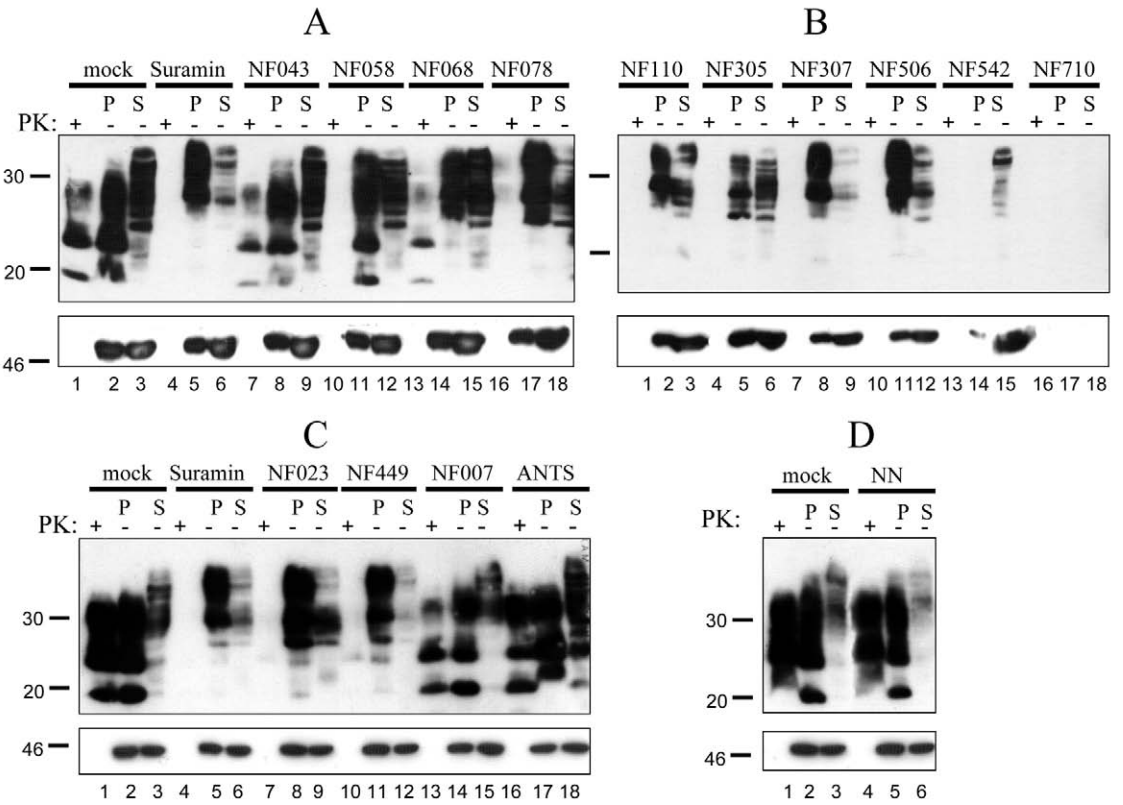

Fig. 2. Suramin and derivatives reduce the amount of PrP^{Sc} in prion-infected cells to undetectable levels and induce insoluble full-length PrP aggregates. (A,B,C) The effect of suramin derivatives on PrP^{Sc} was determined in prion-infected neuroblastoma cells (3F4-ScN2a) using a solubility assay and proteinase K (PK) treatment followed by immunoblot analysis. Cells were seeded on 60 mm plates and treated daily with suramin or with one of its derivatives, as indicated. All compounds were applied at a concentration of 200 μg/ml for 4 days. Mock-treated cells were used as a control. Cells were harvested and postnuclear lysates were either treated with 20 μg/ml PK for 30 minutes at 37°C or subjected to ultracentrifugation at 100,000 ***g*** in the presence of 1% sarcosyl, to separate soluble (S, supernatant) from insoluble (P, pellet) fractions, as indicated above the blots. PrP was visualised by immunoblotting using the monoclonal anti-PrP antibody 4H11. Molecular size markers are depicted on the left. No PK-resistant PrP is detected after treatment of the cells with NF058, NF078 (A, lanes 10,16); NF110, NF305, NF307, NF506 (B, lanes 1,4,7,10); suramin, NF023 or NF449 (C, lanes 4,7,10). NF542 and NF710 were highly cytotoxic. Actin loading controls are shown below each panel.

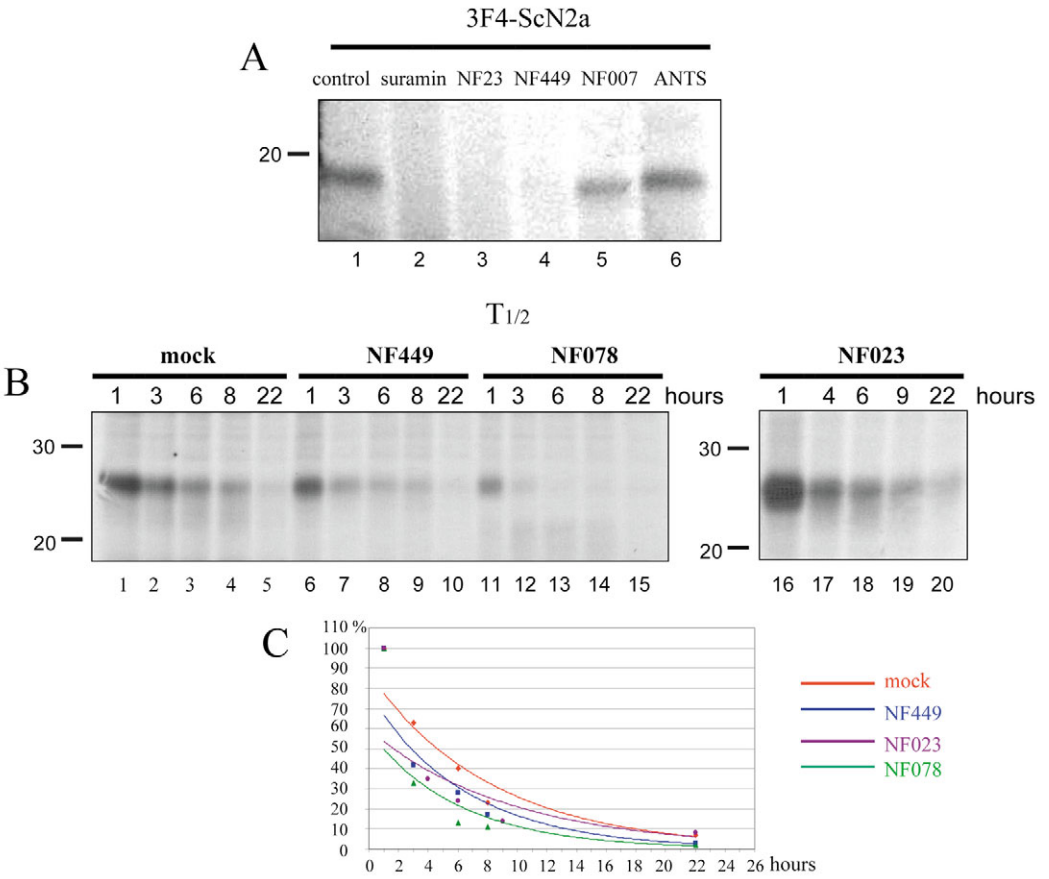

Fig. 3. Suramin derivatives interfere with de novo PrP^{Sc} formation and reduce PrP^{c} half-life. (A) 3F4-ScN2A cells were metabolically labelled with [^{35}S]Met/Cys overnight in the presence of 200 μg/ml suramin or one of its derivatives or without addition of compounds, as a control. Cells were lysed after the pulse and subjected to digestion with 20 μg/ml PK for 30 minutes at 37°C. Lysates were ultracentrifuged in the presence of 1% sarcosyl and the pellets immunoprecipitated with the polyclonal anti-PrP antibody A7. Prion protein was deglycosylated with PNGase F and subjected to SDS-PAGE and autoradiography. De novo formation of PrP^{Sc} is inhibited when the cells are treated with suramin, NF023 or NF449 (lanes 2,3 and 4). NF007 and ANTS show no inhibitory effect on prion conversion (lanes 5 and 6). (B) Confluent wtN2a cells were incubated overnight with 200 μg/ml of the indicated compounds and then metabolically labelled with [^{35}S]Met/Cys for 1 hour (in the presence of suramin derivatives) at 37°C. After the pulse, cells were incubated in culture medium without ^{35}S (with compounds) at 37°C for the indicated chase time points, before harvesting. PrP was precipitated with polyclonal A7 antibody and deglycosylated with PNGase F to facilitate molecular size comparison and quantification. Samples were subjected to SDS-PAGE and autoradiography. Positions of molecular size markers in kDa are shown on the left. (C) Densitometric evaluation of autoradiograms. PrP harvested 1 hour after the radioactive pulse in the presence of suramin derivative (time estimated for proper uptake and activity of compounds) was set as the total PrP population (100 %) (lanes 1,6,11,16 in B; 1 hour chase time). Decrease in protein amounts at following chase points is expressed as a percentage of this total protein and plotted as a function of the chase times. The data points were fitted to an exponential curve using non-linear regression analysis. All the compounds tested reduce the PrP^{c} half-life to different extents.

infected cells treated with suramin, NF023 or NF449, lack of PK-resistant signal (lanes 2,3 and 4) indicates that these compounds abrogate the de novo formation of PrP^{Sc}. Incubation with NF007 and ANTS had no effect on PrP^{Sc} biogenesis (lanes 5 and 6). Taken together, we show that treatment of prion infected cells with symmetrical aromatic suramin derivatives reduces the amount of PrP^{Sc} to undetectable levels in a dose- and time-dependent manner by interfering with its de novo biogenesis.

Treatment with suramin analogues accelerates degradation of PrP^{c} without affecting steady-state cell surface expression of PrP^{c}

To assess whether suramin derivatives also interfere with the metabolism and cellular stability of PrP^{c}, we measured the degradation rate of PrP^{c} in the presence or absence of selected compounds in pulse-chase studies. N2a cells stably overexpressing PrP^{c} were metabolically labelled with [^{35}S]methionine/cysteine and then incubated in ^{35}S-free culture medium in the presence or absence (mock) of 200 μg/ml compound. Cells were harvested 1 hour after the radioactive pulse or chased for up to 22 hours. PrP present in the lysates was immunopurified with the anti-PrP antibody A7, deglycosylated with PNGase F, and analysed by SDS-PAGE (Fig. 3B). The autoradiogram was evaluated by densitometric analysis. PrP harvested 1 hour after the radioactive pulse (time estimated for proper uptake and activity of compounds) was considered as the total PrP population (Fig. 3B, lanes 1,6,11,16) and the specific bands monitored at successive time

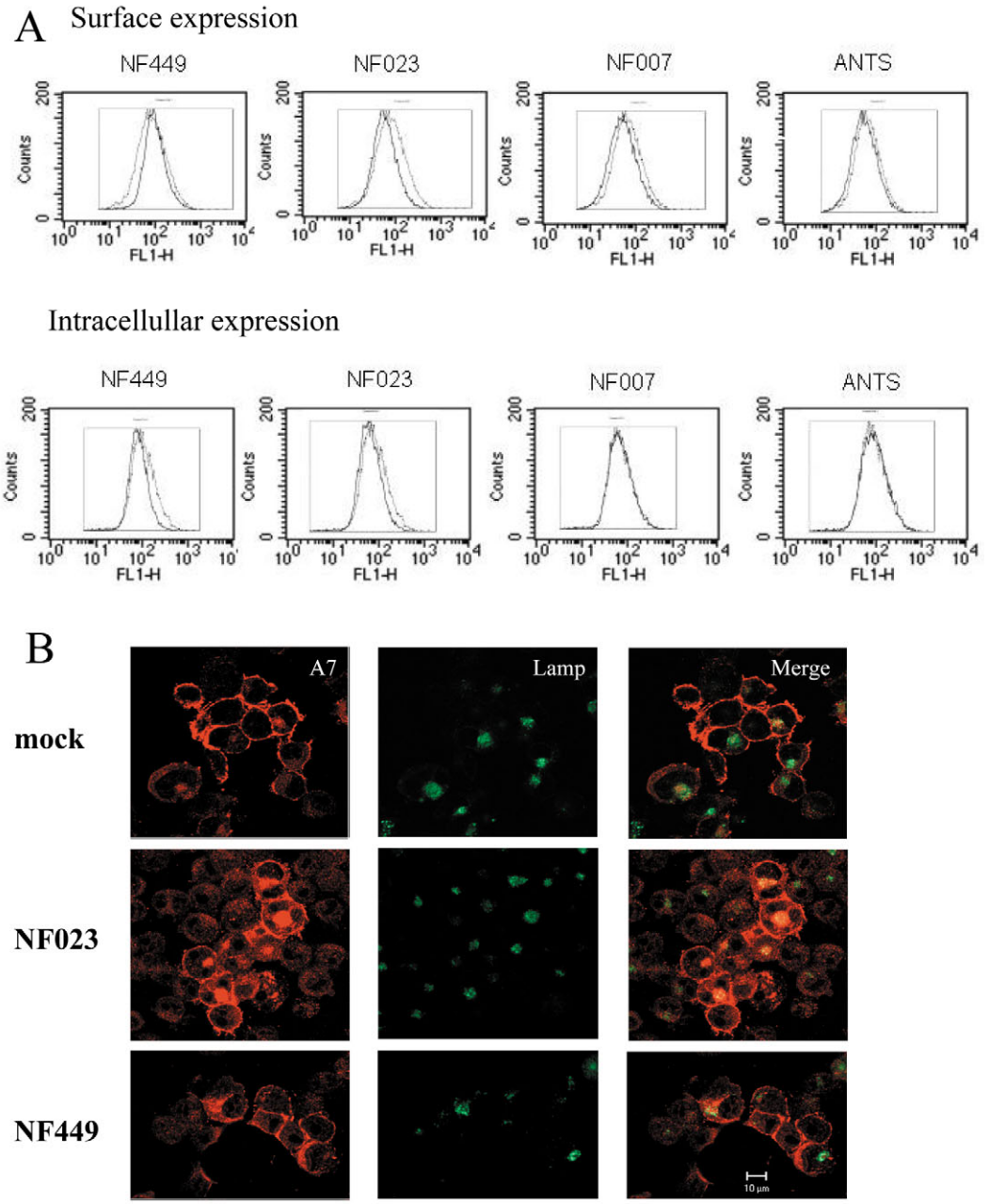

Fig. 4. Treatment with suramin analogues does not downregulate cell surface expression of PrP^c. (A) Expression levels of PrP^c in 3F4-N2a cells treated for 3 days with 200 μg/ml NF023, NF449, NF023 or ANTS was measured by FACS analysis. The histograms depict the cell surface expression in non-permeabilised cells (upper panels) and the intracellular PrP expression in cells permeabilised with saponin buffer. FL1 represents the fluorescence intensity in treated cells (bold line) and in the mock-treated ones (dotted line), plotted against the number of cells (counts). For each cell population 10,000 events were measured. Living and dead cells were separated by staining with propidium iodide and gating. The monoclonal antibody 3F4 was used for detection of PrP^c. No significant change in the intracellular or in the surface expression of PrP^c was detected upon treatment of cells with any of the tested compounds. (B) PrP^c localisation upon exposure to NF023 or NF449 was confirmed by confocal microscopy studies and compared to mock-treated cells in wtN2a cells, stably overexpressing PrP^c. Tested compounds were added daily (48 hour treatment) to the culture medium at a concentration of 200 μg/ml, before fixation and permeabilisation. Staining of PrP and lysosomes was performed using the polyclonal anti-PrP antibody A7 (left-hand column) or a monoclonal antibody directed against the lysosomal protein LAMP-I (middle column), respectively. After treatment with NF023 or NF449, PrP^c could still be detected on the cell surface (middle and lower left panels) at levels comparable to untreated cells (mock). Upon treatment with suramin derivatives, PrP^c also shows increased localisation in intracellular compartments which partially correspond to lysosomes, as seen in an overlay of the two signals (merge). Bar, 10 μm.

points were quantified as fractions of these signals (Fig. 3C). Under these conditions, the half-life of PrP^c in mock-treated cells measured ~4.5 hours. Interestingly, all three compounds tested enhanced degradation of PrP^c compared to mock-treated cells, and the half-lives measured varied between 1.2 and 2.9 hours.

As the results described above show that the compounds analysed in the current study exert inhibitory effect on PrP^{Sc} synthesis, we proceeded in the characterisation of the cellular mechanisms underlying this activity. The cell surface expression of PrP^{c} upon treatment with suramin analogues was investigated by FACS analysis (Fig. 4A shows representative experiments with NF023, NF449, NF007 and ANTS). We detected no significant decrease of the surface localisation of PrP^{c} for any of the compounds tested (Fig. 4A, upper panels). Comparison of the total PrP^{c} amount in treated and mock-treated permeabilised cells did not reveal significant differences in the intracellular expression of PrP^{c} (Fig. 4A, lower panels).

The influence of suramin analogues on the cellular localisation of PrP was also examined by confocal microscopy (Fig. 4B). After a 48 hour treatment of wtN2a cells with NF023 or NF449, the PrP signal at the cell surface (Fig. 4B, middle and lower panels) was still consistent with that detected in untreated cells (Fig. 4B, upper panels). Of note, in a subpopulation of cells, NF023 and NF449 also induced increased partitioning of PrP in intracellular compartments. This intracellular PrP population was mainly present in lysosomes, as shown by co-staining with antibody to the lysosomal membrane protein LAMP-1 (Fig. 4B, right-hand panels). These results show that, in contrast to suramin, the derivatives tested here do not interfere with the cell surface expression of PrP^{c}.

Suramin derivatives induce detergent-insoluble PrP-aggregates

As treatment of prion-infected cells with sulphated suramin derivatives resulted in the formation of detergent-insoluble full-length PrP molecules, we proceeded in the characterisation of this effect on the cellular prion protein in non-infected N2a cells transiently expressing 3F4-tagged wtPrP. When cells were subjected to the described solubility assay, immunoblot analysis of the lysates revealed aggregation of PrP molecules (insoluble pellet) that had been exposed to suramin, NF023, NF078, NF110, NF305 and NF449 (Fig. 5A, lanes 3,5,7,9,11,13). Conversely, PrP^{c} remained soluble and partitioned mainly in the supernatant fraction after incubation with ANTS or NN (lanes 15,21,23), when compared to the control (lane 1,17,19).

For further characterisation of the drug-induced aggregation phenotype, several analogues were tested on a PrP mutant characterised by the substitution of the GPI attachment signal with the transmembrane/cytoplasmic domain of the mouse CD4 molecule. This substitution shifts the raft topology of the prion protein to a clathrin-coated pit trafficking environment (Taraboulos et al., 1995). All of the compounds tested significantly altered the solubility pattern of this molecule in transiently transfected N2a (Fig. 5B, lanes 3,5,7,9,11). Investigation of the specificity of these results led to the performance of the same assay with N2a cells transiently expressing Thy-1, a small cell adhesion GPI-anchored protein of the central nervous system (Morris, 1992; Hollrigel et al., 1998). Of note, PrP and Thy-1 are characterised by the structural diversity of the raft domains they occupy (Madore et al., 1999). Under the conditions used in our study, Thy-1 was soluble in mock-treated cells (Fig. 5C, lane 1) and this biochemical property remained essentially unchanged after treatment with representative suramin derivatives for 3 days (Fig. 5C, lanes 3,5,7,9,11).

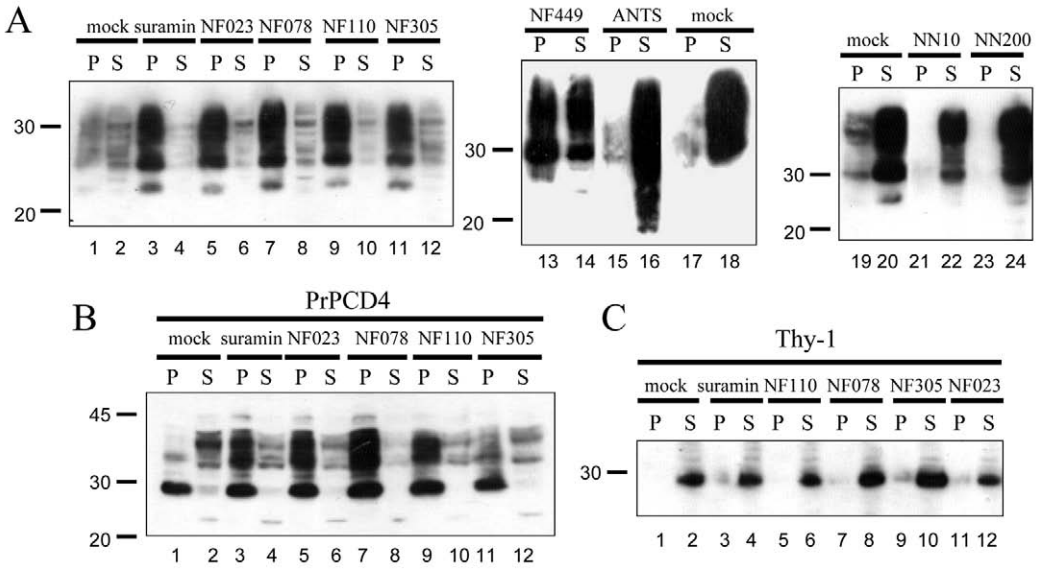

Fig. 5. Effective suramin analogues induce aggregation of PrP^{c} independently of the mode of surface anchorage. (A,B,C) N2a cells were transiently transfected with 3F4-tagged wtPrP, CD4-PrP or Thy-1 and treated with 200 μg/ml of the indicated compounds (10 or 200 μg/ml for NN) for 3 days. Postnuclear lysates were subjected to ultracentrifugation at 100,000 ***g*** in the presence of 1% sarcosyl. Pellets and supernatants were run on SDS-PAGE and analysed by immunoblotting using the monoclonal antibody 3F4 or monoclonal anti-HA antibody to detect Thy-1. With the exception of ANTS and NN, Suramin and all the tested derivatives induce formation of insoluble PrP-aggregates (A, lanes 3,5,7,9,11,13) whereas PrP^{c} remains soluble in cells treated with ANTS (A, lane 16) or NN (A, lanes 22 and 24). CD4-PrP also partitioned in the insoluble fraction upon incubation of cells with suramin derivatives (B, lanes 3, 5, 7, 9, 11), whereas the same treatment had no effect on Thy-1 solubility (C).

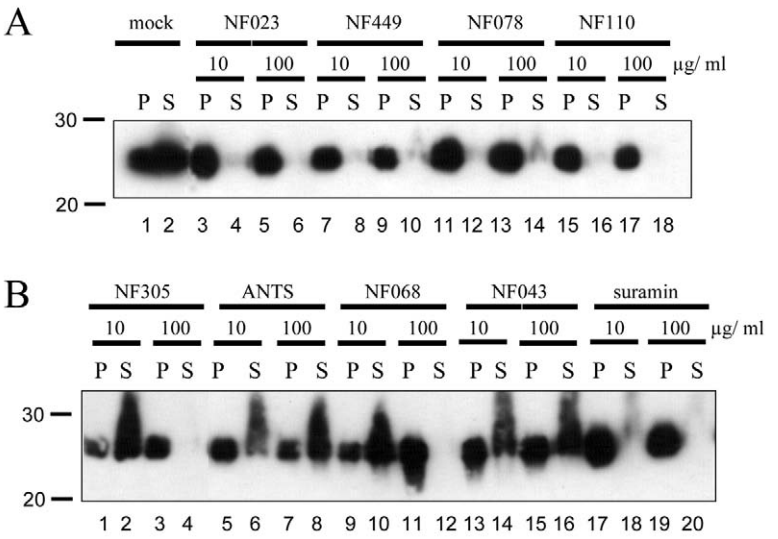

Fig. 6. Suramin analogues induce aggregation of purified recombinant mouse prion protein. (A,B) Recombinant mouse PrP (amino acids 23-231) (50 ng/µl) was incubated overnight with 10 or 100 µg/ml of the suramin analogues indicated in sodium acetate buffer at pH 7. Samples were then subjected to ultracentrifugation in the presence of 1% sarcosyl. Pellets and supernatants were run on SDS-PAGE and analysed by immunoblotting using the mouse monoclonal antibody 4H11. Mock-treated recombinant PrP was detected in the soluble as well as in the insoluble fractions. Upon treatment with NF023, NF449, NF078, NF110, NF305, NF068 and suramin, a clear shift into pellet fractions was detected whereas ANTS (B, lanes 5-8) and NF043 (B, lanes 13-16) did not affect the solubility of PrP.

In summary, the results described show that the compounds tested induce formation of insoluble PrP molecules regardless of the membrane anchoring or of the raft or clathrins-coated pit localisation. As the same compounds do not affect this biochemical property of Thy-1, our results hint at a specificity for certain characteristic features of the prion protein.

Having found that the compounds tested induced aggregation of PrP^{c} (but not of Thy-1) within living cells, we used an in vitro aggregation model to assess the nature of suramin derivative interference with PrP^{c}. Recombinantly expressed and purified mouse PrP was incubated in vitro with 50 or 100 µg/ml of selected compounds overnight (in sodium acetate dialysis buffer, pH 7). The protein was subsequently subjected to ultracentrifugation in detergent. Under these conditions a significant fraction of the untreated PrP partitioned in the insoluble fraction (Fig. 6A, lane 1). Nevertheless, a significant shift into the pellet could be seen upon incubation with even low concentrations of all the substances that had induced insolubility in cell culture (Fig. 6A, lanes 3,5,7,9,11,13,15,17; Fig. 6B, lanes 1,3,17,19). The asymmetric sulfonic drug ANTS and the carbonic one NF043 did not alter the solubility profile of the recombinant PrP (Fig. 6B, lanes 5,7,13,15), again confirming the cell culture data. Of note, high dose of NF068, a substance with phosphonic residues, increased the amount of insoluble protein (Fig. 6B, lane 11). Suramin derivatives therefore can directly bind to PrP^{c} in the absence of the cellular metabolism.

Retention of PrP^{c} in the ER or TGN precludes aggregation by suramin derivatives

We have previously shown that suramin exerts its effect on PrP^{c} mainly in a late-Golgi compartment or in the TGN. To assess whether this mechanism also applies to the derivatives analysed here, 10 µg/ml Brefeldin A (BFA) were added to the culture medium of wtN2a. BFA prevents vesicular and proteins transport from the ER along the secretory pathway (Misumi et al., 1986; Oda et al., 1987; Klausner et al., 1992). After 2 hours, cells were exposed to 200 µg/ml suramin, NF449 or NF023 in the presence of BFA and incubated at 37°C overnight (Fig. 7). Postnuclear lysates of the treated cells were subjected to a solubility assay, as described. Incubation of cells with BFA alone for 16 hours did not change the solubility of PrP^{c}, which partitioned mainly in the supernatant (Fig. 7, lanes 1-4). The size of the heavier glycosylated moieties was reduced, as BFA prevents production of complex N-linked carbohydrates. As previously seen in the absence of BFA, the tested derivatives led to the formation of insoluble PrP aggregates (Fig. 7, lanes 5,9,13). The presence of BFA prevents suramin derivatives from inducing significant amounts of aggregated PrP. (Fig. 7,

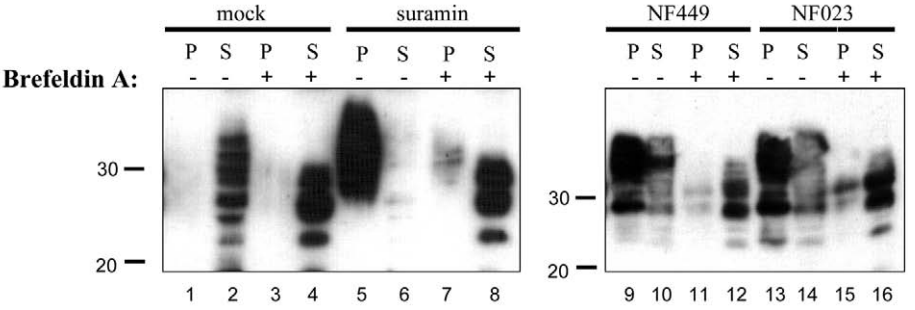

Fig. 7. Treatment with effective compounds does not affect solubility of PrP^{c} when transport of PrP along the secretory pathway is inhibited. 10 µg/ml Brefeldin A (BFA) were added to the culture medium of wtN2a cells. After 2 hours, cells were exposed to 200 µg/ml suramin, NF449 or NF023 in the presence of BFA and incubated at 37°C overnight. Cells were then harvested and subjected to a solubility assay. Soluble (S) and insoluble (P) fractions were analysed by immunoblotting, using the monoclonal anti-PrP antibody 4H11. Positions of molecular size markers are depicted on the left. Treatment of cells with BFA alone did not affect the solubility of PrP^{c} (lane 3). Treatment with BFA in the presence of suramin, NF449 or NF023 significantly decreased the induction of PrP^{c} aggregates (lanes 7,11 and 15).

lanes 7,11,15). The fact that suramin did not cause aggregation of PrP^c when cells were pre-treated with BFA confirms our previous data.

Similar results were obtained upon incubation of wtN2a and N2a cells with derivatives with aluminium fluoride, which, among various trafficking events, inhibits the exit of proteins from the TGN (Kantanen et al., 1995; Sariola et al., 1995; Wolins et al., 1997) (data not shown). These results led to the conclusion that retention along the secretory pathway (in the ER or in the Golgi compartments) makes PrP^c inaccessible by the derivatives tested in this study and suggest that aggregation is induced on the cell surface or along the endocytic pathway.

Suramin derivatives do not affect the solubility of PrP^c in lysosomes and induce aggregation of cell surface PrP

As inhibiting the trafficking of PrP at a pre-Golgi level resulted in a nearly complete abrogation of the activity of the compounds tested, we proceeded in the search for the cellular compartments where suramin derivatives exert their activity. We used a chimeric protein encompassing residues 1-231 of the mouse prion protein expressing the 3F4 epitope (lacking the GPI-attachment signal) fused to the C-terminal transmembrane and cytoplasmic segment of the lysosomal protein LAMP-1. The cytoplasmic tail of this protein comprising 11 residues carries a tyrosine-based sorting motif (-G-Y-X-X-Φ, where Φ represents a hydrophobic residue) required for targeting LAMP-1 molecules from the TGN to lysosomes (Akasaki et al., 1995; Guarnieri et al., 1993; Honing et al., 1996) (supplementary material Fig. S4A). The expression of this chimeric protein was characterised in N2a cells (supplementary material Fig. S4B,C, Fig. S5). Surface biotinylation, flow cytometry and confocal microscopy studies confirmed that fusion of the sorting domain of LAMP-1 to wtPrP lacking the GPI attachment signal abrogated PrP cell surface expression and re-targeted PrP^c to intracellular compartments.

To explore whether suramin derivatives can affect the biochemical properties of PrP-LAMP, N2a cells were transfected with either 3F4-tagged wtPrP or with PrP-LAMP. Cells were treated for 48 hours with 200 μg/ml NF449 or NF023 or were mock treated. Postnuclear lysates were separated into high-speed supernatants and pellets in the presence of 1% sarcosyl and analysed for solubility in an immunoblot. As seen previously, wtPrP partitioned in the pellet fraction upon treatment with NF023 (Fig. 8A, lane 3). Conversely, PrP-LAMP, which was mainly soluble in untreated cells (Fig. 8A, lane 5), was still detected in the supernatant of cells exposed to NF023 or NF449 (Fig. 8A, lanes 7,9). It is of interest that suramin induced the aggregation of PrP-LAMP (Fig. 8A, lane 11), confirming that the intracellular sites of action of suramin are mainly in the TGN and lysosomes. Thus, owing to the fact that NF023 and NF449 did not affect aggregation of intracellular PrP^c in the endosomal/lysosomal pathway, the described results indicate that, in contrast to suramin, these compounds prevent prion propagation by acting mainly on PrP localised at the cell surface.

As the derivatives analysed, although inhibiting prion conversion, did not interfere with the localisation of PrP at the cell surface, we performed a surface biotinylation assay in order to investigate the activity of our compounds on proteins located at the outer layer of the plasma membrane. After surface biotinylation of 3F4-N2a cells, 200 μg/ml suramin, NF449 or NF023, respectively, were added to the culture medium, followed by incubation for 4 hours at 37°C. Mock-treated cells were used as a control. Cells were subsequently lysed, and ultracentrifugation was applied to separate the soluble fraction from the insoluble one. PrP^c was immunoprecipitated using the polyclonal antibody A7. Whereas in the control samples PrP^c was contained almost

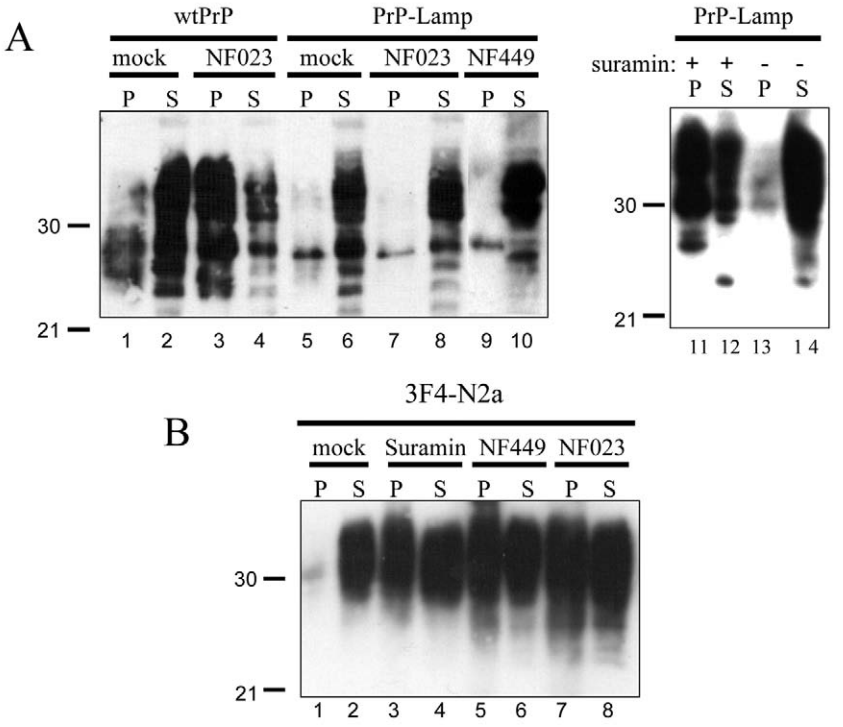

Fig. 8. Suramin derivatives, in contrast to suramin, do not exert their aggregation effect on lysosome-localised LAMP-PrP. (A) N2a cells transiently transfected with wild-type PrP or PrP-LAMP were treated for 48 hours with 200 μg/ml NF449, NF023 or suramin. Cells were then lysed and subjected to ultracentrifugation at 100,000 ***g*** in the presence of 1% sarcosyl. Supernatants (S) and pellets (P) were analysed by immunoblot analysis using the monoclonal antibody 3F4. Suramin (lane 11) but not NF449 or NF023 (lanes 7,9) significantly affected the solubility of lysosome-localised LAMP-PrP, confirming a different site of action of the tested compounds. (B) 3F4-N2a cells were biotinylated for 15 minutes on ice. Suramin, NF449 or NF023 were added to the culture medium and cells were placed at 37°C for 4 hours. Cells were then lysed and subjected to ultracentrifugation at 100,000 ***g*** in the presence of 1% sarcosyl. Supernatants (S) and pellets (P) were precipitated with the polyclonal antibody A7, analysed by immunoblotting, and biotinylated proteins were detected with streptavidin. Although surface-localised PrP^c is soluble in mock-treated cells (lane 1), all tested compounds affect the solubility of this PrP^c population (lanes 3,5 and 7).

exclusively within the soluble fraction (Fig. 8B, lane 2), cells which had been treated with suramin or one of the analysed derivatives harboured visible amounts of insoluble PrP molecules (Fig. 8B, lanes 4,6,8). From these data we conclude that suramin derivatives can interfere with the biochemical properties of cell surface localised PrP^{c}.

Discussion

Despite the wealth of data collected on the effect of anti-prion compounds in cell culture and in in vivo scenarios, the mechanisms and sites of action of many of these treatments remain enigmatic. Understanding the cellular metabolism and the trafficking of PrP^{c} are of fundamental importance for therapeutic and prophylactic strategies against prion diseases. Several approaches have targeted either of the two PrP isoforms, aiming for example to stabilise PrP^{c} conformation, thereby inhibiting initial steps of misfolding and aggregation characteristic of PrP^{Sc}. Other strategies were designed to enhance PrP^{Sc} degradation or to prevent interaction between the cellular and the pathogenic molecules, an essential event in prion biogenesis (Caughey and Raymond, 1993; Shyng et al., 1995; Priola et al., 2000; Tagliavini et al., 2000; Supattapone et al., 2001; Ertmer et al., 2004). We have previously reported that suramin inhibits the de novo formation of PrP^{Sc} by inducing intracellular PrP^{c} aggregates which are targeted from Golgi/TGN compartments to a lysosomal degradation pathway (Gilch et al., 2001). In the present study, we examined the mechanisms of action of a new class of anti-prion agents and analysed molecular requisites for effective clearance of persistently infected cells from PrP^{Sc}.

Suramin analogues prevent PrP^{Sc} biogenesis by inducing aggregation of PrP^{c} at the cell surface and reducing PrP^{c} half-life

The effective suramin derivatives tested in the present work inhibited de novo synthesis of PrP^{Sc} in infected neuroblastoma cells by binding to PrP^{c} and leading to the formation of full-length PrP aggregates which were sensitive to proteolytic digestion. In this respect, these compounds resembled suramin in their molecular structure and in the biochemical activity on PrP^{Sc} biogenesis (Gilch et al., 2001). Analysis of the influence of these compounds on the metabolism and cellular trafficking of PrP^{c}, the precursor of PrP^{Sc}, showed characteristic features that differentiate this class of substances from other anti-prion compounds. Among these, Congo Red, pentosan polysulphate and related drugs seem to exert their prophylactic effect by stimulating endocytosis of PrP^{c} (Shyng et al., 1995; Milhavet et al., 2000). As prion replication is thought to take place either at the cell surface or along the endocytic pathway (Borchelt et al., 1992; Taraboulos et al., 1992), these sulphated glycans probably hamper the production of PrP^{Sc} by shifting PrP^{c} to cellular compartments not eligible for prion conversion. A different mechanism seems to underlie the anti-prion activity of suramin derivatives. The compounds tested do not significantly affect the cell surface localisation of PrP^{c} or its intracellular expression, but influence the solubility state and the half-life of PrP^{c}, inducing aggregates of PrP molecules, which are rapidly degraded. Hence, compounds with related structures do not necessarily exert the same effect on the trafficking of proteins.

Different mechanisms can account for the aggregation of PrP^{c} upon treatment with suramin derivatives. In the previous studies with suramin, misfolding was assumed to be the mechanism underlying a change in the solubility of PrP. Indeed, misfolding of proteins at the cell surface has been seen to compromise recycling and activate quality control mechanisms (Sharma et al., 2004). However, aggregation could also be due to compound-mediated crosslinking. Crosslinking of several surface receptors via antibodies and other ligands results in altered trafficking in the endocytic system. The cellular mechanisms and physiological function of this sorting are still not completely clear and lead to varied effects. As a result, some proteins aggregate at the cell surface and are not internalised, whereas others are downregulated and rapidly degraded, possibly in lysosomes (Lesley and Schulte, 1985; Marsh et al., 1995). Crosslinking of PrP^{c} at the cell surface was described to redistribute its localisation into non-caveolar lipid rafts and to activate signal transduction pathways (Mouillet-Richard et al., 2000; Stuermer et al., 2004). Anti-prion protein antibodies prevent infection of susceptible N2a cells and cure chronically scrapie-infected cell cultures (Enari et al., 2001; Peretz et al., 2001; Gilch et al., 2003). This inhibitory effect was explained by antibodies binding specifically to PrP^{c} molecules on the cell surface and thereby hindering the docking of PrP^{Sc} template or a cofactor critical for the conversion of PrP^{c} to PrP^{Sc}. Indeed anti-PrP antibodies have been described to block PrP^{Sc} replication in cell culture by accelerating PrP^{c} degradation (Perrier et al., 2004). Whether suramin analogues induce misfolding or crosslinking, a model similar to the one described above, that is the inhibition of PrP^{c}/PrP^{Sc} interaction, could also explain their anti-prion effect. Both mechanisms have been suggested to explain the activity of suramin on complement proteins (Saez et al., 1999) and could apply to the compounds tested in our study.

Recently, RNA aptamers, which recognise specific sequences in human PrP, were screened for their ability to reduce PrP^{Sc} synthesis (Proske et al., 2002). The mechanism proposed for this inhibitory activity was the incorporation of aptamers into PrP^{Sc} aggregates. Aptamers therefore prevented formation of high molecular mass aggregates and enhanced susceptibility to proteolytic digestion. A similar model could apply to the insoluble PrP molecules detected upon treatment with suramin derivatives in 3F4-ScN2a. A PrP^{c}-derivative complex might still be incorporated into a PrP^{Sc} seed but this binding is not sufficient to ensure PK resistance and therefore proper conversion. Previous spectroscopic in vitro studies with recombinant PrP showed that in conditions of nearly neutral pH, suramin-induced aggregates still show a predominantly α-helical conformation (Gilch et al., 2001). This finding possibly relates to the PrP aggregates induced by the drugs used in this report and accounts for the lack of PK resistance. In the present study, we show that suramin derivatives also actively bind in vitro to recombinant PrP in the absence of cellular metabolism. They might therefore act by stabilising the α-helical structure of PrP^{c} and making it unsuitable for prion conversion.

Induction of aggregation by suramin analogues might also affect recycling and the endocytic pathway of PrP^{c} molecules. These events could explain reduced half-life in combination with a normal cell surface expression. The cellular prion

protein has been shown to constitutively recycle between the plasma membrane and intracellular compartments before reaching lysosomes for final degradation (Shyng et al., 1993), yet the exact mechanisms of its internalisation remain controversial. Clathrin-mediated endocytosis (Shyng et al., 1994; Stuermer et al., 2004; Sunyach et al., 2003) as well as clathrin-independent mechanisms involving lipid raft or caveolae-like domains (Vey et al., 1996; Kaneko et al., 1997; Peters et al., 2003) or dynamin (Magalhaes et al., 2002) have been discussed for PrP^{c}. The aggregation induced by suramin derivatives might lead to a change in the endocytic pathway and the bypass of the recycling endocytic compartments leading directly to the compartments of degradation. Additionally, these aggregates might also be more easily accessible by lysosomal proteases because of a specific PrP conformation. Interestingly, the transmembrane non-raft localised CD4-PrP aggregated upon incubation with the sulfated compounds, whereas the solubility of Thy-1, a GPI-anchored molecule localised in separate raft domains (Madore et al., 1999; Rudd et al., 2001) was not affected. Suramin derivatives therefore seem to have an affinity for specific features in the PrP structure, regardless of its localisation in rafts or clathrin-coated pits at the cell surface.

Characterisation of intracellular compartment(s) where these substances elicit their activity using drugs that inhibit PrP transport at different steps along the secretory pathway or a PrP-LAMP chimeric protein localised mainly in acidic endocytic compartments and in the Golgi precluded our compounds from altering the chemical properties of PrP. Although suramin itself induced aggregation of PrP-LAMP, the derivatives tested in this assay did not cause its redistribution into the insoluble fraction. On the other hand, bipolar sulphated compounds induced aggregation of cell surface-localised PrP in non-infected cells. It is of importance that suramin derivatives also induce aggregation of endogenous PrP^{c} in N2a cells. This finding demonstrates that the results shown are not merely due to overexpression of the prion protein. The cell surface therefore seems to be the site where suramin analogues mainly (or exclusively) interfere with the prion protein. The finding that suramin, in addition to interfering with the trafficking of PrP^{c} in the mid/trans-Golgi, also induces aggregation at the cell surface was recently described by another group (Kiachopoulos et al., 2004). According to the authors, this phenomenon was due to rapid endocytic uptake induced by misfolded PrP. This is not the case with the compounds tested in our study, as plasma membrane localisation of PrP was not compromised.

Our findings suggest that suramin and its derivatives target different cellular compartments. Alternatively, these large, charged molecules might present reduced cell permeability owing to their size or charged residues, and might therefore only interfere with surface PrP. At least for suramin, uptake and intracellular localisation in low pH intracellular compartments (endosomes, lysosomes and TGN) in concentrations similar to those used in our studies have been described (Fransson et al., 1995; Huang et al., 1997). As the majority of the derivatives have similar molecular weights and similar numbers of sulphated substitutions as suramin, a certain cell permeability cannot be excluded. A more detailed analysis using radioactive or fluorescence labelling should allow evaluation of the possible intracellular accessibility of these compounds.

Despite the effect of suramin derivatives on the aggregation state of PrP^{c}, we cannot exclude the fact that their inhibition of PrP^{Sc} accumulation might also be related to a certain affinity of the drug for the PrP^{Sc} isoform. A similar mechanism has been proposed for the multicyclic anionic molecule Congo Red, which binds to PrP amyloid plaques (Prusiner et al., 1983), thereby possibly sequestering the infectious template (Caughey and Race, 1992; Caspi et al., 1998).

Bipolar aromatic structure and charges are essential for effect of suramin analogues on PrP

Suramin and its numerous derivatives have been extensively studied for their anti-tumour and anti-angiogenic activity (Stein, 1993) and are known to interfere with signal transduction pathways (Kassack et al., 2002; McCain et al., 2004). Most studies agree on the fact that a large symmetrical molecule (urea derivative) with polysulfonated naphthalene groups is a prerequisite for suramin-like activity, as asymmetric molecules with only one acidic group were ineffective (Firsching et al., 1995; Gagliardi et al., 1998; Dhar et al., 2000). Other important factors were the distance between the acid groups and the rigidity of the molecule. Suramin but not ANTS, although also negatively charged (three sulfonated groups) was seen to cause oligomerisation of complement proteins (Saez et al., 1999). These data are in line with those shown in our study. All the compounds we tested which were effective were charged and only those with a bivalent symmetrical structure effectively acted against prion accumulation and affected PrP^{c} solubility. Molecules with phosphonic or carbonic substitutions showed only moderate or no effect on PrP^{Sc} biogenesis and had higher toxic effect on cultured cells. A mere electrostatic interaction of the drugs with PrP or an intermediate factor might not apply to our model. In that case, NF007 and ANTS should also exert some inhibitory effect. The large aromatic molecule NN induced neither aggregation of PrP nor did it reduce the amount of PrP^{Sc}. As this compound is symmetrical but uncharged, these results underline the importance of both bipolar structure and charged sulfonic substitutions for the anti-prion activity of these compounds.

Taken together, our report shows the cellular mechanism of action of a possible new class of anti-prion compounds. Suramin analogues comprise a large number of drugs, only 16 of which were analysed in this report. Testing their prophylactic and therapeutic potential in in vivo scenarios will be necessary, especially considering involvement of PrP^{c} in signalling pathways and that crosslinking PrP^{c} in vivo was found to trigger apoptosis in hippocampal and cerebellar neurons (Mouillet-Richard et al., 2000; Solforosi et al., 2004). We have previously shown that treatment of mice with suramin around the time of peripheral prion inoculation significantly delayed the onset of terminal prion disease. Future in vivo studies will show if the combination of effective doses of suramin with one of the described analogues (or with new compounds based on our data) might result in additive anti-prion effects. Data on the penetration of the blood-brain barrier by the compounds investigated in this study are limited or absent. Although the ability to penetrate the blood-brain barrier is expected to be helpful, a compound might be effective in a prophylactic way by preventing PrP^{Sc} replication and

propagation outside the central nervous system. The pronounced inhibitory effect of suramin derivatives on prion conversion seen in cell culture models and the availability of an enormous number of related substances make these compounds attractive candidates for further studies.

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