Squalestatin Cures Prion-infected Neurons and Protects Against Prion Neurotoxicity*

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A key feature of prion diseases is the conversion of the normal, cellular prion protein (PrPC) into β-sheet-rich disease-related isoforms (PrPSc), the deposition of which is thought to lead to neurodegeneration. In the present study, the squalene synthase inhibitor squalestatin reduced the cholesterol content of cells and prevented the accumulation of PrPSc in three prion-infected cell lines (ScN2a, SMB, and ScG1T cells). ScN2a cells treated with squalestatin were also protected against microglia-mediated killing. Treatment of neurons with squalestatin resulted in a redistribution of PrPC away from Triton X-100 insoluble lipid rafts. These effects of squalestatin were dose-dependent, were evident at nanomolar concentrations, and were partially reversed by cholesterol. In addition, uninfected neurons treated with squalestatin became resistant to the otherwise toxic effect of PrP peptides, a synthetic miniprion (sPrP106) or partially purified prion preparations. The protective effect of squalestatin, which was reversed by the addition of water-soluble cholesterol, correlated with a reduction in prostaglandin E2 production that is associated with neuronal injury in prion disease. These studies indicate a pivotal role for cholesterol-sensitive processes in controlling PrPSc formation, and in the activation of signaling pathways associated with PrP-induced neuronal death.

Prion diseases, or Transmissible Spongiform Encephalopathies (TSEs), are fatal neurodegenerative disorders that include Kuru, Creutzfeldt-Jakob disease (CJD), and Gerstmann-Straussler-Scheinker (GSS) syndrome in man. These diseases are associated with the deposition of aggregates of disease-related isoforms (PrPSc) of a host-encoded protein (PrPC) within the central nervous system (1). During disease, a portion of the α-helix and random coil structure in PrPC is refolded into a β-sheet in PrPSc (2), a conformational change that renders PrPSc poorly soluble in water and resistant to protease digestion, the resultant protease-resistant PrP being designated PrPres (3). Consequently, aggregates of PrPSc accumulate around neurons in affected brain areas (4), a process which is thought to lead to neuronal dysfunction and death, and subsequently the clinical symptoms of infection.

The observation that cellular PrPC is essential for the development of prion diseases (5–7) suggests that the density and cellular location of PrPSc in neurons may influence the production of PrPSc. PrPC is found in lipid rafts or caveolae-like domains (CLDs) (8), specialized membrane compartments that contain high levels of cholesterol and sphingomyelin (9). The formation of these lipid rafts is cholesterol-dependent (10), and drugs that affect cholesterol levels have been shown to influence the formation of PrPSc. Thus, the formation of PrPSc in prion-infected neuroblastoma cells (ScN2a cells) was reduced when cells were treated with lovastatin (11). Lovastatin is a competitive inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the rate-limiting step in cholesterol production (12) and HMG-CoA reductase inhibitors are useful clinical tools used to treat hypercholesterolemia (13) or ischemic heart disease (14–15) in man. However, although HMG-CoA reductase inhibitors reduce cholesterol levels, they exhibit many other effects, for example, they also inhibit the production of isoprenoid precursors (16). Recent studies suggest that many of the effects of HMG-CoA reductase inhibitors are independent of their effects on cholesterol metabolism (17–19). In the present study we investigated the effects of squalestatin, a specific inhibitor of squalene synthase that inhibits cholesterol production without affecting the production of non-sterol products (20) (Fig. 1), on the production of PrPSc in three prion-infected cell lines, and on the levels and distribution of PrPC in non-infected cells. Although the accumulation of aggregated PrPSc in the CNS is a distinguishing feature of prion diseases (21), the precise mechanisms by which PrPSc deposition leads to neuronal damage remain to be fully determined. The processes of neuronal loss have been examined by incubating neuronal cells with either prion preparations (22) or with peptides derived from the protease-resistant core of PrPSc (23). In the present study we used two peptides derived from the human PrP protein, HuPrP(82–146), a synthetic equivalent of a PrPSc fragment present in the brains of patients with GSS (24) and the truncated, neurotropic version HuPrP(106–126) (23), and a synthetic miniprion (sPrP106) (25) to investigate if cholesterol-sensitive microdomains are required for which prions kill infected neurons. We also examined the effects of cholesterol manipulation on neuronal prostaglandin (PG)E2.
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**EXPERIMENTAL PROCEDURES**

**PrP** Production—Scrapie-infected neuroblastoma cells (ScN2a cells) were a gift of Dr. M. Rogers (University College, Dublin, Ireland). These cells produce PrP and infectious agent, and were grown in Ham's F12 medium containing 2 mM glutamine, standard antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin), 2% fetal calf serum (FCS) and 200 nM retinoic acid. SMB cells (TSE Resource Centre, Institute for Animal Health, Compton, UK), which also produce PrP and infectious agent, were grown in RPMI 1640 medium containing standard antibiotics, 2 mM glutamine, 200 nM retinoic acid, and 2% FCS. ScGT1 cells are an immortalized murine hypothalamic neuronal cell line infected by the scrapie Chandler strain and persistently express PrP and were supplied by Dr. Sylvain Lehmann (CNRS-IGH, Montpellier, France) and were grown in optimum supplemented with 2 mM glutamine, 200 nM retinoic acid, 5% FCS, and standard antibiotics.

To measure the effect of squalestatin on the PrP formation, ScN2a, SMB cells or ScGT1 cells were plated at 1 x 10^5 cells/well in 6-well microtiter plates in the presence of 10% FCS. The ScN2a cells were grown in the serum-free neurobasal medium (NBM) (Invitrogen, Paisley, UK) supplemented with 2 mM glutamine and B27 components. Non-infected N2a cells or SMB cells that had been "cured" of infectivity by serial passages in the presence of pentosan polysulphate (PS cells) were used as controls. At the end of the treatment, cells were detached and counted to establish cell numbers. Washed cells were first lysed in distilled water and the insoluble pellet collected. The pellet was suspended in an extraction buffer containing 10 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, and 0.5% sodium deoxycholate and digested with 10 µg/ml proteinase K for 1 h at 37 °C. Digestion was stopped with 5 mM phenylmethylsulfonyl fluoride (PMSF) and the remaining supernatant was split in two. Then the sample was tested in a PrP-specific enzyme-linked immunosorbent assay (ELISA). The second sample was centrifuged at 50,000 x g for 4 h at 4 °C. The pellet was dissolved in 50 µl of Laemmli buffer (Bio-Rad). Samples were boiled for 5 min and 20 µl of each sample was subjected to electrophoresis on a 15% polyacrylamide gel. Proteins were transferred onto a Hybond-P PVDF membrane (Amersham Biosciences) by semi-dry blotting. Membranes were incubated using 10% milk powder in Tris-buffered saline containing 0.2% Tween 20. PrPres was detected by incubation with mab SAF83 (a gift from J. Grassi, CEA, Saclay, France) for 1 h at room temperature, followed by a secondary anti-mouse IgG conjugated to peroxidase (1 h at room temperature). Detection of bound antibody was by the enhanced chemiluminescence kit (Amersham Biosciences). Lysates were also made from the non-infected N2a cells to evaluate PrP content. Cells were treated as above except that proteinase K digestion was excluded.

**Solubility of PrP in Triton X-100**—To determine the effect of squalestatin on the distribution of PrP, uninfected murine N2a neuroblastoma cells were maintained in RPMI 1640 supplemented with 2 mM glutamine, standard antibiotics, and 2% FCS in the presence or absence of 100 nM squalestatin. After 3 days cells were lysed on ice at 1 x 10^5 cells/ml in either (a) an extraction buffer containing 10 mM Tris-HCl, pH 7.8, 100 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, and 5 mM PMSF or (b) a buffer containing 1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, pH 7.8, and 5 mM PMSF. Soluble material was collected after centrifugation at 10,000 x g. The insoluble pellet was dissolved in extraction buffer (a) at 1 x 10^7 cells/ml, and all three supernatants were tested in a PrP ELISA.

**Animals**—129/Ola mice were housed at constant temperature (21 ± 1 °C) and relative humidity (60 ± 10%) and supplied ad libitum with water and a standard diet. Procedures involving animals and their care were conducted in accordance with national and international regulations (Animals (Scientific Procedures) Act, 1986, UK, and EEC Council Directive 86/609, OJ L358, 1, 12 December 1987).

**Primary Neuronal Cultures**—Primary cortical neurons were prepared from embryonic day 15.5 mice as previously described (28). After 2 days, medium was changed to NBM containing B27 components, 2 mM glutamine and 5 µM cytosine arabinoside to prevent the proliferation of astroglia. Mature cultures were treated with 5 mM t-leucine methyl ester to reduce the number of contaminating microglia before further use (31). For experiments, cultures were preincubated for 3 h in media containing 100 nM squalestatin alone or a mixture of 100 nM squalestatin and 1–100 µg/ml water-soluble cholesterol before the addition of peptides. HuPrP-(82–146), HuPrP-(106–146), sPrP106, or a prion extract. After another 3 h, microglia were added to neuronal cultures in the ratio 1 microglia to 10 neurons. After 4 days, cultures were shaken and washed to remove non-adherent microglia, and neuronal viability was determined using the 3-(4,5-diethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method.

**Microglia**—Microglia cultures were prepared from newborn 129/Ola mice as previously described (28). Isolated microglia was added to ScN2a cells or treated neuronal cells in the ratio 1 microglia to 10 neuronal cells. In other studies microglia were added to 96-well flat-bottomed plates at 1 x 10^5 cells/well and allowed to adhere overnight. Microglia were subsequently treated with 100 nM squalestatin or a prion extract for 3 h before the addition of 100 µg/ml lipopolysaccharide. After a further 24 h supernatants were collected and tested for IL-6.

**Neurotoxicity of PrP Peptides or Prion Preparations**—The human neuroblastoma SH-SY5Y cell line (European Collection of Cell Cultures) was grown in RPMI 1640 medium supplemented with 2 mM glutamine, standard antibiotics, and 2% FCS. Cells were plated at 1 x 10^4 cells/well into 96-well microtiter plates and allowed to adhere overnight. The following day, cells were pretreated for 3 h either with 0–100 nM of 5 µM of 100 nM squalestatin alone or with 100 nM squalestatin plus 1–100 µg/ml of water-soluble cholesterol or with 100 nM squalestatin plus 500 µM of mevalonate, before the addition of peptides, sPrP106 or a diluted prion extract. Supernatants were made acid (1 M HCl) and diluted appropriately. Cell viability was determined 24 h later using the MTT method (32). Optical density was measured at 595 nm, and results calculated by reference to untreated cells. PGE2 levels were also determined at this time point.
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Microglia-mediated Toxicity—To investigate the effect of squalestatin on microglia-mediated toxicity, ScN2a cells were plated at $1 \times 10^5$ cells/well in 24-well plates and allowed to adhere overnight. Cells were cultured in the presence or absence of 100 nM squalestatin alone, or in a mixture of 100 nM squalestatin and 1–100 μg/ml water-soluble cholesterol for a further 24 h after which microglia were added in the ratio 1 microglia to 10 ScN2a cells. The survival of ScN2a cells was measured after cells had been co-cultured for 24 h using the MTT method. N2a neuroblastoma cells were used as non-infected controls.

PrP Peptides—For studies using human neuroblastoma (SH-SY5Y) cells, peptides containing amino acids corresponding to residues 106–126 of the human prion protein (HuPrP(106–126)) (23) and a peptide consisting of the same amino acids in a scrambled order (HuPrP-(106–126scrambled)) were synthesized by solid-phase chemistry and purified by reverse-phase HPLC. A longer peptide containing amino acids corresponding to residues 82–146 of the human prion protein (HuPrP(82–146)) (24) was also used, as was a control peptide containing amino acid residues 82–146 in a scrambled sequence (HuPrP-(82–146scrambled)). A synthetic miniprion (sPrP106) derived from the murine PrP sequence was used for studies on murine cortical neurons (25).

Prion Extract Preparations—Partially purified prion extracts were obtained from ScG1T1 cells to reduce the number of potential contaminants that may be present in prion preparations from infected brain material. Washed cells were suspended in an extraction buffer containing 10 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40 and 0.5% sodium deoxycholate and digested with 10 μg/ml of proteinase K for 1 h at 37 °C. Digestion was stopped with 5 mM PMSF and the supernatant was loaded onto a prewashed C18 Sep-Pak column (Waters, Elstree, UK). Bound material was eluted under a gradient of n-propanol in water (5–100%), fractions containing PrP were pooled (samples tested by a PrP ELISA), washed three times in distilled water and resuspended in 0.01 M pH 7.4 phosphate-buffered saline at 100 ng/ml. Control preparations from non-infected N2a cells were produced by lysis in extraction buffer containing 5 mM PMSF. The supernatant was loaded on to a prewashed C18 Sep-Pak column and eluted under a gradient of n-propanol in water. Fractions containing PrP were pooled, washed three times in distilled water, and resuspended in phosphate-buffered saline at 100 ng/ml.

PrP ELISA—Briefly, treated lysates were added to a Nunc Maxisorb Immunoplates precoated with diluted rabbit antisera raised to a PrP peptides (ovine PrP-(27–52) and ovine PrP-(100–111)) conjugated to keyhole limpet hemocyanin (a gift from Dr. J. P. M. Langeveld, Pepscan Systems, Lelystad, The Netherlands). Samples were applied and visualized using mAb SAF83 (gift from Prof. J. Grassi (CEA, Saclay, France), followed by an anti-mouse IgG-alkaline phosphatase conjugate (Sigma) and an appropriate indicator. Plates were read at 450 nm, and results were calculated by reference to a standard curve of recombinant murine PrP (Promics, Zurich, Switzerland).

PGE2 Assay—Analysis of cellular PGE2 levels was determined in SH-SY5Y cells by using an enzyme immunoassay kit (Amer sham Bio-sciences) according to the manufacturer’s instructions. This assay is based on competition between unlabeled PGE2 in the sample and a fixed amount of labeled PGE2 for a PGE2-specific antibody. The detection limit of this assay is 20 pg/ml.

IL-6 ELISA—Levels of interleukin (IL)-6 in cultures containing microglia were assayed in a sandwich ELISA (R&D Systems, Abingdon, UK) as previously described (25b). Plates were read at 450 nm, and results were calculated by reference to a standard curve of recombinant murine IL-6. The detection limit of this assay is 20 pg/ml.

Cholesterol and Protein Content—Cellular cholesterol and protein content were determined on 1 × 107 SH-SY5Y cells, or ScN2a cells grown in the presence or absence of 2% FCS or in serum-free NBM, before and after exposure to 100 nM squalestatin for 3–24 h. At the end of incubation, cells were detached by trypsinization, washed twice with phosphate-buffered saline, and pelleted (550 × g for 10 min). Total lipids were extracted according to Folch et al. (33), and total cholesterol was determined using an enzymatic assay kit (Roche Applied Science). Cellular protein concentration was determined on cell lysates using the Bio-Rad protein assay.

Statistical Analysis—Comparison of treatment effects was carried out using one and two way analysis of variance techniques as appropriate. Post hoc comparisons of means were performed as necessary. For all statistical tests significance was set at the 5% level.

RESULTS

Squalestatin Reduces PrP\textsuperscript{res} Formation in Prion-infected Cells—Previous studies had indicated that ScN2a cells contain protease-resistant PrP (PrP\textsuperscript{res}) and infectious agent (34). Here, we investigated the effect of squalestatin, an inhibitor of squalene synthase that inhibits the production of cholesterol (20), on ScN2a cells. As shown in Fig. 2, the accumulation of PrP\textsuperscript{res} in ScN2a cells was significantly reduced by squalestatin in a dose-dependent manner. The amounts of PrP\textsuperscript{res} were reduced to below detectable levels in ScN2a cells treated with 100 nM squalestatin (Fig. 2 and Table I). This effect was observed in both lipid-depleted NBM medium and in Hams F12 medium containing 2% FCS that contained extracellular cholesterol. The effect of squalestatin on PrP\textsuperscript{res} production was accompanied by a decrease in the cellular cholesterol levels. ScN2a cells grown in medium containing 2% FCS and treated for 24 h with 100 nM squalestatin contained significantly less cholesterol (0.262 ± 0.040 μg of cholesterol/mg protein, mean ± S.D.) than did untreated ScN2a cells (0.509 ± 0.041, $n = 6$, $p < 0.05$). The cholesterol content of ScN2a cells grown in NBM (0.484 ± 0.033) was greatly reduced by treatment with 100 nM squalestatin (0.181 ± 0.025, $n = 6$, $p < 0.05$). There was no significant difference between the cholesterol content of ScN2a cells grown in serum-free medium and ScN2a cells grown in 2% FCS. To investigate whether the protective effect of squalestatin was
mediated by sterol derivatives, the PrP\textsuperscript{res} formation was evaluated in ScN2a cells treated for 24 h with 100 nM squalestatin alone or combined with 1–100 μg/ml of water-soluble cholesterol, or with 500 μM of mevalonate. As reported in Table I, mevalonate did not affect the content of PrP\textsuperscript{res} in squalestatin-treated cells. In contrast, the addition of water-soluble cholesterol partially reversed the effect of squalestatin on PrP\textsuperscript{res} production although it was not fully restored even with the higher dose of 100 μg/ml of cholesterol (Table I).

To confirm the effects of squalestatin on PrP\textsuperscript{res} production, two other prion-infected neuroblastoma cell lines (SMB or ScGT1 cells) was treated with 100 nM squalestatin. The PrP\textsuperscript{res} content of SMB cells treated with a vehicle control (7,405 ± 512 PrP\textsuperscript{res} pg/ml) was significantly higher than that of SMB cells treated with 100 nM squalestatin (<50 pg/ml, n = 8, p < 0.05). Similar results were observed with ScGT1 cells, in that the PrP\textsuperscript{res} content of ScGT1 cells treated with a vehicle control (10,014 ± 845 PrP\textsuperscript{res} pg/ml) was significantly higher than that of ScGT1 cells treated with 100 nM squalestatin (<50 pg/ml, n = 8, p < 0.05).

### Table I

| Squalestatin | Mevalonate | Cholesterol | PrP\textsuperscript{res} |
|--------------|------------|-------------|------------------------|
| nM  | μM  | μg/ml | pg/ml     |
| 0   | 0   | 0     | 1830 ± 145 |
| 100 | 0   | 0     | <50	extsuperscript{a} |
| 0   | 500 | 0     | 1844 ± 147 |
| 0   | 0   | 100   | 1867 ± 193 |
| 100 | 500 | 0     | <50	extsuperscript{a} |
| 100 | 0   | 1     | 92 ± 75	extsuperscript{a} |
| 100 | 0   | 100   | 1033 ± 166	extsuperscript{a} |

\textsuperscript{a} PrP\textsuperscript{res} content of cells significantly less (p < 0.01) than that of untreated cells.

\[ \text{Squalestatin Inhibits Prion Replication and Neurotoxicity} \]

![Fig. 3. Treatment with squalestatin changes the distribution of PrP\textsuperscript{res} in N2a cells. A, non-infected N2a cells were grown in control medium (untreated) or with 100 nM squalestatin (SQ treated) for 72 h. Cells were subsequently lysed in either an extraction buffer or in a buffer containing 1% Triton X-100 at the equivalent of 1 × 10⁶ cells/ml. The amounts of PrP\textsuperscript{res} in whole cellular lysates (shaded bars), soluble in 1% Triton X-100 (open bars) or insoluble in Triton X-100 (striped bars) were determined in an ELISA. Values shown are the mean level of PrP\textsuperscript{res} ± S.D. of triplicate experiments repeated three times (9 observations). B, N2a cells were grown in the presence (+) or absence (−) of 100 nM squalestatin (SQ). Cellular lysates were untreated (PK−) or digested with proteinase K (10 μg/ml for 1 h at 37°C) (PK+). PrP\textsuperscript{res} was demonstrated by immunoblot with mAb SAF83.]

\[ \text{SQ Protects Neurons Against PrP Peptides—The effect of squalestatin on the sensitivity of SH-SY5Y cells to PrP peptides was examined. Cells treated for 3 h with 100 nM squalestatin contained significantly less cholesterol than did untreated ones (0.394 ± 0.024 μg of cholesterol/mg of protein for untreated cells and 0.172 ± 0.033 μg of cholesterol/mg of protein for treated cells, n = 6, p < 0.05). The survival of squalestatin-treated SH-SY5Y cells incubated with 40 μM HuPrP-(106–126), 10 μM HuPrP-(82–146) or 10 μM sPrP106 was significantly greater than that of vehicle treated SH-SY5Y cells incubated with these peptides (Fig. 4A). This protective effect of squalestatin was dose-dependent with an IC\textsubscript{50} -1 nm when tested against 10 μM HuPrP-(82–146) (Fig. 4B).} \]

Previous studies have shown that the neuronal loss is observed after the addition of partially purified prion preparations (22, 28). In the present study the addition of 10 ng/ml PrP extracted from three different prion preparations significantly reduced the survival of untreated SH-SY5Y cells by 50%, whereas the survival of SH-SY5Y cells was not affected by the addition of 10 ng/ml PrP extracted from uninfected cells (Table II). Although these prion preparations were not pure, the neurotoxicity of these preparations was thought to be due to PrP\textsuperscript{res} since these preparations were not toxic for neurons from PrP\textsuperscript{−} deficient mice, and that the toxicity of these preparations was selectively removed following immunoprecipitation with an anti-PrP antibody (data not shown). The survival of cells pre-treated with 100 nM squalestatin, before the addition of the prion extracts, was significantly higher than that of untreated SH-SY5Y cells incubated with the same preparations (Table II). In addition, squalestatin also protected murine primary
SH-SY5Y cells were pretreated for 3 h with a vehicle (Me2SO) (black bars) or with 100 nM squalestatin (open bars). Cells were then incubated for 24 h with different concentrations of HuPrP-(82–146), and the cell viability was evaluated after 2 hours using MTT. Each value is the mean cell survival ± S.D. from triplicate experiments repeated three times (9 observations).

### Table II

|                   | Cell viability |
|-------------------|----------------|
|                   | Vehicle        | Squalestatin  |
|                   | % control      |               |
| Medium            | 100 ± 2        | 100 ± 4       |
| Prion extract - 1 | 92 ± 4         | 98 ± 3        |
| Prion extract - 2 | 49 ± 6         | 96 ± 3        |
| Prion extract - 3 | 48 ± 5         | 93 ± 3        |
| Control extract - 1 | 96 ± 3   | 99 ± 3        |
| Control extract - 2 | 98 ± 3   | 96 ± 2        |
| Control extract - 3 | 99 ± 2   | 100 ± 3       |

*Cell survival significantly less (*p* < 0.01) than that of untreated cells.*

cortical neurons (non-transformed cells) against the toxicity of prion preparations (data not shown).

To determine whether squalestatin-treated cells were completely resistant to PrP peptides, different amounts of HuPrP-(82–146) were added to SH-SY5Y cells treated with 100 nM squalestatin. The addition of HuPrP-(82–146) caused a dose-dependent reduction in the survival of untreated cells, whereas even at high concentrations, HuPrP-(106–146) did not kill cells treated with 100 nM squalestatin (Fig. 5). Further experiments were performed to determine if the addition of cholesterol to squalestatin-treated SH-SY5Y cells would restore their sensitivity to the neurotoxic effect of PrP peptides or to prions. As shown in Fig. 6, the presence of 10 μg/ml of water-soluble cholesterol reversed the protective effect of 100 nM squalestatin on the toxicity induced by 40 μM HuPrP-(106–126), 10 μM HuPrP-(82–146) by a prion extract in SH-SY5Y cells.

**Reduced PGE2 Production in Squalestatin-treated Cells**

The cyclo-oxygenases (COX) are enzymes that convert arachidonic acid to PGs. The observations that the levels of neuronal PGE2 are raised in response to PrP peptides, and that COX-1 inhibitors protect neurons against PrP peptides, suggest that activated COX is involved in neuronal toxicity (35, 28). Thus, in this study, the effects of squalestatin on PGE2 levels from SH-SY5Y cells incubated with PrP peptides or a prion extract were examined. We were unable to detect PGE2 in untreated SH-SY5Y cells, squalestatin-treated cells, or cells incubated with scrambled peptides. Cells exposed to either 40 μM HuPrP-(106–126) to 10 μM HuPrP-(82–146), 10 μM sPrP106, or to a prion extract produced significant amounts of PGE2. SH-SY5Y cells pretreated with 100 nM squalestatin produced significantly less PGE2 when subsequently incubated with these peptides or a prion extract (Table III). The effect of squalestatin was reversed by the addition of 10 μg/ml of water-soluble cholesterol.

**Squalestatin Protects ScN2A Cells Against Microglia-mediated Killing**—The effect of squalestatin on interactions between ScN2a cells and microglia was also examined. As shown in Fig. 7A, the survival of ScN2a cells co-cultured with microglia (ratio of 1 microglia to 10 ScN2a cells) was significantly lower than those of non-infected N2a cells co-cultured with microglia, consistent with previous studies (28). The pretreatment of ScN2a cells with 100 nM squalestatin completely protected them from microglia-mediated toxicity, increasing cell viability from 30 to 100%. This protective effect could be fully...
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Effect of squalestatin on the production of PGE₂ in SH-SY5Y cells

Cells were pretreated for 3 h with a vehicle control (Me₂SO) or 100 nM squalestatin alone or combined with 10 μg/ml water-soluble cholesterol before the addition of peptides (10 μM HuPrP-(82–146), 40 μM HuPrP-(106–126)), 10 μM sPrP106, or a prion extract. PGE₂ production was evaluated after 24 h as shown. Results are the mean levels of PGE₂ ± S.D. from quadruplicate experiments repeated twice (8 observations).

|                      | PGE₂ (pg/ml) |
|----------------------|--------------|
|                      | Vehicle | Squalestatin | Squalestatin + cholesterol |
| Medium               | < 20     | < 20         | < 20                        |
| HuPrP-(106–126)      | 244 ± 74  | < 20         | 262 ± 41                    |
| HuPrP-(82–146)       | 292 ± 70  | < 20         | 303 ± 28                    |
| sPrP106              | 303 ± 48  | < 20         | 288 ± 48                    |
| Prion extract        | 376 ± 48  | < 20         | 365 ± 32                    |

reversed by the addition of 10 μg/ml of water-soluble cholesterol.

The effect of the squalestatin on interactions between PrP-damaged, non-transformed neurons, and microglia was also studied. Thus, primary cortical neurons were treated with 100 nM squalestatin before the addition of 40 μM HuPrP-(106–126), 10 μM HuPrP-(82–146), 10 μM sPrP106 or a prion extract, and microglia then added 3 h later in the ratio of 1 microglia to 10 neurons. The survival of neurons in co-cultures containing squalestatin and PrP peptides or a prion extract, were significantly higher than the survival of untreated co-cultures exposed to these peptides or prion preparations (Fig. 7B). To determine whether squalestatin had a direct effect on microglia, isolated microglia cultures were pretreated with 100 nM squalestatin before the addition of 10 ng/ml LPS. There was no significant difference in the amounts of IL-6 produced by squalestatin-treated microglia (4205 pg/ml ± 221, n = 6) and vehicle-treated microglia (4286 pg/ml ± 161).

DISCUSSION

In the present study, we examined the effect of squalestatin on cellular cholesterol levels, on the accumulation of PrPSc in three prion-infected cell lines, and on two models of PrP-induced neurotoxicity. Squalestatin reduced the accumulation of PrPSc in ScN2a, SMB, and ScG1T1 cells. This effect of squalestatin on PrPSc production was dose-dependent with an IC₅₀ of 5 nM, while even 50 μM squalestatin was not toxic for ScN2a cells. None of the effects of squalestatin were reversed by the addition of mevalonate (previously shown to reverse the effects of HMG-CoA reductase inhibitors such as lovastatin), but were reversed by cholesterol indicating that cholesterol depletion was responsible for the effects of squalestatin. Cholesterol homeostasis in mammalian cells is governed by both cholesterol synthesis and by the influx and efflux of cholesterol from the surrounding medium (36). Recent studies have shown that the statins reduce cerebral cholesterol levels (37) indicating that cholesterol production is important in maintaining neuronal cholesterol levels.

Several observations suggest that the maintenance of cholesterol levels in these neuronal cells is largely influenced by the synthesis of cholesterol rather than by the uptake of cholesterol from the surrounding medium. Firstly, the effects of squalestatin were observed in medium containing FCS (and hence cholesterol), and the addition of 100 μg/ml of normal cholesterol did not affect the neuroprotective effect of squalestatin, or the effect of squalestatin on PrPSc formation (data not shown). In contrast, low concentrations of water-soluble cholesterol (~10 μg/ml) were enough to reverse the neuroprotective effects of squalestatin, restore PrPSc production and restore cellular cholesterol levels. Water-soluble cholesterol is complexed to methyl-β-cyclodextrin that facilitates the insertion of cholesterol into cell membranes while normal cholesterol requires specific uptake mechanisms. Finally, drugs that prevented cellular uptake of cholesterol did not affect either PrPSc formation or the neurotoxicity of PrP peptides.²

Previous studies suggest that the concentration and cellular location of PrPSc might be critical factors in the production of PrPSc (6, 7) since PrPSc formation is thought to be dependent on the supply of PrPSc to an intracellular environment that facilitates the conversion of PrPSc to PrPSc (38). Although cells treated with squalestatin contained significantly less cholesterol than untreated cells, the amounts of PrPSc in untreated and squalestatin-treated cells were not significantly different and, unlike treatment with the cholesterol-binding drug filipin (39), squalestatin did not cause the release of PrPSc into culture medium (data not shown). In the present study squalestatin treatment resulted in the dispersion of PrPSc into Triton X-100 soluble fractions, an observation compatible with previous studies that showed that cholesterol-depleted cells no longer contain typical caveolae (40). PrPSc molecules are normally located in lipid rafts (8) and dispersion from such sites following squalestatin treatment may affect the normal cellular trafficking of PrPSc. The golgi and the endosomal compartments are involved in the trafficking of a GFP-tagged PrPSc (41) and in hippocampal neurons, the trafficking of cholera toxin (whose receptor is known to reside in lipid rafts) from endosomes to the golgi apparatus is blocked by cholesterol depletion (42). While the current results are suggestive, it is not known whether the trafficking of PrPSc is affected in squalestatin-treated cells so as to prevent PrPSc interacting with cellular components required for conversion to PrPSc.

The loss of neurons in response to prions or PrP peptides is thought to be an apoptotic event (43) requiring receptor binding and the activation of specific metabolic pathways. The neurotrophin p75 receptor, located in CLDs (44), is thought to act as a receptor for PrP peptides and is involved in the neuronal damage of HuPrP-(106–126) (45). Thus, the protective effect of squalestatin may be mediated by a destabilizing effect on neurotrophin p75. Since many of the molecules involved in cellular signaling are found in cholesterol-sensitive CLDs (46) the possibility that cholesterol depletion may affect the generation of second messen-

²C. Bate, M. Salmona, L. Diomede, and A. Williams, unpublished observations.
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Fig. 7. Squalestatin protects prion-damaged neurons against microglia. A, ScN2a cells (black bars) or N2a cells (white bars) were pretreated with vehicle (Veh), with 100 μM squalestatin (SQ), or with 100 μM squalestatin and 10 μg/ml water-soluble cholesterol (SQ + Ch) for 24 h before the addition of microglia. Cell viability was evaluated after a further 24 h. Each point represents mean cell survival ± S.D. of triplicate experiments repeated four times (12 observations). B, primary cortical neurons were preincubated for 3 h with vehicle (Me2SO) (black bars) or with 100 μM squalestatin (white bars) or with 100 μM squalestatin and 10 μg/ml water-soluble cholesterol (striped bars) before the addition of a prion extract (Prion), 40 μM HuPrP-(106–126) (106–126), 10 μM HuPrP-(82–146) (82–146), or 10 μM sPrP106. After a further 3 h microglia were added to neuronal cultures in the ratio 1 microglia to 10 neurons, and cell viability was determined 4 days later using the MTT method. Each point represents the mean cell survival ± S.D. of triplicate experiments repeated four times (12 observations).

Authors concluded that the statins were not compatible with use in man, partly due to the high concentrations of lovastatin required, and partly due to the fact that results were only seen in lipid-depleted medium. The concentration of squalestatin required to affect PrPSc formation in three different prion-infected cell lines was shown to be less than 10 nM; considerably lower than that reported for lovastatin (0.3 μM) (11). Moreover, the action of squalestatin was not affected by the cholesterol in the in medium and was only partially restored with high concentrations of water-soluble cholesterol. In contrast, the ability of 100 nM lovastatin to reduce PrPSc production was reversed with 1 μg/ml water-soluble cholesterol (data not shown) suggesting that statins and squalestatin affect different cholesterol pools. The different effects displayed by statins and squalestatin depended on their different pharmacological target (HMG-CoA reductase versus squalene synthase; see Fig. 1) and the effects of these on cholesterol distribution. Time course studies showed that squalestatin produced a prolonged reduction in cholesterol levels (significant reduction after 24 h) that was not observed in lovastatin-treated cells. The greater potency of squalestatin suggests that it may be a more promising candidate than the HMG-CoA reductase inhibitors for use in vivo.

In summary, an understanding of the processes by which prions accumulate in neurons, and how this process leads to neuronal loss, may aid the development of therapies to delay the progression of prion diseases. In the present study, squalestatin had a profound effect on three major aspects of the neuropathogenesis of prion disease. Squalestatin-treated ScN2a, SMB, or ScGT1 cells were cured of PrPSc, ScN2a cells were protected against killing by microglia and neurons treated with squalestatin were resistant to the neurotoxicity of PrP peptides. Furthermore, the observation that squalestatin had protective effects at low concentrations, suggest that the use of this drug to treat prion diseases should be considered.

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Dublin, Ireland). SMB and SMB(PS) cells were obtained from the TSE Resource Centre, Institute for Animal Health, Compton, UK, and ScGT1 cells were obtained from Dr. S. Lehmann (Montpellier, France).

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