



UNIVERSITÀ DEGLI STUDI DI CAGLIARI

Dipartimento di Scienze e Tecnologie Biomediche
Sezione di Microbiologia Generale e Virologia
e Biotecnologie Microbiche

XX Ciclo di Dottorato di Ricerca in: Sviluppo e Sperimentazione di Farmaci Antivirali

**Coordinatore del Dottorato:
Prof. Paolo La Colla**

**Studies on the role of cholesterol in the pathogenesis of
prion diseases:**

**I. Correlation between cell susceptibility to prion
infection/replication and altered cellular cholesterol
distribution**

**Supervisore:
Prof. Pani Alessandra**

**Candidato:
Dott. Cannas Maria Dolores**

TABLE OF CONTENTS

INTRODUCTION	3
• Characteristics and Structure of Prion Protein	4
• Prion Protein Functions	10
• Pathogenesis of Prion Diseases	12
• Aetiology of Human Prion Diseases	15
• Prion Protein and Scrapie	20
• Prions, Membranes and Cholesterol	24
• Diagnosis and Therapeutic Approaches to Lipid-Rafts Diseases	32
• Aim of the Research	36
• Brief description of properties and clinical use of tested Drugs	39
RESULTS	
• Standardization of the protocols for PrPC and PrPres Detection	42
• Cholesterol esters content and effect of cholesterol modulators In mouse neuroblastoma cell lines	43
• Anti-prion activity of cholesterol modulators in mouse neuroblastoma 22L-N2a cells	45
• Cholesterol ester levels in <i>ex vivo</i> skin fibroblasts from sheep with susceptible or resistant prion genotype and comparative inhibition by cholesterol modulators	48
• Cav-1 and PrP expression in sheep skin fibroblasts	50
DISCUSSION	52

MATERIALS AND METHODS

- Chemicals 57
- Cell lines 57
- Sheep 58
- Skin fibroblast cultures 58
- Lipid staining 59
- Detection of PrP^{res} in cell cultures 59
- Cytotoxicity assay 61
- RT-PCR and Southern blotting 61
- Statistical analysis 62

REFERENCES 63

INTRODUCTION

Prion diseases, also known as Transmissible Spongiform Encephalopathies (TSEs), are a group of progressive and invariably fatal neurodegenerative diseases that affect both humans and animals. Most TSEs are characterized by a long incubation period and a neuropathologic feature of multifocal spongiform changes, astrogliosis, neuronal loss, and absence of inflammatory reaction. TSEs in humans include Creutzfeldt-Jakob disease (CJD), kuru, Gerstmann-Straussler-Scheinker syndrome (GSS), Fatal Familial Insomnia (FFI), and new variant CJD (nvCJD).

TSEs described in animals include scrapie in sheep and goats, transmissible mink encephalopathy, chronic wasting disease in deer and elk, bovine spongiform encephalopathy (BSE, commonly known as "mad-cow" disease), exotic ungulate spongiform encephalopathy, and feline spongiform encephalopathy in cats, albino tigers, pumas, and cheetahs. The reported ungulate and feline spongiform encephalopathies appear to represent transmission of the BSE agent to these animals (Belay, 1999).

Prion diseases are biologically unique in that the disease process can be triggered through inherited germline mutations in the human prion protein gene (*PRNP*), infection (by inoculation, or in some cases by dietary exposure) with tissue containing a protease-resistant form of host-derived prion protein, or by rare sporadic events that generate PrP^{Sc} (Wadsworth et al. 2003).

Prion diseases do not characteristically elicit an immune response by the host, and the mechanism of brain damage is poorly understood. However, progressive neuronal accumulation of the disease-associated prions may damage neurons directly, and diminished availability of the normal prion protein may interfere with the presumed neuroprotective effect of the normal prion protein, contributing to the underlying neurodegenerative process (Belay et al. 2005).

The TSE typically have long incubation periods of months (rodents, cats), years (cattle, sheep, deer), or decades (man), so clinical signs usually are evident in older animals (Novakofski et al., 2005).

Characteristics and Structure of Prion Protein

The nature of the transmissible agent has been a subject of intense and heated debate for many years. The initial assumption that it must be viral was challenged, however, both by the failure to directly demonstrate a virus (or an immunological response) and because the transmissible agent was resistant to treatments which inactivate nucleic acids (such as ultraviolet radiation or treatment with nucleases). These remarkable findings led to suggestions in 1966 by Tikvar Alper and others that the transmissible agent may be devoid of nucleic acid and led John Griffith to suggest in 1967 that the transmissible agent may in fact be composed entirely of protein. In this remarkable letter to *Nature*, he proposed three hypothetical mechanisms for propagation of such an agent, one of which closely mirrors current thinking; indeed his model also presciently predicted the existence of distinct strains of agent. Needless to say, such a proposal met with great scepticism at the time, in what was the heyday of the "central dogma" of biology: that DNA encodes RNA that in turn encodes protein. More than a decade later this remarkable proposal was lent biochemical credibility by intensive purification studies allied with laborious rodent bioassay. Progressive enrichment of brain homogenates for infectivity resulted in the isolation of a protease resistant glycoprotein, designated the prion protein (PrP) by Prusiner and co-workers in 1982 (Table 1). This protein was the major constituent of infective fractions and was found to accumulate in affected brains and sometimes to form amyloid deposits. The term prion (from *proteinaceous infectious only*) was proposed to distinguish the infectious pathogen from viruses or viroids. Prions were defined as "small proteinaceous

infectious particles that resist inactivation by procedures which modify nucleic acids" (Collinge, 2005).

Table 1. Essential Chronology of Prion Research

Mid 18th century	Earliest description of scrapie recorded
1898	Neuronal vacuolation discovered in brains of scrapie-sick sheep
1918	Contagious spread of scrapie suspected under natural conditions
1920	First possible cases of CJD described (Creutzfeldt 1920; Jakob 1921).
1937	Scrapie epidemic in Scotland following administration of formalin-treated louping ill vaccine prepared from sheep brain
1939	Experimental transmission of scrapie reported (Cuille and Chelle 1939)
1955–57	Kuru discovered among Fore people of Papua New Guinea (Gajdusek and Zigas 1957)
1959	Similarities between Kuru and scrapie noted (Hadlow 1959)
1961	Multiple strains of scrapie agent described (Pattison and Millson 1961)
1961	Scrapie transmitted to mice (Chandler 1961)
1963	Transmission of Kuru to chimpanzees reported (Gajdusek et al. 1966)
1966	Scrapie agent found to be highly resistant to ionizing radiation and ultraviolet light (Alper et al. 1966; Alper et al. 1967)
1967	First statement of the protein-only hypothesis (Griffith 1967)
1968	CJD transmitted to chimpanzees (Gibbs et al. 1968)
	Description of Sinc gene affecting scrapie incubation period in mice (Dickinson et al. 1968)
1974	First documented iatrogenic prion transmission (corneal graft) (Duffy et al. 1974)
1980	Protease resistant, highly hydrophobic protein discovered in hamster brain fractions highly enriched for scrapie infectivity (Prusiner et al. 1980)
1982	Prion concept enunciated (Prusiner 1982)
1985	Gene encoding PrPC cloned (Chesebro et al. 1985; Oesch et al. 1985)
1986	PrPC and PrPSc isoforms shown to be encoded by same host gene (Basler et al. 1986)
1987	Linkage between Prnp and scrapie incubation period in mice (Westaway et al. 1987)
	First report of BSE in cattle (Wells et al. 1987)
1989	Mutation in PrP linked to Gerstmann–Sträussler syndrome (Hsiao et al. 1989)
	Importance of isologous PrPC/PrPSc interactions established (Scott et al. 1989)
1992	Ablation of Prnp by gene targeting in mice (Büeler et al. 1992)
1993	Prnp0/0 mice are resistant to scrapie inoculation (Büeler et al. 1993; Sailer et al. 1994)
	Structural differences between PrPC and PrPSc isoforms noted (Pan et al. 1993)
1994	Cell-free conversion of PrPC to protease-resistant PrP (Kocisko et al. 1994)
1996	New variant of CJD identified (Will et al. 1996)
	BSE prion strain carries a distinct glyco-type signature (Collinge et al. 1996b)
	First NMR structure of core murine PrPC solved (Riek et al. 1996)
1997	Evidence that nvCJD is caused by the BSE agent (Bruce et al. 1997; Hill et al. 1997)
	B-lymphocytes necessary for peripheral prion pathogenesis (Klein et al. 1997)
1998	Genes controlling incubation period are congruent with Prnp (Moore et al. 1998)
1999	Discovery of the PrPC homolog (Moore et al. 1999)
2000	Temporary depletion of lymphoid follicular dendritic cells impairs prion replication (Montrasio et al. 2000)
	Experimental transmission of BSE in sheep by blood transfusion (Houston et al. 2000)
2001	Complement involved in prion pathogenesis (Klein et al. 2001; Mabbott et al. 2001)
2003	Transgenic expression of soluble PrP inhibits prion replication (Meier et al. 2003)
2005	Inflammation recognized as a modified of pathogenesis (Heikenwalder et al. 2005; Ligios et al. 2005; Seeger et al. 2005)
	Prion detection in blood by cyclic amplification (Castilla et al. 2005b)

(Aguzzi et al. 2006)

Prion protein (PrP^C) is a normal cellular protein that is expressed in the neurons and glia of the brain and spinal cord, as well as in several peripheral tissues and in leukocytes. PrP mRNA is first detectable in the brains of mice and chickens beginning early in embryogenesis, and its level increases as development proceeds. In the adult central nervous system, PrP and its mRNA are widely distributed, with particular concentrations in neocortical and hippocampal neurons, cerebellar Purkinje cells, and spinal motor (Harris, 1999).

The *PRNP* gene is present in most, if not all, wild-type mammals and is highly conserved across species. The presence of the human and mouse *PrP* genes within conserved syntenic groups and the presence of a *PrP* gene in chicken argue that the *PrP* gene existed before the speciation of mammals. In mammals, DNA sequences of the open reading frames (ORFs) encoding PrP generally exhibit ~90% similarity. As expected, the degree of similarity at the amino acid level increases to >95% when PrPs of different primates are compared but is much lower when human PrP is compared with that of a marsupial (~70%). An even lower degree of homology is found when human PrP is compared with that of the chicken (~30%). Attempts to find *PrP*-related genes in lower eukaryotes have, to date, been unsuccessful (Lee et al. 1998)

Endogenous PrP is encoded by a single exon of the *PRNP* gene, based on chromosome 20 in humans and on chromosome 2 in mice (Aguzzi et al., 2000), which codes for a 256- to 264-AA precursor of approximately 28 kDa that is processed by cleavage of a 22- to 24-AA signal peptide. This yields a mature protein of 231 to 253 AA (Novakofski et al 2005).

The structure of mature PrP^C from mice, humans, Syrian hamsters and cattle shares common features: a long, flexible N-terminal region (residues 23-128), three α -helices, and a two-stranded anti-parallel β -sheet that flanks the first α -helix. The second β -sheet and the third α -helix are connected by a large loop with interesting structural properties. α -helices and a short anti-parallel β -sheet

are situated in the C-terminal domain which is stabilized by two cysteine residues at positions 179 and 214 forming a disulfide bond that links helices two and three (Collinge, 2005, Aguzzi et al., 2006).

The N-terminal region contains five repeats of an 8 amino acid sequence (the octapeptide repeat region). Amplification of the number of octa repeats has been found in hereditary prion diseases such as familial Creutzfeldt-Jakob disease and Gerstmann-Straussler-Scheinker syndrome. While unstructured in the isolated molecule, this region is very highly conserved in evolution and contains two tight binding sites for Cu^{2+} ions (Fig. 1). It is proposed that the unstructured N-terminal region may acquire structure following copper binding and a role for PrP in copper metabolism or transport is possible. Disturbance of this function by the conformational transitions between isoforms of PrP could be involved in prion related neurotoxicity.

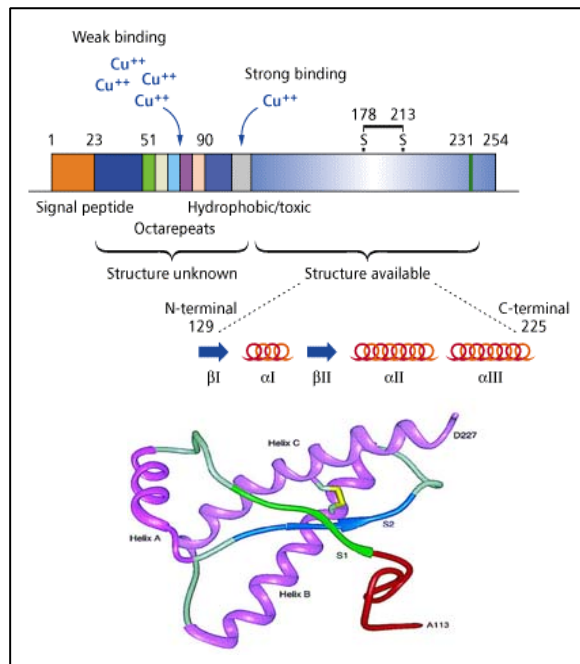


Fig. 1 Anatomy of the prion protein

PrP is a glycoprotein with two asparagine linked glycosylation sites, at position 182 and 198, and is attached to the external cell surface via a glycosyl-phosphatidyl-inositol (GPI) anchor (Collinge, 2005, Aguzzi et al., 2000).

The only difference between the two isoforms appears to be structural. While PrP^C is predominantly α -helical (42%) with little β -sheet structure (3%), the pathological isoform (PrP^{Sc}) is mostly β -sheet (43%) with less α -helical structure (30%) and it has a tendency to polymerise into amyloid fibrils (Fig. 2) (Rymer et al., 2000).

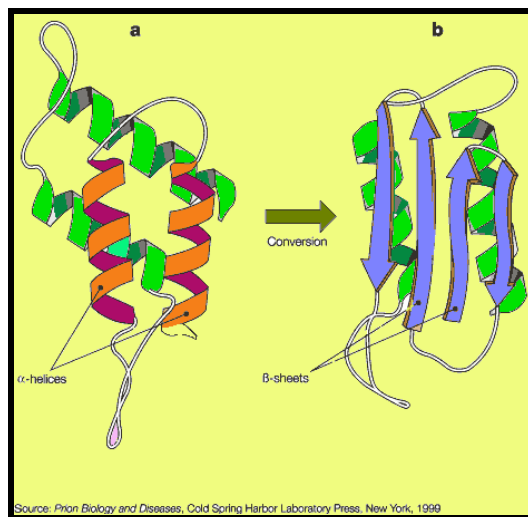


Fig. 2 Conformational structure of PrP^C (a) and PrP^{Sc} (b)

Due to PrP^{Sc} altered conformation its most important hallmark is the partial resistance to degradation by endoproteases, such as proteinase K (PK), and for this reason is also called PrP^{res}. This hallmark is the basis for most current methods of PrP^{Sc} detection (Novakofski et al., 2005).

Depending on the particular prion strain, PK removes 55 to 70 residues from the N-terminal domain of PrP^{Sc} more or less efficiently:

- the PK cleavage fragments of BSE derived PrP^{Sc} is by 1-2 kDa smaller in size than most scrapie-derived PrP^{Sc} fragments, indicating that the amino-terminal cleavage site varies between amino acid at position 85 and 100
- the PK resistance of amino-terminally truncated PrP^{Sc} varies between different prion strains, i.e. BSE PrP^{Sc} is comparatively less resistant towards PK digestion than PrP^{Sc} derived from most classical scrapie strains (Gretzschel et al., 2005).

Classically, after PK treatment PrP^{Sc} exhibits a typical 3-band pattern comprising 18-30 kDa, equivalent to three glycoform: aglycosil, monoglycosil and diglycosil fractions. Nevertheless seems that only a minority of PrP^{Sc} molecules are protease resistant, and this indicates that PrP^{Sc} adopts both protease-resistant and -sensitive conformations (Scott et al., 2004).

The central feature of prion diseases is the post-translational conversion of the normal host-encoded (PrP^C), to the abnormal isoform PrP^{Sc} (Wadsworth et al., 2003). To explain the mechanism by which a misfolded form of PrP could induce the refolding of "native", normal PrP molecules into the abnormal conformation, two distinct models have been postulated (Fig. 3):

- the template assistance or "refolding" model
- the nucleation-polymerization or "seeding" model

In the first model the conformational change is kinetically controlled; a high activation energy barrier prevents spontaneous conversion at detectable rates. Interaction with exogenously introduced PrP^{Sc} causes PrP^C to undergo an induced conformational change to yield PrP^{Sc}.

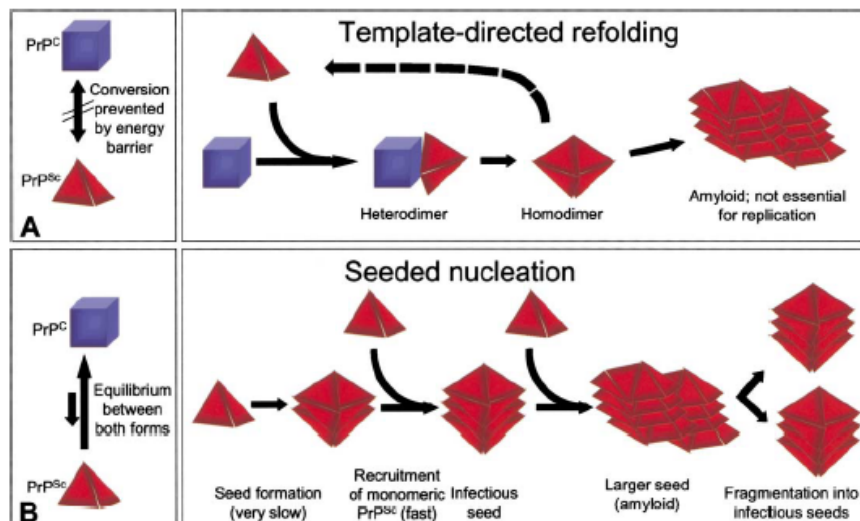


Figure 3. Models for the Conformational Conversion of PrPC into PrPSc. (A) The "refolding" or template assistance model postulates an interaction between exogenously introduced PrPSc and endogenous PrPC, which is induced to transform itself into further PrPSc. A high energy barrier may prevent spontaneous conversion of PrPC into PrPSc. (B) The "seeding" or nucleation-polymerization model proposes that PrPC and PrPSc are in a reversible thermodynamic equilibrium. Only if several monomeric PrPSc molecules are mounted into a highly ordered seed, further monomeric PrPSc can be recruited and eventually aggregates to amyloid. Within such a crystal-like seed, PrPSc becomes stabilized. Fragmentation of PrPSc aggregates increases the number of nuclei, which can recruit further PrPSc and thus results in apparent replication of the agent. (Aguzzi et al., 2004)

This reaction may involve extensive unfolding and refolding of the protein to explain the postulated high energy barrier and could be dependent on an enzyme or chaperone, provisionally designated as Protein X.

In the second model PrP^C and PrP^{Sc} are in equilibrium strongly favouring PrP^C. PrP^{Sc} is only stabilized when it adds onto a crystal-like aggregate of PrP^{Sc} acting as a seed in nucleation-dependent polymerisation process. Consistent with the latter model, cell-free conversion studies indicate that PrP^{Sc} aggregates are able to convert PrP^C into a protease-resistant PrP isoform (Aguzzi et al., 2000).

The latter form can, but does not always, aggregate to form amyloid plaques in the brain. TSE are one among many types of neurodegenerative diseases such as Alzheimer's disease, Huntington's disease, Parkinson's disease, and familial amyloid polyneuropathy that exhibit highly structured proteinaceous aggregates. The relationship between infectivity, PrP^C-converting activity and the size of various PrP^{Sc}-containing aggregates has been systematically investigated. In this analysis, PrP^{Sc} aggregates were partially fragmented, fractionated by size and assessed for infectivity and converting activity. The analysis revealed that 17-27 nm sized (300-600 kDa) particles had the highest infectivity and converting activities, whereas these activities were substantially lower in large fibrils and virtually absent in oligomers containing ± 5 PrP^{Sc} molecules. Therefore, non-fibrillary PrP^{Sc}-containing particles with masses equivalent to 14-28 molecules are the most efficient initiators of prion infection (Aguzzi et al. 2006).

Prion Protein Functions

The normal function of PrP^C remains unknown, although has been implicated in protection from oxidative insults, apoptosis, cellular signalling, membrane excitability and synaptic transmission, neuritogenesis and copper (II) transport or metabolism (Prado et al., 2004). There is some evidence that PrP^C functions as a signal-transducing molecule. Structural similarities between PrP^C and

membrane-anchored signal peptidases led to the suggestion that PrP^C might function as a protease (Glatzel et al., 2005).

It was also hypothesized that human genetic variability of prion protein might be related in the formation of long-term memory (Papassotiropoulos et al., 2005). Defining the physiological role of PrP^C may be relevant to understanding the disease state, since the protein may fail to perform its normal function when it is converted to the PrP^{Sc} isoform. Mice in which the endogenous PrP gene has been disrupted display no gross developmental or anatomical defects but are reported to have electrophysiological and structural abnormalities in the hippocampus, loss of cerebellar Purkinje cells, alterations in circadian rhythm and sleep pattern and changes in learning and memory (Harris, 1999). Although it is not completely clear how conversion occurs from normal PrP^C, into the abnormal and pathogenic isoform PrP^{Sc}, PrP^C expression is absolutely necessary for development of prion disease; since knock out mice for the prion gene (*PRNP*^{0/0}) are resistant to the disease (Milhavet et al., 2001)

A recent work has shown that PrP^C induces polarization in synapse development as well as in neuritogenesis in embryonic hippocampal neuron cultures. PrP^C is also up-regulated after focal cerebral ischemia, and PrP^C overexpression reduces the extent of neuronal loss after ischemic insult, suggesting that PrP^C might confer a neuroprotective effect in certain contexts.

In vivo, was observed that PrP^C is expressed most strongly immediately adjacent to the proliferative region of the sub-ventricular zone but not in mitotic cells. Besides was also find that PrP^C expression increases in fully differentiated, mature neurons. Both *in vivo* and *in vitro*, PrP^C is found in increasing amounts as neuronal differentiation progresses. Besides *in vivo*, PrP^C is found at highest levels in mature neurons, but it is not detected in astroglia (Steele et al, 2006). Furthermore have also shown that PrP^C is expressed on the surface of long-term

repopulating hematopoietic stem cells and that PrP^C-null mice have limited hematopoietic stem cell self-renewal (Zhang et al., 2006).

In cultured nerve cells PrP^{Sc} causes membrane protein alterations, increased membrane microviscosity and abnormal receptor-mediated calcium ion responses. Besides it causes apoptosis of hippocampal neurons and induces hypertrophy and proliferation of astrocytes in vitro (Diomede et al., 2002).

Pathogenesis of Prion Diseases

The natural transmission routes of prions are poorly understood and remain to be clarified, but available evidence indicates that an environmental reservoir of infectivity contributes to the maintenance of these diseases in affected population. By contrast the primary infection pathway for BSE has been via dietary exposure to industrial animals feed and there is little evidence of an environmental component in its spread. There are many unanswered questions relating to sources of TSE infection and the fate and behaviour of TSE infectivity in the natural environment following the death and decomposition of an infected animal. Infected tissues may be introduced into the environment by on-farm carcass burial, mass burial by landfill, TSE infectivity in effluents from rendering plants and in ash following incineration of carcasses or from urinary excretion from infected, nephritic animals. The presence of TSE infectivity in soil may indicate a potential infection pathway via soil ingestion, or via biological vectors considering that it has been shown persistence of prions after burial in soil for at least 3 years (Cooke et al., 2007, Johnson et al., 2006, Genovesiet al., 2007).

Oral infection by ingestion involves transfer of PrP^{Sc} from the digestive tract to the spleen or lympho-reticular system (LRS), and then to the peripheral nervous system and eventually to the brain. Infectivity is established in peripheral lymphoid organs before infective PrP^{Sc} is found in the central nervous system

(CNS), indicating that peripheral conversion of PrP^C to PrP^{Sc} is a necessary step for infection (Novakofski et al 2005). The infectious agent seems to be transported to the central nervous system along two nervous pathways. One route is through the splanchnic nerves, connected to the inter-medio-lateral column (IMLC) of the distal half of the thoracic and the rostral part of the lumbar spinal cord. The other route is through the vagus nerve, connected to the dorsal motor nucleus of the vagus nerve (DMNV) in the medulla oblongata. PrP^{Sc} can also be hematogenously transported, and blood from sheep infected with either bovine spongiform encephalopathy (BSE), or scrapie, has been used to experimentally transmit the disease (Ersdal et al., 2005).

Following ingestion, PrP^{Sc} may be degraded by digestive enzymes, leaving a pathogenic fragment similar to that of a proteinase K-resistant PrP^{Sc} fragment. The PrP^{Sc} fragment may be co-transported across the intestinal epithelial with ferritin. The PrP^{Sc} or PrP^{Sc} fragments are transported from the intestine to secondary lymphoid organs by intestinal dendritic cells, which are specialized to acquire antigen from peripheral tissues.

Dendritic cells from the intestine present PrP^{Sc} to T and B lymphocytes within lymphoid tissues such as Peyer's patches (PPs) of the intestine or follicular dendritic cells (FDC) of the spleen, thymus, and tonsils (Novakofski et al., 2005). Moreover the protease-resistant prion protein accumulate rapidly in gut-associated lymphoid tissues (GALT), and ganglia of the enteric nervous system long before they are detected in the central nervous system (CNS). B lymphocytes play a crucial role in peripheral prion pathogenesis: mice devoid of B lymphocytes do not develop disease after intraperitoneal exposure. This is possibly because B lymphocytes induce maturation of follicular dendritic cells. Early PrP^{Sc} deposition can be detected in FDCs within B cell follicles in lymphoid tissues of patients with vCJD and in rodents inoculated with scrapie by peripheral routes. In mouse spleens, mature FDCs have been shown to be crucial

for both prion replication and PrP^{Sc} accumulation, although prion replication in lymph nodes can occur in the absence of mature FDCs (Fig. 4). In contrast, the role of intestinal B cells in prion pathogenesis following oral challenge is still unclear. B lymphocytes exert an important organogenic role in the GALT and are likely to be involved in the B cell-dependent development of the follicle-associated epithelium (FAE). However, splenic lymphocytes can acquire prion infectivity, and it is unclear whether their role in prion pathogenesis is restricted to the generation and maintenance of FDCs or whether they may also be involved in prion trafficking.

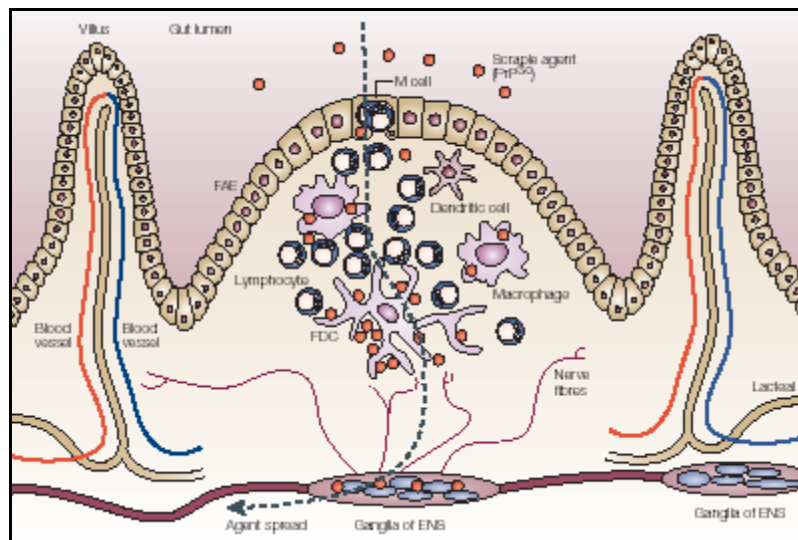


Fig. 4 Possible spread of scrapie infectivity from the gut lumen to the nervous system following oral infection (route indicated by dotted line). Soon after ingestion, the abnormal prion isoform (PrP^{Sc}) is detected readily within Peyer's patches on follicular dendritic cells (FDCs), within macrophages, within cells with morphology consistent with that of M cells and within ganglia of the enteric nervous system (ENS). These observations indicate that, following uptake of scrapie infectivity from the gut lumen, infectivity accumulates on FDCs in Peyer's patches and subsequently spreads via the ENS to the central nervous system. FAE, follicle-associated epithelium (Cashman et al., 2004).

It was shown that prion replication in the GALT and subsequent neuroinvasion was independent of B cells within the mucosa-associated lymphatic tissue and that the remaining M cells are most likely important for this process.

The GALT consists of highly-organized Peyer's patches in the small intestine, and intraepithelial lymphocytes are present throughout the length of the gastrointestinal tract. The intestinal surfaces of PPs are characterized by the presence of "domes," which are regions free of intestinal villi.

At these domes, M cells are able to tunnel pathogens through the cytoplasm to the basal surface, where deep invaginations of their membrane allow close contact with lymphocytes and macrophages (Prinz et al., 2003).

However remains the question how prions are transferred from FDCs to peripheral nerve endings within lymphoid organs given that these cells are not in physical contact. A model requiring cell-to-cell contact for intercellular prion transfer would necessitate additional cell types to bridge the gap between FDCs and neurons. Alternatively prions could be transmitted to the nerves by cell-free, short-range diffusion mechanisms. Data available are consistent with an intercellular prion transfer through direct cell contacts, although another study reported the presence of prion infectivity in the cell culture medium of a prion infected neuronal cell line (Fèvrier et al., 2005).

Aetiology of Human Prion Diseases

The human prion diseases have been traditionally classified into Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker disease (GSS), and kuru. Although these are rare disorders, affecting about one to two people per million world-wide per annum, remarkable attention has been focused on them in recent years. This is because of the unique biology of the transmissible agent or prion, and also because of fears that the epizootic of BSE could pose a threat to public health through dietary exposure to infected tissues. There was considerable interest in the 1950s in an epidemic of a neurodegenerative disease, kuru, characterised principally by a progressive cerebellar ataxia, amongst the Fore linguistic group of the Eastern Highlands of Papua New Guinea. Subsequent field work suggested that kuru was transmitted during cannibalistic feasts. In 1959 Hadlow drew attention to the similarities between kuru and scrapie at the neuropathological, clinical, and epidemiological levels leading to the suggestion that these diseases may also be transmissible. A landmark in the field was the

transmission, by intracerebral inoculation with brain homogenates into chimpanzees, of kuru and then CJD by Gajdusek and colleagues in 1966 and 1968, respectively. Transmission of GSS followed in 1981. This work led to the concept of the "transmissible dementias". The term "Creutzfeldt-Jakob disease (CJD)" was introduced by Spielmeyer in 1922 drawing from the case reports of Creutzfeldt (1920) and Jakob (1921) and was used in subsequent years to describe a range of neurodegenerative conditions, many of which would not meet modern diagnostic criteria for CJD. Interestingly, Jakob suspected that the condition may be transmissible and experimentally inoculated rabbits in an attempt to demonstrate this in the 1920s. This was unsuccessful and we now know that rabbits are unusually resistant to prion infection (Collinge, 2005).

The human prion protein is a product of a single gene located on the short arm of chromosome 20. It is encoded by a single exon of *PRNP*, exon 2. Variation in the gene sequence produces protein variants that are causative of genetic TSE diseases (Sordevila et al., 2006).

Familial Creutzfeldt-Jakob disease (CJD), fatal familial insomnia (FFI) and Gerstmann-Sträussler-Scheinker disease (GSD) are frequently caused by mutations in the prion gene in codons 200, 178 and 102.

CJD can also be sporadic (representing 85% of the cases) or acquired, which includes iatrogenic CJD cases transmitted via human pituitary hormones, human dura mater grafts, corneal grafts and neurosurgical devices; variant CJD which has been causally linked to the bovine spongiform encephalopathy agent; and kuru (Llewelyn et al, 2004). In these cases some polymorphic positions, codons 129 (385A>G: M129V) and 219 (655G>A: E219K) of the *PRNP* are particularly important for susceptibility to prion diseases. Codon 129 is known to be implicated in the development of sporadic, acquired (iatrogenic, kuru, and variant CJD), with increased susceptibility observed for the M/M genotype. This finding was interpreted as suggesting that dimerization of the prion protein is an

important element in the pathogenesis of CJD and that this is more likely to occur in Met homozygotes than in heterozygotes. The relative frequencies of the codon 129 alleles in Europeans were estimated to be 68% M and 32% V. The importance of codon 129 was highlighted in a study, where an increased incidence of sporadic Creutzfeldt-Jakob disease was associated with a high rate of M homozygosity (Fig. 5). These data is the first to relate a high regional incidence rate for sporadic CJD to the distribution of *PRNP* 129 genotypes. To date, all studies of *PRNP* codon 129 have mainly focused on European or some Asian populations, and in all populations the Met allele was always found to be the most frequent (Sordevila et al., 2003).

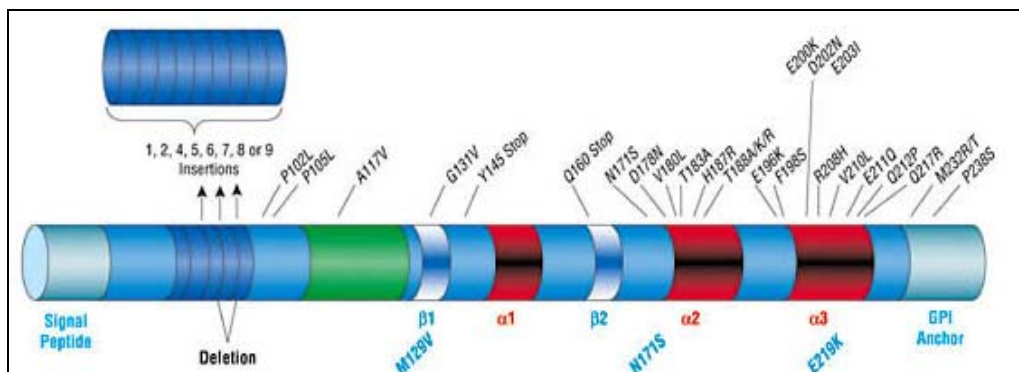


Figure 5. The coding region of the human *PRNP* gene. Mutations that segregate with inherited prion diseases are shown in black and nonpathogenic polymorphisms in blue. The signal peptide is cleaved off during maturation of the cellular prion protein. Octapeptide regions are represented by blue boxes, and pathogenic octarepeat insertions of 8, 16, 32, 40, 48, 56, 64, and 72 amino acids are shown above. Deletion of one octarepeat stretch may segregate with a neurodegenerative disorder. The light green box indicates a conserved region, β -sheet domains are drawn light blue, and α -helical domains (H1, H2, H3) are red. GPI indicates glycosylphosphatidylinositol (Glatzel et al., 2006).

Homozygosity at codon 129 is also a key factor in the resistance or susceptibility to the Kuru prion disease, which was shown to have been transmitted during endo-cannibalistic feasts among the Fore linguistic group in New Guinea (Soldevila et al., 2006). Kuru came to the attention of Western medicine in the 1950s as the affected area of the Eastern Highlands of Papua New Guinea came under Australian administrative control. The Fore and neighboring linguistic groups occupied a remote highland area that had had no direct contact with the outside world prior to this.

It was the practice in these communities for kinship groups to consume deceased relatives at mortuary feasts. From the evidence of local oral history, this practice was not ancient amongst the Fore and is thought to have started around the end of the 19th century. The first remembered case of kuru was around 1920 and the disease rapidly increased in incidence. Adult women and children of both sexes were primarily affected, reflecting their selective exposure-adult males participated little at feasts. At its peak, kuru killed around 1% of the population annually and some villages were almost devoid of young adult women. It is hypothesized that kuru originated from consumption of an individual with sporadic CJD, a disease with a remarkably uniform worldwide incidence of around 1 per million and a lifetime risk of around 1 in 50,000. The ban on cannibalism imposed by the Australian authorities in the mid-1950s led to a decline in kuru incidence, and although rare cases still occur these are all in older individuals and reflect the long incubation periods possible in human prion disease-kuru has not been recorded in any individual born after the late 1950s (Mead, 2003).

A statistically significant excess of heterozygotes for the codon 129 polymorphism was found among these women, implying a heterozygote resistance to the disease. Balancing selection in this generation appeared to be the strongest yet documented in any human population. Based on a wide analysis of *PRNP* sequence and haplotype diversity in a worldwide sample, it was postulated that variation at this locus had been shaped by strong balancing selection related to prion diseases and cannibalism during the evolution of modern humans (Soldevila et al., 2006).

Codon 219 has received somewhat less attention, but it has been suggested that the K allele at codon 219 acts as a protective factor against sporadic CJD, as all individuals with sporadic CJD were 219E homozygotes. This protective allele may inhibit the formation of PrP^{Sc}, the pathological type of PrP, as protein X, that is

thought to accelerate the conversion of the normal type of prion protein into the rogue conformation, could bind to the 219 amino acid residue of PrP (Sordevila et al., 2003).

In an Italian study it was reported that the 219K was absent in healthy controls and CJD patients, showing that Italians are monomorphic for this position (Petraroli and Pocchiari, 1996). There are several different human PrP^{Sc} conformations, referred to as molecular strain types, that can be further classified by the ratio of the three PrP bands seen after protease digestion, corresponding to amino-terminally truncated cleavage products generated from di-, mono-, or non-glycosylated PrP^{Sc}. Four types of human PrP^{Sc} have now been reliably identified using molecular strain typing. Sporadic and iatrogenic CJD are associated with PrP^{Sc} types 1-3, while type 4 human PrP^{Sc} is uniquely associated with vCJD and is characterised by a fragment size and glycoform ratio that is distinct from PrP^{Sc} types 1-3 observed in classical CJD. The methionine/valine polymorphism at codon 129 of *PRNP* is associated with different PrP^{Sc} types. PrP^{Sc} types 1 and 4 have so far only been detected in methionine homozygotes, type 3 cases are predominantly associated with at least one valine allele, while type 2 is seen in any *PRNP* codon 129 genotype. PrP^{Sc} types 1 and 2 are associated with two clinically distinct sub-types of sporadic CJD.

This represents a novel mechanism for post-translational modification of PrP, and for the generation of multiple prion strains in humans. Importantly, the identification of strain-specific PrP^{Sc} structural properties has enabled investigation of the influence of human PrP primary structure, in particular polymorphic residue 129, in determining PrP^{Sc} structure. In addition to the identification of human PrP^{Sc} types 1-4, molecular strain typing has provided insights into the phenotypic heterogeneity seen in inherited human prion diseases. In agreement with existing evidence that human prion strain diversity is generated through variance in PrP^{Sc} conformation and glycosylation, cases of

inherited prion disease caused by point mutations in the *PRNP* gene show glycoform ratios distinct from those observed in sporadic CJD and vCJD. Additionally, individuals with the same *PRNP* mutation can propagate PrP^{Sc} with distinct fragment sizes. Sub-classification of sporadic CJD based upon PrP^{Sc} type immediately allows a more precise molecular classification of human prion disease and re-analysis of epidemiological data using these molecular sub-types may reveal important risk factors obscured when sporadic CJD is analysed as a single entity. For example, it will be important to review the incidence of sporadic CJD associated with PrP^{Sc} type 2 and other molecular sub-types in both BSE-affected and unaffected countries in the light of recent findings suggesting that human BSE prion infection may result in propagation of either type 4 PrP^{Sc} or type 2 PrP^{Sc}. Individuals that propagate type 2 PrP^{Sc} as a result of BSE exposure may present with prion disease that would be indistinguishable on clinical, pathological and molecular criteria from that found in classical CJD (Wadsworth et al. 2003).

Although the link between specific alleles of PrP and susceptibility to the disease has been well documented during the last decade how a few amino acid substitutions can so profoundly affect prion pathogenesis is largely unknown (Sabuncu E. et al., 2003).

Prion Protein and Scrapie

As previously said Scrapie is member of the transmissible spongiform encephalopathy that naturally affects sheep and goats and it's characterized by changes in behaviour, ataxia and pruritus (Goldmann et al., 2005).

The first report of the existence of scrapie appear in 18th and 19th Century literature from England and Germany. The earliest definite record of the occurrence of scrapie was in Great Britain in 1732. Throughout the 1700s and 1800s, scrapie was reported in many breeds of sheep in England as well as in

continental Europe. Spread of reported to have been through the importation of certain breeds of sheep (Detwiler et al., 2003).

The gene that encodes the ovine PrP has three exons (52, 98 and 4028 nucleotides in length) separated by two introns (2421 and 14.031 nucleotides in length). The 3'-untranslated region (UTR) of the sheep *PrP* mRNA is much longer than that of other analyzed species (3246 pb) (Lee et al., 1998).

The sheep PrP gene has two short 5'untranslated exons and a long coding exon III. This arrangement is conserved in goats and cattle splicing of exon I, whereas only the ovine/caprine genes exhibit differential processing (alternative poly-adenylation) of their PrP m-RNAs. The importance of these differences remains to be established. Sheep PrP mRNA is detectable in brain and peripheral tissues from day 98 of gestation and increases strongly towards the newborn and young lambs; the level of expression is then maintained throughout adulthood (Baylis et al., 2004).

The protein-coding region or open reading frame (ORF), of 768 bp (256 codons), is contained entirely within exon 3. DNA sequence analysis of the sheep PrP gene has so far found 25 polymorphic codons that result in an amino acid change (8). The total number of known amino acid substitutions in these 25 codons is 32, while the current number of published haplotypes is 40 (Goldmann et al 2005).

In European domestic sheep breeds the following polymorphic codons have been found: 112, 136, 137, 138, 141, 151, 154, 168, 171, 175, 176, 180 and 211. additional and different variations were found in Asian sheep, such as Mongolian and Chinese breeds at codons 127, 189, and 176. (Baylis et al., 2004).

Only three of these polymorphisms have a clear and significant effect on genetic susceptibility for a sheep to develop scrapie disease:

- at codon 136, amino acid alanine (A) changes to valine (V); valine is associated with high scrapie susceptibility while alanine is associated with low susceptibility, although this might depend on the strain of scrapie agent.

- at codon 154, amino acid arginine (R) changes to histidine (H); arginine (R) is associated with susceptibility while histidine (H) is associated with partial resistance.
- at codon 171, amino acid glutamine (Q) changes to arginine/histidine; glutamine(Q) and histidine (H) are associated with susceptibility while arginine (R) is associated with resistance (Goldmann et al 2005).

The ancestral sheep allele is presumably A₁₃₆R₁₅₄Q₁₇₁ (shortened to ARQ). This allele plus those generated through the substitution of one of its amino acids make up the five most common ovine PrP alleles, namely ARQ, VRQ, AHQ, ARR and ARH. Free permutation of these five alleles leads to 15 possible genotypes in the diploid organism, e.g. heterozygosity for VRQ and ARQ (VRQ/ARQ) or homozygosity for ARR (ARR/ARR) (Saunders et al., 2006).

Sheep homozygous for alanine (AA) have been shown to be more resistant to scrapie than sheep homozygous for valine (VV) or heterozygous (AV) in European studies. However AA sheep are not 100% resistant to scrapie. In the U.S., amino acid changes at codon 136 appear to be less important to scrapie susceptibility than in Europe. This may be due to the different strains of scrapie found in the two regions.

Of the three important codons, amino acid changes at 154 appear to have a slightly less dramatic effect on scrapie susceptibility than do the other two, and the susceptible genotypes are not consistent across studies. In some studies, sheep with the arginine (R) allele have a lowered incidence of scrapie, and in other studies, sheep with the histidine (H) allele have a lowered incidence. At the present time, it appears that screening sheep on the basis of codon 154 genotype has little value in increasing resistance to scrapie.

Amino acid changes at codon 171 has a large effect on scrapie susceptibility in sheep in both Europe and the U.S. Virtually no sheep homozygous for arginine (RR) have been identified with scrapie. The one exception is a single RR Suffolk

in Japan that was diagnosed with scrapie. The frequency of heterozygous (QR) sheep also is very low among scrapie-infected sheep. However, the frequency of sheep homozygous for glutamine (QQ) is very high among scrapie-infected sheep. It should be remembered that a susceptible genotype like QQ at codon 171 does not imply that the animal is scrapie-infected (Thomas).

The susceptibility of a sheep to scrapie depends upon a number of factors, amongst them the age of the animal, the route of infection, the infectious dose, the strain of scrapie, the genotype of the animal and its breed (Baylis et al., 2004). For example, Australia and New Zealand are scrapie-free, but Suffolk, Cheviot, Merino, and Poll Dorset sheep of susceptible genotypes are found in these countries. There is no scrapie in these countries because the scrapie agent is not present (Thomas).

It was repeatedly confirmed that most of the work regarding disease progression has been performed using techniques to detect the partially protease form of the prion protein (PrP^{Sc}) in tissues rather than confirming absolute infectivity. The detection of PrP^{Sc} can be accomplished with greater ease and speed than the detection of infectivity. However, it must be noted that although there have been good correlations between the presence of PrP^{Sc} and infectivity, TSE infections have been reported in the absence of detectable PrP^{Sc} . Epidemiological observations suggest that very young ruminants are more susceptible to TSE infections. These observations are consistent with the known differences between young and old ruminants in the activity of their gut-associated lymphoid tissue and the passage of macromolecules across the gut wall. This does not eliminate the possibility of scrapie infection by other routes of entry. Those which have been shown to be effective experimentally are scarification and via the conjunctiva (Detwiler et al., 2003).

Prions, Membranes and Cholesterol

As previously said PrP is modified by the attachment of a glycosyl-phosphatidyl-inoditol (GPI)-anchor, which is added in the ER after cleavage of the C-terminal hydrophobic segment. GPI-anchor has a core structure common to other glycolipid-anchored proteins, consisting of an ethanolamine residue amide-bonded to the C-terminal amino acid, three mannose residues, an unacetylated glucosamine residue, and a phosphatidyl-inoditol molecule which is embedded in the outer leaflet of the lipid bilayer (Fig. 6). The GPI anchors of both PrP^C and PrP^{Sc} are unusual because their cores are modified by the addition of sialic acid residues. Available evidence indicates that the oligosaccharide chains and GPI anchors of PrP^C and PrP^{Sc} do not differ, although complete structures have been worked out only for PrP^{Sc}.

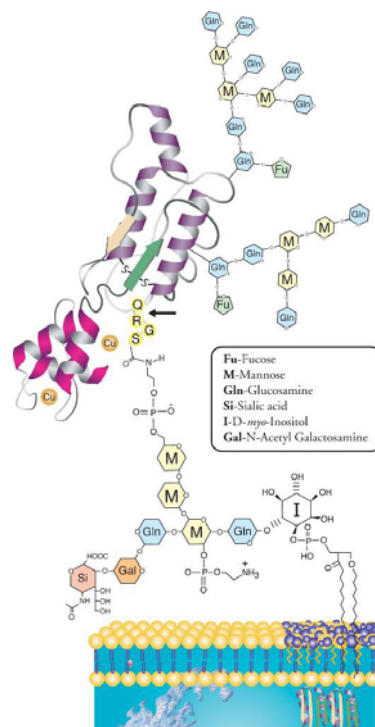


Figure 6 GPI-anchored prion protein

PrP^C does not remain on the cell surface after its delivery there but, rather, constitutively cycled between the plasma membrane and an endocytic compartment. This endocytic recycling pathway could be the route along which

certain steps in the conversion of PrP^C to PrP^{Sc} take place (Harris, 1999). Once PrP^{Sc} is formed, it appears to accumulate in late endosomes, lysosomes, and on the cell surface or in extracellular appears in the form of amorphous deposits, diffuse fibrils or dense amyloid plaque. Recent findings in non-neuronal cell models indicate prion protein association with secreted exosomes. Exosomes correspond to the intraluminal vesicles (ILVs) of multivesicular endosomes, commonly called multivesicular bodies (MVBs) (Fig. 7). Multivesicular endosomes have well known functions as intermediates in the degradation of proteins internalised from the cell surface or sorted from the *trans*-Golgi network. Trans-membrane proteins destined for lysosomal degradation are removed from the limiting membrane of early endosomal vacuoles and subsequently sequestered into the exosomes. Most mature MVBs fuse with pre-existing lysosomes to degrade their contents. Sequestration on the internal membranes allow lysosomal hydrolases to access and degrade all topologic domains of the associated integral membrane proteins. But non all MVBs have fusion with lysosomes.

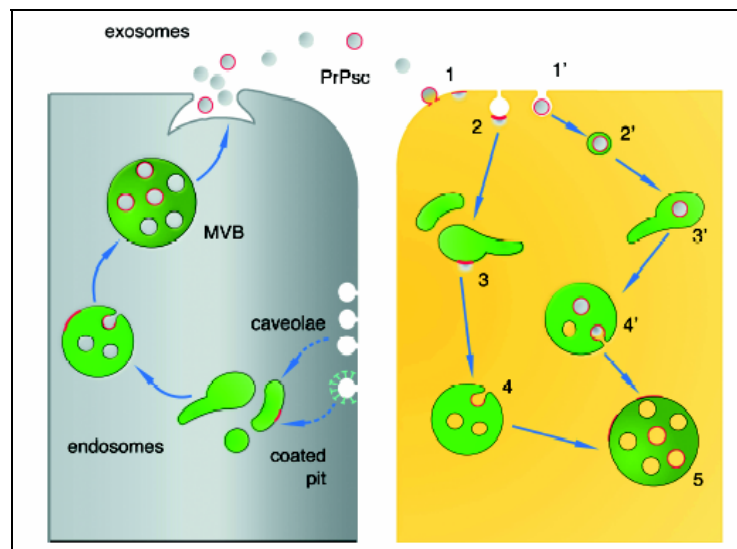


Figure 7 Schematic representation of exosome release from scrapie-infected cells and exosome targeting to recipient cells. PrP^{Sc} (red) transits through endocytic compartments of an infected cell (in gray) and is secreted in association with exosomes in the extracellular milieu. The secreted exosomes could be targeted to noninfected cells (in yellow). As hypothesized in the text, exosomes bearing PrP^{Sc} could fuse with the cell surface (1) which would allow for conversion of PrP^C at the plasma membrane. Internalized PrP^{Sc} (2) could be routed to endosomes (3) and enter ILVs during MVB formation (4 and 5). In an alternative hypothesis, PrP^{Sc}-exosomes could be internalized by the non-infected cell (10, 20) before being transferred to endosomes (30). In forming MVBs, the newly endocytosed exosomes could undergo fusion with the MVB membrane (40). A consequence of such a process would be its incorporation in the limiting membrane (5), permitting conversion of PrP^C in the endocytic pathway (Fèvrier et al., 2005).

Another potential fate in many cell types is fusion with the cell surface. Reticulocytes, which lack lysosomes, were the first cell shown to release their exosomes into the extracellular milieu during exocytic release of MVBs (Fèvrier et al., 2005). Exosomes are secreted by many cell types, including B lymphocytes, dendritic cells, mast cells, platelets, intestinal epithelial cells, melanoma and mesothelioma cells. The protein and lipid composition of exosomes is distinct from that of plasma membrane, reflecting their endosomal origin (Fèvrier et al., 2004). Vesicles with the hallmarks of exosomes are present *in vivo* at the surface of follicular dendritic cells in germinal centres, malignant effusions and serum. These and other studies support the idea that exosomes secretion may be a regulated process and may serve not only as a means to eradicate unwanted molecules, but may also provide vehicles to transfer molecule among cells and even to deliver adhesion signals at distances in normal and pathological states. The association of PrP with exosomal membranes is consistent with features of its intracellular trafficking. Like other membrane proteins, Prp^C is post-translationally processed in the endoplasmic reticulum and Golgi on its way to the plasma membrane.

PrP^C at the surface of numerous cell types is constitutively internalised. Prion protein, in different cell types, appears to be endocytosed by either clathrin-coated pits or caveolae. It could hypothesize that the raft-like nature of exosomal membranes may be a favourable environment for trans-conformation and amyloid fibre formation. PrP^{Sc}, as already said, has been shown to be present at the cell surface of various prion-infected cultured cells. These two sites of accumulation could be reconciled by at least two distinct models. In the first, conversion could occur at the plasma membrane, after which PrP^{Sc} could accumulate in late endosomes and lysosomes due to diversion from a cycling pathway between the plasma membrane and early endosomes.

In the second model, abnormal PrP could be produced in intracellular compartments and then re-exported to the plasma membrane (Fèvrier et al., 2005). However other models must also be considered. PrP^{Sc} has been detected in the nucleus of infected N2a neuroblastoma cells and it has been proposed that conversion of PrP^C to abnormal protein could occur in the cytosol in the absence of membranes. Thus exosomes could constitute vehicles for transmission of the infectious prion protein, bypassing cell-cell contact in the dissemination of prions (Mangé et al., 2004).

As other GPI-anchored proteins PrP^C is normally GPI-anchored to specialized cholesterol-rich domains of the plasma membrane, termed lipid rafts or caveolae. Lipid rafts are sphingolipid- and cholesterol-rich membrane microdomains in the outer leaflet of the plasma membrane. Lipid rafts are first assembled in the Golgi complex in mammalian cells, thus are moved forward to the plasma membrane, where they concentrate but also spread into the endocytic recycling pathways (Simons et al., 2002). The plasma membrane is composed primarily of sphingolipids, phospholipids and cholesterol. Sphingolipids differ from most phospholipids in that they have long, largely saturated acyl chains that allow them to pack tightly in a bilayer, forming a gel phase in which there is very little lateral movement or diffusion. The gel phase of the sphingolipids is altered by the association of cholesterol, which condenses the packing of the sphingolipids by occupying the spaces between the acyl chains. So, cholesterol-containing sphingolipid microdomains exist in a liquid-ordered phase that is significantly more fluid than the gel phase. By contrast, phospholipids are rich in unsaturated acyl chains that tend to be kinked and consequently to pack loosely into a liquid-disordered phase that is considerably more fluid, allowing rapid lateral movement within the bilayer. The different packing of the sphingolipids and phospholipids probably leads to their phase separation in membrane bilayers. Sphingolipid

microdomains float in a phospholipid bilayer, then they lead into the coining of the term 'lipid rafts'(Pierce, 2002).

There is evidence of a role for lipid rafts in a wide array of cellular processes including: trans-cytosis; potocytosis; an alternative route of endocytosis; internalization of toxins, bacteria and viruses; cholesterol transport; calcium homeostasis; protein sorting; and signal transduction. Biochemical analysis of the protein composition of purified lipid rafts in a large number of different cell types shows a striking concentration of signalling molecules within lipid rafts. On the basis of these observations, a role for lipid rafts in mediating signal transduction has been proposed.

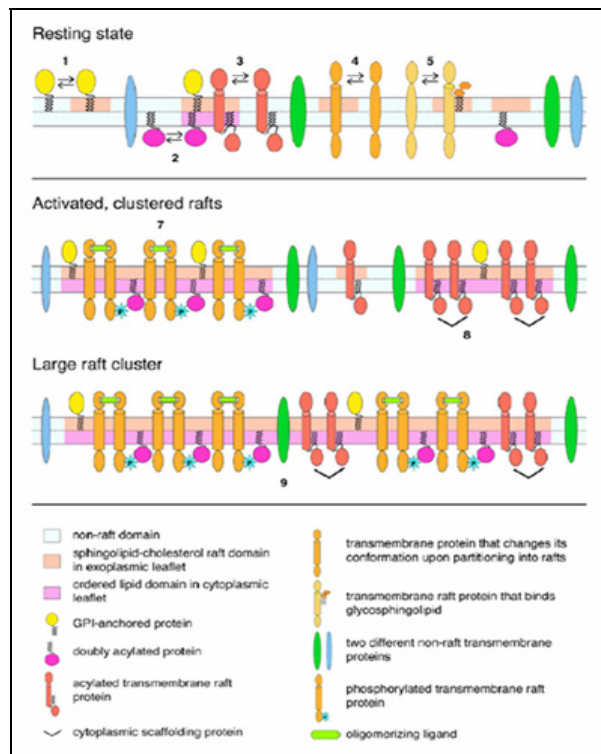


Fig. 8 Resting-state rafts are depicted as dynamic, nanometer-scale assemblies of raft lipids that are metastable (top). These assemblies can form and dissolve again, and proteins and lipids can partition into and out of them. Partitioning of a protein or lipid constituent onto such small raft domains can alter their metastability, effectively bringing them into an "excited" state which favours their growth by diffusion-limited accretion of lipid constituents from the bulk phase or by fusion with other "excited" raft domains. Most raft proteins are either solely lipid anchored, GPI-anchored in the exoplasmic (1) or doubly acylated in the cytoplasmic leaflet (2), or they contain acyl chains in addition to their transmembrane domain (3). A fourth group could undergo a conformational change upon binding to raft lipids, which allows the protein to partition into raft domains (4). Perhaps cytoplasmic raft domains only form opposite an exoplasmic counterpart when there is a slightly more stable assembly of at least a few raft proteins in a given domain (5). Upon oligomerization of raft proteins with themselves (e.g. caveolin), by multivalent ligand (6) or by cytoplasmic scaffolds (7), the minirafts coalesce and become more stable. They may now contain more than one species of raft proteins, as well as cytoplasmic counterparts. These small raft clusters would still have a size below the limits of light microscopic resolution, but could already function as signalling platforms. Large raft clusters are probably only assembled when protein modifications like phosphorylation increase the number of protein-protein interactions, leading to the coalescence of small clusters into larger domains on the scale of several hundred nanometers (8).

In principle, lipid rafts can modulate signalling events in a variety of ways. By localizing all of the components of specific signalling pathways within a membrane compartment, lipid rafts could enable efficient and specific signalling in response to stimuli. Translocation of signalling molecules in and out of lipid rafts could then control the ability of cells to respond to various stimuli. Cross-talk between different signalling pathways could be facilitated if the molecules involved were localized to the same lipid raft. Distinct subpopulations of rafts present on the surface of the same cell might be specialized to perform unique functions. Movement or clustering of lipid rafts could be an efficient means of transporting pre-assembled signalling complexes to specific membrane areas upon stimulation. Formation of higher-order signalling complexes by clustering of one or more types of lipid rafts could allow amplification or modulation of signals in a spatially regulated manner. All of the above mechanisms imply that lipid rafts would play an active role in facilitating efficient and specific signalling. However, lipid rafts might also be involved in negatively regulating signals by sequestering signalling molecules in an inactive state (Zajchowski et al., 2002).

Lipid-rafts are detergent-insoluble regions characterized by the presence of free cholesterol (FC). In normal tissues, with the exception of liver and adrenal gland, approximately 90% of the total cellular cholesterol resides in membrane raft domains as FC, while only a minor amount (approximately 1-10%) is found as cholesterol esters (CE) in a cytoplasmic storage form. Since FC levels are critical to the maintenance of proper membrane fluidity, as well as to the function and/or activation of raft-resident proteins, cells have developed a highly integrated set of homeostatic mechanisms acting in concert to finely control intracellular cholesterol levels. The FC found in cell membranes derives either from *de novo* synthesis in the endoplasmic reticulum, or from lysosomes following receptor-mediated uptake of low-density lipoproteins (LDLs). Membrane FC, however, is in a dynamic state, moving back to the ER in response to changing

homeostatic conditions in the cell. In fact, if cholesterol in the rafts exceeds threshold levels, its excess is promptly transferred to the ER, where it might elicit high-order responses from the control proteins embedded. FC in the ER blocks the function of its cellular sensor, the SREBP-cleavage-activating-protein (SCAP), which is needed for the proteolytic activation of the sterol-regulatory-element-binding-proteins-2 (SREBP-2), a transcriptional factor that promotes the expression of genes involved in cholesterol synthesis [i.e. hydroxyl-methyl-glutaryl-coenzyme A-reductase (HMGCoA-R)] and uptake (i.e LDL receptor). Free cholesterol in the ER can be either used in the assembly of newly formed membrane rafts, and returned to the plasma membrane through vesicular transport, or, if in excess, converted to CE by the Acyl-cholesterol-acyl-transferase (ACAT) and stored in the cytoplasm as neutral lipid droplets. Viceversa, when cell membranes need FC, or when CE droplets exceed a critical threshold value, CE can be reconverted to FC by the neutral-cholesterol-ester-hydrolase (nCEH). FC can then be recycled to the membranes by cholesterol binding protein, such as caveolin-1 (Cav-1), where it can be used to replenish raft domains, or if in excess and where it delivered to extracellular acceptors, namely high-density lipoproteins (HDL), via the ATP-binding-cassette-sub-family A-member 1 (ABCA-1) transporter.

Complex signalling networks are responsible for controlling important cellular functions such as growth, differentiation, adhesion, and motility, and unregulated signalling can lead to many different diseases. Due to their importance in regulating signal transduction, it is not surprising that lipid rafts have been implicated in a wide variety of disorders.

For example: mutations in an isoform of caveolin (caveolin-3) have been linked to a form of limb girdle muscular dystrophy; generation of the β -amyloid peptide from the amyloid precursor protein in Alzheimer's disease has been shown to occur in lipid rafts in a cholesterol-dependent manner. Similarly, efficient

processing of the scrapie isoform of the prion protein requires its targeting to lipid rafts by GPI anchors (Zajchowski et al., 2002).

Increasing evidence indicates that subtle intracellular cholesterol changes affect the intracellular processing/trafficking, function and/or activation of raft-resident proteins, including PrP.

It is important to point out pathological, genetic and mechanistic similarities between TSE and Alzheimer's disease. Alzheimer's disease (AD) is characterized by the presence of extracellular senile plaque and intracellular neurofibrillary tangles within the afflicted brain. The major constituents of senile plaques are the amyloid β ($A\beta$) peptides, which are derived from the proteolytic processing of the amyloid precursor protein (APP). In the amyloidogenic pathway, β -secretase cleavage of APP, on the cell surface and in early endosomes after APP internalisation by clathrin-coated vesicles and/or caveolae. Product of β -secretase cleavage is a soluble N-terminal fragment sAPP, along with a short membrane-bound C-terminal fragment that is subsequently cleaved by γ -secretase (a multi-proteins complex) to release the $A\beta$ peptide. In the alternative, non-amyloidogenic pathway, α -secretase cleaves APP within the $A\beta$ sequence, thus precluding the formation of $A\beta$, and releases a soluble N-terminal fragment sAPP α . α -secretase activity was shown to be associated with members of the ADAM (a disintegrin and metalloprotease) family, particularly ADAM9, 10 and 17. As APP, PrP^C is shed from the cell surface by zinc metalloproteases and is subject to endoproteolysis by ADAM10 and ADAM17. Furthermore, it has been recently shown that PrP^C inhibits the β -secretase cleavage of APP and reduces $A\beta$ formation. This effect is lost in scrapie-infected mouse brain or in cells expressing mutants of PrP associated with human prion disease. The regulation of β -secretase requires PrP^C to be located in lipid rafts and is mediated by the N-terminal polybasic region of PrP^C interacting with β -secretase via glycosaminoglycans (Parkin et al., 2007, Zhang et al., 2006).

Generally Alzheimer disease and Prion diseases are included among rafts neurodegenerative diseases, since the aberrant proteolytic processing of PrP and A-Beta occur in these lipidic membrane domains. Therefore, it is most likely that alterations in the composition of lipid rafts can lead to a variety of disease of lipid metabolism.

Diagnosis and Therapeutic Approaches to Lipid-Rafts Diseases

The ability to secure early diagnosis is vital for therapeutic interventions to be real value. With respect to animals destined for the human food chain, there is the additional demand to determine presence of the prion agent in tissues in asymptomatic organisms, well before the appearance of any clinical symptoms. This applies equally to the detection of prions in humans, who may participate in tissue donation programs (Aguzzi et al., 2004)

Although there have been clinical trials with allegedly prionostatic compounds, the bitter truth is that there is not proven treatment for human prion diseases. Several approaches are currently being investigated to uncover therapeutic mechanisms that prevent the development of prion diseases. These fall into 2 distinct classes of strategies:

- post-exposure prophylaxis, which is aimed at halting the transport of prions to the central nervous system (CNS) following peripheral uptake of the infectious agent.
- curative or palliative. Neurodegenerative diseases, such as prion diseases and Alzheimer's diseases, cause substantial damage to the CNS, thus, the only way to cure a human disease that has manifested itself in the form of a dementia is the replacement of damaged CNS tissue through regeneration or transplantation. Although therapies (i.e. based on stem cells) are still in the experimental phase

- of development, there is hope that protocols based on this technology might be able to cure some of the symptoms elicited by human neurodegenerative diseases.

Palliative approaches, on the other hands, do not have the pretense to cure the causative disease but rather to prolong survival or the decelerate the decline of cognitive functions. Research in this field tends to focus on compounds preventing -directly or indirectly- misfolding of PrP^C to PrP^{Sc} in the case of Prion diseases, or misfolding of APP to A β peptide in case of Alzheimer's disease (Glatzel et al., 2005).

Various compounds are known to interact with PrP^{Sc}, these include anthracycline, Congo red, dextran sulfphate, pentosan polysulphate, and other polyanions and β -sheet breaker peptides. Unfortunately, most of these compounds are only effective if administered well before the onset of clinical disease, and frequently also show either high levels of toxicity, low levels of bioavailability, or both. Although the ability to bind PrP^{Sc}, and prevent further conversion of PrP^C, provides a logical approach to slowing or preventing disease progression, it is unlikely to lead to a cure for prion diseases. The development of therapies that provide a cure will need a clearer understanding of the role of PrP^C, as well as advances in early diagnosis. Currently clinical trials using quinacrine and chlorpromazine treatment in CJD and vCJD patients are underway in both the UK and USA. However, there is no evidence that these drugs are useful against prion disease *in vivo*, and recently quinacrine treatment in a rodent model of CJD demonstrated no efficacy. This has highlighted the difficulty of transferring *in vitro* experiments to the clinical setting. (McKintosh et al., 2003)

Complete knowledge of PrP^C trafficking and biosynthesis and of the conversion of PrP^C to the pathogenetic PrP^{Sc} isoform are fundamental for the elaboration of potential strategies for drug discovery.

One of these approaches involves the inhibition of PrP^C synthesis by antisense oligonucleotides or ribozymes targeted to PrP mRNA and is supported by work on knockout MoPrP mice demonstrating that these animals are resistant to prion disease.

Other researches have worked on the stabilization of the PrP^C molecule making the conformational change energetically less favorable; other strategies involve blocking PrP^C synthesis or stabilizing this PrP isoform, and the manipulation of the PrP^{Sc} molecule. For instance, drugs that destabilize PrP^{Sc}, making it protease sensitive, might enhance clearance of the latter. An important feature for the development a therapeutic treatment for TSE diseases is the conversion of PrP^C to PrP^{Sc}, compounds that bind to PrP^{Sc} and prevent it from serving as a template for the replication of nascent PrP^{Sc} could be effective. Another strategy contemplates prevention of binding of protein X to a PrP^C intermediate (PrPI), thereby inhibiting the conversion of PrP^C to PrP^{Sc} (Koster T. et al.; 2003).

Currently is really difficult diagnostic ante-mortem a prion disease: examination of brain sample is required to confirm the presence of TSE or other neurodegenerative diseases. Biochemical tests using brain samples are based on differential sensitivity of PrP^C vs. PrP^{Sc} to PK digestion. After digestion the PrP^{Sc} may be assayed by conventional techniques such as Western blotting or ELISA. However there is necessity to identify markers allowing early diagnosis in humans as well as in animals, before symptoms development.

A significant advance in prion diagnostics was accomplished in 1997 by the discovery that protease-resistant PrP^{Sc} can be detected in tonsillar tissue of vCJD patients. Furthermore, there have been reports of individual cases showing detection of PrP^{Sc} at pre-clinical stages of the disease in tonsil as well as in the appendix, indicating that lymphoid tissue biopsy may be useful for diagnosing presymptomatic individuals. PrP^{Sc}-positive lymphoid tissue was long considered to be a vCJD-specific feature that would not apply to any other forms of human

prion diseases. However, a recent survey of peripheral tissues of patients with sporadic CJD has identified PrP^{Sc} in as many as one-third of skeletal muscle and spleen samples, as well as the olfactory epithelium of patients suffering from sCJD (Aguzzi et al., 2004)

To date, prion infectivity has not been described to occur in saliva, nasal secretion, respiratory aerosols, urine or feces in animals with naturally occurring scrapie infections. However it has recently been shown that prions are present in the saliva of deer with chronic wasting disease. It has been also found that in sheep with scrapie, PrP^{Sc} deposits are associated with acinar and ductal epithelial cells of the salivary glands and can be detected in gland secretion (Vascellari et al., 2007).

Unfortunately PrP^{Sc} is not always easily detectable in prion diseases. Therefore, the development of highly sensitive assays for biochemical detection of PrP^{Sc} in tissues and body fluids is a top priority. One route to achieve this goal is to develop high-affinity immuno-reagents that recognize PrP^{Sc}. Examples include the 'POM' series of antibodies that recognize various well-defined conformational epitopes in the structured C-terminal region of PrP^C, and linear epitopes in the unstructured N-terminal region. Because of the particular nature of the epitopes to which they are directed, some of these antibodies have affinities for the prion protein in the femto-molar (10^{-15}) range. Protein misfolding cyclic amplification (PMCA) is also a promising method for the sensitive detection of the pathological prion protein. This method relies on the principle of disrupting large PrP^{Sc} aggregates by sonication to generate multiple smaller units. PMCA was recently shown to increase sensitivity 6,600-fold over standard detection methods. Amplifiable PrP^{Sc} was detected in the blood of scrapie-infected hamsters by PMCA during most of the pre-symptomatic phase of disease. Several research efforts have also been directed at identifying proteins that are differentially expressed in the tissues of prion-infected

animals compared with disease-free control animals. Ideally, these surrogate markers should be detectable at preclinical stages of disease and be differentially expressed in easily accessible body fluids such as blood or urine. So far, only one extra-neural gene -the erythroid differentiation related factor- has been identified that is differentially expressed during prion infection of experimentally infected mice, cattle with BSE and sheep with clinical scrapie. The identification of additional surrogate markers would certainly be useful, particularly if they are detectable in body fluids (Aguzzi et al., 2006).

Aim of the Research

As described above, Prion disorders as well as Alzheimer's disease are examples of neurodegenerative disorders whose pathogenesis involve brain deposition of amyloid/misfolded proteins. Several lines of evidence indicate the lipid rafts are at play modulating the amyloidogenic processes and that cholesterol, main component of the raft, is a key molecule (Howland et al., 1998, Gilch et al., 2006). The exact nature of its role, however, remains unclear. Studies on the ability of cholesterol biosynthesis inhibitors (i.e. statins) to inhibit the protein misfolding process gave conflicting results and inconclusive information on their mode of action. While in cell-based prion systems, statins (i.e. squalestatine and lovastatine) were able to inhibit generation of the scrapie-like prion protein (Bate et al., 2004), in experimentally scrapie-infected mice they were reported to prolong the survival time (Mok et al., 2006) by an anti-inflammatory neuroprotective effect, rather than via reduction of brain cholesterol (Kempster et al., 2006). In AD, despite a huge literature on the matter, the reported results are again inconclusive. Numerous evidences suggested that enzymes that generate A β function best in a high-cholesterol environment, possibly by shifting APP metabolism from alfa- to beta-cleavage products (Simons et al., 2002-Arispe et al., 2002). These and other results suggested a mechanism for the observed lowering risk for Alzheimer's disease among the population taking statin therapy and served as a rationale for the use of statins as novel therapy for AD (Jick et al., 2000, Klafki et al., 2006). Statins, by lowering cellular cholesterol content, were suggested to lead to disaggregating membrane raft domains in the Golgi network, thus reducing the collision frequency between β -secretase (BACE) and its substrate APP. However, other studies reported that another statin, atorvastatin, was able to reduce production of neuritic plaque without crossing the blood-brain barrier to a significant degree, raising the possibility that atorvastatin, and statins in general, might protect against AD via

a peripheral mechanism (Wolozin, 2002), perhaps because of their pleiotropic effects on endothelial cell function and as suppressors of inflammation (Maxfield et al., 2005). Finally, in a recent retrospective study, statins were reported to increase the severity of clinical symptoms in AD patients (Rea et al., 2005).

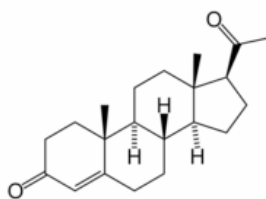
Despite these contrasting findings, there is no doubt that cholesterol influences the processing/trafficking of PrP and APP, and in general of all the raft-resident proteins. Since, as already mentioned, cellular cholesterol is present either as FC, in the membranes, and as CE, in cytoplasmic droplets, we thought possible that, rather than absolute cholesterol level, changes in the cellular content of FC vs. CE pools could be responsible for normal vs. aberrant processing of membrane proteins. In this respect, it is worth to remind that in normal conditions only 5-10% of total cellular cholesterol is in the storage CE form, while more than 90% is in membranes as FC. However, none of the studies on cholesterol and statins discriminated between the different cholesterol pools, limiting analysis to the changes in total cholesterol content.

On these premises, the aim of my research project was to investigate whether cell susceptibility to prion infection/replication was somehow related to alterations in the intracellular cholesterol homeostasis, and in particular whether unbalanced FC vs. CE pools could be found in prion infected/prion susceptible cells with respect to cell conditions of no infection or genetic resistance. To this purpose, I investigated the intracellular content of CE in two prion cell models, namely uninfected and prion-infected mouse neuroblastoma cell lines and ex vivo skin fibroblasts from uninfected and scrapie-affected sheep, and evaluated the effect of a panel of drugs targeting different steps of cholesterol metabolism/trafficking on both CE levels and PrP^{res} formation.

The drugs used were the steroid hormone Progesterone and the calcium-blocking Verapamil that target the MDR1-PgP mediated cholesterol transport from the plasma membrane to the endoplasmic reticulum; Sandoz 58-035, a known inhibitor of the enzyme ACAT-1 which catalyzes the esterification of cholesterol moiety to free fatty acids in the endoplasmic reticulum; owing to its considerable cytotoxicity it can't be used in therapy, but usually it used in research; Pioglitazone, a drug used in the treatment of non-insulin-dependent diabetes mellitus that appears to induce intracellular redistribution of free fatty acids; Everolimus, an immunosuppressant agent that have been reported to inhibit cholesterol esterification in our and other laboratories.

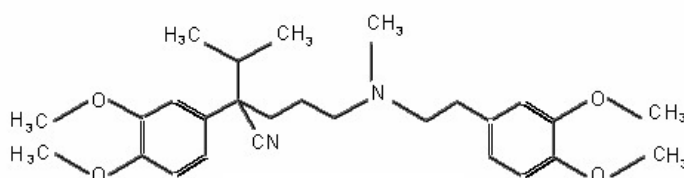
Brief description of properties and clinical use of tested drugs

Progesterone is a C-21 steroid hormone, belonging to the class of progestogens, involved in the female menstrual cycle, pregnancy (supports *gestation*) and embryogenesis of humans and other species. Like other steroids, progesterone consists of four interconnected cyclic hydrocarbons. Its molecule contains ketone and oxygenated functional groups, as well as two methyl branches. Like all steroid hormones, it is hydrophobic. This is mostly due to its lack of very polar functional groups. Normally cells acquire cholesterol through endogenous synthesis and through receptor-mediated uptake of cholesterol-rich low density lipoprotein (LDL). Esterification of LDL-derived cholesterol is catalyzed by acyl-CoA:cholesterol acyltransferase (ACAT) in the endoplasmic reticulum (ER). Progesterone, which has been reported to induce cholesterol storage in the endosomal/lysosomal system (Klingenstein et al., 2006), inhibits esterification, and, although its mechanism of action is not completely understood, this inhibition depends on its ability to influence P-glycoprotein's activity (Metherall et al., 1996).



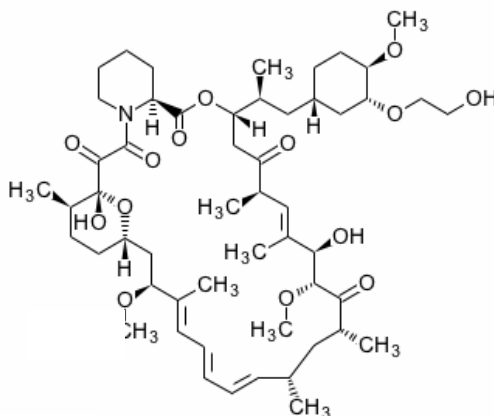
Progesterone

Verapamil is a drug that acts as L-type calcium channel blocker. It is used in the treatment of hypertension, angina pectoris, migraine and some types of arrhythmia. This drug is also used in cell biology as an inhibitor of drug efflux pump proteins such as P-glycoprotein (MDR-1) and is presently evaluated in clinical trials for ability to reverse MDR in cancer patients. (Wikipedia)



Verapamil

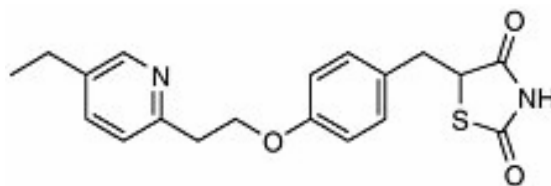
Everolimus is a selective immunosuppressive agent, it is used in organ reject prophylaxis, often in combination with cyclosporine. This drug is also a substrate for the P-glycoprotein.



Everolimus

The medication class of Thiazolidinedione, which includes Pioglitazone, was introduced in the late 1990s as an adjunctive therapy for diabetes mellitus (type II) and related diseases. Thiazolidinediones act by binding to PPARs (Peroxisome Proliferators-Activated Receptors), a group of receptor molecules inside the cellular nucleus, specifically PPAR. The normal ligands for these receptors are Free Fatty Acids (FFAs) and eicosanoids. When activated, the receptor migrates to the DNA, activating transcription of a number of specific genes. By activating PPAR:

- insulin resistance is decreased
- adipocyte differentiation is modified
- VEGF-induced angiogenesis is inhibited
- leptin levels decrease (leading to an increased appetite)
- levels of certain interleukins (e.g. IL-6) fall
- adiponectin levels rise
- Pioglitazone also acts on cholesterol metabolism by capturing fatty acids which were to be esterified with cholesterol molecules, thus influencing cholesterol esterification.



Pioglitazone

RESULTS

Standardization of the protocols for PrP^C and PrP^{res} detection

The cell lines used in this study were mouse neuroblastoma cells (MNB), uninfected and persistently infected with the 22L or RML mouse adapted scrapie strains. These cell lines represent largely used *in vitro* cell models to study prion infection and replication, as well as to identify novel prion inhibitors.

Briefly, RML (Chandler) is a mouse brain adapted scrapie strain (Ward et al., 1974) while the 22L-infected cells were developed by re-infection of RML-infected mouse neuroblastoma cells cured by several passages in 1 μ g of pentosan polysulfate/ml of medium (Kocisko et al., 2003). The cured cells were re-infected by incubation with PrP^{Sc} purified from mouse brains infected with the scrapie strain 22L. Neuroblastoma cells reinfected with 22L have stably expression of PrP^{res} for over 100 passages.

Scrapie infected MNB cells (22L-N2a and RML-N2a), the parental non-infected cell line (N2A) and the protocols used for the immunodetection of PrP^C and PrP^{Sc}, were kindly provided by Dr Byron Caughey from the NIH/NIAID Rocky Mountain Laboratories, USA. These protocols have been adapted to our experimental conditions. Although, NIH protocols indicated the use of an enhanced chemiofluorescent agent (AttoPhos; Promega) for the detection of the prion protein, in our hands Attophos didn't allow an adequate visualization of the PrP protein. Therefore, we decided to use an enhanced chemiluminescent immunodetection technique (ECL). Consequently, in order to visualize the results we used autoradiography and the Scion Image NIH software to quantify the intensity of PrP^C PrP^{res} signal. The primary antibody 6H4 (Prionics) was used, whereas for secondary antibody and the modality of washes of the PVDF membrane, ECL indications were followed.

Western and dot blotting analysis of PrP in parental and prion-infected 22L-N2a and RML-N2a cells, revealed the presence of PK-resistant (PrP^{res}) only in lysates from the infected cells (Fig9 A and B).

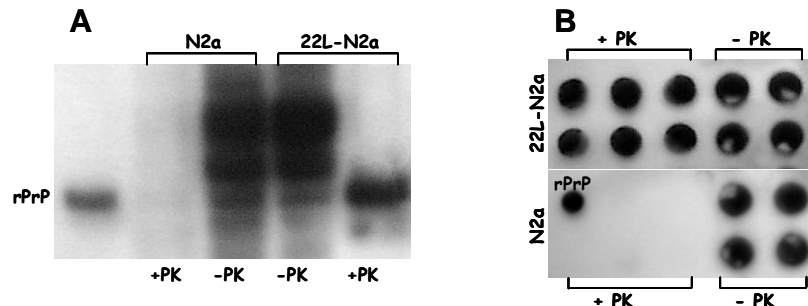


Figure 9. Detection of PrP_c and PrP^{res} in N2a and 22L-N2a mouse neuroblastoma cell lines. Lysate from uninfected and 22L scrapie-infected N2a cell lines were digested or not with PK, 20 µg/ml, and analyzed with 6H4 antibody by Western (A) and Dot (B) blotting procedures (see methods). As control, 0.05 µg of recombinant mouse PrP (rPrP, Prionics) was used.

Cholesterol esters content and effect of cholesterol modulators in mouse neuroblastoma cell lines

To investigate whether prion-infected cells showed an alteration in cholesterol homeostasis, I compared the levels of CE in the N2a cell line with that of the two persistently scrapie-infected sublines, 22L-N2a and RML-N2a, by using the colorimetric Oil Red O (ORO) method which selectively stains cellular neutral lipids (CE) in red color. Interestingly, these experiments revealed markedly higher CE levels in the prion-infected cell lines as compared to the uninfected cells (Table 2).

Cell line	Mean red stain/cell ± SE	
	24 hours	48 hours
N2a	3.1 ± 1.0	4.2 ± 1.5
22L-N2a	8.4 ± 1.8	12.3 ± 1.8
RML-N2a	7.9 ± 1.2	9.8 ± 1.7

Table 2 Neutral lipid content in uninfected and prion-infected mouse neuroblastoma cell lines. Cells seeded at 2x10⁵/ml were stained for intracellular neutral lipids with the ORO method at the indicated times. Values represent the mean ± standard error of red colour intensity per cell. Experiments were performed in duplicate and repeated at least three times. N2a: parental cells; 22L-N2a: N2a subline infected with 22L prion strain; RML-N2a: N2a subline infected with RML prion strain.

In fact, as can be seen (Fig. 10) from the higher red intensity of individual cells as well as from the higher number of more intensively stained cells of the population, CE accumulation was greater in the 22L-N2a than in RML-N2a cells, with 3-fold and 2-fold higher CE levels than N2a cells, respectively.

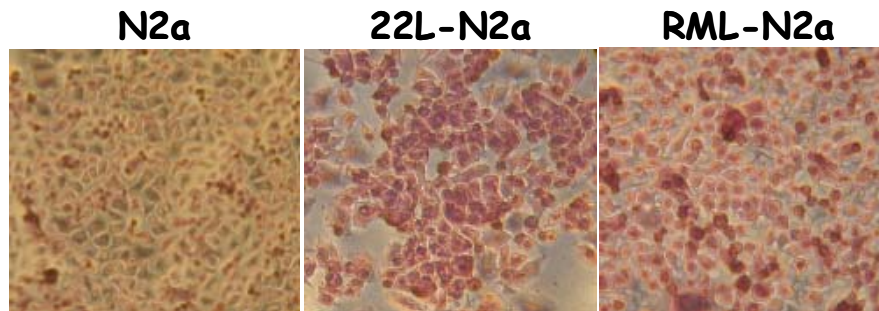
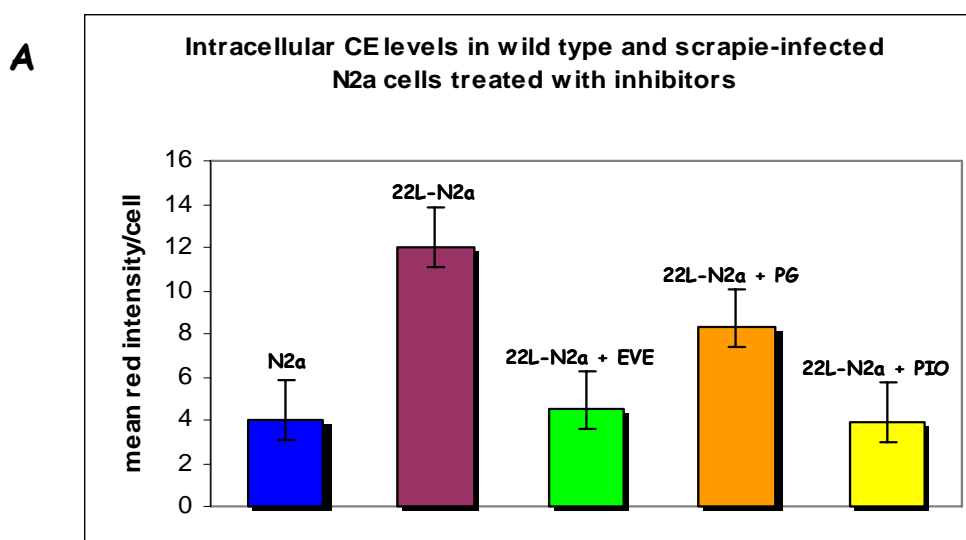


Figure 10. Representative pictures of N2a cultures and N2a cell lines persistently infected with the 22L and RML strain of scrapie. Intracellular neutral lipids were stained with the oil red O method and photographed. (see Materials and Methods for detail)

The experiments performed to investigate the effect of treatments with drugs known to affect cholesterol esterification by targeting different steps of cholesterol metabolism, such as Everolimus (EVE), Pioglitazone (PIO), and Progesterone (PG), showed that, although at different extent, all drugs were able to reduce the CE content in the prion-infected cells. (Figs. 11A and 11B)



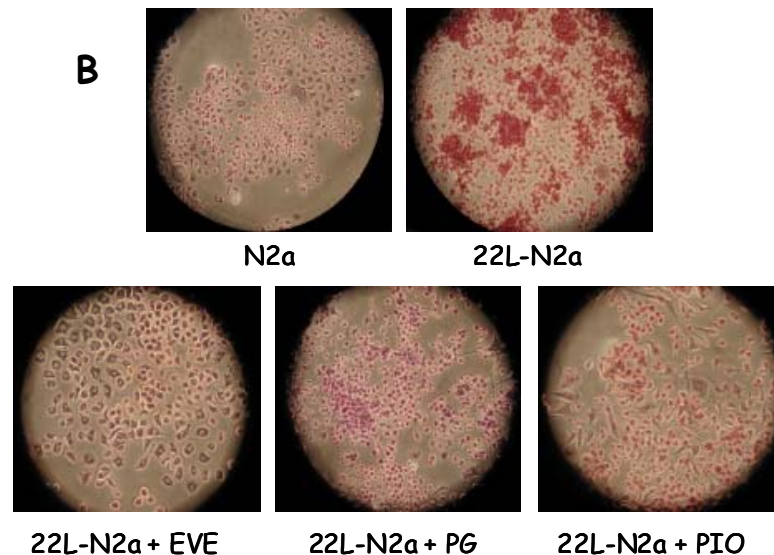


Figure 11. Neutral lipid content in N2a and 22L-N2a mouse neuroblastoma cell lines. A: quantification of the intensity of the lipid-bound stain color was determined by densitometric analysis through the Scion Image software (NIH). B: intracellular neutral lipids in N2a and 22L-N2a cells were stained by the ORO method after 48-hour incubation in the absence or in the presence of Everolimus (EVE, 50 nM), Progesterone (PG, 30 μ M) and Pioglitazone (PIO, 40 μ M). Values represent means \pm SE of quadruplicate determinations from triplicate experiments. * $P < 0.05$ vs 22L-N2a cells.

Anti-prion activity of cholesterol modulators in mouse neuroblastoma 22L-N2a cells.

The results above reported clearly indicated a relationship between prion infection/replication and the presence of an altered regulation of cholesterol metabolism. Therefore, to address more directly this apparent relation, I investigated the influence of modifications in the intracellular cholesterol distribution (i.e. CE) on the PrP misfolding by evaluating the effect of a wider panel of cholesterol modulators on the production of PrP^{res}. Treatments of 22L-N2a cells with Everolimus (EVE), Pioglitazone (PIO), Progesterone (PG), Verapamil (VP) and Sandoz 58-035 (SaH), led to PrP^{res} inhibition in a dose-dependent manner (Fig. 12). Similar results were obtained in the RML-N2a cell line (not shown).

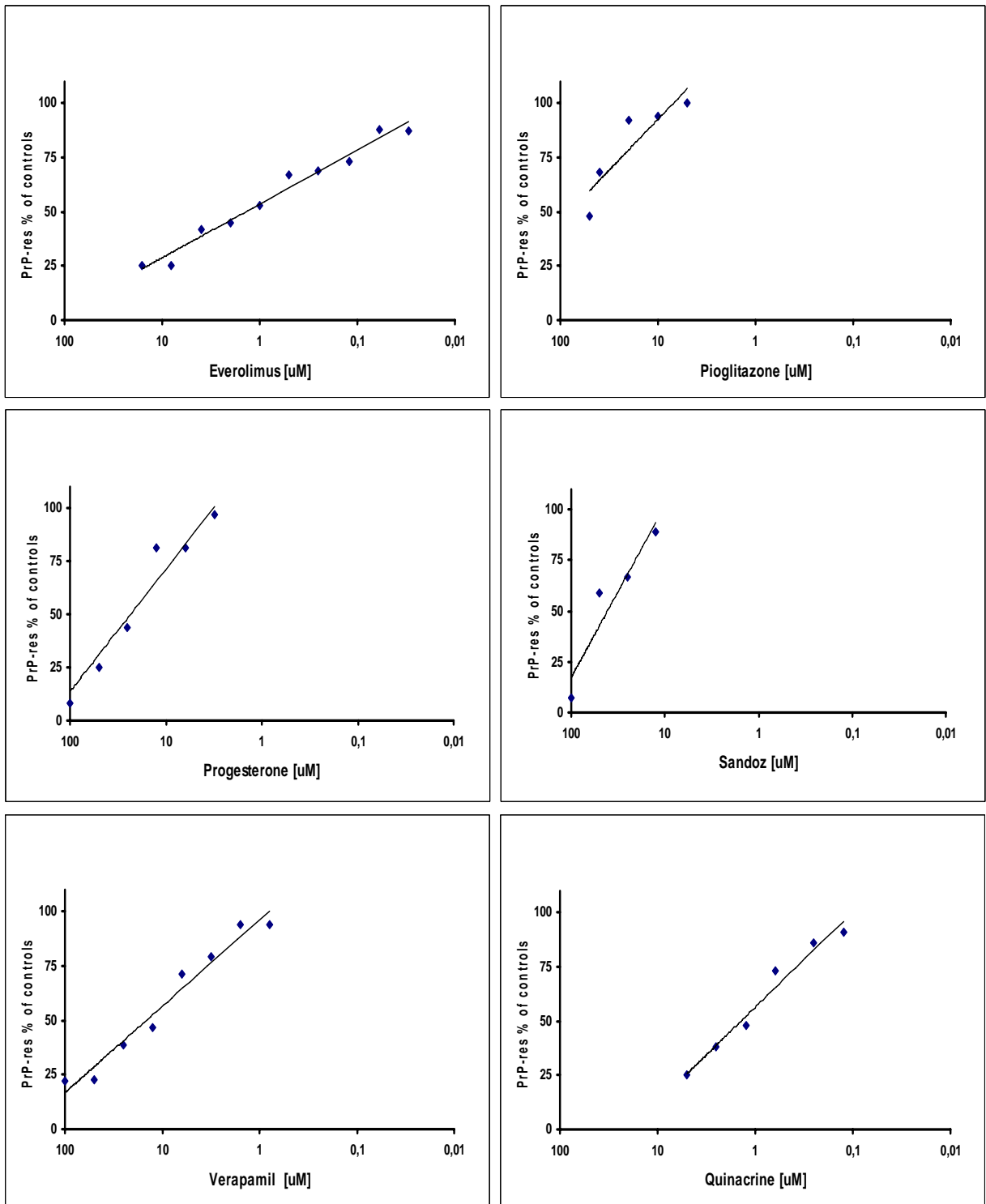


Figure 12 Dose-response curves of anti-prion activity of cholesterol modulators and known prion inhibitor. 22L-N2a cells seeded in growth medium at approximately 5.000 cells/well in 96-well plates were incubated in the absence and in the presence of serial dilutions of the different drugs. After four days cells were lysed and digested with Proteinase K (PK, 20 $\mu\text{g}/\text{ml}$). PrPres in lysates was analyzed by Dot blot procedure with 6H4 antibody and quantized (see methods). The mean value of PrPres at each drug concentration (four wells/concentration) was expressed as percentage of untreated cultures and the concentration resulting in 50% inhibition (EC_{50}) was determined by linear regression analysis. Each drug was tested at least three times.

Although none of the drugs proved either very potent or very selective prion inhibitor, some of them showed anti-prion activity at non-cytotoxic concentrations with EC₅₀ values ranging between 1.4 μM and 40 μM (Table 3). The most potent and selective was Everolimus (EC₅₀ 1.4 μM vs. CC₅₀ >16 μM) followed by Verapamil (EC₅₀ 15 μM vs. CC₅₀ ≥ 100 μM), Progesterone (EC₅₀ 35 μM vs. CC₅₀ 95 μM), and Pioglitazone (EC₅₀ 40 μM vs. CC₅₀ 60 μM), whereas Sandoz 58-035 showed anti-prion activity at concentrations close to the CC₅₀. The dose-response curve of the anti-prion activity of the known prion inhibitor quinacrine (Fig 12), and its 50% inhibitory and cytotoxic concentrations (Table 3), were obtained under the same experimental conditions for comparative purposes.

Cholesterol modulator	CC₅₀ [μM]	PrPres EC₅₀ [μM]	Selectivity Index
Everolimus	> 16	1.4 ± 0.8	11.4
Pioglitazone	60 ± 11	40 ± 5.4	1.5
Progesterone	96 ± 18.6	35 ± 7.8	2.7
Verapamil	≥ 100	15 ± 4.6	6.7
Sandoz	68 ± 8.4	40 ± 6.4	1.7
Androsterone	100	> 100	< 1
Bezafibrate	> 200	> 200	≤ 1
Quinacrine (Control)	4.5 ± 1.3	1 ± 0.9	4.5

Table X. Comparative cytotoxicities and anti-prion activity of cholesterol modulators and prion inhibitor in 22L-N2a cell line. a Values are the mean ± standard deviation of three or four independent experiments.

Cholesterol ester levels in *ex vivo* skin fibroblasts from sheep with susceptible or resistant prion genotype and comparative inhibition by cholesterol modulators.

The ability shown by the cholesterol modulators to parallel inhibition of PrPres to the reduction of CE levels in the prion-infected mouse neuroblastoma cell lines, together with the observed difference in the CE levels of infected vs. uninfected cell lines, prompted us to investigate whether similar differences in CE levels and in the effect of the above drugs could also characterize cells known to carry a susceptible vs. a resistant genotype of the prion protein. To this end, I prepared *ex vivo* cultures of fibroblasts from dermal biopsies of sheep with a susceptible (ARQ) genotype, both uninfected and scrapie-affected, and with a resistant (ARR) genotype. After isolating spindle cells, skin fibroblast cultures were maintained in active growth as described in the Material and Method section, and were used in the experiments between second and fourth passage.

In untreated cultures, the basal content (time 0) of CE was higher in the skin fibroblasts from the susceptible animals than in those carrying the resistant genotype (Fig. 13 A-C, compare controls). Interestingly, cells carrying the same prion susceptible genotype (ARQ) displayed different basal levels of CE according to the presence or absence of scrapie disease: cultures from scrapie-affected sheep showed higher CE content than cells from susceptible but uninfected sheep (Figs 13 B and C). With respect to cultures from scrapie-resistant animals, CE levels increased with incubation time more in skin fibroblasts from all animals carrying the prion susceptible genotype: after 72 hours incubation, the cultures from scrapie-affected sheep doubled the basal CE level (Fig. 13 C).

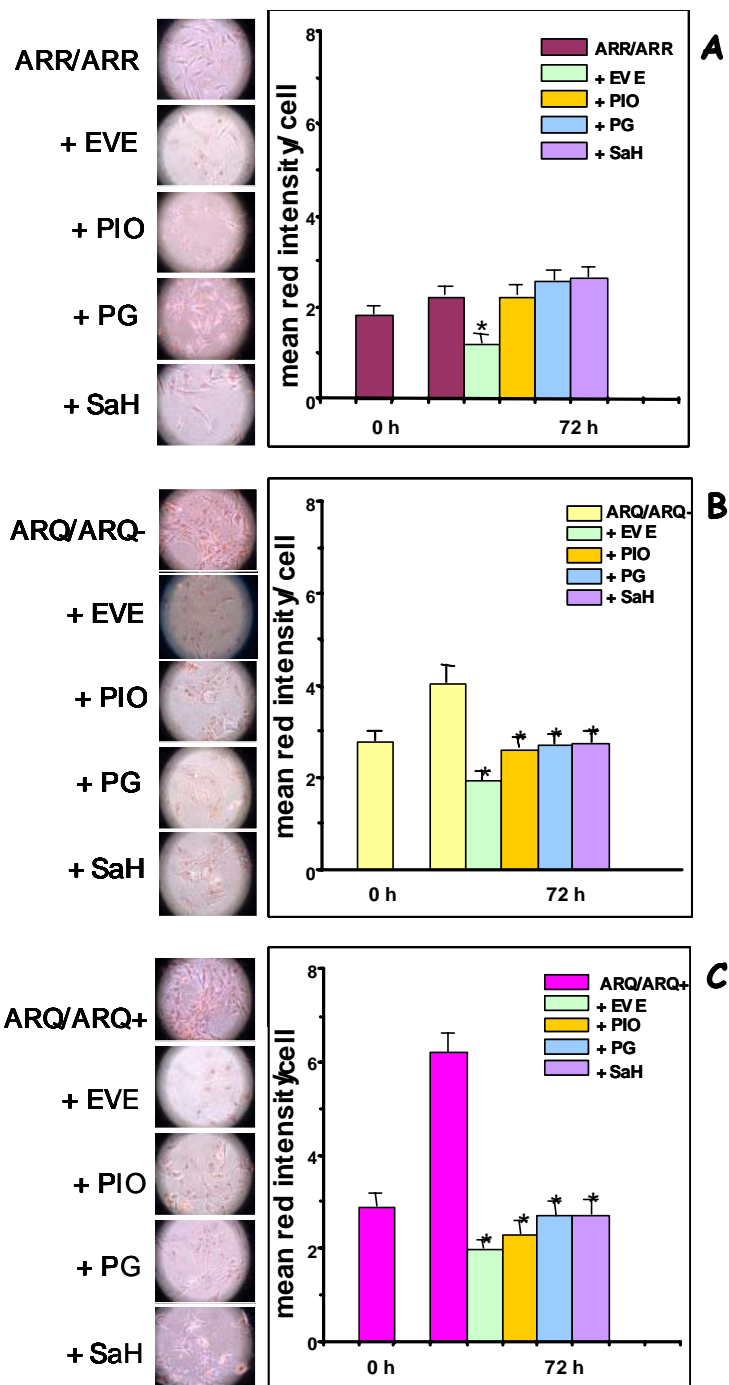


Figure 13. Neutral lipid content of skin fibroblast cultures from sheep with scrapie-susceptible or scrapie-resistant genotype treated *in vitro* with modulators of cholesterol metabolism. Skin fibroblasts were grown in the absence or presence of Everolimus (EVE, 50 nM), Pioglitazone (PIO, 40 μ M), Progesterone (PG, 20 μ M), Sandoz 58-035 (SaH, 40 μ M). The cells were stained by the ORO method and processed for determination of neutral lipid content. Quantification of the intensity of lipid bound red color was determined by densitometric analysis through the Scion Image software (NIH). Values represent the mean \pm SEM of red stain per cell of triplicate determinations from at least three independent experiments with each group of cultures. (A) Skin fibroblast cultures from 4 sheep with scrapie-resistant (ARR/ARR) genotype; (B) Skin fibroblast cultures from 2 sheep with scrapie-susceptible (ARQ/ARQ-) genotype; (C) Skin fibroblast cultures from 8 scrapie-affected sheep with scrapie-susceptible (ARQ/ARQ+) genotype; * $P < 0.05$ was considered statistically significant ($P < 0.05/4 = 0.0125$ after Bonferroni correction). $SP < 0.05$ vs ARQ/ARQ+.

As far as the effect of cholesterol modulators is concerned, in the skin fibroblasts from resistant (ARR/ARR) sheep, only Everolimus (50 nM, EVE) was able to reduce CE levels by about 50%, while Pioglitazone (40 μ M, PIO), Progesterone (20 μ M, PG) and Sandoz 58-035 (40 μ M, SaH) did not show any ability to significantly affect CE content (Fig. 13A).

In fibroblasts from the unaffected-susceptible (ARQ/ARQ-) sheep, all drugs exerted a significant inhibitory effect, ranging from 30% to 50% (Fig. 13B). By contrast, incubation of skin fibroblasts from scrapie-affected (ARQ/ARQ+) sheep in the presence of the cholesterol modulators led to a reduction of intracellular CE levels from about 50% to about 70% (Fig. 13C). In all cultures, Everolimus emerged as the most potent of all inhibitors in terms of effective concentrations (50 nM vs. 20-40 μ M).

Cav-1 and PrP expression in sheep skin fibroblasts.

The results obtained in the two cell-based models of cell susceptibility to prion infection and/or prion replication, i.e. prion-infected vs. uninfected cell lines and scrapie-susceptible/scrapie-affected vs. scrapie-resistant sheep fibroblasts, clearly indicated the presence of a tight connection linking an altered distribution of cellular cholesterol to the susceptibility to prion infection as well as to prion genotype. Given that any biochemical modification in the cellular metabolism is dependent on changes in the expression of the proteins involved in that particular pathway, I evaluated in the skin fibroblast cultures with the different genotypes whether the observed differences in cholesterol distribution (i.e. CE) would also correlate with changes in the gene expression of PrP and Cav-1. It is worth to remind that, as is the case of PrP, also Cav-1 is a raft-protein, its specific function being that of mediating the transport of excess FC from the endoplasmic reticulum to the surface for cholesterol efflux via HDL. With respect to ARR/ARR fibroblasts,

ARQ/ARQ cells associated lower mRNA levels of Cav-1 to higher mRNA levels of PrPc, both at time 0 and after 72 hours of growth (Fig. 14).

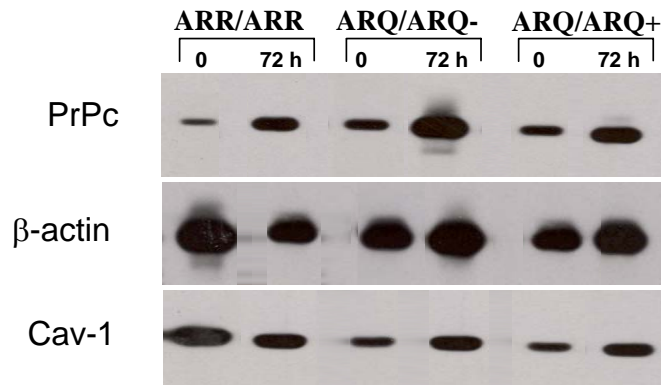


Figure 14. PrPc and Cav-1 mRNA levels in cultured skin fibroblasts from sheep carrying scrapie-resistant (ARR/ARR) or scrapie-susceptible (ARQ/ARQ) genotypes, with (ARQ/ARQ+) or without (ARQ/ARQ-) clinical Scrapie. Analysis were performed at the time of seed (0 hr) and 72 hrs later. The upper parts of panels A and B shows representative autoradiograms of RT-PCR analysis for PrPc and Cav-1, respectively. figure shows densitometric analysis of autoradiographic bands for PrPc and Cav-1 mRNA, normalized for the β -actin mRNA contents..

The significantly higher level of PrPc mRNA detected in fibroblasts from the scrapie-susceptible animals, prompted us to evaluate whether the presence of the scrapie-like PrP protein (PK-resistant PrP, PrPres) could be detected in the ARQ/ARQ fibroblast cultures. Interestingly, Western blot analysis of PrP in extracts of 72 hr FCS-stimulated fibroblasts from the scrapie-susceptible sheep (Fig. 15), whether or not scrapie-infected, revealed the presence of PK-resistant bands of molecular weight comparable to those reported for digested forms of PrPSc.

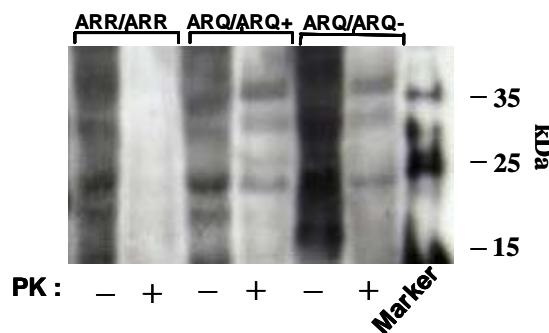


Figure 15. PrPc and PK-resistant PrP (PrPres) profiles of cultured skin fibroblasts carrying scrapie-resistant (ARR/ARR) or scrapie-susceptible (ARQ/ARQ) genotypes, with (ARQ/ARQ+) or without (ARQ/ARQ-) clinical Scrapie. Lysates of 72 hr FCS-stimulated cultures from 1 ARR/ARR, 1 ARQ/ARQ+ and 1 ARQ/ARQ- sheep, were treated or not with 20 μ g/ml PK (ratio 1:900), and analysed by Western blot procedure with 6H4 antiserum.

As seen, no PK-resistant bands were detected in the PK-treated ARR/ARR lysates (Fig. 15). Although the PK-resistant bands could be detected loading extremely high amounts of protein (up to 140 μ g of total protein), and although only two sets of fibroblast cultures from sheep with the different genotypes were so far evaluated, these results confirmed that sheep carrying ARQ/ARQ genotype are more prone to spontaneous PrP structural conversion than those carrying ARR/ARR genotype. These data, if confirmed, coupled to the higher mRNA expression of PrP may likely justify a more consistent PrP^{Sc} accumulation following the natural infection with Scrapie.

DISCUSSION

The results obtained during my research doctorate indicate a strong relationship between abnormal CE accumulation and cell susceptibility to prion infection/replication. Firstly, scrapie-infected mouse neuroblastoma cell lines as well as *ex vivo* fibroblast cultures derived from sheep affected by clinical scrapie, or carrying a susceptible genotype (ARQ), displayed an alteration of cholesterol homeostasis as evidenced by an abnormal intracellular accumulation of CE. In these cultures, CE levels were about 3-fold higher than their respective uninfected or resistant counterparts. Secondly, the modifications of cellular cholesterol pools were accompanied by parallel alterations in the expression levels of gene and protein Cav-1, that is involved in the pathways leading to intracellular cholesterol trafficking, i.e. cholesterol efflux. In particular, a lower Cav-1 expression characterized cells with scrapie-susceptible genotype. Thirdly, fibroblasts carrying the susceptible ARQ/ARQ genotype showed an higher mRNA expression of PrP^C than cultures from scrapie-resistant sheep, and PK-digestion of lysates led to detectable PK-resistant PrP isoforms, suggesting that genetic PrP polymorphisms and altered cholesterol homeostatic

mechanism may act in concert to create a favorable cellular environment to initiation and progression of prion disease. Finally, various drugs able to inhibit cholesterol esterification by targeting different steps of cholesterol metabolism/trafficking markedly reduced CE levels in both prion-infected mouse neuroblastoma and skin fibroblast cultures from scrapie-affected sheep, while had significantly lower or no effects in their uninfected and scrapie-resistant counterparts. Moreover, the cholesterol modulators were able to determine a parallel inhibition of PrPres formation in the prion-infected N2a cell line. Among all cholesterol modulators tested, Everolimus was determined to be the most active drug. It was the most potent inhibitor of cholesterol esterification in terms of effective concentration (50 nM vs. 20-40 μ M), and the most potent anti-prion drug with a 50% inhibitory concentration (EC_{50}) of 1.4 μ M in the 22L-N2a cells. The prion inhibitor Quinacrine, under the same experimental conditions showed a comparable potency with an EC_{50} of 1 μ M. Also the cholesterol modulators Pioglitazone, Progesterone, and Verapamil resulted in a selective, although moderate, anti-prion effect (EC_{50} range 15-40 μ M).

As reported in the Introduction, drugs known to affect the *de novo* cholesterol biosynthesis (i.e. statins) have been reported to inhibit PrPres accumulation (Bate et al., 2004), and more recently, evidence has been presented that quinacrine could exert its anti-prion effect by influencing intracellular redistribution of cholesterol (Klingenstein et al., 2006). However, this is the first time that it is shown a selective anti-prion activity of drugs affecting the overall cholesterol esterification process. It is worth to note that in normal tissues, only a minor amount (1-10%) of total cholesterol is found as CE, the storage form of cholesterol in the cytoplasm, while more than 90% is in the form of free cholesterol (FC) and resides in the cholesterol-rich membrane domains (rafts). Because membrane cholesterol appears critical for the function of raft-resident proteins (e.g. PrP, β - and γ -secretases of APP, growth factor receptors),

cells developed a highly integrated set of homeostatic mechanisms that finely regulate FC vs. CE pools according to the cell's needs and functions. In this context, my results indicate that prion infection and/or replication may be sustained or favoured by alterations in this fine regulatory network that operates to assure the correct content and distribution of cholesterol inside the cell. Several studies (Abid et al., 2006, Critchley et al., 2004, Gilch et al., 2006, Prado et al., 2004, Taraboulos et al., 1995) have pointed out the essential role of cellular cholesterol for the proper folding and trafficking of PrP^C protein, indicating that the conversion rate of PrP^C into PrP^{Sc} may be modulated, at least in part, by cholesterol homeostatic mechanisms. On the other hand, PrP^{Sc} replication itself has been reported (Russelakis-Carneiro et al., 2004) to interfere with intracellular cholesterol metabolism and trafficking by displacing cholesterol binding protein caveolin-1 from the membrane, thus suggesting that PrP perturbations may in turn exacerbate preexisting cholesterol alterations. In our study, sheep were infected *in vivo* and the scrapie agent may have reached, in addition to nervous and lymphatic system, various host tissues, including skin. As a matter of fact, Thomzig et al. (2003) showed PrP^{Sc} accumulation in the muscles and, very recently (2007), also in the skin of experimentally scrapie-infected hamsters as well as in naturally infected sheep.

On the basis of our findings, and in the light of the increasing literature on this matter, we then suggest that abnormal cholesterol esterification could represent a phenotype predisposing a cell to the development of pathologic processes involving abnormal activation, and/or processing, and/or trafficking of membrane resident proteins, and that cholesterol esterification inhibition may be a way to control disease progression.

In agreement with our findings, recent studies by Kovacs' group in models of Alzheimer's disease (AD) have indicated a role for CE as modulators of the amyloidogenic processing of the amyloid precursor protein APP. Inhibition of CE

by RNAi-induced decrease of ACAT expression (Huttunen et al., 2007) or by a novel ACAT inhibitor, CP-113,818, prevented amyloid A β peptide generation (Puglielli et al 2001), and led to more than 90% reduction of cerebral amyloid plaques in a mouse model of AD (Hutter-Paier et al., 2004).

In conclusion, further studies are necessary to elucidate the mechanisms responsible for the cholesterol homeostasis alterations in sheep carrying a prion genotype affecting susceptibility to scrapie, and to unveil the apparent cause-effect relationship between PrP polymorphisms and cholesterol alterations. Nonetheless, the data reported in my study suggest that accumulation of cholesterol esters in peripheral cells, together with altered expression of some proteins implicated in intracellular cholesterol homeostasis, might serve to identify a distinctive lipid metabolic profile associated with increased susceptibility to develop prion disease following natural infection.

Studies are already in progress to establish whether cholesterol esters may truly be a target of clinical interest as well as a biological marker of disease susceptibility applicable to prion diseases as well as to other protein-based neurodegenerative pathologies.

The results of this research are reported in the following papers this year published in international peer-reviewed journals

Pani A, Abete C, Norfo C, Mulas C, Putzolu M, Laconi S, Cannas MD, Orrù CD, La Colla P and Dessi S. *Cholesterol Metabolism in Brain and Skin Fibroblasts from Sarda Breed Sheep With Scrapie-resistant and Scrapie-susceptible Genotypes.* Am J Infect Dis 3(3), 143-150 (2007).

Pani A, Norfo C, Abete C, Mulas C, Putzolu M, Laconi S, Orrù CD, Cannas MD, Vascellari S, La Colla P, and Dessi S. *Accumulation of Cholesterol Esters in ex*

vivo Lymphocytes from Scrapie-susceptible Sheep and in Scrapie-infected Mouse Neuroblastoma Cell Lines. Am J Infect Dis 3(3), 165-168 (2007).

Pani A, Norfo C, Abete C, Mulas C, Putzolu M, Laconi S, Orrù CD, Cannas MD, Vascellari S, La Colla P, and Dessì S. *Anti-Prion Activity Of Cholesterol Esterification Modulators: A Comparative Study In Ex Vivo Sheep Fibroblasts And Lymphocytes And In Mouse Neuroblastoma Cell Lines. Antimicrob Agents Chemother 51, 4141-4147 (2007).*

Pani A, Putzolu M, Mulas C., Orrù CD, Abete C, Norfo C, Cannas MD, Laconi S, La Colla P and Dessì S. *ACAT1, Cav-1, and PrP Expression In Brains And Skin Fibroblasts From Sarda Breed Sheep With Scrapie-Resistant And Scrapie-Susceptible Genotype. Available from Nature Precedings, 2007 (hdl:10101/npre.2007.1211.1).*

ACKNOWLEDGEMENTS

I am indebted with Ciriaco Ligios (Istituto Zooprofilattico Sperimentale, Sassari, Italy) and with Byron Caughey (NIH/NIAID Rocky Mountain Laboratories, USA) for providing sheep samples and mouse neuroblastoma cell lines.

MATERIALS AND METHODS

Chemicals

Verapamil hydrochloride (98%), Quinacrine dihydrochloride, Progesterone, and DMSO (99.5 %), were purchased from Sigma-Aldrich (Italy). Everolimus and Sandoz 58-035 were kindly provided by Novartis (Switzerland), and Pioglitazone by Takeda (Japan). Everolimus was solubilized in 100% ethanol and stored at +4°C. Pioglitazone and Sandoz 58-035 were solubilized in 100% ethanol and stored at room temperature. Stock solutions of the other compounds were prepared in DMSO and stored at -20°C.

Cell lines

The mouse neuroblastoma N2a cell line, and the 22L-N2a and RML-N2a sublines, respectively infected with the mouse-adapted strains of scrapie 22L and RML (Rocky Mountain Laboratory), were a generous gift by Dr. Byron Caughey, NIH/NIAID Rocky Mountain Laboratories, USA. Cells were grown and maintained at 37°C, 5% CO₂, in OptiMEM supplemented with 10% FBS (Gibco, Invitrogen; Italy), 2mM L-glutamine, 50 U/ml penicillin G sodium and 50 µg/ml streptomycin sulphate (Gibco, Invitrogen; Italy) and passaged every 3 or 4 days at a 1:10 or 1:20 dilution, respectively. On occasions, 22L-N2a and RML-N2a sublines were cloned by end point dilution (single-cell dilution) to isolate better PrPres producer populations. Cell lines and subclones were stored in liquid nitrogen and working cultures were replaced at two-three month intervals in order to maintain the same intensity of PrPres signal throughout the experiment. All trials were carried out in cell cultures during exponential growth.

Sheep

Sheep samples were a generous gift from Dr. Ciriaco Ligios and were collected at the Istituto Zooprofilattico Sperimentale of Sardinia, Sassari, Italy. Samples were collected from a total of 14 Sarda breed sheep, 4 carried the scrapie-resistant ARR/ARR genotype, and 10 the scrapie-susceptible ARQ/ARQ genotype. Of the latter, 2 were mock-infected, 1 had natural scrapie, and 7 developed clinical disease following experimental inoculation of scrapie. The 4 scrapie-resistant sheep, which were scrapie-infected in parallel with susceptible animals, and the 2 mock-infected susceptible animals, did not develop any clinical signs and were alive and healthy at the time of this report. With the exception of the sheep affected by natural scrapie, all the animals used were raised in the same environmental conditions and were of the same age and sex in order to reduce physiological differences of experimental determinations. Samples from all sheep were collected at the time of terminal clinical stage of the ill animals.

Skin fibroblast cultures

Tissues fragments from skin biopsies were plated into 6-well plates and allowed to adhere to the bottom of the vessels. After 2 hrs, they were covered with a few drops of Dulbecco's modified Eagle's medium (D-MEM) (Gibco Lab NY, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma), 100 U/ml penicillin/streptomycin (Sigma), and fungizone (Life Technologies, Inc.) and incubated at 37°C in a humidified incubator with 5% CO₂. The following day, tissue fragments were overlaid with culture medium, which was changed every two days. Five to six days later, fibroblasts begun to proliferate from the fragment margins ("halo of cells") and to form a monolayer. After four weeks, fibroblasts were purified by repeated trypsinization (trypsin-EDTA) and passaging to achieve an homogenous population of spindle cells. Purified fibroblasts were washed twice with sterile PBS and centrifuged. 1x10⁶ cells

were then seeded into 25 cm² culture flask and grown to confluence. Then, cells were either used for "in vitro" experiments or resuspended in cryopreservation medium at a density of 1x10⁷ cells/ml and stored in liquid nitrogen. Analytical assays were carried out using fibroblast cultures between the second and fourth passage. Cells were plated at a density of 5x10³ cell/cm² in 6-well plates and brought to proliferative quiescence by incubation for 48 hrs in serum depleted (0.2% FCS) MEM 199. Then, quiescent cells were stimulated to re-enter cell cycle by addition of 10% FCS and incubation at 37 °C for the indicated time intervals.

Lipid staining

Intracellular neutral lipid (i.e. CE) were evaluated with the oil red O method (15,26) at the indicated time points as previously described (30). In brief, cultures were washed three times with PBS and fixed by soaking in 10% formalin. Cells were then treated with isopropyl alcohol (60%), washed, stained with oil red O for intracellular neutral lipid droplets and counterstained with Mayer's hematoxylin. Stained cells were examined by light microscopy and digital images were recorded. Red color intensity in single cells, indicating neutral lipid-bound oil red O, was measured by the NIH Image 1.63 Analysis Software program (Scion Image). Values are expressed as the mean colour intensity per cell calculated on at least 30 single random cells in 6 different microscopic fields.

Detection of PrPres in cell cultures

For dot blot procedure, approximately 5.000 of cells in 100 µl of growth medium were added to each well of a Microtest flat-bottom 96-well plate with a low-evaporation lid (Becton Dickinson, USA). For drug testing, the cells were allowed to settle overnight before addition of 10 µl (10x solutions) of different dilutions of test compounds. DMSO in the cell medium was never higher than 0.5%

(vol/vol). Each drug concentration was tested in quadruplicate. After 4 days at 37°C in 5%CO₂ cells were processed for PrPres as described (24). In brief, cells were lysed with 50 µl of cold lysis buffer (0.5% wt/vol Triton X-100, 0.5% wt/vol sodium deoxycholate, 5 mM Tris-HCl pH 7.4 at 4°C, 5 mM EDTA, and 150 mM NaCl). After 15 min on ice, 25 µl of 0.1 mg/ml proteinase K (PK, Novagen, USA) in TBS (1.4 M NaCl, 1 M Tris-HCl pH 7.6) was added to each well for 60 min at 37°C. A total of 225 µl of 1 mM Pefabloc (Roche, Novagen, USA) was added to the wells of PK-treated and mock treated cultures. Lysates were transferred to a 96-well dot blot apparatus (Schleicher & Schuell) over a 0.45 µm pore-size polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore) and rinsed with TBS. The PVDF membrane was removed, covered with 3M Gdn SCN (Fluka) for 8 min at room temperature, blocked with 5% (wt/vol) non-fat dry milk (Bio Rad, USA) and 0.05% (vol/vol) Tween 20 (USB corporation; USA) in TBS (TBS-T-milk) for 60 min at room temperature, incubated with anti-PrP mouse monoclonal antibody 6H4 (Prionics, Zurich; 1:20.000) in TBS-T for 1 h, and then exposed to horseradish peroxidase labeled anti-mouse IgG antibody (GE Healthcare, UK; 1:50.000) in TBS-T for 1 h. After extensive washings, membrane was soaked for 5 min in ECL-plus reagent (GE Healthcare), and exposed to x-ray film (Hyperfilm ECL, GE Healthcare). Autoradiography films were captured in TIFF format and intensity of each dot was determined by using the Scion Image software (Scion; Frederick, MD). PrPres mean

value at each drug concentration was expressed as percentage of untreated controls and the concentration resulting in 50% reduction (EC₅₀) was determined by linear regression analysis.

For Western blot analysis, 4x10⁶ cell samples were lysed in 500 µl of cold lysis buffer (see above) for 6 min. After centrifugation at 5000 rpm for 5min supernatants were collected and total protein concentration determined by the bicinchoninic acid (BCA) protein assay (Sigma-Aldrich). Samples were digested

with 20 µg/ml PK (Novagen, USA) in TBS at 37°C for 30 min and the digestion was stopped by incubating with 4 mM Pefabloc (Roche, Novagen, USA) for 10 min on ice. PrP^{res} was collected by precipitation with four volumes of methanol at -20°C (7). The resulting pellets were then solubilized by sonication in LDS sample buffer (Invitrogen, USA) and different amount (20-120 µg) of protein samples were loaded onto a 10% NuPage bis-tris-polyacrylamide gel (Invitrogen, USA) just after boiling. Protein bands were electro-blotted onto an Immobilon-P membrane (Millipore, USA) and PrP^{res} was detected as above.

Cytotoxicity assay

Antiproliferative activity was evaluated in exponentially growing cell cultures. One hundred µl of a cell suspension at a density of 5x10⁴ cells/ml was added to each well of flat-bottom 96-well plate 24 hours before addition of 100 µl of 2x dilutions of the test compounds. Each drug concentration was tested in quadruplicate. Cell viability was determined after four days by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Sigma Italy) method as previously described (13). Cell viability at each drug concentration was expressed as percentage of untreated controls and the concentration resulting in 50% viability (CC₅₀) was determined by linear regression analysis.

RT-PCR and Southern blotting

The expression levels of Cav-1 and PrP^c mRNAs were evaluated in brain homogenates and skin fibroblasts by reverse transcription polymerase chain reaction (RT-PCR). mRNA levels for the housekeeping gene β-actin were used to normalize the amount of RNA inputs in the RT-PCR. Total RNA was extracted from 10⁶ cells using TRIZOL reagent (Invitrogen Corporation). Equal amounts of total RNA (1 µg) were reverse transcribed into cDNA using the random hexamer method and amplified by PCR in the presence of specific primers, according to

the instructions provided by the manufacturer (GeneAmp RNA PCR Kit, Perkin-Elmer Cetus). PCR was performed using the following ovine-specific primers and conditions: for Cav-1 (258 bp) forward: 5'- GATTAACAGTGGGTACGATA-3', reverse: 5'-TATGTAGTCTTGCGTTATCC-3'; 94 °C for 30 sec, 59 °C for 30 sec and 72 °C for 45 sec, for 30 cycles. For PrP, (341 bp.) forward: 5'- ATTGTCACCTAGCAGATAGA-3', reverse: 5'-TTGTTTCAGTAGCTCAAGTCT-3'; 94 °C for 30 sec, 58°C for 1 min, and 72 °C for 45 sec, for 30 cycles. For β -actin (217 bp) forward: 5'-GATCATGTTTGAGACCTTC-3', reverse: 5'-GAGGATCTTCATGAGGTAGT-3'; 96 °C for 30 sec, 60 °C for 59 sec, and 72 °C for 45 sec, for 20 cycles. Sub-saturation levels of cDNA templates, needed to produce a dose-dependent amount of PCR product, were defined in initial experiments by testing a range of template concentrations. Amplicons were labeled during PCR with Digoxigenin-11-dUTP (DIG; Roche Applied Science), immuno-detected with anti-digoxigenin antibodies conjugated to alkaline phosphatase (Roche Applied Science) and visualized with the chemiluminescent substrate CSPD®. The intensity of the autoradiographic bands was measured after exposure to X-ray film with the Kodak Digital Science Band Scanner Image Analysis System containing HP ScanJet, ID Image Analysis Software. The overall procedure was normalized by expressing the amount of PCR products for each target mRNA relative to the amount of PCR products obtained for the housekeeping gene β -actin.

Statistical analysis

All values are presented as the mean \pm SD (Table) or mean \pm SEM (Figures). Statistic analysis was performed with the Student *t*-test. For multiple comparisons all significance values were corrected by the Bonferroni method for multiple tests. Significance was set at $p < 0.05$.

REFERENCES

- Abid K., Soto C.**, 2006. The intriguing prion disorders. *Cell. Mol. Life Sci.*, **63**:2342-2351
- Aguzzi A.**, 2006. Prion diseases of humans and from animals: epidemiology, genetics, and pathogenesis. *Journal of Neurochemistry*. **97**:1726-1739
- Aguzzi A., Heikenwalder M.**, 2006. Pathogenesis of prion diseases: current status and future outlook. *Nature Reviews Microbiology*. **4**:765
- Aguzzi A., Heikenwalder M., Miele G.**, 2004. Progress and problems in the biology diagnostics, and therapeutics of prion diseases. *J. Clin. Invest.* **114**(2):153-160
- Aguzzi A., Heppner F.**, 2000. Pathogenesis of prion diseases: a progress report. *Cell Death and Differentiation* **7**: 889-902
- Aguzzi A., Polymenidou M.**, 2004. Mammalian prion biology: one century of evolving concepts. *Cell*. **116**:313-327
- Ariste N., Doh M.**, 2002. Plasma membrane cholesterol controls the cytotoxicity of Alzheimer's disease Abeta (1-40) and (1-42) peptides. *Faseb J.* **16**:1526-1536
- Bate C., Salmona M., Diomedea L., Williams A.**, 2004. Squalenstatin cures prion-infected neuron and protects against prion neurotoxicity. *J. Biol. Chem.* **279**:14983-14990
- Baylis M., Goldmann W.**, 2004. The genetics of scrapie in sheep and goats. *Current Molecular Medicine*. **4**:385-396
- Belay E.D.**, 1999. Transmissible spongiform encephalopathies in humans. *Annu. Rev. Microbiol.* **53**:283-314
- Belay E.D., Schonberger L.B.**, 2005. The public health impact of prion diseases. *Annu. Rev. Public Health*. **26**:191-212
- Cashman N.R., Caughey B.**, 2004. Prion diseases—Close to effective therapy? *Nature Reviews* **3**: 874-884

- Colline J.**, 2005. Molecular neurology of prion disease. *J. Neural Neurosurg Psychiatry*. **76**:906-919
- Cooke C.M., Rodger J., Smith A., Fernie K., Shaw G., Somerville R.A.**, 2007. Fate of prions in soil: detergent extration of PrP from soils. *Environ. Sci. Technol.* **41**:811-817
- Critchley, P., Kazlauskaitė J., Eason R., Pinheiro T.J..**, 2004. Binding of prion protein to lipid membranes. *Biochem Biophys Res Commun.* **313**:559-567.
- Detwiler L.A., Baylis M.**, 2003. The epidemiology of scrapie. *Rev. Sci. Tech. Int. Epiz.* **22(1)**:121-143
- Diomede L., Forloni G., Bugiani O., Tagliavini F., Salmona M.**, 2002. The prion protein and cellular cholesterol homeostasis. *Neurobiology of Lipids* **1,3**
- Ersdal C., Ulvund M.J., Espenes A., Benestad L., Sarradin P., Landsverk T.**, 2005. Mapping PrP^{Sc} propagation in experimental and natural scrapie in sheep with different PrP genotypes. *Vet. Pathol.* **42**:258-274
- Fèvrier B., Vilette D., Laude H., Raposo G.**, 2005. Exosomes: a bubble ride for prions? *Traffic*. **6**: 10-17
- Genovesi S., Leita L., Sequi P., Andrighetto I., Sorgato M.C., Bertoli A.**, 2007. Direct detection of soil-bound prions. *PLoS ONE*. **2(10)**:e1069
- Gilch S., Kehler c., Schatzl H.M.**, 2006. The prion protein requires cholesterol for cell surface localization. *Mol. Cell Neurosci.* **31**:346-353
- Glatzel M., Stoeck K., Seeger H., Lührs T., Aguzzi A.**, 2005. Human prion diseases: molecular and clinical aspects. *Arch. Neurol.* **62**:545-552
- Goldmann W., Baylis M., Chihota C., Stevenson E., Hunter N.**, 2005. Frequencies of PrP gene haplotypes in British sheep flocks and the implications for breeding programmes. *Journal of Applied Microbiology*. **98**:1294-1302
- Gretzschel A., Buschmann A., Eiden M., Ziegler U., Lühken G., Erhardt G., Groschup M.H.**, 2005. Strain typing of German Transmissible Spongiform Encephalopathies field cases in small ruminants by biochemical methods. *J. Vet. Med.* **B52**:55-63

Harris D.A., 1999. Cellular biology of prion diseases. *Clinical Microbiology Reviews*. **12**:429-444

Howland D.S., Trusko S.P., Savage M.J., 1998. Modulation of secreted beta-amyloid precursor protein and amyloid beta-peptide in brain by cholesterol. *J. Biol. Chem.* **273**:16576-16582

Hutter-Paier B., Huttunen H.J., Puglielli L., Eckman C.B., Kim D.Y., Hofmeister A., Moir R.D., Domnitz S.B., Frosch M.P., Windisch M., Kovacs D.M., 2004. The ACAT inhibitor CP-113,818 markedly reduces amyloid pathology in a mouse model of Alzheimer's disease. *Neuron*. **44**(2):227-38.

Huttunen H.J., Greco C., Kovacs D.M., 2007. Knockdown of ACAT-1 reduces amyloidogenic processing of APP. *FEBS Letters*, **581**:1688-1692

Jick H., Zornberg G.L., Jick S.S., Drachman D.A., 2000. Statins and the risk of dementia. *Lancet*. **356**:1627-1631

Johnson C.J., Phillips K.E., Schramm P.T., McKenzie D., Aiken J.M., 2006. Prions adhere to soil minerals and remain infectious. *PloS Pathogens*. **2**(4):e32

Kempster S., Bate C., Williams A., 2006. Simvastatin treatment prolong the survival of scrapie-infected mice. *Neuroreport*. **18**:479-482

Klafki HW., Staufenbiel M., Kornhuber J., Wiltfang J., 2006. Therapeutic approaches to Alzheimer's disease. *Brain*. **129**:2840-2855

Klingenstein R., Lober S., Kujala P., Godsave S., Leliveld S.R., Gmeiner P., Peters P.J., Korth C., 2006. Tricyclic antidepressants, quinacrine and a novel, synthetic chimera thereof clear prions by destabilizing detergent-resistant membrane compartments. *J. Neurochem.* **98**(3):748-59

Kocisko D.A., Baron G.S., Rubenstein R., Che J., Kuison S., Caughey B., 2003. New inhibitors of scrapie-associated prion protein formation in a library of 2,000 drugs and natural products. *J. Virol.* **77**(19):10288-10294

Koster T., Singh K., Zimmermann M., Gruys E., 2003. Emerging therapeutic agents for transmissible spongiform encephalopathy. *J. Veter. Pharm. Applic. Therap.* **12**:315-326

Lee I.Y., Westaway D., Smit A.F.A., Wang K., Seto J., Chen L., Acharya C., Ankener M., Baskin D., Cooper C., Yao H., Prusiner S.B., Hood L.E., 1998. Complete genomic sequence and analysis of the prion protein gene region from three mammalian species. *Genome Res.* **8**:1022-1037

Mangé A., Crozet C., Lehmann S., Béranger F., 2004. Scrapie-like prion protein is translocated to the nuclei of infected cells independently of proteasome inhibition and interacts with chromatin. *Journal of Cell Science.* **117**:2411-2416

Maxfield F.R., Tabas I., 2005. Role of cholesterol and lipid organization in disease. *Nature.* **438**:36-45

McKintosh E., Tabrizi S.J., Collinge J., 2003 Prion diseases *Journal of NeuroVirology.* **9**:183-193

Mead S., Stumpf M.P.H., Whitfield J., Beck J.A., Poulter M., Campbell T., Uphill J.B., Goldstein D. Alpers M., Ficher D.M.C., Collinge J., 2003. Balancing selection at the prion protein gene consistent with prehistoric kurulike epidemics. *Science.* **300**:640-643

Metherall, J.E., Waugh K., Li H., 1996. Progesterone inhibits cholesterol biosynthesis in cultured cells. Accumulation of cholesterol precursors. *J Biol Chem.* **271(5)**:2627-33

Milhavet O., Lehmann S., 2002. Oxidative stress and the prion protein in transmissible spongiform encephalopathies. *Brain Research Reviews* **38**: 328-339

Mok et al., 2006. Simvastatin prolongs survival times in prion infections of the central nervous system. *Biochem. Biophys. Res. Comm.* **348**:697-702

Novakofski J., Brewer M.S., Mateus-Pinilla N., Killefer J., McCusker R.H. 2005. Prion biology relevant to bovine spongiform encephalopathy. *J.Anim. Sci.* **83**:1455-1476

Papassotiropoulos A., Wollmer M.A., Aguzzi A., Hock C., Nitsch R.M., de Quervain D.J.F., 2005. The prion gene is associated with human long-term memory. *Human Molecular Genetics* **14(15)**: 2241-2246

Parkin E.T., Watt N.T., Hussain I., Eckman E.A., Eckman C.B., Manson J.C., Baybutt H.N., Turner A.J., Hopper N.M., 2007. Cellular prion protein regulates β -secretase cleavage of the Alzheimer's amyloid precursor protein. *PNAS*. **104(26)**:11062-11067

Petraroli R, Pocchiari M., 1996 Codon 219 polymorphism of PRNP in healthy Caucasian and Creutzfeldt-Jakob disease patients (Letter). *American Journal of Human Genetics*. **58**:888-889.

Pierce S. K., 2002, Lipid rafts and B-cell activation. *Nature Reviews Immunology*. **2**:96-105

Prado M.A.M., Alves-Silva J., Magalhaes A.C., Prado V.F., Linden R., Martins R., Brentani R.R., 2004. PrP^C on the road: trafficking of the cellular prion protein. *J. Neurochem*. **88**:769-781

Prinz M., Huber G., Macpherson A.J.S., Heppner F.L., Glatzel M., Eugster H.P., Wagner N., Aguzzi A., 2003. Oral prion infection requires normal numbers of Peyer's patches but not of enteric lymphocytes. *American Journal of Pathology* **4**:1103-1111

Puglielli, L., Konopka G., Pack-Chung E., Ingano L.A., Berezovska O., Hyman B.T., Chang T.Y., Tanzi R.E., Kovacs D.M., 2001. Acyl-coenzyme A: cholesterol acyltransferase modulates the generation of the amyloid beta-peptide. *Nat. Cell. Biol.*, **3 (10)**:905-912

Rea T.D., Breitner J.C., Psaty B.M., Fitzpatrick A.L., Lopez O.L., Newman A.B., 2005. Statin use and the risk of incident dementia: the Cardiovascular Health Study. *Arch. Neurol.* **67**:1047-1051

Russelakis-Carneiro M., Hetz C., Maundrell K., Soto C., 2004. Prion replication alters the distribution of synaptophysin and caveolin 1 in neuronal lipid rafts. *Am. J. Pathol.* **165**:1839-1848

Rymer D.L., Good T.A., 2000. The role of prion peptide structure and aggregation in toxicity and membrane binding. *J. Neurochem.* **75**: 2536-2545

Sabuncu E., Petit S., Le Dur A., Lan Lai T., Vilotte J.L., Laude H., Vilette D., 2003. PrP polymorphisms tightly control sheep prion replication in cultured cells. *Journal of Virology*. **77(4)**:2696-2700

Saunders G.C., Cawthraw S., Mountjoy S.J., Hope J., Windl O., 2006. PrP genotypes of atypical scrapie cases in Great Britain. www.socgenmicrobiol.org.uk

Scott M.R., Peretz D., Nguyen H.O.B., DeArmond S.J., Prusiner S.B., 2005. Transmission barriers for bovine, ovine, and human prions in transgenic mice. *Journal of Virology*. **79**:5259-5271

Simons K., Eehalt R., 2002. Cholesterol, lipid rafts, and disease. *J. Clin. Invest.* **110**:597-603

Sordevila M., Andrés A.M., Ranirez-Soriano A., Marquès-Bonet T., Calafell F., Navarro A., Bertranpetit J., 2006. The prion protein gene in humans revisited: Lessons from a worldwide resequencing study. *Genome Res.* **16**:231-239

Sordevila M., Calafell F., Andrés A.M., Yagüe J., Hergason A., Stefansson., Bertranpetit J., 2003. Prion susceptibility and protective alleles exhibit marked geographic differences. *Human Mutation*. DOI:10.1002/humu.9157

Steele A., Emsley J.G., Özdinler H., Lindquist S., Macklis J.D., 2006. Prion protein (PrP^C) positively regulates neural precursor proliferation during developmental and adult mammalian neurogenesis. *PNAS*. **103(9)**:3416-3421

Taraboulos A., Scott M., Semenov A., Avrahami D., Laszlo L., Prusiner S.B., 1995. Cholesterol depletion and modification of COOH-terminal targeting sequence of the prion protein inhibit formation of the scrapie isoform. *J. Cell. Biol.* **129(1)**:121-32

Thomas D.L. Genetics of scrapie resistance in sheep. www.edu/ces/animalscience/sheep/etn_01/nov/scrapiegenetics.doc

Thomzig A., Kratzel C., Lenz G., Kruger D., Beekes M., 2003. Widespread PrP^{Sc} accumulation in muscles of hamsters orally infected with scrapie. *EMBO reports*, **4(5)**:530-533.

Thomzig A., Schulz-Schaeffer W., Wrede A., Wemheuer W., Brenig B., Kratzel C., Lemmer K., Beekes M., 2007. Accumulation of pathological prion protein PrP^{Sc} in the skin of animals with experimental and natural scrapie. *PLoS Pathogens*, **3 (5)**:659-667

Vascellari M., Nonno R., Mulinelli F., Bagolaro M., Di Bari M.A., Melchiotti E., Marcon S., D'agostino C., Vaccai G., Conte M., De Grossi L., Rosone F., Giordani F., Agrimi U., 2007. PrP^{Sc} in salivary glands of scrapie-affected sheep. *Journal of Virology*. **81(9)**:4872-4876

Wadsworth J.D.F., Hill A.F., Beck J.A., Collinge J., 2003. Molecular and clinical classification of human prion disease. *British Medical Bulletin*. **66**:241-254

Ward R.L., Porter D.D., Stevens J.G., 1974. Nature of the scrapie agent: evidence against a viroid. *J. Virol.* **14(5)**:1099-1103

Will R.G., 2004. Possible transmission of variant Creutzfeldt-Jakob disease by blood transfusion. *Lancet*. **363**:417-421

Wolozin B., 2002. Cholesterol and Alzheimer's disease. *Biochem. Soc. Transact.* **30(4)**:525-529

Zajchowski L., Robbins S.M., 2002. Lipid rafts and caves. Compartmentalized signalling in membrane microdomains. *Eur. J. Biochem.* **269**:737-752

Zhang C.C., Steele A.D., Lindquist S., Lodish H.F., 2006. Prion protein is expressed on long-term repopulating hematopoietic stem cells and is important for their self-renewal. *PNAS*. **103(7)**:2184-2189

Zhang M., Haapsalo A., Kim D.Y., MacKenzie Ingano L.A., Pettingel W.H., Kovacs D.N., 2006. Presenilin/ γ -secretase activity regulates protein clearance from the endocytic recycling compartment. *FASEB J.* **20**:E271-E280