Prion Misfolding

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Cholesterol Secosterol Adduction Inhibits the Misfolding of a Mutant Prion Protein Fragment that Induces Neurodegeneration**

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Novel infectious particles termed prions, composed solely of single proteins, are considered the causative agents in a group of transmissible spongiform encephalopathies that produce a lethal decline in motor and cognitive functions.[1–3] Prion diseases are infectious, inherited, or sporadic in nature but perhaps the most unsettling aspect of these protein-only diseases is that they arise at their pathogenic state by misfolding of a protein from its normal state that may exist with ubiquitous distribution throughout tissue. Thus, the prion diseases are now generally accepted to occur by the presence of an exogenous gene, *Prnp*, encoding a dimorphic protein that can exist in a normal (PrP^C) or disease-causing "scrapie" form (PrP^{Sc}).[4]

At present there is no therapy, either small molecule or vaccine-based, for the treatment of prion diseases. However, molecules that inhibit PrP^{Sc} formation from PrP^C are viewed as potential therapeutics for these diseases. Small molecules such as sulphated polyanions,[5] congo red,[6] heparin sulfate,[7] pentosan polysulfate[8] and cyclic tetrapyrroles[9] inhibit PrP^{Sc} formation. In addition, short peptides comprised of residues from the hydrophobic core of PrP^C also inhibit prion protein aggregation.[10,11]

Recently we have discovered a process that we are studying in the context of a number of disease-related sporadic amyloidoses. We have shown that in vitro, certain inflammatory-derived lipid aldehydes, when adducted to pro-amyloidogenic proteins in their native state, can induce misfolding and aggregation of the native protein sequences.[12] Thus, we have shown that cholesterol 5,6-secosterols such as **1**, that are derived from oxidation of cholesterol by activated leukocytes, accelerate the in vitro misfolding of $apoB_{100}$, the protein component of low-density lipoprotein (LDL) (Figure 1b).[13]

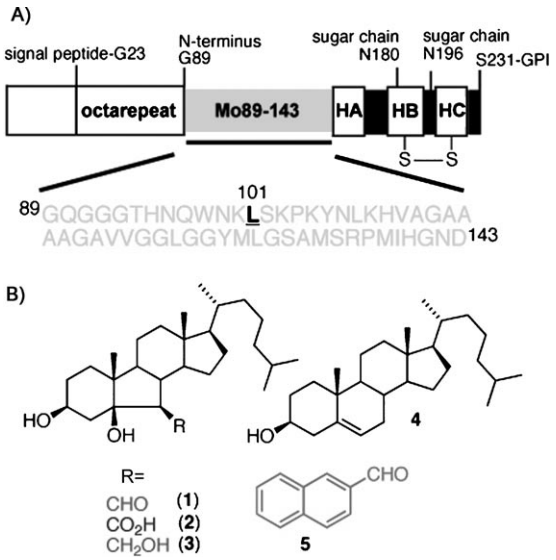

Figure 1. A) Representation of an open reading frame of the mouse prion protein (MoPrP) and the synthetic core mutant peptide MoPrP(89-143, P101L) that initiates GSS in transgenic mice that we have synthesized and studied in this work. The mutant leucine residue 101 is black and underlined. HA, HB and HC represent helices A, B and C, respectively. GPI represents the glycosylphosphatidyl inositol (GPI) anchor. B) Chemical structures of cholesterol 5,6-secosterol **1** (atheronal-B), carboxylic acid **2**, primary alcohol **3**, cholesterol (**4**), and 2-naphthaldehyde (**5**).

In addition, we have shown that 4-hydroxynonenal and **1** accelerate the in vitro amyloidogenesis of β-amyloid peptides ($A\beta_{1-40}$ and $A\beta_{1-42}$)[14] through a process that involves a site-specific modification of Aβ.[15] More recently, aldehyde **1** has been shown to accelerate the aggregation of antibody light chains[16] and α-synuclein.[17]

Cholesterol secosterol **1** arises in vivo as a product of chronic inflammation, and it has been shown that inflammation is a risk factor for prion diseases. Organs that are undergoing an inflammatory response are more vulnerable to prion infection, and prion-type infections arise earlier in inflamed tissue than in the central nervous system (CNS).[18] Therefore, as part of our ongoing investigation into lipid aldehyde-induced protein misfolding we wondered whether aldehyde **1** may accelerate the formation of PrP^{Sc} from PrP^c in vitro. Remarkably, what we show herein is exactly the opposite effect: atheronal-B (**1**) inhibits the misfolding of a truncated murine prion protein MoPrP(89-143, P101L). It is the first example where adduction of a lipid-derived aldehyde to a protein inhibits its misfolding, and as such offers an insight into a potential new class of molecular structures that may serve as therapeutics.

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The model prion protein selected for study was a 55-residue peptide, MoPrP(89-143, P101L), from a murine mutant prion protein that can be refolded into at least two distinct conformations, the β- and α-forms (Figure 1a).[19] Critically, injection of the β-form of this peptide intracerebrally into mice induces neuropathological changes that are hallmarks of Gerstmann–Sträussler–Scheinker (GSS) disease, whereas injection of the α-form causes no neuropathological effects.[19]

MoPrP(89-143, P101L) was synthesized in multimilligram amounts using standard Fmoc/*t*Bu solid phase peptide synthesis (SPPS) using the Rink amide linker resulting in a C-terminal amide (Figure 1a; for full methods see Supporting Information) and purified to > 98% purity by reversed-phase HPLC. Aldehyde **1** was synthesised as described previously;[23] carboxylate **2** and alcohol **3** were prepared by oxidation and reduction of **1**, respectively (see Supporting Information).

The aqueous buffer conditions for studying the effect of cholesterol secosterol **1** on MoPrP(89-143, P101L) misfolding, phosphate buffered saline (PBS, pH 7.4) and 30% v/v 2,2,2,-trifluoroethanol (TFE), were selected to ensure that the conformation of the protein at the start of the studies comprised a high proportion of α-helix. Preliminary studies revealed that MoPrP(89-143, P101L) is almost completely α-helical in structure when dissolved in TFE (Supporting Information, Figure S2). Upon dilution of the protein solution (in TFE) into PBS (pH 7.4), the fraction of α-helical structures decreases as a function of TFE concentration, with the protein being almost completely random coil at ca. 10% v/v TFE in PBS. At 30% TFE in PBS, the conditions selected for the misfolding assays, the peptide contains considerable, ca. 30%, α-helical structure, as determined by far-UV circular dichroism (CD) (Figure S2B).

Incubation of MoPrP(89-143, P101L) (20 μM) in PBS (pH 7.4) with TFE (30% v/v) at 37 °C with shaking (900 rpm) leads to the time-dependent formation of thioflavin T (ThT)-binding conformers and aggregates with the kinetic profile of a nucleation-dependent polymerisation process with a lag phase of ca. 4 d, the time taken to reach 50% of the plateau intensity (t_{50}) of 5 d and plateau being reached at ca. 6 d (Figure 2A). In addition, the MoPrP(89-143, P101L) aggregated in this process is resistant to proteinase K digestion, a feature of scrapie-like prion aggregates (Figure S3).

When the protein (20 μM) is incubated with increasing concentrations of atheronal-B (**1**) (10–150 μM) the ThT fluorescence profile reveals a concentration-dependent decrease in plateau fluorescence intensity and an increase in the t_{50} (Figure 2A). With increasing concentration of the lipid aldehyde, the lag phase also becomes longer (ca. 10 d). At the highest concentration of **1** (150 μM), PrP55 does not show any ThT fluorescence within the measured timeframe (19 d), demonstrating a complete inhibitory effect of **1** on the misfolding/aggregation of PrP55.

Far-UV CD confirms that in the buffer system selected to study PrP55, in the absence of **1**, PrP is initially rich in α-helices ($t = 0$ d), with two characteristic local minima (206 nm and 222 nm) (Figure 2B and Figure S4). Upon heating and agitation (37 °C, 900 rpm), a gradual loss in secondary structure is observed ($t = 3$ d) followed by conversion into a β-sheet-rich conformation ($t = 11$ d) characterised by a mean molar ellipticity minimum at 217 nm. These changes in the far-UV CD spectra of PrP55 are slowed in the presence of atheronal-B (**1**), with conversion into β-sheet only occurring at 15 d (Figure 2C). The far-UV CD data supports the ThT fluorescence analysis, insofar the atheronal-B induced changes in protein secondary structure observed by far-UV CD occur in the same time frame as the observed changes in ThT fluorescence.

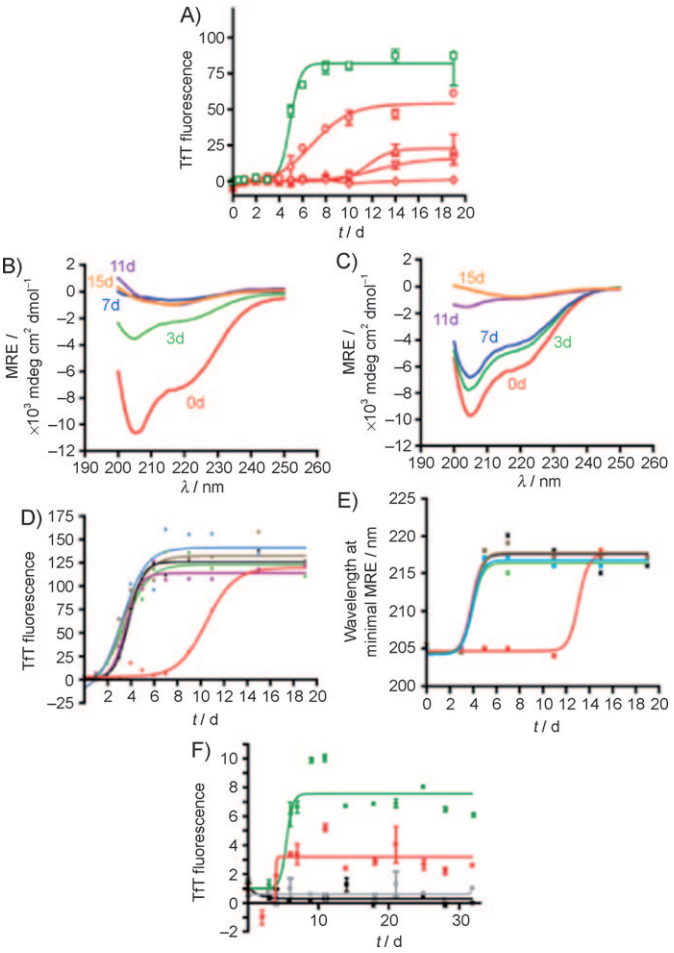

Figure 2. Inhibition of moPrP55 misfolding by atheronal-B (**1**). A) ThT fluorescence of PrP55 misfolding in the presence of varying concentrations of **1**. PrP55 (20 μM) was incubated with shaking (900 rpm) at 37 °C with **1**; □ 0 μM, ○ 10 μM, △ 50 μM, ▽ 100 μM, ◇ 150 μM). B, C) Far-UV CD spectra (0–15 days) of PrP55 (100 μM) incubated in the absence (B) or presence (C) of **1** (100 μM). D) ThT fluorescence of PrP55 (100 μM) misfolding in the presence of compounds **1**–**5** (100 μM). PrP55 alone (——), **1** (——), **2** (——) **3** (——), **4** (——), **5** (——). E) Far-UV CD kinetics of PrP55 aggregation in the presence of structurally related compounds **1**–**5** plotted as wavelength at minimal MRE (mean residue ellipticity) in CD spectra vs. time. Color scheme as in (D). F) ThT fluorescence of the aggregation of PrP55 (25 μM) in the presence of cholesterol–sphingomyelin liposomes (400 μM of each lipid) with or without **1** (100 μM). —— **PrP55** in the presence of cholesterol–sphingomyelin liposomes; —— PrP55 in the presence of cholesterol–sphingomyelin–**1** liposomes; —— cholesterol–sphingomyelin–**1** liposomes; —— cholesterol–sphingomyelin liposomes.

To assess the structural aspects of atheronal-B induced inhibition of prion protein misfolding we next studied how the misfolding of MoPrP(89-143, P101L) was affected by a panel of structural analogs of **1** (compounds **2**–**5**, Figure 1B). These studies showed that only aldehyde **1** causes any change in the misfolding of PrP55 as shown by both time-dependent ThT fluorescence and far-UV CD spectra (Figures 2D and E and Figure S4).

Given that the carboxylate and hydroxy analogs of atheronal-B (**2** and **3**, respectively) as well as cholesterol (**4**), which all lack an aldehyde group but are otherwise structurally very similar to **1**, do not demonstrate an inhibitory effect, it becomes apparent that the aldehyde function present in **1** is a critical moiety required for the inhibition process to occur. Furthermore, the fact that possessing close structural simile with **1**, as does the hydroxy compound **3**, is not sufficient to induce a measurable change in the aggregation kinetics of the protein, suggests that any binding event associated with the secocholesterol core is secondary and minor to an initial presumed covalent interaction between the aldehyde of **1** and the protein. In addition, the fact that the hydrophobic aldehyde 2-naphthaldehyde (**5**) has no discernible effect on the misfolding properties of PrP55 suggests that, while the aldehyde group of **1** is essential for its effect, not all aldehyde-containing hydrophobic compounds will induce this effect.

Previous studies with amyloid-β ($A\beta_{1-40}$) have shown that the aldehyde of secosterol **1** binds covalently to lysine residues Lys16 and Lys28 and the N-terminal amine of Asp1, but it is only when Lys16 is adducted that amyloidogenesis occurs.[25] Given that PrP55 contains a cluster of four lysine residues (100, 103, 105 and 109) (Figure 1A), we investigated whether this same adduction reaction is affecting the aggregation of PrP55. Thus, PrP55 was quiescently incubated in the presence of **1** for 3 h in PBS (pH 7.4) followed by the addition of an excess of $NaBH_4$. Sodium borohydride was selected for Schiff base reduction because it would simultaneously reduce unreacted aldehyde **1** in buffer and thus prevent any adduction occurring during the reduction and centrifugation process. After ultracentrifugation, the pellet was separated from supernatant. Both the pellet (dissolved in 30% CH_3CN/H_2O) and the supernatant were then subjected to analytical HPLC, the peaks collected and analyzed by MALDI-TOF mass spectrometry (Figure 3 and Figure S5). The supernatant contained only one peptide species, i.e. unreacted peptide (77% by area), whereas the pellet contained unmodified PrP55 (9%) as well as several atheronal-B adducted peptides (14% combined). The maximum number of molecules detected in the aggregates was two molecules of **1** attached to PrP55. While it is likely that atheronal-B (**1**) can attach to PrP55 at more than two loci, such multiply adducted forms may be below the detection limit of the analytical HPLC and mass spectrometry. However, an alternative explanation could be that specific mono- and bis-adducted forms of PrP55 get trapped into the misfolded protein during inhibition of aggregation and hence appear in the pellet. As described above, this phenomenon of only certain adducted forms of a protein being amyloidogenic was something we observed with $A\beta_{1-40}$.

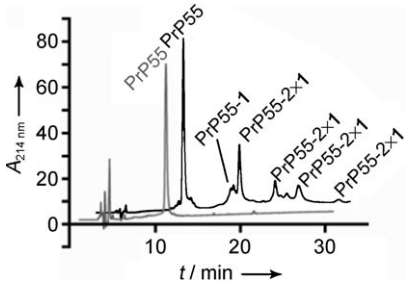

Figure 3. HPLC traces of the supernatant (——) and the pellet (——) after sodium borohydride reduction of MoPrP(89-143, P101L) (150 μM) incubated with **1** (150 μM). After reduction, the sample was subjected to ultracentrifugation and the pellet and the supernatant evaluated by analytical HPLC and MALDI-TOF mass spectrometry. In the pellet, adductions of up to two molecules of **1** per molecule of PrP55 could be detected.

We next investigated whether secosterol **1** could inhibit misfolding of MoPrP(89-143, P101L) in lipid rafts. Lipid rafts are cholesterol and sphingolipid-rich membrane domains, where both GPI-anchored and phosphatidylinositol-specific phospholipase C (PI-PLC) processed prion locates on cell membranes, and where the conformational conversion of PrP^C to PrP^{Sc} is thought to occur.[26] We prepared unilamellar liposomes[27] incorporating cholesterol, sphingomyelin and **1** (molar lipid ratio 4:4:1) and studied the misfolding of PrP55 (25 μM) in the presence of these liposomes in PBS (pH 7.4) by ThT fluorescence. Liposomes that contain atheronal-B (**1**) inhibit aggregation of PrP55 and lead to a reduced ThT fluorescence plateau compared to PrP55 in the presence of liposomes lacking **1** (Figure 2F).

The data obtained from ThT assays and far-UV CD demonstrates that atheronal-B is able to retard the misfolding of the PrP55 truncated prion protein. The CD spectra revealed that the α-helical form of PrP55 is first converted into a random coil-rich structure before formation of β-sheets. Given that not only the switch from random coil to β-sheets is retarded by aldehyde **1** but also the initial loss in secondary structure, it seems plausible that the inhibitory effect due to adduction of **1**, results in stabilisation of the initial α-helical secondary structure. However, both thermal and urea denaturation studies of PrP55 have shown no measurable difference in the overall thermodynamic stability of the PrP55 in the presence of atheronal-B (**1**) and so the search for this inhibitory effect is still ongoing (Figure S6). One can imagine a scenario where adduction of **1** at specific sites on a protein will lead to a local increase in hydrophobicity due to the cholesterol secosterol structure. If this added hydrophobicity stabilizes a proximate protein domain, a reduction in the free energy of the α-form of the protein ($\Delta G\alpha$, the ground state of the protein) will occur and the rate of transformation into the β-form will be reduced, because the $\Delta\Delta G$ between the $\Delta G\alpha$ and the free energy of the transition state for unfolding/misfolding ($\Delta G^{\neq}$) will be increased. Alternatively, for a folded protein to undergo partial unfolding before refolding into a more thermodynamically stable form, as occurs in the α to β transformation of the MoPrP(89-143, P101L) protein, the protein has to pass through a number of kinetic free energy

barriers. If adduction of **1** leads to an elevation in the highest $\Delta G^{\ddagger}$ (free energy of the transition state) or raises another energy barrier above the level of the transition state in the absence of **1**, then the rate at which the α to β transformation occurs will also be lowered. Our denaturing studies are only able to offer partial information into these two possible mechanisms, because while we are starting from the α-form, denaturation leads to a completely unfolded form, not the β-form. However, given that the denaturation profile from the α-form of MoPrP(89-132, P101L) either heat or urea-mediated is essentially unchanged in the presence of **1**, this suggests atheronal-B is not lowering the ΔGα. This drives us to our present conclusion that atheronal-B is elevating a kinetic barrier, $\Delta G^{\ddagger}$, on the pathway to the β-form.

In conclusion, we have shown that the misfolding of a truncated murine prion protein is retarded in the presence of the lipid aldehyde atheronal-B (**1**), but not by structurally and functionally related compounds (**2**–**5**). This effect was seen both in preparations where atheronal-B was added into solution and where it was present in liposomes, mimicking membrane lipid raft domains. This work builds upon our work studying the effect of lipid-derived aldehydes, that arise from lipid peroxidation, and their effects on amyloidogenic peptides and proteins. In all previous studies lipid aldehydes, when adducted to proamyloidogenic peptides accelerate or trigger the misfolding event. This acceleration occurs with proteins that are both natively unfolded, such as $A\beta_{1-40}$ and $A\beta_{1-42}$, and natively folded forms such as $apoB_{100}$, α-synuclein and antibody light chains. The fact that aldehyde **1** is able to inhibit the misfolding of the MoPrP(89-143, P101L) is an exciting and unexpected result but expands the potential impact and scope of lipid aldehydes and their impact on protein misfolding in vivo. One wonders for example, whether these aldehydes, generated as a by-product of the immune response in addition to being iatrogenic, could in fact play a role in protection against certain unwanted infections, such as prion disease. If such a case were to be true then the right levels and loci for production would differentiate the iatrogenic from beneficial effects.

Arguably, the most important aspect of this study is that if the data for the murine prion fragment can be transposed to proteins that induce prion disease in humans, atheronal-B will offer a new molecular scaffold on which to start structure–activity studies to develop a new class of compounds that may prove to be useful to treat prion disease in vivo.

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