Published in final edited form as:

Angew Chem Int Ed Engl. 2009 ; 48(50): 9469–9472. doi:10.1002/anie.200904524.

Cholesterol seco-sterol adduction inhibits the misfolding of a mutant prion protein fragment that induces neurodegeneration

Johanna C. Scheinost,

The Scripps-Oxford Laboratory, Dept. of Biochemistry, University of Oxford, South Parks Rd., Oxford OX13QU (UK)

Daniel P. Witter,

The Scripps-Oxford Laboratory, Dept. of Biochemistry, University of Oxford, South Parks Rd., Oxford OX13QU (UK)

Grant E. Boldt,

The Scripps-Oxford Laboratory, Dept. of Biochemistry, University of Oxford, South Parks Rd., Oxford OX13QU (UK)

John Offer, and

The Scripps-Oxford Laboratory, Dept. of Biochemistry, University of Oxford, South Parks Rd., Oxford OX13QU (UK)

Paul Wentworth Jr

The Scripps-Oxford Laboratory, Dept. of Biochemistry, University of Oxford, South Parks Rd., Oxford OX13QU (UK), Fax: (+44) 1865-285-329, paul.wentworth@bioch.ox.ac.uk, Homepage: <http://www2.bioch.ox.ac.uk/wentworthlab>

Department of Chemistry and the Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 N. Torrey Pines Road, La Jolla CA 92037 (USA)

Abstract

Cholesterol *seco*sterol aldehydes inhibit the misfolding of a prion protein fragment that induces GSS in mice. Atheronal-B completely inhibits the α to β -form transformation of MoPrP(89-143, P101L) *via* a mechanism that involves adduction to the protein. This result offers a paradigm shift in lipid aldehyde induced protein misfolding and offers a new molecular scaffold on which to develop new potential prion disease therapeutics

Keywords

prion protein; protein misfolding; atheronal; cholesterol oxidation

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.[1,2][3] Prion diseases are infectious,

Correspondence to: Paul Wentworth, Jr.

Supporting information for this article is available on the WWW under <http://www.angewandte.org> or from the author.

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 poly(anions)[5], congo red[6], heparin sulfate[7], pentosan poly(sulfate)[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-*seco*-sterols such as **1**, that are derived from oxidation of cholesterol by activated leukocytes, accelerate the *in vitro* misfolding of apoB₁₀₀, the protein component of low-density lipoprotein (LDL) (figure 1b).[13]

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

Cholesterol *seco*sterol **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 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 potential therapeutics.

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 (Fig. 1a).[19] Critically, injection of the β -form of this peptide intracerebrally into mice induces neuropathological changes that are hallmarks of Gerstmann-Straussler-Scheinker (GSS) disease, whereas injection of the α -form causes no neuropathological effects.

MoPrP(89-143, P101L) was synthesised in multimilligram amounts using standard Fmoc/tBu solid phase peptide synthesis (SPPS) using the Rink amide linker resulting in a C-terminal amide (Fig. 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 hydroxyl **3** were prepared by oxidation and reduction of **1** respectively (see supporting information).

The aqueous buffer conditions for studying the effect of cholesterol *seco*sterol **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 (SI figure 2). Upon dilution of the protein solution (in TFE) into PBS (pH 7.4), the α -helical structure decreases as a function of TFE concentration, with the protein being almost completely random coil at ~10 % v/v TFE in PBS. At 30 % TFE in PBS, the conditions selected for the misfolding assays, the peptide contains considerable, ~ 30 %, α -helical structure, as determined by far u.v. CD (SI figure 2B).

Incubation of MoPrP(89-143, P101L) (20 μ M) 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 ~ 4 d, the time taken to reach 50 % of the plateau intensity (t_{50}) of 5 d and plateau being reached at ~ 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 (SI figure 3).

When the protein (20 μ M) is incubated with increasing concentrations of atheronal-B (**1**) (10 – 150 μ M) 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 (~ 10 d). At the highest concentration of **1** (150 μ M), 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 for study PrP55, in the absence of **1**, is initially rich in α -helices ($t = 0$ d), with two characteristic local minima (206 nm and 222 nm) (figures 2 B, and SI figure 4). 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 *vide supra*, in that 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.

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 2E and SI figure 4).

Given that the carboxylate and hydroxyl 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, possessing close structural simile with **1**, as does the hydroxyl 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 *seco*cholesterol core is secondary and minor to an initial presumed covalent interaction between the aldehyde of **1** and the protein. Furthermore, 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 *seco*sterol **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 triggering 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 SI figure 5). The supernatant contained only one peptide species; 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 $A\beta_{1-40}$.

We next investigated whether *seco*sterol **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 incorporating[27] cholesterol, sphingomyelin and **1** (molar lipid ratio 4:4:1) and studied the misfolding of PrP_{55} (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 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 stabilization of the initial α -helical secondary structure. However, both thermal and salt 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 (SI figure 6). 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 *seco*sterol structure. If this added hydrophobicity stabilizes a proximate protein domain a reduction in the free energy of the α -form of the protein (ΔG_α , 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 ΔG_α and the free energy of the transition state for unfolding/misfolding ($\Delta G^\ddagger$) 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 slowed. 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 thermal or salt-mediated is essentially unchanged in the presence of **1**, this suggests atheronal-B is not lowering the ΔG_{α} . 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 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 β _{1–40} and A β _{1–42}, and natively folded forms such as apoB₁₀₀, 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 transposes can be transposed to proteins that induce prion disease in humans, atheronal-B will offer a new molecular scaffold on which to start SAR studies to develop a new class of compounds that may prove to be useful for prion disease *in vivo*.

Acknowledgments

This work was supported by the NIH AG028300 (PW) and by a grant from The Scripps Research Institute. The authors would like to thank Mr. R. P. Troseth (TSRI) for synthesis of **1**; and Dr. P. R. Antrobus (OU) for high resolution ES-MS of the synthetic peptides.

References

1. Horwich AL, Weissman JS. Cell. 1997; 89:499. [PubMed: 9160742]
2. Brown P, Gajdusek DC. Curr Top Microbiol Immunol. 1991; 172:1. [PubMed: 1687378]
3. Bruce ME, Will RG, Ironside JW, McConnell I, Drummond D, Suttie A, McCordle L, Chree A, Hope J, Birkett C, Cousens S, Fraser H, Bostock CJ. Nature. 1997; 389:498. [PubMed: 9333239]
4. Prusiner SB. Science. 1991; 252:1515. [PubMed: 1675487]
5. Caughey B, Raymond GJ. J. Virol. 1993; 67:643. [PubMed: 7678300]
6. Caughey B, Race RE. J Neurochem. 1992; 59:768. [PubMed: 1352803]
7. Gabizon R, Meiner Z, Halimi M, Ben-Sasson SA. J Cell Physiol. 1993; 157:319. [PubMed: 7901226]
8. Diringier H, Ehlers B. J Gen Virol. 1991; 72(Pt 2):457. [PubMed: 1704414]
9. Priola SA, Raines A, Caughey WS. Science. 2000; 287:1503. [PubMed: 10688802]
10. Chabry J, Caughey B, Chesebro B. J Biol Chem. 1998; 273:13203. [PubMed: 9582363]

11. Chabry J, Priola SA, Wehrly K, Nishio J, Hope J, Chesebro B. *J Virol.* 1999; 73:6245. [PubMed: 10400714]
12. Bieschke J, Zhang Q, Bosco DA, Lerner RA, Powers ET, Wentworth P Jr. Kelly JW. *Acc Chem Res.* 2006; 39:611. [PubMed: 16981677]
13. Wentworth P Jr. Nieva J, Takeuchi C, Galve R, Wentworth AD, Dilley RB, DeLaria GA, Saven A, Babior BM, Janda KD, Eschenmoser A, Lerner RA. *Science.* 2003; 302:1053. [PubMed: 14605372]
14. Zhang Q, Powers ET, Nieva J, Huff ME, Dendle MA, Bieschke J, Glabe CG, Eschenmoser A, Wentworth P Jr. Lerner RA, Kelly JW. *Proc. Natl. Acad. Sci. U.S.A.* 2004; 101:4752. [PubMed: 15034169]
15. Scheinost JC, Wang H, Boldt GE, Offer J, Wentworth PJ. *Angew. Chemie Int. Ed.* 2008 in press.
16. Nieva J, Shafon A, Altobelli LJ 3rd, Tripuraneni S, Rogel JK, Wentworth AD, Lerner RA, Wentworth P Jr. *Biochemistry.* 2008; 47:7695. [PubMed: 18578541]
17. Bosco DA, Fowler DM, Zhang Q, Nieva J, Powers ET, Wentworth P Jr. Lerner RA, Kelly JW. *Nat Chem Biol.* 2006; 2:249. [PubMed: 16565714]
18. Heikenwalder M, Zeller N, Seeger H, Prinz M, Klohn P-C, Schwarz P, Ruddle NH, Weissmann C, Aguzzi A. *Science.* 2005; 307:1107. [PubMed: 15661974]
19. Kaneko K, Ball HL, Wille H, Zhang H, Groth D, Torchia M, Tremblay P, Safar J, Prusiner SB, DeArmond SJ. *Journal of Molecular Biology.* 2000; 295:997. [PubMed: 10656806]
20. Taraboulos A, Rogers M, Borchelt DR, McKinley MP, Scott M, Serban D, Prusiner SB. *Proc Natl Acad Sci U S A.* 1990; 87:8262. [PubMed: 1978322]
21. Rogers M, Yehiely F, Scott M, Prusiner SB. *PNAS.* 1993; 90:3182. [PubMed: 8475059]
22. Laws DD, Bitter H-ML, Liu K, Ball HL, Kaneko K, Wille H, Cohen FE, Prusiner SB, Pines A, Wemmer DE. *PNAS.* 2001; 98:11686. [PubMed: 11562491]
23. Wentworth P Jr. Nieva J, Takeuchi C, Galve R, Wentworth AD, Dilley RB, DeLaria GA, Saven A, Babior BM, Janda KD, Eschenmoser A, Lerner RA. *Science.* 2003; 302:1053. [PubMed: 14605372]
24. Roland R, Simone H, Gerhard W, Rudi G, Kurt Wt. *FEBS letters.* 1997; 413:282. [PubMed: 9280298]
25. Zhang Q, Powers ET, Nieva J, Huff ME, Dendle MA, Bieschke J, Glabe CG, Eschenmoser A, Wentworth P Jr. Lerner RA, Kelly JW. *PNAS.* 2004; 101:4752. [PubMed: 15034169]
26. Pinheiro TJJ. *Chemistry and Physics of Lipids.* 2006; 141:66. [PubMed: 16647049]
27. Wachtel E, Bach D, Epand RF, Tishbee A, Epand RM. *Biochemistry.* 2006; 45:1345. [PubMed: 16430232]

Figure 1.

(A) Graphical representation of an open reading frame of the 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-secoesterol **1** (atheronal-B), carboxylic acid **2**, and primary alcohol **3**. cholesterol (**4**) and 2-naphthaldehyde (**5**).

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 μ M) was incubated with shaking (900 rpm) at 37 °C with 1; $\square$ 0 μ M, $\circ$ 10 μ M, $\triangle$ 50 μ M, ∇ 100 μ M, $\diamond$ 150 μ M). (B and C) Far-UV circular dichroism spectra (0–15 days) of PrP55 (100 μ M) incubated in the absence (B) or presence (C) of 1 (100 μ M). (D) ThT fluorescence of PrP55 (100 μ M) misfolding in the presence of compounds 1–5 (100 μ M). PrP55 alone (—), 1 (—), 2 (—), 3: (—), 4: (—), 5 (—). (E) Far-UV CD Kinetics of PrP55 aggregation in the presence of SAR 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 μ M) in the presence of cholesterol-sphingomyelin liposomes (400 μ M of each lipid) with or without **1** (100 μ M). — **PrP55** in the presence of cholesterol-sphingomyelin liposomes; — PrP 55 in the presence of cholesterol-sphingomyelin-**1** liposomes; — cholesterol-sphingomyelin-**1** liposomes; — cholesterol-sphingomyelin.

Figure 3.

HPLC traces of the supernatant (—) and the pellet (—) after sodium borohydride reduction of MoPrP(89-143, P101L) (150 μ M) incubated with **1** (150 μ M). 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.