Alzheimer's Disease

DOI: 10.1002/ange.200705922

Cholesterol *seco*-Sterol-Induced Aggregation of Methylated Amyloid-β Peptides—Insights into Aldehyde-Initiated Fibrillization of Amyloid-β**

*Johanna C. Scheinost, Hong Wang, Grant E. Boldt, John Offer, and Paul Wentworth, Jr.**

The nucleation and aggregation of amyloid-β (Aβ) peptides, Aβ(1–40) and Aβ(1–42), into neurotoxic oligomers is considered a primary event in Alzheimer's disease (AD) pathogenesis.[1,2] The vast majority of this disease is sporadic in origin (> 85 %),[3] involving the oligomerization of native Aβ peptide oligomers, therefore, research is ongoing to classify the in vivo environmental triggers of AD onset that facilitate intracellular and extracellular nucleation, aggregation and deposition of native Aβ peptide.

As part of our ongoing research in this area,[4] we discovered cholesterol *seco*-sterol aldehyde **1** (termed atheronal-B) in vivo.[5] Aldehyde **1** is quantifiable in all human plasma and central nervous system (CNS), but is significantly elevated in the plasma and inflamed arteries of patients with advanced atherosclerosis, and in the CNS of patients with inflammatory neurological disease.[5,6] We have further shown that adduction of **1** to apoB-100, a protein component of low-density lipoprotein (LDL), causes this protein to misfold in vitro,[5] a misfolding process that renders LDL particles susceptible for uptake into macrophages.[7] In addition, we have shown that **1** accelerates the aggregation of Aβ(1–40) and Aβ(1–42) in vitro[8] hinting that **1** could be a plausible chemical factor linking the known AD association with atherosclerosis.[9] An important and largely unanswered question regarding lipid aldehyde-induced protein misfolding is the nature of the interaction between the aldehyde and the protein and how this facilitates protein aggregation.[4] Herein we show, by kinetic analyses of the atheronal-B (**1**)-induced oligomerization and fibrillization of a panel of synthetic mono-, bis- and tris-*N*,*N*-dimethylamine-containing Aβ(1–40) protein sequences **2b–f** (Figure 1b), that the aggregation of

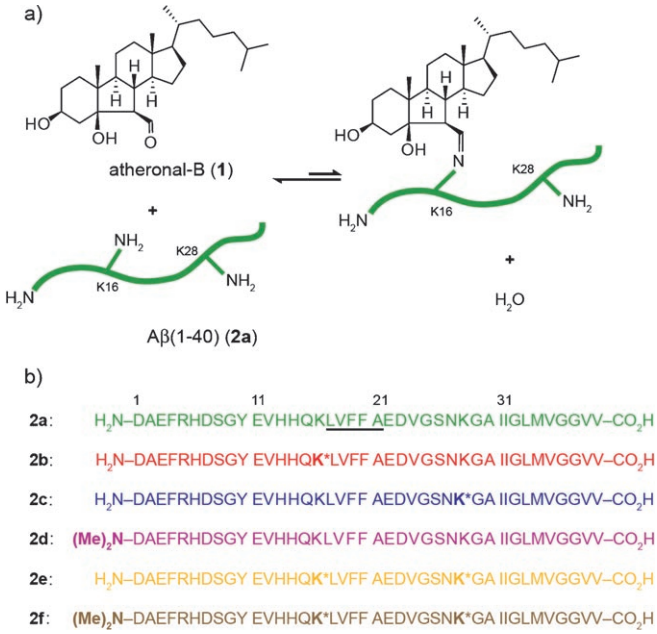

Figure 1. Atheronal-B (**1**)-induced aggregation of Aβ(1–40) peptide (**2a**). a) Schiff-base equilibrium between Lys16 of Aβ(1–40) (**2a**) and aldehyde **1**. Similar equilibria exist for Schiff-base formation at the ε-amino group of Lys28 and the α-amino group of Asp1. b) Amino acid sequences of Aβ(1–40) (**2a**) and *N*,*N*-dimethylamino-containing peptide sequences (**2b–f**) synthesized for these studies. The central hydrophobic cluster (CHC) of Aβ is underlined. **K*** = N^{ε},N^{ε}-dimethyl Lys.

Aβ(1–40) peptide **2a** is accelerated by **1** only when the aldehyde adducts to the ε-amino group of Lys16; no initiation in oligomerization of **2a** is observed when aldehyde **1** adducts to either the ε-amino group of Lys28 or the α-amino group amine of Asp1. In addition, the atheronal-B-induced aggregation of peptide Aβ(1–40) **2a** is inhibited by cholesterol. Both data combine to suggest that the atheronal-B-induced aggregation of Aβ(1–40) involves a high degree of structural recognition between the lipid and the peptide that involves, in part, binding of **1** into the putative cholesterol-binding domain of **2a**.

Atheronal-B (**1**) was synthesized as outlined previously.[5] Peptides **2a–f** were synthesized by Boc/benzyl solid phase peptide synthesis (SPPS) using in situ neutralization.[10] N^{ε},N^{ε}-Dimethyl Lys (**K***) substitutions were incorporated using Boc-Lys$(Me)_2$-CO_2H. The N^{α},N^{α}-dimethyl Asp group required for peptides **2d** and **2f** was incorporated using Me_2N-Asp(OcHx)-CO_2H, prepared by reductive amination

[*] J. C. Scheinost, Dr. G. E. Boldt, Dr. J. Offer, Prof. P. Wentworth, Jr.
The Scripps-Oxford Laboratory
Dept. of Biochemistry, University of Oxford
South Parks Rd., Oxford OX1 3QU (UK)
Fax: (+44) 1865-285-329
E-mail: paul.wentworth@bioch.ox.ac.uk
Homepage: http://www2.bioch.ox.ac.uk/wentworthlab
Dr. H. Wang, Prof. P. Wentworth, Jr.
Department of Chemistry and The Skaggs Institute for Chemical Biology, The Scripps Research Institute
10550 N. Torrey Pines Rd., La Jolla CA 92037 (USA)

[**] This work was supported by a grant from The Scripps Research Institute. The authors thank R. P. Troseth (TSRI) for synthesis of **1**, Dr. M. Wood for EM images, and Dr. P. R. Antrobus (UO) for high resolution ES-MS of peptides **2a–f**.

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

of H_2N-Asp(OcHx)-CO_2H (for full synthetic details see the Supporting Information).

The Aβ(1–40) peptide analogs (**2b–f**, Figure 1b) incorporate tertiary amines that replace the primary amines present in native Aβ(1–40) (**2a**) at specific loci along the peptide chain. Tertiary amines are unable to form Schiff-base adducts with aldehydes, and are isoelectronic with primary amines at physiological pH (7.4), rendering them ideal for a study to elucidate putative site-specific modification of peptide primary amines by aldehydes. However, *N*,*N*-dimethylation of primary amine-containing peptides may lead to increased basicity and hydrophobicity of the resulting peptide,[11] coupled with an impaired ability to form H-bonds. Therefore, the impact of the non-isostructural *N*,*N*-dimethylamino modifications on oligomerization and fibrillization propensity of peptides **2b–f** was determined using a combination of TEM (Figure 2 and Figure S2 in the Supporting Information) and

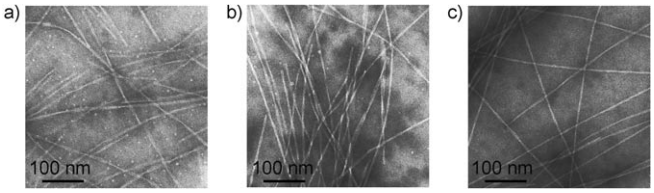

Figure 2. Representative TEM images of fibrils of *N*,*N*-dimethylated amyloid-β peptides: a) **2b** (K*16), b) **2c** (K*28), and c) **2f** (Me_2N-D1, K*16, K*28).

thioflavin-T fluorescence (Figure S3). Fibrillization of the methylated Aβ peptide analogs **2b–f** (100 μM) was induced by incubation with shaking in phosphate buffered saline (PBS) (pH 7.4) containing NaN_3 (0.02% w/v) at 37 °C. TEM analysis of the protein aggregates revealed that the methylated Aβ analogues (**2b–f**) form a classical network of non-branched fibrillar aggregates, several micrometer in length, indistinguishable from native Aβ(1–40) peptide **2a** (Figure 2 and Figure S2 in the Supporting Information).

A comparison of the kinetic profile of aggregation of the methylated peptides **2b–f** relative to native **2a** was determined from a timecourse of thioflavin T (ThT) fluorescence of a deseeded peptide solution (100 μM) in sodium acetate buffer (20 mM, pH 5.0) and NaCl (100 mM) by incubating with standing at 37 °C (see Supporting Information for experimental details).[12] This analysis revealed that the methylated peptides **2b–f** have a classical sigmoidal aggregation profile, indicative of a nucleation-dependent polymerization process,[13,14] with all the peptides having a lag phase similar in duration to that observed with native **2a**, about 1 h (Figure S3 in the Supporting Information).

Timecourses of ThT binding and fluorescence of peptides **2a–f** (100 μM) in the presence or absence of atheronal-B (100 μM) were then measured (Figure 3a–f) using a modification of our previous method.[8] In brief, peptides **2a–f** were deseeded in hexafluoroisopropanol (HFIP),[15] dissolved in dimethylsulfoxide (DMSO) and added into buffer (PBS, pH 7.4). The aggregation was initiated by addition of aldehyde **1** in isopropyl alcohol (IPA) and then proceeded quiescently at 37 °C. Aliquots were removed periodically, added to a ThT solution and the fluorescence of the ThT solution was measured. The concentration of peptide **2a–f** (100 μM) was selected such that no measurable oligomerization of protein in the absence of **1** would occur (Figure 3a–f).

Incubation of atheronal-B (**1**) with peptide **2a** leads to rapid formation of ThT-positive aggregates (time to half maximal fluorescence, $t_{50} = 14$ h, Figure 3a) in a process that is thermodynamically favoured from the outset,[8,16] a so-called "downhill polymerization", that has no measurable lag phase (Figure 3a).[17] Aldehyde **1** also generates ThT-positive aggregates when incubated with the *N*,*N*-dimethylated peptides **2c** (K*28, $t_{50} = 25$ h, Figure 3c) and **2d** (Me_2N-D1, $t_{50} = 13$ h, Figure 3d) in a manner similar to that of **2a**; thus, the ThT profile in each case has no lag phase, with oligomerization proceeding immediately. In contrast, aldehyde **1** does not initiate the formation of ThT-positive aggregates when incubated with *N*,*N*-dimethylated peptides **2b** (K*16, Figure 3b), **2e** (K*16, K*28, Figure 3e) and **2f** (Me_2N-D1, K*16, K*28, Figure 3f).

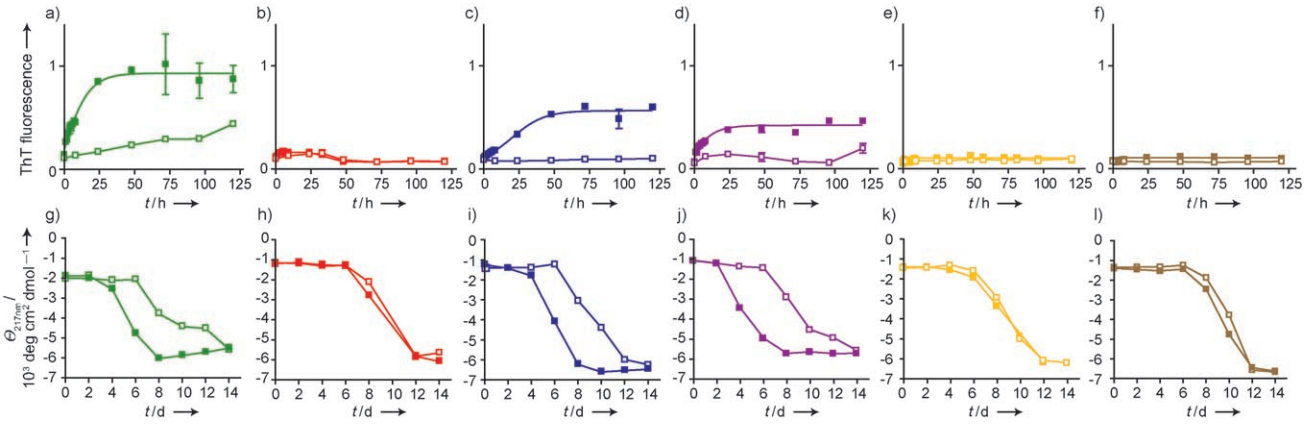

Figure 3. Kinetics of atheronal-B-induced aggregation of amyloid-β peptides **2a–f**. a–f) ThT analyses, ex: 440 nm and em: 485 nm reported as mean ± SD. g–l) Far-UV CD analyses; mean residue ellipticity [Θ] at 217 nm reported as average of three scans of peptides **2a** (——, wild-type); **2b** (——, **K*16**); **2c** (——, **K*28**); **2d** (——, **Me_2N-D1**); **2e** (——, **K*16**, **K*28**); **2f** (——, **Me_2N-D1**, **K*16**, **K*28**). In each case, the peptide (100 μM) is incubated quiescently in the presence (■) or absence (□) of aldehdye **1** (100 μM) at 37 °C.

We next investigated the secondary structure characteristics during atheronal-B-induced fibrillization of peptides **2a–f** spectroscopically using far-UV circular dichroism (CD). For the CD analyses, the deseeding protocol was modified from that used in the ThT assay to remove the DMSO co-solvent. Thus, a typical aggregation involved deseeding in urea (8 M) and glycine/NaOH (10 mM, pH 10). This stock solution was then diluted into PBS (final pH 7.4) containing NaN_3 (0.02 % w/v) in the presence or absence of **1** (100 μM) in IPA (0.5 % v/v) and the aggregation mixtures were then incubated quiescently at 37 °C. Fibrillization was followed by the time-dependent change in the mean residue ellipticity [Θ] at 217 nm (the wavelength minimum for β-strand) (Figure 3). All the peptides **2a–f** were random coil (RC) at $t = 0$ (Figure 3g–l) and in the absence of **1**, all the peptides remained in this form until ca. day 6 when the RC→β-strand transition started to occur; the classic conformational change that occurs during fibrillization of Aβ peptides (for full CD spectra see Figure S5).[18]

For all peptides **2a–f**, the amount of β-strand increases from day 6 until the plateau phase is reached by ca. day 12. The time to half maximal [Θ] at 217 nm, $t_{50}\Theta$, for all the peptides was very similar, ranging from 8–10 d.

The kinetics of the far-UV CD of peptides **2a–f** in the presence of **1** supported the ThT fluorescence data (Figure 3g–l). Specifically, atheronal-B accelerates the onset of β-strand formation when incubated with peptides **2a** [$t_{50}\Theta =$ 5.5 d, (8.0 d in the absence of **1**), Figure 3 g], **2c** [**K*28**, $t_{50}\Theta =$ 6.0 d (9.5 d), Figure 3i] and **2d** [**Me_2N-D1**, $t_{50}\Theta = 4.0$ d (9.0 d), Figure 3j]. In contrast, atheronal-B has no effect on the onset of β-strand formation of peptides, **2b** [**K*16**, $t_{50}\Theta = 9$ d (9 d), Figure 3h], **2e** [**K*16**, **K*28**, $t_{50}\Theta = 9$ d (9 d), Figure 3k] and **2f** [**Me_2N-D1**, **K*16**, **K*28**, $t_{50}\Theta = 7.5$ d (8 d), Figure 3l].

During the CD analyses, the atheronal-induced aggregation of **2a**, **2c**, and **2d** proceeds with a measurable lag phase, indicative of a nucleated polymerization process (Figure 3g, i, and j). This observation is in contrast to the ThT fluorescence kinetic data, where ThT-positive aggregates start to form immediately (Figure 3a, c, and d). In our previous reports of lipid aldehyde **1** induced Aβ(1–40) fibrillization, quiescent aggregation protocols, such as those used throughout this study, lead to a downhill polymerization with no lag phase.[8,16] AFM analysis has revealed that quiescent aggregation Aβ(1–40) with **1** yields spherical aggregates, not fibrils.[8] However, if agitation is applied during the incubation of atheronal **1** with Aβ(1–40) (**2a**), fibrils are formed in a two stage process that does proceed through a nucleation process that has a measurable lag phase.[16] To help answer the quandary as to why with quiescent aggregation conditions we observe a lag phase in the atheronal-induced aggregation of **2a**, we repeated the ThT fluorescence analysis of the aggregation of **2a** (100 μM) in the presence and absence of **1** (100 μM), but using the deseeding method and buffer conditions employed for the CD assay. This approach led to a profile of aldehyde-induced ThT-positive aggregate generation indicative of a nucleation polymerization with a measurable lag phase (see Figure S4 in the Supporting Information). Clearly, the process by which atheronal-B (**1**) is able to obviate Aβ nucleation and form spherical aggregates, considered to be the main neurotoxic species in AD,[1] is sensitive not only to the physical environment (quiescent or shaking) but also the chemical environment in which the aggregation is occurring.

The data presented here adds further support to our original hypothesis, that covalent modification of Aβ-peptides by lipid aldehyde **1** is a key event that facilitates fibrillization. This is emphasized with peptide **2f**, that has no free primary amines with which to form a Schiff base with aldehyde **1**, and whose aggregation is not initiated with atheronal-B (Figure 3l).

However, the most significant result to come from this work is the clear observation that the fibrillization of Aβ-peptides **2a–f** is only accelerated by atheronal-B (**1**), if the ε-amino group of Lys 16 is available for covalent modification. This observation is the most dramatic in the case of peptide **2b** (**K*16**) in which the ε-amino group of Lys 28 and the α-amino group of Asp 1 are both available for adduction, but there is still no initiation in fibrillization by atheronal-B (Figure 3b and h).

In line with current thinking on how hydrophobic peptide mutations contribute to peptide fibrillization through burial of hydrophobic surface,[19,20] we had initially speculated that covalent modification of the ε-amino group of Lys 16, Lys 28 and the α-amino group of Asp 1 with the hydrophobic aldehyde **1** would be sufficient to trigger amyloidogenesis. The hydrophobic effect may still impact amyloidogenesis once aldehyde **1** is adducted to Aβ, but what is clear is that this process is specific to Lys 16. Increasing local hydrophobicity at Lys 28 and Asp 1 by adduction of atheronal-B is not sufficient to trigger fibrillization.

Lys 16 sits at the N-terminus of the central hydrophobic cluster (CHC) which has been suggested to be a cholesterol binding domain of Aβ (Figure 1b).[21] The binding of cholesterol by Aβ has been linked to a role of membrane stabilization.[22,23] Given that atheronal-B (**1**) and cholesterol share structural simile, it seems plausible that upon adduction to Lys 16 binding of the *seco*-sterol **1** in the CHC may occur. We investigated such a hypothesis by studying the effect of cholesterol on atheronal-B(**1**)-induced fibrillization of Aβ(1–40) (**2a**) (Figure 4). Cholesterol exhibits a concentration-dependent reduction of the ability of atheronal **1** to induce fibrillization of Aβ(1–40) **2a**, with an EC_{50} (effective concentration that reduces the maximum ThT-positive aggregates to 50 % of the untreated) of ca. 30 μM. This is the first example of

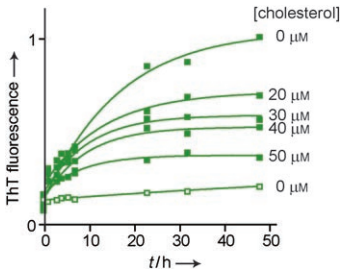

Figure 4. Cholesterol inhibits aldehyde **1**-induced Aβ(1–40) fibrillization. ThT fluorescence of Aβ(1–40) (**2a**) (100 μM) in the presence (■) or absence (□) of **1** (100 μM) and cholesterol (0–50 μM) (PBS, pH 7.4 and 37 °C). Each point is the mean ± SD of duplicate measurements.

inhibition of atheronal-induced Aβ aggregation by a molecule that does not trap the aldehyde of atheronal-B with a nucleophile.[4,8,16] Cholesterol could be impacting aldehyde-induced fibrillization of Aβ by competing with atheronal-B (**1**) for the CHC domain or alternatively, by binding in the CHC domain cholesterol may block adduction of atheronal-B to Lys16. Further studies are ongoing to unravel this effect, but clearly there are molecular recognition events occurring between atheronal-B and Aβ that go beyond Schiff-base formation with Lys16 and could well form the basis for the development of inhibitors as potential therapeutics for Alzheimer's disease.

In summary, we have discovered that cholesterol *seco*-sterol-**1**-induced aggregation of Aβ involves a site-specific adduction of the aldehyde to the ε-amino group of Lys16, suggesting that Lys16 is a hot spot for atheronal-induced fibrillization of Aβ. This process can be inhibited by molecules that compete for the CHC binding domain. Although in this report we have focused on the interaction between **1** and **2a**, the implications are more wide reaching for protein misfolding and disease. We have previously shown that lipid aldehydes cause the misfolding of other disease-related proteins such as apoB-100[5] and α-synuclein.[6] This study suggests that there may be specific hot spots on misfolding prone proteins at which molecular recognition events with specific lipid aldehydes may bind, and in such cases this process may be amenable to competition and inhibition by small molecules that may ultimately lead to new therapeutic targets.

Received: December 22, 2007
Revised: January 26, 2008
Published online: April 11, 2008

Keywords: alzheimer's disease · amyloids · lipid peroxidation · methylation · protein misfolding

[1] C. Haass, H. Steiner, *Nat. Neurosci.* **2001**, *4*, 859.
[2] W. L. Klein, W. B. Stine, Jr., D. B. Teplow, *Neurobiol. Aging* **2004**, *25*, 569.
[3] F. Chiti, C. M. Dobson, *Annu. Rev. Biochem.* **2006**, *75*, 333.
[4] J. Bieschke, Q. Zhang, D. A. Bosco, R. A. Lerner, E. T. Powers, P. Wentworth, J. W. Kelly, *Acc. Chem. Res.* **2006**, *39*, 611.
[5] P. Wentworth, Jr., J. Nieva, C. Takeuchi, R. Galve, A. D. Wentworth, R. B. Dilley, G. A. DeLaria, A. Saven, B. M. Babior, K. D. Janda, A. Eschenmoser, R. A. Lerner, *Science* **2003**, *302*, 1053.
[6] D. A. Bosco, D. M. Fowler, Q. Zhang, J. Nieva, E. T. Powers, P. Wentworth, Jr., R. A. Lerner, J. W. Kelly, *Nat. Chem. Biol.* **2006**, *2*, 249.
[7] C. Takeuchi, R. Galve, J. Nieva, D. P. Witter, A. D. Wentworth, R. P. Troseth, R. A. Lerner, P. Wentworth, *Biochemistry* **2006**, *45*, 7162.
[8] Q. Zhang, E. T. Powers, J. Nieva, M. E. Huff, M. A. Dendle, J. Bieschke, C. G. Glabe, A. Eschenmoser, P. Wentworth, Jr., R. A. Lerner, J. W. Kelly, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 4752.
[9] I. Casserly, E. J. Topol, *Lancet* **2004**, *363*, 1139.
[10] M. Schnolzer, P. Alewood, A. Jones, D. Alewood, S. B. Kent, *Int. J. Pept. Protein Res.* **1992**, *40*, 180.
[11] S. Hyun, H. J. Kim, J. N. Lee, K. H. Lee, Y. Lee, D. R. Ahn, K. Kim, S. Jeong, J. Yu, *J. Am. Chem. Soc.* **2007**, *129*, 4514.
[12] H. Levine III, *Protein Sci.* **1993**, *2*, 404.
[13] J. D. Harper, P. T. Lansbury, Jr., *Annu. Rev. Biochem.* **1997**, *66*, 385.
[14] D. B. Teplow, *Amyloid* **1998**, *5*, 121.
[15] W. B. Stine, Jr., K. N. Dahlgren, G. A. Krafft, M. J. LaDu, *J. Biol. Chem.* **2003**, *278*, 11612.
[16] J. Bieschke, Q. Zhang, E. T. Powers, R. A. Lerner, J. W. Kelly, *Biochemistry* **2005**, *44*, 4977.
[17] A. R. Hurshman, J. T. White, E. T. Powers, J. W. Kelly, *Biochemistry* **2004**, *43*, 7365.
[18] H. Y. Shao, S. C. Jao, K. Ma, M. G. Zagorski, *J. Mol. Biol.* **1999**, *285*, 755.
[19] G. Bitan, S. S. Vollers, D. B. Teplow, *J. Biol. Chem.* **2003**, *278*, 34882.
[20] F. Chiti, M. Stefani, N. Taddei, G. Ramponi, C. M. Dobson, *Nature* **2003**, *424*, 805.
[21] T. J. Nelson, D. L. Alkon, *J. Biol. Chem.* **2005**, *280*, 7377.
[22] N. A. Avdulov, S. V. Chochina, U. Igbavboa, C. S. Warden, A. V. Vassiliev, W. G. Wood, *J. Neurochem.* **1997**, *69*, 1746.
[23] Z.-X. Yao, V. Papadopoulos, *FASEB J.* **2002**, *16*, 1677.

