

# New Class of Inhibitors of Amyloid- $\beta$ Fibril Formation

IMPLICATIONS FOR THE MECHANISM OF PATHOGENESIS IN ALZHEIMER'S DISEASE\*<sup>[S]</sup>

Received for publication, July 2, 2002

Published, JBC Papers in Press, August 6, 2002, DOI 10.1074/jbc.M206593200

Hilal A. Lashuel<sup>‡§</sup>, Dean M. Hartley<sup>‡</sup>, David Balakhaneh<sup>¶</sup>, Aneel Aggarwal<sup>||</sup>, Saul Teichberg<sup>\*\*</sup>,  
and David J. E. Callaway<sup>§¶</sup>

From the <sup>‡</sup>Center for Neurologic Diseases, Brigham and Women's Hospital and Department of Neurology, Harvard Medical School, Cambridge, Massachusetts 02139, <sup>¶</sup>Center for Neurosciences, North Shore/Long Island Jewish Research Institute, Manhasset, New York 11030, <sup>||</sup>Department of Physiology and Biophysics, Mount Sinai School of Medicine, New York, New York 10010, and <sup>\*\*</sup>Department of Pathology, North Shore University Hospital, Manhasset, New York 11030

The amyloid hypothesis suggests that the process of amyloid- $\beta$  protein (A $\beta$ ) fibrillogenesis is responsible for triggering a cascade of physiological events that contribute directly to the initiation and progression of Alzheimer's disease. Consequently, preventing this process might provide a viable therapeutic strategy for slowing and/or preventing the progression of this devastating disease. A promising strategy to achieve prevention of this disease is to discover compounds that inhibit A $\beta$  polymerization and deposition. Herein, we describe a new class of small molecules that inhibit A $\beta$  aggregation, which is based on the chemical structure of apomorphine. These molecules were found to interfere with A $\beta$ 1–40 fibrillization as determined by transmission electron microscopy, Thioflavin T fluorescence and velocity sedimentation analytical ultracentrifugation studies. Using electron microscopy, time-dependent studies demonstrate that apomorphine and its derivatives promote the oligomerization of A $\beta$  but inhibit its fibrillization. Preliminary structural activity studies demonstrate that the 10,11-dihydroxy substitutions of the D-ring of apomorphine are required for the inhibitory effectiveness of these aporphines, and methylation of these hydroxyl groups reduces their inhibitory potency. The ability of these small molecules to inhibit A $\beta$  amyloid fibril formation appears to be linked to their tendency to undergo rapid autoxidation, suggesting that autoxidation product(s) acts directly or indirectly on A $\beta$  and inhibits its fibrillization. The inhibitory properties of the compounds presented suggest a new class of small molecules that could serve as a scaffold for the design of more efficient inhibitors of A $\beta$  amyloidogenesis *in vivo*.

Alzheimer's disease (AD)<sup>1</sup> is a progressive neurodegenerative disease that is characterized by the presence of extracellular amyloid plaques and intraneuronal neurofibrillary tan-

gles in the brains of AD patients (1–3). Biochemical analysis of amyloid plaques revealed that the main constituent of amyloid plaques is fibrillar aggregates of a 39–42-residue peptide referred to as the amyloid- $\beta$  (A $\beta$ ) protein (4). Several lines of evidence point toward a central role for the process of A $\beta$  amyloid fibril formation in the etiology of AD. Transgenic animals overexpressing mutant forms of the amyloid precursor protein develop amyloid plaques, one of the major histopathologic hallmarks of AD (5). Several pathogenic AD mutations have been shown to effect the processing of amyloid precursor protein, resulting in increased A $\beta$  levels, in particular the more amyloidogenic A $\beta$ 42 (6). These data implicate the process of amyloid formation as the cause of neurodegeneration and disease progression in AD. Thus, a small molecule that reduces A $\beta$  production or slows and/or inhibits A $\beta$  aggregation would be considered a useful therapeutic strategy for slowing and/or preventing the progression of AD (7–10).

Although a causal link between amyloid fibril formation and AD is supported by genetic, neuropathologic, and biochemical evidence, the mechanism by which A $\beta$  assemblies cause neurodegeneration is controversial (11). Fibrillization of A $\beta$  occurs via a complex multistep-nucleated polymerization mechanism and proceeds via oligomeric intermediates called protofibrils. The neurotoxicity of A $\beta$  is highly dependent on its conformation, quaternary structure, and the morphology of the aggregates formed (12–15). Monomeric A $\beta$  is thought to be not neurotoxic, whereas both protofibrillar and fibrillar species of A $\beta$  exhibit neurotoxicity in cell-based assays (16–18). Although A $\beta$  toxicity has been reported to be associated with A $\beta$ -mediated oxidative stress (generation of free radicals and reactive oxygen species independent of the aggregation state of A $\beta$ ) (19, 20), recent reports suggest that monomeric A $\beta$ 1–40 and A $\beta$ 1–42 acts as a natural antioxidant and protects neurons against metal-induced oxidative damage, whereas aggregated A $\beta$  acts as a free radical generator, implying an aggregation-dependent biological function of A $\beta$  (21).

Several therapeutic strategies have been proposed for the treatment of amyloid-related disorders caused by the misfolding and aggregation of a precursor protein or a fragment thereof (10, 22, 23). For example, the stabilization of the native three-dimensional structure of the amyloidogenic protein transthyretin (TTR) by its natural ligands (thyroxine, retinol-binding protein) or analogues will inhibit TTR fibril formation *in vitro* (24, 25). Fibril formation by TTR or TTR variants has been linked to the etiology of systemic amyloidosis and familial amyloid polyneuropathy (26). The fact that A $\beta$  is unstructured and its natural partner(s) has not been found makes structural-based design of compounds that stabilize the native nontoxic conformation a challenging task. However, limited screening

\* This work was supported by a grant from the Picower Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at <http://www.jbc.org>) contains supplementary Figs. 1 and 2.

§ To whom correspondence may be addressed. Tel.: 516-562-9425; Fax: 516-365-5090; E-mail: hlashuel@hms.harvard.edu or DCallawa@nshs.edu.

<sup>1</sup> The abbreviations used are: AD, Alzheimer's disease; A $\beta$ , amyloid- $\beta$ ; TTR, transthyretin; SVAU, sedimentation velocity analytical ultracentrifugation; EM, electron microscopy; ThT, Thioflavin T; HMW, high molecular weight.

has resulted in several compounds that inhibit A $\beta$  fibrillogenesis and/or A $\beta$ -associated neurotoxicity *in vitro* including small molecules, proteins, antibodies, and small synthetic peptides (27–30).

In this paper, we report a new class of inhibitors of A $\beta$  fibrillization based on the parent compound apomorphine. *In vitro* studies indicate that these compounds strongly interfere with the fibrillization of A $\beta$ 1–40. Previously, the high susceptibility of apomorphine to autoxidation was thought to limit its efficacy (31–33). However, our results demonstrate that autoxidation of apomorphine and its derivatives is critical for their inhibitory activity, suggesting that one or several of the autoxidation products of apomorphine interacts with monomeric and protofibrillar forms of A $\beta$  in a manner that leads to inhibition of fibril formation. Electron microscopy and sedimentation velocity studies demonstrate that apomorphine and several of its derivatives bind to A $\beta$  and promote its oligomerization, resulting in the accumulation of protofibrillar intermediates (34, 35). These compounds can stabilize protofibrils for approximately 2 weeks at 4 or 25 °C, producing a novel method for investigating the biophysical and toxic properties of protofibrils. More importantly, these compounds may provide useful tools for evaluating the role of A $\beta$  fibrillogenesis in the pathogenesis of AD.

#### EXPERIMENTAL PROCEDURES

Apomorphine (HBr), (*R*)-(-)norapomorphine hydrobromide (D040), (*R*)-(-)-2,10,11-trihydroxyaporphine hydrobromide (D029), (*R*)-(-)-propyl-norapomorphine hydrochloride (D027), (*R*)-(-)-2,10,11-trihydroxy-*N*-propyl-norapomorphine hydrobromide (D030), bulbocapnine hydrochloride, (*R*)-(-)-10,11-methylenedioxy-*N*-*n*-propylnoraporphine hydrochloride, (*R*)-(-)-apocodine hydrochloride, isocrydine hydrochloride, dopamine, and norepinephrine were purchased from Sigma or ICN. Thioflavin T and Congo Red were purchased from Aldrich.

**Preparation of A $\beta$  Protein for Fibrillation Studies**—Purified A $\beta$ 1–40 was purchased from California Peptides (Lot No. SF104). A $\beta$ 1–40 stock solutions were prepared by dissolving the peptide in cold twice distilled water to yield 2 mg/ml solutions. The peptide was processed within 10–30 min of dissolution by diluting to 100  $\mu$ M with Tris-HCl buffer (50 mM Tris, 150 mM NaCl, pH 7.4) to afford a final peptide concentration of 100  $\mu$ M. The samples were incubated at 37 °C without agitation for 1–10 days, depending on the experiment. Drugs were dissolved in water or in 1–1.5% Me<sub>2</sub>SO solutions at 5–7 mM.

**Congo Red Binding Assay for Fibril Formation**—Congo Red was prepared as described previously (25, 26) and diluted to 10  $\mu$ M in 50 mM Tris-HCl, 150 mM NaCl, pH 7.4. Congo Red binding was carried out by combining 10  $\mu$ l of 100  $\mu$ M A $\beta$  solutions with 90  $\mu$ l of 10  $\mu$ M Congo Red solution mixed and incubated for 5–10 min. The binding was determined by using the UV-visible spectra (300–700 nm) collected on a Beckman UV-visible spectrophotometer in a mini-cuvette with a 1-cm pathlength.

**Thioflavin T (ThT) Fluorescence Assay for Fibril Formation**—ThT binding assays were performed by combining 50  $\mu$ l of 100  $\mu$ M A $\beta$  solutions (previously incubated at 37 °C in the absence and presence of drugs) to 450- $\mu$ l solution of 10  $\mu$ M ThT in 10 mM phosphate, pH 7.4, and 100 mM KCl. Fluorescence measurements were recorded in a Biosystems spectrofluorometer at 25 °C using a 1-cm pathlength quartz cell. The excitation wavelength was set to 450 nm (slit width = 4 nm), and emission was monitored from 460–630 nm (slit width = 8 nm). The relative fluorescence at 482 nm was used as a measure of the amount of fibrillar aggregates formed in solution.

**Molecular Weight Determination of A $\beta$ 1–40 Monomer and Oligomers by Analytical Ultracentrifugation Methods**—Sedimentation equilibrium was performed at a rotor speed of 3,000–20,000 rpm using a double sector cell with charcoal-filled epon centerpieces and sapphire windows on 150- $\mu$ l samples of 100  $\mu$ M A $\beta$  in the absence and presence of drugs at pH 7.4. All of the scans were performed at 280 nm or at 330 nm (for solutions containing apomorphine or its derivatives) with a step size of 0.001 cm and 25 averaged scans. Samples were allowed to equilibrate for 24–30 h, and duplicate scans of 3-h apart were overlaid to determine that equilibrium had been reached. The data were analyzed by a non-linear least squares analysis using the Origin software (Beckman Instruments). The concentration profiles obtained at equilibrium were initially fit to a single ideal species model to determine the best fitting molecular weight as shown in Equation 1,

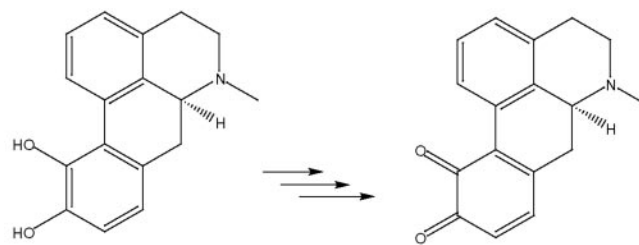


FIG. 1. Chemical structure of apomorphine and one of its possible oxidation products.

$$A_r = \text{Exp}[\ln(A_0) + (M\omega^2(1-\bar{v}\rho)/2RT)\cdot(x^2 - x_0^2)] + E \quad (\text{Eq. 1})$$

where  $A_r$  is the absorbance at radius  $x$ ,  $A_0$  is the absorbance at a reference radius  $x_0$  (usually the meniscus),  $\bar{v}$  is the partial specific volume of A $\beta$  (ml/g),  $\rho$  is the density of the solvent (g/ml),  $\omega$  is the angular velocity of the rotor (radian/sec),  $E$  is the base-line error correction factor,  $M$  is the molecular weight,  $R$  is the universal gas constant ( $8.314 \times 10^7$  erg/mol), and  $T$  was the temperature (Kelvin). The partial specific volume of A $\beta$ 1–40 (0.734 cm<sup>3</sup>/g) was estimated based on the partial specific volumes of the component amino acid residues, whereas the density of the buffers was calculated using polynomial equations and tables of coefficient solution (36, 37).

**Sedimentation Velocity Analytical Ultracentrifugation (SVAU)**—To probe the mechanism by which apomorphine and its derivatives inhibit A $\beta$  fibril formation, sedimentation velocity was used to determine the distribution and molecular size of A $\beta$  quaternary structures in the presence and absence of drugs. A $\beta$  samples (100  $\mu$ M) in 50 mM Tris-HCl, 150 mM NaCl, pH 7.4, were incubated at 37 °C for 1–10 days in the presence and absence of inhibitors and then transferred to 25 °C for 1–3 h before the sedimentation experiments were carried out at 20 °C. For heterogeneous samples containing multiple species, the sedimentation velocity absorbance profiles were analyzed to obtain the apparent distribution of sedimentation coefficients  $g(s^*)$  for all of the quaternary structures in solution using the DCDT software provided by Philo (38, 39). The molecular weight can then be estimated by combining the Svedberg equation and Stokes equation to obtain Equation 2, which utilizes the estimated sedimentation coefficient.

$$(\text{MW})^{\frac{2}{3}} = \frac{100s \cdot \bar{v}^{\frac{1}{3}}}{(1 - \bar{v}\rho)} \quad (\text{Eq. 2})$$

**A $\beta$  Monomer Determination**—SVAU was also used to determine the amount of monomeric A $\beta$  and soluble aggregates in solution after sedimentation of A $\beta$  fibrils. To determine the amount of unsedimented A $\beta$  in solution, two radial scans were collected at 3,000 rpm (only fibrils are sedimentable at this speed) and 20,000–30,000 rpm (soluble high  $M_r$  (HMW) oligomers of A $\beta$  sediment but not monomer). The percent of monomeric species in solution was calculated after complete sedimentation of A $\beta$  high  $M_r$  oligomeric species using Equation 3 at 50,000 rpm.

% Unsedimented A $\beta$  = 1

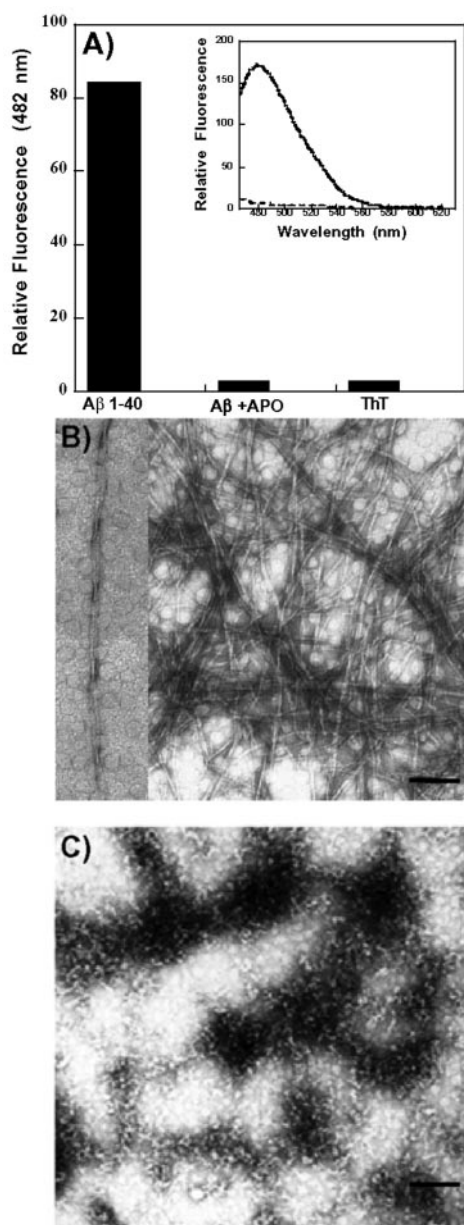
$$- (A_{260 \text{ nm}, 3000 \text{ rpm}} - A_{260 \text{ nm}, 20,000-30,000 \text{ rpm}}) \times 100 \quad (\text{Eq. 3})$$

Sedimentation equilibrium studies were also carried out on the unsedimented species to determine whether a monomer, dimer, or a mixture was present in solution.

**Electron Microscopy**—Transmission electron microscopy was used to analyze the size and structural morphology of A $\beta$  in the presence and absence of drugs at 37 °C. The transmission electron microscopy samples were prepared by placing 5  $\mu$ l of the A $\beta$  solution on a carbon-coated grid for 2 min before removing excess solution. The grid was then washed once with distilled water before staining the sample with 1% fresh uranyl acetate for another 2 min. The grids were thoroughly examined to get an overall evaluation of the structures present in the sample. All electron micrographs were taken at 100 kV using a Phillips CM-100 electron microscope.

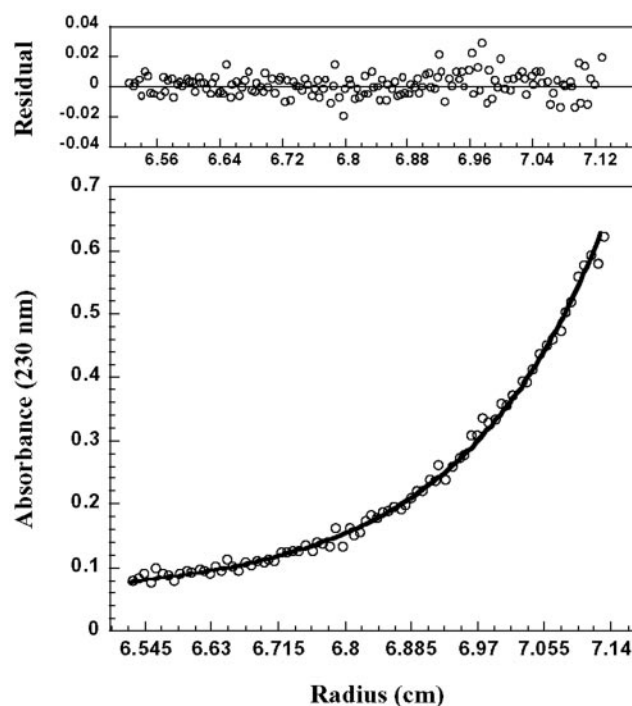
#### RESULTS

**Effect of Apomorphine on Amyloid- $\beta$  Protein Fibrillization**—Solutions of A $\beta$ 1–40 (100  $\mu$ M) were incubated at 37 °C for 1–3 days in the absence and presence of apomorphine (Fig. 1) at



**FIG. 2. Apomorphine inhibits ThT binding and fibril formation.** A, Thioflavin T in the presence of fibrillar aggregates exhibits an emission maximum at 482 nm, characteristic of amyloid fibrils (excited at 450 nm). *Inset*, emission spectra of solutions of A $\beta$  (100  $\mu$ M) alone (*solid line*) and A $\beta$  incubated in the presence of apomorphine (100  $\mu$ M) (*dashed line*). Solutions of A $\beta$  containing apomorphine did not exhibit any fluorescence in the presence of ThT. Negatively stained electron microscopy performed on a sample of A $\beta$ 1–40 (100  $\mu$ M) in the absence (B) or presence of apomorphine (100  $\mu$ M) (C) after incubation at 37 °C for 3 days. A $\beta$  incubated alone showed long twisted helical fibrils (B, *inset*). When incubated with apomorphine, short nonfibrillar oligomeric assemblies are the major species in solution. C, no fibrils could be detected in A $\beta$  solutions containing apomorphine. Scale bar represents 100 nm.

equimolar concentration. Within 2–3 days, visible (by eye) and sedimentable aggregates (15,000  $\times$  g) were observed in samples containing A $\beta$ 1–40 alone. In contrast, A $\beta$ 1–40 (100  $\mu$ M) incubated in the presence of apomorphine (100  $\mu$ M) did not show any visible aggregates. ThT fluorescence is enhanced upon binding to amyloid fibrils, which are proportional to the amount of fibrils in solution (40). Solutions of A $\beta$  only exhibited a greatly enhanced emission at 482 nm, a characteristic for ThT bound to amyloid fibrils (Fig. 2A). Conversely, the combi-



**FIG. 3. Sedimentation equilibrium concentration profile of unsedimented A $\beta$ 1–40 revealed a  $M_r$  consistent with a monomer.** A solution of A $\beta$ 1–40 (100  $\mu$ M) alone was incubated at 37 °C (2 days), the fibrils were spun out by centrifugation (20,000 rpm), and sedimentation equilibrium was performed on the supernatant containing unsedimented A $\beta$  (50,000 rpm). The equilibrium concentration profile (*open circle*) gave an excellent fit (*solid line*) to a single ideal species model as indicated by the residuals and yielded a  $M_r$  of 4,137  $\pm$  280.

nation of A $\beta$  + apomorphine did not show an increase in ThT fluorescence. In addition, the amyloid-specific Congo Red dye showed no increase in the maximal absorbance or change in the red-shifted maximum for A $\beta$  solutions containing apomorphine after 3 days at 37 °C (data not shown). It is noteworthy that A $\beta$ 1–40 solutions containing apomorphine exhibited a change in color over time toward an intensive green color upon incubation at 37 °C; the color change was significantly less than that of apomorphine alone. This color formation is known to be the result of a complicated multistep oxidation process with the autoxidation of apomorphine being the initial step (41).

To further investigate the efficacy of apomorphine and its mechanism of action, EM was used to monitor A $\beta$ 1–40 fibril formation in the absence and presence of apomorphine. A $\beta$  alone revealed unbranched fibrils (~11 nm in diameter) of indeterminate length as the major species (Fig. 2B). The fibrils were observed to exhibit a characteristic helical twist with varying periodicity (Fig. 2B, *inset*). In contrast, co-incubation (3 days, 37 °C) of A $\beta$ 1–40 (100  $\mu$ M) with apomorphine (100  $\mu$ M) resulted in the accumulation of predominantly soluble nonfibrillar assemblies (Fig. 2C). These results suggest that apomorphine is acting as an effective inhibitor of A $\beta$ 1–40 fibril formation *in vitro*.

*Investigating the Molecular Mechanism of Apomorphine Inhibition of A $\beta$  Fibril Formation by Analytical Ultracentrifugation—SVAU and EM experiments performed on solution of A $\beta$ 1–40 (100  $\mu$ M) alone (2 days, 37 °C) indicated that 85–95% A $\beta$ 1–40 exist as fibrils, which rapidly sediment at 3,000 rpm. Sedimentation equilibrium studies were carried out to determine the  $M_r$  of the unsedimented material (~5–15% of the total protein as determined using Equation 3) (see “Experimental Procedures”). The fitting of the equilibrium concentration profile using a single species model yielded a  $M_r$  of 4,137  $\pm$  280,*

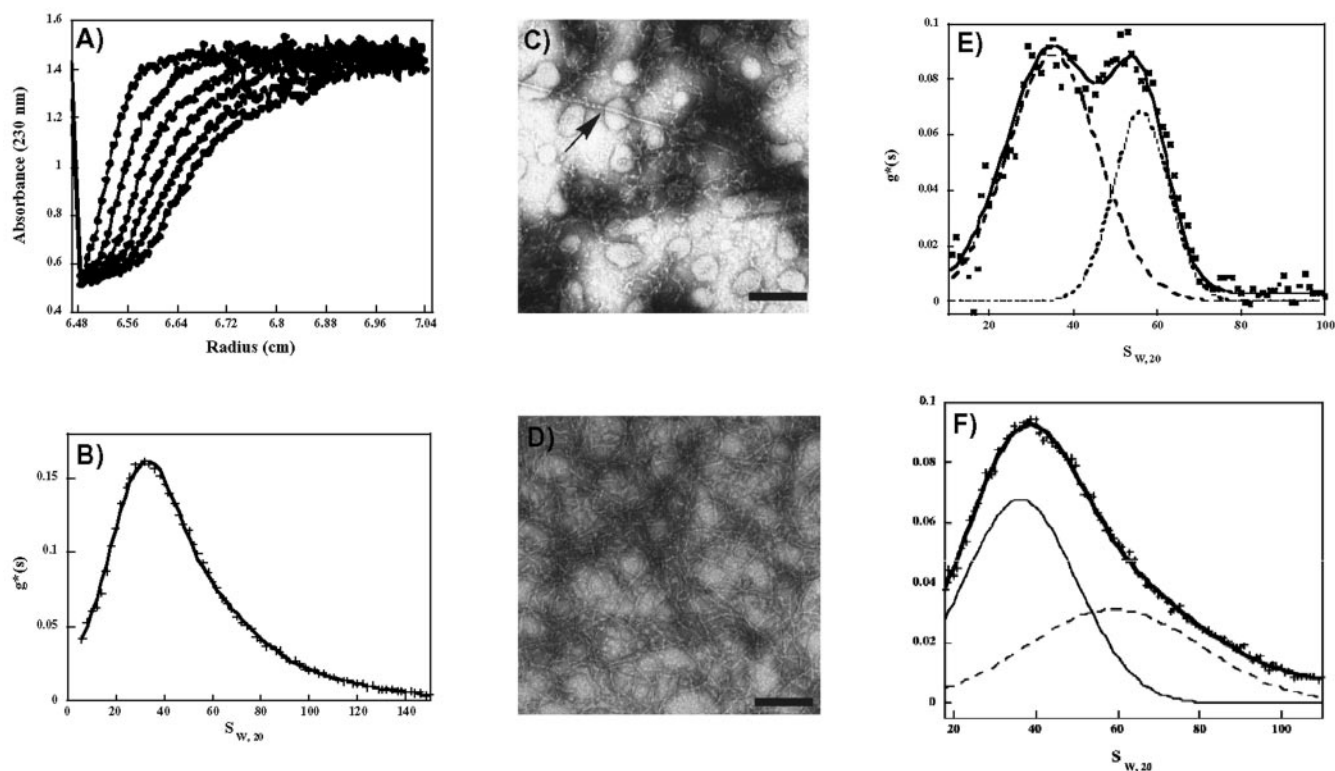


FIG. 4. *A*, sedimentation velocity profiles of a sample of A $\beta$  (100  $\mu$ M) after incubation for 3 days in pH 7.4 buffer (50 mM Tris, 150 mM NaCl) in the presence of apomorphine (100  $\mu$ M). Scans were acquired at 1-min intervals; for clarity, scans were acquired every 4 min as shown. The absorbance base line at 0.6 represents absorbance of unsedimented A $\beta$  and excess apomorphine in solution. *B*, analysis of the sedimentation profiles in *A* using the time-derivative (dc/dt) method. The sedimentation coefficient distribution plot  $g(s^*)$  reveals the presence of heterogeneous distribution of high  $M_r$  A $\beta$ -apomorphine aggregates. The *solid line* represents the best least square fit of the experimental data (+) to a two-sedimenting species model using a Gaussian function. *C* and *D*, negatively stained electron micrograph performed on a sample of A $\beta$ 40 (100  $\mu$ M) in the presence of apomorphine at 50  $\mu$ M (*C*) and 25  $\mu$ M (*D*). At apomorphine concentrations of <50  $\mu$ M, significant fibrils were formed after 3–6 days of incubation at 37  $^{\circ}$ C as seen in *D*. *E*, sedimentation coefficient distribution plot obtained by using the time-derivative analysis revealing two populations of oligomeric species of A $\beta$  (formed in 50  $\mu$ M apomorphine) with average sedimentation coefficients of 35 and 56 S. *F*, similar to *E* but aggregation was carried out in the presence of 25  $\mu$ M apomorphine, ~50% of the sample sediments to the bottom of the centrifuge cell as fibrils at 3,000 rpm. *Scale bar* in *C* and *D* represent 100 nm.

corresponding to the  $M_r$  of monomeric A $\beta$ 1–40 (Fig. 3). In the presence of apomorphine at an equimolar ratio, no sedimentation of A $\beta$ 1–40 was observed at 3,000 rpm, consistent with the absence of fibril formation as verified by EM. Upon increasing the speed to 30,000 rpm, HMW-fast sedimenting species could be detected as observed from the sedimentation profiles (Fig. 4A). An analysis of the sedimentation profiles reveals a heterogeneous distribution of A $\beta$ -apomorphine oligomeric species with a 30 S species being the dominant sedimenting species, indicative of soluble HMW assemblies as the dominant species in solution (Fig. 4B). The results obtained from SVAU experiments are consistent with the EM results, which showed non-fibrillar assemblies as the major species present in A $\beta$  solution containing apomorphine.

At an apomorphine concentration of 50  $\mu$ M (0.5:1, apomorphine:A $\beta$ ), both long unbranched fibrils and short nonfibrillar assemblies were observed by electron microscopy (Fig. 4C). However, the nonfibrillar assemblies remained the dominant species in solution (~70–85% of the total protein) over the incubation period of 3 days at 37  $^{\circ}$ C. At concentrations below 50  $\mu$ M, apomorphine is not effective as an inhibitor, and the long unbranched fibrils become the predominant species (Fig. 4D). SVAU experiments carried out on the same samples used for EM revealed the presence of two oligomeric species of A $\beta$ 1–40 complexed to apomorphine with average sedimentation coefficients of 35 and 56 S with the 35 S species being the dominant species (Fig. 4E). At an apomorphine concentration of 25  $\mu$ M (0.25:1, apomorphine:A $\beta$ ), 40–50% of the sample, which was

incubated at 37  $^{\circ}$ C for 3 days, sediments rapidly at 3,000 rpm, reflecting the presence of amyloid fibrils. An analysis of the sedimentation velocity profiles of the species remaining in solution also revealed at least two populations of HMW species with average sedimentation coefficients of 36 and 58 S (Fig. 4F). These results suggest that a 1:1 molar ratio of A $\beta$  to apomorphine is required for complete inhibition of A $\beta$  fibril formation *in vitro* (3–6 days).

*The Hydroxyl Groups on Apomorphine Are Critical for Inhibiting A $\beta$ 1–40 Fibril Formation*—The structure of apomorphine is characterized by the presence of two adjacent hydroxyl groups on the D-ring of the molecule. Several structural analogues of apomorphine were examined to evaluate the role of the dihydroxy substitutions (10, 11) of the D-ring and the *N*-alkyl groups in modulating the inhibitory effectiveness of these aporphines (Fig. 4). The compounds shown in Fig. 5 were incubated with A $\beta$ 1–40 (100  $\mu$ M) under conditions that favor fibril formation (pH 7.4, 37  $^{\circ}$ C for 3 days). The fibril formation was evaluated by ThT fluorescence, EM, and sedimentation velocity. Fig. 6 shows the amount of ThT binding to A $\beta$  in the presence of apomorphine and its derivatives after incubation for 3 days (37  $^{\circ}$ C). Apomorphine, D027, D029, D030, and D040 did not exhibit any increase in ThT fluorescence, whereas bulbocapnine, *R*-(-)apocodeine, isocrydine, and M121 showed ThT binding similar in magnitude to that of A $\beta$ 1–40 alone. SVAU and EM studies revealed that D027, D029, D030, and D040 inhibited the formation of A $\beta$ 1–40 fibrils in a similar manner as apomorphine + A $\beta$  (data not shown). In support, these

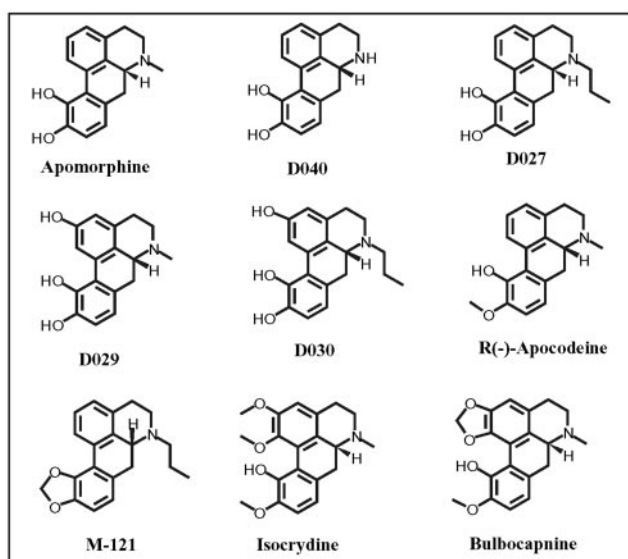


FIG. 5. Structures of apomorphine and apomorphine derivatives used to evaluate the role of the dihydroxy and *N*-alkyl substitutions in modulating the ability of apomorphine to inhibit  $A\beta$  fibril formation.

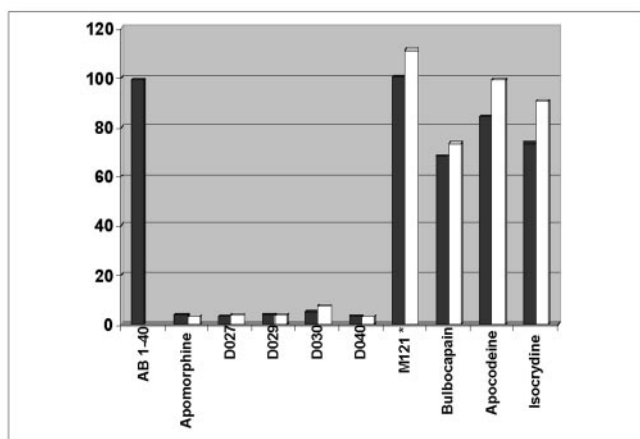


FIG. 6. Bar graph depicting the extent of  $A\beta$  (100  $\mu$ M) fibril formation in the absence and presence of apomorphine and apomorphine derivatives (see Fig. 5). The bar represents the extent of fibril formation based on a quantitative Thioflavin T binding assay in the presence of drugs at 50  $\mu$ M (black bars) or 100  $\mu$ M (white bars).

samples showed the presence of numerous short oligomeric structures with an average length of 100–400 nm and an average diameter of 6–7 nm, suggesting that these compounds stabilize oligomeric intermediates and block fibril formation. SVAU studies revealed that apomorphine, D027, D029, D030, and D040 stabilize  $A\beta$  oligomeric species of similar size, a class of 30–35 S species and a class of 58–65 oligomeric species. In all cases, the compounds at 50 and 100  $\mu$ M were effective at inhibiting  $A\beta$ 1–40 (100  $\mu$ M) fibril formation over a 3-day period with the exception of D030, which required 100  $\mu$ M (1:1,  $A\beta$ :D030). Unlike apomorphine, bulbocapnine, *R*(-)-apocodeine, isocrydine, and M121 have one or both of the hydroxyl groups methylated. These methylated derivatives of apomorphine showed no evidence of inhibiting  $A\beta$ 1–40 fibril formation as discerned from the strong ThT signal, fast sedimentation, and fibril-positive EMs. Together, these results suggest that the hydroxyl groups on the D-ring are required for the inhibitory effectiveness of these compounds.

*Apomorphine-stabilized Nonfibrillar Assemblies Undergo a Conversion into Amyloid Fibril after Longer Incubations*—We

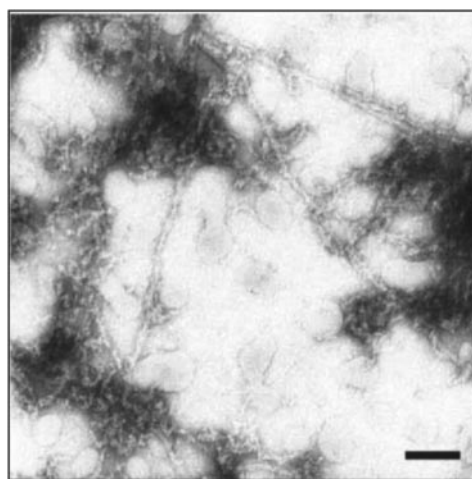


FIG. 7. Electron micrograph of negatively stained quaternary structures deposited from a solution of  $A\beta$  (100  $\mu$ M) at pH 7.4 (50 mM Tris, 150 mM NaCl) after incubation for 9 days at 37  $^{\circ}$ C in the presence of D030, demonstrating the presence of some long fibrils in solution. Scale bar represents 100 nm.

carried out a time-dependent study of  $A\beta$  aggregation to determine whether the nonfibrillar assemblies formed in the presence of apomorphine, D027, D029, D030, and D040 can be converted to amyloid fibrils. To address this possibility, the 37  $^{\circ}$ C incubation of  $A\beta$  + drugs was extended from 3 to 9 days, and the samples were evaluated by EM, SVAU, and ThT fluorescence. Nonfibrillar assemblies remain the dominant species during the incubation period of 1–3 days in the presence of D030 and 1–6 days in the presence of apomorphine, D027, D029, and D040. However, long fibrils appeared, for example, with D030 during the 6–9-day period, which were absent in the EM micrographs at 3 days (Fig. 7). Nevertheless, even after 9 days (37  $^{\circ}$ C), the soluble nonfibrillar assemblies remained in the dominant species and long fibrils composed only ~10–15% total  $A\beta$  in the sample. The slow conversion of nonfibrillar intermediates into fibrils suggests that the short nonfibrillar assemblies complexed to  $A\beta$  could be an on-pathway intermediate of  $A\beta$  amyloid fibril formation.

*Apomorphine, D027, D029, D030, and D040 Promote  $A\beta$  Oligomerization but Inhibit Fibrillization*—We evaluated the rate of oligomeric intermediate(s) formation of  $A\beta$  in the presence and absence of apomorphine, D027, D029, D030, and D040 over the time period of 20–90 h. The incubation of  $A\beta$ 1–40 alone for 20 h (37  $^{\circ}$ C) results in the population of predominantly oligomeric intermediates (15–20%, see Supplementary Fig. 1), which resemble previously describe protofibrillar  $A\beta$  (Fig. 8) and sediment with an average sedimentation coefficient of 20 S. Equilibrium studies revealed that the remaining 80–85%  $A\beta$  (unseeded  $A\beta$ ) in solution exist in its monomeric form. Further incubation (40–72 h) leads to the disappearance of monomeric as well as protofibrillar  $A\beta$  and the appearance of fibrils as the predominant species in the sample (data not shown). In contrast,  $A\beta$  (100  $\mu$ M) samples incubated for 20 h at 37  $^{\circ}$ C in the presence of apomorphine and derivatives (100  $\mu$ M) showed the accumulation of similar nonfibrillar assemblies resembling protofibrils as the major species in solution. The amount of protofibrils formed after 20-h incubation of  $A\beta$  alone was ~30% of that obtained in the presence of apomorphine, D027, D029, D030, and D040. Table I presents a summary of the time-dependent SVAU studies of  $A\beta$  in the presence of inhibitors over a period of 4 days. SVAU analysis indicates the  $A\beta$ -inhibitor complex sediment as a broad boundary of species with average sedimentation coefficients of 33, 35,

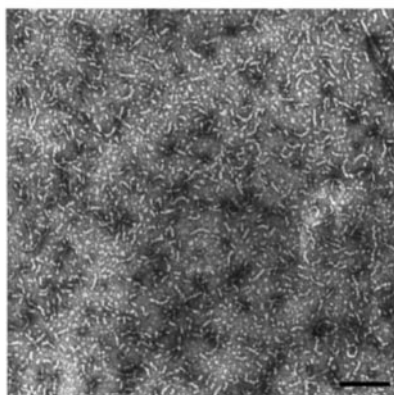


FIG. 8. Electron micrograph of negatively stained quaternary structures deposited from a solution of A $\beta$  (100  $\mu$ M) at pH 7.4 (50 mM Tris, 150 mM NaCl) after incubation for 18 h at 37 °C, demonstrating the presence of protofibrils as the predominant form of A $\beta$  quaternary structures. Scale bar represents 100 nm.

TABLE I

Summary of the time-dependent SVAU studies of A $\beta$  in the presence of inhibitors over a period of 4 days

A $\beta$ 1–40 (100  $\mu$ M) was incubated (37 °C) alone or in the presence of apomorphine derivatives (100  $\mu$ M), and 400  $\mu$ l of the samples were taken at 20, 50, and 90 h for analysis by sedimentation velocity analytical ultracentrifugation. The S values represent the predominant populations of oligomeric A $\beta$ 1–40 species as determined by fitting the experimental data (the sedimentation coefficient distribution plots  $g(s^*)$ ) to a one-, two-, or three-species model using a Gaussian function.

Sample	20 h	50 h	90 h
A $\beta$ 1–40	20 S	Fibrils	Fibrils
A $\beta$ 1–40 + D027	33 S	31 S, 65 S	30 S, 50 S, 76 S
A $\beta$ 1–40 + D029	35 S	36 S, 70 S	26 S, 51 S, 70 S
A $\beta$ 1–40 + D030		23 S, 103 S	
A $\beta$ 1–40 + D040	31 S	36 S, 61 S	36 S, 67 S

33, and 31 S in the presence of D027, D029, D030, and D040, respectively. Further incubation resulted in the population of a second HMW oligomeric species in the presence of apomorphine and its derivatives (Table I). These results suggest that apomorphine-related compounds are unique anti-amyloidogenic agents. Interestingly, our data suggest that these compounds alter the kinetics of fibrillogenesis by enhancing oligomerization while inhibiting fibril formation.

**Apomorphine and Its Structural Analogues Binding to A $\beta$  Monomer Versus Oligomeric Intermediates**—The absorbance of apomorphine and its derivatives at 300–340 nm (A $\beta$ 1–40 does not show any absorbance at this wavelength) enabled us to selectively monitor the sedimentation and estimate the molecular size of soluble A $\beta$  inhibitor aggregates. SVAU studies showed that after centrifugation, 5–15% A $\beta$  remained in the supernatant in its monomeric form. To investigate whether apomorphine binds to monomeric A $\beta$ , we carried out a sedimentation equilibrium experiment on the remaining unsedimented A $\beta$  species by monitoring at 330 nm where apomorphine is the sole contributor to the absorbance at this wavelength. The equilibrium concentration distribution profile produced an excellent fit to a single ideal species model and yielded a  $M_r$  of  $4,430 \pm 360$  g/mol, corresponding to the  $M_r$  of monomeric A $\beta$  (4,329 g/mol) (see Supplementary Fig. 2). Apomorphine is too small (334.2 g/mol) to sediment at 50,000 rpm, and monomeric A $\beta$  should not be detected at 330 nm unless it is bound to apomorphine.

We also investigated the ability of apomorphine and its analogues to act on late intermediates on the pathway of A $\beta$  aggregation and prevent fibril formation. Apomorphine, D027, D029, D030, and D040 (100  $\mu$ M) were added to preformed protofibrils.

Protofibrils were made by preincubating A $\beta$  at 37 °C for 18–20 h, producing a distribution of monomer (80–85%) and protofibrils (15–20%). The samples, A $\beta$  + drugs, were further incubated for 3–9 days (37 °C), after which the solutions were examined by EM and SVAU. All five compounds were shown to interfere with amyloid fibril formation even when added after the protofibrils had formed. For example, the co-incubation of monomeric A $\beta$  with D027 resulted in the accumulation of two populations of assemblies with average sedimentation coefficients of 31 and 65 S, respectively (Fig. 9A). In comparison, D027 added to preformed protofibrils resulted in the accumulation of a wider distribution of quaternary structures (at least three populations of nonfibrillar species with average sedimentation values of 32, 48, and 78 S) and no fibril formation (Fig. 9B–C). Apomorphine, D029, and D040 exhibited a similar behavior with a slightly different size of assemblies being populated. In all cases, fibril formation (*i.e.* protofibrils assembling into amyloid fibrils) was inhibited. D030 was shown to be the least effective compound for inhibiting protofibril assembly into fibrils as observed using EM. These similar binding affinities and our EM studies suggest that protofibrils and the nonfibrillar assemblies formed in the presence of apomorphine and its analogues are similar if not identical.

**Apomorphine Autoxidation Is Required for Inhibiting Aggregation**—The rapid autoxidation of apomorphine, which occurs within the first 1–3 h of incubation at 37 °C (50 mM Tris-HCl + 150 mM NaCl, pH 7.4) (33), suggests that one of the oxidation intermediates or products may be responsible for inhibiting fibril formation. To test this hypothesis, we carried out an A $\beta$  fibril formation assay using a one-month-old (4 °C) inhibitor stock solution (oxidized, colored solution) or a freshly prepared stock solution (clear solution) with or without the reducing agent sodium metabisulfite (1% (v/v), clear solution) (42). The samples were incubated at 37 °C, pH 7.4, for 3 days before being evaluated by ThT and EM. An evaluation by ThT reveals similar inhibition of fibril formation for both the oxidized compounds and the freshly dissolved compounds, which rapidly oxidizes, consistent with the idea that the inhibitory effect is associated with an autoxidation product(s) (Fig. 10). An addition of sodium metabisulfite to A $\beta$  solution samples containing apomorphine, D027, D029, D030, and D040 resulted in the restoration of the ThT signal (similar to A $\beta$  alone), indicating the presence of fibrils. Further support is provided by the EM examination of A $\beta$  samples incubated with either aged or freshly prepared stock solutions of apomorphine or D027, revealing an effective inhibition of A $\beta$  fibril formation in both cases over a 3-day period (37 °C) (Fig. 11). Conversely, an examination of the samples containing sodium metabisulfite revealed long unbranched fibrils similar to those observed for A $\beta$  alone as the major species (data not shown). These results suggest that autoxidation is required for the anti-fibrillization activity observed for these compounds.

## DISCUSSION

The accumulation of A $\beta$  deposits in the form of amyloid plaques in the brain parenchyma is thought to play a critical role in the pathogenesis of AD. Recent studies from several laboratories suggest that the process of A $\beta$  aggregation *in vivo* is responsible for triggering a cascade of physiological events that are critical for the initiation and progression of AD. Therefore, inhibiting the aggregation or deposition of A $\beta$  is thought to be a promising therapeutic strategy to combat AD.

The recent application of analytical ultracentrifugation (sedimentation velocity and sedimentation equilibrium) in parallel with imaging methods (EM and atomic force microscopy) to probe the mechanism of fibril formation has allowed for the

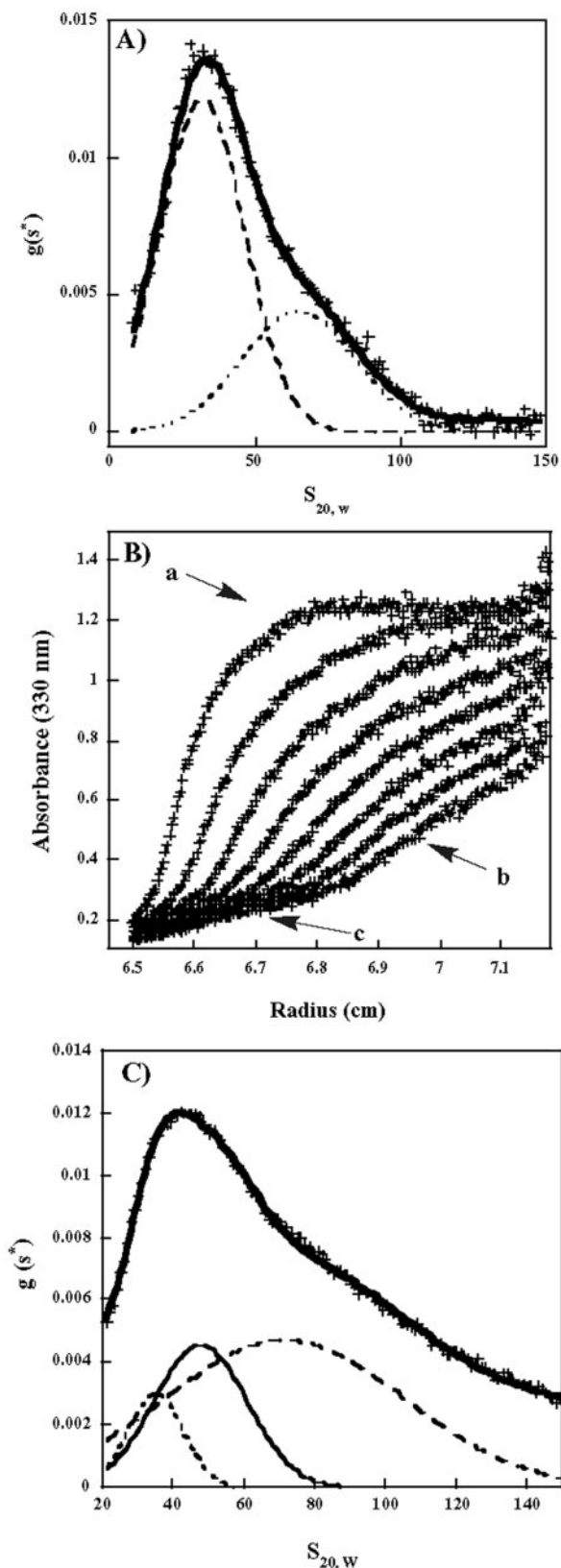


FIG. 9. A, sedimentation coefficient distribution of a sample of A $\beta$  (100  $\mu$ M) at pH 7.4 (50 mM Tris, 150 mM NaCl) after incubation for 3 days at 37  $^{\circ}$ C in the presence of D027 (100  $\mu$ M) obtained from a two-sedimenting species fit (solid line) of the experimental data (+). B, sedimentation velocity profiles of a solution of A $\beta$  (100  $\mu$ M), which was preincubated at 37  $^{\circ}$ C for 18 h, after which D027 was added and the sample was further incubated for 9 days at 37  $^{\circ}$ C. The sedimentation profiles reveal at least three sedimenting boundaries (see arrows a, b, c). C, sedimentation velocity analysis of the profiles shown in B obtained by fitting the data to three-sedimenting species model.

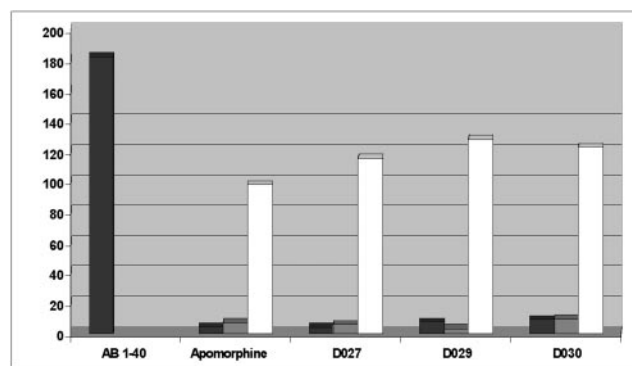
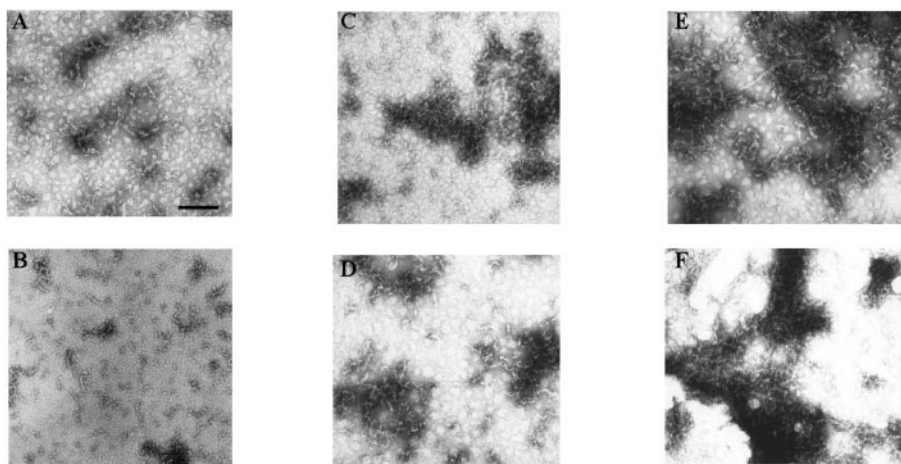


FIG. 10. Bar graph depicting the extent of A $\beta$  fibril formation in the absence and presence of apomorphine, D027, D029, and D030 using freshly prepared stock solutions (dark bars), aged stock solutions (gray bars), and stock solution of the compounds containing 1% (v/v) sodium metabisulfite. The bar represents the extent of fibril formation based on a quantitative Thioflavin T emission (emission = 482 nm; excitation = 450 nm).

observation and characterization of soluble HMW oligomeric intermediates formed during fibrillogenesis for several amyloidogenic proteins including A $\beta$  (34, 43–47). In this paper, analytical ultracentrifugation and EM were employed to probe the mechanism by which catecholamines such as apomorphine and its derivatives interact with A $\beta$  and inhibit fibril formation *in vitro*. Our SVAU studies clearly demonstrate that apomorphine and its structural analogues bind to early oligomeric intermediates of A $\beta$ , resulting in a significant delay in fibril formation and the accumulation of protofibrillar intermediates. The protofibrillar intermediates stabilized by apomorphine, D027, D029, D030, and D040 exhibit molecular mass distribution and structural morphologies that resemble to some extent those observed in the case of A $\beta$  alone as determined by SVAU and EM studies.

Although current evidence implicates the process of A $\beta$  aggregation in the pathogenesis of AD, it remains to be resolved whether a misfolding intermediate, protofibrils, amyloid fibrils, or some combination of species are the toxic assemblies associated with the pathogenesis of AD. Understanding the relative importance of protofibrillar intermediates and fibrils in the pathogenesis of AD is critical for elucidation of the molecular etiology of AD. Studies by several groups suggest a potential pathological role for preamyloid oligomeric intermediates in the pathogenesis of AD (17, 35, 46, 48, 49). Therefore, the development of a small molecule-based strategy for manipulating the amyloid fibril formation pathway, such that each of these species can be populated exclusively, is critical for the elucidation of their relative toxicity and the development of drugs that block their formation as a potential therapeutic strategy for treating AD. If amyloid fibrils are the toxic species, small molecules that inhibit amyloid fibril formation could yield a potential therapy for delaying and/or preventing amyloid-associated neurotoxicity. A delay in amyloid fibril formation may allow for a more efficient clearance of toxic preamyloid intermediates as well as preformed amyloid fibrils, producing an affective therapy. On the other hand, if protofibrillar species such as those stabilized by apomorphine and its derivatives are the toxic agents in AD, *in vivo* conditions or small molecules that promote their formation and accumulation should produce a more severe AD phenotype and accelerate disease progression. Catecholamines including apomorphine, dopamine, norepinephrine, and epinephrine have been shown to enhance A $\beta$  toxicity in cultured hippocampal neurons, most probably because of the formation of reactive oxygen species, semiquinones, quinones, and melanin-like pigments,

FIG. 11. Electron micrographs of negatively stained quaternary structures deposited from solutions of A $\beta$  (100  $\mu$ M) at pH 7.4 (50 mM Tris, 150 mM NaCl) in the presence of apomorphine from aged stock solution (A) and freshly prepared stock solution (B) and in the presence of D027 from aged stock solution (C) and freshly prepared D027 stock solution (D). Panels E and F show electron micrographs from solutions of A $\beta$  incubated with apomorphine and D027, respectively, for 4 days at 37 °C. Scale bar represents 100 nm.



all of which are associated with the autoxidation of these compounds and are thought to mediate their cytotoxicity (50, 51). In addition, A $\beta$ -peptides have been shown to enhance the oxidation of hydroxylamine derivatives without the formation of peptide-derived free radicals (52). The results presented in this paper suggest that autoxidation products of apomorphine and its derivatives could exacerbate A $\beta$  toxicity by stabilizing toxic A $\beta$  protofibrillar intermediates. Recent evidence has begun to suggest that soluble intermediates are neurotoxic; therefore, reagents such as apomorphine may become a valuable tool to test this hypothesis *in vivo*.

Apomorphine is known to undergo rapid autoxidation in aqueous solutions, suggesting that an oxidation product is probably responsible for the observed anti-amyloidogenic properties of apomorphine. In support, we have found that blocking the autoxidation of these molecules either through modification of the hydroxyl groups or by using stabilizing agents such as sodium metabisulfite results in the loss of the inhibitory effect of these molecules. Compounds such as D030, which possess an additional hydroxyl group on the adjacent ring exhibited, reduced inhibitory activity most probably because of their increased susceptibility to additional oxidation processes and suggest that a specific autoxidation product is an effective inhibitor of A $\beta$  fibril formation. An interesting property of apomorphine and its active derivatives is that they seem to target protofibrillar intermediates on the fibril formation pathway of A $\beta$ . Although protofibrillar intermediates were observed in all A $\beta$  incubations, they were present longer in incubations containing apomorphine and its derivatives containing unmodified hydroxyl groups. Indeed, Lansbury and coworkers (53) have recently reported that catechol-containing compounds including dopamine (L-DOPA), norepinephrine, and apomorphine inhibit  $\alpha$ -synuclein fibrillization and result in the kinetic stabilization of protofibrillar intermediates (53). Similar to our observations with A $\beta$ , the inhibitory activity of these compounds was abolished by the addition of antioxidant (sodium metabisulfite), suggesting that oxidation is critical for inhibiting fibril formation. To investigate whether the catechol moiety is sufficient for inhibiting A $\beta$  fibril formation, the activity of the naturally occurring catecholamines, dopamine and norepinephrine, was compared with apomorphine and its derivatives. Apomorphine and its derivatives were shown to be more effective inhibitors of A $\beta$  (100  $\mu$ M) fibril formation at 50  $\mu$ M (~80–92% inhibition) than dopamine (49%) and norepinephrine (32%) (Fig. 12). The addition of apomorphine and its derivatives at higher concentrations (100–200  $\mu$ M) resulted in >95% inhibition of fibril formation, whereas dopamine and norepinephrine resulted in ~82 and 65% inhibition of fibril forma-

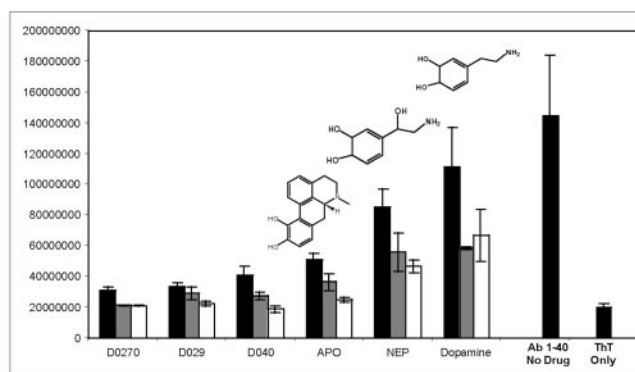


FIG. 12. A comparison of the inhibition of amyloid fibril formation of naturally occurring catecholamines, dopamine and norepinephrine, versus apomorphine and its derivatives. The bar represents the extent of fibril formation based on a quantitative Thioflavin T binding assay in the presence of each of the drugs listed at 50  $\mu$ M (black bars), 100  $\mu$ M (gray bars), and 200  $\mu$ M (white bars).

tion, respectively. These results demonstrate that the catechol moiety contributes significantly to the anti-amyloid activity of these compounds, but it is not the only factor. Apomorphine has an additional ring structure that distinguishes it from naturally occurring catechols making it more hydrophobic, which could increase its affinity for binding especially with A $\beta$ , which has a hydrophobic core. In addition, the nature and reactivity of the intermediate oxidation products have been demonstrated to vary considerably among catechols, suggesting that the active autoxidation species could be different for each class of catechols. Dopamine-derived orthoquinone was shown to modify a small portion of the  $\alpha$ -synuclein monomer and inhibit  $\alpha$ -synuclein aggregation. Although apomorphine has been observed to modify proteins such as albumin (54), we have not been able to detect any covalent modification of A $\beta$  by these compounds using high pressure liquid chromatography and liquid chromatography-tandem mass spectrometry methods.<sup>2</sup> However, an analysis of the A $\beta$  solutions incubated in the presence of apomorphine by liquid chromatography-tandem mass spectrometry suggests that the process of apomorphine oxidation results in the oxidation of the methionine in A $\beta$ . Recent findings by several laboratories demonstrate that oxidation of the amyloidogenic proteins A $\beta$ 40 (55), A $\beta$ 42 (56), and  $\alpha$ -synuclein (57) attenuates their oligomerization properties and inhibits fibrillization. Taken together, these re-

<sup>2</sup> H. A. Lashuel, D. M. Hartley, D. Balakhaneh, A. Aggarwal, S. Teichberg, and D. Callaway, unpublished results.

sults suggest that catecholamine-mediated oxidation of methionine and catecholamines binding to A $\beta$  monomer and protofibrils could have a synergistic effect in inhibiting amyloid fibril formation. It is possible that apomorphine and its derivatives may have the same effect on stabilizing protofibrils generated from other amyloidogenic proteins and may be a very useful tool for understanding the role of protofibrils in other amyloid diseases.

In addition to its apparent anti-amyloidogenic properties, apomorphine is known to act as a potent antioxidant and protect lipids and proteins from radical damage (58). Also, neurotrophic properties have also been reported for apomorphine and its derivatives, giving these compounds multiple biological activities (59). Radical scavengers seem to have a high potential for attenuating neurodegenerative diseases. Apomorphine as a drug is known to quickly pass through the nasal and intestinal mucosa and cross the blood brain barrier and has been approved by the FDA for treating patients with Parkinson's disease, primarily to manage the on-off fluctuation associated with L-DOPA treatment (60). Apomorphine could be an important candidate as a potential neuroprotective agent in the treatment of AD, assuming amyloid fibril formation and oxidative stress are causative (19). Conversely, if apomorphine does stabilize protofibrils, treatment may potentially exacerbate neurodegenerative diseases that produce neurotoxic protofibrils. Therefore, apomorphine may be a valuable tool in animal models to test the hypothesis that protofibrils, at least in part, mediate neurotoxicity. Together, our studies are consistent with previous studies, which demonstrate that the aggregation pathway of A $\beta$  can be manipulated by small molecules or peptidomimetics that stabilize intermediates on the aggregation pathway, ultimately slowing down or preventing amyloid fibrils from being formed (16, 61–65). The ability to use small molecules, such as the those presented in this paper, to selectively interfere and/or block the aggregation of A $\beta$  at different stages on the amyloid fibril formation pathway is critical for investigating the relationship between preamyloid intermediates and fibrils in the pathogenesis of AD. Additional studies are currently underway to identify the regions in A $\beta$  with which these compounds interact. Studies on different derivatives of apomorphine are needed to identify more stable and potent inhibitors of A $\beta$  aggregation and to establish the exact mechanism by which these compounds interfere with A $\beta$  fibril formation. It is probable that these compounds function either through modulating the kinetics of conformational changes required for fibril growth or by decreasing the rate of monomer addition to preamyloid assemblies. Together, our results suggest that the use of apomorphine-like molecules will contribute to a better understanding of the underlying mechanism(s) of A $\beta$  fibrillogenesis in animal models and neurodegeneration. Moreover, these reagents may provide the basis for the development of new aggregation inhibitors as a therapeutic strategy for delaying or treating AD.

*Note Added in Proof*—After submission of this paper, we observed that the relative efficacy of the inhibitors reported varied depending on the lot and source of the peptide when the stocks were prepared in water. However, dissolving the peptide in Me<sub>2</sub>SO + H<sub>2</sub>O + Tris prevented this lot to lot variation. Peptides were dissolved in neat Me<sub>2</sub>SO at a concentration of 5% (A $\beta$ 1–40/vol Me<sub>2</sub>SO) and then in water and in Tris, pH 7.4. Final concentrations of peptide, Me<sub>2</sub>SO, and Tris were 100  $\mu$ M, 2%, and 10 mM, pH 7.4, respectively.

## REFERENCES

- Selkoe, D. J. (1997) *Science* **275**, 630–631
- Castano, E. M., and Frangione, B. (1988) *Lab. Invest.* **58**, 122–132
- Selkoe, D. J. (2000) *Ann. N. Y. Acad. Sci.* **924**, 17–25
- Wang, R., Sweeney, D., Gandy, S. E., and Sisodia, S. S. (1996) *J. Biol. Chem.* **271**, 31894–31902
- Janus, C., Phinney, A. L., Chishti, M. A., and Westaway, D. (2001) *Curr. Neurol. Neurosci. Rep.* **1**, 451–457
- Lichtenthaler, S. F., Ida, N., Multhaup, G., Masters, C. L., and Beyreuther, K. (1997) *Biochemistry* **36**, 15396–15403
- Schenk, D., Games, D., and Seubert, P. (2001) *J. Mol. Neurosci.* **17**, 259–267
- Wolfe, M. S. (2002) *Curr. Top. Med. Chem.* **2**, 371–383
- Yang, D. S., Serpell, L. C., Yip, C. M., McLaurin, J., Christiti, M. A., Horne, P., Boudreau, L., Kisilevsky, R., Westaway, D., and Fraser, P. E. (2001) *Amyloid* **8**, Suppl. 1, 10–19
- LeVine, H. (2002) *Curr. Med. Chem.* **9**, 1121–1133
- Koo, E. H., Lansbury, P. T., Jr., and Kelly, J. W. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 9989–9990
- Simmons, L. K., May, P. C., Tomaselli, K. J., Rydel, R. E., Fuson, K. S., Brigham, E. F., Wright, S., Lieberburg, I., Becker, G. W., Brems, D. N., and Li, W. Y. (1994) *Mol. Pharmacol.* **45**, 373–379
- Howlett, D. R., Jennings, K. H., Lee, D. C., Clark, M. S., Brown, F., Wetzel, R., Wood, S. J., Camilleri, P., and Roberts, G. W. (1995) *Neurodegeneration* **4**, 23–32
- Buchet, R., Tavitian, E., Ristig, D., Swoboda, R., Stauss, U., Gremlich, H. U., de La Fourniere, L., Staufienbiel, M., Frey, P., and Lowe, D. A. (1996) *Biochim. Biophys. Acta* **1315**, 40–46
- Pike, C. J., Burdick, D., Walencewicz, A. J., Glabe, C. G., and Cotman, C. W. (1993) *J. Neurosci.* **13**, 1676–1687
- Lorenzo, A., and Yankner, B. A. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 12243–12247
- Hartley, D. M., Walsh, D. M., Ye, C. P., Diehl, T., Vasquez, S., Vassilev, P. M., Teplow, D. B., and Selkoe, D. J. (1999) *J. Neurosci.* **19**, 8876–8884
- Walsh, D. M., Klyubin, I., Fadeeva, J. V., Cullen, W. K., Anwyl, R., Wolfe, M. S., Rowan, M. J., and Selkoe, D. J. (2002) *Nature* **416**, 535–539
- Butterfield, D. A., Drake, J., Pocernich, C., and Castegna, A. (2001) *Trends Mol. Med.* **7**, 548–554
- Varadarajan, S., Yatin, S., Aksenova, M., and Butterfield, D. A. (2000) *J. Struct. Biol.* **130**, 184–208
- Zou, K., Gong, J. S., Yanagisawa, K., and Michikawa, M. (2002) *J. Neurosci.* **22**, 4833–4841
- Cutler, N. R., and Sramek, J. J. (2001) *Prog. Neuropsychopharmacol. Biol. Psychiatry* **25**, 27–57
- Jacobsen, J. S. (2002) *Curr. Top. Med. Chem.* **2**, 343–352
- Mirog, G. J., Lai, Z., Lashuel, H. A., Peterson, S. A., Strang, C., and Kelly, J. W. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 15051–15056
- White, J. T., and Kelly, J. W. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 13019–13024
- Kelly, J. W., Colon, W., Lai, Z., Lashuel, H. A., McCulloch, J., McCutchen, S. L., Mirog, G. J., and Peterson, S. A. (1997) *Adv. Protein Chem.* **50**, 161–181
- Findeis, M. A. (2002) *Curr. Top. Med. Chem.* **2**, 417–423
- McLaurin, J., Golomb, R., Jurewicz, A., Antel, J. P., and Fraser, P. E. (2000) *J. Biol. Chem.* **275**, 18495–18502
- Soto, C. (1999) *Mol. Med. Today* **5**, 343–350
- Frenkel, D., Solomon, B., and Benhar, I. (2000) *J. Neuroimmunol.* **106**, 23–31
- Linde, H. H., and Ragab, M. S. (1968) *Helv. Chim. Acta* **51**, 683–687
- Lundgren, P., and Landersjö, L. (1970) *Acta. Pharmacol. Suec.* **7**, 133–148
- Sam, E., Augustijns, P., and Verbeke, N. (1994) *J. Chromatogr. B Biomed. Appl.* **658**, 311–317
- Harper, J. D., Wong, S. S., Lieber, C. M., and Lansbury, P. T., Jr. (1999) *Biochemistry* **38**, 8972–8980
- Walsh, D. M., Hartley, D. M., Kusumoto, Y., Fezoui, Y., Condron, M. M., Lomakin, A., Benedek, G. B., Selkoe, D. J., and Teplow, D. B. (1999) *J. Biol. Chem.* **274**, 25945–25952
- Durchschlag, H. (1986) in *Thermodynamic Data for Biochemistry and Biotechnology* (Hinz, H.-J., ed), p. 45, Springer-Verlag New York Inc., New York
- Perkins, S. J., Ashton, A. W., Boehm, M. K., and Chamberlain, D. (1998) *Int. J. Biol. Macromol.* **22**, 1–16
- Philo, S. J. (1997) *Biophys. J.* **72**, 435–444
- Stafford, W. F. (1994) *Methods Enzymol.* **240**, 478–501
- LeVine, H., III (1999) *Methods Enzymol.* **309**, 274–284
- Kaul, P. N., and Brochmann-Hassen, E. (1961) *J. Pharmacol. Sci.* **50**, 266–267
- Wilcox, R. E., Humphrey, D. W., Riffée, W. H., and Smith, R. V. (1980) *J. Pharmacol. Sci.* **69**, 974–976
- Lashuel, H. A., Lai, Z., and Kelly, J. W. (1998) *Biochemistry* **37**, 17851–17864
- Snyder, S. W., Lador, U. S., Wade, W. S., Wang, G. T., Barrett, L. W., Matayoshi, E. D., Huffaker, H. J., Krafft, G. A., and Holzman, T. F. (1994) *Biophys. J.* **67**, 1216–1228
- Harper, J. D., Lieber, C. M., and Lansbury, P. T., Jr. (1997) *Chem. Biol.* **4**, 951–959
- Lambert, M. P., Barlow, A. K., Chromy, B. A., Edwards, C., Freed, R., Liosatos, M., Morgan, T. E., Rozovsky, I., Trommer, B., Viola, K. L., Wals, P., Zhang, C., Finch, C. E., Krafft, G. A., and Klein, W. L. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 6448–6453
- Hatters, D. M., Minton, A. P., and Howlett, G. J. (2002) *J. Biol. Chem.* **277**, 7824–7830
- Lambert, M. P., Viola, K. L., Chromy, B. A., Chang, L., Morgan, T. E., Yu, J., Venton, D. L., Krafft, G. A., Finch, C. E., and Klein, W. L. (2001) *J. Neurochem.* **79**, 595–605
- Nilsberth, C., Westlind-Danielsson, A., Eckman, C. B., Condron, M. M., Axelman, K., Forsell, C., Sten, C., Luthman, J., Teplow, D. B., Younkin, S. G., Naslund, J., and Lannfelt, L. (2001) *Nat. Neurosci.* **4**, 887–893
- Fu, W., Luo, H., Parthasarathy, S., and Mattson, M. P. (1998) *Neurobiol. Dis.* **5**, 229–243
- dos Santos El-Bacha, R., Daval, J., Koziel, V., Netter, P., and Minn, A. (2001) *Biochem. Pharmacol.* **61**, 73–85
- Dikalov, S. I., Vitek, M. P., Maples, K. R., and Mason, R. P. (1999) *J. Biol. Chem.* **274**, 9392–9399

53. Conway, K. A., Rochet, J. C., Bieganski, R. M., and Lansbury, P. T., Jr. (2001) *Science* **294**, 1346–1349
54. Smith, R. V., Velagapudi, R. B., McLean, A. M., and Wilcox, R. E. (1985) *J. Med. Chem.* **28**, 613–620
55. Palmblad, M., Westlind-Danielsson, A., and Bergquist, J. (2002) *J. Biol. Chem.* **277**, 19506–19510
56. Hou, L., Kang, I., Marchant, R. E., and Zagorski, M. G. (2002) *J. Biol. Chem.*, in press
57. Uversky, V. N., Yamin, G., Souillac, P. O., Goers, J., Glaser, C. B., and Fink, A. L. (2002) *FEBS Lett.* **517**, 239–244
58. Ubeda, A., Montesinos, C., Paya, M., Terencio, C., and Alcaraz, M. J. (1993) *Free Radic. Res. Commun.* **18**, 167–175
59. Gassen, M., Glinka, Y., Pinchasi, B., and Youdim, M. B. (1996) *Eur. J. Pharmacol.* **308**, 219–225
60. Corboy, D. L., Wagner, M. L., and Sage, J. I. (1995) *Ann. Pharmacother.* **29**, 282–288
61. Wood, S. J., MacKenzie, L., Maleeff, B., Hurler, M. R., and Wetzel, R. (1996) *J. Biol. Chem.* **271**, 4086–4092
62. Gordon, D. J., Sciarretta, K. L., and Meredith, S. C. (2001) *Biochemistry* **40**, 8237–8245
63. Poduslo, J. F., Curran, G. L., Kumar, A., Frangione, B., and Soto, C. (1999) *J. Neurobiol.* **39**, 371–382
64. Pallitto, M. M., Ghanta, J., Heinzelman, P., Kiessling, L. L., and Murphy, R. M. (1999) *Biochemistry* **38**, 3570–3578
65. Howlett, D. R., Perry, A. E., Godfrey, F., Swatton, J. E., Jennings, K. H., Spitzfaden, C., Wadsworth, H., Wood, S. J., and Markwell, R. E. (1999) *Biochem. J.* **340**, 283–289