New Class of Inhibitors of Amyloid-β Fibril Formation

IMPLICATIONS FOR THE MECHANISM OF PATHOGENESIS IN ALZHEIMER’S DISEASE[S]

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The amyloid hypothesis suggests that the process of amyloid-β protein (Aβ) 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 Aβ polymerization and deposition. Herein, we describe a new class of small molecules that inhibit Aβ aggregation, which is based on the chemical structure of apomorphine. These molecules were found to interfere with Aβ1–40 fibrillation 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 Aβ but inhibit its fibrillation. 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 Aβ amyloid fibril formation appears to be linked to their tendency to undergo rapid autoxidation, suggesting that autoxidation product(s) acts directly or indirectly on Aβ and inhibits its fibrillation. 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 Aβ amyloidogenesis in vivo.

Alzheimer’s disease (AD)† is a progressive neurodegenerative disease that is characterized by the presence of extracellular amyloid plaques and intraneuronal neurofibrillary tangles 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-β (Aβ) protein (4). Several lines of evidence point toward a central role for the process of Aβ 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 Aβ levels, in particular the more amyloidogenic Aβ42 (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 Aβ production or slows and/or inhibits Aβ 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 Aβ assemblies cause neurodegeneration is controversial (11). Fibrillation of Aβ occurs via a complex multistep-nucleated polymerization mechanism and proceeds via oligomeric intermediates called protofibrils. The neurotoxicity of Aβ is highly dependent on its conformation, quaternary structure, and the morphology of the aggregates formed (12–15). Monomeric Aβ is thought to be not neurotoxic, whereas both protofibrillar and fibrillar species of Aβ exhibit neurotoxicity in cell-based assays (16–18). Although Aβ toxicity has been reported to be associated with Aβ-mediated oxidative stress (generation of free radicals and reactive oxygen species independent of the aggregation state of Aβ) (19, 20), recent reports suggest that monomeric Aβ1–40 and Aβ1–42 acts as a natural antioxidant and protects neurons against metal-induced oxidative damage, whereas aggregated Aβ acts as a free radical generator, implying an aggregation-dependent biological function of Aβ (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 (thyroxin, 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 Aβ 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
has resulted in several compounds that inhibit Aβ fibrillogenesis and/or Aβ-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 Aβ fibrillization based on the parent compound apomorphine. In vitro studies indicate that these compounds strongly interfere with the fibrillation of Aβ1–40. 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 Aβ in a manner that leads to inhibition fibril formation. Electron microscopy and sedimentation velocity studies demonstrate that apomorphine and several of its derivatives bind to Aβ 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 Aβ fibrillogenesis in the pathogenesis of AD.

EXPERIMENTAL PROCEDURES

Apopomorphine (HBr), (R)-(+)-norapomorphine hydrobromide (D040), (R)-(+)-2,10,11-trihydroxyapomorphine hydrobromide (D029), (R)-(+)-1-propyl-norapomorphine hydrochloride (D027), (R)-(+)-2,10,11-trihydroxy-N-propyl-norapomorphine hydrobromide (D030), bulbcapeine hydrochloride, (R)-(+)-10,11-methyleneoxy-N-propynorapomorphine hydrochloride, (R)-(-)-apocodeine hydrochloride, isocysteine hydrochloride, dopamine, and norepinephrine were purchased from Sigma or ICN. Thioflavin T and Congo Red were purchased from Aldrich.

Preparation of Aβ Protein for Fibrillation Studies—Purified Aβ1–40 was purchased from California Peptides (Lot No. SF104). Aβ1–40 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 μM with Tris-HCl buffer (50 mM Tris, 150 mM NaCl, pH 7.4) to afford a final peptide concentration of 100 μM. 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% Me2SO 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 μM in 50 mM Tris-HCl, 150 mM NaCl, pH 7.4. Congo Red binding was carried out by combining 10 μM of 100 μM Aβ solutions with 90 μM of 100 μM Congo Red solution mixed and incubated for 5–10 min. The binding was determined 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 μl of 100 μM Aβ solutions (previously incubated at 37 °C in the absence and presence of drugs) to 450-μl solution of 10 μM 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 Aβ1–40 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-μl samples of 100 μM Aβ 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 nonlinear 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.

\[ A = \frac{\text{Exp}(\text{ln}(A_o) + (Mw/(1 - \nu \rho)) \times (x^2 - x_0^2))) + E \] (Eq. 1)

where \( A_o \) is the absorbance at radius \( x \), \( A \) is the absorbance at a reference radius \( x_0 \) (usually the meniscus), \( \nu \) is the partial specific volume of Aβ (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 \((8.314 \times 10^7 \text{ erg/mol})\), and \( T \) is the temperature (Kelvin). The partial specific volume of Aβ1–40 \((0.734 \text{ cm}^3/\text{g})\) 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 Aβ fibrillation, sedimentation velocity was used to determine the distribution and molecular size of Aβ quaternary structures in the presence and absence of drugs. Aβ 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 \(g(n^2)\) 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 Sedwdberg equation and Stokes equation to obtain Equation 2, which utilizes the estimated sedimentation coefficient.

\[ (MW)^2 = \frac{1000 \times v^2 \times r^2}{(1 - w \rho)} \] (Eq. 2)

Aβ Monomer Determination—SVAU was also used to determine the amount of monomeric Aβ and soluble aggregates in solution after sedimentation of Aβ fibrils. To determine the amount of unsedimented Aβ 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 Aβ sediment but not monomer). The percent of monomeric species in solution was calculated after complete sedimentation of Aβ high \( M_r \) oligomeric species using Equation 3 at 50,000 rpm.

\[ \% \text{ Unsedimented Aβ} = 1 - \left( \frac{A_{350 \text{ nm, } 3000 \text{ rpm}} - A_{350 \text{ nm, } 20,000–30,000 \text{ rpm}}}{A_{350 \text{ nm, } 3000 \text{ rpm}} \times 100 \right) \] (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 Aβ in the presence and absence of drugs at 37 °C. The transmission electron microscopy samples were prepared by placing 5 μl of the Aβ 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 Philips CM-100 electron microscope.

RESULTS

Effect of Apomorphine on Amyloid-β Protein Fibrillization—Solutions of Aβ1–40 (100 μM) were incubated at 37 °C for 1–3 days in the absence and presence of apomorphine (Fig. 1) at
equimolar concentration. Within 2–3 days, visible (by eye) and sedimentable aggregates (15,000 \times g) were observed in samples containing Aβ1–40 alone. In contrast, Aβ1–40 (100 μM) incubated in the presence of apomorphine (100 μM) 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 Aβ only exhibited a greatly enhanced emission at 482 nm, a characteristic for ThT bound to amyloid fibrils (Fig. 2A). Conversely, the combi-

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 Aβ (100 μM) alone (solid line) and Aβ incubated in the presence of apomorphine (100 μM) (dashed line). Solutions of Aβ containing apomorphine did not exhibit any fluorescence in the presence of ThT. Negatively stained electron microscopy performed on a sample of Aβ1–40 (100 μM) in the absence (B) or presence of apomorphine (100 μM) (C) after incubation at 37 °C for 3 days. Aβ 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 Aβ solutions containing apomorphine. Scale bar represents 100 nm.

nation of Aβ + 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 Aβ solutions containing apomorphine after 3 days at 37 °C (data not shown). It is noteworthy that Aβ1–40 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 Aβ1–40 fibril formation in the absence and presence of apomorphine. Aβ 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 Aβ1–40 (100 μM) with apomorphine (100 μM) resulted in the accumulation of predominantly soluble nonfibrillar assemblies (Fig. 2C). These results suggest that apomorphine is acting as an effective inhibitor of Aβ1–40 fibril formation in vitro.

Fig. 3. Sedimentation equilibrium concentration profile of unsedimented Aβ1–40 revealed a Mr consistent with a monomer. A solution of Aβ1–40 (100 μM) 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 Aβ (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 Mr of 4,137 ± 280.
corresponding to the $M_r$ of monomeric Aβ1–40 (Fig. 3). In the presence of apomorphine at an equimolar ratio, no sedimentation of Aβ1–40 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 Aβ-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 nonfibrillar assemblies as the major species present in Aβ solution containing apomorphine.

At an apomorphine concentration of 50 μM (0.5:1, apomorphine:Aβ), 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 °C. At concentrations below 50 μM, 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 Aβ1–40 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 μM (0.25:1, apomorphine:Aβ), 40–50% of the sample, which was incubated at 37 °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 35 and 56 S. F, similar to E but aggregation was carried out in the presence of 25 μM 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.

The Hydroxyl Groups on Apomorphine Are Critical for Inhibiting Aβ1–40 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 Aβ1–40 (100 μM) under conditions that favor fibril formation (pH 7.4, 37 °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 Aβ in the presence of apomorphine and its derivatives after incubation for 3 days (37 °C). Apomorphine, D027, D029, D030, and D040 did not exhibit any increase in ThT fluorescence, whereas buncapnine, R(-)epocodine, isocryside, and M121 showed ThT binding similar in magnitude to that of Aβ1–40 alone. SVAU and EM studies revealed that D027, D029, D030, and D040 inhibited the formation of Aβ1–40 fibrils in a similar manner as apomorphine + Aβ (data not shown). In support, these
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 Aβ 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 μM were effective at inhibiting Aβ1–40 (100 μM) fibril formation over a 3-day period with the exception of D030, which required 100 μM (1:1, Aβ: 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 Aβ1–40 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 carried out a time-dependent study of Aβ 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 °C incubation of Aβ + 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 °C), the soluble nonfibrillar assemblies remained in the dominant species and long fibrils composed only ~10–15% total Aβ in the sample. The slow conversion of nonfibrillar intermediates into fibrils suggests that the short nonfibrillar assemblies complexed to Aβ could be an on-pathway intermediate of Aβ amyloid fibril formation.

**Apomorphine, D027, D029, D030, and D040 Promote Aβ Oligomerization but Inhibit Fibrillization**—We evaluated the effectiveness of these compounds to convert Aβ fibrils to amyloid fibrils. To address this possibility, the nonfibrillar assemblies formed in the presence of apomorphine, D027, D029, D030, and D040 were incubated for 20 h at 37 °C in the presence of apomorphine, D027, D029, D030, and D040 over the time period of 20–90 h. The incubation of Aβ1–40 alone for 20 h (37 °C) results in the population of predominantly oligomeric intermediates (15–20%, see Supplementary Fig. 1), which resemble previously described protofibrillar Aβ (Fig. 8) and sediment with an average sedimentation coefficient of 20 S. Equilibrium studies revealed that the remaining 80–85% Aβ (unsedimented Aβ) in solution exist in its monomeric form. Further incubation (40–72 h) leads to the disappearance of monomeric as well as protofibrillar Aβ and the appearance of fibrils as the predominant species in the sample (data not shown). In contrast, Aβ (100 μM) samples incubated for 20 h at 37 °C in the presence of apomorphine and derivatives (100 μM) 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 Aβ 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 Aβ in the presence of inhibitors over a period of 4 days. SVAU analysis indicates the Aβ-inhibitor complex sediment as a broad boundary of species with average sedimentation coefficients of 33, 35,
New Inhibitors of Amyloid-β Fibril Formation

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 Analогues Binding to Aβ Monomer Versus Oligomeric Intermediates—**The absorbance of apomorphine and its derivatives at 300–340 nm (Aβ1–40 does not show any absorbance at this wavelength) enabled us to selectively monitor the sedimentation and estimate the molecular size of soluble Aβ inhibitor aggregates. SVAU studies showed that after centrifugation, 5–15% Aβ remained in the supernatant in its monomeric form. To investigate whether apomorphine binds to monomeric Aβ, we carried out a sedimentation equilibrium experiment on the remaining unsedimented Aβ 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 $M_c$ of 4,430 ± 360 g/mol, corresponding to the $M_c$ of monomeric Aβ (4,329 g/mol) (see Supplementary Fig. 2). Apomorphine is too small (334.2 g/mol) to sediment at 50,000 rpm, and monomeric Aβ 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 Aβ aggregation and prevent fibril formation. Apomorphine, D027, D029, D030, and D040 (100 μM) were added to preformed protofibrils. These results suggest that 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 Aβ 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 Aβ solution samples containing apomorphine, D027, D029, D030, and D040 resulted in the restoration of the ThT signal (similar to Aβ alone), indicating the presence of fibrils. Further support is provided by the EM examination of Aβ samples incubated with either aged or freshly prepared stock solutions of apomorphine or D027, revealing an effective inhibition of Aβ 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 Aβ alone as the major species (data not shown). These results suggest that autoxidation is required for the anti-fibrilization activity observed for these compounds.

**DISCUSSION**

The accumulation of Aβ 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 Aβ 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 Aβ 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...
observation and characterization of soluble HMW oligomeric intermediates formed during fibrillogenesis for several amyloidogenic proteins including Aβ (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 Aβ and inhibit fibril formation in vitro. Our SVAU studies clearly demonstrate that apomorphine and its structural analogues bind to early oligomeric intermediates of Aβ, 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 Aβ alone as determined by SVAU and EM studies.

Although current evidence implicates the process of Aβ 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 affecting 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 Aβ toxicity in cultured hippocampal neurons, most probably because of the formation of reactive oxygen species, semiquinones quinones, and melanin-like pigments.
all of which are associated with the autoxidation of these compounds and are thought to mediate their cytotoxicity (50, 51). In addition, Aβ-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 Aβ toxicity by stabilizing toxic Aβ 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 Aβ 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 Aβ. Although protofibrillar intermediates were observed in all Aβ 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 a-synuclein fibrillization and result in the kinetic stabilization of protofibrillar intermediates (53). Similar to our observations with Aβ, 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 Aβ fibril formation, the activity of 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 Aβ (100 μM) fibril formation at 50 μM (~80–92% inhibition) than dopamine (49%) and norepinephrine (32%) (Fig. 12). The addition of apomorphine and its derivatives at higher concentrations (100–200 μM) resulted in >95% inhibition of fibril formation, whereas dopamine and norepinephrine resulted in ~82 and 65% inhibition of fibril formation, 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 Aβ, 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 α-synuclein monomer and inhibit α-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 Aβ by these compounds using high pressure liquid chromatography and liquid chromatography-tandem mass spectrometry methods. However, an analysis of the Aβ 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 Aβ. Recent findings by several laboratories demonstrate that oxidation of the amyloidogenic proteins Aβ40 (55), Aβ42 (56), and α-synuclein (57) attenuates their oligomerization properties and inhibits fibrillization. Taken together, these re-

![Fig. 11. Electron micrographs of negatively stained quaternary structures deposited from solutions of Aβ (100 μM) 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 Aβ incubated with apomorphine and D027, respectively, for 4 days at 37 °C. Scale bar represents 100 nm.](http://www.jbc.org/)

![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 μM (black bars), 100 μM (gray bars), and 200 μM (white bars).](http://www.jbc.org/)
New Inhibitors of Amyloid-β Fibril Formation

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New Class of Inhibitors of Amyloid-β Fibril Formation: IMPLICATIONS FOR THE MECHANISM OF PATHOGENESIS IN ALZHEIMER'S DISEASE
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J. Biol. Chem. 2002, 277:42881-42890.
doi: 10.1074/jbc.M206593200 originally published online August 6, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M206593200

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