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Carboxyfullerenes as neuroprotective agents

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ABSTRACT **Two regioisomers with C_3 or D_3 symmetry of water-soluble carboxylic acid C_{60} derivatives, containing three malonic acid groups per molecule, were synthesized and found to be equipotent free radical scavengers in solution as assessed by EPR analysis. Both compounds also inhibited the excitotoxic death of cultured cortical neurons induced by exposure to *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), or oxygen-glucose deprivation, but the C_3 regioisomer was more effective than the D_3 regioisomer, possibly reflecting its polar nature and attendant greater ability to enter lipid membranes. At 100 μM, the C_3 derivative fully blocked even rapidly triggered, NMDA receptor-mediated toxicity, a form of toxicity with limited sensitivity to all other classes of free radical scavengers we have tested. The C_3 derivative also reduced apoptotic neuronal death induced by either serum deprivation or exposure to $A\beta_{1-42}$ protein. Furthermore, continuous infusion of the C_3 derivative in a transgenic mouse carrying the human mutant (*G93A*) superoxide dismutase gene responsible for a form of familial amyotrophic lateral sclerosis, delayed both death and functional deterioration. These data suggest that polar carboxylic acid C_{60} derivatives may have attractive therapeutic properties in several acute or chronic neurodegenerative diseases.**

Since their discovery in 1985, the pure carbon spheres of C_{60} (buckminsterfullerene) have generated increasing interest from many different branches of science and engineering, culminating in presentation of the 1996 Nobel Prize in Chemistry to Kroto, Smalley, and Curl for their identification of these unique molecules. Subsequently, investigation into the chemical and physical properties of C_{60} (and larger fullerenes) has yielded an extensive amount of information about C_{60}, including its avid reactivity with free radicals (1). Buckminsterfullerenes, for example, are capable of adding multiple radicals per molecule; the addition of as many as 34 methyl radicals to a single C_{60} sphere has been reported, leading Krusic *et al.* (1) to characterize C_{60} as a "radical sponge." However, native C_{60} is soluble in only a limited number of organic solvents, such as toluene or benzene. We have been interested in the possibility that the potent innate antioxidant properties of C_{60} could be harnessed for use in biological systems by adding functional groups aimed at enhancing its water solubility.

Glutamate receptor-mediated excitotoxicity has been implicated in the pathogenesis of neuronal loss in the central nervous system in several disease states, including hypoxia-ischemia, epilepsy, and trauma (2–5). Oxygen or nitric oxide radicals are produced as a consequence of glutamate receptor overstimulation (6–10), and free radical scavengers have been shown to attenuate, but not to block, excitotoxic neuronal death (11–15). We recently reported promising neuroprotective effects of antioxidant polyhydroxylated derivatives of C_{60} on cultured cortical neurons (16). However, further testing revealed considerable synthesis lot-to-lot variability in both water solubility and biological effects, presumably reflecting uncontrolled differences in the number and location of hydroxyl and hemiketal moieties ending up on the C_{60} shell. To refine this strategy, we have turned to malonic acid derivatives of C_{60}, ($C_{63}((COOH)_2)_3$), synthesized and purified as two specific regioisomers with C_3 and D_3 symmetry (Fig. 1) and demonstrated that they are effective neuroprotective antioxidants *in vitro* and *in vivo*. Although a recent commentary in *Science* by C. Holden (17) suggested that, "Buckyballs have not lived up to their early promise. . . . (in applications)," our findings suggest that water-soluble derivatives of C_{60} may have a novel application as neuroprotective agents.

METHODS

Synthesis and Characterization of Malonic Acid (Carboxy) Derivatives of C_{60}. Malonic acid derivatives of C_{60} were synthesized as described by Lamparth and Hirsch (18). Briefly, diethyl bromomalonate was added to a solution of C_{60} in toluene, followed by the addition of 1,8-diazobicyclo[5,4,0]undec-7-ene, which resulted in a color change from violet to dark red. After stirring for 4 days, the solvent was removed *in vacuo*, and the blackish residue was chromatographed on silica gel (270–230 mesh) using toluene-hexane (1:1 by volume) as eluent. The unreacted C_{60} was obtained first, followed by a brown band that corresponds to the diester. The eluent was changed to toluene-hexane (4:1). A brown band (tetraester) was obtained after a narrow yellow band (tetraester, para addition) followed by a deep red band (tetraester). The eluent was changed again to toluene-hexane (9:1), and a red band was collected that corresponds to the D_3 isomer as the major component. Toluene (100%) then was used to elute three bands. The third band corresponded to semipure C_3 as the major component. The fractions containing semipure C_3 or D_3 were rechromatographed on silica gel (230–400 mesh) using toluene as the mobile phase to give the purified C_3 or D_3 isomers. To a solution of either C_3 or D_3 (100 mg in 100 ml toluene, ≈ 0.1 mmol) was added NaH (80%, 60 mg, 2 mmol), and the mixture was refluxed for 1 hr. After the heating source was removed, MeOH (5 ml) was added immediately to quench the reaction. Red powder soon precipitated and was collected by centrifugation. The powder was washed with toluene twice and hexane four times. The red solid was dissolved in water to which HCl (4 M) was added. A red amorphous precipitate was formed immediately, which was collected by centrifugation. The solid again was washed with HCl (4 M), and then by water twice. The solid was dissolved in MeOH, and the solvent was removed *in vacuo* to give the powdery pure isomer acid (red for C_3 and brownish red for D_3).

EPR Spectroscopy: Radical Reactivity and Membrane Interactions of Carboxyfullerenes. The reactivity of carboxyfullerenes with oxygen radicals was assessed by EPR spectroscopy. Aqueous

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Abbreviations: NMDA, *N*-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; FALS, familial amyotrophic lateral sclerosis.

¶D.W.C. and T.-S.L. contributed equally to this work.

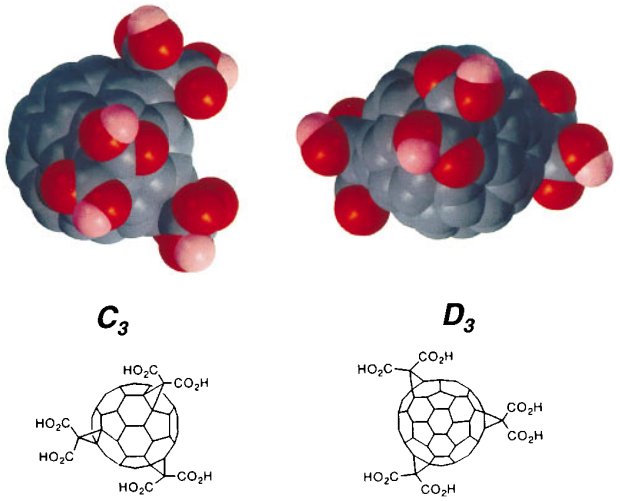

FIG. 1. Structures of carboxyfullerenes showing the paired carboxyl groups on the C_{60} sphere. The three-dimensional models demonstrate the polar distribution of the carboxyl groups on C_3 and the equatorial distribution on D_3.

samples were loaded into a quartz flat cell (60 × 10 × 0.25 mm) and analyzed on a Bruker 200 X-band spectrometer, with experimental conditions as described in the legend for Fig. 2. Signal averaging was performed on some samples.

A second set of experiments was designed to evaluate the degree of membrane interaction of the two carboxyfullerene isomers. Mouse brain lipids were extracted (19) and aliquoted into test tubes. Spin-labeled lipids, 5-doxyl or 16-doxyl ketostearic acids, were added at a ratio of 1:50, and dried under N_2. Tris saline (25 mM, pH 7.4), with or without C_3 or D_3, was added to each tube and vortexed. C_3 and D_3 were tested at concentrations of 10–300 nmol (1:30–1:1 compared with lipids). Settings for the above EPR experiments were power = 1.6 mW, modulation = 1 G, field modulation = 100 Hz, receiver gain = 3.2×10^5. Low-temperature (77°K) EPR also was performed on H_2O_2-oxidized carboxyfullerenes to determine whether oxidation produces a paramagnetic (radical) species.

Media and Reagents. Cell culture reagents and supplies were obtained from standard sources. Laboratory reagents were purchased from Sigma. Media stock consisted of minimal essential media without glutamine, containing 20 mM glucose.

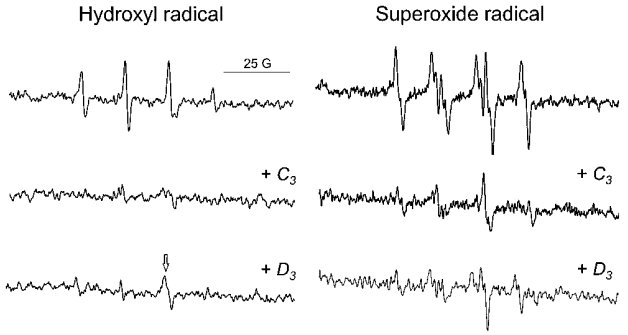

FIG. 2. Water-soluble carboxylic C_{60} compounds are effective free radical scavengers. EPR spectra of hydroxyl ($\cdot$OH) (from 100 μM H_2O_2 with 10 μM Fe^{2+} via the Fenton reaction) and superoxide $O_2^{\cdot-}$ (from 25 μM xanthine + 10 mU/ml xanthine oxidase) radicals with 100 mM 5,5-dimethyl-1-pyrolline-N-oxide (DMPO) as the spin-trapping agent: $\cdot$OH in the presence of DMPO alone, in the presence of 4 μM C_3, or in the presence of 4 μM D_3, $O_2^{\cdot-}$ in DMPO alone, in the presence of 40 μM C_3, or in the presence of 40 μM D_3. The arrow indicates a spurious signal due to an unknown radical in the cavity. The sample was analyzed in a quartz flat cell (60 × 10 × 0.25 mm) in a Bruker 200, X-band EPR spectrometer. Settings were power, 1.6 mW; modulation, 1 G; field modulation, 100 Hz; receiver gain, 3.2×10^5.

Generation of Cell Cultures. Mouse neocortical cultures were prepared as neuronal-astrocyte cocultures or as pure neuronal cultures (<1% astrocytes) from Swiss–Webster mice as described previously (20–21).

Determination of *in Vitro* Neuroprotection by Carboxyfullerenes. Stock solutions of purified C_3 or D_3 carboxyfullerenes (25 mM or 50 mM) were freshly prepared in sterile water. Brief exposure to *N*-methyl-D-aspartate (NMDA) was carried out as described (16). The culture media was exchanged twice with Hepes, bicarbonate-buffered balanced salt solution (9), and then NMDA (200 μM) was added alone or with each of the carboxyfullerenes (30 μM-1 mM) for 10 min. Exposure was terminated by exchanging the medium four times with media stock. The cells were returned to the 37°C (5% CO_2) incubator for 24 hr, when injury was assessed. To determine whether carboxyfullerenes could block NMDA receptor-mediated calcium entry, cells were exposed to NMDA for 10 min in the presence of tracer $^{45}Ca^{2+}$ (0.5 μCi/well; New England Nuclear), and the amount of intracellular $^{45}Ca^{2+}$ determined as previously described (22).

Cultures were exposed to α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) (8 μM), with or without carboxyfullerenes, in media stock containing MK-801 (10 μM MK-801; Merck, Sharp and Dohm) to block activation of NMDA receptors by released endogenous glutamate. Combined oxygen, glucose deprivation was performed as described (23). Cultures were placed in an anoxic chamber (O_2 < 2 mm Hg), and the media was exchanged three times with a balanced salt solution (BSS_0) lacking glucose and oxygen. After 60 min, the cells were washed back into oxygen, glucose-containing medium and returned to the aerobic culture incubator for 24 hr.

To induce apoptosis, pure neuronal cultures containing less than 1% astrocytes were deprived of serum on day *in vitro* 7 by exchanging the medium with serum-free media stock (20). Washed controls were returned to medium with serum. Exposure to $A\beta_{1-42}$ was performed on day *in vitro* 10 mixed cultures by application of 20 μM $A\beta_{1-42}$ (K-Biologicals, Rancho Cucamonga, CA) for 48 hr.

Cell death was assessed by phase contrast microscopy, assay of lactate dehydrogenase in the bathing medium, and manual counting of dead cells stained with propidium iodide (10 μM for 7 min).

Confocal Microscopy and Assessment of Reactive Oxygen Species. Cultures were loaded with 15 μM dihydrorhodamine 123 (Molecular Probes) at the onset of serum deprivation. Cells were imaged 0.5, 4, and 8 hr later on a laser scanning confocal microscope (Noran), using Exλ = 488 nm, Emλ > 515 nm as previously described (9, 24).

***In Vivo* Neuroprotection in a Mouse Model of Amyotrophic Lateral Sclerosis.** Mice from the G93A SOD1 G_1 strain developed by Gurney *et al.* (25) as a model for familial amyotrophic lateral sclerosis (FALS) were treated with carboxyfullerene beginning at 73 ± 2 days of age. Carboxyfullerene containing a mixture of isomers (>85% C_3, 10% D_3, with trace amounts of other Tris malonic acid C_{60} adducts) was dissolved in sterile 0.9% saline, and loaded into mini-osmotic pumps (no. 2004, Alza, 28-day delivery) as per manufacturer's instructions. Pumps were soaked in sterile normal saline for 40 hr and implanted into the peritoneum through a small midline incision. Before implantation of the second pump 4 weeks later, the depleted first pump was removed, and the volume remaining transferred to a tube and measured to verify that drug delivery had occurred at the specified rate of 6.6 μl/day, to deliver 15 mg/kg per day. Mice were maintained in the colony until moribund, determined by inability of the mouse to right itself in 30 sec after being placed on its side. This correlated well with inability to drink spontaneously or to respond to offered food or water. Determination of whether an animal was moribund was determined by a blinded observer, and the date of death recorded.

Motor performance was assessed using a scale developed by Basso *et al.* (26) to evaluate function after spinal cord injury.

Mice were videotaped weekly starting at 89 days of age, and their gait scored by a blinded observer using this 21-point scale, which rates spontaneous ambulation, including leg position and coordination. Mice were evaluated more informally daily.

RESULTS

Characterization of Carboxyfullerenes. Mass spectroscopy of the two purified trisadduct regioisomer esters demonstrated a peak in each sample at 1,194/1,195 mass units, as previously described (18). Identity and purity of both the intermediate ester and the final malonic acid product of each regioisomer were confirmed by ^{13}C NMR spectroscopy and mass spectrometry (18). The final malonic acids, C_3 and D_3, were soluble in water to at least 75 mM. Control experiments verified that the compounds did not interfere with the colorimetric lactate dehydrogenase assay, and solutions to at least 25 mM failed to alter the pH of the experimental solutions.

Carboxyfullerenes Are Potent Free Radical Scavengers. Both C_3 and D_3 isomers were unusually potent scavengers of hydroxyl radical ($\cdot OH$) and superoxide anion ($O_2^{\cdot-}$) in solution. They were able to completely eliminate $\cdot OH$ at concentrations as low as 4–5 μM (Fig. 2), 10- to 100-fold less than required for most other free radical scavengers. Both isomers also effectively scavenged superoxide radical ($O_2^{\cdot-}$), although 10-fold higher concentrations were required to do this (Fig. 2).

Neuroprotection Against Excitotoxic Injury *in Vitro*: The C_3 and D_3 Regioisomers Show Differential Efficacy. Both the C_3 and D_3 isomers produced a dose-dependent decrease in death of cortical neurons exposed to NMDA or AMPA (Fig. 3 *A* and *B*), although the C_3 compound was both more potent and more effective than D_3 (Fig. 3 *A* and *B*). The C_3 compound provided essentially complete neuroprotection against NMDA receptor-mediated neuronal death. Although the degree of protection afforded by C_3 against AMPA-induced neurotoxicity was generally somewhat less, coapplication of C_3 still reduced neuronal death by over 80%. The concentration of carboxyfullerene required to produce maximal protection against these excitotoxic insults varied somewhat with the degree of injury; i.e., insults that resulted in 100% neuronal death required slightly higher concentrations of carboxyfullerene for maximal neuroprotection. The C_3 isomer also reduced neuronal death after combined oxygen-glucose deprivation for 45–60 min (Fig. 4), an injury mediated in large part by NMDA receptors (23); the D_3 compound was not tested. To exclude the possibility that C_3 could antagonize NMDA receptors, the cellular uptake of $^{45}Ca^{2+}$ from the bathing medium induced by NMDA was assessed; coapplication of 30–300 μM C_3 did not affect Ca^{2+} uptake (data not shown).

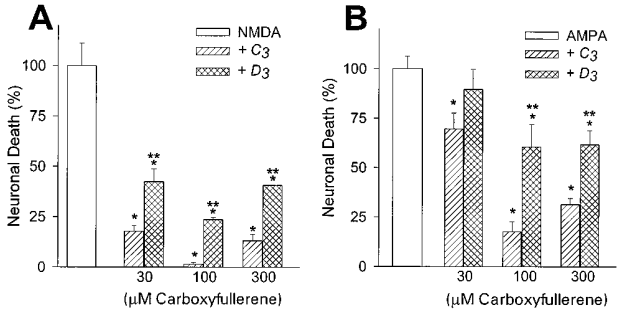

FIG. 3. The C_3 isomer was a more effective neuroprotective agent than the D_3 isomer. C_3 provided better protection than D_3 against excitotoxic neuronal death induced by exposure to NMDA (200 μM for 10 min, *A*) or AMPA (8 μM for 24 hr, *B*). In all figures, the data were normalized to the injury condition (NMDA, AMPA, etc.) without C_3, and were expressed as percent of the injury without C_3. *, $P < 0.05$ vs. untreated injury condition, using ANOVA followed by Student–Newman–Keuls test for multiple comparisons. Values are mean ± SEM, n = 8–16 cultures per condition. *, different from excitotoxin alone at $P < 0.05$, using ANOVA followed by Student–Newman–Keuls test for multiple comparisons. **, different from C_3.

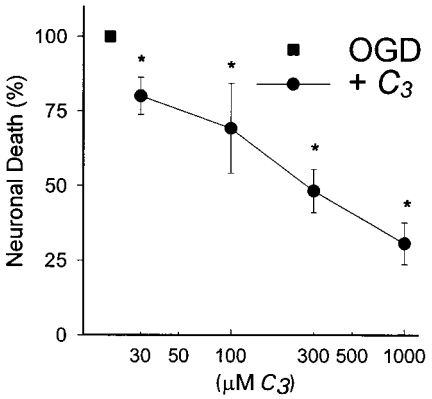

FIG. 4. C_3-reduced neuronal cell death produced by 60 min of combined oxygen-glucose deprivation; n = 8–12 per condition. The data were normalized to oxygen-glucose deprivation without C_3 and were expressed as percent of this injury. *, $P < 0.05$ vs. oxygen-glucose deprivation without C_3, using ANOVA followed by Student–Newman–Keuls test for multiple comparisons.

C_3 and D_3 Regioisomers Show Different Membrane Interactions. We hypothesized that the difference in biological activity between the isomers might reflect a difference in dipole moment and resultant ability to intercalate into lipid bilayers. To test the hypothesis that C_3 can enter cell membranes better than D_3, we used EPR spectroscopy of nitroxide spin-labels incorporated into a mixture of mouse brain lipids (Fig. 5). Three parameters of carboxyfullerene-lipid interaction were determined from the EPR measurements: the order parameter (S), correlation time (t_c), and lipid/aqueous partition factor. C_3 decreased the order parameter (measured with the spin-labeled 16-doxyl lipid) more than D_3, suggesting that C_3 interacts with the interior of the bilayer to a greater degree than D_3. This is supported by the observed changes in t_c and the partition factor for C_3, which indicate substantial entry of C_3 into the bilayer. On the other hand, D_3 caused opposite changes in t_c and the partition factor, which imply interaction by D_3 with the headgroup region of the bilayer, but little entry into the membrane. The results, taken together, are consistent with greater intercalation of C_3 compared with D_3 into brain lipid membranes (Table 1).

Carboxyfullerenes Limit Apoptotic Neurodegeneration from Serum Deprivation or Aβ_{1-42}. To examine whether the carboxyfullerenes also could inhibit neuronal apoptosis, we turned to two paradigms: (*i*) serum deprivation, and (*ii*) exposure to the Alzheimer disease amyloid peptide, Aβ_{1-42}. Cortical neurons cultured without glia underwent apoptosis 24–48 hr after removal of serum (Fig. 6 *A* and *B Top*). As with sympathetic neurons deprived of nerve growth factor, this serum deprivation-induced neuronal apoptosis was associated with an enhancement of intracellular free radical production (Fig. 6*B*, *Bottom*), detectable by oxidation of dihydrorhodamine to fluorescent rhodamine 123. Application of C_3 at the onset of serum deprivation reduced subsequent free radical production and apoptosis (Fig. 6 *A* and *B*). In addition, application of C_3 also reduced apoptotic death of cortical neurons induced by 24–48 hr exposure to Aβ_{1-42} (Fig. 7).

Carboxyfullerenes Are Neuroprotectve in a Mouse Model of FALS. Mice that received C_3 carboxyfullerene via intraperitoneal mini-osmotic pumps for 2 months, beginning just after 2 months of age, showed delayed onset of symptoms, improved functional performance, and delayed death as compared with saline-treated controls. C_3-treated mice exhibited a delay in deterioration of about 10 days, and scored 4.6 ± 2.2 points (mean ± SEM, P = 0.048, by t test) better than controls on weekly testing. Death in the treated mice also was delayed by 9.0 ± 3.3 days (mean ± SEM, P = 0.023, by t test) (Fig. 8).

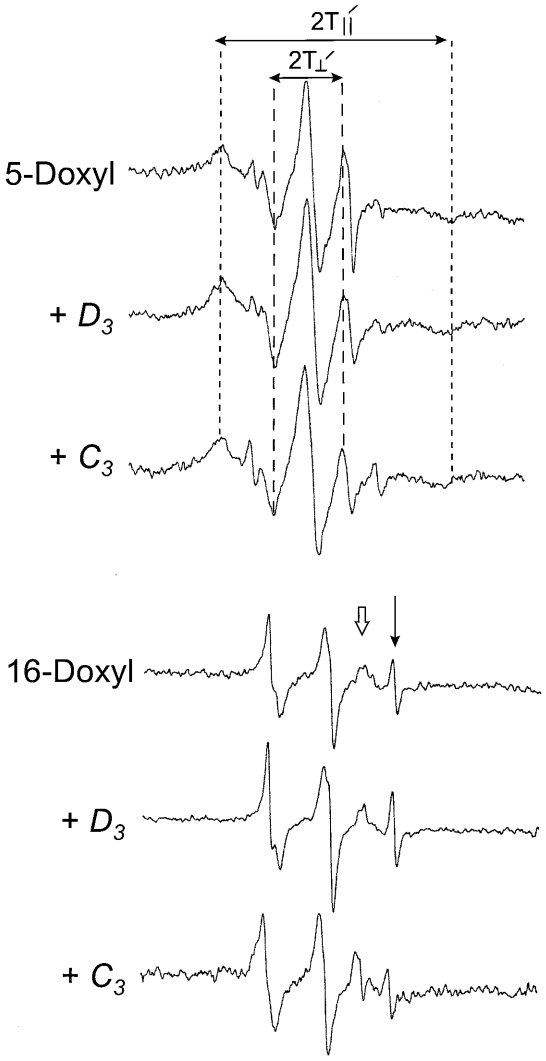

FIG. 5. EPR spectra of spin-labeled lipids (5- or 16-doxyl ketostearic acid) incorporated into mouse brain lipid micelles, with either C_3 or D_3. Both isomers produce a shift in the order parameter (S) of the 5-doxyl group (see Table 1), but C_3 produced a greater change in S. C_3 also altered the lipid (⇓)/aqueous (↓) partition factor, detected by 16-doxyl ketostearic acid, to a greater extent than D_3. Both results suggest that C_3 enters the lipid bilayer to a greater extent than D_3.

DISCUSSION

Carboxyfullerenes effectively reduced neuronal death resulting from exposure to glutamate receptor agonists, NMDA or AMPA. C_{60} derivatives are the only class of antioxidant compounds that we have worked with to date that can fully block intense, rapidly triggered, NMDA receptor-mediated excitotoxicity in our cortical neuronal cultures. In this system, many benchmark scavengers show little ability to attenuate rapidly triggered NMDA receptor-mediated excitotoxicity, and the previous best scavenger we have tested, 21-aminosteroids, reduced neuronal death by less than half (16). Indeed, the C_3 compound provided a level of neuroprotective efficacy comparable to that of NMDA receptor antagonists. The C_3 isomer also protected neurons from combined oxygen glucose deprivation injury, an insult mediated in part through overactivation of NMDA receptors. Ca^{2+} flux studies confirmed that carboxyfullerenes are not NMDA receptor antagonists.

Previous studies in our cultures have indicated that the above neuronal deaths, induced by the addition of exogenous excitotoxins, or by oxygen-glucose deprivation, are necrosis deaths (21, 27–28). Apoptosis, which occurs normally during nervous system development, also has been implicated in several forms of pathological neuronal loss (29–31), and oxidative stress has been implicated as both a trigger and a mediator of apoptosis (32–34). To examine whether the carboxyfullerenes also could inhibit neuronal apoptosis, we tested them in two apoptotic insults: (*i*) serum deprivation and (*ii*) exposure to $A\beta_{1-42}$. Degeneration of cultured cortical neurons after removal of trophic factors (serum deprivation) is a delayed event with morphological features consistent with an apoptotic process, including cell body shrinkage, fragmentation of neuronal processes, and chromatin condensation (20–21). Exposure of neurons to $A\beta_{1-42}$ also results in a delayed injury with features of apoptosis (35). Carboxyfullerenes were able to block neuronal death in both of these apoptotic injuries. Thus, our data support the emerging concept that free radicals contribute to neuronal death in excitotoxic insults as well as injuries that result in apoptosis.

Table 1. Results of EPR spectroscopy of nitroxide spin-labels incorporated into a mixture of mouse brain lipids

Compound	Order parameter*	Correlation time, ns†	Lipid/aqueous partition factor‡
Control	0.88 ± 0.01	5.1 ± 0.5	0.8 ± 0.2
$+C_3$	0.84 ± 0.01	5.7 ± 0.5	1.0 ± 0.2
$+D_3$	0.86 ± 0.01	4.5 ± 0.5	0.6 ± 0.2

*Order parameter, $S = a(T_\parallel' - T_\perp')/(T_\parallel - T_\perp)$, where $T_\parallel'$ is the measured hyperfine splitting for the parallel orientation in 5-doxyl ketostearic acid spin label/phospholipids, and $T_\perp'$ the perpendicular orientation. We assume $a = 1$, and $T_\parallel - T_\perp = 25$ G.

†Correlation time, $t = 6.5 \times 10^{-10}\, w_o[(h_o/h_{-1})^{1/2} - 1]$, where w_o is the width of the mid-field line in gauss, h_o and h_{-1} are peak height of mid- and high-field lines on the first derivative absorption measured for 5-doxyl ketostearic acid.

‡Lipid/aqueous partition factor, $f = h_L/h_A$, where h_L and h_A are the peak height of lipid (⇓) and aqueous phases (↓) measured for 16-doxyl ketostearic acid. We used 1/2 of the full height of h_A for a standard comparison.

Data from our EPR studies confirmed that the carboxyfullerenes retained the potent free radical scavenging ability of the parent C_{60} molecule. The higher potency of both carboxyfullerenes as ·OH scavengers versus O_2^- scavengers may reflect the greater ability of C_{60} to accept multiple ·OH compared with O_2^- moieties. We speculate that after the first ·OH radical is added onto C_{60}, the newly generated hydroxyl carboxyfullerene radical may take up another ·OH to form a diol, a nonradical diamagnetic adduct. If an odd number of ·OH radicals are added to a pentagon of the fullerene framework, a three-membered ring epoxide might be formed (2), due to loss of H by reaction with another nearby ·OH, thus preserving the diamagnetic character of the compound. Addition of two O_2^- radicals on the same pentagon, on the other hand, would be less favorable, due to generation of an adduct with two negative charges in close proximity.

The excellent neuroprotective efficacy of the carboxyfullerenes may reflect their ability to react with superoxide radical in addition to hydroxyl radical. We speculate that because the half-life of ·OH is extremely short (10^{-9} sec) it may be difficult for compounds that are only capable of scavenging ·OH to achieve sufficient concentration near the site of ·OH generation to out-compete endogenous targets of ·OH attack (lipids, proteins, DNA, and other macromolecules). On the other hand, O_2^-, which can be a major source of ·OH via the Haber–Weiss reaction, is a relatively stable radical, with a $\tau_{1/2}$ generally measured in seconds. It may be that the ability of carboxyfullerenes to eliminate O_2^- before its conversion to ·OH is an important aspect of their biologically relevant antioxidant properties.

While the two carboxyfullerene isomers show similar free radical scavenging potency when compared under similar "test tube" experimental conditions, they demonstrated different neuroprotective potency and efficacy in cortical cultures. We hypothesized that the C_3 isomer, because of its amphiphilic (dipole) nature, might intercalate into biological membranes better than the D_3 isomer, which lacks a dipole moment (with

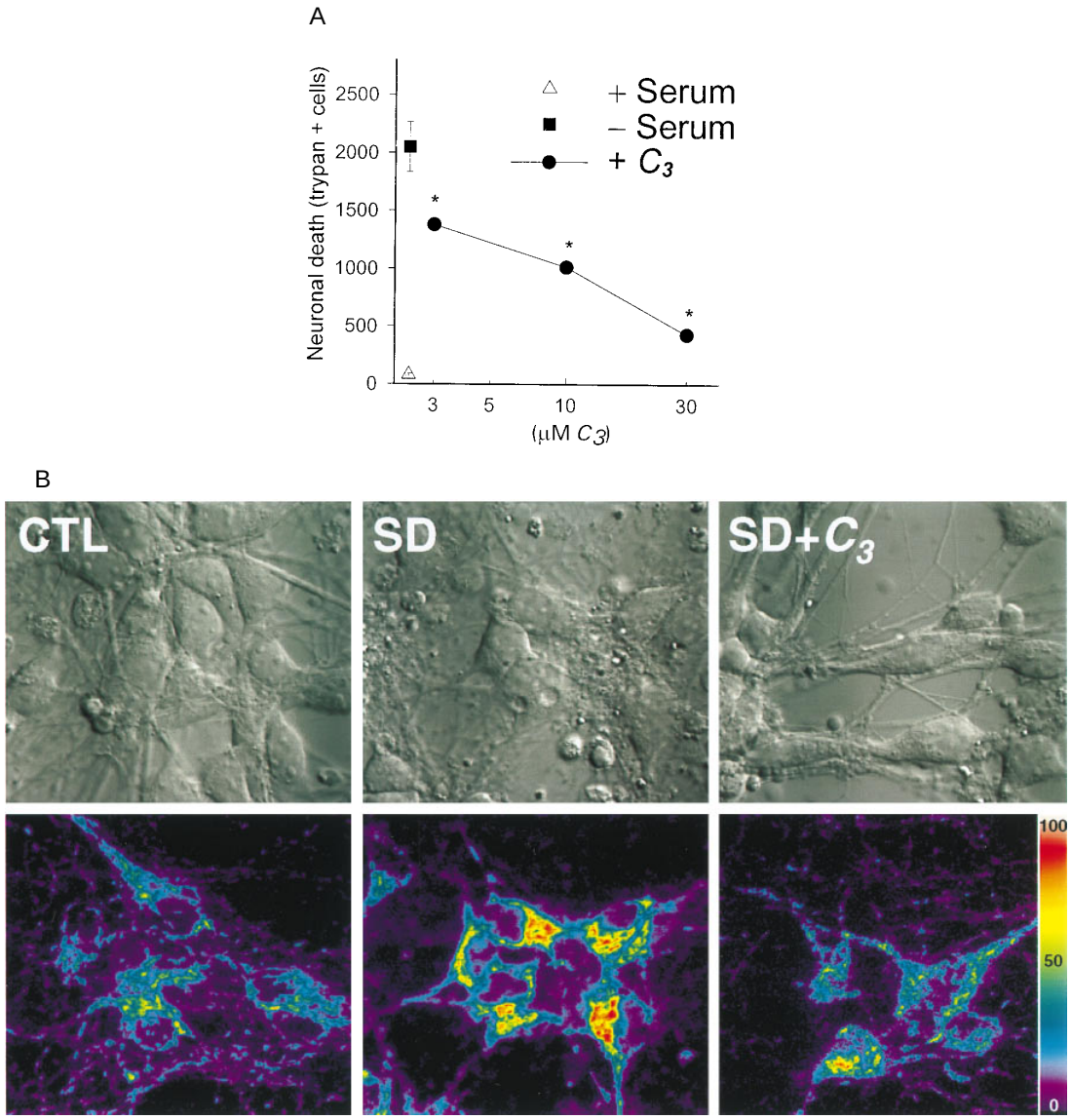

FIG. 6. C_3 attenuated neuronal death (*A*) and dihydrorhodamine oxidation (*B*) induced by serum deprivation. Cell death was determined by manual cell counts of trypan-stained neurons 48 hr after onset of serum deprivation. *, $P < 0.05$ vs. serum deprivation, using ANOVA followed by Student–Newman–Keuls test for multiple comparisons. Values are mean ± SEM, $n = 4$–8 per condition. Figure is representative of two additional replicates. Confocal images (*B*) of cortical neurons showing Nomarski images (*Upper*) of neurons before (CTL) and 8 hr after the onset of serum deprivation (SD). Neurons in the SD condition demonstrate typical apoptotic features, including membrane blebbing and condensation of nuclear contents. (*Lower*) Concurrent photomicrographs show increased fluorescence due to oxidation of preloaded, nonfluorescent dihydrorhodamine to fluorescent rhodamine 123. Rhodamine fluorescence is quantified with a linear pseudocolor scale corresponding to arbitrary fluorescence intensity units.

carboxyl groups evenly spaced around the C_{60} equator). EPR studies using spin-labels in aqueous dispersions of mouse brain lipids demonstrated that the C_3 isomer intercalates more deeply into the lipid bilayer than the D_3 isomer, as indicated by the higher lipid/aqueous partition factor for the 16-doxyl compound, in which the spin-label is at carbon 16 in the bilayer. Results for the 5-doxyl-labeled lipid suggest that both C_3 and D_3 interact with the surface of the bilayer, although, again, the increased correlation time seen with the C_3 isomer suggest it is actually embedded in the bilayer. While we have not yet been able to confirm these results in live cells, it is likely that entry of C_3 into membranes would improve its ability to limit lipid peroxidation and to gain access to the interior of the cell.

Finally, to test C_3 for *in vivo* efficacy, we administered the compound systemically to transgenic mice carrying a human disease gene for FALS, an adult onset neurodegenerative disorder characterized by progressive motor neuron death and motor weakness. Mice overexpressing the mutant human Cu, Zn superoxide dismutase (SOD1) gene (*G93A*) appear normal at birth, but go on to develop hind limb motor impairment at about 100–110 days of age, and progress over the next 2–3 weeks to paralysis and death accompanied by degeneration of lower motorneurons (25). Transgenic mice carrying other human disease SOD1 mutations also have developed comparable features (36–37). While the exact connection between the mutant gene and selective degeneration of the motor system has not yet been defined, participation of free radicals in the neurodegenerative process is suspected (38). Supporting this connection, administration of the free radical scavenger, vitamin E, delayed the onset of symptoms, although not death, in *G93A* SOD1 transgenic mice (39).

Neuronal degeneration in this mouse FALS model occurs in a sharply compressed fashion. Pathological evidence has suggested that lower motor neuron degeneration becomes detectable at 8 weeks of age and then progresses over the next several weeks to near-complete motor neuron loss. Functional impairment of motor function is apparent only 2–3 weeks before death. The 10-day delay in symptom onset induced by C_3 represents a 15%

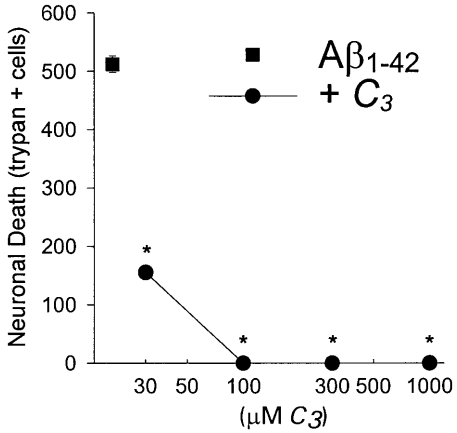

FIG. 7. C_3-blocked neurodegeneration produced by application $A\beta_{1-42}$, (20 μM). Manual cell counts of trypan 48 hr after application of $A\beta_{1-42}$ were graphed as mean ± SEM, n = 10–16 per condition.

increase in symptom-free life, and the 8-day improvement in survival is nearly half the 2–3 week symptomatic period. Greater beneficial effects might be produced in situations where neurodegeneration is less aggressive.

Present data thus provide substantial extension of our earlier suggestion that C_{60} derivatives might constitute antioxidant compounds useful in biological systems. The unique efficacy of the C_3 carboxyfullerene against excitotoxic necrosis, as well as its powerful protective effects against two forms of neuronal apoptosis, provides support for the evolving idea that oxidative stress is a critical downstream mediator in disparate necrotic and apoptotic neuronal deaths. In addition, the present direct comparison between C_3 and D_3 regioisomers suggests that amphiphilicity is a desirable feature, increasing intercalation into brain membranes and neuroprotective efficacy. Finally, we provide here a direct demonstration that C_{60} derivatives can indeed function as neuroprotective drugs *in vivo*.

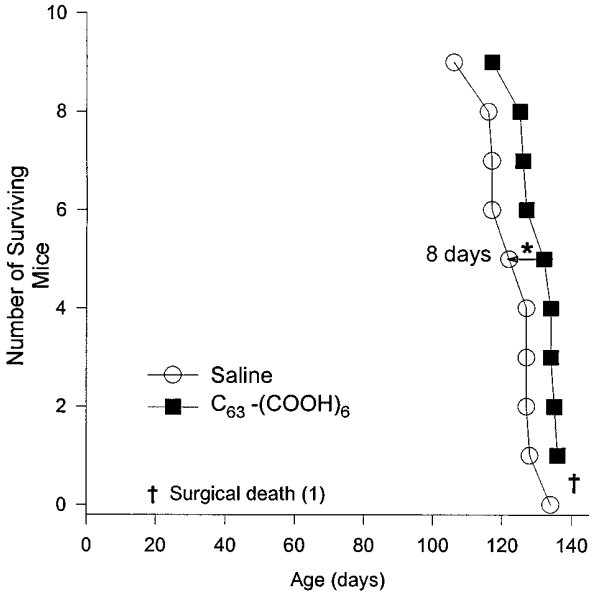

FIG. 8. Survival curves for FALS mice treated with continuous intraperitoneal infusion of saline or 15 mg/kg per day C_3 carboxyfullerene via implanted mini-osmotic pumps for 2 months, starting at age 2 months. Animals treated with carboxyfullerene had increased survival (P = 0.023 by t test). Three surgical deaths occurred during pump implantation, two in the C_3-treated group (after 1 month of treatment, before symptom onset), and one in the control group (with initial pump placement). All procedures conformed to Washington University institutional guidelines for animal welfare.

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