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Cytoprotective activities of water-soluble fullerenes in zebrafish models

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Using zebrafish (Danio rerio) embryos as a model system, we compared the antioxidant and cytoprotective effects of a series of new water-soluble fullerenes **1**–**12**. Since zebrafish embryos are transparent during the first week of life, the effects of fullerenes on multiple organ systems, including CNS, PNS, and heart, could be assessed *in situ*. Both positively charged and negatively charged water-soluble fullerenes were added at concentrations between 1 and 500 μM to 96 well plates containing zebrafish embryos at 24–120 hours post fertilization (hpf). Direct toxicity of each fullerene compound was assessed by LC50. In addition, we assessed the ability of each fullerene to protect against toxicity induced by known chemical toxins in this system. Four different drug/chemical toxicity models were used in our study: (i) protection of neuromast hair cells from gentamicin-induced toxicity; (ii) protection of neuromast hair cells from cisplatinum-induced toxicity; (iii) protection of tyrosine hydroxylase-containing dopaminergic CNS neurons from 6-hydroxydopamine toxicity; and (iv) protection of total CNS neurons from 6-hydroxydopamine toxicity. Our results indicate that (i) positively charged water-soluble fullerenes tend to exhibit greater toxicity than negatively charged fullerenes with similar structures; (ii) toxicity varies considerably among negatively charged fullerenes from very low to moderate, depending on structural features; (iii) dendrofullerenes **2**–**7** (monoadducts of C_{60}) show stronger protection against cisplatinum toxicity in neuromast hair cells while then the e,e,e-trismalonic acid **1** (so called C3) shows stronger protection against gentamicin-induced cytotoxicity in the same cells; (iv) C3 (**1**) is relatively unstable in all aqueous solvents tested and breaks down mainly through decarboxylation reactions to form penta, tetra and tris carboxylated forms, which exhibit increased toxicity *in vivo* compared with C3 (**1**). Our findings indicate that water-soluble fullerenes can protect against chemical toxin-induced apoptotic cell death in a vertebrate, whole-animal model that may be useful for predicting the efficacy and toxicity of these compounds in mammals. Furthermore, the relative potential for pharmacologic use of these compounds varies considerably with respect to stability.

Keywords: Fullerenes; Dendrimers; Antioxidants; Superoxide; Neuroprotection; Otoprotection

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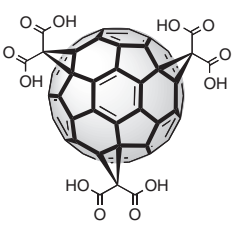
1. Introduction

Water-soluble fullerenes have been shown to be effective antioxidants against reactive oxygen species [1–14], including superoxide [15] and hydroxyl radical and appear to protect cells and tissues from oxidative injury associated with chemical oxidants [16], as well as UV, X-rays, and gamma irradiation [17–19]. Two different mechanisms through which fullerenes provide this protection have been proposed. The first involves covalent attachment of oxygen radicals to the fullerene carbon framework, resulting in fullerene radical adducts (stoichiometric process), which could in some cases after a sequence of subsequent addition and elimination steps convert back to the parent water-soluble derivative (catalytic process). The second mechanism appears to involve a sequence of electron transfer processes between the fullerene and reactive oxygen species. The concentrations required for effective biological protection in mammalian cell cultures appears to be in the 10–100 micromolar range [1, 5, 18, 20–24]. Data using antibodies against fullerenes indicate that water-soluble fullerenes readily enter many cell types, and may be located preferentially with mitochondria [25], although it is not clear whether they can enter the mitochondria to any significant extent.

One of the leading candidates for a pharmacologically active form of fullerenes is the e,e,e-trismalonic acid **1** (so called C3) (figure 1). C3 (**1**) has been shown to protect against oxidative injury and cell death in a variety of cell culture and animal models [7, 15, 18–20, 23, 25–28]. Moreover, C3 fullerenes appear to cross the blood-brain-barrier in detectable amounts and are eliminated almost entirely from the body via the liver and kidneys.

We have recently synthesized and characterized various new families of water-soluble fullerenes that can be prepared in scalable amounts and have compared their activities against C3 (**1**) [29]. We observed broad differences among the various modified fullerenes with respect to both superoxide quenching and interaction with cytochrome C. One factor linked to the overall antioxidant capabilities was net charge of the modified fullerene. Anionic fullerenes give rise to stonger binding of cytochrome C. On the other hand monoadducts (e.g. dendrofullerenes **2**–**7**) tend to have enhanced activity of superoxide quenching compared with higher adducts such as C3-like fullerenes (**1**, **8**–**10**) or oxo-aminofullerenes **11** and **12** (figure 1, table 1) [29]. However other, as yet unknown, factors appear to play a role as well.

We have extended our observations on the cytoprotective antioxidant effects of a subset of our water-soluble fullerenes **1**–**12** (figure 1) to zebrafish (*Danio rerio*), a well-characterized vertebrate animal model for studying general and specific cytotoxicity of small molecules and biologicals. Zebrafish embryos develop easily recognizable and functional organs, including heart, CNS and eye within 24 hours after fertilization, and toxicity to these organs from small molecules and radiation mimics similar effects observed in many mammals, including humans. Moreover, since zebrafish embryos are small (they can be studied qualitatively and quantitatively in a single well of a 96-well plate), and transparent up to ten days post fertilization, the toxic and cytoprotective effects of small molecules can be studied *in situ* during this period. Finally, since the morphological structure of zebrafish embryos has been mapped to the single-cell level, the toxic, apoptotic and cytoprotective effects on various organs can be tracked to approximately the single-cell level *in situ* [30].



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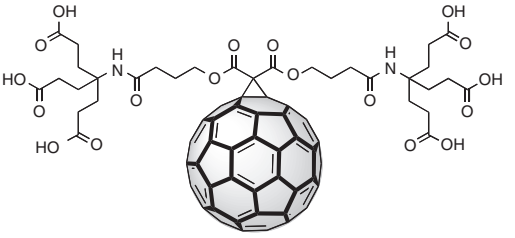

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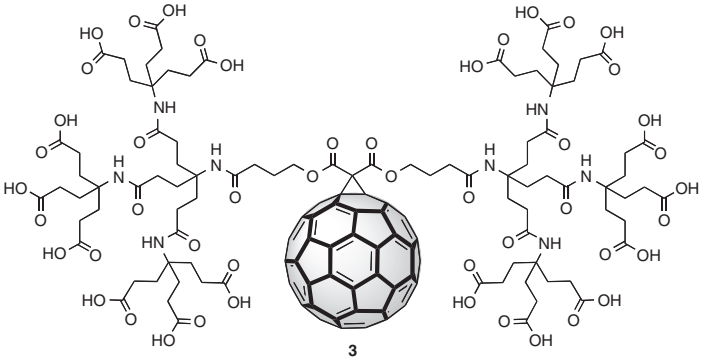

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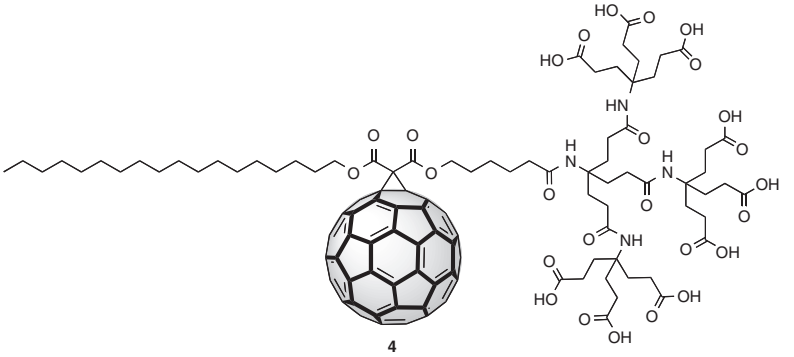

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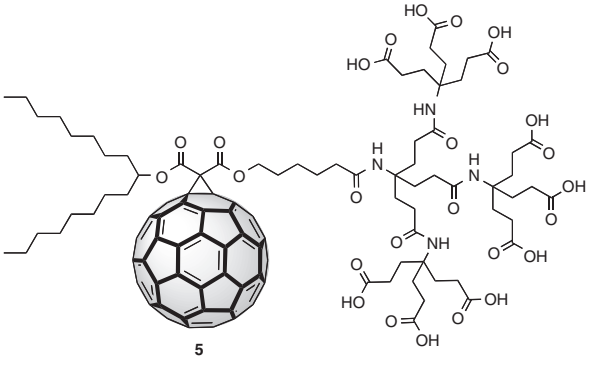

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Figure 1. Structures of various water-soluble fullerenes **1**–**12**.

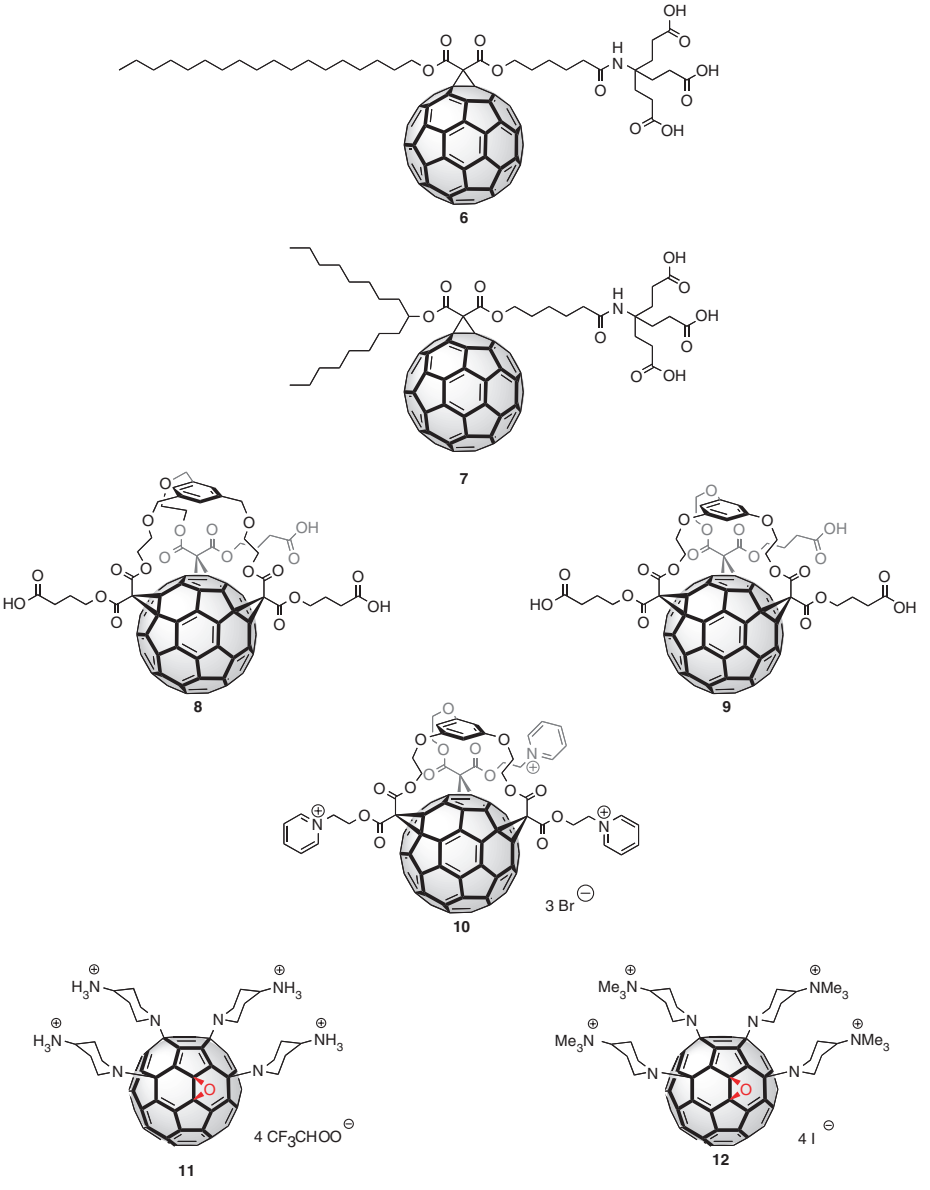

Figure 1. Continued.

Our results indicate that many of our anionic water-soluble fullerenes generally exhibit little overall toxicity at concentrations of 400 micromolar or greater, while cationic fullerenes exhibit significant toxicity at 100 micromolar or less. We also observed that anionic fullerenes provide significant protection against apoptosis induced by three known chemical cytotoxins, cisplatinum, gentamicin and 6-hydroxydopamine, which produce similar patterns of cell injury and death in zebrafish and humans.

Table 1. IC50 values for the superoxide quenching activities of fullerene derivatives **1–12** in Xanthine/Xanthine Oxidase assays.

Compound	Class	IC50 Superoxide [μM]
1	C3-like fullerenes	18.5
2	Dendrofullerenes	6.2
3	Dendrofullerenes	11.0
4	Dendrofullerenes	15.4
5	Dendrofullerenes	14.7
6	Dendrofullerenes	24.0
7	Dendrofullerenes	26.1
8	C3-like fullerenes	56.0
9	C3-like fullerenes	35.0
10	C3-like fullerenes	202.0
11	Oxo-amino fullerenes	35.0
12	Oxo-amino fullerenes	45.4

2. Materials and methods

Compounds. Water soluble fullerenes **1–12** were prepared according to our previously reported synthesis protocols [29] and references cited therein. In order to carry out the biological tests 25 mM–100 mM stock solutions in 10% DMSO and 0.1 N HCl of **1–12** were prepared. All stocks were stored in −20°C and diluted before use.

Standard procedures for embryo collection

Embryos were generated by natural pair-wise mating, as described in the Zebrafish Handbook [31a]. Four to five pairs of adult zebrafish were set up for each mating, and, on average, 100–150 embryos per pair were generated. Embryos were maintained at 28°C in fish water [200 mg Instant Ocean Salt per liter of deionized water; pH 6.6–7.0 maintained with 2.5 mg/liter Jungle pH Stabilizer (Jungle Laboratories Corporation, Cibolo, TX); conductivity 670–760 μS]. Embryos were cleaned (dead embryos removed) and sorted by developmental stage [31b] at 6 and 24 hours post-fertilization (hpf). Because the embryo receives nourishment from an attached yolk sac, no feeding was required for seven days post fertilization (dpf).

2.1. *Determination of LC50 and organ toxicity*

2.1.1. Treatment of embryos with 1–12. Zebrafish embryos at 24 hpf were distributed into 96-well cell culture plates, one embryo per well in 100 μl fish water containing the compound. Embryos were exposed from day 1 to day 5 post-fertilization (pf). Typically, the fish water contains PBS at a final concentration of 10%. Treated embryos were compared with untreated and 0.1% DMSO-treated controls.

2.1.2. Measurement of LC50 and lethality curves. Mortality was recorded every 24 hrs. At 120 hpf, total mortality was used to generate the concentration-response curves.

The data were averaged from multiple experiments. Best-fit concentration-response curves were generated using KaleidaGraph software (Synergy Software, Reading, PA, USA) using the equation: $Y = M1 + \{(M2 - M1)/[1 + (X/M3)\hat{}M4]\}$, where $M1$ = maximum Y value (100% in this case), $M2$ = minimum Y value (0% in this case), $M3$ = the concentration corresponding to the value midway between $M1$ and $M2$ (LC_{50}), $M4$ = slope of the curve at $M3$ (best fit, generating R^2 closest to 1), X = concentration of compound, Y = percent lethality.

2.1.3. Visual toxicity assessment of developing embryos. At 5 dpf, organs in 5 randomly selected embryos were inspected by light microscopy. Body morphology, liver, intestine, and heart were assessed. Since drug treatment was initiated at 24 hpf, which is after circulation and heart beat are present, but before the liver and intestine are developed, observations are for effects on developing organs.

2.1.4. Body morphology. Defects in the development of midline structures, including notochord and floor plate, often result in abnormal body shape in zebrafish. To assess the potential drug effects on midline development, abnormalities in body shape, including small body size, bent or missing tail were examined.

2.2. *Determination of otoprotective activity*

2.2.1. Hair cell assessment. To damage inner hair cells, 5-day zebrafish were treated with gentamicin (2.5 μg/ml) or cisplatinum (10 μM) for 24 hours to induce apoptosis in inner hair cells. To test drug effects on protection of hair cell damage, test compounds were administered at 0, 0.1, 1, 10, 100 and 250 μM concentrations with either the gentamicin or cisplatinum treatment. The inner hair cells in the lateral neuromasts were examined by 2,4-dimethyl-aminostyryl-*N*-ethyl pyridinium iodide (DASEPI or 2,4 Di-Asp) staining.

2.2.2. DASPEI staining. Zebrafish were incubated with DASPEI solution (1 mM) for 2 hours, then rinsed thoroughly in fish water. Zebrafish were anaesthetized with MESAB (0.5 mM 3-aminobenzoic acid ethyl ester, 2 mM Na_2HPO_4), and mounted in methylcellulose in depression slide for observation.

2.2.3. Morphometric analysis. Fluorescent signals (SS) were quantified as: SS = staining area × staining intensity of neuromasts by particle analysis (Scion Image, Scion Corporation, Frederick, Maryland). Images of lateral sides in each animal were obtained by the same exposure time and fluorescent gain (anterior on the left; posterior on the right; dorsal on the top). The size of neuromasts were defined and specified for particle analysis. Five animals for each treatment were quantified and intensity of fluorescent signals was averaged.

2.2.4. Statistics. All data were presented as mean ± standard deviation (SD). Student's *t*-Test was used to compare vehicle-treated and drug treated zebrafish. Significance was defined as $P < 0.05$, $n = 5$.

2.3. *Determination of general CNS neuroprotective activity against 6-hydroxdopamine-(6-OHDA) induced neuronal apoptosis*

2.3.1. Microinjection of 6-OHDA. 6-OHDA is extremely unstable in solution, it was prepared fresh for each injection. 3-day zebrafish were anesthetized in MESAB (0.5 mM 3-aminobenzoic acid ethyl ester, 2 mM Na_2HPO_4) and 500 mM 6-OHDA was microinjected into the midbrain region. The estimated amount of injection was about 1–2 pmole per compound. Zebrafish were rapidly transferred to fresh fish water after injection, allowed to recover for 30 minutes, and incubated with neuroprotective anti-oxidants. Water alone was injected into zebrafish brain region as a vehicle control.

2.3.2. Treatment of embryos with 1–12. Embryos were exposed to water-soluble fullerenes at 1, 10, 100 and 250 μM for 24 hours, then microinjected with 6-OHDA. Twenty embryos were treated with each concentration of each compound.

2.3.3. Morphometric analysis. Fluorescent signals (SS) were quantified as: SS = staining area × staining intensity of neuromasts by particle analysis (Scion Image, Scion Corporation, Frederick, Maryland). Images of dorsal sides in each animal were obtained by the same exposure time and fluorescent gain. The positive staining was defined by size and fluorescent intensity evaluation, and specified for particle analysis. Five animals for each treatment were quantified and intensity of fluorescent signals was averaged.

2.3.4. Fluorescence microscopy and image analyses. All fluorescence microscopy studies were performed using a Zeiss M2Bio fluorescence microscope (Carl Zeiss Microimaging Inc., Thornwood, NY) equipped with a rhodamine cube and a green FITC filter (excitation: 488 nm, emission: 515 nm), and a chilled CCD camera (Axiocam MRM, Carl Zeiss Microimaging Inc., Thornwood, NY). Screens were routinely done using a 1.6X, 10X and 20X objective archromats and 10X eye pieces. Our system was also equipped with a *z*-motorized stage, deconvolution software and 4-D reconstruction software (Axiovision, Carl Zeiss Microimaging Inc., Thornwood, NY) which permits reconstruction of 3-D objects and analyzes Z-stacks. Images were analyzed with Axiovision software Rel 4.0 (Carl Zeiss Microimaging Inc., Thornwood, NY), the Adobe Photoshop 6.0 computer program (Adobe, San Jose, CA) and NIH image software (Bethesda, MD). The patterns and the intensity of staining were recorded and quantitated.

2.3.5. Acridine orange staining. At 48, 72 and 120 hpf, five embryos were immersed in 0.5 μg/ml acridine orange (acridinium chloride hemi- [zinc chloride]) in PBS for 60 minutes and rinsed thoroughly twice in 10 ml of fresh fish water. Stained embryos are

anesthetized with MESAB (0.5 mM 3-aminobenzoic acid ethyl ester, 2 mM Na_2HPO_4), and mounted in methycellulose in a depression slide for observation using fluorescent microscopy. Effects of water-soluble fullerenes apoptosis in the hatching glands, retina, lateral neuromasts, and olfactory pits were examined.

2.4. *Determination of dopaminergic CNS neuroprotective activity against 6-hydroxydopamine-induced apoptosis*

2.4.1. Treatment of embryos with 1–12. 2-day zebrafish were treated with 1% DMSO for vehicle control and treated with 1% DMSO plus 250 μM 6-OHDA for DA-loss control. For compound testing, 2-day embryos were exposed to a mixture of 250 μM 6-OHDA and fullerenes for 72 hours.

2.4.2. Antibody staining. Embryos were fixed in 4% paraformaldehyde overnight at 4°C. Fixed embryos were permeabilized with cold acetone at −20°C for 20 minutes and rehydrated in step-wise descending ethanol/PBS solutions (95, 79, 50, 25, 0% ethanol/PBS mix, ten minutes for each step). Embryos were then stained with anti-tyrosine hydroxylase antibody (mouse anti-human, Sigma, St Louis, MO) at 4°C overnight. The next day, samples were washed with PBS-T (0.1% Tween 20), incubated with secondary antibody (goat anti-mouse) and color was developed using ABC reagent (Vector Labs, Burlingame, CA) according to manufacturer's instructions. Stained embryos were further flat-mounted on a glass slide and five randomly selected embryos were examined for DA neuron loss.

2.4.3. Light microscopy and image analyses. All microscopy studies were performed using a Zeiss light microscope (Carl Zeiss Microimaging Inc., Thornwood, NY) equipped with a Spot camera (Diagnostic instruments, Sterling, MI). The patterns and the intensity of staining were recorded and quantified.

2.4.4. Fluorescence microscopy. All fluorescence microscopy studies were performed using a Nikon Eclipse E600 fluorescence microscope (Nikon Inc., Melville, NY) equipped with a 200 watt mercury/xenon lamp and a green FITC filter (excitation: 488 nm, emission: 515 nm), and a C5985 chilled CCD camera (Hamamatsu Photonics, Hamamatsu City, Japan). Images were analyzed with the Adobe Photoshop 6.0 computer program (Adobe, San Jose, CA).

2.4.5. Vertebrate animal care and safety. The Office of Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH) has approved Phylonix' Animal Welfare Assurance #A4191-01, effective 7/14/03, for a five year approval period. We euthanize zebrafish of all ages by over-exposure to tricaine methanesulfonate; adult fish are then rapidly frozen. These procedures are consistent with the American Veterinary Medical Association's (AVMA) Panel on Euthanasia. Approximately 1500 animals were used in this study.

3. Results

3.1. *General remarks and* in vitro *properties*

Three different classes of water-soluble fullerenes, dendrofullerenes (**2**–**7**), C3-like fullerenes (**1**, **8**–**10**) and oxo-amino fullerenes (**11**, **12**) (figure 1) were tested in five different zebrafish assays to assess both overall toxicity (measured by LC50) as well as efficacy in protecting against three different tissue-specific chemical cytotoxins: cisplatinum, gentamicin and 6-hydroxydopamine. Cisplatinum is a widely used and highly effective anti-cancer drug and also causes apoptosis in non-cancerous hair cells of auditory system and renal tubular cells in mammals. A similar pattern of cytotoxicity was observed in lateral-line neuromast hair cells of zebrafish embryos after exposure to cisplatinum. Gentamicin and related aminoglycoside antibiotics induce apoptosis and cell loss in auditory hair cells in mammals and dorsal mechanoreceptor hair cells in zebrafish and represents a major cause of acquired deafness in children [32]. 6-Hydroxydopamine is taken up by CNS neurons, particularly by tyrosine hydroxylase-containing dopaminergic neurons in the central nervous system and causes oxidative injury and apoptotic death [33]. The preferential destruction of dopaminergic neurons in mammals has led to widespread use of 6-hydroxydopamine as a model system for induction and study of Parkinson's-like syndromes in experimental animals. The data in table 1 indicates that both dendrofullerenes **2**–**7** and C3-like anionic fullerenes **8** and **9** show substantial antioxidant activity against superoxide whereas the activity of the cationic derivatives **10**–**12** are considerably lower [29]. The results in table 1 were obtained using xanthine/xanthine oxidase-generated superoxide. Similar results have been obtained with potassium superoxide and direct spectroscopic detection of superoxide [34]. The fact that anionic fullerenes interact much stronger with the positively charged cytochrome C than cationic derivatives demonstrates that electrostatic interactions are the major driving force for this binding process [29].

3.2. *Stability of the C3 fullerene 1*

The e,e,e-trismalonic acid **1** (C3) was the first water-soluble fullerene derivative with a defined structure whose antioxidant properties were studied in detail. However, when comparing the *in vitro* and *in vivo* antioxidant, neuroprotective and toxic properties of C3 (**1**) with **2**–**12** and other water-soluble fullerenes we recognized the C3 can easily degrade and that the degradation products can be comparatively toxic. In a systematic investigation we studied the stability of purified C3 (**1**) in crystalline form as well as after suspension in several different buffers. The results are summarized in figure 2. In crystalline powder form, C3 (**1**) degrades approximately 0.5% per week at room temperature, but is stable at −20°C. In solution, the stability of C3 (**1**) is highly variable, depending upon the suspending medium. In DMSO at room temperature, C3 (**1**) is degraded completely within a few minutes; the same is true in DMSO with immediate freezing at −20°C. C3 solutions of distilled water, phosphate-buffered Tyrode's solution and 5% glucose exhibit a degradation rate of approximately 4% per week at room temperature, but all three solutions are completely stable at −20°C for at least 10 weeks. C3 solutions in polyethylene glycol (PEG) at room temperature exhibit rapid degradation rates of approximately 40% per week.

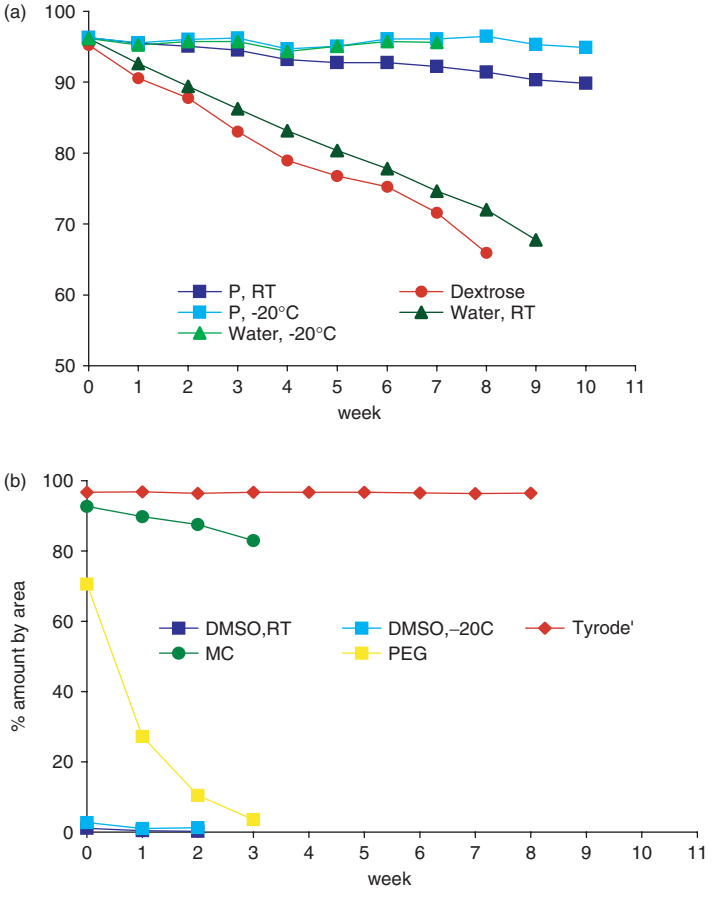

Figure 2. Stability of C3 (**1**): (a) as powder (P) and in water and dextrose; (b) in DMSO, Tyrode's, MC. The amount of **1** was determined by the corresponding peak areas in the HPLC profiles.

HPLC analysis of C3 degradation products indicates that decarboxylation reactions of the malonyl adducts represents the major pathway for degradation of C3 (**1**). Three major decomposition products, namely the mono-, bis- and tris-decarboxylation products C3-penta, C3-tetra and C3-tris could be identified (figure 3). The initial breakdown product is mostly C3-penta, but with continued degradation, significant amounts of C3-tetra and C3-tris are observed as well. In the presence of DMSO, complete degradation to C3-tris appears complete within 1–2 minutes at room temperature.

3.3. *Toxicity of water-soluble fullerenes in zebrafish embryos*

We compared the overall toxicity of our water-soluble fullerenes in zebrafish embryos, and the results are summarized in table 2. Fullerenes were added to the water in

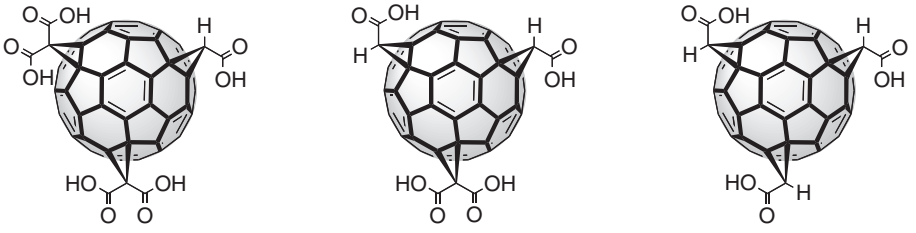

Figure 3. Schematical representation of the C3 decarboxylation products C3-penta, C-tetra and C-treat. Only one stereoisomer each is represented although they were formed as mixtures of isomers (NMR).

Table 2. LC50 of fullerenes **1**–**12** in zebrafish.

Compound	Class	LC50 Zebrafish [μM]
1	C3-like fullerenes	596
2	Dendrofullerenes	> 500 (30% lethality at 500 μM)
3	Dendrofullerenes	> 500 (20% lethality at 500 μM)
4	Dendrofullerenes	ND
5	Dendrofullerenes	ND
6	Dendrofullerenes	≫250 (0% lethalityat 250 μM)
7	Dendrofullerenes	ND
8	C3-like fullerenes	≫500 (0% lethalityat 250 μM)
9	C3-like fullerenes	≫500 (0% lethalityat 250 μM)
10	C3-like fullerenes	117
11	Oxo-amino fullerenes	104
12	Oxo-amino fullerenes	30

single-wells of a 96-well plate, each containing a single zebrafish embryo at 24 hpf, and lethality along with any observed morphologic abnormalities were scored at 120 hpf. In most cases, fullerenes were tested at varying concentrations up to 500 μM, but in some cases the maximum concentration tested was 250 μM due to solubility issues at higher concentrations. LC50 was calculated as the fullerene concentration at which 50% lethality is observed at 120 hpf. For the three cationic fullerenes **10**–**12** tested the LC50 was less than 120 μM, and in the case of **12**, as low as 30 μM. For the anionic fullerenes, the LC50 values were considerably higher reaching values up to 0% lethality at 500 μM for **8** and **9**. In several cases, morphological abnormalities such as shortened body length and abnormal body curvature were detected in embryos treated with fullerene concentrations at or near the LC50. For example, we observed shortened body length after treatment with C3 (**1**) at 250 μM (figure 4(a), Panel B) and slight curvature of the body after treatment with **3** at 500 μM (figure 4(a), Panel C). The majority of fullerenes tested showed no observable body length or curvature abnormalities (see for example figure 4(b)). Higher resolution microscopy of individual organs within developing zebrafish embryos showed abnormal cardiac chambers in the presence of 250 μM C3 (**1**) (figure 5(b)), enlargement of liver and intestines in the presence of **3** at 500 μM (figure 5(c)), and underdevelopment of liver and intestines in the presence of **2** at 500 μM (figure 5(f)). In addition, embryos treated with **8** (500 μM) showed slight

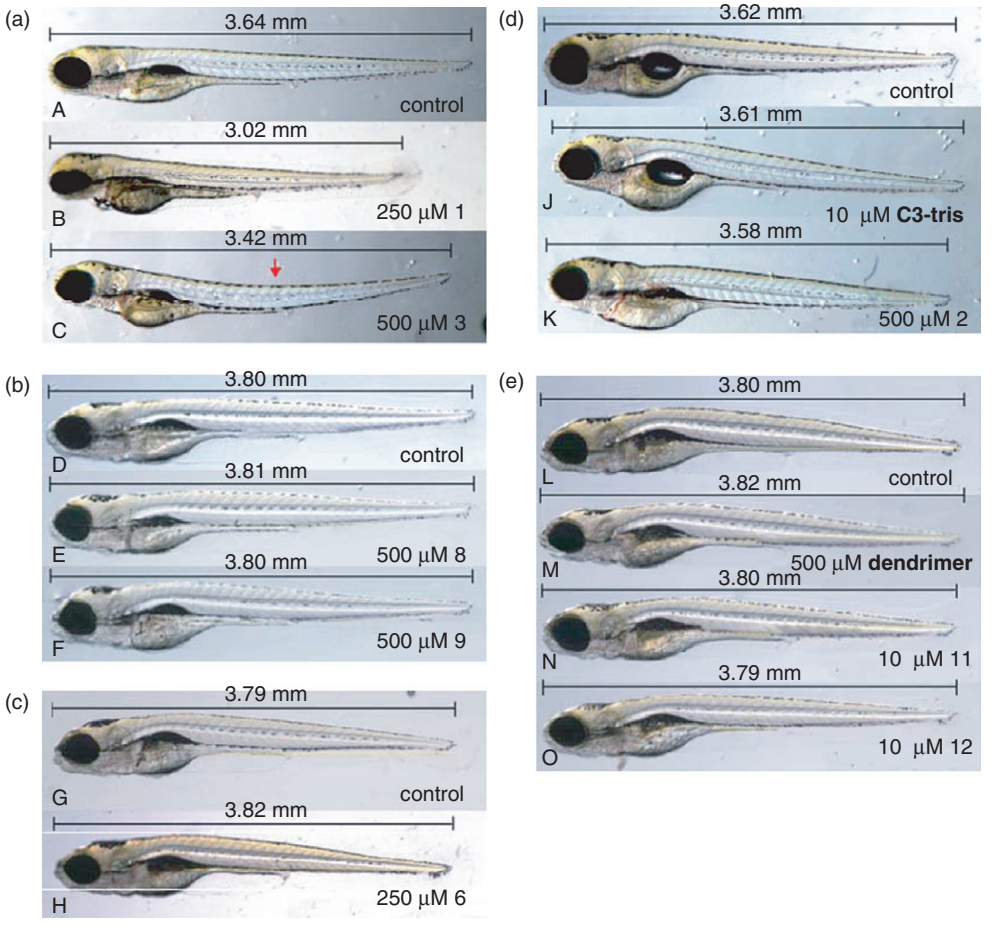

Figure 4. Morphological body-length abnormalities caused by doses of fullerenes C3 (**1**), C3-tris, **2**, **3**, **6**, **8**, **9**, **11**, **12**. For comparison the corresponding free G-2 dendron was investigated (e, panel M).

enlargement of the heart (figure 5(h)). These results suggest that at high concentrations, some water-soluble fullerenes may exhibit teratogenic effects, but this has not yet been tested in mammals.

The decarboxylation breakdown products of C3 (**1**) represent an important exception with respect to our generalization of low toxicity for anionic fullerenes. With progressive decarboxylation steps, each resulting C3 breakdown product shows increasing toxicity with respect to the parent C3 (**1**) (table 3). The LC50 for C3 (**1**) is 596 µM, followed by C3-penta at 373 µM, C3-tetra at 134 µM and C3-tris at 10 µM. Interestingly, the increasing toxicity of the progressive C3 decarboxylation products was not observed with cultured murine endothelial cells, at least for C3-tris (Data not shown), suggesting that the toxic effects of C3 decarboxylation products may be limited to a specific cell, tissue or organ type that is critical for overall survival. To further investigate this possibility, we initiated a series of experiments to study

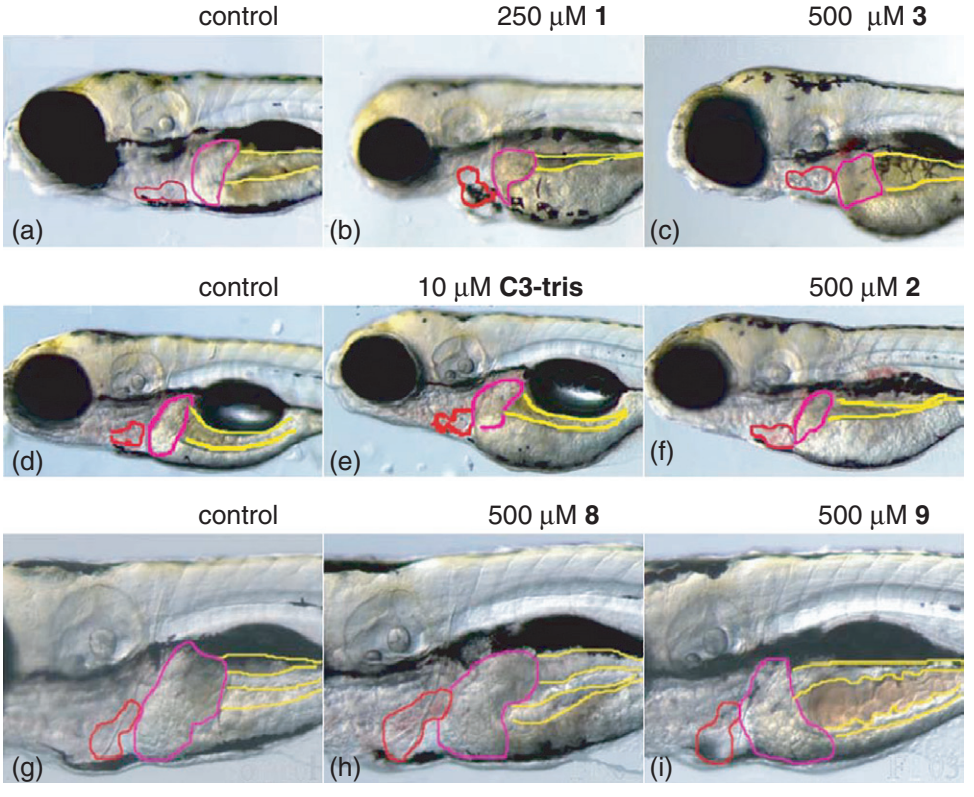

Figure 5. Morphological organ abnormalities in the development of liver, intestine and heart caused by doses of fullerenes C3 (**1**), C3-tris, **2**, **3**, **8** and **9** near LC50. The liver, intestine, heart are outlined by magenta, yellow and red lines respectively for easy visual assessment. Compared to controls (a, b and g), 250 μM C3-treated zebrafish exhibited unfolded cardiac chambers, underdeveloped liver and intestine (b); 500 μM **3** treated zebrafish exhibited enlarged liver and intestine (c); 500 μM **2** treated zebrafish exhibited underdeveloped liver and intestinal tract (f). No obvious defects on liver, intestine, and heart were observed for 10 μM C3-tris (e), 500 μM **8** (h) or 500 μM **9** (I). However, slightly enlarged cardiac chambers were observed for **8**. No organ necrosis was observed with any of the treatments.

Table 3. Toxicity of C3 decarboxylation products in zebrafish and cell culture.

	1	C3-penta	C3-tetra	C3-tris
Antioxidant activity *in vitro* (Superoxide)	174 μM	62 μM	48 μM	44 μM
Zebrafish toxicity	596 μM	373 μM	134 μM	10 μM
Cell Culture toxicity	350 μM	ND	ND	325 μM

the effects of C3 (**1**) and its decarboxylated breakdown products on cardiac conduction in zebrafish, since previous work had shown that native fullerenes can dramatically effect cardiac conduction by binding to the ZERG potassium channel in fish (analogous to the HERG potassium channel in humans). We were unable to measure the ECG directly in fish and therefore chose as a surrogate induction of

bradycardia by various water soluble fullerenes, including C3 (**1**), **3** and C3-tris. Figure 6 indicates that C3-tris induces bradycardia at very low doses of 1 μM or less. In contrast, no significant bradycardia is observed at **3** or C3 concentrations of 100 μM. The extent of bradycardia and associated cardiac abnormalities is probably sufficient to account for the increased toxicity of C3 decarboxylation products in the LC50 assay. Figure 6 also indicates that cardiac injury is not characteristic of water soluble fullerenes in general.

3.4. *Protection of zebrafish embryos from CNS injury due to 6-hydroxydopamine (6-OHDA)*

Previous reports in the literature [1, 4, 12, 20, 21, 23, 27, 35–38] have suggested that C3 (**1**) and related fullerenes may be useful in the treatment of Parkinson's disease as well as other CNS degenerative disease related to neuronal apoptosis. CNS dopaminergic neurons are particularly sensitive to the toxic effects of 6-OHDA, and we therefore tested the ability of various fullerenes to protect from 6-OHDA-induced apoptosis of CNS dopaminergic neurons in developing zebrafish. Dopaminergic CNS neurons are easily identified by the presence of tyrosine-hydroxylase, which can be detected by immunohistochemistry on whole zebrafish embryos.

Fullerenes **1**–**4** were tested in various combinations for their ability to protect zebrafish embryos against damage to both general CNS neurons and tyrosine-hydroxylase CNS neurons against 6-hydroxydopamine (6-OHDA). 6-OHDA crosses the blood-brain barrier and is taken up preferentially by dopaminergic CNS neurons, and to a lesser extent by non-dopaminergic CNS neurons. Intracellular 6-OHDA

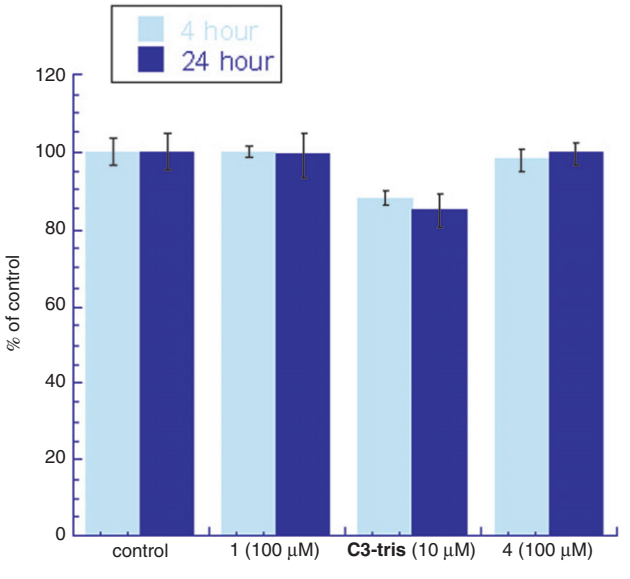

Figure 6. Cardiac bradycardia induced by C3-tris compared with C3 (**1**) and **4**.% of control (heart rate) was plotted for control (0.1% DMSO), C3 (**1**) (100 μM), C3-tris (1 μM) and **4** (100 μM) at four hours (light blue bar) and 24 hours (blue bar).

induces cellular apoptosis and death, at least in part through oxidative injury [39]. Dendrofullerene **3** showed the highest levels of general CNS neuroprotection against 6-OHDA injury, and was able to protect 47% of total CNS neurons, while C3 (**1**) and (**2**) were able to block 42 and 23%, respectively of 6-OHDA-induced total CNS neuronal death (table 4). With respect to specific protection of tyrosine hydroxylase-positive dopaminergic neurons in the diencephalon, **3** (100 μM) protected 100% of dopaminergic neurons, while C3 (**1**) (100 μM) and **4** (250 μM) each protected 60%. Figure 7 and table 5 show results obtained with **4**, in protecting CNS dopaminergic neurons against 6-OHDA-induced cell death.

3.5. *Blocking developmental apoptosis in zebrafish embryos*

Water-soluble fullerenes appear to be highly effective at blocking apoptotic cell death in the presence of known chemical toxins and electromagnetic radiation [40]. We wanted to explore whether they could also block apoptosis that occurs naturally during embryonic development. Zebrafish embryos were exposed to fullerenes (C3 (**1**),

Table 4. Protection provided by fullerenes **1**-**4** against general CNS apoptosis induced by 6-OHDA.

Compound	Maximum protection CNS apoptosis	Maximum protection dopaminergic CNS apoptosis
1	23%	ND
2	47%	100%
3	ND	60%
4	42%	60%

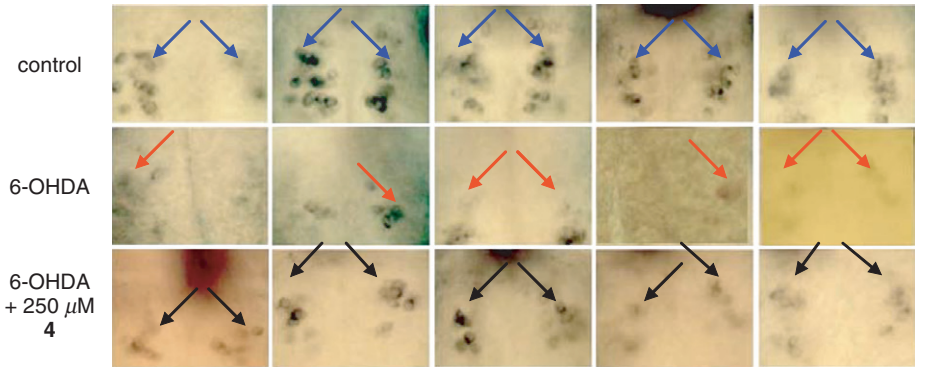

Figure 7. Protection of CNS dopaminergic neurons from 6-OHDA-induced apoptosis by **4**. Untreated (top panels), 6-OHDA plus vehicle treatment (middle panels), and 6-OHDA plus 250 μM **4** (lower panels) were stained with anti-tyrosine hydroxylase antibody. Five images of each group were examined; the number of TH-immunoreactive cells clustered in diencephalons (between the eyes) was examined. At untreated animals, 20–40 TH-cells were observed (blue arrows). After 6-OHDA treatment, decrease in the number of TH-immunoreactive cells was observed (1–17) (red arrows). After 250 μM **4** treatment, three of five **4**-treated animals showed a normal number of TH-immunoreactive cells with normal morphology (black arrows).

C3-tris, **2** and **3**) from day 1 to day 5 post-fertilization, and whole embryos were subsequently stained with acridine orange to detect apoptotic cells in control and fullerene-treated embryos. Table 6 shows that **3** and C3-tris block embryogenesis-associated apoptosis, while no effects were observed in these experiments with C3 (**1**) or (**2**). Figure 8 shows acridine orange staining for C3-tris and **3**, demonstrating reduction in number of positively stained cells in hatching glands and olfactory pits for C3-tris and in retina and neuromast cells for **3**. It is notable that zebrafish embryos appear viable after treatment with these agents based on lethality, although developmental abnormalities in body and organ development have been noted in preceding sections.

3.6. *Protection of zebrafish embryos from mechanoreceptor hair cell injury and death induced by cisplatinum and gentamicin*

In zebrafish, the dorsal mechanoreceptor hair cells are responsible for sensing position, orientation, balance and movement and are biochemically and morphologically similar to outer hair cells of the inner ear in mammals. Using DASPEI (2-(4-dimethyl-aminostyryl)-*N*-ethyl pyridinium iodide), a vital dye that stains mechanoreceptor cells specifically, the fate of each lateral-line neuromast cell can be tracked in zebrafish embryos after exposure to chemical toxins which induce apoptosis in these cells [41]. Cisplatinum is a widely used and highly effective chemotherapy agent. The major side-effects are damage to renal tubular cells and outer hair cells of the inner ear, leading to renal failure and loss of hearing acuity or deafness, respectively. The mechanism by which cisplatinum induces cell death is believed to be primarily direct DNA damage, although perturbation of cellular redox pathways may also play a role [42]. In zebrafish embryos, cisplatinum rapidly induces apoptotic cell death in all

Table 5. Protection provided by **4** against dopaminergic CNS apoptosis induced by 6-OHDA.

Treatment	Animal 1	Animal 2	Animal 3	Animal 4	Animal 5
control	28	29	30	26	32
6-OHDA	7	8	10	1	3
6-OHDA + 250 μM 4	22	14	21	18	26

Table 6. Ability of C3 (**1**), C3-tris, **2** and **3** to block apoptosis during normal development of zebrafish embryos.

		Reduction of Apaptosis			
Compound	c [μM]	Hatching glands	Olfactory pits	Retina	Lateral neuromasts
1	250	N	N	N	N
C3-tris	10	Y	N	N	Y
2	500	Y	Y	Y	Y
3	500	N	N	N	N

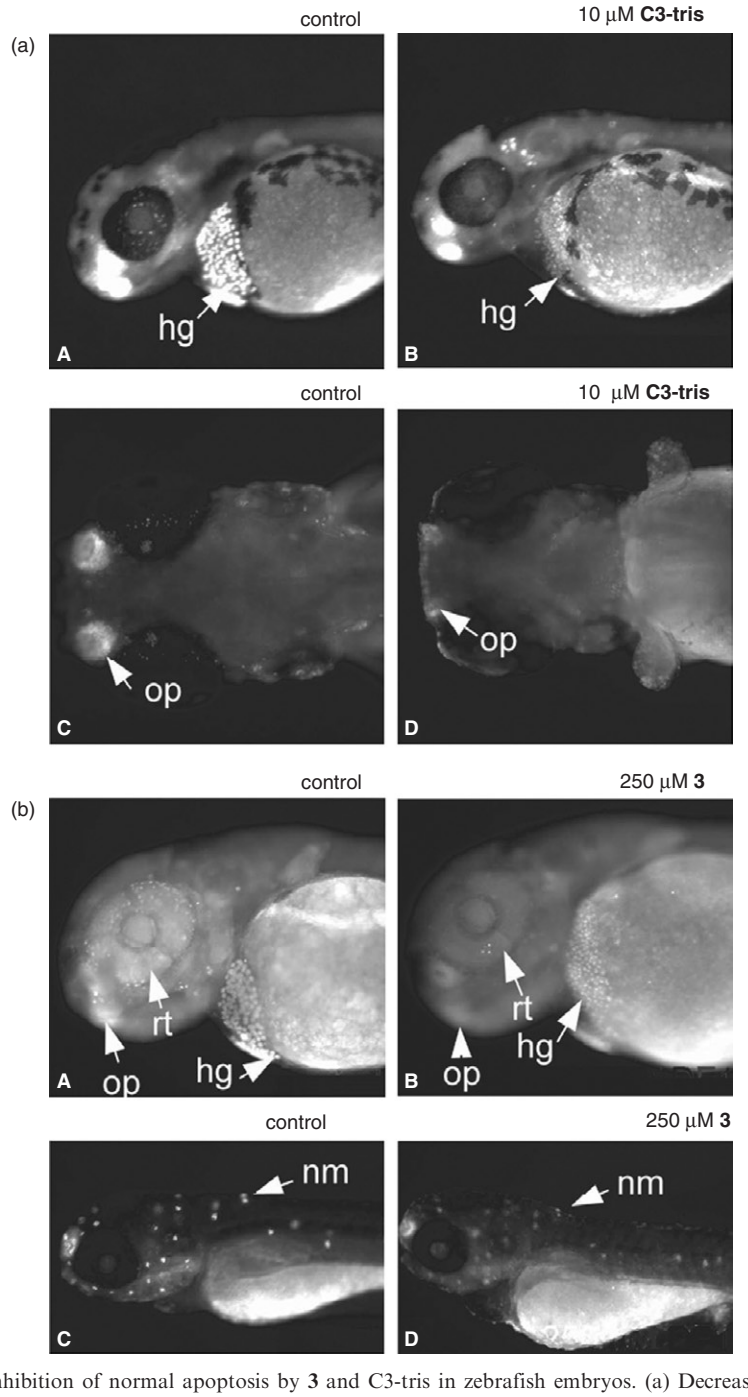

Figure 8. Inhibition of normal apoptosis by **3** and C3-tris in zebrafish embryos. (a) Decreased apoptosis after C3-tris treatment. Compared to controls (A) and C), 10 μM C3-tris caused decreased apoptosis in the hatching gland (hg) at 2 dpf (B) and olfactory pits (op) at 4 dpf (D), (b) decreased apoptosis after treatment with **3**. Compared to controls (A and C), decreased apoptosis in the hatching gland (hg), olfactory pits (op), retina (rt) was observed at 2 dpf (B). Decreased apoptosis in neuromasts (nm) were observed at 5 dpf (D).

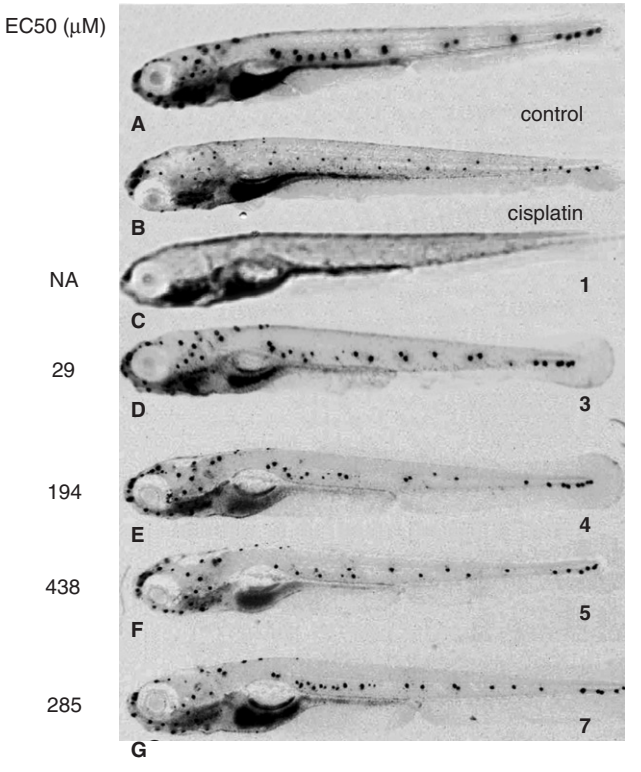

Figure 9. Protection of hair cells from cis-platinum damage by C3 (**1**), (**3**–**5**) and (**7**).

of the dorsal mechanoreceptor hair cells detectable by DASPEI staining. We assessed the comparative ability of our water-soluble fullerenes to block cisplatinum-induced mechanoreceptor cell apoptosis in zebrafish embryos. The results, displayed in figure 9, indicate an unexpectedly wide variation among various anionic fullerenes. Fullerene **3** displayed the highest efficacy, blocking 50% of cisplatinum-induced apoptosis at low concentrations (29 μM), followed by **4** (194 μM), **7** (285 μM) and **5** (438 μM). C3 (**1**), though highly effective against the superoxide assay (table 1), was almost completely unable to block apoptosis induced by cisplatinum, achieving only 5% protection at 500 μM.

Gentamicin and related aminoglycoside antibiotics are also widely used against a variety of serious infections in both adult and pediatric populations. High dose gentamicin exposure is occasionally required with life-threatening infections, but may lead to permanent damage to outer hair cells of the inner ear and result in hearing loss or deafness [43]. In zebrafish embryos, exposure to gentamicin or the related antibiotic neomycin also induces apoptosis and complete loss of DASPEI-stained dorsal mechanoreceptor hair cells in a pattern indistinguishable for that described for cisplatinum. We therefore assessed the comparative ability of our water-soluble fullerenes to block gentamicin-induced hair cell apoptosis and cell loss. Surprisingly, our results contrast substantially with those observed in the same cells using cisplatinum-induced cell loss (figure 10). C3 (**1**), (**4**) and (**6**) are most effective in

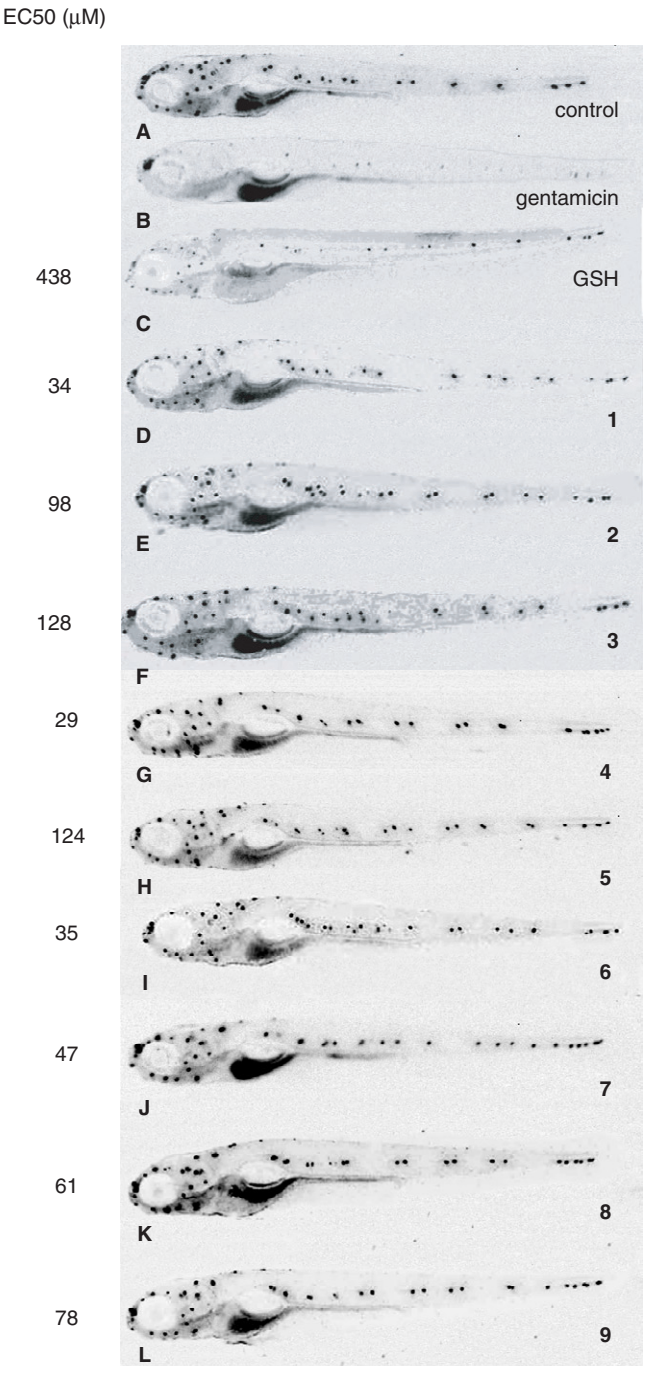

Figure 10. Protection of hair cells from gentamicin damage by various fullerenes **1**–**9**.

protecting against gentamicin-induced hair cell loss, with EC50 values of 34 μM, 29 μM and 35 μM respectively, followed closely by **5** (47 μM), **8** (60.7 μM) and **9** (78 μM). Dendrofullerene **3**, the most effective fullerene against cisplatinum-induced hair cell loss ranks far behind in protection of the same cells from gentamicin-induced apoptosis (128 μM), along with **2** (97.5 μM) and **7** (124 μM) μM. It should be mentioned that even the least effective fullerenes in this group are 3–4 fold more effective than glutathione (EC50 = 438 μM) [44], which has been used successfully in animal models to protect against aminoglycoside-induced hearing loss and renal injury.

4. Discussion

Anionic water-soluble fullerenes have previously been shown to exhibit cytoprotective and antioxidant effects in both cell culture and in various animal models [1–8, 12–20, 22, 23, 25, 27–29, 36, 37, 45–51]. Our current results described above indicate that at least in a highly accessible zebrafish embryo model system, water-soluble anionic fullerenes can block apoptosis of specific cell types induced by chemical toxins and commonly used drugs whose toxicity is well characterized in mammalian systems, including in some cases, humans. However, our results also indicate that the mechanisms by which various modified fullerenes can protect cells from apoptosis are not monolithic and depend both upon the specific mechanism of toxicity, even in a single cell type. Clearly, cisplatinum and gentamicin have very different binding and chemical activities, and the differential ability of fullerenes to protect against these two toxins may reflect the intracellular localization of each fullerene, the chemical reactivity against unknown reactive compounds or intermediates, or the ability to interact with proteins or other biological molecules involved in apoptosis signaling and regulation. In this context, the ability of some anionic fullerenes to bind to cytochrome C is of particular interest. Cytochrome C is located within mitochondria and is released to the cytosol after apoptosis-inducing signals are activated either at the cell surface or within the cell. Cytoplasmic cytochrome C facilitates that activation of cytoplasmic caspase cascades, triggering irreversible steps in the apoptosis pathway.

Our current results do not address whether or not fullerenes actually interact directly or indirectly with cytochrome C or related proteins nor do they elucidate whether this could occur in mitochondria, cytoplasm or both. They do however indicate that fullerenes are capable of interacting with cytochrome C *in vitro* [29]. Moreover, our results suggest that the ability of various fullerenes to interact with cytochrome C is somehow linked to the ability to protect against apoptosis induced by cisplatinum, but not gentamicin, in mechanoreceptor hair cells. Some of our water-soluble fullerenes, such as **4**, exhibit cytoprotective activity against both cisplatinum and gentamicin-induced apoptosis and may prove to be useful as candidates for anti-apoptosis drugs.

While some water-soluble fullerenes can exhibit cytoprotective activities, others show predominantly toxic effects. For example, C3 (**1**) is largely protective and relatively non-toxic, but C3 decarboxylation products show increasing lethality in zebrafish with increasing extent of decarboxylation. Much of this toxicity appears due to effects mediated primarily at the level of the cardiovascular system, including rhythm disturbances in the zebrafish heart. Native fullerenes and unmodified single-walled

carbon nanotubes have been shown to inhibit the ZERG potassium channel in zebrafish by binding to the hydrophobic external channel and blocking conduction through the channel [52, 53]. It is possible that decarboxylated forms of C3 (**1**) are small enough to fit into the 1nm ZERG channel, while fullerenes with more bulky adducts such as C3 (**1**) and dendritic fullerenes are prevented from binding to the hydrophobic ZERG channel by steric hindrance. However, the current study does not directly measure fullerene binding to the ZERG channel, and the proposed mechanism of toxicity remains speculative.

5. Conclusions

We have investigated the toxicity and the cytoprotective activities of three different families of water-soluble fullerenes. Using zebrafish (*Danio rerio*) embryos as a model system we assessed the ability of each fullerene to protect against toxicity induced by known chemical toxins in this system. We discovered that (i) positively charged water-soluble fullerenes tend to exhibit greater toxicity than negatively charged fullerenes with similar structures; (ii) toxicity varies considerably among negatively charged fullerenes from very low to moderate, depending on structural features; (iii) dendrofullerenes **2**–**7** (monoadducts of C_{60}) show stronger protection against cisplatinum toxicity in neuromast hair cells while then the e,e,e-trismalonic acid **1** (so called C3) shows stronger protection against gentamicin induced cytotoxicity in the same cells; (iv) C3 (**1**) is relatively unstable in all aqueous solvents tested and breaks down mainly through decarboxylation reactions to form penta, tetra and tris carboxylated forms, which exhibit increased toxicity in vivo compared with C3 (**1**). These findings demonstrate that water-soluble fullerenes can protect against chemical toxin-induced apoptotic cell death in a vertebrate, whole-animal model that may be useful for predicting the efficacy and toxicity of these compounds in mammals. Since we have established straightforward synthesis protocols for various families of water-soluble fullerenes allowing for the fine-tuning of pharmacological properties and bioavailability by using suitable combinations of building blocks as exohedral addends, we believe that water-soluble fullerenes represent a promising platform for the development of new potent antioxidant drugs.

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