**In vivo** Radioprotection by the Fullerene Nanoparticle DF-1 as Assessed in a Zebrafish Model

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**Abstract**

**Purpose:** We have previously shown that zebrafish (Danio rerio) embryos can be used as an in vivo model to validate modifiers of the radiation response. Here, we evaluated the radioprotective effect of the nanoparticle DF-1, a fullerene with antioxidant properties, in zebrafish embryos. **Experimental Design:** Zebrafish embryos were exposed to different doses of ionizing radiation ranging from 20 to 80 Gy in the presence and absence of DF-1. Toxicity and radioprotective effects were assessed by monitoring overall survival and morphology as well as organ functions by employing assays to measure kidney excretory function and development of sensory nerve cells (neuromasts). Antioxidant properties of DF-1 were assessed in whole fish. **Results:** DF-1 had no apparent adverse effects on normal zebrafish morphology or viability throughout the concentration range tested (1-1,000 μmol/L). Ionizing radiation (10-40 Gy) caused time-dependent and dose-dependent perturbations of normal zebrafish morphology and physiology, notably defective midline development resulting in dorsal curvature of the body axis ("curly-up"), neurotoxicity, impaired excretory function, and decreased survival of the exposed embryos. DF-1 (100 μmol/L) markedly attenuated overall and organ-specific radiation-induced toxicity when given within 3 hours before or up to 15 minutes after radiation exposure. By contrast, DF-1 afforded no protection when given 30 minutes after ionizing radiation. The degree of radioprotection provided by DF-1 was comparable with that provided by the Food and Drug Administration—approved radioprotector amifostine (4 mmol/L). Protection against radiation-associated toxicity using DF-1 in zebrafish embryos was associated with marked reduction of radiation-induced reactive oxygen species. **Conclusion:** The fullerene DF-1 protects zebrafish embryos against deleterious effects of ionizing radiation due, in part, to its antioxidant properties.

The zebrafish (Danio rerio), a small vertebrate species, has become a powerful model system to study human disease because many of the key genes involved in developmental processes, cell cycle progression and proliferation, and differentiation are highly conserved between the two species (1, 2). Unlike other vertebrate species, however, zebrafish are rapidly and prolifically bred and easily maintained in the laboratory. In addition, zebrafish embryos and early adults are optically transparent, a characteristic that facilitates direct observation of the effects of toxic agents on internal organs. In previous work, we assessed radiation-associated toxicity in zebrafish embryos and showed that the established radiation protector amifostine exerts protective effects in zebrafish embryos as it does in humans (3, 4).

In the present study, we used zebrafish embryos to explore in vivo the putative radioprotective effects of DF-1 (C-Sixty, Inc., Houston, TX), a water-soluble antioxidant based on the hollow nanostructure of fullerenes (5). Fullerenes represent a family of molecules that contain 20, 40, 60, 70, or 84 carbon atoms. C₆₀ fullerene is the most frequently used member of this family (5). DF-1 is a C₆₀ fullerene derivative (dendrofullerene) containing 18 carboxylic groups designed to enhance water solubility (6). Fullerenes have the potential to scavenge reactive oxygen species (ROS), including hydrogen peroxide, hydroxyl radical, hydroperoxy radicals, and superoxide (7). In this study, we tested the hypothesis that the powerful in vitro antioxidant effects of DF-1 may alleviate radiation toxicity in irradiated zebrafish embryos in vivo in a manner similar to the Food and Drug Administration–approved radioprotector amifostine.

**Materials and Methods**

**Embryo harvesting and maintenance.** Zebrafish (use and handling approved by the Institutional Animal Care and Use Committee at
Thomas Jefferson University) were mated in embryo collection tanks. Viable embryos were washed and sorted (10 per well in standard six-well plates) at the one- to two-cell developmental stage and maintained under normoxic conditions at 28.5°C. Embryo medium was changed at 24, 72, and 120 hours post fertilization (hpf).

**Radiation exposure and drug treatments.** DF-1 was dissolved in embryo medium containing no more than 0.4% DMSO. Embryo medium containing 0.4% DMSO was used as a vehicle control in all experiments. Amifostine (MedImmune Oncology, Inc., Gaithersburg, MD) served as a positive control in select experiments and was used at 4 mmol/L as described by us previously (4). Unless stated otherwise, embryos were exposed to ionizing radiation ranging in dose from 0 to 40 Gy at 24 hpf using a 137Cs radiation source (Mark 1 irradiator, JL Shepherd Associates, San Fernando, CA). DF-1 toxicity analysis was conducted using a dilution series of 0, 10, 100, and 1,000 μmol/L. DF-1 in the absence of radiation. To determine modulation of radiation-induced toxicity, DF-1 was added at 100 μmol/L to embryos for 3 hours and 30, 15, or 5 minutes before radiation exposure and 5, 15, or 30 minutes after radiation exposure. After irradiation, zebrafish embryos were maintained at 28.5°C for up to 6 days post fertilization (dpf) to monitor effects of treatments on survival, morphology, and organ-specific toxicity.

**Analysis of treatment effects on zebrafish morphology and survival.** Dechorionated embryos at 72 hpf were anesthetized with 0.004% tricaine and immobilized by placing them on 3% methylcellulose on a glass depression slide. Morphology was assessed visually using a light transmission microscope (Olympus BX51, Olympus, Melville, NY) at ×40 to ×100 magnification, and representative images were recorded using a SPOT camera and SPOT Advanced software (SPOT Diagnostic Instruments, Sterling Heights, MI). Similarly, survival of embryos was assessed visually at 24-hour intervals up to 144 hpf by light microscopy. The criterion for embryonic survival was the presence of cardiac contractility.

**Renal function assay.** Clearance of tetramethylrhodamine-labeled, 10-kDa dextran from the cardiac area was determined as described previously (8). Briefly, zebrafish embryos at 24 hpf were exposed to ionizing radiation and maintained in embryo medium. At 72 hpf, embryos were anesthetized using a 1:100 dilution of 4 mg/mL tricaine methanesulfonate (Sigma, St. Louis, MO) and dorsally positioned on 3% methylcellulose gel. Tetramethylrhodamine-labeled, 10-kDa dextran (Molecular Probes, Eugene, OR) was injected into the cardiac venous sinus; embryos were kept at 28.5°C and imaged 1, 5, and 24 hours following microinjection. The average fluorescence emission at 590 nm following excitation at 570 nm was detected at the center of the cardiac area, and the relative intensity was measured using a Leica microscope (Leica Mikroskope and Systeme GmbH, Wetzlar, Germany). Images were transformed into grayscale and evaluated with NIH ImageJ software as described by Hentschel et al. (8).

**Neurotoxicity assay.** Neuromast development was assessed as described by Harris et al. (9), using the fluorescent vital dye 2-[4-(dimethylamino)styryl]-N-ethylpyridinium iodide DASPEI (Molecular Probes). Zebrafish embryos at 5 dpf were exposed to 80 Gy. Neuromast staining was done 24 hours later by incubation with 0.005% 2-[4-(dimethylamino)styryl]-N-ethylpyridinium iodide in embryo medium for 15 minutes. Embryos were rinsed once with

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**Fig. 1.** A, toxicity profile of DF-1 as assessed by survival of zebrafish embryos. The time-dependent survival of embryos exposed to DF-1 at 24 hpf at concentrations ranging from 10 to 1,000 μmol/L. B, dose-dependent effects on survival (at day 6 of development) of zebrafish embryos exposed to 20 Gy ionizing radiation at 24 hpf in the presence of increasing concentrations of DF-1. DF-1 was given 3 hours before irradiation. C and D, comparison of radioprotection afforded by DF-1 and amifostine administration to zebrafish embryos exposed to 20 or 40 Gy ionizing radiation at 24 hpf. Drugs were given 3 hours before irradiation, and overall survival was scored for up to 6 dpf. ●, vehicle (DMSO, 0.4 %) control; ○, DF-1 (100 μmol/L); ▲, amifostine (4 mmol/L).
embryo medium and anesthetized for 5 minutes. The fluorescent emission at 515 nm following excitation at 450 to 490 nm was detected using a fluorescence microscope (Olympus BX51, Olympus). Neuromast staining was evaluated as follows: each neuromast on one side of the body was given a 2-[4-(dimethylamino)styryl]-N-ethyl-pyridinium iodide score of +2 for normal staining, +1 for reduced staining, or +0 for no staining. Values were normalized to the maximum possible score of 54 (27 neuromasts).

**Histopathology and evaluation of embryos.** Zebrafish embryos were evaluated histopathologically for morphologic alterations of ionizing radiation exposure and the potential radioprotective effects of DF-1. Briefly, embryos at 4 dpf were exposed to 0, 20, or 20 Gy plus DF-1 (100 μmol/L) given 3 hours before radiation exposure. Following sacrifice using a 1:100 dilution of 4 mg/mL tricaine methanesulfonate (Sigma), embryos (six per treatment group) were initially fixed and preserved in Davidson’s solution (Electron Microscopy Sciences, Hatfield, PA) for 24 hours and then rinsed and placed in 10% neutral buffered formalin for a minimum of 4 days. All specimens were processed in graded alcohol (70-100%), cleared twice at 10 minutes each in Clear-Rite 3 (Richard Allen, Kalamazoo, MI), and infiltrated with paraffin (Paraplast, McCormick Scientific, St. Louis, MO). Sections were embedded in paraffin, and transverse whole-body sections (4.6-μm thickness; 100-120 sections per fish) were microtomed (Leica RM 2135 rotary microtome, Leica, Inc., Wetzlar, Germany) serially from the rostral-most aspect of the head to the mid-trunk region of each fish. All sections were stained with modified Mayer’s hematoxylin 2 and eosin-Y (Richard Allen), mounted (Permount, Fisher Scientific, Fairlawn, NJ) on glass slides, and coverslipped. Sections were examined by light microscope (Olympus BX51, Olympus) at ×4 to ×40 magnification, and representative images were obtained using a SPOT camera and SPOT Advanced software (SPOT Diagnostic Instruments).

**Detection of ROS.** Production of ROS was measured in dechorioated zebrafish embryos at 24 hpf in 96-well plates. Embryos (one per well) were treated with either vehicle (0.4% DMSO in embryo medium) or DF-1 (100 μmol/L) and 5-(and-6)-chloromethyl-2′,7′-dihydrodichlorofluorescein diacetate (CM-H2DCF; Molecular Probes). DF-1 (100 μmol/L) was added to zebrafish embryos at 3 hours before ionizing radiation at 24 hours, the fluorescent dye CM-H2DCF (500 ng/mL) was added 1 hour before radiation exposure. The average fluorescence emission at 530 nm following excitation at 490 nm was detected 5 minutes and 2 hours after ionizing radiation exposure using a microplate fluorescent reader (BIO-TEK FL 600; BIO-TEK Instruments, Inc.; Winooski, VT). To account for radiation-induced ROS in the embryo medium results were corrected by subtraction of values obtained in wells containing medium and either vehicle or DF-1 with and without irradiation.

**Statistical analysis.** All experiments were done at least thrice with ≥10 embryos per experimental group. Statistical analysis was done by one-tailed Student’s t test analyses.

### Results and Discussion

**Toxicity profile of DF-1 in zebrafish embryos.** The unmodified Buckminster fullerene C60 reportedly exerts toxic effect in vitro and in several in vivo systems, including the aquatic largemouth bass (10). This toxicity has been attributed, in part, to lipid peroxidation by C60. In contrast to the unmodified C60, DF-1 has been structurally altered to increase water solubility and thereby potentially reduce C60-associated toxicity. To determine DF-1-associated toxicity, we exposed zebrafish embryos at 24 hpf to increasing concentrations of DF-1 and scored the effects of this treatment on morphologic appearance and survival up to day 6 of development. DF-1 administration (<1 mmol/L) was not toxic to zebrafish embryos because neither viability nor gross morphology were adversely affected (Fig. 1).

**Fig. 2.** Mitigating effects of DF-1 on radiation-induced lethality in zebrafish embryos. DF-1 (100 μmol/L) was given 5, 15, or 30 minutes after irradiation (20 Gy), and survival was assessed for 5 days. When given 30 minutes after radiation, DF-1 did not affect embryonic survival after exposure. Vehicle control (○), 100 μmol/L DF-1, 5 minutes (▲), 15 minutes (▼), and 30 minutes (△) after irradiation.

**Fig. 3.** DF-1 protects against radiation-induced defects in midline development. Radiation-induced morphologic changes in body axis were assessed at 3 dpf. Representative pictures of ionizing radiation – induced dorsal curvature (cup phenotype) in zebrafish and attenuation of this effect by DF-1 (100 μmol/L) given 3 hours before ionizing radiation (24 hpf). Quantitative representation of the results after 20 or 40 Gy ionizing radiation as indicated. *P = 0.0077, statistically significant differences between control and experimental groups.
Effects of DF-1 on survival of zebrafish embryos exposed to ionizing radiation. We next focused on the radioprotective effects of DF-1 as determined by viability assessment of irradiated zebrafish embryos pretreated with the fullerene.Embryos were exposed at 24 hpf to either 20 or 40 Gy based on our previous observation that, at this stage of development, 20 Gy represents the LD₅₀, whereas 40 Gy causes death in 100% of irradiated embryos within 5 days after radiation exposure (4). In addition, organogenesis has commenced by 24 hpf; thus, this time point is a preferred developmental stage to assess radiation-induced effects on most major organs as described in more detail below. DF-1 given before radiation afforded significant survival advantage to zebrafish embryos when exposed to either 20 or 40 Gy. This effect was maximal at a concentration of 100 μmol/L (Fig. 1B). At 40 Gy, protection was only partial as survival was extended, but all embryos eventually died within the observation period of up to 6 dpf (Fig. 1D). DF-1 (100 μmol/L) provided a level of radioprotection, which was similar to that provided by 4 mmol/L amifostine (Fig. 1C and D). Time course experiments showed that maximal rescue of radiation-associated lethality and gross morphologic defects was achieved when DF-1 was given 3 hours, compared with 30, 15, or 5 minutes, before ionizing radiation treatment. We also assessed whether DF-1 could be used to mitigate radiation effects on survival when given after radiation exposure. DF-1 did not confer a survival advantage to zebrafish embryos when given 30 minutes after 20 Gy ionizing radiation exposure. However, DF-1 significantly enhanced survival when given concurrently or up to 15 minutes after irradiation (Fig. 2; data not shown). Collectively, these experiments illustrate that, as expected for an agent with antioxidant properties, DF-1 protects against and, to a limited degree, mitigates radiation-induced lethality in zebrafish embryos.

DF-1 alleviates radiation-induced defects in midline development. To obtain a more detailed view of DF-1-mediated radioprotection, we evaluated the effects of this drug on organ-specific, radiation-induced damage. We first assessed the effects

Fig. 4. DF-1 attenuates radiation-associated reduction in dextran clearance. A, representative photomicrographs illustrating clearance of tetramethylrhodamine-labeled, 10-kDa dextran within 24 hours after cardiac injection in irradiated and control fish embryos. Radiation (20 Gy) led to delayed clearance measured at 24 hours. Quantitative representation of results obtained by using the NIH Image J software according to Hentschel et al. (8). Reduction of dextran clearance by radiation exposure (20 Gy) was significant \( (P = 0.029) \) and abrogated by DF-1 (100 μmol/L) given 3 hours before irradiation. Ionizing radiation was associated with renal glomerular changes that are not mitigated by DF-1 treatment. B, representative section of pronephric glomerulus (pg) from zebrafish embryo exposed to 0 Gy ionizing radiation at 4 dpf (control). C, pronephric glomerulus from embryo exposed to 20 Gy ionizing radiation at 4 dpf is relatively smaller than control and the glomerular tuft is less cellular. D, treatment with DF-1 does not lessen ionizing radiation – associated changes in pronephric glomerulus in embryo exposed to 20 Gy at 24 hpf. All sections were taken from 4 dpf embryos, stained with H&E. Bar, 25 μm.
of DF-1 on a commonly observed and easily scored phenotype apparent within 1 to 2 days following ionizing radiation exposure of zebrafish. This phenotype consists of dorsal curvature of the body axis previously described as “curly-up” or cup and ascribed to defects in midline development of zebrafish embryos (11). Although the mechanism leading to cup is presently unknown, the ease with which this phenotype can be observed makes it an attractive variable to score radiation toxicity in zebrafish embryos (Fig. 3). We observed that throughout the dose range tested, DF-1 markedly reduced the incidence of cup. Depending on the ionizing radiation dose, it either reduced the severity or abolished the dorsal curvature altogether (Fig. 3).

**DF-1 attenuates radiation-induced renal function defects.** Radiation exposure of embryos produced extensive edema in the developing fish, and this effect was reversed by DF-1 treatment (data not shown). We hypothesized that this phenotype was due to impaired renal function after radiation, an effect well documented in mammals, including humans (12). To address this issue, we used an excretory function assay, which measures clearance of a fluorescent dextran within 24 hours after injection into the cardiac venous sinus (8). Compared with non-irradiated controls, exposure to 20 Gy ionizing radiation markedly reduced clearance of this agent. By contrast, pretreatment of embryos with 100 μmol/L DF-1 restored clearance to levels indistinguishable from those observed in non-irradiated fish embryos (Fig. 4A). Thus, DF-1 appears to restore compromised excretory function in irradiated zebrafish embryos. To determine whether this effect was due to protection of renal tissue against radiation effects, we assessed the effects of different treatments on renal tissue architecture. Compared with unirradiated control fish, embryos exposed to 20 Gy ionizing radiation exhibited minimal to moderate apoptotic-type individual cell death involving a variety of tissues; however, in the kidney, 20 Gy ionizing radiation exposure was associated with only a subtle but consistent decrease in the size and overall cellularity of pronephric glomeruli compared with controls (Fig. 4B and C). This particular morphologic effect did not appear to be mitigated by DF-1 treatment (Fig. 4D). We, thus, tested whether the profound edema in zebrafish embryos following ionizing radiation exposure was secondary to reduced cardiac function (13). Time-lapse microscopy of cardiac contractility in control and irradiated fish embryos revealed only marginal effects of 20 Gy ionizing radiation on heart rate and blood flow (data not shown). These experiments show that radiation reduces clearance of dextran in zebrafish embryos, although it is presently unclear which cells or tissues are responsible for this effect.

**DF-1 protects against neurotoxicity in irradiated zebrafish.** Neurotoxicity represents a dose-limiting toxicity associated with radiation therapy of the central nervous system. We investigated whether nerve cell development was affected by ionizing radiation in zebrafish and whether DF-1 treatment could reduce radiation-induced nerve cell damage. To this end, we made use of a dye, which exclusively stains neuromasts, the equivalent of inner ear nerve hair cells. To measure effects of radiation on neuromast development, embryos were exposed at 5 dpf to 80 Gy ionizing radiation in the presence or absence of DF-1. In these experiments, 80 Gy was given because 20, 40, or 60 Gy did not produce a measurable neurotoxic effect. Significantly reduced staining of neuromasts was apparent following exposure to 80 Gy, an effect dramatically inhibited by DF-1 pretreatment (Fig. 5). This result shows that DF-1 protects against radiation-induced damage of developing nerve cells in zebrafish.
Reduction of ROS in irradiated zebrafish embryos treated with DF-1. C-60 fullerenes are known to have antioxidant effects in vitro (7). To investigate whether DF-1 exhibits similar effects in vivo, levels of ROS were determined in developing zebrafish embryos after ionizing radiation. Measurement of ROS in zebrafish revealed that free radicals following radiation exposure (20 Gy) were significantly diminished by DF-1 pretreatment (100 μmol/L) and indistinguishable from non-irradiated controls (Fig. 6). This result reveals ROS scavenging activity of DF-1 in vivo and suggests a putative mechanism for the observed radioprotective action of this fullerene in zebrafish. Note that similar studies cannot be done with amifostine due to molecular interactions between amifostine and the fluorescent dye, which lead to false-positive values in the assay used.

Conclusion

Collectively, the results presented in this report underscore that zebrafish embryos provide a versatile model system to assay radioprotectors/radiomitigators in a vertebrate organism, both on a systemic and organ-specific basis. Furthermore, we provide evidence that the modified fullerene DF-1 provides radioprotection to several target tissues and organs and acts as an oxygen radical scavenger in this in vivo model system.

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References

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