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Nuclear factor κB inhibitors alleviate and the proteasome inhibitor PS-341 exacerbates radiation toxicity in zebrafish embryos

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Abstract

Inflammatory changes are a major component of the normal tissue response to ionizing radiation, and increased nuclear factor κB (NF-κB) activity is an important mediator of inflammatory responses. Here, we used zebrafish embryos to assess the capacity of two different classes of pharmacologic agents known to target NF-κB to modify radiation toxicity in the vertebrate organism. These were proteasome inhibitors, including lactacystin, MG132, and PS-341 (Bortezomib/VELCADE), and direct inhibitors of NF-κB activity, including ethyl pyruvate (EP) and the synthetic triterpenoid CDDO-TFEA (RTA401), among others. The proteasome inhibitors either did not significantly affect radiation sensitivity of zebrafish embryos (MG132, lactacystin) or rendered zebrafish embryos more sensitive to the lethal effects of ionizing radiation (PS-341). Radiosensitization by PS-341 was reduced in fish with impaired p53 expression or function but not associated with enhanced expression of select p53 target genes. In contrast, the direct NF-κB inhibitors EP and CDDO-TFEA significantly improved overall survival of lethally irradiated zebrafish embryos. In addition, direct NF-κB inhibition reduced radiation-induced apoptosis in the central nervous system, abrogated aberrations in body axis development, restored metabolization and secretion of a reporter lipid through the gastrointestinal system, and improved renal clearance compromised by radiation. In contrast to amifostine, EP and CDDO-TFEA not only protected against but also mitigated radiation toxicity when given 1 to 2 hours postexposure. Finally, four additional IκB kinase inhibitors with distinct mechanisms of action similarly improved overall survival of lethally irradiated zebrafish embryos. In conclusion, inhibitors of canonical pathways to NF-κB activation may be useful in alleviating radiation toxicity in patients. [Mol Cancer Ther 2009;8(9):2625–34]

Introduction

Normal tissue damage limits the dose of ionizing radiation that can be safely administered to treat neoplastic disease. A well-known example of this problem is inflammation of the oral mucosa and of the lining of the gastrointestinal tract in tumor patients receiving chemotherapy or radiation (1). Depending on the area of the body treated with radiation, other organ sites including the lungs and the pericardium also manifest radiation-induced inflammation. A pervasive feature of ionizing radiation–associated inflammation is the increased presence of proinflammatory cytokines including tumor necrosis factor-α (TNF-α) and interleukin-6, both locally and in the circulation (2). In contrast to intracellular regulators of the DNA damage response, these and other inflammatory mediators act in a paracrine fashion affecting diverse cell types in the tissue microenvironment or even at a distance (3). This circumstance highlights the necessity to use animal models to investigate the relative contribution of inflammatory changes to the overall response to radiation-induced cell and tissue injury in a multicellular organism. In recognition of this need, we recently established zebrafish embryos as a facile vertebrate *in vivo* system to monitor the effects of radiation protectors on normal tissues during development (4).

The nuclear factor κB (NF-κB) family of transcription factors represents a diverse and shared signaling mechanism activated during cell stress responses (5). In addition, deregulated NF-κB signaling has been implicated in the malignant phenotype and treatment resistance of select tumor forms (6–10). The canonical pathway to NF-κB activation leads to IκB kinase β (IKKβ)-dependent phosphorylation and subsequent proteasomal degradation of the NF-κB inhibitor IκB, increased nuclear presence of NF-κB dimers, and enhanced NF-κB–dependent transcriptional activity (5).

Whole-body radioprotection through anti-inflammatory agents has very recently been shown in animal models. Specifically, certain triterpenoids (CDDO and derivatives thereof) have been shown to selectively protect normal mouse tissues against the deleterious effects of ionizing radiation (11). Furthermore, ethyl pyruvate (EP), a derivative of the

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end product of glycolysis, similarly protects normal cells against the deleterious effects of radiation both *in vitro* and in mice (12). Among other molecular targets, both drugs inhibit activation of NF-κB. EP inhibits NF-κB signaling through direct molecular interaction with a reactive cysteine of the p65 subunit of NF-κB (13) whereas CDDO-TFEA binds to a reactive cysteine (Cys179) of IKKα, thus inhibiting its kinase activity (14). However, these drugs also target other signaling molecules and pathways of potential relevance to the radiation response, including signal transducers and activators of transcription 3 and Jaks (15, 16). In addition to these agents proteasome inhibitors have been shown to inhibit NF-κB–dependent transcription, and one of these (PS-341; Bortezomib; VELCADE) has been Food and Drug Administration–approved for clinical use in patients afflicted with multiple myeloma (for review see refs. 17, 18). It is presently unknown whether and how proteasome inhibitors affect whole-body radiation sensitivity.

Collectively, these results raised the question whether inhibition of NF-κB activity by different pharmacologic agents contributes to the protection of normal cells and tissues against damage induced by ionizing radiation. Here, we addressed this issue using zebrafish embryos as an *in vivo* model system. We observed that the NF-κB inhibitors EP and CDDO-TFEA afforded protection to zebrafish embryos against the lethal effects of radiation in the pre-exposure and postexposure settings, i.e., when administered hours after radiation exposure. Radiation protection extended to multiple organ sites including the gastrointestinal system and, importantly, was also observed when using additional IKK inhibitors with different modes of action. In contrast, several proteasome inhibitors, including PS-341, did not protect against, but rather moderately exacerbated radiation-associated normal tissue toxicity in zebrafish embryos. These results predict a favorable therapeutic index for the use of inhibitors of canonical pathways to NF-κB activation in combination with radiation therapy.

Materials and Methods

Embryo Harvesting and Maintenance

Zebrafish were mated in embryo collection tanks. Viable embryos were washed and sorted (25 embryos per 60-mm dish) at the one- to two-cell developmental stage, and maintained under normoxic conditions at 28.5°C to enable normal development. Embryo medium was changed at 24, 72, and 120 h postfertilization (hpf). All procedures using live zebrafish were approved by the Institutional Animal Care and Use Committee at Thomas Jefferson University. In select experiments, embryos (24 hpf) were dechorionated by placement in embryo medium supplemented with 50 μg/mL pronase (Sigma) for approximately 10 min at room temperature, then gently agitated with a plastic pipette until the embryos were liberated from the disrupted chorions. After dechorionation, the embryos were rinsed thoroughly with embryo medium, and placed in fresh embryo medium.

Radiation Exposure and Drug Treatments

Pharmacological agents [EP was kindly provided by CDDO-TFEA was from Reata Pharmaceuticals; IKK inhibitor 2 (Weldelolactone), IKK inhibitor 3 (BMS-345541), IKK-2 inhibitor 4, and IKK-2 inhibitor 5(IMD-0354) were from Calbiochem; MG132 was from Sigma; PS-341 was from Millennium Pharmaceuticals; and lactacystin was from Calbiochem) were dissolved in embryo medium containing <0.1% DMSO. Embryo medium was used as a vehicle control in all experiments. Unless stated otherwise embryos were exposed to ionizing radiation ranging in dose from 0 to 20 Gy at 24 hpf using an X-ray machine (Gulmay Medical) or a ^{137}Cs radiation source. Toxicity analyses for EP (<10 mmol/L), CDDO-TFEA (<10 μmol/L), PS-341 (<10 μmol/L), MG132 (<50 μmol/L), or lactacystin (<10 μmol/L) were conducted by monitoring survival and development of zebrafish embryos for 7 d in the absence of radiation. To determine modulation of radiation-induced toxicity, EP (1 mmol/L) or CDDO-TFEA (1 μmol/L) was added to embryos either 1 h before or up to 3 h after radiation exposure at 24 hpf. The proteasome inhibitors were added to zebrafish embryos 1 h prior to ionizing radiation. After irradiation, zebrafish embryos were maintained at 28.5°C for up to 7 d postfertilization to monitor effects of treatments on survival, morphology, and organ-specific toxicity.

Analysis of Treatment Effects on Zebrafish Survival and Gross Morphology

Dechorionated embryos at 72 hpf were anesthetized with a 1:100 dilution of 4 mg/mL tricaine methanesulfonate (Sigma) 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) at 40 to 100× magnification, and representative images recorded using a QIMAGING camera and QIMAGING Advanced software (QIMAGING Diagnostic Instruments). Similarly, survival of embryos was assessed visually at 24-h intervals up to 7 days by light microscopy. The criterion for embryonic survival was the presence of cardiac contractions.

Apoptosis Assay

Zebrafish embryos were incubated for 1 h in embryo medium containing modifiers of the radiation response and exposed to 20 Gy at 24 hpf. Six hours after radiation exposure, embryos were stained for 15 min using 5 μg/mL of acridine orange dye (Sigma) and rinsed five times with embryo medium as described previously (19). Zebrafish embryos were imaged with QIMAGING camera and iVision software; the images were analyzed using ImageJ software.

Detection of ROS

Reactive oxygen species (ROS) levels were measured in dechorionated zebrafish embryos in 96-well plates. Embryos (1 embryo/well) were treated with either vehicle (embryo medium) or EP (1 mmol/L) or CDDO-TFEA (1 μmol/L) in the presence of 5-(and-6)-chloromethyl-2′,7′-dihydrodichlorofluorescein diacetate (CM-H_2DCFA; 500 ng/mL; Molecular Probes) followed by radiation

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exposure at 24 hpf. The average fluorescence emission at 530 nm following excitation at 490 nm was detected immediately and 2 h after ionizing radiation exposure using a microplate fluorescent reader (BIO-TEK FL 600, BIO-TEK Instruments Inc.). To account for radiation-induced ROS in the embryo medium results were corrected by subtraction of values obtained in wells not containing fish in the presence and absence of pharmacologic agents.

Renal Function Assay

Time-dependent clearance of tetramethylrhodamine-labeled 10-kDa dextran (Molecular Probes) was determined as described previously with minor modifications (20). 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) and dorsally positioned on 3% methylcellulose gel. Tetramethylrhodamine-labeled 10-kDa dextran was injected into the cardiac venous sinus; embryos were kept at 28.5°C, and imaged at 1 and 24 h 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 Mikroskopie & Systeme GmbH). Images were transformed into grayscale and evaluated with NIH ImageJ software as described (20).

Morphologic Analysis of the Gastrointestinal System

The functional and morphologic integrity of the developing gastrointestinal system was assessed in zebrafish embryos using PED6, a fluorescent reporter of phospholipase A2 (PLA_2) activity. PED6 is a fluorogenic substrate for PLA_2, which contains a BODIPY FL dye-labeled acyl chain and a dinitrophenyl quencher group (21). The cleavage of the dye-labeled acyl chain by PLA_2 within cells lining the intestine unquenches the dye and leads to detectable fluorescence in the lumen of the developing gastrointestinal tract. PED6 was added to zebrafish embryos at 5 dpf followed by imaging the fish at 6 dpf with the average fluorescence emission at 540 nm excitation at 505 nm. Images were taken at 6 dpf using a Leica microscope and analyzed using the ImageJ software.

Histopathology and Evaluation of Tissue Morphology

Zebrafish embryos were evaluated histopathologically for morphologic alterations induced by radiation exposure and potential radioprotective effects of EP and CDDO-TFEA with special emphasis on the gastrointestinal morphology. Briefly, embryos at 24 hpf were exposed to 0 or 12 Gy in the presence or absence of either CDDO-TFEA or EP administered 1 h prior to ionizing radiation. Embryos were sacrificed, fixed by immersion in 4 % paraformaldehyde for 24 h, and then rinsed and placed in 10× PBS for another 24 h. Sections were embedded in paraffin, and coronal, transverse, and sagittal whole-body sections (4 μm thickness) were generated. All sections were stained with H&E, mounted on glass slides, and examined by light microscope; representative images were taken using a QIMAGING camera and iVision software.

NF-κB Reporter Assay

NF-κB reporter assay was done as described by us previously (22) with minor modifications. HeLa cells were seeded at 7.5×10^4/mL in DMEM supplemented with 10% fetal bovine serum. The cells were cotransfected with the pSEAP2-NF-κB vector (BD BioSciences) encoding a secreted form of human placental alkaline phosphatase driven by a NF-κB–responsive promoter and a β-galactosidase expression vector for control purposes. Forty-eight hours posttransfection, different NF-κB inhibitors (0.5 μmol/L velcade, 5 μmol/L MG-132, 1 mmol/L EP, 1 μmol/L CDDO) were added to the cells in serum-free media for 24 h. NF-κB–dependent transcription in the absence and presence of recombinant TNF-α (10 ng/mL; R&D Systems) was determined 72 h posttransfection using the Great EscAPe SEAP Reporter System 3, which is based on detection of secreted alkaline phosphatase in cell supernatants normalized to β-galactosidase activity using the luminescent β-gal detection kit (BD Biosciences).

Reverse Transcription PCR Analysis

Zebrafish total RNA was isolated from 100 embryos per experimental condition at 30 hpf (6 h post radiation) using the RNeasy mini kit (QIAGEN Sciences) and stored at −80°C. For reverse transcription, total RNA was annealed with Oligo(dT) primer (Roche) at 70°C for 5 min followed by the incubation at 42°C for 1 h. Reverse transcription reaction products were boiled for 2 min followed by incubation on ice for 2 min before use. Primer sequences used for amplification of *bax, mdm2, p21/waf-1*, and *β-actin* zebrafish sequences are provided in Supplementary Table S1. PCR reaction conditions were 94°C, 60°C, 72°C for 30 s, 30 s, 1 min, respectively, and 35 cycles with 7 min extension time after the last cycle. Thermo Fisher Scientific Taq-polymerase was used in 50 μL PCR reaction mix containing 1 μL reverse transcription reaction. PCR reactions were analyzed by 1.5% agarose gel electrophoresis.

Statistical Analysis

All experiments were done at least three times with at least 75 embryos total per experimental group. To determine statistically significant differences between groups χ^2 tests were done.

Results

Proteasome Inhibitors Radiosensitize Zebrafish Embryos

The proteasome inhibitor PS-341 (Bortezomib/VELCADE) is presently the only Food and Drug Administration–approved drug with well-characterized inhibitory effects on NF-κB activity (18). PS-341 is a small, cell-permeable molecule inhibiting proteasome activity in a reversible manner. In addition to reducing the activation state of NF-κB by inhibiting proteasomal degradation of IκB, PS-341 affects many other pathways and targets, leading to high expression levels of several proapoptotic proteins in certain experimental conditions (23). *In vitro*, PS-341 has been found to enhance antitumor cell effects of select chemotherapeutic agents (6, 24), tumor cell targeting antibodies

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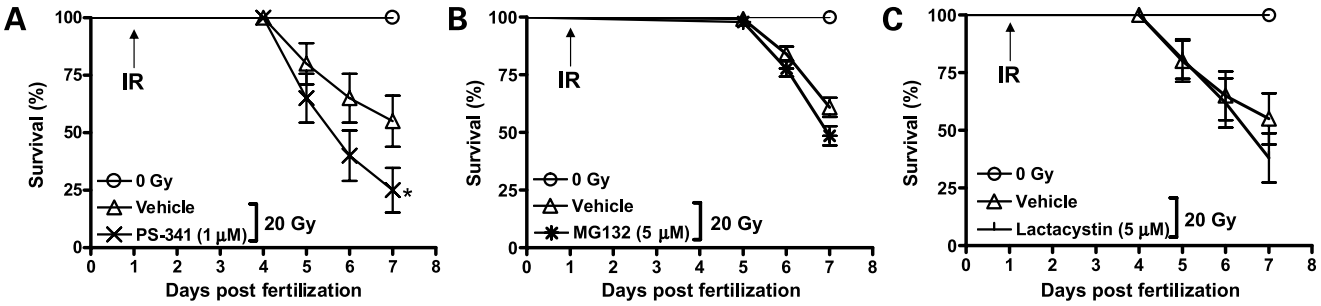

Figure 1. Effects of the proteasome inhibitors PS-341 (**A**), MG132 (**B**), and lactacystin (**C**) on the radiation sensitivity of zebrafish embryos. Embryos were irradiated at 24 hpf and survival was scored every day up to 7 dpf. Results shown represent mean ± SD of triplicate experiments. *, statistically significant difference in survival at 6 to 7 dpf.

(25), and ionizing radiation (26). Yet, little is known about the combined effects of PS-341 and ionizing radiation on normal cells and tissues of vertebrate organisms. To address this issue we used PS-341 in zebrafish embryos exposed to high doses of ionizing radiation as described by us previously (4, 27, 28). We first established that treatment of zebrafish with PS-341 alone (dose range, 0–10 μmol/L) was nontoxic as assessed by embryo survival and gross morphology during the first 7 days after fertilization (Supplementary Fig. S1). In contrast, PS-341 (1 μmol/L) markedly sensitized zebrafish embryos to the lethal effects of ionizing radiation when administered 1 hour prior to radiation (Fig. 1A). In these experiments zebrafish embryos were exposed at 24 hpf to 20 Gy, previously determined to kill 50% of irradiated zebrafish embryos by day 7 of development (27). In HeLa cells, at the same concentration (1 μmol/L) PS-341 abrogated the TNF-α–induced NF-κB activity, whereas it did not significantly affect the basal activity (Supplementary Fig. 2A and B).

To ascertain whether radiation sensitization by PS-341 could be replicated using other inhibitors of the proteasome we next tested the effects of MG132, a nonboronated small molecule inhibitor of the 26S proteasome (29), on zebrafish survival in the presence and absence of ionizing radiation. Similar to PS-341, MG132 was remarkably nontoxic when applied as a single agent to zebrafish embryos (dose range, 0–50 μmol/L) yet efficiently inhibited TNF-α–induced but not the baseline NF-κB activity in HeLa cells when used at 5 μmol/L (Supplementary Fig. 2A and B). At this concentration, however, MG132 marginally sensitized zebrafish embryos to the lethal effects of 20 Gy ionizing radiation albeit to a lesser degree than PS-341 (Fig. 1B). An irreversible proteasome inhibitor (lactacystin) at a nontoxic concentration (5 μmol/L) also slightly radiosensitized zebrafish embryos in a manner similar to MG132 (Fig. 1C).

These results show that several proteasome inhibitors do not protect normal cells and tissues in the developing fish larvae against the deleterious effects of radiation. As p53 is a major target of proteasomal degradation and enhances ionizing radiation–associated tissue damage in mice (30–32) and zebrafish (33, 34) we asked whether the deleterious effects of proteasome inhibitors could be linked to p53 stabilization and subsequent induction of target genes. Consistent with our earlier observations, ablating p53 expression by antisense morpholino oligodeoxynucleotide (35) or p53 function by PFT-α (1 μmol/L) given to zebrafish embryos at 24 hpf (34) markedly improved zebrafish survival after radiation either alone or in combination with PS-341 (not shown). However, reverse transcription-PCR analysis did not reveal increased steady-state mRNA levels of the p53 targets *p21/WAF1*, *bax*, or the zebrafish ortholog of *mdm2* in PS-341–treated embryos whereas ionizing radiation led, as expected, to elevated transcript levels for these genes (Supplementary Fig. S3). Thus, the molecular target(s) responsible for radiosensitization by PS-341 and their relationship, if any, to the p53 response remain to be identified.

Radiation Protection of Zebrafish Embryos by the NF-κBp65 Inhibitor Ethyl Pyruvate

In consideration of the fact that proteasome inhibitors affect multiple intracellular pathways in addition to NF-κB and to pinpoint the functional contribution of NF-κB to the radiation response of zebrafish embryos, we tested the effects of a series of pharmacologic inhibitors of NF-κB activity with different mechanisms of action on the radiation response of zebrafish embryos. Reducing NF-κB activity by expression of upstream regulator IκB has previously been shown to cause severe embryonal malformations in zebrafish (36, 37) and, thus, was not further considered. In addition, knockdown of the NF-κBp65 subunit by antisense morpholinos similarly caused severe morphologic defects (no tail phenotype) during the first 3 days of development (Supplementary Fig. S4) consistent with published results (37) and, thus, was not informative in the context of assaying the radiation response. Instead, we used pharmacologic inhibitors that disrupt the canonical pathway to NF-κB activation and could be used at concentrations that do not interfere with embryonal development. First, we tested EP, a ROS scavenger and inhibitor of NF-κBp65 (38). EP inhibits the DNA binding activity of NF-κBp65 by binding to a reactive cysteine in the DNA binding site (Cys 38) of NF-κBp65 (13), which is shared between humans and zebrafish (Supplementary Fig. S2C). EP has very recently been shown to mitigate deleterious effects of total body

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irradiation in mice (12). We observed that EP similarly not only protected against but also mitigated lethality associated with whole body irradiation of zebrafish embryos (Fig. 2A, C, and F). EP was administered at various time points ranging from 1 hour prior to radiation exposure to 3 hours postirradiation. The ROS scavengers amifostine and DF-1 served as positive controls in these experiments as we observed marked protection of embryos by these two compounds in earlier work (4, 27). Whereas amifostine and DF-1 afforded protection against deleterious effects of ionizing radiation when administered prior to or concurrent with radiation, neither compound could mitigate the lethal effects of radiation when given beyond 15 minutes after ionizing radiation (27). In marked contrast, EP administered up to 2 hours after radiation significantly reduced ionizing radiation–associated lethality (Fig. 2F).

The IKK Inhibitor CDDO-TFEA Mitigates Radiation Effects in Zebrafish Embryos

To further address the relevance of NF-κB activation in modulating radiation sensitivity of zebrafish embryos we used CDDO-TFEA that inhibits NF-κB signaling by interacting with Cys179 of IKKβ, inhibiting its activity and preventing phosphorylation and proteasomal degradation of IκB

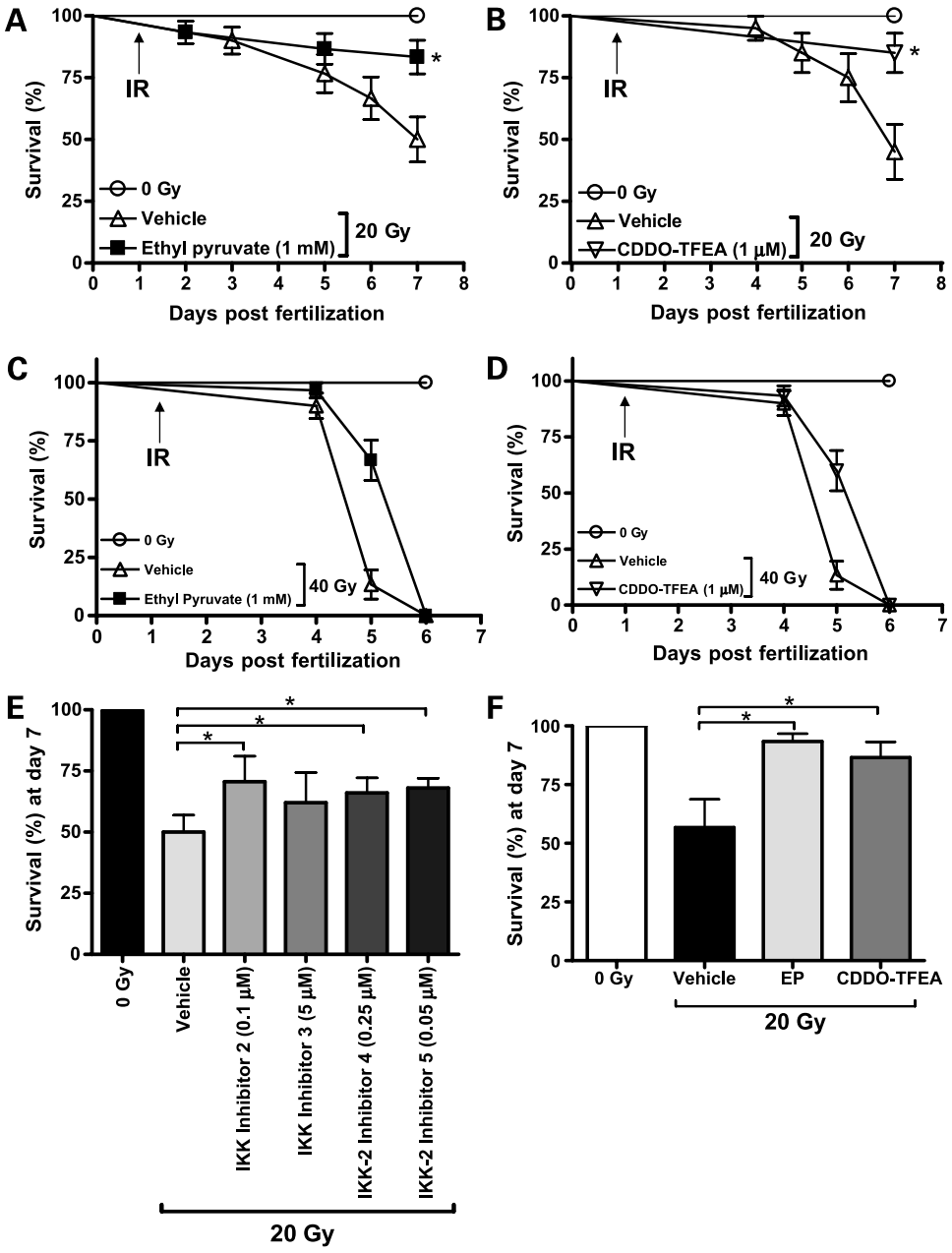

Figure 2. Protection against and mitigation of lethal effects of radiation by direct NF-κB inhibitors. Differential survival of zebrafish embryos pretreated with EP (**A** and **C**) or CDDO-TFEA (**B** and **D**) and exposed to either 20 or 40 Gy as indicated. **E,** radiation protection of zebrafish embryos exposed to 20 Gy and treated for 1 h prior to irradiation with different IKK inhibitors. **F,** increased survival of irradiated (20 Gy) zebrafish embryos treated with EP (1mmol/L) or CDDO-TFEA (1 μmol/L) 2 h post radiation at 24 hpf. Survival was scored at 7 dpf. All results shown represent mean ± SD of triplicate experiments. *, statistically significant differences in survival at 7 dpf between drug-treated and vehicle-treated groups.

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(39) and, thus, through a molecular mechanism distinct from EP. The amino acid sequence around this reactive Cys179 is also highly conserved in zebrafish (Supplementary Fig. 2C). CDDO-TFEA protected against and mitigated overall lethal effects of radiation in zebrafish embryos in a manner similar to EP (Fig. 2B, D, and F). We next determined whether mitigation of radiation effects cosegregated with the capacity of the compounds under investigation to act as ROS scavengers. This was based on the findings that, in addition to directly binding to IKKβ, CDDO has been described to induce expression of enzymes catalyzing antioxidant reactions in peripheral blood mononuclear cells due to increased nuclear accumulation of Nrf2, an oxidant-responsive bZIP transcription factor (40, 41). Whereas EP is an effective ROS scavenger in irradiated zebrafish embryos, CDDO-TFEA did not reduce ROS levels measured 2 hours after radiation exposure (Supplementary Fig. S5). Thus, at least the effect of CDDO-TFEA on radiation mitigation cannot be ascribed to ROS scavenging, whereas in the case of EP, ROS scavenging and NF-κB inhibition may be jointly responsible for the beneficial effects of EP in the mitigation setting. Of note, the ROS scavengers with no known effect on NF-κB signal transduction (amifostine and DF-1) do not mitigate radiation effects if administered beyond 15 minutes after ionizing radiation (27). To further probe whether IKK inhibition is radioprotective we tested four additional small molecule IKK inhibitors, i.e. Wedelolactone (IKK inhibitor 2), BMS-345541 (IKK inhibitor 3) and IKK-2 inhibitors 4 and 5. All four agents protected zebrafish embryos against the lethal effects of radiation in a manner similar to CDDO-TFEA and EP (Fig. 2E). Moreover, unlike EP or CDDO-TFEA, these agents are not known to have antioxidant properties and primarily inhibit IκBα phosphorylation by IKKβ (i.e., IKK-2). On balance, these results suggest that prolonged and excessive activation of the canonical NF-κB pathway is a major contributor to radiation

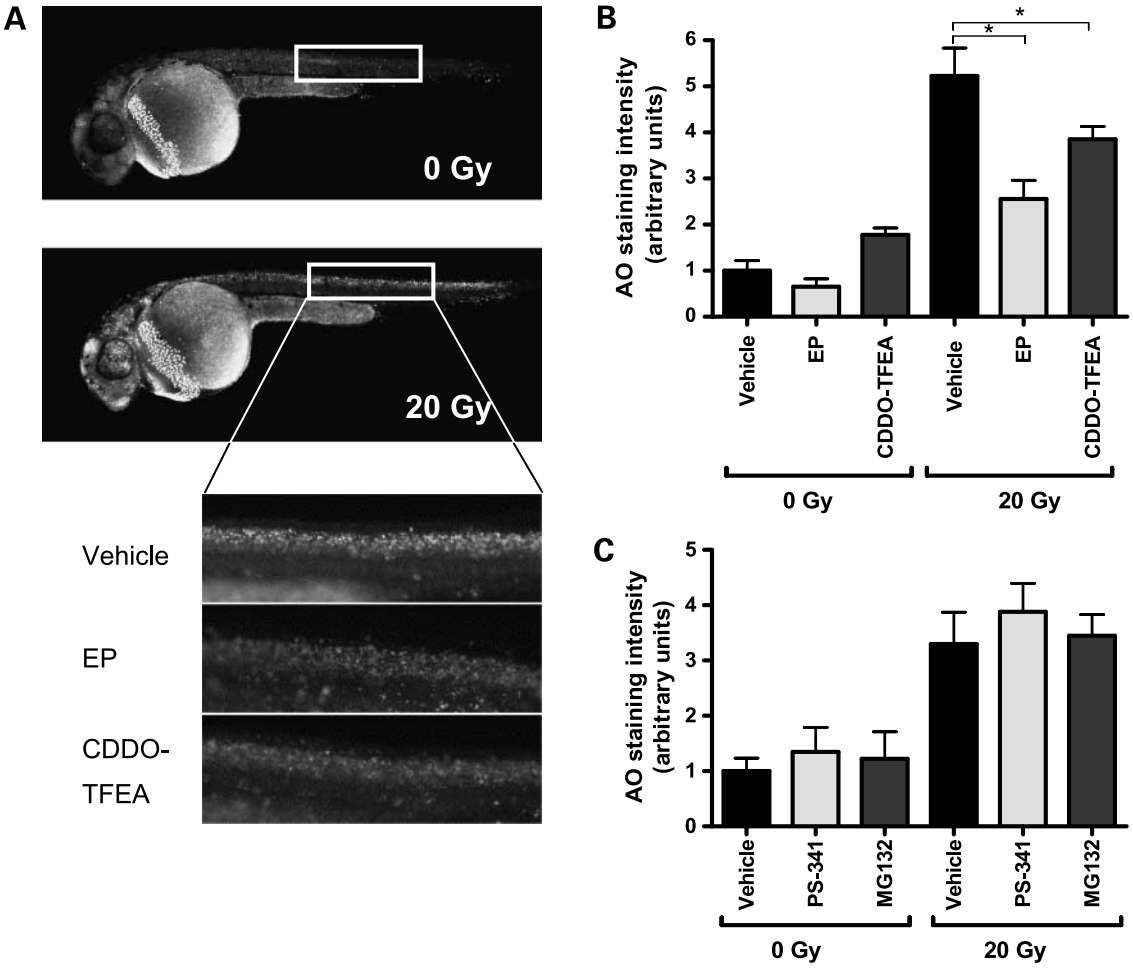

Figure 3. Reduction of organism-wide apoptosis by EP and CDDO-TFEA treatment preradiation and postradiation. Acridine orange (AO) staining of whole embryos was done 6 h postradiation at 30 hpf. **A,** representative examples of control or irradiated fish revealing strong AO staining in the central nervous system and along the body axis induced by radiation (20 Gy). Regions selected for quantitative evaluation are boxed. **B,** reduced AO staining in CDDO-TFEA- and EP-treated embryos exposed to ionizing radiation. **C,** PS-341 or MG132 treatment does not significantly affect AO staining of embryos exposed to ionizing radiation.

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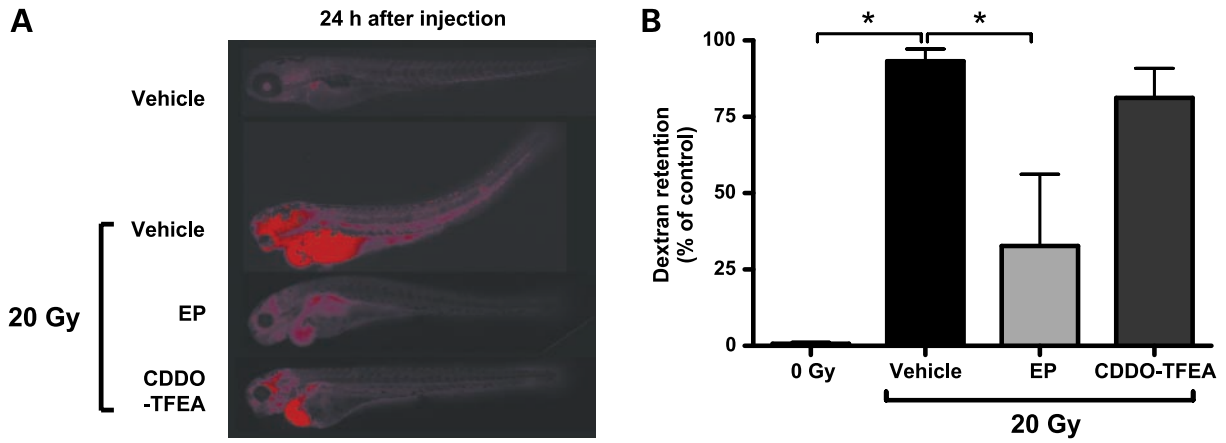

Figure 4. Effects of EP and CDDO-TFEA on radiation-induced kidney damage as determined by dextran clearance. **A,** Representative images of dextran retention in irradiated embryos and effects of EP and CDDO-TFEA on this phenomenon. **B,** quantitative representation of dextran retention in embryos treated with either EP or CDDO-TFEA as indicated; results are expressed relative to vehicle-treated, nonirradiated controls. *, statistically significant differences between the experimental groups indicated by brackets.

toxicity in the developing vertebrate organism and that inhibiting this pathway may protect the organism against deleterious effects of radiation.

Organ-Specific Radiation Protection by CDDO-TFEA and EP

Having established that EP and CDDO-TFEA provide whole-body protection against lethal doses of radiation and in consideration of the fact that these compounds are in preclinical development, we next determined organ-specific radiation protective effects of these two NF-κB inhibitors. First, we assessed, by acridine orange staining, organism-wide apoptosis in zebrafish embryos determined 6 hours after radiation. Consistent with earlier reports (33), we observed increased acridine orange staining in the central nervous system and along the body axis of irradiated embryos. Both NF-κB inhibitors markedly reduced radiation-induced acridine orange staining (Fig. 3). We previously reported that ionizing radiation compromised zebrafish kidney function as determined by delayed excretion of a fluorescent dextran injected intracardially (27). Treatment with EP but not CDDO-TFEA significantly reversed the effect of ionizing radiation on dextran clearance of irradiated embryos to near normal levels, suggesting protection against ionizing radiation–induced kidney damage (Fig. 4). It is currently unknown whether this effect reflects differences in ROS scavenging capacity between the two compounds as described above (Supplementary Fig. S5) or is due to differences in pharmacokinetics or pharmacodynamics. In addition, radiation of zebrafish embryos is associated with a high incidence of a body axis malformation called "curly-up" to describe aberrant dorsal curvature of the fish tail. Both CDDO-TFEA and EP reduced the incidence of curly-up significantly (Fig. 5).

Finally, we determined the effects of radiation on the developing gastrointestinal system. This was done in consideration of several prior reports suggesting that NF-κB

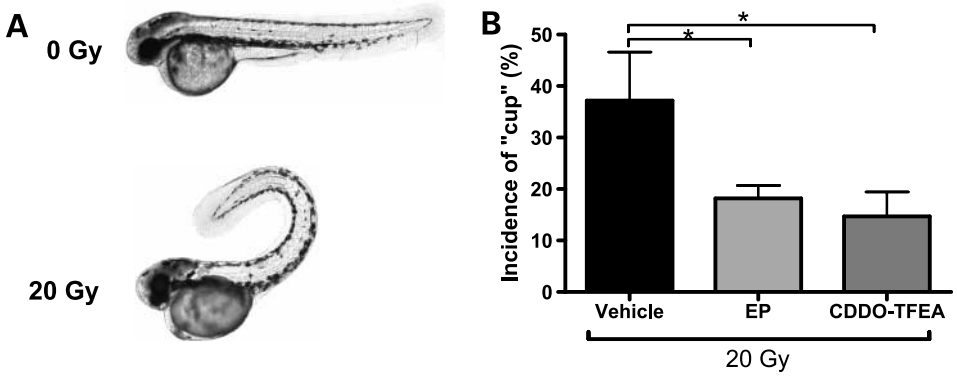

Figure 5. EP and CDDO-TFEA alleviate radiation-induced malformations of the body axis. **A,** representative micrographs showing dorsal curvature ("curly-up"; *cup*) in irradiated (20 Gy) embryos at 72 hpf relative to control nonirradiated fish embryos. **B,** quantification of curly-up incidence in CDDO-TFEA- and EP-treated fish relative to vehicle-treated controls. *, statistically significant differences between the experimental groups indicated by brackets.

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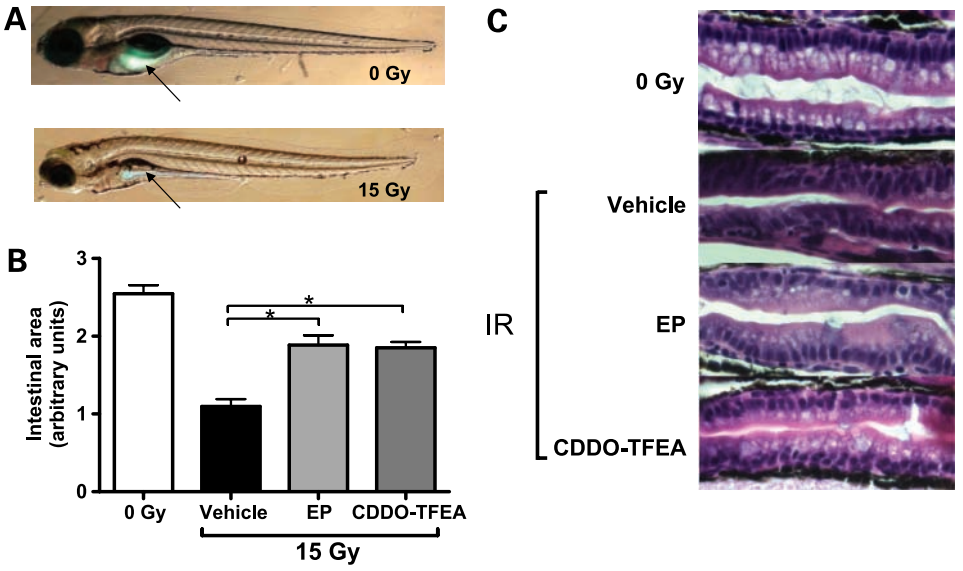

Figure 6. Effects of EP and CDDO-TFEA on radiation-induced alterations of the gastrointestinal system. **A,** improved PLA_2 activity and gastrointestinal lumen formation in irradiated zebrafish treated with either EP or CDDO-TFEA. Dequenched fluorescence reflects endogenous PLA_2 activity and transport of cleavage products through the lumen of the developing gastrointestinal system. **B,** quantitative evaluation of gastrointestinal lumen formation as determined by fluorescent dye content at 6 dpf. **C,** representative histologic sections of hindgut proximal to the cloaca at 6 dpf [5 d post (12 Gy)].

activation protects the gastrointestinal tract of higher vertebrates against acute radiation damage (42, 43). Radiation protection of the gastrointestinal system was determined in several ways. First, we assayed overall gastrointestinal function by scoring "long-term" survival of fish irradiated in the presence and absence of EP or CDDO-TFEA (up to 15 dpf). Fish larvae become dependent on external food sources at approximately 6 dpf when the contents of the yolk sac are depleted. Significant functional damage to the gastrointestinal system will thus lead to death by starvation within 10 days after conception (19). Conversely, survival of fish beyond two weeks indicates establishment of a functionally adequate gastrointestinal system. Both EP and CDDO-TFEA increased extended survival of zebrafish larvae (Supplementary Fig. S6) although this effect was statistically significant only in the case of CDDO-TFEA. To address the combined effects of radiation and EP or CDDO-TFEA treatment on the developing gastrointestinal system further, we determined gastrointestinal lumen formation by use of a fluorescent reporter (PED6; ref. 21) that is metabolized and excreted through the gastrointestinal system. This analysis revealed severely impaired lumen formation of the gastrointestinal system induced by ionizing radiation (15 Gy) and partial restoration of lumen formation and fluorescent dye excretion by treatment with either EP or CDDO. These functional results were complemented by examining the histologic appearance of the gastrointestinal system 5 days after radiation exposure in the presence and absence of the NF-κB inhibitors under investigation (Fig. 6). The hindgut mucosal epithelium immediately proximal to the cloaca revealed distinct cellular changes associated with sublethal ionizing radiation exposure (12 Gy), including irregular shape and disorganization of the columnar absorbing cells with redistribution of nuclei away from the basal orientation. In addition, decreased goblet cell numbers were observed. By contrast, EP and CDDO-TFEA pretreatment of irradiated embryos restored, in part, the columnar structure of absorbing cells and basal location of nuclei.

Discussion

Our results show that 6 of 6 pharmacologic inhibitors with different chemical structures and mode of actions inhibit the canonical pathway of NF-κB activation (consisting of IKKβ/IκB/NF-κBp65) and provide protection against radiation-induced overall lethality and damage to multiple organ systems of the developing zebrafish. By contrast, 3 of 3 proteasome inhibitors did not afford radiation protection, but radiosensitized zebrafish embryos to the lethal effects of ionizing radiation. Taking into account that each of the pharmacologic agents used in this study is likely to affect targets other than NF-κB, it is remarkable that radioprotection cosegregated with interference with activation of the canonical pathway to NF-κB. This observation suggests that NF-κB may be the relevant target for radiation protection by pharmacologic IKK/NF-κB inhibition.

Currently, there is no consensus about the functional contribution of NF-κB activation to the radiation response (44). Abundant reports of radiosensitization of tumor cells *in vitro* and *in vivo* by NF-κB inhibition are contrasted by relatively few such reports dealing with normal cells. The use of genetically engineered mouse models to monitor NF-κB dysfunction in normal tissues has been limited due to embryonal lethality observed in IKKβ (45) and NF-κBp65 (46) knockout animals. In cases where either conditional knockouts were made or transgenic mice were generated by forced expression of dominant negative regulators to modulate NF-κB activation, the interpretation of results is further complicated by compensatory adjustments of homeostasis (for review see ref. 47). The present study sidesteps the problems inherent to using genetic models by examining the effects of pharmacologic agents used

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at concentrations that reduce but do not abrogate NF-κB activity. The ease of our "assay system," i.e., observation of overall effects of ionizing radiation on zebrafish survival as well as effects on specific target organs, allowed us to monitor the effects of drug classes grouped according to target specificity and mechanisms of action. This approach had the advantage to minimize confounding effects due to unknown, off-target effects of any pharmacologic agent. By contrast, and as expected, ablating NF-κB activity by targeting IKKβ or NF-κBp65 expression using antisense approaches produced a dramatically different outcome as these interventions were associated with embryonic lethality even in the absence of genotoxic stress (see Supplementary Fig. S4 and ref. 46). This result is consistent with the view that inhibition of excess NF-κB activity after lethal irradiation is beneficial whereas blocking NF-κB expression and/or activation altogether, as in genetic knockout/knockdown models, is deleterious (even in the absence of radiation). This contention is further supported by our finding that EP and CDDO-TFEA at the nontoxic concentration used here disrupted TNF-α–induced NF-κB activation but not basal NF-κB activity in HeLa cells *in vitro* (Supplementary Fig. S2). Importantly, CDDO-TFEA and EP not only protected against but also mitigated the lethal effects of radiation. This result is of interest as it points to the importance of sustained NF-κB activation consistent with inflammatory responses rather than the burst of NF-κB activity observed immediately after radiation exposure. It remains to be seen whether other anti-inflammatory agents may be used to mitigate radiation damage to normal tissues in the developing embryo.

Interestingly, radiation protection of zebrafish embryos by NF-κB inhibitors extended to the gastrointestinal system whereas previous work using genetically modified mice (42) and the TLR5 ligand flagellin (43) has implicated NF-κB activation in radiation protection of gastrointestinal stem cells. The reason(s) for this difference are unclear at this point. However, the TLR5 ligand flagellin exerts pleiotropic stimulatory effects on multiple signaling pathways that include NF-κB but also p38, Erk/mitogen-activated protein kinase, and potentially, signal transducers and activators of transcription (48). It has not been reported which of these multiple effects alone or in combination is at cause for radioprotection provided by flagellin (43). In addition, the NF-κB inhibitory effects of both EP and CDDO-TFEA are completely reversible, whereas genetic ablation is not and this circumstance could affect outcomes of NF-κB activation in reference to gastrointestinal function. Our findings are further consistent with the view that excessive NF-κB activation, as observed in the context of chronic inflammation, is potentially deleterious to the gastrointestinal system (49) and, thus, down-modulating NF-κB activity but not ablating it altogether can be advantageous in certain settings (50). Although the details of these diverse outcomes in different model systems remain to be sorted out, our results clearly show that reducing NF-κB activity with a variety of compounds with different mechanisms of action diminishes radiation-induced damage to several organ systems in the developing zebrafish embryo.

In conclusion, the most salient finding of this study is that direct inhibitors of NF-κB activity provided effective protection and mitigation against overall lethality and specific organ damage caused by ionizing radiation in zebrafish embryos. Direct NF-κB inhibitors also exert antineoplastic effects in select model systems as shown extensively for CDDO-TFEA and derivatives thereof (51–57). These findings are consistent with a favorable therapeutic window for NF-κB inhibitors when used in combination with radiation and, potentially, chemotherapeutic drugs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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