EVALUATION OF AGENTS THAT CAN MODIFY THE RADIATION RESPONSE

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ABBREVIATIONS

AO: acridine orange, **bZIP:** basic leucin zipper, **CNS**: central nervous system, **cup:** curly-up phenotype characterized by dorsal curvature of the body axis, **DMSO:** dimethyl sulphoxide, **dpf:** days post-fertilization, **EP:** ethyl pyruvate, **hpf:** hours post-fertilization, **IKKβ:** IκB kinase β, **IR:** ionizing radiation, **LD50:** lethal dose 50, **LET:** linear energy transfer, **MO:** morpholino, **MnSOD:** manganese superoxide dismutase, **NF-κB:** nuclear factor kappa B, **Nrf2:** nuclear factor erythroid 2-related factor 2, **OER:** oxygen enhancement ratio, **ROS:** reactive oxygen species, **RT-PCR:** reverse transcription polymerase chain reaction, **TMZ:** temozolomide, **TNF-α:** tumor necrosis factor-alpha

1. INTRODUCTION

Zebrafish

Zebrafish embryos (*Danio rerio*) are emerging as a powerful vertebrate model system for research. Zebrafish embryos offer several important advantages when compared to cell cultures or other conventional *in vivo* experimental animal models: high fecundity, high number of zebrafish progeny, easy upkeep at minimal cost, large sample sizes, and availability of many conventional cell culture techniques. The transparency of the embryo permits a visual observation of the ex utero development. There is high similarity between zebrafish and mammals, and the entire zebrafish genome is sequenced. The organs including the gut and the vasculature are in place by 48 hours post fertilization (hpf). Zebrafish embryos are permeable to small molecules, drugs, dyes as well as peptides, and can be transfected to express genes in a tissue-specific manner. Knockdown of individual mRNAs is easily achieved by morpholino antisense oligodeoxinucleotides.

In addition, we have established zebrafish embryos as an *in vivo* vertebrate system to evaluate toxic effects of ionizing radiation (IR) and modifiers of IR.

Radiation-induced damage

The mechanisms leading to the deleterious effects of ionizing radiation are multifactorial.

IR can cause damage through energy deposition. Radiation can damage cells directly and indirectly. Many molecules in cells will be altered due to direct energy deposition or because of indirect effects as a consequence of energy transfer from one molecule to another. With low LET (linear energy transfer) radiation, as photons (gamma-or x-ray), mostly indirect effects occur, leading to single strand breaks. Charged particles, such as protons and heavy ions, can cause direct damage to cancer cell DNA through high LET and have an antitumor effect independent of tumor oxygen supply because these particles act mostly via direct energy transfer, usually causing double-strand DNA breaks.

The interactions between low LET radiation and cells result in energy deposition in the tissues, generating free radicals. Free radicals derive from water in cells, which then damage the macromolecules. The medical use of ionizing radiation can cause a variety of side effects during treatment (acute side effects) due to inflammatory cytokine release, in the months or years following treatment (long-term side effects) as a consequence of cell death or after re-treatment (cumulative side effects).

Tissue damage associated with inflammatory changes often limits the dose of ionizing radiation that can be safely delivered during radiotherapy. The gastrointestinal tract is particularly sensitive to radiation. A well-known example of this is inflammation of the oral mucosa or the intestinal lining following chemo - and/or radiation therapy. A hallmark of IR-associated inflammation is the increased liberation of pro-inflammatory cytokines including TNF-α and IL-6, both locally and systematically. 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.

Radiation sensitivity of normal tissues

Cells are intrinsically sensitive to irradiation. The nature, severity, and longevity of radiation effects depend on the cell type, proliferation, intracellular and microenvironmental factors as well as on the type of radiation, dose, and fractionation. Radiation sensitivity to low LET radiation also depends on the oxygen level of the tissue. The more oxygenated the more sensitive the tissue is. Radiation sensitivity reaches its maximum at an oxygen tension of 30 mmHg and does not significantly change by increasing the oxygen tension. Since normal tissues have an oxygen tension ranging from 40 to 120 mmHg; therefore, the oxygen tension does not interfere with the radiation sensitivity in healthy normal tissues.

Radiation modifiers

There is a great interest in developing radioprotectors to prevent normal tissue toxicity during chemo- and/or radiotherapy or nuclear accidents. On the other hand, the most successful way to treat certain cancers is through multimodality therapy. In one of our studies, we put emphasis on the postoperative management of patients with primary glioblastoma multiforme. Groups of patients treated with combinations of different therapeutic modalities were compared to each other. Temozolomide (TMZ) was used as a chemotherapeutic agent. The alkylating agent TMZ, when administered simultaneously with radiation therapy, can improve the effectiveness of treatment by making tumor cells more sensitive to radiation. At the same time, sparing healthy tissues of the central nervous system (CNS) from radiation injury and increasing radiation tolerance in normal tissues of the CNS play an essential role in improving the therapeutic index. To develop approaches to prophylaxis/protection, mitigation and treatment of radiation injuries, appropriate models are needed, which integrate the complex events that can occur in the organism exposed to radiation. While the spectrum of agents in clinical use or preclinical development is limited, new research findings promise improvements in survival after whole-body irradiation and reductions in the risk of adverse effects of radiotherapy. The approaches include protective agents that act on the initial radiochemical events, mitigating agents that prevent or reduce progression of the radiation damage, and agents that facilitate recovery from radiation injuries.

Ideally, radioprotectors can prevent normal tissues from the deleterious effects of ionizing radiation; whereas radiation sensitizers can make tumor cells more sensitive to radiation, enhancing the efficacy of radiotherapy. Generally, radioprotectors work on low linear energy transfer and only have a protective effect as great as the oxygen enhancement ratio (OER). Mostly, radioprotectors afford protection against ionizing radiation when given prophylactically.

Aims

The aim of our study was to assess agents that can modify the radiation response. We evaluated whether the agents under investigation could prevent normal tissues from the deleterious effects of total body irradiation and whether any of these compounds could mitigate the deleterious effects of irradiation.

2. MATERIALS and METHODS

Embryo harvesting and maintenance

Wild-type zebrafish were mated in embryo collection tanks. Viable embryos were washed and plated at the one- to two-cell developmental stage and kept under normoxic conditions at 28.5 ˚C. Embryo medium was changed at every 24 hours.

DF-1

DF-1 is a hydrophilic antioxidant based on the hollow nanostructure of fullerenes. Fullerenes represent a family of molecules composed of 20, 40, 60, 70, or 84 carbon atoms. Fullerenes have the potential to scavenge reactive oxygen species (ROS), including hydrogen peroxide, hydroxyl radicals, hydroperoxy radicals, and superoxide, but their basic structures are not water-soluble without modification. C60 fullerene is the most frequently used member of this family. DF-1 is a C60 fullerene derivative (dendrofullerene) that contains 18 carboxylic groups to enhance water solubility. DF-1 has been known to exert powerful antioxidant effects *in vitro*.

DF-1 was dissolved in embryo medium containing no more than 0.4% dimethyl sulphoxide (DMSO). Embryo medium containing 0.4% DMSO was used as a vehicle control in the DF-1 experiments since DMSO is known to possess radioprotective effects. Amifostine was used as a positive control in select experiments and was used at 4 mmol/L, which is the efficient concentration previously described for radioprotection. Embryos were exposed to a single dose of ionizing radiation ranging dose from 0 to 40 Gy at 24 hpf using either a ^{137}Cs radiation source or an X-ray machine. DF-1 toxicity analysis was conducted using a dilution series of 0, 10, 100, and 1000 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 kept at 28.5 C for up to 7 days post fertilization to monitor effects of treatments on survival, morphology, and organ-specific toxicity.

Direct inhibitors of NF-κB and proteasome inhibitors

The nuclear factor κB (NF-κB) family of transcription factors represents a diverse and shared signaling mechanism activated during cell stress responses. Deregulated NF-κB signaling has been implicated in

the malignant phenotype and treatment resistance of select tumor forms. 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.

Here, we tested ethyl pyruvate (EP) and CDDO-TFEA. Both CDDO-TFEA and EP inhibits activation of NF-κB. EP inhibits NF-κB signaling through direct molecular interaction with a reactive cysteine of the p65 subunit of NF-κB whereas CDDO-TFEA binds to a reactive cysteine (Cys 179) of IKKα, thus inhibiting its kinase activity. Additionally, four pharmacologic IKK inhibitors with different modes of action were also tested.

In addition to these agents, proteasome inhibitors (PS-341, MG132 and Lactacystin) were also evaluated.

Pharmacologic agents (EP; CDDO-TFEA; IKK inhibitors: IKK Inhibitor 2 (Weldelolactone), IKK Inhibitor 3 (BMS-345541), IKK-2 Inhibitor 4 and IKK-2 Inhibitor 5 (IMD-0354); MG132; PS-341, and Lactacystin) were dissolved in EM containing <0.1% DMSO. EM was used as a vehicle control in all experiments. Unless stated otherwise embryos were exposed to ionizing radiation ranging in dose from 0-20 Gy at 24 hpf using an X-ray machine or a ^{137}Cs radiation source.

Toxicity analyses for EP (≤10 mM), CDDO-TFEA (≤10 μM), PS-341 (≤10 μM), MG132 (≤50 μM) or Lactacystin (≤10 μM) were conducted by monitoring survival and development of zebrafish embryos for 7 days in the absence of radiation. To determine modulation of radiation-induced toxicity, EP (1 mM) or CDDO-TFEA (1 μM) was added to embryos either 1h before or up to 3 h after radiation exposure at 24 hpf. The proteasome inhibitors were added to zebrafish embryos 1 h prior to IR. After irradiation, zebrafish embryos were maintained at 28.5 ˚C for up to 7 days post fertilization to monitor effects of treatments on survival, morphology and organ-specific toxicity.

Manganese superoxide dismutase mimetics

We used zebrafish embryos to explore the putative radioprotective effects of two manganese superoxide dismutase (MnSOD) mimetics; M40403 and MnTE-2-PyP5+. M40403 is a stable, non-peptidyl, synthetic manganese superoxide dismutase mimetic (MnSOD) with a low molecular weight, which eliminates the superoxide anions ($^{\cdot}O_2^-$) without interfering with other radicals known to be involved in inflammatory responses. MnTe-2-PyP5+ is also a synthetic SOD mimetic.

Radioprotection was assessed by determining drug effects on IR-associated mortality. To evaluate *in vivo* toxicity of these agents, zebrafish embryos at 24 hours post fertilization (hpf) were exposed to log-fold dilution series until 144 hpf. Based on the toxicity profiles, zebrafish embryos (24 hpf) were exposed to IR (20 Gy) with either MnTE-2-PyP5+ (2.5 μM) or M40403 (100 μM) or Amifostine (4 mM)

administered 3 h prior to ionizing radiation. Embryos were evaluated at 24-h intervals until 168 hpf for viability and radiation-induced gross morphological alterations.

Analysis of treatment effects on zebrafish morphology and survival

Dechorionated embryos at 72 hpf were anesthetized with 1:100 dilution of 4 mg/mL tricaine methanesulfonate and immobilized by placing them on 3% methylcellulose on a glass depression slide. Morphology was assessed visually using a light transmission microscope at 40 to 100X magnification, and representative images were recorded. Similarly, survival of embryos was assessed visually at 24-hour intervals up to 168 hpf by light microscopy. The criterion for viability was the presence of cardiac contractility.

Renal function assay

Clearance of tetramethylrhodamine-labeled, 10-kDa dextran from the cardiac area was determined. Briefly, zebrafish embryos at 24 hpf were exposed to ionizing radiation and maintained in embryo medium. At 72 hpf, zebrafish embryos were anesthetized using a 1:100 dilution of 4 mg/mL tricaine methanesulfonate 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 ºC and imaged 1 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. Images were transformed into grayscale and evaluated with NIH ImageJ software.

Histopathology and evaluation of embryos treated with DF-1

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, embryos were initially fixed and preserved in Davidson's solution 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, and infiltrated with paraffin Sections were embedded in paraffin, and transverse whole-body sections (4-6 μm thickness; 100-120 sections per fish) were made serially from the rostral 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, mounted on glass slides, and coverslipped. Sections were examined by light microscope at 4 to 40X magnification, and representative images were recorded using a SPOT camera and SPOT Advanced software.

Detection of ROS

Production of ROS was measured in dechorionated whole 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 the agent to be tested (namely DF-1 at 100 µmol/L or EP at 1 mmol/L or CDDO-TFEA at 1 µmol/L) in the presence of 5-(and-6)-chloromethyl 2,7-dihydrodichlorofluorescein diacetate (CM-H2DCFA) followed by radiation exposure at 24 hpf. Zebrafish embryos were incubated in EM containing one of the above-mentioned agents before ionizing radiation at 24 hours; the fluorescent dye CM-H2DCFA (500 ng/mL) was added 1 hour before radiation exposure. The average fluorescence emission at 530 nm following excitation at 490 nm was detected immediately and 2 hours after ionizing radiation exposure using a microplate fluorescent reader. 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.

Apoptosis assay

Quantitative apoptosis measurements were performed by staining with the vital dye acridine orange (AO) that permeates dying cells to bind to chromatin. Zebrafish embryos were incubated for 1 h in EM containing one of the radiation modifiers and irradiated with 20 Gy at 24 hpf. Six hours later, zebrafish embryos were manually dechorionated and stained for 15 minutes using 5 µg/mL of Acridine Orange dye and rinsed five times with EM. Zebrafish embryos were then imaged with QIMAGING camera and iVision software, and the images were then analyzed using ImageJ software.

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. To evaluate intestinal lipid processing, PED6 was added to zebrafish at day 5 and images were taken at day 6 with the average fluorescence emission at 540 nm following excitation at 505 nm. PED6 is a fluorogenic substrate for PLA_2, which contains a BODIPY FL dye–labeled acyl chain and a dinitrophenyl quencher group. 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 in zebrafish embryos treated with either EP or CDDO-TFEA

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 10x 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 Ren et al. with minor modifications. HeLa cells were seeded at 7.5 x 10^4/ml in DMEM medium supplemented with 10% fetal bovine serum. The cells were co-transfected with the pSEAP2- NF-κB vector 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 post transfection, different NF-κB inhibitors (Velcade, 0.5 μ M; MG-132, 5 μM; EP, 1mM; CDDO-TFEA, 1 μM) were added to the cells in serum-free media for 24 hours. NF-κB-dependent transcription in the absence and presence of recombinant TNF-α (10 ng/ml; R&D Systems) was determined 72 hours post transfection 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.

Reverse transcription polymerase chain reaction (RT-PCR) analysis

Zebrafish total RNA was isolated form 100 embryos per experimental condition at 30 hpf (6 h post radiation) using the RNeasy mini kit (QIAGEN Sciences, Maryland, USA) and stored at -80 °C. For reverse transcription, total RNA was annealed with Oligo(dT) primer at 70°C for 5 min and then incubated 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 were used for amplification of *bax*, *mdm2*, *p21/waf-1* and *beta-actin* zebrafish sequences. PCR reaction conditions were 94 ˚C, 60 ˚C, 72 ˚C for 30 sec., 30 sec., 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 RT reaction. PCR reactions were analyzed by 1.5% agarose gel electrophoresis.

Use of morpholinos

Morpholino antisense oligonucleotides (MO) have commonly been used to achieve sequence-specific gene knockdown. Zebrafish embryos were injected at 1-2 cell stage with an approximately 3-5 ng MO per embryo to study signaling in developing zebrafish embryos. Control embryos were mock-injected with control MO.

Statistical analysis

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

3. RESULTS

Effects of the fullerene DF-1

We demonstrated radioprotection by the fullerene DF-1 in our *in vivo* zebrafish model.

DF-1 administered prior to irradiation afforded significant survival advantage to zebrafish embryos exposed to either 20 Gy or 40 Gy. DF-1 was found to significantly enhance survival when given concurrently or up to 15 minutes after irradiation. DF-1 alleviated radiation-induced defects in the midline development of zebrafish embryos. It attenuated radiation-induced renal function defects.

Effects of the MnSOD mimetics

Administration of M40403 (100 μM) or MnTE-2-PyP5+ (2.5 μM) markedly increased overall survival

Effects of the proteasome inhibitor PS-341 (Bortezomib/VELCADE)

PS-341 (1 μmol/L) markedly sensitized zebrafish embryos to the lethal effects of ionizing radiation when administered 1 hour prior to radiation.

Effects of the NF-κBp65 inhibitor ethyl pyruvate

We observed that EP not only protected against but also mitigated lethality associated with whole-body irradiation of zebrafish embryos. EP given up to 2 hours after radiation significantly reduced ionizing radiation-associated lethality. EP was also found to be an effective ROS scavenger in irradiated zebrafish embryos.

Effects of the IKK inhibitor CDDO-TFEA

CDDO-TFEA protected against and mitigated overall lethal effects of radiation in zebrafish embryos in a manner similar to ethyl pyruvate. However, CDDO-TFEA, at least at the concentration (1 μM) applied in our experimental model, did not demonstrate ROS scavenging properties.

Organ-specific radiation protection by CDDO-TFEA and EP

Both of them markedly reduced radiation-induced apoptosis. Treatment with EP but not CDDO-TFEA significantly reversed the effect of ionizing radiation on dextran clearance of irradiated zebrafish embryos to near normal levels, suggesting protection against ionizing radiation-induced kidney damage. Both reduced the incidence of curly-up phenotype significantly. We assayed overall gastrointestinal function by evaluating long-term survival (up to 15 days) of zebrafish embryos irradiated in the presence and absence of either CDDO-TFEA or EP. Both compounds increased extended survival of zebrafish larvae. We also determined gastrointestinal lumen formation by use of the fluorescent reporter PED6. Pretreatment with EP or CDDO-TFEA restored, in part, the gastrointestinal lining of zebrafish embryos exposed to sublethal ionizing radiation exposure (12 Gy).

4. DISCUSSION

DF-1

The experiments with DF-1 illustrated that, as expected for an agent with antioxidant properties, DF-1 protected against and, at least to a limited extent, mitigated radiation-induced lethality 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. This toxicity has been attributed, in part, to lipid peroxidation by C60. In contrast to unmodified C60, DF-1 has been structurally altered to increase water solubility and, thereby potentially reduce C60-associated toxicity.

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.

Manganese superoxide dismutase mimetics

The degree of radioprotection provided by the SOD mimetics M40403 and MnTE-2-PyP5+ was comparable to that provided by Amifostine, but they proved to be effective at much lower concentrations, especially MnTE-2-PyP5+. Our finding is consistent with another report demonstrating the survival benefit of M40403 in mice exposed to lethal whole-body irradiation. MnTE-2-PyP5+ was also reported to selectively protect normal cells but not tumor cells against radiation-induced damage.

Direct inhibitors of NF-κB and proteasome inhibitors

Our results show that 6 of 6 pharmacologic inhibitors with different chemical structures and mode of actions inhibited the canonical pathway of NF-κB activation (consisting of IKKβ/IκB/NF-κBp65) and provided 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 made zebrafish embryos more sensitive to the lethal effects of ionizing radiation. Taking into account that each of the pharmacologic agents tested in this study is likely to affect targets other than NF-κB, it is remarkable that radioprotection co-segregated 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. Numerous 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 embryonic lethality observed in IKKβ and NF-κBp65 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. This study sidesteps the problems inherent to using genetic models by examining the effects of pharmacologic agents applied at concentrations that reduce but do not abrogate NF-κB activity. 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. As expected, ablating NF-κB activity by targeting IKKβ or NF κBp65 expression using antisense approaches led to a dramatically different outcome as these interventions were associated with embryonic lethality even in the absence of genotoxic stress. This result is consistent with the view that inhibition of excess NF-κB activity following 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 irradiation. This contention is also supported by our finding that EP and CDDO-TFEA at the nontoxic concentration used here disrupted TNF-α–induced NF-κB activation but not the basal NF-κB activity in HeLa cells *in vitro*. 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.

Interestingly, radiation protection of zebrafish embryos by NF-κB inhibitors extended to the gastrointestinal system whereas previous work using genetically modified mice and the TLR5 ligand flagellin 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. It has not yet been reported which of these effects alone or in combination can result in radioprotection provided by flagellin.

In addition, the NF-κB inhibitory effects of both EP and CDDO-TFEA are completely reversible, whereas genetic ablation is not, and this condition could affect the 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 and, thus, down-modulating NF-κB activity but not abolishing it altogether can be beneficial in certain settings. Although the details of these diverse outcomes in different model systems remain to be sorted out, these 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 embryos.

In conclusion, the most salient finding of this study with NF-κB inhibitors 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. These findings are consistent with a favorable therapeutic window for NF-κB inhibitors when used in combination with radiation and, potentially, chemotherapeutic drugs.

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