A novel multifunctional radioprotective strategy using P7C3 as a countermeasure against ionizing radiation-induced bone loss

Fei Wei[1], Zewen Kelvin Tuong[2,3], Mahmoud Omer[1], Christopher Ngo[1], Jackson Asiatico[4], Michael Kinzel[4], Abinaya Sindu Pugazhendhi[1], Annette R Khaled[5], Ranajay Ghosh[4], Melanie Coathup[1]

[1]Biionix Cluster, and Department of Internal Medicine, College of Medicine, University of Central Florida, Orlando, FL, 32827
[2]Molecular Immunity Unit, Department of Medicine, University of Cambridge, Cambridge, UK, CB2 2QQ
[3]Cellular Genetics, Welcome Sanger Institute, Hinxton, UK, CB10 1SA
[4]Department of Mechanical and Aerospace Engineering, University of Central Florida, Orlando, FL, USA, 32816
[5]Burnett School of Biomedical Sciences, College of Medicine, University of Central Florida, Orlando, FL, 32827

Purpose: Cancer remains a leading cause of death in every country in the world. A combination of surgical resection, radiotherapy, and chemotherapy are commonly used in the treatment of various local and metastasizing cancer cells. One half to nearly two thirds of cancer patients will be exposed to controlled, radiotherapeutic levels of ionizing radiation (IR) at some point during their care. Therefore, radiotherapy is a critical component of cancer care but does cause osteoporosis and pathological insufficiency fractures to surrounding, and otherwise healthy bone. This occurs *via* a complex series of events, which culminate in increased bone resorption and loss. Presently, no effective countermeasure exists. As such, the burden of IR-induced damage to healthy bone is a persistent and substantial source of functional impairment, pain, disability, and morbidity. Therefore, the purpose of this study was to investigate a small molecule aminopropyl carbazole named P7C3 as a novel radioprotective strategy.

Methods: This study used human bone marrow-derived mesenchymal stromal cells (hBMSCs), murine-derived macrophages (RAW 264.7 cells), human osteosarcoma (hOS), metastatic prostate cancer (PC3), and triple negative breast cancer cells (MDA-MB-231/GFP). hBMSCs and RAW 264.7 cells were used for the *in vitro* proliferation and differentiation studies in order to investigate the potential radioprotective effect of P7C3. For *in vivo* study, rat hind limbs were subjected to a local fractionated X-Ray dose of 8 Gy on days 1, 3 and 5 (a total dose of 24 Gy). P7C3 (20 mg/kg) was administered prior to IR and then daily via intraperitoneal (*i.p.*) injection for 7 days. Whole-mount histological staining and Nano-CT scanning were performed for bony structure analysis. Tartrate resistant alkaline phosphatase (TRAP) staining was performed for osteoclastic activity. Bone strength was evaluated via 3-point bending. Bone marrow adiposity was measured via Sudan black B staining and oil red O staining. Blood samples were collected for cytokine array analysis.

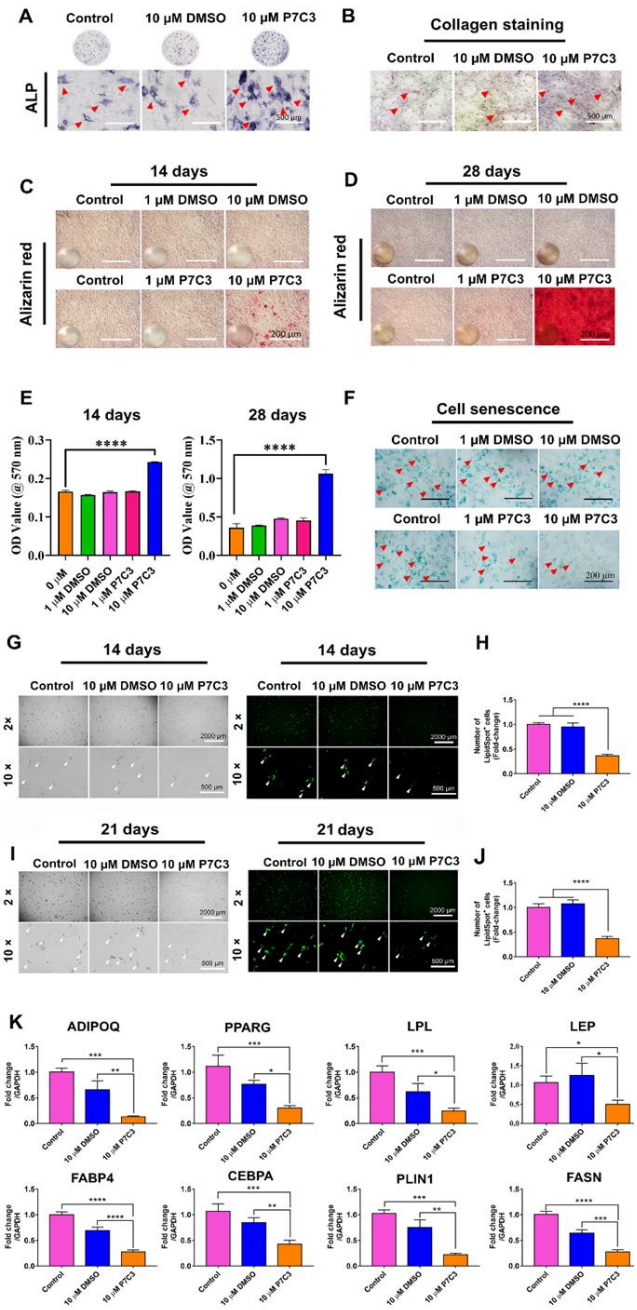

Figure 1. P7C3 promotes hBMSC osteogenesis and inhibits adipogenesis following IR-induced functional damage *in vitro*. [A] ALP-stained micrographs depicting IR-damaged hBMSCs cultured in osteogenic differentiation medium supplemented with DMSO or P7C3. [B] Representative micrographs displaying collagen formation levels. [C-D] Representative micrographs of Alizarin red S staining for mineralization. Mineral deposition appeared bright red in color. [E] Quantification of Alizarin red S staining: ****$p < 0.0001$. [F] Representative micrographs showing senescent cells in IR-damaged hBMSCs with or without P7C3 treatment. [G] Representative phase contrast (left panel) and LipidSpot™ Lipid Droplet staining images (right panel) of hBMSCs cultured in adipogenic induction medium supplemented with 10 μM DMSO (solvent control) or 10 μM P7C3 at 14 days. [H] Quantification of LipidSpot™-positive cells at 14 days. ****$p < 0.0001$. [I] Representative phase contrast (left panel) and LipidSpot™ Lipid Droplet staining images (right panel) at 21 days. [J] Quantification of LipidSpot™-positive cells at 21 days. ****$p < 0.0001$. [K] qRT-PCR showing gene expression of adipogenic-related marker genes at 12-day post-irradiation. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$.

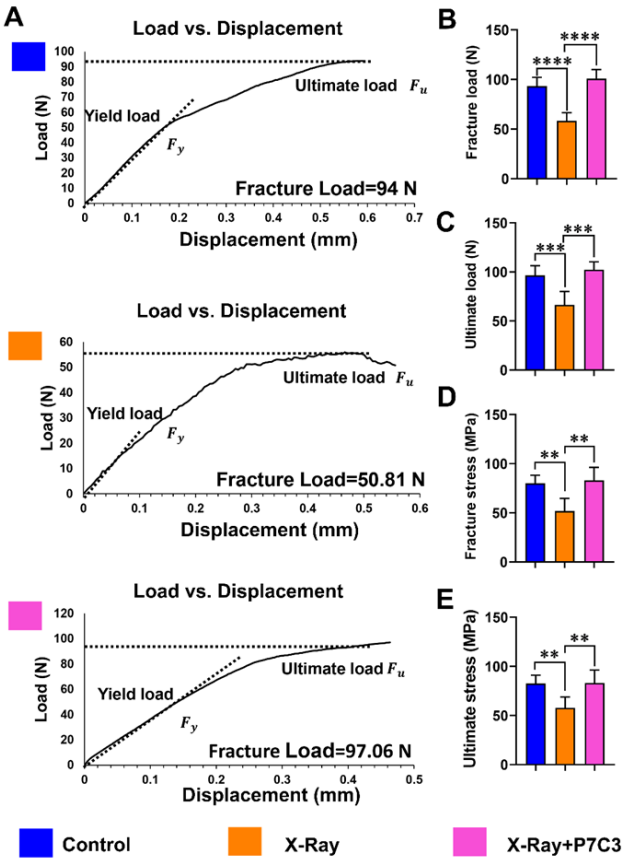

Figure 2. P7C3 maintains the strength of bone despite exposure to harmful levels of irradiation (n=6). [A] Representative load-displacement curves of non-irradiated healthy control, IR exposed, and IR+P7C3 groups. [B-E] The mechanical properties of fracture load [B], ultimate load [C], fracture stress [D], and ultimate stress [E] at the tibial mid-point, varied between the different groups. Animals receiving 20 mg/kg *i.p.* treatment showed higher fracture load/stress and ultimate load/stress, suggesting P7C3 protects bone strength despite exposure to harmful irradiation. **p < 0.01, ***p < 0.001, ****p < 0.0001.

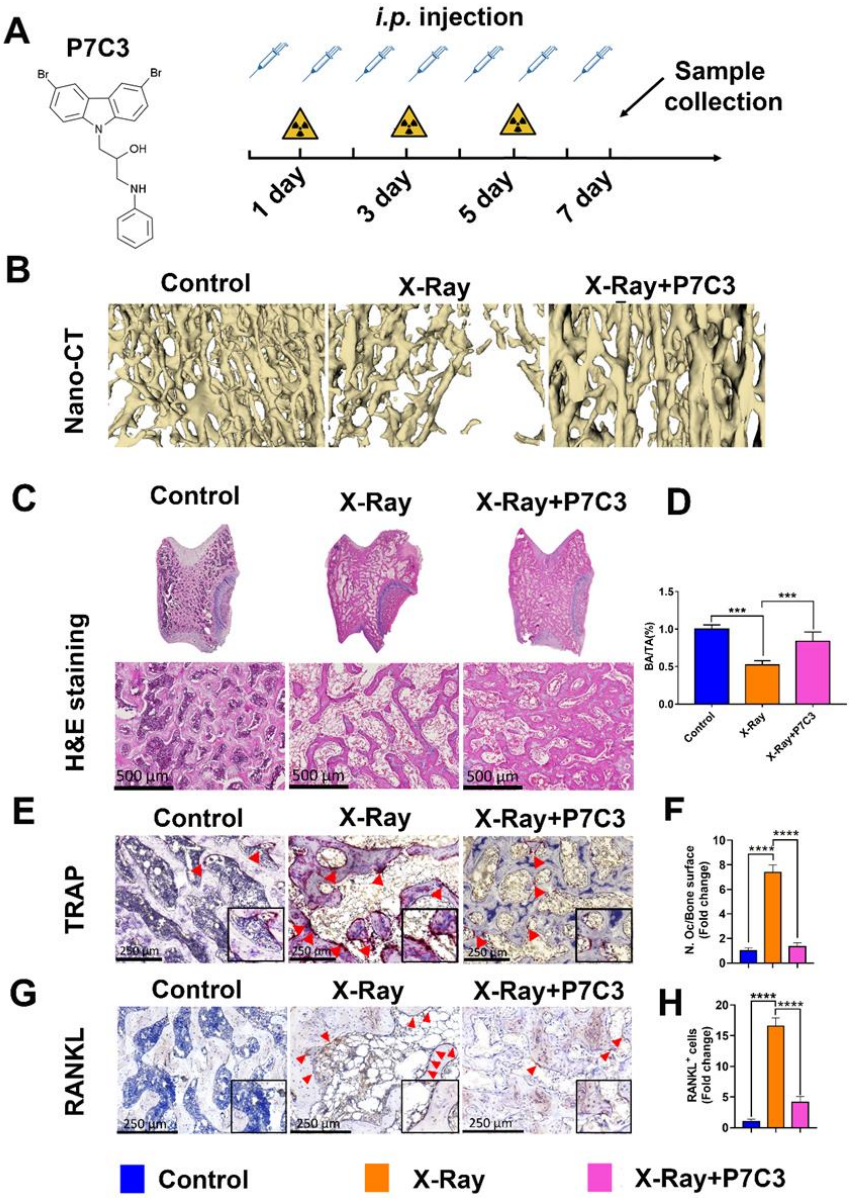

Figure 3. Analysis of the radioprotective effect of *i.p.* given P7C3 (20 mg/kg) following exposure to irradiation *in vivo*. [A] A flow chart of the animal experiment. [B] 3D models of the proximal tibia were generated using 3D Slicer™ (v4.11.20210226; Brigham and Women's Hospital and Massachusetts Institute of Technology). The IR-treated control group showing osteoporosis. Bone architecture and area was maintained in P7C3+IR treated rats. [C] Representative images of H&E-stained transverse sections through the femoral condyle. Rats exposed to harmful levels of irradiation resulted in bone loss and osteoporosis as indicated by reduced trabecular connectivity, thinner and shorter trabeculae, and increased bone marrow adiposity. P7C3 treatment (20 mg/kg) markedly protected against bone loss. [D] BA/TA% quantification. ***$p < 0.001$. [E] Multinucleated osteoclasts were identified by TRAP staining. Red arrows indicate TRAP$^+$ cells (purple-red color). [F] The number of osteoclasts *per* unit of bone surface (cells/mm^2) was quantified *via* bone histomorphometric analyses (5 images/rat, n=6). ****$p < 0.0001$. [G] Representative images of RANKL immunohistochemical staining. Red arrows indicate RANKL$^+$ cells. [H] Quantification for RANKL$^+$ cells. RANKL strongly expressed in irradiation exposed rats, while 20 mg/kg P7C3 treatment reduced RANKL expression. ****$p < 0.0001$.

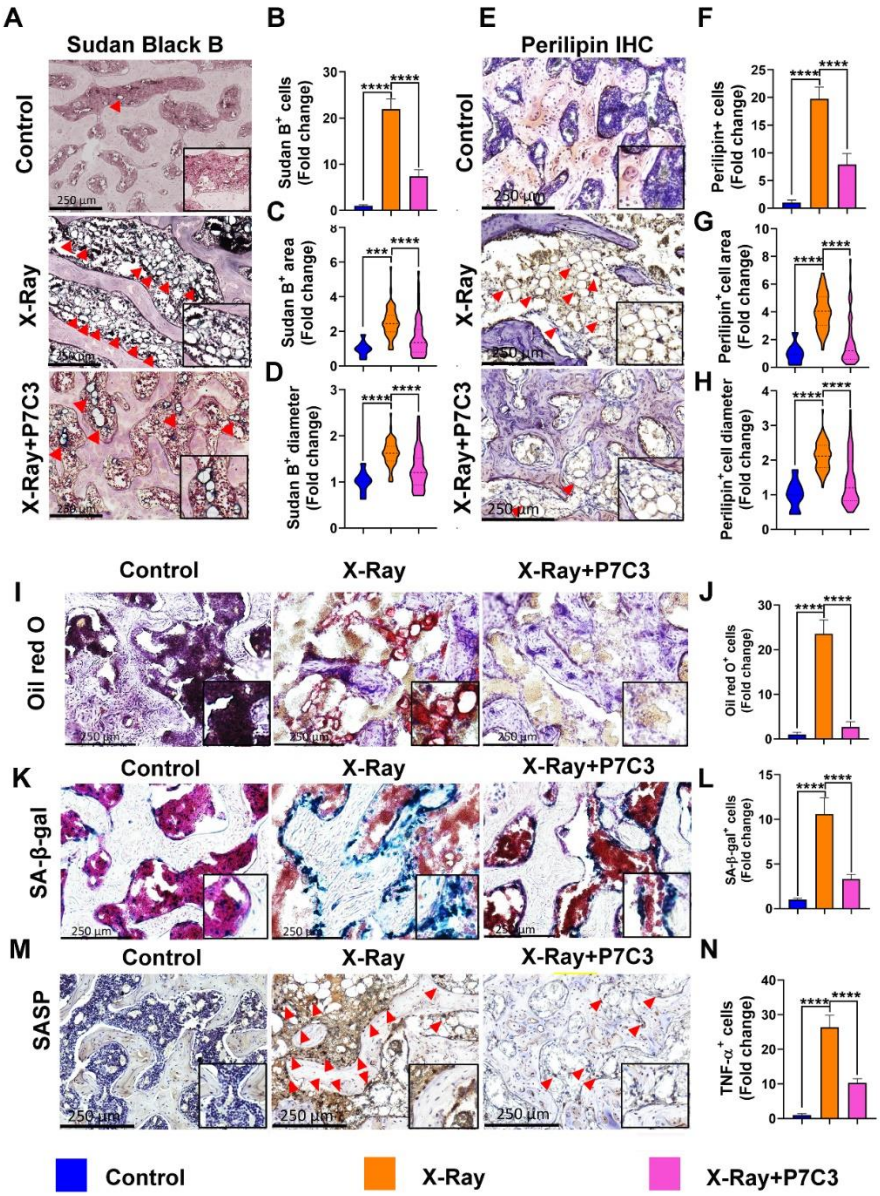

Figure 4. P7C3 treatment (20 mg/kg) reduces bone marrow adiposity and cell senescence following exposure to harmful irradiation (*n*=6). [A] Representative photomicrographs of Sudan Black B staining for adipocytes and lipids (stained black). The nuclei were counterstained with nuclear fast red (pink). [B] Quantification of Sudan Black B$^+$ cell number in each of the groups. [C] Quantification of Sudan Black B$^+$ cellular area, and [D] cell diameter. [E] Representative photomicrographs of perilipin immunohistochemical staining. Red arrows indicate perilipin$^+$ cells. [F] Quantification of perilipin$^+$ cells. [G] Perilipin$^+$ cellular area. [H] Perilipin$^+$ cell diameter. [I] Representative micrographs of Oil Red O staining of the rat bone sections. P7C3 reduced IR-induced bone marrow adiposity. [J] Quantification of Oil Red O staining *via* absorbance measurement at 560 nm. [K] Representative photomicrographs of SA-β-Gal staining for senescent cells (stained blue). [L] Quantitative analysis of SA-β-Gal$^+$ cells intensity. [M] Representative images of senescence-associated secretory phenotype (SASP) TNF-α$^+$ cell staining. Red arrows indicate TNF-α$^+$ cells. [N] Quantification of TNF-α$^+$ cells in each of the groups. ***$p < 0.001$, ****$p < 0.0001$.

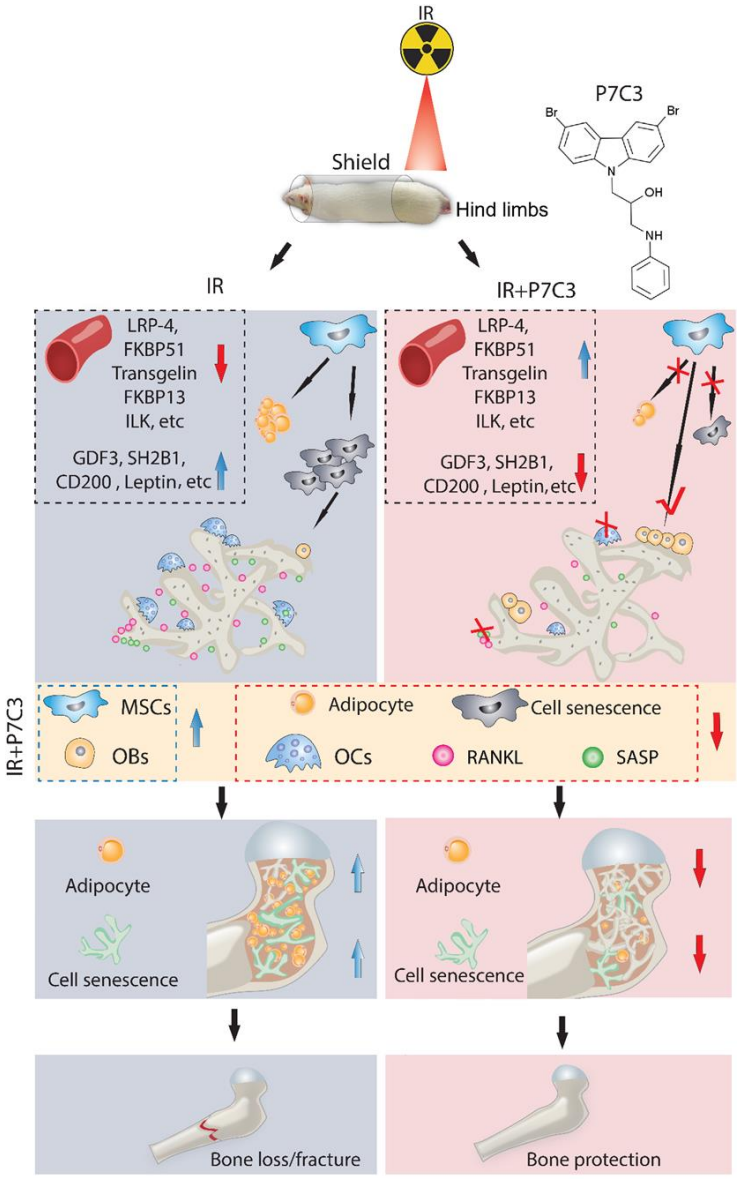

Figure 5. Schematic diagram highlighting the protective effect provided by P7C3 against IR-induced bone loss. Exogenous administration of 20 mg/kg P7C3 shifts the pathological environment induced by irradiation from favoring osteoclastogenesis into osteogenesis and reduces bone marrow adipogenesis and cell senescence, thereby significantly protecting bone from IR-mediated bone loss and fracture *in vivo*.

Results: Our studies revealed that P7C3 repressed IR-induced osteoclast-like macrophage activity, inhibited adipogenesis (Figure 1), and promoted osteoblastogenesis and mineral deposition *in vitro* (Figure 1). We also demonstrated that rodents exposed to clinically equivalent hypofractionated levels of IR *in vivo*, develop weakened, osteoporotic bone (Figure 2). However, the administration of P7C3 maintained its mechanical strength (Figure 2), mitigated bone loss (Figure 3), and inhibited osteoclastic activity (Figure 3). Our results also revealed that administration of P7C3 reduced lipid formation, bone marrow adiposity, and cell senescence induced by IR (Figure 4). In addition, our findings uncovered significant upregulation of cellular macromolecule metabolic processes, myeloid cell differentiation, and the proteins LRP-4, TAGLN, ILK, and Tollip, with downregulation of GDF-3, SH2B1, and CD200 (Figure 5). These proteins are key in favoring osteoblast over adipogenic progenitor differentiation, cell matrix

interactions, shape and motility, facilitate inflammatory resolution, and suppress osteoclastogenesis, potentially *via* Wnt/β-catenin signaling. In the end, we found that at the same protective P7C3 dose, a significant reduction in triple negative breast cancer, and osteosarcoma cell metabolic activity was measured *in vitro*.

Conclusions: Our results indicate that P7C3 is a previously undiscovered key regulator of adipo-osteogenic progenitor lineage commitment and may serve as a novel multifunctional therapeutic strategy leaving IR an effective clinical tool while diminishing the risk of adverse post-IR complications (Figure 5).

Relevance to CIRMS: Due to its high calcium content, bone tissue is estimated to absorb 30-40% more radiation than other tissues, making it a common site for serious ancillary tissue damage in cancer survivors. This work represents part of the therapeutic candidates pursed by the first author to seek novel radiation protection countermeasures that could mitigate IR-induced osteoporotic bone fractures while maintaining bone mechanical strength. This work strongly relates to the CIRMS mission, as ionizing radiation exposure is relevant to cancer patients undergoing radiotherapy, astronauts, radiation workers and victims of nuclear accidents, etc. The first author aims to become an internationally recognized scholar for seeking novel radioprotective therapies and enhancing the understanding of radiobiology. I am currently working in the laboratory with a focus on the detailed molecular mechanism of P7C3's radioprotective effect.