Dose-Dependent Growth Delay of Breast Cancer Xenografts in the Bone Marrow of Mice Treated with Radium-223: The Role of Bystander Effects and their Potential for Therapy

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**ABSTRACT**

**Rationale.** The role of radiation-induced bystander effects in radiation therapy remains unclear. With renewed interest in therapy with alpha-particle emitters, and their potential for sterilizing disseminated tumor cells (DTC), it is critical to determine the contribution of bystander effects to the overall response so they can be leveraged for maximum clinical benefit.

**Methods.** Female Foxn athymic nude mice were administered 0, 50, or 600 kBq/kg $^{223}$RaCl₂ to create bystander conditions. At 24 h post-administration, MDA-MB-231 or MCF-7 human breast cancer cells expressing luciferase were injected into the tibial marrow compartment. Tumor burden was tracked weekly via bioluminescence.

**Results.** The MDA-MB-231 xenografts were observed to have a 10 d growth delay in the 600 kBq/kg treatment group only. In contrast, MCF-7 cells had 7 and 65 d growth delays in the 50 and 600 kBq/kg groups, respectively. Histological imaging of the tibial marrow compartment, alpha camera imaging, and Monte Carlo dosimetry modeling revealed DTC both within and beyond the range of the alpha particles emitted from $^{223}$Ra in bone for both MCF-7 and MDA-MB-231 cells.

**Conclusions.** Taken together, the results support the participation of $^{223}$Ra-induced antiproliferative/cytotoxic bystander effects in delayed growth of DTC xenografts. They indicate that the delay depends on the injected activity and therefore is dose-dependent. They suggest using $^{223}$RaCl₂ as an adjuvant treatment for select patients at early stages of breast cancer.

**Keywords:** alpha particle, irradiation, bystander effect, breast cancer, Radium-223
INTRODUCTION

There has been renewed interest in radiopharmaceutical therapy since the FDA approved Xofigo® (223RaCl₂), an alpha particle-emitting radiopharmaceutical, for palliative treatment of metastatic castration-resistant prostate cancer (mCRPC). Patients given 223RaCl₂ demonstrated decreases in bone pain and, unlike previous bone-metastatic palliative radiotherapies like the beta-emitting ⁸⁹Sr, increased survival (1). To understand these responses, and guide clinical use of 223RaCl₂, detailed studies on its pharmacokinetics and dosimetry have been conducted (2-6). In addition, its clinical benefits have initiated other trials investigating the efficacy of 223RaCl₂ against other bone-metastatic cancers, such as breast cancer.

Breast cancer is the most common cancer diagnosed in women (7). A difficulty in successfully treating this cancer is the prevention and treatment of metastatic disease, with ~20% of 5-year-survivors ultimately relapsing 5-10 y post-treatment (8). The formation of metastases involves circulating tumor cells (CTC) shedding from the primary tumor and gaining access to the circulatory system (9). While the majority of CTC are rapidly eliminated, a small number survive and disseminate to various niches. These disseminated tumor cells (DTC) may sustain active proliferation and develop into macrometastases, or remain dormant for years before becoming active. Using animal models, it was estimated that 2.5% of shed CTC survive to become DTC, and ~0.01% may progress to macrometastases (10). While the overall breast cancer 5-year survival rate is ~89%, patients with bone-metastatic tumors only had a median survival of 19-25 months from diagnosis (11). In one third of patients with Stage I-III breast cancer, DTC were found in bone marrow biopsies (12). Yet, despite the standard use of adjuvant therapy to address the issue of DTC, 20% of breast cancer patients suffer from relapse (8). Recent studies have found that DTC can occur early in cancer development, in contrast to the concept that tumor metastasis occurs in late-stage cancer only (13). DTC are a significant risk factor in reducing patients’ life-expectancy (9, 14).
Therefore, a key goal for radionuclide therapies of cancer is to develop strategies to sterilize DTC before they become micrometastases in bone.

A benefit of using $^{223}$RaCl$_2$ for treatment of DTC is the emission of several short-range high-linear-energy-transfer (LET) alpha particles (Supplemental Table 1). In comparison to low LET radiation, only a few traversals across the cell nucleus is sufficient to cause cell death (15). This is due to the alpha particle’s ability to cause clustered DNA damage, including complex DNA double strand breaks (16). In addition, its principal localization in bone and the short track length of the alpha particles (<70 µm in water) allows for better sparing of bone marrow compared to competing therapeutics that emit beta particles with ranges up to a cm or more depending on their energy (3, 17). Combined with its calcium mimetic properties that allow for rapid and preferential uptake to osseous surfaces at marrow interfaces, $^{223}$RaCl$_2$ is an alpha-emitter therapy well-suited to treat tumor cells within the bone microenvironment. However, due to the alpha particles’ short track length, it is unlikely that every DTC in bone marrow is traversed by an alpha particle (Fig. 1), suggesting additional mechanisms of action for the observed clinical benefits of longer survival. Cancer cells outside the scope of the radiation field are known to be indirectly affected by bystander effects: DTC beyond the range of the alpha particles may suffer toxic effects due to signals propagated by alpha particle-irradiated cells. These signals can be transmitted by secreted factors and direct intercellular communication (18-21). The bystander effect has been shown to affect cells 1 mm away from the cells directly hit by radiation, many times farther than the range of the alpha particle (22). This suggests that the biological effects of alpha therapy may extend beyond the range of the alpha particle. However, how bystander effects impact the therapeutic efficacy of alpha therapy and whether they can be leveraged is unclear (23). Animal studies have been conducted to demonstrate the capacity of $^{223}$RaCl$_2$ to treat prostate cancer metastases at various stages of disease in both preventive and therapeutic capacities, but the mechanisms underlying its efficacy have not been explored (24, 25). Therefore, using a mouse model of DTC in bone, the present study was conducted to determine whether
bystander effects play a role in $^{223}$RaCl$_2$ therapy, and, if so, can they be leveraged to treat DTC at early stages of disease?
MATERIALS AND METHODS

Tumor Cells and Animals

MCF-7-luc-F5 (ER+) and MDA-MB-231-luc-D3H1 (ER-, PR-, Her2/neu-) human breast cancer cells were obtained from Caliper Life Sciences and cultured in Leibovitz L-15 medium (Sigma-Aldrich) supplemented with 2 mM L-glutamine (Gibco), 100 IU/mL penicillin and 100 μg/mL streptomycin (Corning), and 10% (vol/vol) fetal bovine serum (Gibco). Cells were grown in 175 cm² flasks and passaged weekly. The cell lines were authenticated by ATCC using Short Tandem Repeat analysis. MDA-MB-231-luc-D3H1 had 86% match to ATCC cell line HTB-26 (MDA-MB-231), with the allelic loss at loci D7S820 and vWA. MCF-7-luc-F5 had 100% match to ATCC cell line HTB-22 (MCF-7), with 14 of 14 alleles matching.

Female Foxn1nu athymic nude mice (4-6 wk old, 18-23 g, Envigo) were housed in rectangular opaque M.I.C.E. cages in groups of 4 on wood shavings with no enrichment. Room temperature was 21-23°C with 12 h light/dark cycle. Mice were provided irradiated Purina 5053 Picolab and water ad libitum. Moribund animals and those in apparent pain were euthanized with CO2 followed by cervical dislocation. All procedures were approved by Rutgers IACUC.

Creation of Bystander Conditions in the Tibial Marrow Compartment

Measurement of Radioactivity in Hind Limbs. Previous data (26) suggested that the marrow compartment is largely free of 223Ra and daughters by 24 h post-injection, and therefore a suitable location at that time for studies of radiation-induced bystander effects (Fig. 1). This was confirmed as follows. First, mice were administered 200 μL 5 mM citrate-buffered saline containing 600 or 1200 kBq/kg 223RaCl₂ (Xofigo®, Cardinal Health) via tail vein injection at 4:00-6:00 pm. At 24 or 48 h post-injection, the mice were euthanized by CO2 asphyxiation. The tibiae and femurs were resected and the activity in each was measured with a Packard Cobra automatic gamma counter for 2 min. Calibration (DPM=CPM/51.1)
was conducted with an aliquot from a known concentration of clinical grade $^{223}$RaCl$_2$. The resected tibiae were then processed to ascertain activity in cortical bone versus marrow. The tibial epiphyses were cut off, and the low-level activity in the cortical bone shaft determined with a calibrated low-background HpGe well counter (Canberra) using the 154.2 keV photopeak of $^{223}$Ra (yield=0.057, efficiency=0.47). The bone marrow was purged from the diaphysis with 1 mL phosphate buffered saline (PBS) using a 1 mL syringe with a 1.27-cm 27G needle. Air was blown through the diaphyseal shaft, followed by centrifugation at 600xg for 10 min to ensure all of the flush was purged. Activities in the marrow and the flushed shaft were then measured and the %activity in marrow determined.

Alpha Imaging of Activity Distribution in the Tibia. To determine detailed spatial distribution of $^{223}$Ra and daughter decays within the tibial and femoral bone and marrow, mice were administered $^{223}$RaCl$_2$ as above. At 72 h post-injection, the mice were euthanized and their hind limbs placed in ethanol. The limbs were then frozen in cryoprotective gel using liquid nitrogen-cooled isopentane, cryosectioned (12 µm-thick) using a tape-transfer method (CryoJane, Leica), and imaged using the Alpha Camera as described elsewhere (27). For each alpha image section, a consecutive section was made, stained with hematoxylin and eosin (H&E) and used for morphological identifications.

Effective Clearance Half-time. To determine the effective half-time $T_e$ in the tibia for radiation dosimetry, mice were administered 600 kBq/kg $^{223}$RaCl$_2$, euthanized 1, 8, and 21 d post-injection, and tibial activities measured. The $T_e$ was determined by fitting data (Sigmaplot13) to $A(t) = A_0 e^{-0.693t/T_e}$.

Inoculation of Tumor Cells into the Tibial Marrow Compartment

The breast cancer cells maintained in culture as adherent monolayers were used in experiments when 80-90% confluent. They were detached by trypsinization and their concentration determined with a Coulter ZM. Cells were pelleted at 2000 RPM for 3 min in 14 mL centrifuge tubes; the media was
discarded leaving a concentrated slurry of cells that were placed on ice. The mice were anesthetized with 2% isoflurane in O₂, and a 29-gauge insulin syringe was used to drill a hole into the tibial marrow compartment via a transpatellar approach. A chilled 31-gauge 10 μL Neuros syringe (Hamilton) loaded with 10⁶ MDA-MB-231 cells or 4×10⁵ MCF-7 cells in a 3 μL volume was inserted through the hole, and the cells were inoculated into the tibial marrow compartment. The larger size of MCF-7 cells led to fewer cells in the 3 μL slurry. At 24 h post-xenograft, mice were anesthetized with 2% isoflurane and injected subcutaneously with 200 μL of 15 mg/mL luciferin. To confirm successful inoculation and to determine initial bioluminescence intensity (IVIS 200, Perkin Elmer) serial images were taken until maximum bioluminescence intensity was observed.

**Measurement of Tumor Cell Proliferation**

To examine whether bystander effects modulate proliferation and/or killing of DTC in vivo, the ²²³RaCl₂ must be administered before tumor cell inoculation and the inoculation must take place after the circulating ²²³Ra clears. Accordingly, mice were injected with 0, 50, or 600 kBq/kg ²²³RaCl₂ on Day -1 and followed by intratibial inoculation of cells on Day 0 (Fig. 2). On Day 1, and weekly thereafter, the bioluminescence intensity was measured with IVIS. The normalized bioluminescence intensity (NB) was taken as the ratio of the average luminescence intensities in test and control groups. A 2-way ANOVA with post-hoc Bonferonni comparison determined significance (p<0.05) between treatment groups.

**Spatial Location of Tumor Cells in Tibial Marrow Compartment**

Mice were treated with 0 or 600 kBq/kg ²²³RaCl₂ on Day -1. On Day 0, an 80-90% confluent breast cancer cells were washed twice with PBS, labeled with 10 mL 1 μM CellTracker Green® (Thermo Fisher) in unsupplemented L-15 medium for 45 min. The cells were then trypsinized and inoculated intratibially. IVIS on Days 1 and 3 confirmed successful inoculation. Upon detection of luminescence signal, the mice were euthanized and the tibiae resected. The resected tibiae were imaged with IVIS to confirm that the previous
signal observed was within the tibial marrow compartment. The tibiae were placed in 7 mL 4% PFA and fixed for 48 h at 4°C. Samples were then decalcified in 14% EDTA for 2 wk, followed by paraffin embedment. Transverse sections (5 µm-thick) were cut with a microtome and stained with H&E. A Nikon A1R confocal microscope was used to image the fluorescence (517 nm peak) emitted by the CellTracker Green®-labeled breast cancer cells.

Absorbed Dose Profile in the Tibial Bone and Marrow

A section of a mouse tibia was modeled using the Monte Carlo simulation toolkit Geant4 (28). First, a series of micro-Computed Tomography (µCT) images of a mouse tibia was acquired (Bruker Skyscan 1172) with 5.0 µm voxel size. Second, the images were processed to extract the endosteal and the periosteal surfaces that were then converted into tessellated solids suitable for transport by Geant4. Third, the marrow and cortical bone compartments where subdivided into 2.0-µm layers from the endosteal surface. Details regarding the software and parameters are provided in Supplemental Table 2.

Five million $^{223}$Ra decays and ensuing daughter decays were simulated in a 400-µm long transverse section of the tibial diaphysis. Because redistribution of the daughters is <1% (26), daughter decays were simulated at the $^{223}$Ra parent decay sites. The emitted radiations were transported with Geant4 and energy depositions were scored in a 1600-µm long region surrounding the decay region. Based on activity measurements reported herein (see Results), 99.2% of decays were in the cortical bone and only 0.8 % in the marrow cavity. Exponential distributions based on alpha camera activity quantitation (see Results) were used to concentrate decays along the bone surface with a half-thickness of 75.0 µm within the bone tissue and 37.0 µm within the marrow tissue. The ICRP tissue composition for cortical bone and soft tissue were used for the bone and marrow compartments, respectively (29).
RESULTS

Measurement of Radioactivity in Hind Limbs

Measurements of femoral $^{223}$Ra activity yielded no significant difference in the counts in femurs harvested at 24 and 48 h post-injection of $^{223}$RaCl$_2$ (Supplemental Fig. 1). This is in agreement with published data for mice (26, 30). Therefore, it was concluded that maximum bone uptake was achieved by 24 h post-injection and supported the selection of this time to inoculate the tibia with breast cancer cells. Uptake of $^{223}$Ra in the hind limbs at 24 h was linearly dependent on the injected activity (Supplemental Fig. 2). Measurements of tibial activity with the HpGe detector determined that the epiphyses, diaphysis, and bone marrow contained 63.0±3.1%, 36.7±3.4%, and 0.30%±0.12% of the total tibial activity, respectively, and diaphyseal bone marrow contained 0.80%±0.73% of the activity in the diaphysis (Supplemental Fig. 3).

Alpha imaging of tibiae showed that $^{223}$Ra was concentrated to bone surfaces and active growth locations, particularly in the proximal epiphysis (Fig. 3A-B). Quantification by digital post-processing showed that the emitted alpha particles are detected predominantly in bone with very few events (2.1±1.3%) in the marrow (Fig. 3C). This percentage is similar to the 0.80%±0.73% obtained with the HpGe measurements. The HpGe derived value is used for dosimetry calculations because it represents an average over the relevant portion of the marrow compartment. Finally, least squares fits of the radial activity distribution to exponential functions yielded half-thicknesses of 75.0 μm and 37.0 μm in the diaphyseal bone and marrow, respectively (Supplemental Fig. 4).
Effective and Biological Half-lives of $^{223}$Ra in the Tibia

Using a least-squares fit of the data to a monoexponential decay function, the $T_e$ of $^{223}$Ra in the tibia and femur were 10.0 and 10.3 d, respectively (Supplemental Fig. 5). The biological half-times were determined using $1/T_e = 1/T_p + 1/T_b$, where $T_p$ and $T_b$ represent the physical half-life and biological half-time, respectively. The $T_p$ for $^{223}$Ra is 11.4 d and the calculated $T_b$ was 81 d and 107 d for the tibia and femur, respectively. These values indicate that the effective clearance of $^{223}$Ra from the tibia is driven primarily by physical decay, with a small contribution from biological clearance.

Tumor Cells in Marrow Lie Within and Beyond the Range of Alpha Particles Emitted from Bone

Histological sections of tibiae, harvested from both 0 and 600 kBq/kg treatment groups at 24 and 72 h post-inoculation, showed breast cancer cells within and beyond the 70 µm range of the alpha particles (Fig. 4, Supplemental Figs. 6 and 7). Therefore, the in vivo model contains breast cancer cells that will be directly irradiated by the alpha particles, as well as a bystander population of breast cancer cells farther from the bone endosteum and unlikely to be traversed by an alpha particle.

Absorbed Dose Profile in the Tibial Bone and Marrow

The µCT-derived tessellated solids representing the tibia, and the absorbed dose distribution obtained with the Geant4 Monte Carlo simulation of $5\times10^6$ decays, are presented in Fig. 5. The average absorbed doses in the bone and marrow compartments were 37.0 ($D_\alpha=36.4$, $D_\beta=0.77$) and 2.59 ($D_\alpha=2.36$, $D_\beta=0.23$) Gy, respectively for $5\times10^6$ decays, with a statistical error <0.1%. Inside the marrow cavity, the dose is concentrated within 70 µm of the endosteum, which corresponds to the maximum range of the alpha particles emitted from $^{223}$Ra and daughter decays in the bone. In the bystander region, the alpha
particles emitted by the few decays that occur in the marrow cavity, and the beta particles emitted by
\(^{223}\text{Ra}\) daughters in the bone compartment, contribute to an absorbed dose of less than 0.8 \((D_a=0.56, \ D_\beta=0.27)\) Gy. The \(5\times10^6\) decays correspond to complete decay of an initial activity of 4.0 Bq of \(^{223}\text{Ra}\) in the
modeled 400-\(\mu\)m region. The measured activity corresponding to the modeled 400-\(\mu\)m region was 2.3 Bq
for the 600 kBq/kg administration. Accordingly, given this, and the linear uptake depicted in Supplemental
Fig. 2, the mean absorbed dose per unit injected activity to diaphyseal bone and marrow was \(3.5\times10^{-2}\) and
\(2.4\times10^{-3}\) Gy (kBq/kg)\(^{-1}\), respectively. The absorbed dose to the bystander region ranged from \(3.8\times10^{-4}\) to
\(7.7\times10^{-4}\) Gy (kBq/kg)\(^{-1}\). Absorbed doses for the different injected activities, and initial dose rates \((r_0 = D(t=\infty)/1.44T_\circ)\) to the bystander region, are summarized in Table 1.

**Tumor Cell Proliferation**

Tumor burden is given as normalized bioluminescence intensity (NB) in Fig. 6. The growth delays
for each treatment group, defined by Demidenko as the difference of times required for treated and
untreated tumors to double their initial size \((31)\), are given in Table 1. The MDA-MB-231 cells
demonstrated a modest delay in tumor progression for the 600 kBq/kg group \((p<0.001)\), and no significant
difference for the 50 kBq/kg group when compared to control. MCF-7 cells appeared to respond to both
the 50 \((p=0.09)\) and 600 kBq/kg \((p<0.001)\); only the 600 kBq/kg treatment was significant. The MCF-7 600
kBq/kg group showed a marked decrease in tumor burden of approximately 70% that was not observed
in other groups (Fig. 6). In all treated groups, exponential tumor growth rate \((NB = ae^{bt})\) returned
eventually to that of control \((\text{MCF-7}: b = 0.053 \pm 0.011, 0.064 \pm 0.021, \text{and } 0.052 \pm 0.031 \text{ day}^{-1}\) for 0, 50,
and 600 kBq/kg; \text{MDA-MB-231}: \(b = 0.080 \pm 0.003, 0.149 \pm 0.029, \text{and } 0.103 \pm 0.007 \text{ day}^{-1}\)).
Discussion

The tumor progression studies in Fig. 6 show that MDA-MB-231 and MCF-7 responded very differently when placed into the marrow compartment that is surrounded by an in vivo alpha particle irradiator formed by the $^{223}\text{Ra}$ in the bone. Proliferation of MCF-7 cells was curtailed in the presence of $^{223}\text{Ra}$ more robustly than MDA-MB-231 cells at both the low (50 kBq/kg) and high injected activities (600 kBq/kg) (Table 1). Given that tumor cells are found both near and far from the bone surface relative to the range of the alpha particles, this could simply be a consequence of differences in absorbed doses received by the cells and/or differences in sensitivity to direct irradiation and/or induced bystander effects.

There are several factors arguing against either location or sensitivity to direct irradiation as primary contributors: First, the distribution of tumor cells in the tibial marrow compartment is similar for both MCF-7 and MDA-MB-231 (Fig. 4, Supplemental Figs. 6-7), thus they were exposed to the same absorbed dose profiles (Fig. 5). Second, the maximum mean absorbed dose delivered to the bystander region by alpha particles is 0.32 Gy for complete decay of the 600 kBq/kg administration (Table 1). According to Charlton et al., this will result in only about 20% of the cell nuclei being hit (any chord length) by an alpha particle after 10 d, 30% in 20 d, and 40% for complete decay (Fig. 7 of Ref. (32)). Third, extensive study of these two cell lines, wherein responses to ionizing radiation were conducted in parallel under well-controlled conditions, demonstrated very similar sensitivities to high dose rate exposures as evidenced by clonogenic cell survival (33-35). Third, MCF-7 do not demonstrate low dose hypersensitivity (36), and their radioresistance is increased at low dose rate exposures (37). Taken together, these points suggest that the different responses of these two cell lines is likely due to different sensitivities to radiation-induced stressful bystander effects that cause decreased proliferation and/or cell death (Fig. 6).

These findings are supported by our published in vitro studies showing high-LET radiation-induced lethal...
bystander effects are more robust for MCF-7 than MDA-MB-231 (38), and the antiproliferative bystander effect observed in vivo by Xue et al. (39).

The shape of the tumor response curves in Fig. 6 and the growth delays reported in Table 1 support a bystander response that depends on dose and dose rate. The dose rate decrease half-time is equal to the effective clearance half-time of $^{223}$Ra from the tibia which is 10 d (Supplemental Fig. 5). The nadir of MCF-7 tumor burden occurs at 40 d for 600 kBq/kg, at which time dose rate has dropped about 16-fold and the rate of reduction in tumor burden begins to diminish. The dose rate produced by 600 kBq/kg injection at this time ($t=40$ d) is about the same as produced by 50 kBq/kg at $t=0$, and their effect on tumor burden is similar, suggesting that an injected-activity-dependent bystander effect occurred within the bone microenvironment that affects both tumor cell burden and growth, depending on breast cancer phenotype.

Strictly, no single definition fits the type of bystander conditions that are present in $^{223}$Ra therapy due to its nonuniform activity distribution and complex mixture of emitted radiations ($\alpha$, $\beta$, $\gamma$, X). Bystander effects have been defined classically as responses of unirradiated cells to signals emanating from irradiated cells (40), and cohort effects as those responses that occur within an irradiated cell population not attributed to effects caused by direct radiation hits (40, 41). Both can be transmitted via gap junctional intercellular communication and/or released factors, and are thus not attributed to the radiation hits directly. Abscopal effects arise in an entirely different locale that is distant from the region irradiated (40). Here, all are three classes of effects are possible, tempting the attribution of the observed responses as “non-targeted” in the sense that radiation hits are not directly responsible for some of the observed effect. While “non-targeted” is a widely used term in external beam radiobiology, the use of “targeted” in nuclear medicine refers to the localization of the radiopharmaceutical to a specific population of cells or treatment region. Therefore, the use of “non-targeted” effects is inappropriate in
this context and we simply call the effect observed in the present work as a bystander effect in the sense that irradiated cells (e.g. tumor cells, marrow constituents, osteocytes, osteoblasts, etc. in the skeleton) are responsible for imparting effects to tumor cells in the marrow compartment that may or may not have been irradiated. This is consistent with clinical implementation of radiopharmaceutical therapy where cells are irradiated while the radiopharmaceutical circulates throughout the body and concentrates in the tumor, making it unlikely that there are cells in the marrow that are not irradiated at all. Key to this and recognized by both Blyth & Sykes and Brady et al. (23, 40), is the understanding that a bystander is a cell that may have been either irradiated or unirradiated, and a bystander effect is one imparted from irradiated cells. With the recent shift in targeted, high LET radiotherapy, being able to kill bystanders will be highly beneficial in the design of future treatment plans for radiopharmaceuticals alone, or combined with external beam therapy.

The increased magnitude of the bystander effect in the present study suggests that higher injected activities may better sterilize undetected dormant or slow-growing DTC in the bone marrow microenvironment. Thus, $^{223}$RaCl$_2$ may be a potential adjuvant therapy. Indeed, past studies suggest that there may be some benefit to escalating injected activity when treating advanced stages of disease (42). While $^{223}$RaCl$_2$ is currently approved at 50 kBq/kg, tolerance has been demonstrated at activities as high as 200 kBq/kg per injection in patients and higher amounts have not been explored (6). While a single treatment of $^{223}$RaCl$_2$ in the current study was insufficient to sterilize all DTC in the bone marrow, multiple treatments may yield a more durable tumor response. Additionally, the different in vivo responses of the MCF-7 and MDA-MB-231 cells suggests that some patient subsets with breast cancer DTC may derive greater benefit from $^{223}$RaCl$_2$ therapy than others (38). Characterization of the DTC within the bone marrow may be required to determine which patients are likely to benefit most from $^{223}$RaCl$_2$ therapy. Further work is required to determine the molecular mechanism(s) by which bystander cells are killed or inhibited in growth, and which DTC phenotypes would likely benefit from early $^{223}$RaCl$_2$ administration.
Given the difference in responses of the MCF-7-\textit{luc}-F5 (ER+) and MDA-MB-231-\textit{luc}-D3H1 (ER-, PR-, Her2/neu-) human breast cancer cells to bystander signals elicited by $^{223}$RaCl$_2$, and the success of $^{223}$RaCl$_2$ against mCRPC (1), the potential for leveraging these bystander effects should be further explored experimentally and clinically in the context of hormone-related cancers.
Conclusions

Taken together, the data indicate that radiation-induced bystander effects play an important role in the response of some DTC to $^{223}\text{RaCl}_2$, and the response depends on injected activity. These results support the use of $^{223}\text{RaCl}_2$ as an adjuvant treatment for select patients at early stages of breast cancer.

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KEY POINTS

**QUESTION:** Can radiation induced bystander effects, produced by radium-223, be used to treat disseminated tumor cells in bone marrow?

**PERTINENT FINDINGS:** Radium-223 induced bystander effects contribute to significant growth delays of human breast cancer xenografts in the bone marrow of nude mice. The magnitude of the effect depends on dose and the breast cancer xenograft.

**IMPLICATIONS FOR PATIENT CARE:** The results suggest using $^{223}\text{RaCl}_2$ as an adjuvant treatment for select patients at early stages of breast cancer.
References


FIGURE 1. Depiction of marrow (pink) in bone (gray), comparing traversal lengths of $\beta$ particles (blue arrows) and $\alpha$ particles (red arrows). Radium-223 is localized on bone surface (yellow). Cells within the blue dotted circle are untouched by the short-range $\alpha$ particles. The ranges of the $\beta$ particles vary due to their polyenergetic emission spectra.
FIGURE 2. In vivo experimental design. The experimental timeline for measuring proliferation of disseminated tumor cells. Animals were administered $^{223}$RaCl$_2$ intravenously on Day -1. Breast cancer cells were administered on Day 0. Luminescence imaging was conducted on Day 1 and then weekly.
FIGURE 3. Images of a longitudinal section of a mouse tibia harvested 24 h post-administration of 1200 kBq/kg of $^{223}$RaCl$_2$. (A) Bright field image of the unstained section. (B) Merge of bright field image (H&E stained) and low-resolution multiple-event alpha camera image. (C) High-resolution single-event post-processed alpha camera image that identifies precise locations of alpha particle events.
FIGURE 4. Histological images of transverse sections of mouse tibiae that were harvested 24 h after inoculation with CellTracker Green® labeled MDA-MB-231 or MCF-7 breast cancer cells. Bright field H&E and green fluorescence images of adjacent sections Note that tumor cells are present within and beyond the 70 µm range of the alpha particles emitted from the bone surfaces. Additional tibial sections are shown in Supplemental Figs. 6 and 7, respectively (600 kBq/kg, 24 h post-inoculation; 0 and 600 kBq/kg, 72 h post-inoculation).
FIGURE 5. (A) Three-dimensional model of a mouse tibia reconstructed from μCT images. The section used for the Monte-Carlo simulation corresponds to the green box. Radioactivity was restricted to the 400-μm thick section delineated by the two red lines: bone volume = 0.27 mm³, bone mass = 0.52 mg, marrow volume = 0.39 mm³, marrow mass = 0.40 mg. (B) Transverse cross section of the 400-μm thick region showing the surfaces of endosteum and periosteum. (C) Absorbed dose distribution in the marrow cavity (red curve) and cortical bone (blue curve) versus distance from endosteum after $5 \times 10^6$ $^{223}$Ra decays and ensuing daughter decays. The gold curve shows the activity distribution based on analysis of alpha camera data (Supplemental Fig. 4).
FIGURE 6. Tumor burden, measured with luminescence imaging, of MDA-MB-231 (left) and MCF-7 (right) cells under bystander conditions induced by radiation in vivo. Cells were inoculated into mouse tibiae 1 d post-administration of 0, 50, or 600 kBq/kg $^{223}$RaCl$_2$. Values are given as average (n=6) ±SEM. Black horizontal dashed line delineates two-fold increase in initial normalized luminescence intensity. Dashed gray lines are exponential least squares fits to the terminal data points for each data set. Representative luminescence images of tumor burden in the 0 and 600 kBq/kg treatment groups at various times post-inoculation are shown in Supplemental Figs. 8 and 9, respectively.
Table 1. Growth Delay of Human Breast Cancer Xenografts*.

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<tr>
<th>Treatment (kBq/kg)</th>
<th>$D_{\text{bone}}$ (Gy)**</th>
<th>$D_{\text{marrow}}$ (Gy)**</th>
<th>$D_{\text{bystander}}$ (Gy)**</th>
<th>$r_0,\text{bystander}$ (Gy/h)***</th>
<th>MCF7 Doubling time (days)</th>
<th>MDA-MB-231 Doubling time (days)</th>
<th>MCF7 Growth Delay (days)</th>
<th>MDA-MB-231 Growth Delay (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>23</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>50</td>
<td>$D_a=1.74$</td>
<td>$D_a=0.11$</td>
<td>$D_a=0.01$</td>
<td>0.58-1.2×10^{-4}</td>
<td>27</td>
<td>9.4</td>
<td>7</td>
<td>-14</td>
</tr>
<tr>
<td></td>
<td>$D_b=0.04$</td>
<td>$D_b=0.01$</td>
<td>$D_b=0.01-0.03$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$D_{\text{tot}}=1.8$</td>
<td>$D_{\text{tot}}=0.12$</td>
<td>$D_{\text{tot}}=0.02-0.04$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>600</td>
<td>$D_a=20.9$</td>
<td>$D_a=1.36$</td>
<td>$D_a=0.12-0.32$</td>
<td>0.67-1.3×10^{-3}</td>
<td>86</td>
<td>33</td>
<td>65</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>$D_b=0.44$</td>
<td>$D_b=0.13$</td>
<td>$D_b=0.11-0.16$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$D_{\text{tot}}=21$</td>
<td>$D_{\text{tot}}=1.5$</td>
<td>$D_{\text{tot}}=0.23-0.48$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Doubling time and growth delay as defined by Demidenko (31).

**Absorbed dose for complete decay (Integrated to $t=\infty$), tot = .

***Initial dose rate $r_0$ to the bystander compartment.
Supplemental Table 1. Principal* alpha particles and beta particles emitted in the decay chain $^{223}\text{Ra} \rightarrow ^{219}\text{Rn} \rightarrow ^{215}\text{Po} \rightarrow ^{211}\text{Pb} \rightarrow ^{211}\text{Bi} \rightarrow ^{207}\text{Tl}$ in order of descending yield per $^{223}\text{Ra}$ decay.

<table>
<thead>
<tr>
<th>Radionuclide</th>
<th>Radiation type</th>
<th>Yield* (per $^{223}\text{Ra}$ decay)</th>
<th>Energy** (MeV)</th>
<th>Range in Water*** (µm)</th>
<th>Range in Bone*** (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\rightarrow ^{215}\text{Po}$</td>
<td>$\alpha$</td>
<td>0.999</td>
<td>7.39</td>
<td>70</td>
<td>40</td>
</tr>
</tbody>
</table>
| $\rightarrow \\
| $\rightarrow ^{211}\text{Bi}$ | $\alpha$ | 0.835 | 6.62 |  |  |
| $\rightarrow ^{219}\text{Rn}$ | $\alpha$ | 0.794 | 6.82 |  |  |
| $^{223}\text{Ra}$ | $\alpha$ | 0.516 | 5.72 |  |  |
| $\rightarrow \\
| $\rightarrow ^{211}\text{Bi}$ | $\alpha$ | 0.252 | 5.61 |  |  |
| $\rightarrow ^{219}\text{Rn}$ | $\alpha$ | 0.162 | 6.28 |  |  |
| $\rightarrow \\
| $\rightarrow ^{207}\text{Tl}$ | $\beta^-$ | 0.995 | 0.496 | 1770 | 1050 |
| $\rightarrow \\
| $\rightarrow ^{211}\text{Pb}$ | $\beta^-$ | 0.912 | 0.476 | 1640 | 980 |

*Only particles with yields > 10% are shown. Values taken from the MIRD Decay Scheme monograph (1).
**Beta particle energies are mean values.
***Ranges were determined from tables in ICRU Reports 37 and 49 for $\beta^-$ and $\alpha$, respectively (2, 3).
$\rightarrow$The number of arrows represents the number of decays from the starting parent $^{223}\text{Ra}$.

Supplemental Table 2. Monte Carlo simulation: Software and parameters used during the study.

<table>
<thead>
<tr>
<th>Parameter name</th>
<th>Value / Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Software</td>
<td>Geant4</td>
</tr>
<tr>
<td>Version</td>
<td>10.1 with patch 02</td>
</tr>
<tr>
<td>Execution time</td>
<td>30 hours on an Intel® Xeon® CPU E5-1620 v3 @ 3.5 GHz</td>
</tr>
<tr>
<td>Geometry</td>
<td>Endosteum: tessellated solid (19,852 triangles) Periosteum: tessellated solid (22,282 triangles)</td>
</tr>
<tr>
<td>Material composition</td>
<td>Bone matrix: G4_BONE_CORTICAL_ICRP Marrow cavity: G4_TISSUE_SOFT_ICRP Surrounding tissue: G4_TISSUE_SOFT_ICRP</td>
</tr>
<tr>
<td>Source</td>
<td>$^{223}\text{Ra}$ decay (including daughters)</td>
</tr>
<tr>
<td>Physics</td>
<td>G4EmLowEPPPhysics</td>
</tr>
<tr>
<td>Cutoff range</td>
<td>2 µm</td>
</tr>
<tr>
<td>Number of histories</td>
<td>5 million</td>
</tr>
<tr>
<td>Quantities scored</td>
<td>Absorbed energy and absorbed dose vs. distance to endosteum</td>
</tr>
</tbody>
</table>
Supplemental Figure 1. Radium-223 activity (counts per minute) in the mouse femur at 24 (n=5) and 48 h (n=8) post-administration of $^{223}$RaCl$_2$. Values are given as mean ± standard error of the mean.

Supplemental Figure 2. Femoral $^{223}$Ra activity at 24 h post-injection of different activities of $^{223}$RaCl$_2$. A linear least squares fit to the data yielded $A = 0.363 \pm 0.008$ IA Bq per kBq/kg, where A is the activity per femur and IA is the injected activity.
Supplemental Figure 3. Distribution of $^{223}$Ra in mouse hind limbs. **Left:** Mean $^{223}$Ra activity in the tibial epiphyses, diaphysis, and bone marrow 24 h post-administration of 600 kBq/kg $^{223}$RaCl$_2$. **Right:** Percent of diaphyseal activity in that lies within the marrow compartment at 24 h post-administration of $^{223}$RaCl$_2$. Data was obtained by removing epiphyses at the proximal and distal ends, purging the marrow from the remaining diaphysis, and measuring activity in the epiphyses, purged diaphysis and marrow. Values are given as mean ± SE. n=4 animals, 2 limbs per animal.

Supplemental Figure 4. (A) Relative alpha particle density in cortical bone as a function of distance from surface of cortical bone. (B) Relative alpha particle density in marrow as a function distance from the endosteal surface of cortical bone. Alpha particle density (particles/cm$^3$) was obtained by acquiring alpha camera images of transverse sections (12-µm thick) of tibiae resected from mice 24 h after administering 600 kBq/kg $^{223}$Ra intravenously. The number of alpha particles was scored in regions of interest (ROI) drawn as concentric elliptical shells of thickness 4-µm. The alpha particle density was obtained for each shell by dividing the number counted by the volume of the shell. Finally, the relative density was calculated to use in concert with the absolute activity data obtained with the HpGe detector. A least squares fit to the data in graph A with $y = \exp(-0.693x / X_{1/2})$ yielded $X_{1/2} = 75$ µm. This represents the decrease in activity concentration as one moves from the cortical bone surface into the bone. In graph B, a fit with $y = y_0 + a*\exp(-0.693x / X_{1/2})$ yielded $X_{1/2} = 37$ µm. These fitted parameters, and the HpGe data in Supplemental Fig. 3, were used to represent the activity distribution in the model shown in Fig. 5.
Supplemental Figure 5. Effective clearance of $^{223}$Ra from the tibia (●) and femur (▲). $^{223}$Ra activity in the limbs were measured at 1, 8, and 21 d post-injection. The fraction of activity relative to day 1 are plotted as a function of time post-injection. A monoexponential decay function was fitted to the tibial data (black line). Up error bars and down error bars represent standard deviations of the mean for the tibiae (n=5 animals) and femurs (n=6 animals) respectively.
Supplemental Figure 6. Histological images of transverse sections of mouse tibiae that were inoculated with MDA-MB-231 cells (CellTracker Green) 24 h after mice were administered $^{223}$RaCl$_2$ intravenously. (A) 0 kBq/kg at 24 h after cells were injected; (B) 0 kBq/kg at 72 h after cells were injected; (C) 600 kBq/kg at 24 h after cells were injected; and (D) 600 kBq/kg at 72 h after cells were injected.
**Supplemental Figure 7.** Histological images of transverse sections of mouse tibiae that were inoculated with MCF7 cells (CellTracker Green) 24 h after mice were administered $^{223}$RaCl$_2$ intravenously. (A) 0 kBq/kg at 24 h after cells were injected; (B) 0 kBq/kg at 72 h after cells were injected; (C) 600 kBq/kg at 24 h after cells were injected; and (D) 600 kBq/kg at 72 h after cells were injected.
Supplemental Figure 8. Time course of representative luminescence images of mice injected intravenously with $^{223}$RaCl$_2$ and then inoculated with MDA-MB-231 cells 1 d later. Color represents luminescence intensity on a log scale. Control mice (0 kBq/kg): A) Day 1; B) Day 22, and C) Day 43. Treated mice (600 kBq/kg): D) Day 1; E) Day 22, F) Day 43.

Supplemental Figure 9. Time course of representative luminescence images of mice injected intravenously with $^{223}$RaCl$_2$ and then inoculated with MCF-7 cells 1 d later. Color represents luminescence intensity on a log scale. Control mice (0 kBq/kg): A) Day 1; B) Day 22, and C) Day 43. Treated mice (600 kBq/kg): D) Day 1; E) Day 22, F) Day 43.

References

3. ICRU. Stopping Powers and Ranges for Protons and Alpha Particles. Bethesda, MD: International Commission on Radiation Units and Measurements, Bethesda, MD; 1993. 49.p