ARTICLE

Survival Benefit With Radium-223 Dichloride in a Mouse Model of Breast Cancer Bone Metastasis

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Background	Bone metastases are associated with increased morbidity and poor prognosis in breast cancer patients. Radium-223 dichloride is a calcium mimetic that localizes to bone, providing targeted therapy for skeletal metastasis.
Methods	We investigated the mode of action of radium-223 dichloride using breast cancer cell, osteoclast, and osteoblast cultures as well as a mouse model of breast cancer bone metastasis. A single dose of radium-223 dichloride was used in three different settings mimicking the prevention or treatment of bone metastasis. Disease progression was monitored using fluorescence and radiographic imaging and histological analyses. The effect of radium-223 dichloride alone and in combination with doxorubicin or zoledronic acid on survival of mice was analyzed by Kaplan-Meier methods. All statistical tests used were two-sided.
Results	Radium-223 dichloride incorporated into bone matrix and inhibited proliferation of breast cancer cells and differentiation of osteoblasts and osteoclasts (all P values < .001) in vitro. In an established bone metastasis setting, radium-223 dichloride prevented tumor-induced cachexia (0/14 vs 7/14 control mice) and decreased osteolysis by 56% and tumor growth by 43% (all P values < .05). Radium-223 dichloride induced double-strand DNA breaks in cancer cells in vivo. Finally, radium-223 dichloride extended survival as a monotherapy (29.2 days, 95% confidence interval [CI] = 26.6 to 31.8 days, P = .039) and in combination with zoledronic acid (31.4 days, 95% CI = 28.8 to 34.0 days, P = .004) or doxorubicin (31.5 days, 95% CI = 29.5 to 33.5 days, P < .001) compared to the vehicle group (24.9 days, 95% CI = 23.4 to 26.4 days). Similar but even more pronounced effects were observed when radium-223 dichloride was administered in a preventive or micrometastatic setting.
Conclusions	Our findings strongly support the development of radium-223 dichloride for the treatment of breast cancer patients with or at high risk of developing bone metastases.
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Breast, prostate, and various other cancers have a strong predilection to metastasize to bone and subsequently cause clinically significant morbidity, chronic pain, and pathologic fractures in patients. Bone metastasis secondary to several tumor entities have been shown to be associated with poor prognosis (1). To date, therapies to manage bone metastases have been only palliative. Therefore, there is a high unmet need for effective treatment options that have a favorable safety profile for patients suffering from bone metastases (2).

Radium-223 dichloride (Algeta ASA, Oslo, Norway and Bayer Pharmaceuticals, NJ) is a calcium mimetic and localizes to bone metastases where the emission of alpha particles provides targeted therapy (3). In a preclinical model of osteolytic breast cancer bone metastasis in nude rats, radium-223 dichloride increased symptom-free survival (4). Radium-223 dichloride also demonstrated

promising phase II clinical data for the treatment of predominately osteoblastic bone metastases in prostate cancer (5,6). Recently, radium-223 dichloride was shown to improve overall survival in a phase III clinical study (ALSYMPCA) on the treatment of castration resistant prostate cancer with bone metastases (7). In an open-label phase IIa study on breast cancer patients with bone-dominant disease, who have progressed on endocrine therapy, radium-223 dichloride treatment consistently reduced biochemical markers of bone metabolism during the 16-week treatment period (8).

In this study, we investigated the mode of action of radium-223 dichloride in the vicious cycle of bone metastasis and osteolysis using breast cancer cell, osteoclast, and osteoblast cultures in vitro. Furthermore, we studied the effects of radium-223 dichloride in mouse models mimicking the prevention and treatment of breast cancer bone metastasis.

Materials and Methods

In Vitro Cell Assays

MDA-MB-231(SA) cell proliferation assay was performed as previously described (9). Mouse KS483 cells were used to study the effects of radium-223 dichloride on osteoblast maturation and bone formation as well as for clarifying the mechanism of radium-223 dichloride incorporation into bone matrix (10). In vitro effects of radium-223 dichloride on osteoclast differentiation and activity were studied by culturing primary human osteoclast precursor cells as previously described (11). Modifications and specific materials used in the in vitro breast cancer, osteoblast, and osteoclast assays are described in detail in the Supplementary Methods (available online).

In Vivo Mouse Models

The animal studies were approved by the Animal Experiment Board of Finland and performed according to European Union directive 2010/63/EU. Mice were kept under pathogen-free and controlled conditions and fed a soy-free radiated 2016 Teklad Global diet (Harlan Laboratories, B.V., Horst, the Netherlands). The in vivo effects of radium-223 dichloride on breast cancer metastasis to bone were studied using three different settings: 1) preventive, 2) micrometastatic, and 3) established bone metastasis models. In addition, survival studies were performed. In all studies, 10^5 human MDA-MB-231(SA) cells (12) transfected with green fluorescent protein (GFP) (pTurboGFP-N vector, Evrogen JSC, Moscow, Russia) were inoculated into 5-week-old female nude mice (Hsd:Athymic nude-*Foxn1^nu*, Harlan Laboratories) via left cardiac ventricle (12,13). For the preventive and micrometastatic settings, the mice were randomized to groups by their body weights (n = 7 per group or n = 12 per group for the survival study). For the established bone metastasis setting, the mice were randomized to groups based on their body weight and presence of osteolytic lesions on day 14 (n = 15 per group or n = 9–10 per group for the survival study). Mice were administered intravenously with either vehicle (sodium citrate) or a single dose of radium-223 dichloride (300 kBq/kg) both 5 mL/kg on day –1 (preventive setting), day 2 (micrometastatic setting), or day 15 (established metastases). Prior to these experiments, a dose-finding study comparing three different doses of radium-223 dichloride was performed in a model of established metastases (300 kBq/kg, n = 7; 600 kBq/kg, n = 6; 1200 kBq/kg, n = 7; vehicle control, n = 5). In addition, three vehicle-treated mice were killed on day 2 for the immunohistochemical demonstration of tumor cells in the bone marrow in the micrometastatic setting. For the comparative analysis of the effects of radium-223 dichloride, doxorubicin, and zoledronic acid as monotherapy and combination therapies, vehicle, radium-223 dichloride (300 kBq/kg), and/or zoledronic acid (0.1 mg/kg, subcutaneous injection; Novartis Pharma GmbH, Nürnberg, Germany) was administered on day 15 and doxorubicin (5 mg/kg, intraperitoneal injection; Ebewe Pharma GmbH, Unterach, Austria) once weekly (n = 9–10 per group). The mice were given analgesic once before the intracardiac inoculation and for the last 5 days of the experiment (0.1 mg/kg buprenorphine subcutaneously twice a day or 0.02 mg/mL in drinking water at the end of the experiment).

Mice were considered cachectic when two of the following three conditions were met: curved spine, dehydration, and/or a 20% or more reduction from the maximum weight. Radiography and fluorescence imaging were performed on day 14 and at euthanasia (days 22–25), as explained in detail in the Supplementary Methods (available online). Mice were killed by cervical dislocation under anesthesia. Tissue samples from hind limbs (left and right tibiae and femora) were collected for histology. Histologic stainings and histomorphometry were performed as explained in the Supplementary Methods (available online). Osteoblasts were counted in the metaphysis of femur and tibia, 3 mm below the growth plate. Blood samples were collected from the saphenous vein before the inoculation of cancer cells and on days 14 and 24 (or when killed). Serum tartrate-resistant acid phosphatase (TRACP) 5b and N-terminal propeptide of type I procollagen (PINP) were analyzed using the MouseTRAP and mouse PINP enzyme-linked immunosorbent assay kits, respectively (IDS Ltd).

Statistical Analysis

The data were analyzed using SPSS (version 14.0 or later) and R (version 2.14). All the measured variables were analyzed as continuous and the treatments as categorical variables. The statistical differences between the groups were assessed predominantly using Student t test/one-way analysis of variance (for normally distributed parameters with homogenous variance) or Kruskal-Wallis test (if the assumptions were not fulfilled even after logarithmic, square root, or reciprocal data transformations). For the statistically significant findings ($P < .05$), pairwise tests were performed using Dunnett test or nonparametric Mann-Whitney U test, correspondingly. The analyses were performed as two-sided tests. The frequency data were analyzed using the Fisher exact test, time to euthanasia by Kaplan-Meier survival analysis (log-rank test), and the proliferation curves using fixed-effects linear models.
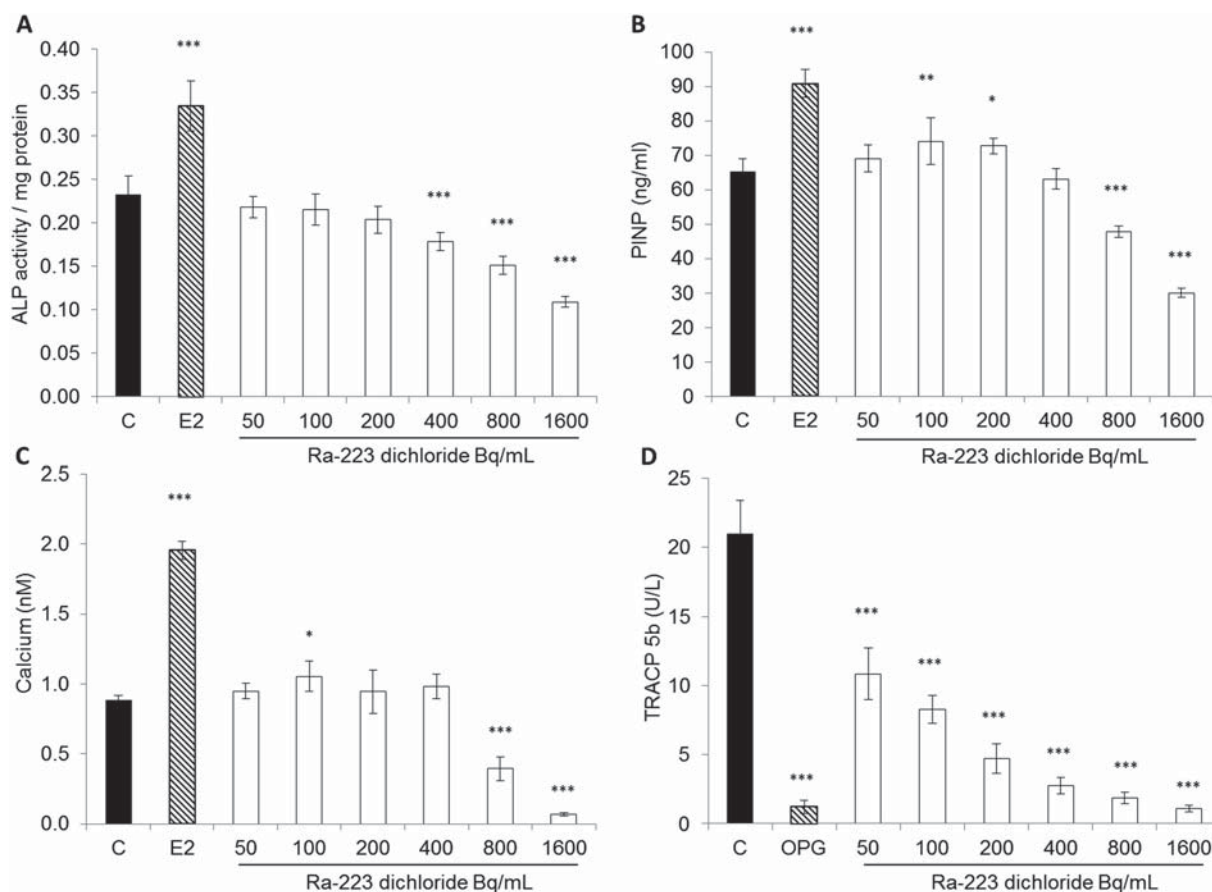
RESULTS

Effects of Radium-223 Dichloride on Proliferation of Breast Cancer Cells and Differentiation of Osteoblasts and Osteoclasts In Vitro

Radium-223 dichloride showed a dose-dependent inhibition of MDA-MB-231(SA) breast cancer cell proliferation at concentrations of 400 (contrast value 0.043, 95% CI = 0.010 to 0.075, $P = .010$), 800 (contrast value 0.135, 95% CI = 0.103 to 0.168, $P < .001$), and 1600 Bq/mL (contrast value 0.237, 95% CI = 0.204 to 0.269, $P < .001$) compared to the control in a 5-day in vitro proliferation assay (Figure 1).

In order to study the effects of radium-223 dichloride on the differentiation and activity of osteoblasts in vitro, KS483 mouse osteoprogenitor cell cultures were used. Radium-223 dichloride inhibited osteoblast differentiation dose dependently at concentrations 400, 800, and 1600 Bq/mL, as indicated by the decreased alkaline phosphatase values ($P < .001$, Figure 2A), and at concentrations 800 and 1600 Bq/mL decreased the PINP and calcium values in the osteoblast activity assay ($P < .001$, Figure 2, B and C). Conversely, 100 Bq/mL radium-223 dichloride increased both the PINP ($P = .009$) and calcium values ($P = .039$), and 200 Bq/mL radium-223 dichloride increased PINP values
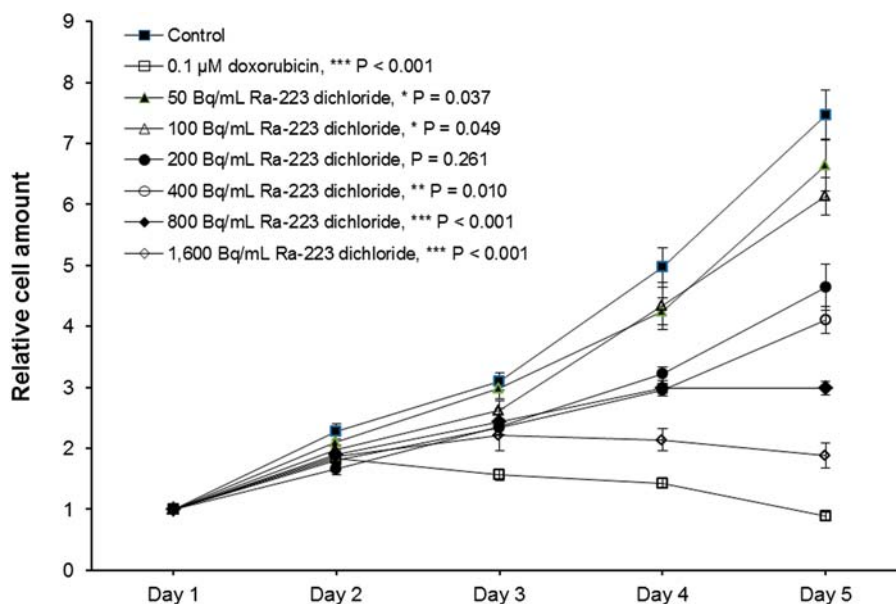
Figure 1. The effects of radium-223 (Ra-223) dichloride on MDA-MB-231(SA) cell proliferation (means; error bars represent 95% confidence intervals). The results of all other groups ($P < .001$ between all groups) were compared separately with the results of the control group using a linear model with a fixed effect for the treatment, a continuous covariate for the day, and an interaction term for the treatment and the day. The statistical tests were two-sided.

Figure 2. The effects of radium-223 (Ra-223) dichloride on mouse osteoblasts and human osteoclasts in vitro (means; error bars represent 95% confidence intervals). **A**) Osteoblast differentiation assay. The results are shown as cellular alkaline phosphatase (ALP) activity per milligram of protein. **B**) Bone-forming activity of osteoblasts measured as N-terminal propeptide of type I procollagen (PINP) in the culture medium on day 11. **C**) Bone-forming activity of osteoblasts measured as calcium deposition on day 13. **D**) Osteoclast differentiation assay measured as tartrate-resistant acid phosphatase (TRACP) 5b activity in the culture medium on day 7. $P < .001$ for all groups. Statistical analysis was performed by one-way analysis of variance ($P < .001$) followed by Dunnett test. The tests were two-sided. *$P < .05$, **$P < .01$, ***$P < .001$. C = control (no compounds added); E2 - 17β-estradiol (10 nM); OPG = osteoprotegerin (100 ng/mL).

(P = .036), indicating a stimulatory effect on osteoblast activity at lower concentrations. Radium-223 dichloride showed high incorporation into the bone matrix during the bone formation process by osteoblasts. Radioactivity of bone matrix measured by liquid scintillation counter after a 13-day bone formation assay was 1343 counts per minute (cpm) (95% CI = 1254.3 to 1431.7) in the radium-223 dichloride (100 Bq/mL) and 0.1 cpm (95% CI = 0.07 to 0.15) in the control wells.

The effects of radium-223 dichloride on the differentiation and activity of osteoclasts in vitro were evaluated using human bone marrow–derived osteoclast precursor cells. Radium-223 dichloride showed a dose-dependent inhibition of osteoclast differentiation (P < .001, Figure 2D) but did not affect the resorption activity of osteoclasts (data not shown).

Treatment of Established Breast Cancer Bone Metastases With Radium-223 Dichloride

We investigated the potential of radium-223 dichloride for the treatment of breast cancer bone metastasis using the MDA-MB-231 bone metastasis model. The mice were administered with radium-223 dichloride 15 days after the intracardiac inoculation of MDA-MB-231(SA) cells. In the first dose-finding study, 300, 600, and 1200 kBq/kg radium-223 dichloride was applied. Because a statistically significant decrease in total tumor burden, total osteolytic area, and lesion count was already observed at 300 kBq/kg; this dose was used in all subsequent experiments. This dose is 12% of the severely toxic dose of radium-223 dichloride to 10% of the mice (STD10) after single administration.

Our data showed that radium-223 dichloride completely prevented the tumor-associated cachexia in the model of established bone metastases: 0 of 14 of the radium-223 dichloride–treated mice as opposed to 7 of 14 (50%) of the vehicle control group were cachectic. Radium-223 dichloride also delayed the loss of body weight observed in the vehicle group (Figure 3A). Importantly, as quantified by fluorescence imaging, radium-223 dichloride treatment decreased the whole-body tumor burden by 43% (17.8 mm², 95% CI = 9.8 to 25.9) as compared to the vehicle group (32.0 mm², 95% CI = 19.4 to 44.6, P = .035, Figure 3B). Radiographic analysis revealed a 56% decrease in the osteolytic lesion area in the radium-223 dichloride–treated group (2.41 mm², 95% CI = 1.38 to 3.45) as compared to the vehicle (5.53 mm², 95% CI = 3.29 to 7.77, P = .012, Figure 3, C–E). This inhibitory effect of radium-223 dichloride on tumor-induced bone destruction was confirmed as larger trabecular (P = .007) and cortical (P < .001) bone area in the radium-223 dichloride-treated as compared to the vehicle group (Figure 3F, Supplementary Figure 1A, available online). Based on histologic sections of bone metastases, the number of osteoclasts at tumor-bone interface was smaller (vehicle 1.24, 95% CI = 0.87 to 1.61 vs radium-223 dichloride 0.64, 95% CI = 0.49 to 0.79, P = .007; Figure 4, A and B). According to the microscopic evaluation, some necrotic tumor foci were observed in the bone metastatic areas in approximately half of the mice in both groups, but this was more profound in the radium-223 dichloride-treated mice suggesting stimulation of tumor cell necrosis (Figure 4C). Therefore, the efficacy of radium-223 dichloride in inducing double-strand breaks in cancer cells was evaluated by immunohistochemical staining of the γ-H2AX molecules (14).

A 3-fold increase in the number of tumor cells with double-strand breaks in the radium-223 dichloride–treated mice as compared with the vehicle control mice (P < .001; Figure 4, D and E) was revealed. Radium-223 dichloride treatment did not substantially affect serum TRACP 5b activity (bone resorption marker) measured on day 25 (Supplementary Figure 2A, available online) but increased serum PINP values (bone formation marker) (P = .019, Supplementary Figure 2B, available online).
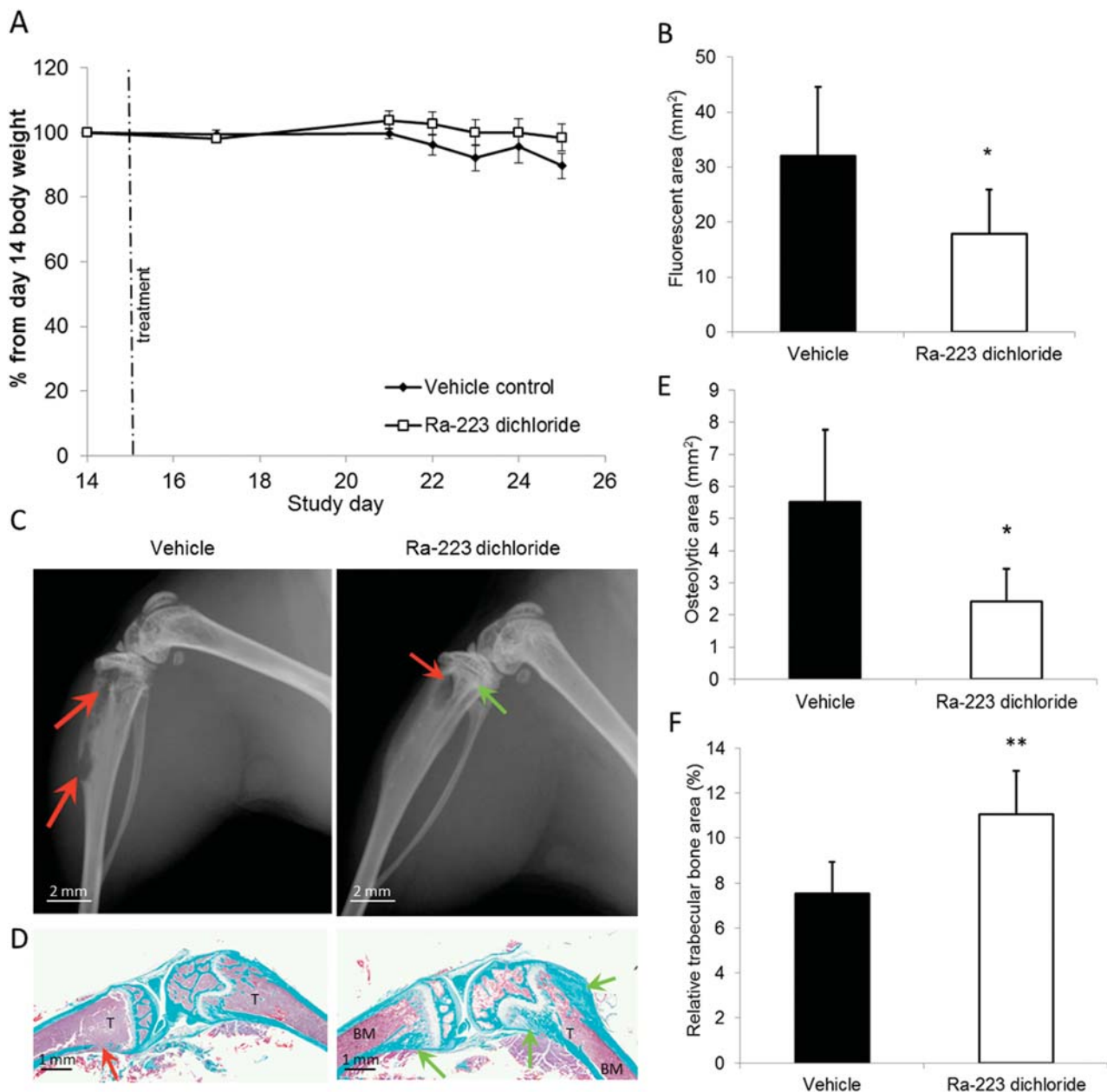
Effects of Radium-223 Dichloride on Tumor Growth and Osteolysis in Bone

We also studied the effects of radium-223 dichloride on breast cancer bone metastasis in preventive and micrometastatic settings. The mice were dosed with radium-223 dichloride one day before (preventive) or two days after (micrometastatic) the intracardiac inoculation of MDA-MB-231(SA) cells. To verify the presence of disseminated tumor cells in the bone marrow, three control mice were killed for histologic analysis on day 2. Tumor cells were observed in all (18/18) histologic sections. Representative images are shown in Supplementary Figure 3 (available online). Radium-223 dichloride prevented cachexia: none of the radium-223 dichloride–treated mice as opposed to 6 of 7 (86%) of the control group were cachectic. The mouse body weights were better maintained in both radium-223 dichloride treatment settings (data not shown). Radium-223 dichloride also decreased whole-body tumor burden as quantified by fluorescence imaging by 81% (10.4 mm², 95% CI = 1.6 to 19.1, P < .001) and 84% (8.6 mm², 95% CI = 0.9 to 16.2, P = .001) when administered on days –1 or 2, respectively, as compared to control (54.9 mm², 95% CI = 36.4 to 73.5), and no tumor foci were detected in histological bone sections (Figure 5, A–C). Radium-223 dichloride decreased osteolysis by 98% (0.071 mm², 95% CI = 0.014 to 0.128, P < .001) and 99.6% (0.018 mm², 95% CI = 0.004 to 0.032, P < .001) when administered on days –1 or 2, respectively as compared to control (4.008 mm², 95% CI = 1.039 to 6.977, Figure 5, E and F), and increased trabecular and cortical bone area (P < .001, Figure 5D, Supplementary Figure 1B). Radium-223 dichloride treatment did not affect the number of osteoblasts nor serum TRACP 5b activity or PINP values measured on day 25 (data not shown).

To see whether radium-223 dichloride treatment affects the survival of mice developing breast cancer bone metastases, we performed an additional in vivo study in preventive and micrometastatic settings. Our results demonstrated that radium-223 dichloride treatment increased the time to death in both treatment settings (P < .001, Figure 6).

Survival Benefit of Radium-223 Dichloride Alone or in Combination With Zoledronic Acid or Doxorubicin in Established Bone Metastasis Model

The effects of radium-223 dichloride on survival compared to and in combination with doxorubicin or zoledronic acid treatment were determined using the model with established bone metastases. First, our data demonstrated that, similar to the preventive and micrometastatic settings, radium-223 dichloride treatment also extended time to death in mice with established bone metastases (vehicle: 24.9 days, 95% CI = 23.4 to 26.4 vs radium-223 dichloride: 29.2 days, 95% CI = 26.6 to 31.8, P = .039). Second,
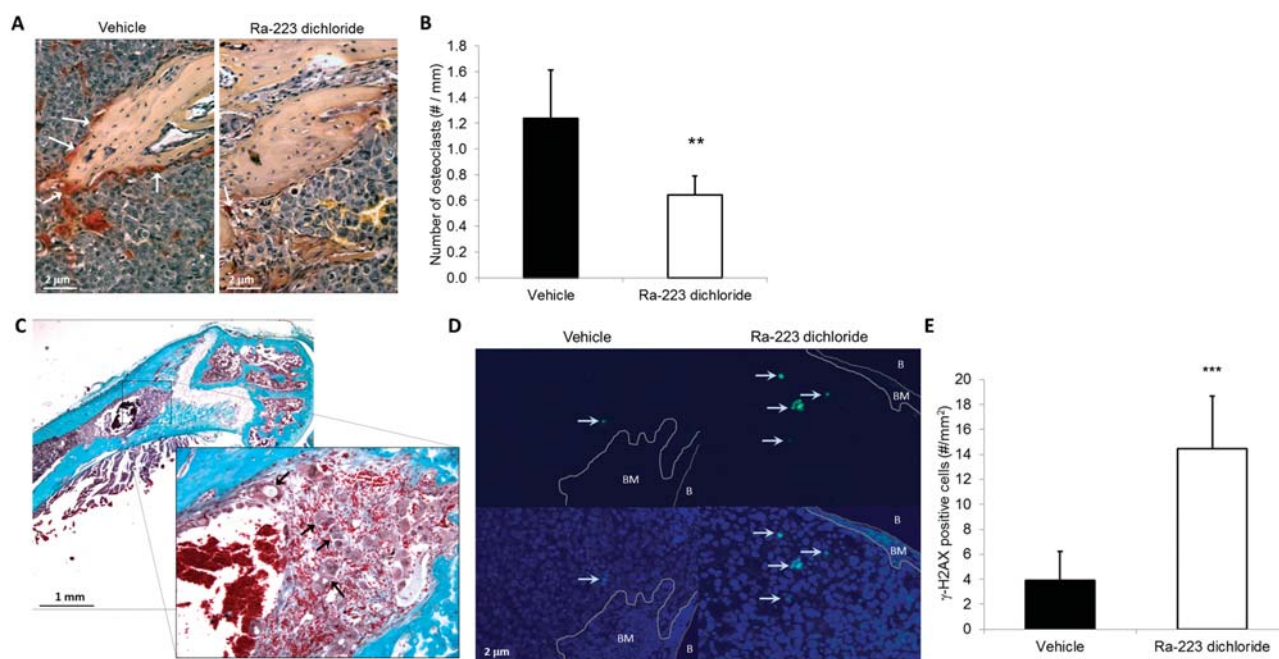
Figure 3. The effects of radium-223 (Ra-223) dichloride in mice with established bone metastases. **A**) Change in mouse body weight shown as percentage of day 14 body weight (means; error bars represent 95% confidence intervals [CIs]). **B**) Whole-body tumor burden analyzed from fluorescent images (means; error bars represent 95% CIs). *P = .035 (Student t test after logarithmic transformation). **C**) Representative radiographic images of hind legs of Ra-223 dichloride-treated and vehicle mice. **Red arrows**: osteolytic lesions, **green arrow**: very dense bone. **D**) Representative histological images of hind legs of Ra-223 dichloride-treated and vehicle control mice. **Red arrow**: osteolytic lesion, **green arrows**: very dense bone, BM = bone marrow; T = tumor. Original magnification × 25. **E**) Total osteolytic area analyzed from radiographic images from both hind legs (means; error bars represent 95% CIs). *P = .012 (Student t test after logarithmic transformation). **F**) Trabecular bone area relative to the bone marrow area analyzed from histological sections (means; error bars represent 95% CIs). **P = .007 (Student t test). The statistical tests were two-sided.

survival benefit was also observed in both radium-223 dichloride combination treatment groups vs the vehicle group (radium-223 dichloride + doxorubicin: 31.5 days, 95% CI = 29.5 to 33.5, P < .001, and radium-223 dichloride + zoledronic acid: 31.4 days, 95% CI = 28.8 to 34.0, P = .004) and third, survival in the doxorubicin or zoledronic acid monotherapy groups was not different from the vehicle group. Finally, combining radium-223 dichloride treatment with either doxorubicin or zoledronic acid did not confer an additional survival benefit as compared to the

radium-223 dichloride monotherapy (Figure 7). Serum TRACP 5b activity was lower in both groups receiving zoledronic acid on day 25 (Supplementary Figure 2C, available online). Serum PINP values were higher in the radium-223 dichloride monotherapy group (P = .041), lower in the zoledronic acid monotherapy group (P = .003) and did not differ from control in the radium-223 dichloride and zoledronic acid combination group (Supplementary Figure 2D, available online). Serum TRACP 5b and PINP values measured when mice were killed were lower in

Figure 4. Histologic evaluation of the effects of radium-223 (Ra-223) dichloride on osteoclasts, necrosis, and breast cancer cells in bone metastasis. **A**) Osteoclasts at the tumor–bone interface (**white arrows**). **B**) Number of osteoclasts at the tumor–bone interface (means; error bars represent 95% confidence intervals [CIs]). ****** P = .007 (Student t test). **C**) Representative image of Masson-Goldner trichrome-stained histologic section of femur. Necrotic foci were observed at bone metastatic sites in Ra-223 dichloride-treated mice with established bone metastases. The figure shows macrophages cleaning up the necrotic debris (**arrows**). Magnification ×25, inset ×200. **D**) Representative images of the γ-H2AX (histone H2A) staining of the bone metastasis sections from the established bone metastasis model. **Left panel** shows the vehicle control and right panel the Ra-223 dichloride-treated. **Upper panel** shows the γ-H2AX staining, and **lower panel** is an overlay with the nuclear counterstain (DAPI). Arrows indicate cells counted as γ-H2AX positive. B = bone, BM = bone marrow. Original magnification ×200. **E**) The number of γ-H2AX–positive cells relative to tumor area (mean + 95% CI). Sample size was 9 per group. ******* P < .001 (Student t test). The statistical tests were two-sided.

mice receiving both radium-223 dichloride and zoledronic acid (P < .001, Supplementary Figure 2, E and F, available online).

Discussion

In this study, we provide insights into the mechanisms of action of radium-223 dichloride, starting from its osteoaffinity to its dual effects on both tumor growth and osteolysis in bone metastases.

It has been suggested that osteoblasts actively incorporate radium-223 dichloride to hydroxyapatite, a major mineral component of bone, during mineral formation (15). We were able to verify this bone-binding mechanism in our osteoblast in vitro studies. Interestingly, lower doses of radium-223 dichloride transiently stimulated osteoblast activity, a phenomenon earlier reported for calcium (16). In addition to active incorporation by osteoblasts, passive binding of radium-223 dichloride as a calcium mimetic to hydroxyapatite might also play a role in areas of high bone turnover.

Previously, it was shown that radium-223 dichloride treatment prolongs paralysis free survival in a rat model of breast cancer bone metastasis (4). Our results from preclinical models reflecting different stages of breast cancer dissemination to bone confirm and extend these previous observations. Although we used a relatively aggressive bone metastasis model, a single dose of radium-223 dichloride corresponding to only 12% of the severely toxic dose of radium-223 dichloride to 10% of the mice completely prevented the tumor-associated cachexia and delayed the loss of body weight in the established metastasis setting. Furthermore, the mice treated

with radium-223 dichloride had decreased bone destruction and tumor burden as compared to the vehicle control group. Finally, these effects translate into a survival advantage. Histologic examination revealed that radium-223 dichloride treatment induced tumor cell necrosis in bone metastases, and active cleaning of the necrotic cell debris by macrophages was observed. The α-emitters, such as radium-223 dichloride, induce double-strand DNA breaks and are therefore predicted to have superior efficacy in cancer treatment as compared to β- and γ-emitting radionuclides (17). Using γ-H2AX staining, we demonstrated for the first time that radium-223 dichloride induces double-strand breaks in cancer cells in vivo, contributing at least partly to the necrosis observed in the radium-223 dichloride group. Furthermore, radium-223 dichloride treatment dose-dependently inhibited osteoclast differentiation in vitro. This could partly explain the reduced number of osteoclasts at the tumor-bone interface observed in vivo. Therefore, both the in vivo and in vitro findings support the dual effect of radium-223 dichloride on the vicious cycle by targeting both osteoclasts and tumor cells in primarily osteolytic metastases. Inhibition of osteoblast differentiation was observed with higher doses of radium-223 dichloride. This in vitro finding might partially explain the high efficacy observed in hormone refractory prostate cancer patients with bone metastases (5–7).

The effect and safety of radium-223 dichloride were also studied in preventive and micrometastatic settings, simulating two distinct clinical situations. Radium-223 dichloride treatment completely prevented tumor-induced cachexia and increased body weight and
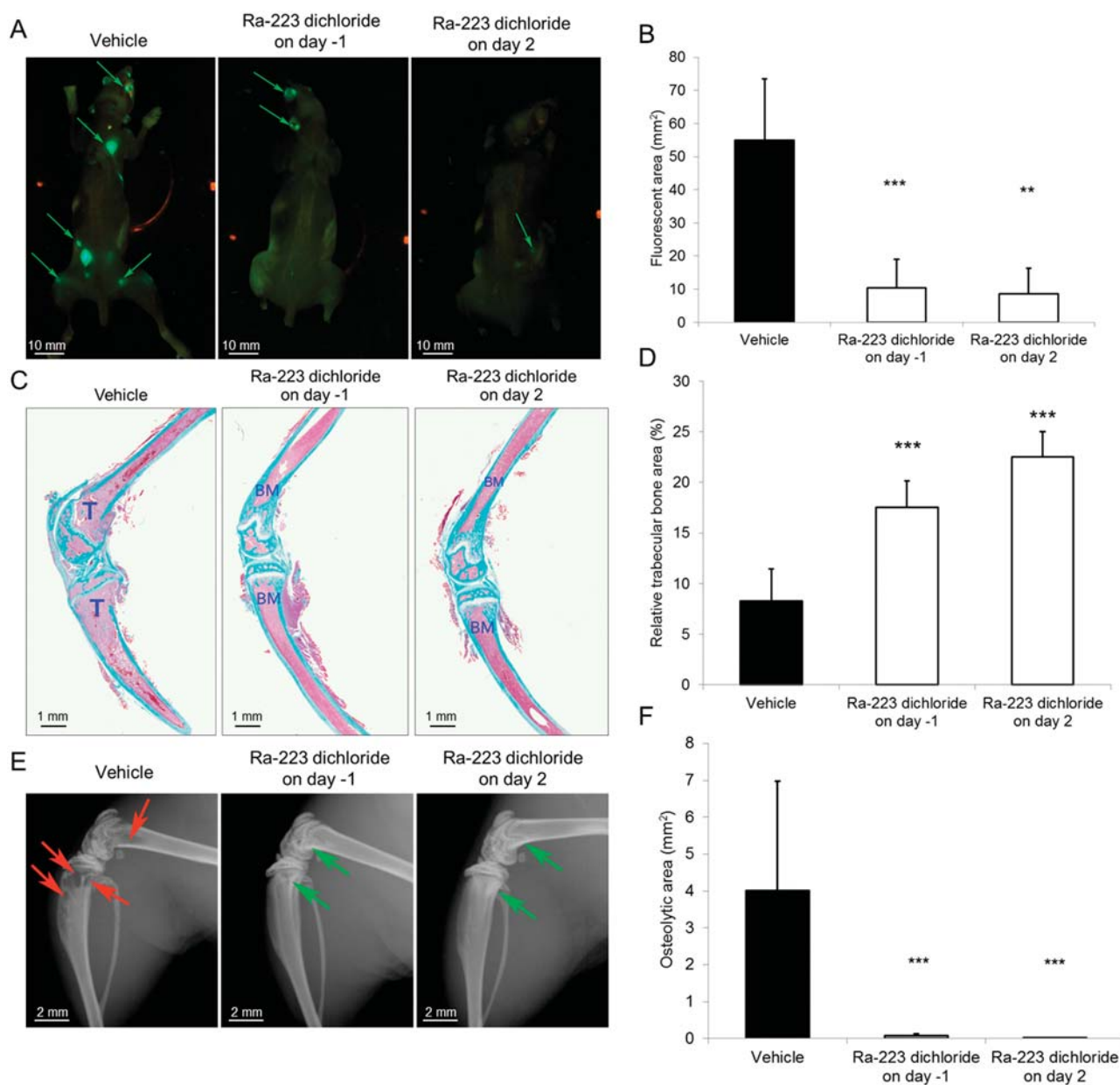
Figure 5. The effects of radium-223 (Ra-223) dichloride on tumor growth and osteolysis in preventive and micrometastatic settings. **A)** Representative images of mice with green fluorescent protein–expressing metastases on day 25. **B)** Whole-body tumor burden analyzed from fluorescent images (means; error bars represent 95% confidence intervals [CIs]). ***$P < .001$, **$P = .001$ (analysis of variance [ANOVA] followed by Dunnett t test). **C)** Representative images of histology showing tumor burden and the amount of bone in the hind limbs of mice. Hind limbs of both treatment groups were completely devoid of tumor. T = tumor, BM = bone marrow. Treatment groups (from left to right): vehicle control, Ra-223 dichloride dosed on day −1 and Ra-223 dichloride dosed on day 2. **D)** Trabecular bone area relative to the bone marrow area analyzed from histological sections (means; error bars represent 95% CIs). ***$P < .001$ (ANOVA followed by Dunnett t test). **E)** Representative radiographic images (from left to right): vehicle control, Ra-223 dichloride dosed on day −1 and radium-223 dichloride dosed on day 2. The images were obtained on day 25. **Red arrows:** osteolytic lesions, **green arrows:** very dense bone. **F)** Total osteolytic area analyzed from radiographic images from both hind legs (means; error bars represent 95% CIs). ***$P < .001$ (Kruskal-Wallis test followed by Mann-Whitney U test). The statistical tests were two-sided.

survival in both settings. Interestingly, in the preventive setting, 25% of the mice survived until the preplanned termination day of the study, and the majority of them had only minimal residual disease at the time of killing. Furthermore, the treatment increased the cortical and trabecular bone area and prevented tumor progression in skeletal sites in both settings.

In previous animal studies, no signs of bone marrow toxicity or body weight loss were observed with therapeutic doses of radium-223 dichloride (4), and even high doses did not completely inactivate the blood-producing cells (18). The high tolerance observed in experimental models has been reflected as a highly favorable side effect profile in clinical trials (5–8,19) and is likely due to the short range of the alpha particles, minimizing unwanted radiation to bone marrow cells surrounding bone metastases (20). The combination of exceptional efficacy and tolerability observed in animal studies calls for clinical evaluation of radium-223 dichloride in breast cancer patients with high risk of developing bone metastasis.
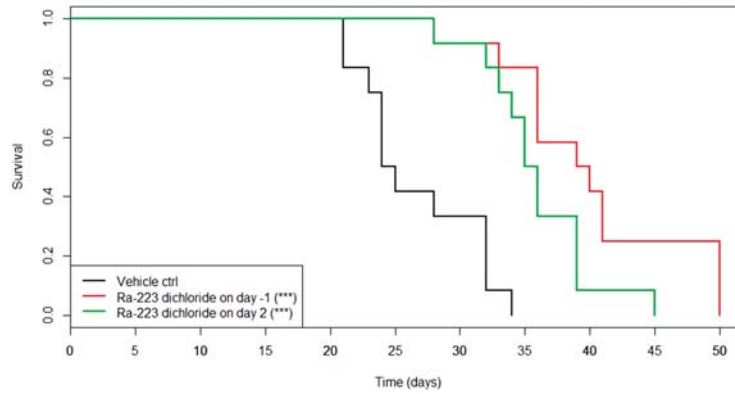
Number at risk

Vehicle ctrl	12	12	12	12	12	6	5	0	0	0	0
Ra-223 dichloride on day -1	12	12	12	12	12	12	12	11	6	5	3
Ra-223 dichloride on day 2	12	12	12	12	12	12	12	8	4	1	0

Figure 6. Survival (time to death) of the mice treated with radium-223 (Ra-223) dichloride (300 kBq/kg) one day before or two days after the intracardiac inoculation of MDA-MB-231(SA) cells. The mice were killed when they became paraplegic, had breathing difficulties, or their weight loss was >20%. ***$P < .001$ (Kaplan-Meier survival analysis, log-rank test). The statistical tests were two-sided. ctrl = control.
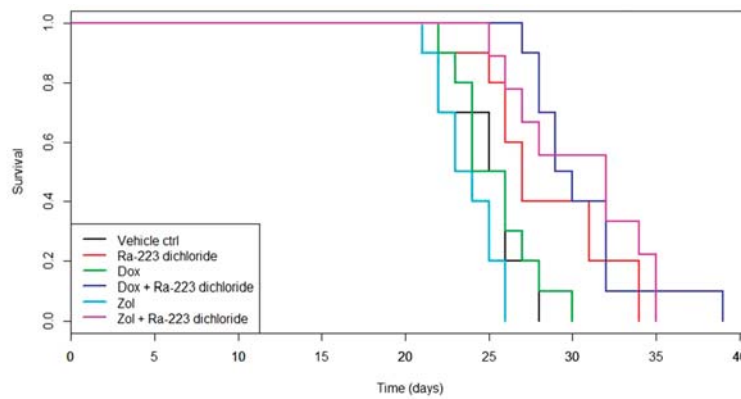
Number at risk

Vehicle ctrl	10	10	10	10	10	7	0	0	0
Ra-223 dichloride	10	10	10	10	10	9	6	0	0
Dox	10	10	10	10	10	8	1	0	0
Dox + Ra-223 dichloride	10	10	10	10	10	10	5	4	0
Zol	10	10	10	10	10	4	0	0	0
Zol + Ra-223 dichloride	9	9	9	9	9	9	6	2	0

Figure 7. Survival (time to death) of the mice with established bone metastases with single or combination treatment of radium-223 (Ra-223) dichloride, doxorubicin (Dox), and/or zoledronic acid (Zol). Ra-223 dichloride (300 kBq/kg) and/or zoledronic acid (0.1 mg/kg, subcutaneous) were administered on day 15 and doxorubicin (5 mg/kg, intraperitoneal) was administered once weekly (n = 9–10 per group). The mice were killed when they became paraplegic, had breathing difficulties, or their weight loss was >20%. Ra-223 dichloride extended time to death both as a monotherapy ($P = .039$) and in combination of either doxorubicin or zoledronic acid ($P < .001$ and $P = .004$, respectively). The combination treatments with doxorubicin and zoledronic acid did not differ from Ra-223 dichloride monotherapy ($P = .324$ and .168, respectively). Doxorubicin and zoledronic acid monotherapies had no effect on survival ($P = .715$ and .118, respectively). Statistical analysis for overall difference was performed by Kaplan-Meier survival analysis, log-rank test. The tests were two-sided.

Currently, the standard treatments for preventing skeletal-related events in cancer patients are bisphosphonates and denosumab (21). Our results demonstrate that radium-223 dichloride treatment is superior to zoledronic acid or doxorubicin in improving survival. Treatment with radium-223 dichloride in combination with either zoledronic acid or doxorubicin did not have a negative effect on survival, confirming the findings of a previous study where combination therapy with the bisphosphonate pamidronate did not affect the radium-223 dichloride-induced delay in time to onset of paralysis in a rat bone metastasis model (4). Furthermore, radium-223 dichloride treatment consistently reduced biochemical markers of bone metabolism in a phase II study on breast cancer

patients with bone metastases despite the concomitant use of bisphosphonate (8), and, correspondingly, we found a decrease in serum bone formation and resorption markers in the radium-223 dichloride/bisphosphonate combination treatment arm in mice. These findings indicate that binding of both radium-223 dichloride and zoledronic acid to bone does not undermine treatment efficacy and safety in primarily osteolytic metastases. The radium-223 dichloride phase III clinical trial on patients with hormone refractory prostate cancer and bone metastases (ALSYMPCA) met its primary endpoint of improving overall survival as compared to the placebo group receiving standard of care, including bisphosphonates (7). Of note, bisphosphonates, denosumab, and β-emitting

bone-targeting agents do not improve overall survival in this indication (21). A decrease in serum bone markers was also observed in prostate cancer patients (6).

There were some limitations to this study. For the widely used in vivo model of breast cancer bone metastasis, MDA-MB-231 cells are inoculated into young mice whose bones are still growing. This sets some limitations to the interpretation of the results. The mode of action of radium-223 dichloride at the cellular level remains to be elucidated in different animal models, including models reflecting the primarily osteoblastic nature of prostate cancer bone metastasis. In addition, further studies with larger treatment groups are needed to evaluate the possible additive or synergistic effects of radium-223 dichloride combination treatment with zoledronic acid or doxorubicin and other treatment modalities used in breast cancer patients with bone metastases.

In conclusion, radium-223 dichloride inhibits tumor growth and osteolysis in bone and increases survival in preventive, micrometastatic, and established breast cancer bone metastasis mouse models via dual action by targeting tumor growth and osteolysis, both important players in the destructive vicious cycle of bone metastasis. Phase II and III clinical trials of radium-223 dichloride in patients with HRPC showed an improvement in survival as compared to placebo, with no hematologic toxicity (5–7). Furthermore, a phase IIa study in breast cancer patients has revealed promising results (8). Taken together, these results strongly support the clinical development of radium-223 dichloride for breast cancer patients with bone metastases as well as for patients at high risk of developing bone metastases.

References

1. Gonzalez-Angulo AM, Morales-Vasquez F, Hortobagyi GN. Overview of resistance to systemic therapy in patients with breast cancer. *Adv Exp Med Biol*. 2007;608:1–22.
2. Coleman R, Gnant M, Morgan G, et al. Effects of bone-targeted agents on cancer progression and mortality. *J Natl Cancer Inst*. 2012;104(14):1059–1067.
3. Bruland OS, Nilsson S, Fisher DR, et al. High-linear energy transfer irradiation targeted to skeletal metastases by the alpha-emitter 223Ra: adjuvant or alternative to conventional modalities? *Clin Cancer Res*. 2006;12(20 Pt 2):6250s–6257s.
4. Henriksen G, Breistol K, Bruland OS, et al. Significant antitumor effect from bone-seeking, alpha-particle-emitting (223)Ra demonstrated in an experimental skeletal metastases model. *Cancer Res*. 2002;62(11):3120–3125.
5. Nilsson S, Franzen L, Parker C, et al. Bone-targeted radium-223 in symptomatic, hormone-refractory prostate cancer: a randomised, multicentre, placebo-controlled phase II study. *Lancet Oncol*. 2007;8(7):587–594.
6. Nilsson S, Strang P, Aksnes AK, et al. A randomized, dose-response, multicenter phase II study of radium-223 chloride for the palliation of painful bone metastases in patients with castration-resistant prostate cancer. *Eur J Cancer*. 2012;48(5):678–686.
7. Parker C, Nilsson S, Heinrich D, O'Sullivan JM, Fossa SD, Chodacki A, Wiechno PJ, Logue JP, Seke M, Widmark A, Johannessen DC, Hoskin P, Bottomley D, Coleman RE, Vogelzang NJ, O'Bryan-Tear CG, Garcia-Vargas JE, Shan M, Sartor AO. Updated analysis of the phase III, double-blind, randomized, multinational study of radium-223 chloride in castration-resistant prostate cancer (CRPC) patients with bone metastases (ALSYMPCA). Paper presented at ASCO Annual Meeting; June 2012; Chicago, IL.
8. Coleman R, Flamen P, Naume B, Jerusalem G, Garcia C, Piccart M, O'Bryan-Tear CG, Aksnes A-K. An open-label, phase IIa, non-randomized study of radium-223 in breast cancer patients with bone dominant disease no longer considered suitable for endocrine therapy. Paper presented at San Antonio Breast Cancer Symposium; December 2011; San Antonio, TX.
9. Fagerlund KM, Rissanen JP, Seppänen JA, Suominen MI, Vuorikoski H, Halleen JM. Validation of in vitro assays for identifying compounds that affect breast and prostate cancer cells. Paper presented at ASBMR Annual Meeting; September 2011; San Diego, CA.
10. Dang ZC, van Bezooijen RL, Karperien M, et al. Exposure of KS483 cells to estrogen enhances osteogenesis and inhibits adipogenesis. *J Bone Miner Res*. 2002;17(3):394–405.
11. Rissanen JP, Ylipahkala H, Fagerlund KM, et al. Improved methods for testing antiresorptive compounds in human osteoclast cultures. *J Bone Miner Metab*. 2009;27(1):105–109.
12. Guise TA, Yin JJ, Taylor SD, et al. Evidence for a causal role of parathyroid hormone-related protein in the pathogenesis of human breast cancer-mediated osteolysis. *J Clin Invest*. 1996;98(7):1544–1549.
13. Arguello F, Baggs RB, Frantz CN. A murine model of experimental metastasis to bone and bone marrow. *Cancer Res*. 1988;48(23):6876–6881.
14. Redon CE, Nakamura AJ, Sordet O, et al. Gamma-H2AX detection in peripheral blood lymphocytes, splenocytes, bone marrow, xenografts, and skin. *Methods Mol Biol*. 2011;682:249–270.
15. Salmon PL, Onischuk YN, Bondarenko OA, et al. Alpha-particle doses to cells of the bone remodeling cycle from alpha-particle-emitting bone-seekers: indications of an antiresorptive effect of actinides. *Radiat Res*. 1999;152(6 suppl):S43–47.
16. Ahlstrom M, Pekkinen M, Riehle U, et al. Extracellular calcium regulates parathyroid hormone-related peptide expression in osteoblasts and osteoblast progenitor cells. *Bone*. 2008;42(3):483–490.
17. Kassis AI, Adelstein SJ. Radiobiologic principles in radionuclide therapy. *J Nucl Med*. 2005;46(suppl 1):4S–12S.
18. Larsen RH, Saxtorph H, Skydsgaard M, et al. Radiotoxicity of the alpha-emitting bone-seeker 223Ra injected intravenously into mice: histology, clinical chemistry and hematology. *In Vivo*. 2006;20(3):325–331.
19. Nilsson S, Larsen RH, Fossa SD, et al. First clinical experience with alpha-emitting radium-223 in the treatment of skeletal metastases. *Clin Cancer Res*. 2005;11(12):4451–4459.
20. Elgqvist J. Targeted alpha therapy: part I. *Curr Radiopharm*. 2011;4(3):176.
21. Autio KA, Scher HI, Morris MJ. Therapeutic strategies for bone metastases and their clinical sequelae in prostate cancer. *Curr Treat Options Oncol*. 2012;13(2):174–188.

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