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Radiotoxicity of the Alpha-emitting Bone-seeker ^{223}Ra Injected Intravenously into Mice: Histology, Clinical Chemistry and Hematology

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Abstract. *Background: The alpha-emitter ^{223}Ra, which localizes in osteoblastic active zones, including on skeletal surfaces and in osteoblastic metastases, has recently been introduced as a potential therapeutic agent against skeletal metastases. Here, the adverse effects of high dosages in animals were investigated. Materials and Methods: Balb/c mice received intravenously (i.v.) either 1250, 2500, or 3750 kBq/kg of dissolved $^{223}RaCl_2$ and were followed in the initial toxicity phase. At the 4-week end-point, the animals were sacrificed and blood samples were collected to study the effects on clinical chemistry and hematological parameters. Selected organs were weighed and tissue samples examined by microscopy. Results: Treatment with ^{223}Ra caused a dose-related minimal to moderate depletion of osteocytes and osteoblasts in the bones. Furthermore, a dose-related minimal to marked depletion of the hematopoietic cells in the bone marrow, and a minimal to slight extramedullary hematopoiesis in the spleen and in the mandibular and mesenteric lymph nodes were observed. The LD_{50} for acute toxicity, defined as death within 4 weeks of receiving the substance, was not reached. Conclusion: This study demonstrated that high doses of the bone-seeker ^{223}Ra did not completely inactivate the blood-producing cells. The relatively high tolerance to skeletal alpha doses was probably caused by the surviving pockets of red bone marrow cells beyond the range of alpha particles from the bone surfaces, and the recruitment of peripheral stems cells.*

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Alpha-emitting radionuclides are currently being evaluated for applications in cancer therapy (1). They have the ability to deliver a target-specific radiation dose due to a short and well-defined track length (1, 2). The high linear energy transfer (high-LET) properties of alpha-emitters also bring other radiobiological dimensions into play, since high-LET radiation can be therapeutically effective in cells with low sensitivity to low-LET radiation and chemotherapy, as well as dormant tumor cells residing in the G_0-phase. This is because high-LET radiation is associated with a high proportion of non-rejoining double-strand breaks of the DNA (3). Another important aspect of alpha-emitters is their strong cytotoxicity at both low and high-dose rates (4). This may be an important aspect since modest-dose rates are believed to limit the therapeutic efficacy of beta-emitting radionuclides (5).

Only a few alpha-emitters are considered suitable candidates for therapeutic use in cancer patients. Clinical trials have been conducted with ^{213}Bi ($t_{1/2}$=46 min), ^{211}At ($t_{1/2}$=7.2 h) and ^{223}Ra ($t_{1/2}$=11.43 days), while ^{212}Pb ($t_{1/2}$=10.6 h), ^{212}Bi ($t_{1/2}$=60 min), ^{225}Ac ($t_{1/2}$=10.0 days) and ^{227}Th ($t_{1/2}$=18.7 days) have been studied preclinically (1, 6). In addition, ^{224}Ra ($t_{1/2}$=3.66 days) has been used for pain palliation in non-cancer bone diseases for a number of years (7). Recent investigations have indicated that the alpha-emitter ^{223}Ra may have therapeutic potential against skeletal metastases (8, 9). The ^{223}Ra series produces four alpha- and two beta-particles (Table I). Intravenously-administered ^{223}Ra, in the form of dissolved radium chloride, has a very high affinity for the calcium hydroxyapatite crystals in bone and osteoblastic skeletal metastases. A phase I study, conducted in patients with skeletal involvement from breast and prostate cancer,

Table I. *Principal emissions of ^{223}Ra and decay progeny (radon-219, polonium-215, lead-211, bismuth-211 and thallium-207).*

Nuclide (half-life)	Decay	Yield (Bq^{-1} sec^{-1})	Mean energy (MeV)	Dose constant Δ (Gy kg Bq^{-1} sec^{-1})
^{223}Ra (11.43 days)	α	0.525	5.72	$4.80x10^{-13}$
		0.242	5.61	$2.17x10^{-13}$
		0.095	5.75	$8.74x10^{-14}$
		0.091	5.54	$8.07x10^{-14}$
		0.023	5.43	$2.00x10^{-14}$
		0.009	5.87	$8.45x10^{-15}$
	X or gamma	0.154	0.081	$2.00x10^{-15}$
		0.256	0.084	$3.44x10^{-15}$
		0.030	0.094	$4.51x10^{-16}$
		0.057	0.095	$8.66x10^{-16}$
		0.030	0.098	$4.70x10^{-15}$
		0.012	0.122	$2.34x10^{-16}$
		0.037	0.144	$8.52x10^{-16}$
		0.060	0.154	$1.48x10^{-15}$
		0.136	0.269	$5.85x10^{-15}$
		0.037	0.324	$1.92x10^{-15}$
		0.026	0.338	$8.79x10^{-16}$
		0.013	0.445	$9.26x10^{-16}$
^{219}Rn (3.96 sec)	α	0.808	6.82	$8.82x10^{-13}$
		0.115	6.55	$1.21x10^{-13}$
		0.075	6.43	$7.72x10^{-14}$
	X or gamma	0.099	0.271	$4.30x10^{-15}$
		0.065	0.402	$4.25x10^{-15}$
^{215}Po (1.78 msec)	α	1.000	7.39	$1.18x10^{-12}$
^{211}Pb (36.1 min)	β	0.997	0.449	$7.16x10^{-14}$
	X or gamma	0.038	0.405	$2.46x10^{-15}$
		0.014	0.427	$9.56x10^{-16}$
		0.028	0.832	$3.73x10^{-15}$
^{211}Bi (2.17 min)	α	0.834	6.62	$8.83x10^{-13}$
		0.164	6.28	$1.65x10^{-13}$
	X or gamma	0.008	0.071	$9.02x10^{-17}$
		0.013	0.073	$1.52x10^{-16}$
		0.130	0.351	$7.30x10^{-15}$
^{207}Tl (4.77 min)	β	1.00	0.491	$7.86x10^{-14}$
^{207}Pb	stable			

From Nuclide Explorer data sheets, Institute for Transuranium Elements, Karlsruhe, Germany. European Commission, Joint Research Centre, Program Version 1.00 (1999).

indicated promising clinical effects of ^{223}Ra including pain palliation in the majority of patients and a consistent reduction in alkaline phosphatase levels (10). Gamma scintigraphy, by use of the photons associated with the ^{223}Ra series, indicated a skeletal uptake similar to that observed with ^{99m}Tc-MDP, including an increased concentration in osseous metastases. The scintigrams also showed that the unbound compound is mainly cleared *via* the intestinal route. The toxicity in patients was generally mild, even at doses significantly higher than the level required for antitumor activity in a skeletal metastases model in animals (8, 10). The animal and human studies evaluated doses of about 300 kBq/kg or less.

Here, a toxicity study, which included blood profiles and histological and pathological examinations of tissues, in mice that had received *i.v.*-administered doses surpassing previous levels by four to twelve times, was conducted to explore the acute and subacute effects in the first month after treatment.

Materials and Methods

Production of ^{223}Ra. Radium-223 was produced by using AC-resin to trap ^{227}Ac and ^{227}Th and to subsequently elute ^{223}Ra in 1 M HCl, as previously described (11). The Ra was thereafter absorbed on a cation exchange column (AG 50W-X12, Biorad, Hercules, CA, USA) and subsequently eluted in 8 M HNO_3. Following evaporation to dryness, ^{223}Ra was dissolved in a sodium chloride/sodium citrate solution.

^{223}Ra in concentrated form was shipped to The Radiopharmacy Laboratories at the Institute for Energy Technology, Kjeller, Norway, where the raw product was sterile-filtered and the pH and activity concentration were adjusted to 7.4 and 500 kBq/ml, respectively, to yield a pathogen-free and pyrogen-free product, supplied in a 20-ml ready-to-use glass vial with a rubber cap.

Radioactivity measurements. The ^{223}Ra radioactivity was determined using a calibrated Ge-detector (Gem 15-P, EG&G Ortec, Oak Ridge, TN, USA) as a primary detector. ^{223}Ra samples, in equilibrium with daughters, with activity levels determined by the Ge-detector, were used in the calibration of a dose calibrator (CRC-127R, Capintec Inc., Ramsey, NJ, USA). The final product vial was measured in the dose calibrator.

Mice. SPF Balb/C mice, male and female, were supplied by Taconic M&B, Ejby, Denmark. An acclimatization period of 5 days allowed rejection of animals in poor condition or with abnormal body weight. On arrival, the animals were in the weight range of 17-27 g.

Animal housing. The study was performed in dedicated animal rooms supplied with filtered air with a relative humidity of 40-70%. The temperature was kept within the range of 18-24˚C. The rooms were illuminated from 06:00 to 18:00. The mice were kept in transparent polycarbonate (Macrolone type III) cages with a floor area of 810 cm^2 and bedding of softwood sawdust, one animal per cage. The animals received a diet of pellets for growing animals, Altromin 1314 (Chr. Petersen AS, Ringsted, Denmark), and domestic quality drinking water, acidified to pH 2.5 using HCl to prevent microbial contamination, *ad libitum*.

Animal treatment. Prior to the study, the animals were allocated to four groups, each group including five animals of each sex. A punched earmark identified each animal. The study animals received either a control injection without radioactivity (0.9%

NaCl), or 1250, 2500 or 3750 kBq/kg of body weight of ^{223}Ra. The product, Alpharadin™ (0.5 MBq/ml $^{223}RaCl_2$ dissolved in 30 mM sodium citrate/ 0.9% NaCl) was supplied as a sterile and non-pyrogenic solution in ready-to-use glass vials. The Alpharadin™ was administered by injection into the tail vein using injection volumes of 2-6 μl/g of body weight. The investigation was performed in accordance with the OECD principles of good laboratory practice. The animals were treated according to international guidelines for animal welfare.

Follow-up. The animals were followed for 30 days after receiving ^{223}Ra. The body weight was measured on day 1 and weekly thereafter. The weight at the time of necropsy was also measured. On a per cage basis, the food consumption was measured weekly. The ^{223}Ra solution was administered on day 1 and, in general, the animals were sacrificed on day 30. Any visible signs of health deterioration or abnormal behavior were registered on a daily basis. In cases of deteriorating health, the animals were sacrificed before day 30 if required.

Terminal procedures. On day 30, prior to termination, the animals were weighed, examined externally and anesthetized with Hypnorm and Dormicum supplemented with an intraperitoneal injection of barbiturate (penthothal natrium), if needed. At termination, during anesthesia, blood samples were drawn from the orbital venous plexus. Approximately 300 μl of blood was collected in EDTA-coated tubes and used for hematological analyses. As much blood as possible was collected in serum tubes for clinical chemistry analyses. One or two mice per group were sequentially sacrificed by exsanguination and necropsied. The following hematological parameters were measured: hemoglobin (Hb), red blood cell count (RBC), hematocit (HT), mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), white blood cell count (WBC), differential leucocyte count (NEUTRO, LYMPHO, EOS, BASO, MONO) and platelet count (Plt).

Clinical chemistry. The following parameters were evaluated: alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), alkaline phosphatase (ALP), bilirubin (total) (BILI), gamma-glutamyl transferase (GGT), cholesterol (CHOL), triglycerides (Trig), carbamide (UREA), creatinine (CREAT), glucose (GLUC), sodium (Na), potassium (K), calcium (Ca), magnesium (Mg), inorganic phosphorus (P), chloride (Cl), protein (total) (PROT), albumin (ALB), globulin (G) and the albumin/globulin (ALB/G) ratio.

Necropsy and organ sample preservation. An initial macroscopic examination was performed following opening of the cranial, thoracic and abdominal cavities, by observing the appearances of tissues and organs *in situ*. Macroscopic abnormalities were reported in the PathData electronic record with details such as location, size, shape, color *etc.*

The eyes and testes were fixed in Davidson's fixative and Bouin's fixative, respectively. All other organ and tissue samples were fixed in 4% formaldehyde in phosphate buffer (pH ~ 7) and representative specimens were used for histological processing. These specimens were embedded in paraffin, cut to a nominal thickness of approximately 5 μm and stained with hematoxylin and eosin. Paired organs were processed together and examined under a light microscope. Pathological findings were entered directly into a computer using the PathData electronic system.

Histological alterations were graded on a five-grade system: Grade 1, minimal/very few/very small; Grade 2, slight/few/small; Grade 3, moderate/moderate number/moderate size; Grade 4, marked/many/large; Grade 5, massive/extensive number/extensive size.

In the case of ungradable findings, the following term would be used: present, finding present/severity not scored.

The following organs and tissues were subjected to histological examination: i) All organs and tissues from the control group and the high-dose group. ii) Tissues and samples showing treatment-related changes in the high-dose group were evaluated in the other treatment groups. iii) All organs and tissues from animals dying or sacrificed during the follow-up period of the study. iv) All gross lesions from all animals.

Statistics. Group mean values and standard deviations were calculated. Each continuous variable was tested for homogeneity of variance using Bartlett's test. If any significant differences were detected, possible inter-group differences were evaluated using Dunnett's test. If the variance was heterogeneous, each variable was tested for normality by employing the Shapiro-Wilk method. In the case of normal distribution, possible inter-group differences were determined by the Student's *t*-test. Otherwise the possible inter-group differences were assessed by the Kruskal-Wallis's test. If any significant inter-group differences were detected, the subsequent identification of the groups was carried out with the Wilcoxon Rank Sum Test. The level of significance was defined as $p<0.05$.

Results

Survival. Three animals, all females, did not complete the follow-up period. One animal from each of the 2500 kBq/kg and the 3750 kBq/kg groups was sacrificed due to clinical signs of piloerection, hunched posture and passive behavior. One additional animal in the 3750 kBq/kg dose group, that had shown similar behavior, was found dead. All other animals survived the study period. Adverse clinical signs were not observed among the other animals.

Body weight changes vs. injected dose. The body weight curves of the animals are shown in Figure 1. A significantly reduced weight gain was observed for males in the 2500 and 3750 kBq/kg groups *vs.* the control group. On comparing the 1250 kBq/kg group with the control group, the difference in weight gain was not statistically significant. In the female groups, only a tendency of reduced weight gain was seen without being statistically significant.

Food consumption. The food consumption was significantly reduced in the male groups receiving ^{223}Ra *vs.* the control in the third and fourth weeks of follow-up. Among the female groups, no significant reduction was observed.

Hematology. A significant increase in the MCV was observed for the 3750 kBq/kg male group and the 1250 and 3750 kBq/kg female groups. The lymphocytes and WBC were significantly reduced in the male groups

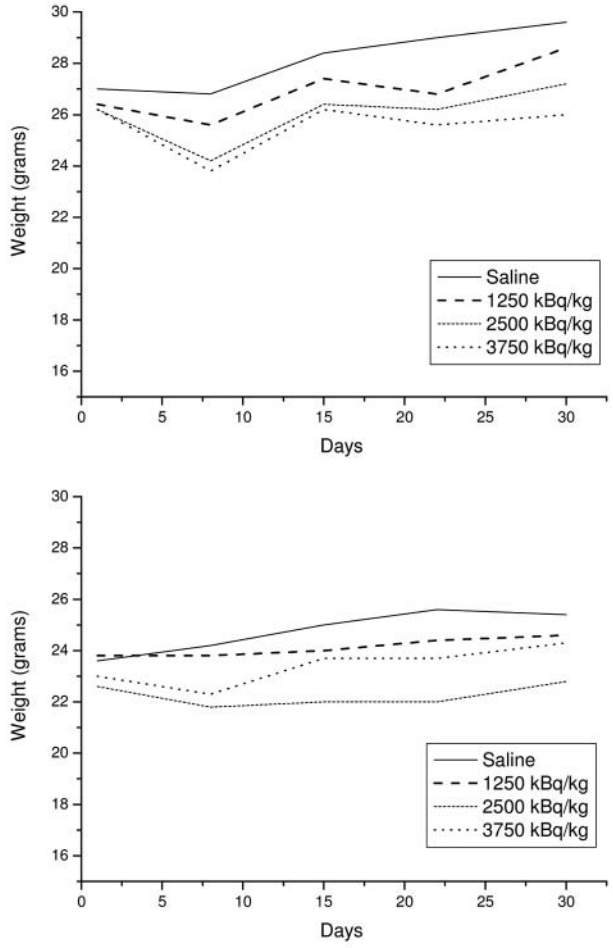

Figure 1. *Body weight curves for male (upper graph) and female (lower graph) SPF Balb/C mice after treatment.*

Table II. *Effects on the spleen of SPF Balb/C mice measured on day 30 after i.v. injection of ^{223}Ra.*

Dosage (kBq/kg)	0 (Saline)		1250		2500		3750	
Gender	M	F	M	F	M	F	M	F
Number of animals	5	5	5	5	5	5	5	5
SPLEEN								
Hematopoiesis increased								
Total	-	-	5	5	5	4	5	2
Grade 1	-	-	2	2	-	-	1	-
Grade 2	-	-	3	3	5	4	4	2
SPLEEN								
Mean group weight±SD (mg)	102 ±19	114 ±14	139 ±19	147 ±23	156 ±13	146* ±11	146 ±17	131§ ±27

*For the four animals surviving the follow-up period.
§For the three animals surviving the follow-up period. M-male, F-female.

receiving 1250 and 2500 kBq/kg and the female group receiving 2500 kBq/kg, while reduced RBC was observed in the female group receiving 1250 kBq/kg. The Plt was significantly reduced in the 2500 and 3750 kBq/kg female groups. In the male animals, only a tendency towards reduced RBC was seen.

Clinical chemistry. A significant reduction in the ALP levels was seen in the male groups receiving ^{223}Ra *vs.* the control. The ALP levels also tended to be reduced in the female groups. No particular changes were noticed for the other chemical parameters evaluated.

Macroscopic examination of tissues. The weight of the organs showed some variations *vs.* the control, but without any striking differences. The exception was the spleen, which showed a significant increase in size for the ^{223}Ra groups *vs.* the controls (Table II). The three animals that did not complete the follow-up period had organ weights for the liver, spleen, ovaries, thymus and uterus which were typically below 50% of those of the animals surviving the follow-up period.

Microscopic findings. Changes related to ^{223}Ra were observed in the bones, bone marrow, spleen, and the mandibular and mesenteric lymph nodes. A dose-related depletion of the osteoblasts and osteocytes was found in the bones of almost all animals receiving ^{223}Ra. The depletion was more pronounced in the trabecular bone of the metaphysis of the femur (Figure 2) and the cancellous bone of the sternum. Some depletion was also observed in the compact bone in the shaft of the femoral diaphysis and in the cortical bone of the sternum (Table III).

A proliferative fibro-osseous lesion (minimal to slight) was observed in some animals in the two highest ^{223}Ra dose-groups, which could reflect a reactive change to the treatment. A dosage-related minimal to marked depletion of the hematopoietic cells was seen in almost all animals of both sexes that had received ^{223}Ra. The depletion was more pronounced in the metaphysis and epiphysis of the femur and around the cancellous bone of the sternum (Table IV). Minimal to slight extramedullary hematopoiesis was observed in the spleen in all treated groups (Table II). Signs of extramedullary hematopoesis were also observed in the mandibular and mesenteric lymph nodes in treated animals. This is an adaptive response.

The No-Observed-Adverse-Effect-Level (NOAEL) was considered to be below 1250 kBq/kg, since some adverse effects were observed at all dose-levels studied. The LD_{50} for acute toxicity was not reached.

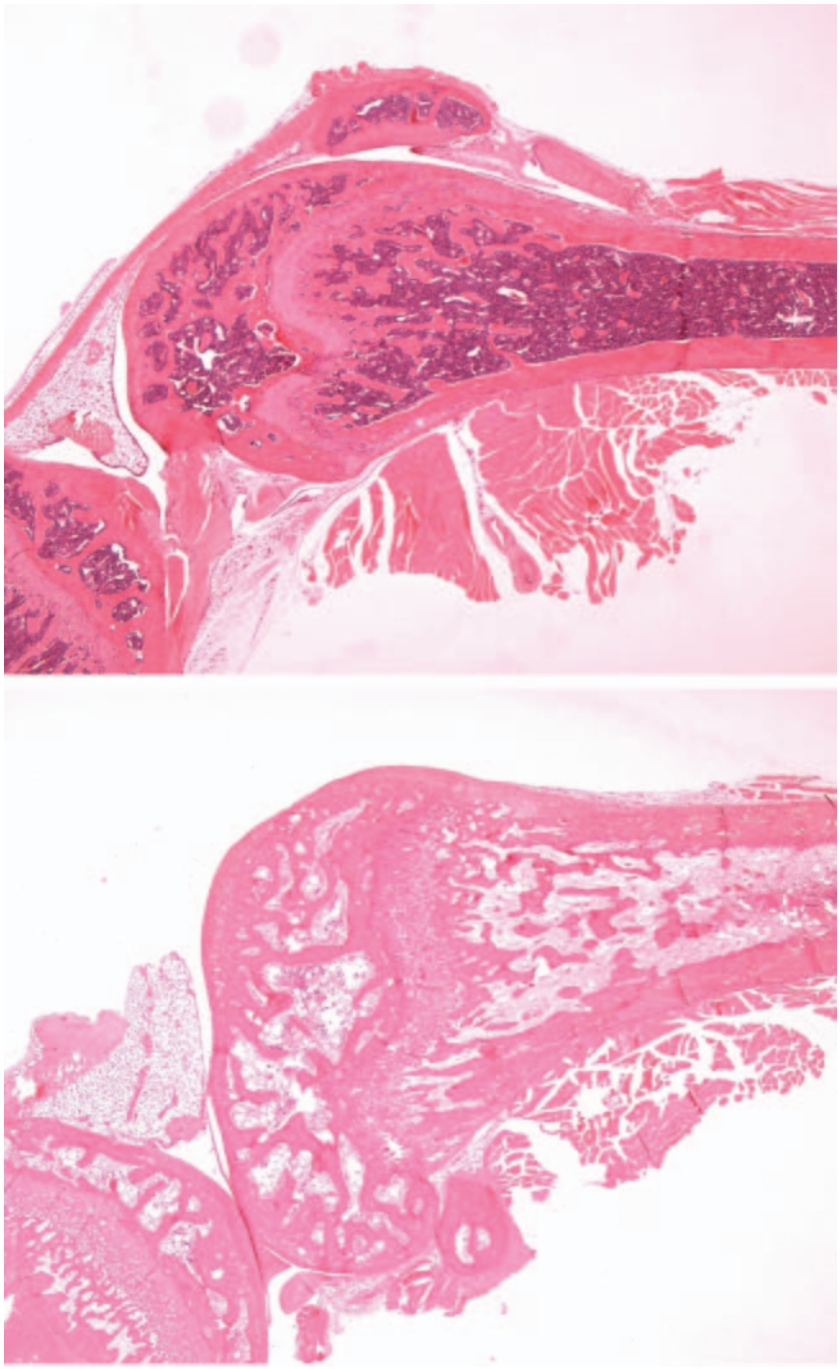

Figure 2. *Micrographs of SPF Balb/C femurs on day 30 after receiving i.v. injected saline (upper frame) or 3750 kBq/kg ^{223}Ra (lower frame).*

Discussion

The current study was designed to investigate the short-term acute toxicity from ^{223}Ra and its decay products. Thirty days of follow-up was considered to be a relevant time-period as the major dose fraction is delivered in 2 weeks, although the effective half-life would be somewhat less than the physical half-life of 11.43 days due to some biological clearance of the radionuclide (9). This follow-up period was also expected to encompass the time of nadir for blood values, which was found to be 2-4 weeks after administration in humans (10). It is noteworthy that

Table III. *Evaluation of changes in bone and bone-forming cells of SPF Balb/C mice measured on day 30 after i.v. injection of ^{223}Ra.*

Dosage (kBq/kg)	0 (Saline)		1250		2500		3750	
Gender	M	F	M	F	M	F	M	F
Number of animals	5	5	5	5	5	5	5	5
FEMUR								
Depletion osteocytes/ osteoblasts								
Total	-	-	5	5	5	4	5	4
Grade 1	-	-	5	5	1	4	-	3
Grade 2	-	-	-	-	4	-	5	1
STERNUM								
Depletion osteocytes/ osteoblasts								
Total	-	-	4	3	5	4	5	3
Grade 1	-	-	4	3	-	-	-	-
Grade 2	-	-	-	-	5	4	3	2
Grade 3	-	-	-	-	-	-	2	1
FIBRO-OSSEOUS LESION								
Total	-	-	-	-	3	1	4	1
Grade 1	-	-	-	-	3	1	2	1
Grade 2	-	-	-	-	-	-	2	-

Table IV. *Effects on the bone marrow of SPF Balb/C mice measured on day 30 after i.v. injection of ^{223}Ra.*

Dosage (kBq/kg)	0 (Saline)		1250		2500		3750	
Gender	M	F	M	F	M	F	M	F
Number of animals	5	5	5	5	5	5	5	5
FEMUR								
Depletion of hematopoietic cells								
Total	-	-	5	5	5	5	5	4
Grade 1	-	-	1	2	-	-	-	-
Grade 2	-	-	4	3	1	3	4	1
Grade 3	-	-	-	-	4	1	1	1
Grade 4	-	-	-	-		1	-	2
STERNUM								
Depletion of hematopoietic cells								
Total	-	-	4	2	5	5	5	3
Grade 1	-	-	4	2	-	2	-	-
Grade 2	-	-	-	-	-	2	-	-
Grade 3	-	-	-	-	1	1	-	-
Grade 4	-	-	-	-	4	-	5	3

the majority of the animals survived the 30-day follow-up period even at the highest dose level of 3750 kBq per kg of body-weight. This activity level corresponds to an average skeletal dose of 79 Gy for the 30-day period, assuming the biodistribution and kinetics as described for Balb/C mice (9). This would give an initial dose rate of approximately 4.7 Gy/day. The average bone surface alpha-particle dose would be about seven to ten times the average skeletal dose, *i.e.*, a total of 553-790 Gy (initial dose rate: 32.6-46.6 Gy/day). At this rather extreme alpha-particle dose-level, the beta-particle and gamma dose component from the ^{223}Ra series would come into play, accounting for an average of 3.6 % *vs.* the alpha-particle dose, *i.e.*, in the order of 2.8 Gy to the bone. This was delivered at a relatively low dose-rate (initially approximately 0.17 Gy/day), but could probably affect the bone marrow cells since beta-radiation from a surface-deposited source would reach the marrow cells that are out of range of the alpha-particles. As indicated by previous rat studies (8), there may be some uptake of ^{223}Ra in the bone marrow in the early distribution phase, and this could also be a factor which could cause suppression of the bone marrow cells outside the range of the bone surface-deposited alpha-emitters. The relatively moderate effect on the blood parameters that was observed at these extreme doses were encouraging with respect to the clinical use of ^{223}Ra as a bone-seeking agent and were possibly linked to compensatory responses from peripheral stem cells.

The ALP isoenzyme was greatly affected by the treatment, indicating that zones of high osteoblastic activity were targeted. This could be beneficial in treatment of patients with osteoblastic skeletal metastases, since these metastases are distinguished by their elevated osteoblastic activity compared to normal bone.

On comparing the toxicity of ^{223}Ra to that of other alpha-emitters, one has to take into account differences in the half-lives, biological retention and micro-distribution of the radionuclides. In terms of injected activity, ^{223}Ra is assumed to be very toxic compared to other alpha-emitters considered for radionuclide therapy, partly because of its significant half-life and partly because of the decay chain, which produces a total of four alpha-particles and two beta-particles. Also, the fact that ^{223}Ra is a bone-seeker has previously raised questions about its safety because of possible bone marrow toxicity. At therapeutically relevant doses, the beta and gamma components are not expected to play a significant role in terms of toxicity, since the activity concentration administered, being limited by high-energy radiation delivered from the alphas, would be too low to produce

radiotoxic doses of beta- and gamma-radiation. In terms of half-life and number of alpha-particles in the decay chain, ^{225}Ac has several similarities with ^{223}Ra, *i.e.*, the half-life is 87.5% of that of ^{223}Ra and the decay chain gives rise to four alpha-particles. Some toxicity data exist for the use of ^{225}Ac in mice, but not as a bone-seeker. When ^{225}Ac was used as a radioimmunoconjugate, toxic death within the first 4 weeks occurred at an activity level of about 750 kBq/kg (12). This indicates that alpha-emitting sources, with a prolonged circulation half-life in blood, may be more toxic than sources with long retention half-lives in the skeleton.

In conclusion, the acute toxicity of high activity levels of the bone-seeking alpha-emitter ^{223}Ra was surprisingly modest in Balb/C mice. This is an indicator of the individual behavior of alpha-emitting compounds and that the toxicity may depend more on the microscopic source distribution rather than the number of radioactive transformations taking place *in vivo*. The data are notable considering the extreme radiation doses, in the order of several hundred Grays, delivered to the bone surfaces. This demonstrates that high radiation doses delivered to the skeletal surfaces do not completely inactivate the blood-producing cells when using ^{223}Ra as a bone-seeking alpha-emitter. The high tolerance for skeletal alpha doses could be caused by recruitment of peripheral stems cells. This would be plausible since *i.v.*-injected radium rapidly clears the blood (>90% in an hour), causing very limited alpha exposure to cells circulating in the blood. The data presented in this study may help in predicting which tissues are at risk from alpha-particle exposure when ^{223}Ra is used clinically.

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