First *In Vivo* Evaluation of Liposome-encapsulated ^{223}Ra as a Potential Alpha-particle-emitting Cancer Therapeutic Agent

THORA J. JONASDOTTIR[1], DARRELL R. FISHER[2], JØRGEN BORREBÆK[3], ØYVIND S. BRULAND[4] and ROY H. LARSEN[3]

[1]*The Norwegian School of Veterinary Science, Department of Companion Animal Clinical Sciences, Small Animal Section, P.O. Box 8146 Dep., N-0033 Oslo, Norway;*
[2]*Pacific Northwest National Laboratory, Richland, Washington, 99354 U.S.A.;*
[3]*Algeta ASA, P.O. Box 54 Kjelsås, N-0411 Oslo;*
[4]*Department Group of Clinical Medicine, University of Oslo and Department of Oncology, The Norwegian Radium Hospital, Montebello, N-0310 Oslo, Norway*

Abstract. *Background: Liposomes carrying chemotherapeutics have had some success in cancer treatment and may also be suitable carriers for therapeutic radionuclides. This study was designed to evaluate the biodistribution and to estimate the radiation doses of the alpha emitter ^{223}Ra loaded into pegylated liposomes in selected tissues. Materials and Methods: ^{223}Ra was encapsulated in pegylated liposomal doxorubicin (PLD) by ionophore-mediated loading. The biodistribution of liposomal ^{223}Ra was compared to free cationic ^{223}Ra in Balb/C mice. Results: Liposomal ^{223}Ra circulated in the blood with an initial half-life in excess of 24 hours, which agreed well with that reported for PLD in rodents, while the blood half-life of cationic ^{223}Ra was considerably less than an hour. When liposomal ^{223}Ra was catabolized, the released ^{223}Ra was either excreted or taken up in the skeleton. This skeletal uptake increased up to 14 days after treatment, but did not reach the level seen with free ^{223}Ra. Pre-treatment with non-radioactive PLD 4 days in advance lessened the liver uptake of liposomal ^{223}Ra. Dose estimates showed that the spleen, followed by bone surfaces, received the highest absorbed doses. Conclusion: Liposomal ^{223}Ra was relatively stable in vivo and may have potential for radionuclide therapy and combination therapy with chemotherapeutic agents.*

The application of alpha-emitting radionuclides for targeted tumor therapy is an exciting new field of cancer research. A number of candidate compounds have been clinically tested (1-3), and recently the first phase II study with radium-223 (^{223}Ra), as a dissolved dichloride salt, in the targeted therapy of calcified bone metastases was completed (4).

^{223}Ra is a promising radionuclide for tumor therapy because it can be produced in large quantities relatively inexpensively (compared to other alpha emitters), its half-life of 11.4 days allows sufficient time for preparation, shipment and handling (4), and it has a favorable decay chain with short-lived daughters that emit three additional alpha-particles (Table I). To extend the use of ^{223}Ra to applications other than bone metastases, it would have to be incorporated in a carrier compound with tumor-seeking properties. However, efforts to prepare conjugates, *e.g.,* for radioimmunotherapy, with proven *in vivo* applicability have been unsuccessful to date (5).

Liposomes carrying a chemotherapeutic agent have been successfully introduced into cancer therapy (6). Recently, we presented a study demonstrating that liposome-encapsulated radium and actinium could be prepared from pre-formed liposomes by ionophore-mediated loading (7). The ^{223}Ra was incorporated with a good loading yield and was stably retained for several days when incubated at 37˚C in serum.

The current work represents the first evaluation of liposome-encapsulated ^{223}Ra *in vivo*. Biodistribution and blood clearance measurements were performed with a liposome formulation (Caelyx™/Doxil™; Schering Plough, Kenilworth, NJ, USA) having well-characterized properties *in vivo*. The *in vivo* behavior and stability of liposome-encapsulated ^{223}Ra was also compared to that of dissolved $^{223}RaCl_2$.

Materials and Methods

Preparation of radionuclide. ^{223}Ra was produced from ^{227}Ac ($t_{1/2}$=22 years) and ^{227}Th ($t_{1/2}$=18.7 days) according to previously described methods (8). Briefly, ^{223}Ra was separated from the actinides ^{227}Ac and ^{227}Th by the use of Ac-resin, followed by cation exchange chromatography and filtration through a sterile filter

Correspondence to: Roy H. Larsen, Ph.D., Algeta ASA, P.O. Box 54 Kjelsås, N-0411 Oslo, Norway. Tel: +47 23007990, Fax: +47 23007991, e-mail: roy.larsen@algeta.com

Key Words: Alpha-emitting ^{223}Ra, pegylated liposomes, *in vivo* evaluation.

Table I. *Summary of effective energy and dose constants for ^{223}Ra and progeny for all emissions combined.*

Radionuclide (half-life, decay mode)	Effective energy[1] (MeV)	Dose constant Δ (Gy kg Bq^{-1} s^{-1})
^{223}Ra (11.43 days α)	5.99[1]	9.58x10^{-13}
	5.56[2]	8.90x10^{-13}
^{219}Rn (3.96 sec α)	6.95[1]	1.11x10^{-12}
	6.72[2]	1.08x10^{-12}
^{215}Po (1.78 microsec, α)	7.53[1]	1.20x10^{-12}
	7.39[2]	1.18x10^{-12}
^{211}Pb (36.1 min, β)	0.518[1]	8.29x10^{-14}
^{211}Bi (2.17 min, α)	6.75[1]	1.08x10^{-12}
	6.57[2]	1.05x10^{-12}
^{207}Tl (4.77 min, β)	0.494[1]	7.90x10^{-14}
Total	28.2[1]	4.5x10^{-12}
	26.4[2]	4.2x10^{-12}

From Nuclide Explorer data sheets, Institute for Transuranium Elements, Karlsruhe, Germany. European Commission, Joint Research Centre, Program Version 1.00 (1999).
[1]Includes alpha, beta, photon, X-ray and electron energies.
[2]Includes only alpha particle energies.
Branching of less than 1% was not considered.

(Millex GV 0.22 μm; Millipore Ireland B.V., Tullagreen, Carrigtwohill, Co. Cork, Ireland). The properties of ^{223}Ra are presented in Table I.

Loading of ^{223}Ra into liposomes. Pegylated liposomal doxorubicin (PLD), Caelyx™, which consists of liposomes averaging about 80 nm in diameter, and corresponding to 2 mg doxorubicin per mL, was used. The liposomes were subjected to buffer exchange with a buffer containing 20 mM HEPES and 300 mM sucrose, adjusted with NaOH to pH 7-8. The lipsomes were added to the buffer and concentrated three times in a centrifuge concentration cartridge (Millipore UFV2BTK10, 30 KNMWL membrane, 15 mL maximum volume; Millipore, Bedford, IL, USA) inserted into an Eppendorf Centrifuge 5810R (Eppendorf, Germany), operated at 20˚C and 1400 relative centrifugal force (rcf). The calcium-ionophore (Calcimycin; Sigma, St. Louis, MO, USA) was dissolved to a concentration of 1 mg per mL in chloroform. Approximately 15 μL was added to a 2-mL vial and the chloroform evaporated off in a stream of argon to generate a thin film of Ca-ionophore on the inner surface of the vial. The ^{223}Ra solution was diluted in a solution of sucrose (300 mM) and HEPES (20 mM). The solution was preheated to 60˚C and 200 μL of concentrated PLD was added. The loading mixture was shaken for 45 minutes on a Thermomixer (Eppendorf) followed by the addition of 200 μL 10 mM EDTA solution. After further shaking for 5-10 minutes, the mixture was transferred to a Sephadex G-25 PD-10 column (Pharmacia, Lund, Sweden) and eluted with 0.9% NaCl solution. The fraction containing the liposomes, recognized by its red color, was collected, and 10% of a 10x Dulbecco's Modified Eagle's Medium (Sigma) was added. Subsequently, inside a sterile hood, the liposomes were sterile-filtered through a 0.22-μm sterile filter (Millex GV 0.22 μm; Millipore Ireland B.V.) into a 10-ml sterile vial that was immediately capped with a metal-and-rubber cap. The vial was stored for at least 3 hours to reach equilibrium between ^{223}Ra and its daughter nuclides. A Capintec CRC-15R dose calibrator (Capintec Inc., Ramsey, NJ, USA), which was calibrated for the ^{223}Ra decay chain in equilibrium with the daughter nuclides, was used to quantify the radioactivity.

Animal model. Healthy white Balb/C mice of both genders (around 12 weeks old and weighing 18-25 g) were used to determine the biodistribution of liposomal ^{223}Ra. The research protocol fulfilled the requirements of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123) issued by the Council of Europe and approved by The Norwegian Animal Research Authority. The mice were housed under standard condition with access to food and water *ad libitum*.

Biodistribution study. The mice were divided into four test groups. Each group consisted of six or seven animals of both genders. Six mice in each group received an intravenous (*i.v*) pre-treatment with doxorubicin liposomes (8.1 mg/kg) 4 days prior to the main treatment with 375 kBq/kg ^{223}Ra and doxorubicin liposomes (0.9 mg/kg) *i.v.* to reduce the uptake of liposomal ^{223}Ra by the reticulo-endothelial system and to achieve the highest possible blood: liver ratio. One mouse in each of groups 2, 3 and 4 was left untreated and used to measure the possible re-assimilation of the liposomal ^{223}Ra from the feces or bedding. For each group, all available blood was drawn by cardiac puncture under anesthesia (Enflurane; Abbott Laboratories, Ltd., Queen Borough, Kent, UK) followed by euthanasia. On dissection, all assessable blood was carefully wiped off the organs. At least 12 hours after dissection, when ^{223}Ra would be in equilibrium with the daughter nuclides, the radioactivity in the blood, urine and different tissues was measured in a Crystal II Multidetector System (Packard Instrument Company, Downers Grove, IL, USA), and was compared with the injection standards at 1 hour, 24 hours, 6 days and 14 days post-injection with liposomal ^{223}Ra.

Comparative biodistribution study of free ^{223}Ra. To study the difference between liposomally-encapsulated ^{223}Ra and free ^{223}Ra, one group of mice was injected *i.v.* with dissolved $^{223}RaCl_2$. Five animals were sacrificed at 1 hour, 24 hours and 14 days post-injection, tissue samples were removed, and the radioactivity per unit tissue mass was determined, as described above for the biodistribution study on liposomal ^{223}Ra. Localization indices (LI) for an organ or tissue at a specific time were calculated for liposomal *vs.* free ^{223}Ra by the following equation:
LI=[% administered activity/g tissue for liposomal ^{223}Ra]/[% administered activity/g tissue for free ^{223}Ra].

Dosimetry calculations. Radiation absorbed doses were calculated for the major organs, tissues, and for the whole body using methods previously described (9). An equilibrium dose-constant of 4.51x10^{-12} Gy kg/Bq-sec was used, which assumes equilibrium between ^{223}Ra and its decay products in each tissue. An energy-absorbed fraction of 1.0 was assumed, since the primary contributor to the dose is the short-range alpha-particles, and since the relative

Table II. *Biodistribution, as percent of administered activity per gram of tissue[1], of liposomal ^{223}Ra in Balb/C mice (corrected for decay).*

Tissue	1 hour	24 hours	6 days	14 days
Blood	30.51±3.19	17.21±1.88	1.08±0.77	0.05±0.02
Lung	11.89±2.17	8.37±1.63	1.29±0.58	0.19±0.10
Heart	3.85±2.54	2.85±0.59	0.38±0.25	ND[2]
Liver	4.59±0.80	3.19±0.37	0.53±0.07	0.17±0.04
Spleen	13.78±2.32	32.36±7.04	42.46±16.92	29.47±12.16
Kidney	17.41±2.34	9.07±0.87	3.11±0.82	0.53±0.39
Stomach	1.90±0.29	2.50±0.52	0.88±0.24	0.32±0.12
Small intestine	2.22±0.25	2.12±0.24	0.39±0.12	0.06±0.02
Large intestine	1.27±0.20	2.97±1.04	0.63±0.11	0.09±0.04
Muscle	0.61±0.13	0.46±0.15	0.26±0.14	0.18±0.15
Brain	0.71±0.21	0.42±0.15	ND	ND
Skull	4.31±1.55	6.38±1.25	15.05±4.56	12.38±3.57
Femur	5.82±1.73	7.63±0.74	15.63±3.90	16.99±3.85
Skin	0.69±0.55	1.96±1.14	ND	ND

[1]Mean±SD, n=5 to 6 animals.
[2]ND=not determined (because of low activity counts *vs.* background counts).

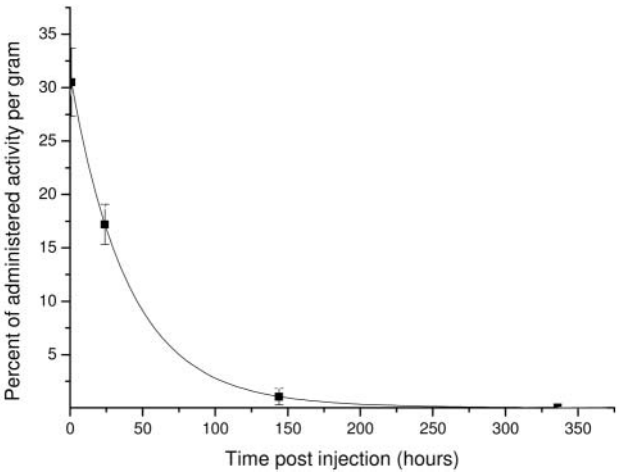

Figure 1. *Blood clearance of liposomal ^{223}Ra in Balb/C mice.*

contributions to the organ dose from cross-organ irradiation by beta-particles and γ-rays is negligible for ^{223}Ra decay-chain emissions.

To validate the assumed equilibrium between ^{223}Ra and the daughter nuclides, one animal, injected 24 hours earlier with liposomal ^{223}Ra, was sacrificed by cervical dislocation and dissected. Count rates for ^{223}Ra, ^{211}Pb and ^{211}Bi were measured according to their characteristic γ-rays using a solid-state photon detector (GEM-50; ORTEC, Oak Ridge, TN, USA) coupled to a digital γ-ray spectrometer (Dispec Jr., ORTEC) and analyzed using the computer software Gammavision-32, version 6.01 (ORTEC). For ^{223}Ra, the 324-keV γ-ray (3.65% abundance) was chosen, while for ^{211}Pb and ^{211}Bi, the 405-keV (3.84%) and 351-keV (13.0%) γ-rays (Nuclide Explorer 2000, Version 1.2, European Communities 2000, Institute for Transuranium Elements, Karlsruhe, Germany) were chosen to determine the levels of the different radionuclides which were present shortly after the animal had been sacrificed.

Results

Loading yield. The amount of ^{223}Ra loaded into the Caelyx™ liposomes was 51% to 67% for three individual experiments. A control experiment using ^{223}Ra and liposomes under identical conditions, but without the ionophore, showed less than 1% of the ^{223}Ra in the liposome fraction eluted from the Sephadex G-25 D-10 column.

Animal studies. The data for the biodistribution study are shown in Table II and Figures 1 and 2. These results showed that the blood clearance of injected liposomal radium was relatively slow, as expected for pegylated liposomes (Figure 1). Among soft-tissue organs, the highest uptake was observed in the liver (Figure 2). The uptake as a percent of the administered activity per gram was comparatively low in the liver relative to the substantial uptake in the spleen, particularly at later time-points (Table II, Figure 2). Skeletal uptake, reflected by activity in the femur and the skull, increased with time, probably due to gradual catabolism of the liposomes and release of free ^{223}Ra. No significant differences were observed in the radioactivity uptake between marrow-containing samples (femur) and those with no marrow (skull). It appeared that ^{223}Ra bone uptake reflected the free radium and not an accumulation of liposomal radium in the bone marrow. When the results from the biodistribution study were analyzed for gender differences, only one tissue at 6 days and one tissue at 14 days indicated significant differences ($p<0.05$, Student's paired *t*-test). At 6 days, females had a significantly higher splenic uptake ($p<0.05$, Student's paired *t*-test), while at 14 days the males had significantly higher colonic uptake ($p<0.05$, Student's paired *t*-test).

Liposomal ^{223}Ra LI were determined for various tissues and, on comparison with cationic ^{223}Ra at 1 hour, 24 hours and 14 days after injection (Figure 3), distinct differences were observed. The LI were particularly high for blood as a result of much slower blood clearance of liposomal ^{223}Ra *vs.* free ^{223}Ra. The LI for most soft tissues were significantly more than 1.0, whereas the indices for bone samples were less than 1.0.

Assessment of daughter radionuclide distribution. Data concerning the daughter radionuclide biodistributions are shown in Table III. Because of the short half-lives of ^{219}Rn ($t_{1/2}$=4 sec) and ^{215}Po ($t_{1/2}$=1.8 microsec), the first three alpha-particle emissions in the ^{223}Ra decay series take place at or near the site of parent ^{223}Ra deposition. The final alpha decay in the series (^{211}Bi, $t_{1/2}$=2.17 min) follows the

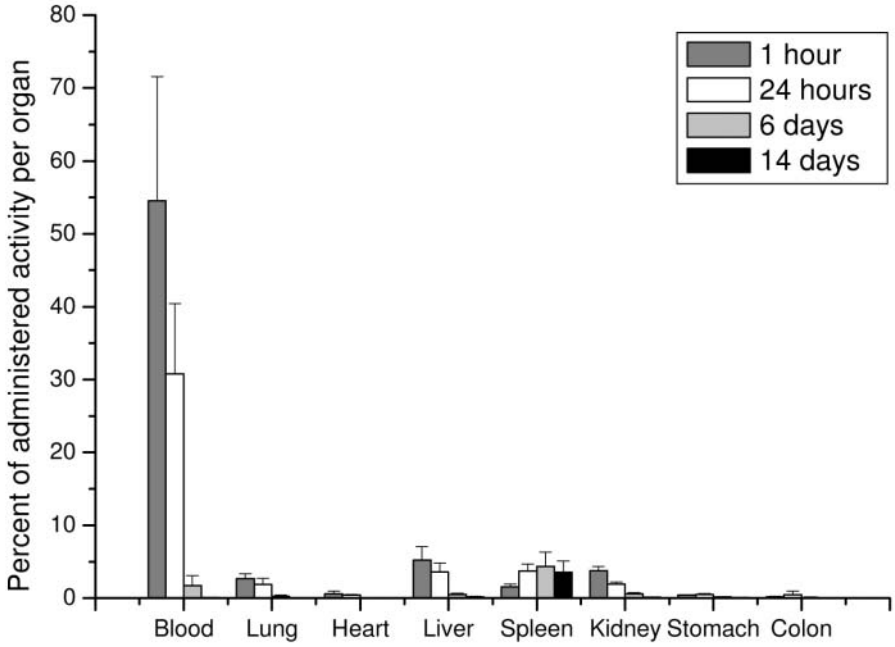

Figure 2. *Biodistribution of liposomal ^{223}Ra for selected tissues of Balb/C mice after intravenous injection.*

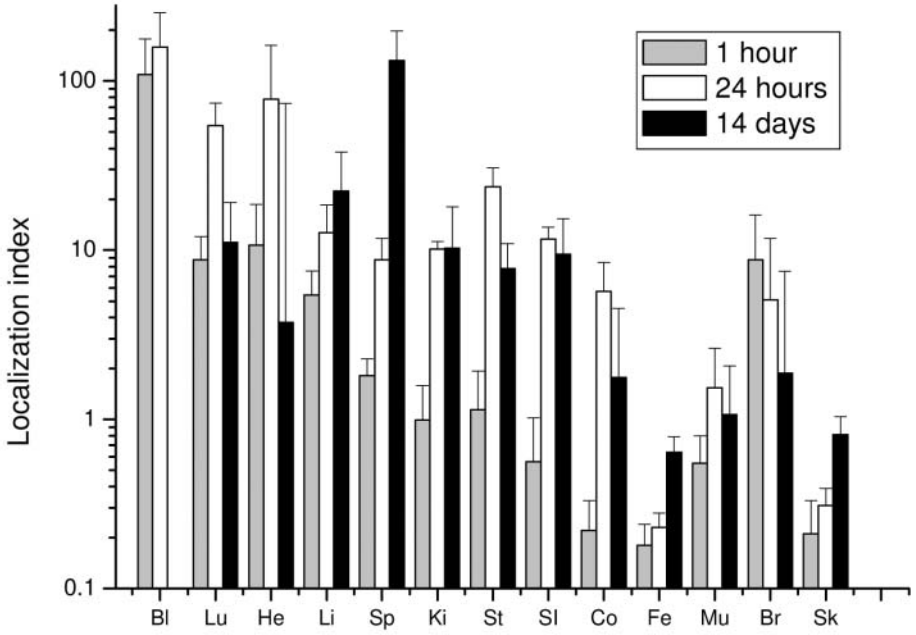

Figure 3. *Localization indices LI=[percent of administered activity per gram for liposomal ^{223}Ra]/[percent of administered activity per gram for free ^{223}Ra] for a tissue at a specific time-point in Balb/C mice. Bl – blood; Lu – lung; He – heart; Li – liver; Sp – spleen; Ki – kidney, St – stomach; SI – small intestine; Co – colon; Fe – femur; Mu – muscle; Br – brain; Sk – skull.*

decay of ^{211}Pb ($t_{1/2}$=36.1 min) and, therefore, redistribution away from the site of ^{223}Ra is possible. Table III shows the relative γ peak activity determinations for ^{223}Ra, ^{211}Pb and ^{211}Bi in samples (urine, blood, kidneys, femur, liver and spleen). Measurements indicated depletion of ^{211}Pb/^{211}Bi in the urine and spleen and some accumulation of ^{211}Pb/^{211}Bi in the kidneys. For blood, depletion of ^{211}Pb but not of ^{211}Bi was found. The assumed equilibrium between ^{223}Ra

Table III. *Relative levels of ^{211}Pb and ^{211}Bi compared to ^{223}Ra in mouse samples 24 h after intravenous injection of liposomal ^{223}Ra.*

Sample	Time (min) from sacrifice to measurement	Theoretical physical ingrowth[1] ^{211}Pb	Theoretical physical ingrowth[1] ^{211}Bi	Measured ratio ^{211}Pb *vs.* ^{223}Ra	Measured ratio ^{211}Bi *vs.* ^{223}Ra
Urine	1	0.017	0.002	0.39	0.36
Blood	3	0.054	0.019	0.43	1.20
Kidney	7	0.124	0.075	1.42	1.57
Femur	12	0.204	0.155	0.86	1.00
Liver	17	0.277	0.155	1.23	1.36
Spleen	22	0.343	0.301	0.60	0.44

[1]Ratio of daughter radionuclides to ^{223}Ra in a sealed source of pure ^{223}Ra from time of sacrifice to time of measurement.

Table IV. *Fraction[1] of administered ^{223}Ra retained per gram organ or tissue in Balb/C mice from 1 to 226 h after intravenous injection of liposomal ^{223}Ra (not corrected for decay).*

Organ or tissue	Time (h) 1 h	24 h	144 h	226 h
Blood	0.304	0.162	0.00750	0.00021
Lung	0.119	0.0788	0.00896	0.00081
Heart	0.0384	0.0268	0.00264	ND[2]
Liver	0.0458	0.0300	0.00368	0.00073
Spleen	0.137	0.305	0.295	0.126
Kidney	0.174	0.0854	0.0216	0.00227
Stomach	0.0190	0.0235	0.00611	0.00137
Small intestine	0.0221	0.0200	0.00271	0.00026
Large intestine	0.0127	0.0280	0.00438	0.00038
Muscle	0.00608	0.00433	0.00181	0.00077
Brain	0.00708	0.00395	ND	ND
Skull	0.0430	0.0600	0.105	0.0529
Femur	0.0580	0.0718	0.109	0.0726
Skin	0.00688	0.0185	ND	ND

[1]Accounts for both physical decay and biological clearance.
[2]ND- not determined.

Table V. *Biokinetic parameters[1] for ^{223}Ra in organs and tissues of Balb/C mice receiving liposomal ^{223}Ra.*

Organ or tissue	a	b	r	T_{eff}	T_{bio}	Residence time, τ, (μCi-hours per μCi administered)
Lung	0.104	0.0149	0.991	46.5	56.0	7.00
Heart	0.0398	0.0189	0.999	36.6	42.3	2.10
Liver	0.0366	0.0124	0.965	56.1	70.5	2.96
Spleen	0.295	0.00443	1.000	156.0	364.0	108.0
Kidney	0.138	0.0124	0.991	55.8	70.1	11.1
Stomach	0.0212	0.00900	0.990	77.0	107.0	2.86
Small intestine	0.0231	0.0137	0.995	50.8	62.3	1.70
Large intestine	0.0257	0.0136	0.997	50.8	62.4	2.36
Muscle	0.00519	0.00597	0.971	116.0	201.0	0.870
Brain	0.00708	0.0254	1.000	27.3	30.4	0.279
Skull	0.105	0.00355	1.000	195.0	681.0	40.6
Femur	0.0726	0.00253	1.000	274.0	>821[2]	58.5
Skin	0.0185	0.00253	1.000	274.0	>821	7.59

[1]Retention in organs according to the following equation: $y=ae^{-bx}$ (a=intercept at y-axis, b=slope, x=time in hours). r=correlation coefficient, T_{eff}=effective half-life, T_{bio}=biological half-time, τ=residence time, an integral corresponding to the the total number of radioactive transformations in a tissue per unit administered activity.
[2]Corresponding to three physical half-lives of ^{223}Ra.

and its daughters is, therefore, quite relevant for blood, femur and liver. For the kidneys, we observed more ^{211}Bi than estimated, while for the spleen the observed levels were less than estimated.

Liposomal ^{223}Ra biokinetics and dosimetry. Radionuclide clearance was fitted to a single exponential equation for all tissues according to the equation: $y=ae^{-bx}$ (x=time in hours, a=intersection at the y-axis, b=slope), except for blood, where clearance was best fitted to a two-exponential equation of the form: $y=ae^{-bx} + ce^{-dx}$. For blood, the coefficients a=0.299, b=0.0256, c=0.0597 and d=1.56 were obtained, corresponding to blood clearance half-times of 0.44 hour (6%) and 27.1 hours (64%). The remaining 30% did not clear from the blood, but rather was redistributed *via* fluids and was retained in soft tissues and the skeleton. Tables IV and V and Figure 4 show the effective (sum of the physical and biological clearance rates) retention parameters, and absorbed dose estimates for individual tissues containing liposomal ^{223}Ra and daughters. The absorbed doses were calculated for injections of 375 kBq/kg (approximately 8.1 kBq/animal). These results showed that the spleen among the soft tissues received the highest absorbed dose, *i.e.*, 14.1 Gy, a value even higher than for the femur and the skull (Figure 4). The liver and intestines received quite modest doses. The dose to the blood was calculated using microdosimetry methods (10), assuming the

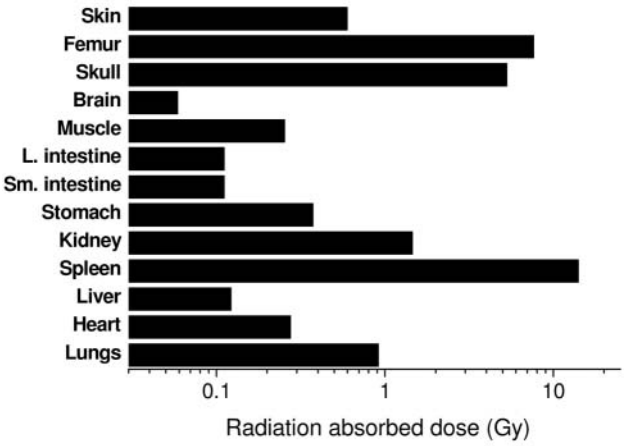

Figure 4. *Radiation absorbed doses in various tissues after injection of liposomal ^{223}Ra in Balb/C mice. It was assumed that ^{223}Ra was in equilibrium with the daughter nuclides. The values were calculated according to an administered activity of 375 kBq/kg (approximately 8.1 kBq per animal).*

mean blood vessel and capillary diameter to be about 25 μm (yielding an average energy-absorbed fraction of approximately 0.13). From this assumption, and by integrating the time-activity curves for ^{223}Ra in equilibrium with the daughter products in blood, an absorbed dose to circulating blood of 0.2 Gy was calculated.

Discussion

This work is the first *in vivo* study of *i.v.* injected alpha-emitting radio-liposomes. The observed half-time in blood of liposome-encapsulated ^{223}Ra differed significantly from that of dissolved $^{223}RaCl_2$. The observed half-times agreed well with values reported for Caelyx™/Doxil™ in rodents. These results showed that ^{223}Ra was largely retained in the liposomes, a finding compatible with the *in vitro* stability testing presented for doxorubicin-containing liposomes in this study and also to that reported previously for empty pegylated liposomes loaded with ^{223}Ra (7).

The metabolic activity of the reticulo-endothelial system, and thus the biodistribution of liposomes, could depend significantly on the immune status of the animal. Therefore, this study was performed using immuno-competent Balb/C mice without tumors. Because of the promising blood half-time of liposomal ^{223}Ra observed in the current study, a tumor uptake study in immuno-suppressed animals with tumor xenografts is warranted to evaluate whether sufficient tumor targeting can be achieved.

To use radioactive liposomes in cancer therapy, it would be important to minimize the soft tissue exposure and, in particular, exposure to tissues involved with uptake in the reticulo-endothelial system. As previously shown (11), pre-treatment with conventional PLD reduced the reticulo-endothelial system uptake. Pre-treatment with PLD was therefore included in our study. A commercially available liposome formulation was employed as the carrier compound, instead of using drug-free liposome, for the following reasons: (i) PLD represents the state of the art in terms of clinically approved liposomal tumor therapy (6, 12), which (ii) made it possible to use the same liposomal formulation in the pre-treatment/pre-loading regimen to help diminish reticulo-endothelial system activity.

The current data indicate that the reticulo-endothelial system interacted with the lipsomes. Even though pre-treatment with non-radioactive liposomes was performed, the spleen had the highest uptake and thus received a considerable radiation dose. ^{223}Ra uptake in the spleen was relatively high in terms of percent of administered activity per gram, even though the splenic uptake did not exceed 5% of the total administered activity. Some preliminary data from biodistribution in dogs indicated that splenic uptake is less in dogs than in mice. In addition, the skeleton, probably mostly the bone surfaces, received significant radiation doses. The latter was probably caused by the release of cationic ^{223}Ra when the liposomes were metabolized, since the biodistribution at later time-points more closely resembled that of injected free $^{223}RaCl_2$.

Knowledge of the chemical integrity of PLD containing ^{223}Ra, prior to or at administration, is important for interpreting *in vivo* data. In the current work, the PLD could be loaded with ^{223}Ra without affecting the release of doxorubicin. This was evident as both ^{223}Ra and the bright-red-colored doxorubicin were observed in the same high molecular fractions when eluted from the gel exclusion column. Control experiments with liposomes heated to over 80˚C resulted in elution of the bright-red doxorubicin in the low molecular weight fractions. We also confirmed that cationic ^{223}Ra would elute in the low molecular weight fraction unless the proper procedure for loading had been performed. PLD with ^{223}Ra stored for several weeks in the injection buffer had a gel exclusion elution profile indicating an intact product. These observations indicate that co-injection of substantial amounts of free ^{223}Ra or doxorubicin could be ruled out.

In accordance with previously published data for the *in vitro* serum stability of liposomal ^{223}Ra (7), we confirmed (data not shown) that ^{223}Ra was not released from the doxorubicin-containing liposomes when incubated in fetal calf serum for up to 7 days at 37˚C. Thus, any generation of cationic ^{223}Ra *in vivo* could not be ascribed to a general chemical instability. As observed in the biodistribution study, liposomal ^{223}Ra slowly cleared from the blood and was metabolized, probably by macrophages in the reticulo-endothelial system. This process gradually generated free, cationic ^{223}Ra, which was eliminated either by intestinal or renal clearance, or was incorporated onto bone surfaces. A

similar clearance pattern with time was observed for liposomal ^{223}Ra in the majority of tissues, except for the spleen and bone. In the skull and femur, an increased ^{223}Ra uptake was observed during the first 6 days, and this activity level was maintained up to 14 days after injection, indicating that most of the radium was released from the liposomes within the first 6 days. Free $^{223}Ra^{2+}$ uptake in bone could cause bone marrow toxicity, but recent studies have suggested that skeletal tolerance to ^{223}Ra is higher than previously expected (13, 14) and would, therefore, probably not be dose-limiting for long-circulating liposomal ^{223}Ra. On the other hand, the significant levels of circulating liposomal ^{223}Ra during the first few days could cause considerable bone marrow exposure. Therefore, a marrow toxicity assessment should be included in future studies. The LI showed that, at 14 days, less ^{223}Ra was observed in bone in the group given liposomal ^{223}Ra *vs.* cationic ^{223}Ra. This finding could not be accounted for by the splenic uptake alone, but was probably due to retention in the reticulo-endothelial system.

For maximum utility, the physical half-life of any therapeutic radionuclide should match the *in vivo* retention half-time of its carrier compound. The reported plasma half-lives of Doxil™ (US brand name for Caelyx™) is 24-35 hours in the rat and 23-27 hours in the dog (12). This closely matched the blood half-times that were observed in the current study. With liposomes, the physical half-life of the radionuclide should not be too short (less than 1 day), since concentration of the liposomal radionuclide in tumor tissue may require a few days.

Only a few radionuclides appear suitable for liposomal encapsulation. One possibility is ^{212}Pb ($t_{1/2}$=10.6 hours), a beta emitter that decays to the alpha emitter ^{212}Bi ($t_{1/2}$=60 minutes). ^{212}Pb is an *in vivo* generator of alpha particles since the beta transformation does not seem to cause a significant release of ^{212}Bi from liposomes (15). Because of its short half-life, the use of ^{212}Pb would probably be limited to regional delivery where rapid targeting is anticipated.

For systemic therapy against solid tumors, where the time to achieve a maximum tumor uptake could be 24 hours or more, longer-lived radionuclides would be more appropriate than ^{212}Pb. Alpha emitter candidates with suitable half-lives are ^{225}Ac ($t_{1/2}$=10.0 days), ^{224}Ra ($t_{1/2}$=3.6 days) and ^{223}Ra $t_{1/2}$=11.4 days). Each decays *via* multiple steps, releasing four alpha-particles. Unless micrometer-sized liposomes are used, the recoiling nucleus from the first alpha decay would escape from the liposomes (16). Given that the optimum size for *i.v.* liposomes is within the range of 100 nm or less, the use of these radionuclides in liposomal delivery may be problematic and the properties of the decay product must be considered. ^{225}Ac decays to ^{221}Fr ($t_{1/2}$=4.9 minutes) in the first decay, so that only the first alpha decay would be associated with the liposome if nano-sized liposomes were used, as is true for ^{224}Ra which decays to ^{220}Rn ($t_{1/2}$=55.6 seconds). For ^{223}Ra, however, the three first alpha decays would decay within or in the close vicinity of the liposome because the two decays following the ^{223}Ra transformation are from alpha-emitting daughters with half-lives of 3.96 seconds (^{219}Rn) and 1.78 microseconds (^{215}Po), respectively. Thus ^{223}Ra appears to be the best suited for liposomal delivery among the longer-lived alpha emitters which have been considered for radionuclide therapy.

In this work, the fate of the daughter nuclides were studied in an animal at the 24-hour point. Measurements of ^{223}Ra daughters are difficult due to their short half-lives. We focused on the blood, liver, spleen, kidneys and bone for analysis of ^{211}Pb and ^{211}Bi. It was found that ^{223}Ra was quite close to equilibrium with ^{211}Bi in the blood, liver and femur, while less ^{211}Pb and ^{211}Bi were measured in the spleen and more in the kidneys. Depletions of ^{211}Pb and ^{211}Bi were observed in urine, which may account for the increased levels in the kidney. Since the kidney was measured several minutes after sacrifice, the precise amount of ^{211}Bi in the kidney at the time of death remains unknown. If, for purposes of dosimetry, one assumed the same difference between ^{211}Pb and ^{211}Bi uptakes in the kidney, as described by Ando *et al.* (17), the level of ^{211}Bi may be about three times that of ^{223}Ra at that point, suggesting a two-fold increase in the alpha-particle dose to the kidney compared to the dose obtained on the basis of an assumed parent/daughter equilibrium.

Tissue samples were routinely counted, using the non-discriminating multi-well counter, within 3 hours post-mortem, and were recounted after 12 hours or more when radium was in equilibrium with its daughters. When the decay-corrected data were analyzed, the blood counts increased with time. This was probably due to release of some ^{211}Pb (half-life=36.1 minutes) from liposomes, as indicated by the γ peak measurement at the 24-hour point.

Conclusion

Liposome-encapsulated ^{223}Ra was found to display favorable physical and radiological characteristics for use as a therapeutic agent in cancer therapy, including *in vivo* stability as a carrier and promising biodistribution properties in mice. Further studies in tumor-bearing animals are warranted to evaluate the potential targeting of soft-tissue tumors with liposome-encapsulated ^{223}Ra.

Acknowledgements

This work was financially supported by the Norwegian Research Council Grant E53544/110 and Algeta ASA, Oslo, Norway. We thank Solveig Garman-Vik, Animal Department, the Norwegian Radium Hospital, Oslo, Norway, for assistance with the animal experiment, and Gro Salberg, Algeta ASA, Oslo, Norway, for help with counting the radioactive samples.

References

1 Zalutsky, MR, Zhao XG, Alston KL and Bigner DD: High-level production of α-particle-emitting ^{211}At and preparation of ^{211}At-labeled antibodies for clinical use. J Nucl Med *42*: 1508-1515, 2001.

2 Jurcic JG, Larson SM, Sgouros G, McDevitt MR, Finn RD, Divgi CR, Ballangrud ÅM, Hamacher KA, Ma D, Humm JL, Brechbiel MV, Molinet R and Scheinberg DA: Targeted α-particulate immunotherapy for myeloid leukemia. Blood *100*: 1233-1239, 2002.

3 Nilsson S, Larsen RH, Fosså SD, Baltesgard L, Borch KW, Westlin JE, Salberg G and Bruland ØS: First clinical experience with α-emitting radium-223 in the treatment of skeletal metastases, Clin Cancer Res *11*: 4451-4459, 2005.

4 Bruland ØS, Nilsson S, Fisher DR and Larsen RH: High-linear energy-transfer irradiation targeted to skeletal metastases by the alpha emitter ^{223}Ra: adjuvant or alternative to conventional modalities? Clin Cancer Res, in press, 2006.

5 Henriksen G, Hoff P and Larsen RH: Evaluation of potential chelating agents for radium. Appl Rad Isot *56*: 667-671, 2002.

6 Gabizon AA: Stealth liposomes and tumor targeting: one step further in the quest for the magic bullet. Clin Cancer Res *7*: 223-225, 2001.

7 Henriksen G, Schoultz BW, Michaelsen TE, Bruland ØS and Larsen RH: Sterically stabilized liposomes as a carrier for alpha-emitting radium and actinium radionuclides. Nucl Med Biol *31*: 441-449, 2004.

8 Henriksen G, Hoff P, Alstad, J and Larsen RH: ^{223}Ra for endoradiotherapeutic applications prepared from an immobilized ^{227}Ac/^{227}Th source. Radiochim Acta *89*: 661-666, 2001.

9 Henriksen G, Fisher DR, Roeske JC, Bruland ØS and Larsen RH: Targeting of osseous sites with alpha-emitting ^{223}Ra: comparison with the beta-emitter ^{89}Sr in mice. J Nucl Med *44*: 252-259, 2003.

10 Fisher DR and Harty R: The microdosimetry of lymphocytes irradiated by alpha particles. Int J Radiat Biol *41*: 315-324, 1982.

11 Gabizon A, Tzemach D, Mak L, Bronstein M and Horowitz AT: Dose dependency of pharmacokinetics and therapeutic efficacy of pegylated liposomal doxorubicin (DOXIL) in murine models. J Drug Target *10*: 539-548, 2002.

12 Gabizon A, Shmeeda H and Barenholz Y: Pharmacokinetics of pegylated liposomal doxorubicin: review of animal and human studies. Clin Pharmacokinet *42*: 419-436, 2003.

13 Nilsson S, Larsen RH, Fosså SD, Baltesgard L, Borch KW, Westlin JE, Salberg G and Bruland ØS: First clinical experience with α-emitting radium-223 in the treatment of skeletal metastases. Clin Cancer Res *11*: 4451-4459, 2005.

14 Larsen RH, Saxtorph H, Skydsgaard M, Borrebaek J, Jonasdottir TS, Bruland ØS, Klastrup S, Harling R and Ramdahl T: Radiotoxicity of the alpha-emitting bone-seeker ^{223}Ra injected intraveneously into mice: histology, clinical chemistry and hematology. In Vivo *20*: 325-332, 2006.

15 Henriksen G, Schoultz BW, Hoff P and Larsen RH: Potential *in vivo* generator for alpha-particle therapy with ^{212}Bi: presentation of a system to minimize escape of daughter nuclide after decay of ^{212}Pb to ^{212}Bi. Radiochim Acta *91*: 109-113, 2003.

16 Sofou S, Thomas JL, Lin HY, McDevitt MR, Scheinberg DA and Sgouros G: Engineered liposomes for potential alpha-particle therapy of metastatic cancer. J Nucl Medic *45*: 253-260, 2004.

17 Ando A, Ando I, Hiraki T and Hisada K: Relation between the location of elements in the periodic table and various organ-uptake rates. Nucl Med Biol *1*: 57-80, 1989.

Received April 28, 2006
Accepted May 30, 2006