

Targeted cancer therapy with a novel low-dose rate α -emitting radioimmunoconjugate

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α -emitting radionuclides are highly cytotoxic and are of considerable interest in the treatment of cancer. A particularly interesting approach is in radioimmunotherapy. However, α -emitting antibody conjugates have been difficult to exploit clinically due to the short half-life of the radionuclides, low production capability, or limited source materials. We have developed a novel technology based on the low-dose rate α -particle-emitting nuclide

²²⁷Th, exemplified here using the monoclonal antibody rituximab. In vitro, this radioimmunoconjugate killed lymphoma cells at Becquerel per milliliter (Bq/mL) levels. A single injection of ²²⁷Th-rituximab induced complete tumor regression in up to 60% of nude mice bearing macroscopic (32-256 mm³) human B-lymphoma xenografts at Becquerel per gram (Bq/g) levels without apparent toxicity. Therapy with ²²⁷Th-rituximab was sig-

nificantly more effective than the control radioimmunoconjugate ²²⁷Th-trastuzumab and the standard β -emitting radioimmunoconjugate for CD20⁺ lymphoma, ⁹⁰Y-tiuxetan-ibritumomab. Thorium-²²⁷ based constructs may provide a novel approach for targeted therapy against a wide variety of cancers. (Blood. 2007;110:2049-2056)

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Introduction

Monoclonal antibodies conjugated to the β -emitting isotopes, ¹³¹I and ⁹⁰Y, are currently standard treatment options for patients with CD20-expressing B-cell non-Hodgkin lymphoma, and have produced long-term responses in up to 86% of patients.¹⁻⁵ However, the long path lengths of the β and γ emissions from these radionuclides generate dose-limiting myelosuppression at conventional doses, and cardiopulmonary toxicities at higher doses when used with stem cell transplantation.² Furthermore, the low energy transfer characteristics of the β -particles result in suboptimal killing of tumor cells, ultimately leading to relapse in most treated patients.

α -particle radiation, on the other hand, yields high linear energy transfer and confers potent cytotoxicity within a radius of less than 100 μ m of the decaying radionuclide. Monoclonal antibodies labeled with α -emitting radionuclides ²¹³Bi, ²¹¹At, and ²²⁵Ac show promise for radioimmunotherapy.⁶⁻⁸ However, ²¹³Bi and ²¹¹At are challenging to apply in clinical settings because of their short half-lives (46 minutes and 7.2 hours, respectively) and their limited production capability with currently available technology. ²²⁵Ac ($t_{1/2}$ = 10.0 days) has been effective when conjugated to monoclonal antibodies, but the supply of source material for the preparation of this nuclide is very limited.^{8,9} Cationic ²²³Ra ($t_{1/2}$ = 11.4 days) has recently shown considerable promise in clinical trials in patients with skeletal metastases,¹⁰ and may also be used with liposomes,¹¹ but cannot be stably conjugated to monoclonal antibodies.¹²

Here, we suggest that ²²⁷Th, the precursor of ²²³Ra, may be an attractive radionuclide to explore for radioimmunotherapy based on its physical and chemical properties and its availability.^{13,14}

²²⁷Th has a half-life of 18.7 days and can be produced in practically unlimited amounts from ²²⁷Ac, which is generated by thermal neutron irradiation of ²²⁶Ra.¹⁵ Consequently, long-term operating generators for ²²⁷Th can be prepared relatively easily and affordably. The long half-life of ²²⁷Th should permit conjugation, administration and targeting of a ²²⁷Th-labeled radioimmunoconjugate (RIC) before a significant amount of ²²³Ra is generated. The relatively long half-life of ²²³Ra, in turn, assures that this nuclide would be largely excreted or trapped in skeletal hydroxyapatite before decay occurs.¹⁰ Although a considerable amount of ²²³Ra is taken up in bone,¹⁶ this probably will not cause bone marrow toxicity at relevant levels because of the short range of the α -particles as indicated by recent evaluation of this radionuclide in animals and humans.^{17,18} Thus, a therapeutic window permitting treatment with ²²⁷Th with acceptable toxicity may exist. Furthermore, the relatively modest γ components of ²²⁷Th and the ²²³Ra series reduce the necessity for patient shielding, allowing α -radioimmunotherapy to be given on an outpatient basis. The γ -photons emitted by ²²⁷Th are suitable for imaging and may facilitate dosimetry evaluation in patients.

Since antibody that binds to the tumor target will remain in the body for a longer time than antibody that is not bound, the longer half-life of ²²⁷Th also leads to a higher tumor-to-normal organ ratio of absorbed radiation than would be delivered by a nuclide with a shorter half-life. Micrometastases and disseminated malignancies have been proposed as the most suitable indications for radioimmunotherapy with α -emitters because of the short effective ranges of α particles, since minimal disease permits more rapid targeting and has limited penetration barriers. However, the considerations above

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suggest that ^{227}Th -labeled antibodies might also be effective for treatment of macroscopic tumors. These considerations motivated us to compare a standard β -emitting anti-CD20 RIC, ^{90}Y -tiuxetan-ibritumomab, with our α -emitting counterpart, ^{227}Th -DOTA-p-benzyl-rituximab, in a lymphoma xenograft model.

Materials and methods

Preparation of ^{227}Th -DOTA-p-benzyl-rituximab

^{227}Th , ^{227}Th -p-isothiocyanato-benzyl-DOTA complexes, and ^{227}Th -DOTA-p-benzyl-rituximab (F. Hoffman-LaRoche AG, Basel, Switzerland) were prepared as previously described.^{13,16,19} The specific activity of the RIC was in the range of 650 to 5300 Bq/ μg . The quality of the RIC was assessed using lymphoma cells and a modified Lindmo and Bunn method.²⁰ Cell concentrations of up to 10^8 Raji cells/mL were used to compensate for the modest specific activity of the RIC.^{13,16} The immunoreactive fractions of conjugates used in the current study were between 56% and 65%.

Measurement of cell growth

Cells of the CD20-expressing B-cell lymphoma line Raji were used. Single-cell suspensions were grown in a humidified atmosphere with 95% air/5% CO_2 in RPMI 1640 (PAA, Linz, Austria) supplemented with 13% heat-inactivated FCS (PAA), 1% L-glutamine (PAA), and 1% penicillin-streptomycin (PAA). A total of 10^6 cells were seeded in 10 mL medium in 25-cm² bottles at day 0. In half of the bottles, the cells were blocked by 10 $\mu\text{g}/\text{mL}$ unlabeled rituximab. The ^{227}Th -DOTA-p-benzyl-rituximab was added in amounts equivalent to 0, 100, 200, or 400 Bq/mL. At days 1, 4, 6, and 9, a 2.5-mL sample was taken for cell counting by a Coulter counter.²¹ Subsequently, the cells were centrifuged, medium was removed so that 5 mL was left, and 5 mL fresh medium was added. The cells were resuspended and seeded out in the same bottles. In the bottles with blocked cells, 10 $\mu\text{g}/\text{mL}$ unlabeled rituximab was added.

Animals

Institutionally bred female Balb/C nu/nu (NCR) mice that were 4 to 8 weeks old and had body weights in the range of 18 to 22 g at the start of the experiment were used. The animals were maintained under pathogen-free conditions, and food and water were supplied ad libitum. All procedures and experiments involving animals in this study were approved by the National Animal Research Authority and carried out according to the European Convention for the Protection of Vertebrates Used for Scientific Purposes. Mice were anesthetized with 200 μL Tiletamin-Zolazepam mix (Virbac, Carros Cedex, France) diluted 1:5 with saline and given intraperitoneally before implantation with about $2 \times 2 \times 2$ -mm pieces of Raji B-lymphoma tumor tissue.

Biodistribution experiments

The biodistributions of ^{227}Th -DOTA-p-benzyl-rituximab, ^{227}Th -DOTA-p-benzyl-trastuzumab (F. Hoffman-LaRoche AG) and ^{125}I -ibritumomab-tiuxetan (Schering AG, Berlin, Germany) were studied in mice with tumor sizes of 32 to 256 mm³ at the start of the study. Ibritumomab-tiuxetan was labeled with ^{125}I in an IODOGEN Pre-Coated Iodination Tube (Pierce, Rockford, IL) according to the manufacturer's description. The preparations were administered by tail vein injection of 100 μL solution to each animal. A mean activity of 1100 kBq/kg, 620 kBq/kg, or 1250 kBq/kg was injected for ^{227}Th -DOTA-p-benzyl-rituximab, ^{227}Th -DOTA-p-benzyl-trastuzumab, and ^{125}I -ibritumomab-tiuxetan, respectively. For ^{125}I -ibritumomab-tiuxetan, the measured values were adjusted for the decay of ^{125}I and ^{90}Y . A total of 6 to 8 animals were used per time point. Autopsies were performed after cervical dislocation at various time points after injection. The weight of each tissue sample was determined, and ^{227}Th and ^{223}Ra were measured by their most characteristic γ rays using the solid-state photon detector GEM-50 (Ortec, Oak Ridge, TN) coupled to a digital γ -ray spectrometer (Dispec Jr; Ortec) and analyzed using the computer software Gammavision-32 Version 6.01 (Ortec). For ^{227}Th , the 235.97 keV γ ray (11.6% probability) was chosen, and for ^{223}Ra , the 154.21 keV γ ray (5.62% probability) was chosen. The count rates in the samples were also routinely measured using a γ counter (Crystal II

Multidetector RIA System; Packard Instrument Co, Downers Grove, IL). The ^{125}I -ibritumomab-tiuxetan samples were measured with a γ counter (Cobragamma; Packard Instrument Co). Samples of the injectates were used as references in the measurement procedures.

Calculation of absorbed radiation dose

The absorbed radiation doses were calculated assuming dose contributions coming only from α -particle emissions for the ^{227}Th conjugates. Mean α -energies of 5.9 MeV for ^{227}Th and 26.4 MeV for ^{223}Ra with α -emitting daughters in equilibrium were used (Nuclide Explorer 2000 (1.00), Institute for Transuranium Elements, Karlsruhe, Germany). It was assumed that there was 100% absorption of the α particles in the tissues measured. The biodistribution data were normalized to an injection of 200 kBq/kg body weight for the ^{227}Th conjugates. It was assumed that the radionuclides were uniformly distributed in the various tissues. For blood, 100% absorption of the α -particles was assumed. This is a simplification, since there will be some escape of α -particles beyond the blood in the capillaries.

To determine the dose after ^{90}Y -ibritumomab-tiuxetan treatment, the activities of ^{125}I -ibritumomab-tiuxetan measured at different time points, t , were divided by $e^{-\ln 2/T_{1/2}(^{125}\text{I}) \cdot t}$, where $T_{1/2}(^{125}\text{I})$ is the half-life of ^{125}I and then multiplied by $e^{-\ln 2/T_{1/2}(^{90}\text{Y}) \cdot t}$, where $T_{1/2}(^{90}\text{Y})$ is the half-life of ^{90}Y . It was assumed that only the β particle emissions contributed to the dose, and that the radionuclides were distributed uniformly in the tissue. A mean β -energy of 0.9336 MeV was used.²² The biodistribution data were normalized to an injection of 7.5 MBq/kg. It was assumed that there was 100% absorption of the β particles in the tissues measured.

The total number of disintegrations from the time of the injection of the preparation until no activity was left in the body was calculated by area under curve (AUC) estimate of the activity concentrations in various tissues versus time.²³ Thus, the total dose to each organ could be calculated by Equation 1: (1)

$$\text{Dose} = \text{AUC}_0^\infty \cdot E_\alpha(^{227}\text{Th}) + \text{AUC}_0^\infty \cdot E_\alpha(^{223}\text{Ra} + \text{daughters})$$

$$\text{Dose} = \text{AUC}_0^\infty \cdot E_\beta(^{90}\text{Y})$$

Experimental therapy studies

Treatment started when macroscopically growing tumors (32-256 mm³) had formed. Control mice were injected with 100 μL NaCl or with 100 μL PBS containing 20, 100, or 250 μg cold rituximab. For radioimmunotherapy, mice were injected with 50, 200, 400, or 1000 kBq/kg ^{227}Th -DOTA-p-benzyl-rituximab; 200 or 400 kBq/kg ^{227}Th -DOTA-p-benzyl-trastuzumab; or 7.5, 15, or 30 MBq/kg of ^{90}Y -tiuxetan-ibritumomab, which correspond to 50%, 100%, or 200% of the recommended clinical human dosage of ^{90}Y -tiuxetan-ibritumomab (Schering AG). Clinical grade ^{90}Y -tiuxetan-ibritumomab was obtained from the Department of Nuclear Medicine at the Norwegian Radium Hospital. Tumor growth and mouse survival were assessed 3 times a week in the first week before injection and throughout the first month after injection; thereafter, growth and survival were assessed 2 times a week. Mice with tumor diameters larger than 20 mm were killed. The different treatment groups were compared by the Mantley-Cox log-rank test. Treatment-induced growth delays, the differences in time to reach a size of 1000 mm³, were calculated after nonlinear regression of mean growth versus time and compared using the Student t test.

Hematologic evaluation

Balb/C nu/nu mice without tumors, weighing 25 to 30 g, were used for blood sampling at the 1- to 8-week time points. Animals used in therapy experiments and which had complete tumor regression to the treatment were compared with similar aged untreated animals at 14, 19, 30, and 36 weeks. The mice were divided into 5 groups, each containing 12 to 15 mice receiving either saline or 200, 400, or 1000 kBq/kg ^{227}Th -DOTA-p-benzyl-rituximab. At 1, 2, 3, 4, 5, 14, and 30 weeks after injection, approximately 200 μL blood was collected from the vena saphena lateralis in 0.5-mL EDTA-coated tubes (Microtainer; Becton Dickinson, Franklin Lakes, NJ). At least 3 weeks separated each blood sampling of an individual mouse. In addition, at 6, 7, 8, 19, and 36 weeks after injection, the mice were anesthetized using sevoflurane (Abbott, Abbott Park, IL), blood was

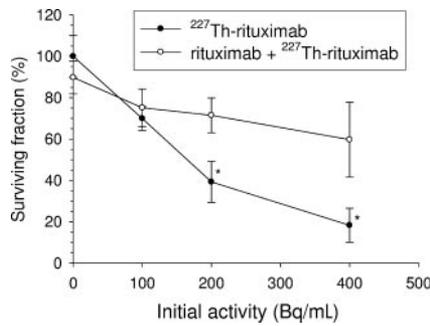


Figure 1. In vitro cytotoxicity. Relative number of surviving Raji cells treated with increasing activities of ²²⁷Th-p-benzyl-DOTA-rituximab. The relative cell number was calculated by dividing the number of treated cells by the number of control cells at day 8 after start of incubation. On days 1, 4, and 6, half of the medium was changed to simulate the elimination of RIC that occurs in the body and to supply the cells with nutrients. ○ shows that binding of ²²⁷Th-p-benzyl-DOTA-rituximab to the cells was blocked by incubation with cold rituximab (10 μg/mL). ● indicates without cold rituximab. *Significantly different from corresponding blocked cells (*t* test, *P* < .05). Error bars represent SE from 3 to 5 experiments.

collected by heart puncture, and the mice were killed by cervical dislocation. In weeks 1 and 5 the samples were counted by a hemolysis method²⁴: 100 mL blood were mixed with 1 mL lysing solution (VersaLyse; Beckman Coulter, Fullerton, CA) for at least 15 minutes to remove red blood cells. Subsequently, the white blood cells were counted in an automatic viability analyzer (Vi-Cell-XR; Beckman Coulter). At all other time points, the blood samples were evaluated for complete blood counts (CBCs) according to standard procedures by the Central Laboratory, Norwegian School of Veterinary Science.

Results

Single-cell cancer model in vitro

Incubation with ²²⁷Th-DOTA-p-benzyl-rituximab for 8 days significantly inhibited growth of CD20⁺ human Raji B-lymphoma cells

(Figure 1). On days 1, 4, and 6 of the experiment, half of the medium was changed to simulate the elimination of RIC that occurs in the body and to supply the cells with nutrients. Cold rituximab alone had no significant effect. Cultures containing cells that were blocked with cold rituximab prior to the addition of ²²⁷Th-DOTA-p-benzyl-rituximab demonstrated some growth inhibition due to nonspecific radiation from the medium. The doubling time increased from 43 hours for untreated cells to 93 hours for cells treated with 400 Bq/mL ²²⁷Th-DOTA-p-benzyl-rituximab. It should be noted that the RIC-specific activity was low in these experiments, typically between 0.5 and 1.0 MBq/mg.

Biodistribution of RICs

To assess binding of ²²⁷Th-rituximab and retention of the daughter ²²³Ra in vivo, the radioactivity per gram of tissue was measured in various normal organs and in tumors at various time points after injection of ²²⁷Th-DOTA-p-benzyl-rituximab in nude mice with CD20-expressing Raji xenografts (Figure 2A,B). There was a large difference between the amount of activity of ²²⁷Th-DOTA-p-benzyl-rituximab in tumor and in normal organs. The uptake of ²²⁷Th-DOTA-p-benzyl-rituximab in Raji xenografts reached a maximum 4 days after injection (Figure 2A). Previously, we measured the biodistribution of ²²⁷Th-DOTA-p-benzyl-rituximab in Balb/C mice without tumors,¹⁶ and similar normal tissue dose-distribution profiles were observed. The control antibody trastuzumab, which does not bind to Raji cells, was also conjugated to ²²⁷Th and injected into nude mice with Raji xenografts to assess nonspecific tumor uptake (Figure 2C-D). The uptake of ²²⁷Th-DOTA-p-benzyl-trastuzumab in tumor was significantly less than with ²²⁷Th-DOTA-p-benzyl-rituximab (*t* test, *P* < .05). The daughter nuclide, ²²³Ra, mainly localized to bone (eg, femur and skull) for both RICs (Figure 2B,D), although some tumor retention was seen of ²²³Ra produced from ²²⁷Th-DOTA-p-benzyl-rituximab decaying in the tumors (Figure 2B). The activity of ¹²⁵I-tixetan-ibratumomab in tumor was

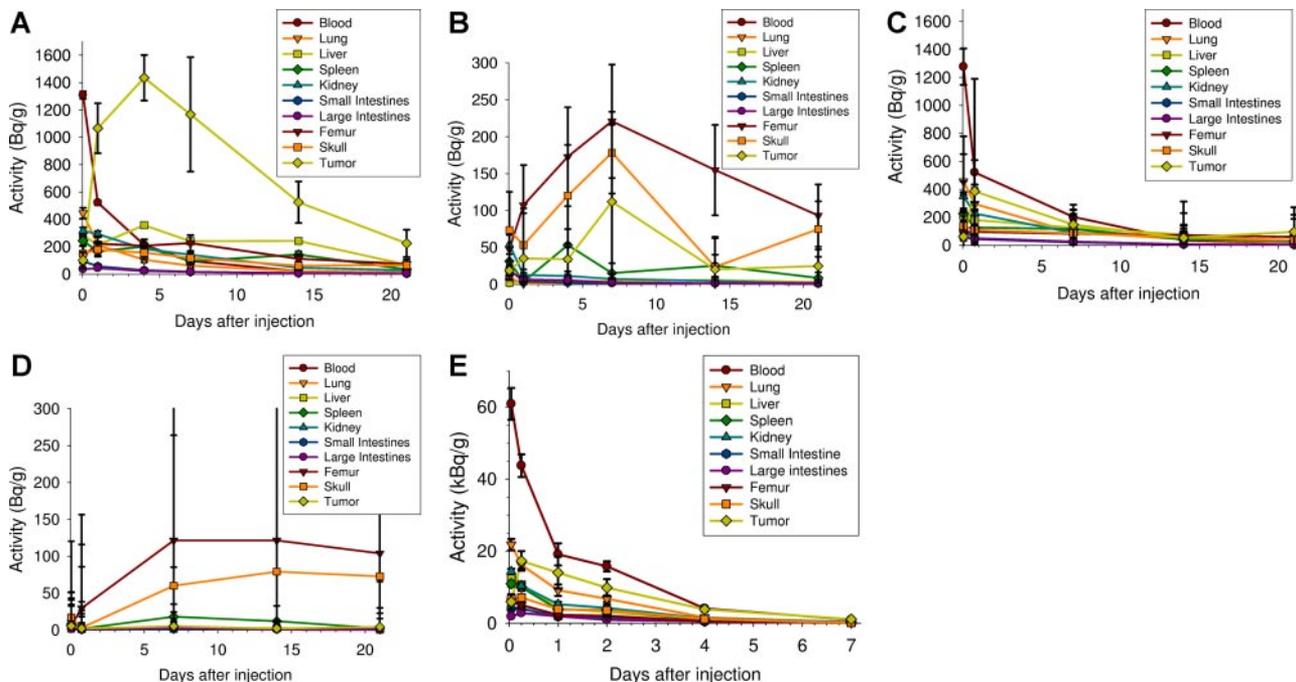


Figure 2. Biodistribution of RICs in mice with CD20⁺ Raji xenografts. Uptake and retention of ²²⁷Th-p-benzyl-DOTA-rituximab (A-B), ²²⁷Th-p-benzyl-DOTA-trastuzumab (C-D) and ¹²⁵I-ibratumomab-tixetan (E) in tumor xenografts and normal tissues were measured at various time points after injection into athymic mice bearing Raji xenografts. The activity per gram was measured using the 236-keV line of ²²⁷Th (A,C) and a germanium detector. (B,D) Activity per gram of ²²³Ra measured using the 154-keV line of ²²³Ra. The measured ²²⁷Th activities were normalized to an injection of 200 kBq/kg body weight. The activity of ¹²⁵I was measured using a γ counter and normalized to an injection of 7.5 MBq ⁹⁰Y/kg body weight. Error bars represent SE. A total of 6 to 8 mice were used.

lower than the activity in blood for all time points, and after the initial uptake it decreased with similar rate as the activity in blood (Figure 2E). Thus, the tumor specificity was higher and the retention time in tumor was longer for ^{227}Th -DOTA-p-benzyl-rituximab.

The radiation doses to normal tissues and to tumor for ^{227}Th -DOTA-p-benzyl-rituximab (Figure 3A) and ^{227}Th -DOTA-p-benzyl-trastuzumab (Figure 3B) were determined by calculating the area under the biodistribution curves and multiplying the resulting cumulated activity with the mean α -particle energy from ^{227}Th or ^{223}Ra and daughters. It was assumed that only α particles contributed to dose and that the radium daughters decayed in the same tissue as radium. When normalized to 200 kBq injected dose per kilogram of body weight, the doses to tumor were approximately 0.5, 2, and 4 Gy for injected dosages of 50, 200, and 400 kBq/kg ^{227}Th -DOTA-p-benzyl-rituximab, respectively. Injection of ^{227}Th -DOTA-p-benzyl-trastuzumab resulted in a significantly lower radiation dose to tumor than injection of ^{227}Th -DOTA-p-benzyl-rituximab. The radiation doses to other tissues were similar to the doses obtained with ^{227}Th -DOTA-p-benzyl-rituximab.

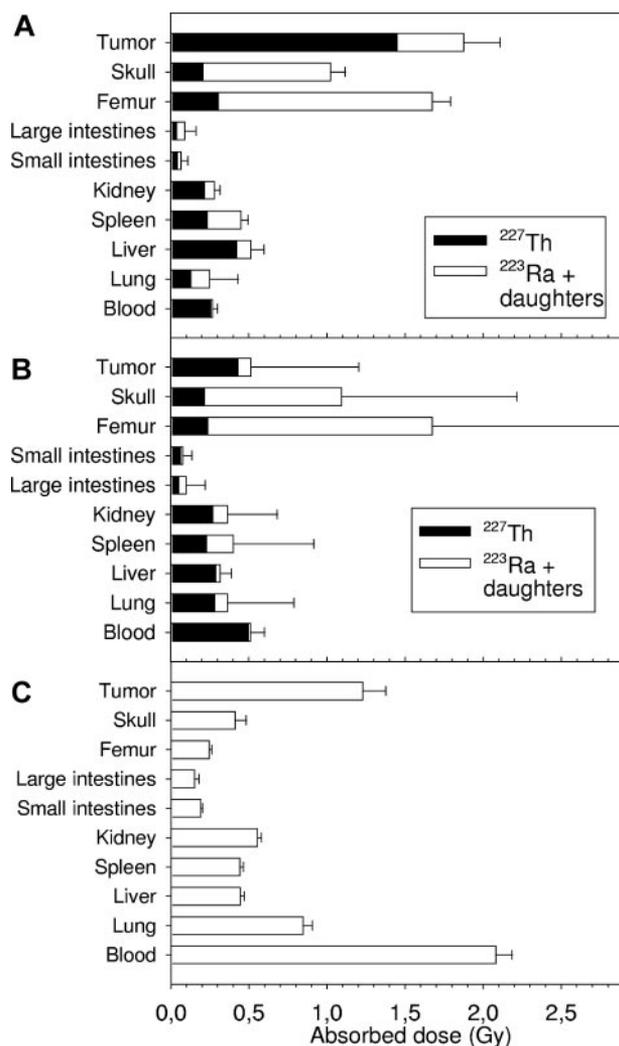


Figure 3. Absorbed doses to tumor xenografts and normal tissues. Cumulated activities were calculated from biodistribution curves and multiplied with the mean energy of the α particles from ^{227}Th or from ^{223}Ra and daughters in order to estimate the absorbed dose after injection of ^{227}Th -p-benzyl-DOTA-rituximab (A) or ^{227}Th -p-benzyl-DOTA-trastuzumab (B). For ^{90}Y -ibritumomab-tiuxetan (C), cumulated activities were calculated from the biodistribution curves of ^{125}I -ibritumomab-tiuxetan adjusted for decay of ^{125}I . The activities of ^{227}Th -p-benzyl-DOTA-rituximab and ^{227}Th -p-benzyl-DOTA-trastuzumab were normalized to an injection of 200 kBq/kg and the activities of ^{90}Y -ibritumomab-tiuxetan were normalized to an injection of 15 MBq/kg. Data are mean (\pm SE).

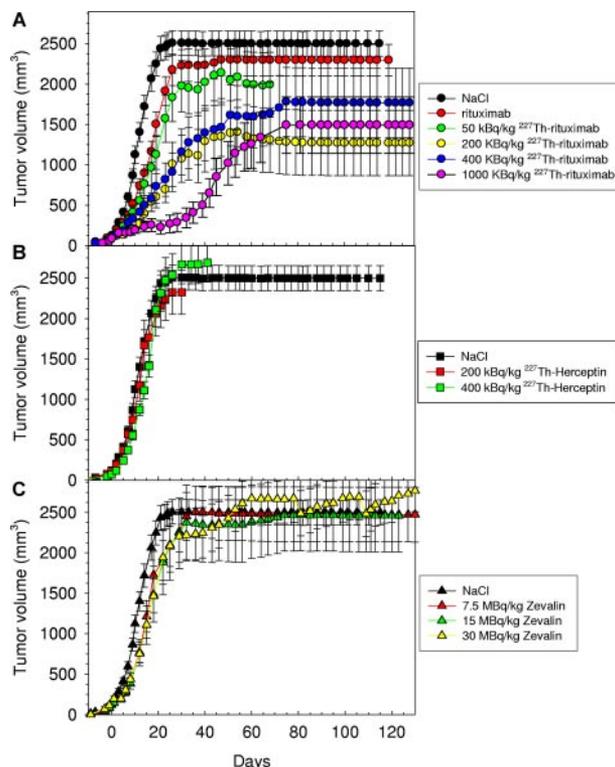


Figure 4. Effect of α -based radioimmunotherapy on growth of Raji xenografts. Mean tumor size versus time after intravenous injection of cold rituximab, 50, 200, and 400 kBq/mL ^{227}Th -p-benzyl-DOTA-rituximab (A); intravenous injection of 200 and 400 kBq/mL ^{227}Th -p-benzyl-DOTA-trastuzumab (B); or intravenous injection of 7.5, 15, and 30 MBq/kg ^{90}Y -ibritumomab-tiuxetan (C). The data for cold rituximab were pooled from groups of mice injected with 20, 100, and 250 μg rituximab. Error bars represent SE. The tumors were not removed from the calculation of mean tumor size when the mice were censored.

For ^{90}Y -tiuxetan-ibritumomab (Figure 3C), the doses to normal tissues and to tumor were calculated using the biodistribution curves of ^{125}I -tiuxetan-ibritumomab. It was assumed that all the β -particles were absorbed by the tumor and that the mean β -energy was 933.6 keV.²³ The radiation doses to tumor were 0.6, 1.2, and 2.5 Gy for injected dosages of 7.5, 15, and 30 MBq/kg ^{90}Y -tiuxetan-ibritumomab. For a given tumor radiation dose, the doses to normal tissues were compared: the radiation doses to the liver and the spleen were similar, while the radiation doses to the blood, lung, kidney, and intestines were more than 100% higher for ^{90}Y -tiuxetan-ibritumomab than for ^{227}Th -DOTA-p-benzyl-rituximab. For the skull and the femur, the radiation doses were more than 80% lower for ^{90}Y -tiuxetan-ibritumomab than for ^{227}Th -DOTA-p-benzyl-rituximab.

Therapy of mice with Raji lymphoma xenografts

The efficacy of ^{227}Th -DOTA-p-benzyl-rituximab for treating macroscopic tumors was assessed by measuring the growth of Raji lymphoma xenografts after radioimmunotherapy and comparing the results with control groups treated with saline, cold rituximab, ^{227}Th -DOTA-p-benzyl-trastuzumab, and the β -emitting RIC ^{90}Y -tiuxetan-ibritumomab (Figures 4-5). Treatment with 200 to 1000 kBq/kg ^{227}Th -DOTA-p-benzyl-rituximab resulted in a pronounced delay in tumor growth and in a significant increase in survival compared with all other treatments (Table 1; Figures 4-6). Treatment with 50 kBq/kg ^{227}Th -DOTA-p-benzyl-rituximab and cold rituximab had no significant effect on tumor growth or mean survival. The

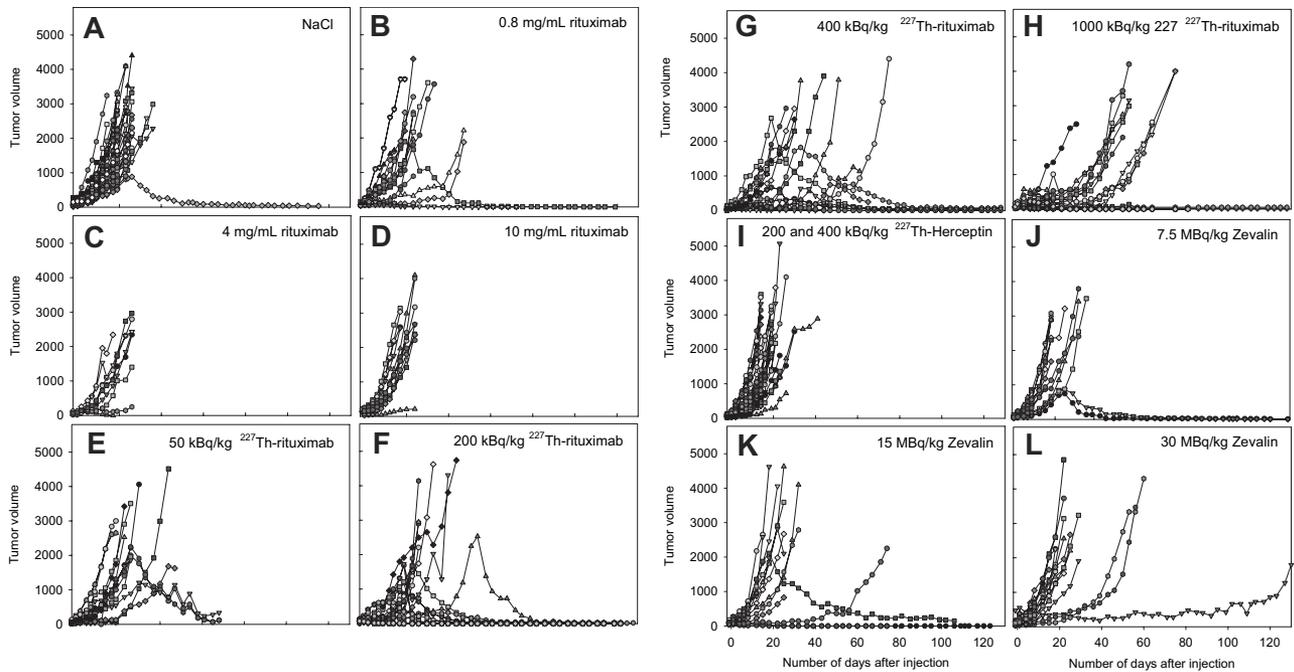


Figure 5. Effect of α-based radioimmunotherapy on growth of individual Raji xenografts. Individual tumor sizes versus time after intravenous injection of NaCl (A); 20, 100, and 250 μg cold rituximab (B-D); 50, 200, 400, and 1000 kBq/mL ²²⁷Th-p-benzyl-DOTA-rituximab (E-H); intravenous injection of 200 and 400 kBq/mL ²²⁷Th-p-benzyl-DOTA-trastuzumab (I); or intravenous injection of 7.5, 15, and 30 MBq/kg ⁹⁰Y-ibritumomab-tiuxetan (J-L).

efficacy of the ²²⁷Th-DOTA-p-benzyl-rituximab treatment improved in a significant and dosage-dependent fashion in the range from 50 to 200 kBq/kg, but there was no further increase in therapeutic effect from 200 to 400 kBq/kg. By using 1000 kBq/kg ²²⁷Th-DOTA-p-benzyl-rituximab, a significant increase in growth delay as compared with 200 kBq/kg (*t* test, *P* = .003) was obtained, although the long-term survival tended to be lower for 1000 kBq/kg than for 200 kBq/kg (Table 1).

There was no significant effect of cold rituximab (Figures 5B-D, 6A). The highest dosage of cold rituximab used (10 mg/kg) is comparable to the clinical dosage used in humans (8.7 mg/kg).²⁵ One animal treated with isotonic NaCl exhibited tumor shrinkage (Figure 5A). We speculate that tumor regression in this animal was related to an immunologic reaction induced by a wound on the tumor.

Radiolabeled trastuzumab, which targets HER2/neu, did not bind specifically to Raji tumors (Figure 2C) and had no significant therapeutic effect (Table 1; Figures 4B, 5I, 6C).

The commercially available β-emitting RIC, ⁹⁰Y-tiuxetan-ibritumomab, which also targets CD20 presenting cells, had significantly less effect than ²²⁷Th-DOTA-p-benzyl-rituximab on tumor growth and survival (Table 1; Figures 4, 5J-L, 6D). However, despite the fact that treatment with ⁹⁰Y-tiuxetan-ibritumomab resulted in successful inhibition of tumor growth in some cases (Figure 5J-L), it was much less striking than the effects of 200 to 400 kBq/kg of ²²⁷Th-DOTA-p-benzyl-rituximab. There was also a tendency to improved survival after treatment with ⁹⁰Y-tiuxetan-ibritumomab, but the effect was not statistically significant when compared with the control group.

Toxicity of ²²⁷Th-DOTA-p-benzyl-rituximab

There was a dosage related reduction in the white blood cell (WBC) count after treatment with ²²⁷Th-DOTA-p-benzyl-rituximab (Figure 7). The WBC nadir was around 3 weeks following injection with maximum 1-log reduction in WBC count. In the control mice, the WBC count varied from 2.5 × 10⁹ to above 10 × 10⁹ per L blood. In the group receiving a therapeutic dosage of 200 kBq/kg, bone marrow suppression was modest, and after 4 weeks, the WBC count was within the range of the control mice. The mice receiving 400 kBq/kg showed more signs of bone marrow suppression, but after 7 weeks, the WBC count was within the range of the control mice. In the group receiving 1000 kBq/kg, the hemotoxicity was more pronounced, and 1 of 37 animals had to be killed due to fatigue and weight loss. However, only 2 mice in the 2- to 3-week time interval had lower WBC counts than 1.3 × 10⁹/L, and the WBC count for animals in the 1000 kBq/kg group reached the level of the control mice after 14 weeks. The number of platelets was within the reference limits for most of the samples. A total of 3 mice in the group receiving 1000 kBq/kg had slightly lowered platelet counts 3 weeks after treatment (not

Table 1. Growth delay and median survival for all treatments

Treatment	Growth delay, d ± SE*	Median survival, d ± SE
NaCl	—	21 ± 1
rituximab (pooled)	3 ± 1	44 ± 10
50 kBq/kg ²²⁷ Th-rituximab	5 ± 1	26 ± 3
200 kBq/kg ²²⁷ Th-rituximab†	17 ± 1	> 119
400 kBq/kg ²²⁷ Th-rituximab†	15 ± 1	> 81
1000 kBq/kg ²²⁷ Th-rituximab†	40 ± 7	75 ± 11
200 kBq/kg ²²⁷ Th-trastuzumab	0 ± 6	15 ± 2
400 kBq/kg ²²⁷ Th-trastuzumab	2 ± 1	20 ± 1
7.5 MBq/kg Zevalin®	2 ± 2	33 ± 4
15 MBq/kg Zevalin®	3 ± 1	26 ± 1
30 MBq/kg Zevalin®	3 ± 1	23 ± 3

— indicates not applicable.

*Delay in days to reach a mean tumor size of 1000 mm³ as compared with treatment with NaCl.

†Growth delay significantly different from NaCl and rituximab (*t* test, *P* < .05) and median survival significantly different from NaCl and rituximab (Mantley-Cox log rank test, *P* < .001).

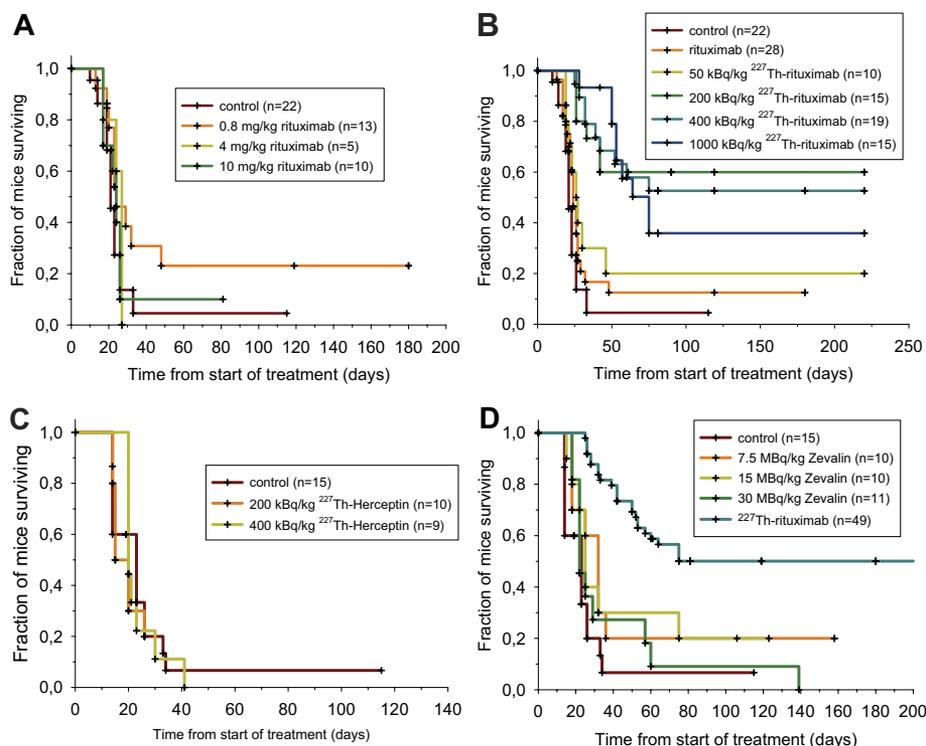


Figure 6. Effect of α -based radioimmunotherapy on survival of athymic mice with Raji xenografts. The mice were intravenously injected with saline, 20, 100, and 250 μ g cold rituximab (A); 50, 200, 400, and 1000 kBq/kg ^{227}Th -p-benzyl-DOTA-rituximab (B); 200 and 400 kBq/kg ^{227}Th -p-benzyl-DOTA-trastuzumab (C); or 7.5, 15, and 30 MBq/kg ^{90}Y -ibritumomab-tiuxetan (D). Mice with tumor diameters greater than 20 mm were killed. Median survival times of treated groups were compared with control and cold rituximab (pooled) using the Mantley-Cox log-rank test: $P < .001$ for 200, 400, and 1000 kBq/kg ^{227}Th -p-benzyl-DOTA-rituximab. The rest of the treatments were not significantly different from the control group (NaCl). The groups receiving 200, 400, and 1000 kBq/kg ^{227}Th -p-benzyl-DOTA-rituximab had also significantly longer survival than the group receiving 20 μ g cold rituximab ($P < .05$, Mantley-Cox log-rank test). The groups receiving 200, 400, and 1000 kBq/kg ^{227}Th -p-benzyl-DOTA-rituximab treatment groups were pooled in panel D, and the pooled group had significantly longer survival than all the ^{90}Y -ibritumomab-tiuxetan treatment groups ($P < .01$, Mantley-Cox log-rank test). One mouse in the 1000 kBq/kg ^{227}Th -p-benzyl-DOTA-rituximab group died after 42 days. n indicates the number of mice per treatment.

shown). In conclusion, even at the highest dosage level, the hematologic toxicity was moderate.

Discussion

We have demonstrated that ^{227}Th -DOTA-p-benzyl-rituximab targets CD20-expressing Raji B-lymphoma tumor cells in vitro and in vivo, successfully inhibits tumor growth, and improves survival in mice, even though very small amounts of isotope were used. Since the therapeutic efficacy was so high with 200 kBq/kg ^{227}Th -DOTA-p-benzyl-rituximab, the minimal required activity was probably between 50 to 200 kBq/kg, which can be extrapolated to 2.5 to 10 MBq for a 50-kg patient. Treatment with ^{227}Th -DOTA-p-benzyl-rituximab was significantly superior to treatment with clinically relevant dosages of ^{90}Y -tiuxetan-ibritumomab and rituximab. The upper dosage with rituximab and the middle dosage with ^{90}Y -tiuxetan-ibritumomab correspond to the recommended dosages for clinical use in

humans on a milligram per kilogram (mg/kg) and mega-Becquerel per kilogram (MBq/kg) scale, respectively. The safe human dosage of ^{227}Th -DOTA-p-benzyl-rituximab is not known, and therefore a range of dosages was explored with this compound. The range of dosages selected for testing with ^{227}Th -DOTA-p-benzyl-rituximab was based upon reported dosages in preclinical radioimmunotherapy with ^{225}Ac ,⁸ and the knowledge that 250 kBq/kg of the thorium daughter ^{223}Ra is hematologically safe in humans.¹⁸ Also, in our study there was no evidence of delayed bone marrow toxicity, including myelodysplasia, even at the highest dosage level of ^{227}Th -DOTA-p-benzyl-rituximab.

The in vitro cell growth data demonstrated that it was possible to obtain specific single-cell killing with low dose rate α radioimmunotherapy with ^{227}Th . The mean number of α -particle hits per lymphoma cell necessary to inactivate more than 99% of the cells incubated with ^{211}At -rituximab has been calculated to be from 15 to 50.^{26,27} Figure 1 shows that 400 Bq/mL of ^{227}Th -DOTA-p-benzyl-rituximab significantly inhibited cell growth. At this activity concentration, 22 μ Bq was bound to each cell,¹⁶ corresponding to 1.9 α -disintegrations per day, or about 15 after 8 days. However, the amount of RIC bound to the cells will decrease with increasing incubation time.²⁶ Furthermore, only a third of the α -particles are expected to hit the nucleus of lymphoma cells, assuming a cellular radius of 5.5 to 8.5 μm and a nuclear radius of 4.5 to 7 μm .²⁸ Thus, with an initial activity of 400 Bq/mL, the nucleus of each cell was on the average hit by approximately 5 targeted α -particles in 8 days. In addition, the cells were hit by α -particles from unbound RIC, from RIC bound to neighboring cells, and from α -particles from daughter nuclides generated during the incubation period. However, it is likely that some of the cells were not affected because of the long half-life of ^{227}Th . These cells divide with a speed similar to that of the control cells. Furthermore, the number of viable cells was probably overestimated, since cells undergoing early phases of apoptosis, necrosis, and mitotic

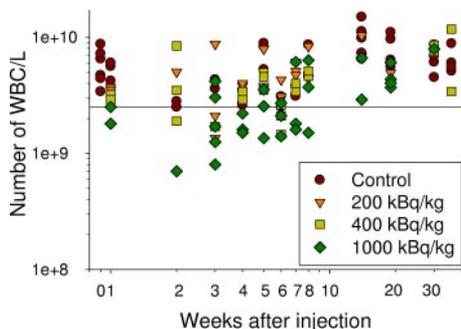


Figure 7. Assessment of bone marrow toxicity of ^{227}Th -p-benzyl-DOTA-rituximab. The number of WBCs was counted each week for 8 weeks and at 14, 19, 30, and 36 weeks after injection of NaCl and 200, 400, and 1000 kBq/kg ^{227}Th -p-benzyl-DOTA-rituximab. In weeks 1 and 5, the number of WBCs was counted using a hemolysis procedure; in all other time points, CBCs were obtained. (Horizontal line is lowest value of controls.)

catastrophe could not be distinguished from viable cells. Consequently, low dose rate α -radioimmunotherapy has significant cytotoxicity against single cells and, probably, micrometastatic disease.

To assess the ability to treat macroscopic tumors, ^{227}Th -DOTA-p-benzyl-rituximab was injected into nude mice bearing macroscopic (32–256 mm³) human B-lymphoma xenografts. There was a large difference between the amount of activity of ^{227}Th -DOTA-p-benzyl-rituximab in tumor and in normal organs, which indicates a large therapeutic window. From Figure 2, it can be estimated that a 0.5-g tumor receives 820 million α -hits if 200 kBq/kg ^{227}Th -DOTA-p-benzyl-rituximab is injected. The density of the cells is approximately 1 mg/mm³, and the cell radius is approximately 7 μm (data not shown). We assume that 50% of the tumor is intercellular space. Thus, such a tumor would consist of 175 million cells, which corresponds to a mean of 4.7 α -particle hits per tumor cell nuclei if all α -particles are absorbed by tumor cell nuclei and an α -particle only impacts 1 nucleus. The uptake in the periphery of a large tumor is higher than in the central region of the tumor, but redistribution of RIC from inactivated cells in the periphery to viable cells in the core during the course of treatment may counteract heterogeneous tumor uptake.

Treatment with 1000 kBq/kg ^{227}Th -DOTA-p-benzyl-rituximab had a significantly better effect on the early phase of tumor growth than 200 and 400 kBq/kg ^{227}Th -DOTA-p-benzyl-rituximab, while the effect on long-term survival tended to be better at 200 kBq/kg. This apparent contradiction might be related to the higher hematologic toxicity of the 1000 kBq/kg dosage. It may be beneficial for long-term tumor control to have active immune cells, while short-term tumor control is more dependent on dosage level.

Several tumors had large necrotic cores, as determined by hematoxylin-eosin (HE) staining and microscopy. About 50% of the tumors had necrotic cores in the control mice, while approximately 90% of the tumors had necrotic cores in the mice treated with ^{227}Th -DOTA-p-benzyl-rituximab. Thus, the high potency of ^{227}Th may be attributed, at least in part, to its long half-life. Retention of the daughter nuclides, which also are α -emitters, in the tumor tissue may also have contributed to the favorable therapeutic effect.

Low-dose rate α -radioimmunotherapy seems to be beneficial against macroscopic tumors as well as single tumor cells. There may be both advantages and disadvantages of using low dose rates. Disadvantages may include tumor tissue repair due to proliferation and possible DNA repair, although the latter is less likely since α radiation causes mainly irreparable double-strand breaks in the DNA.²⁹ The therapeutic level of ^{227}Th found to be effective in this study was quite modest. The amount of ^{223}Ra generated would probably not limit the use of ^{227}Th , as indicated by the modest toxicity shown in recent clinical data on ^{223}Ra in patients with prostate and breast cancer.¹⁰

The β -emitting, commercially available RIC ^{90}Y -tiuxetan-ibritumomab, which also targets CD20 presenting cells, had significantly less effect than ^{227}Th -DOTA-p-benzyl-rituximab. The uptake of ^{125}I -ibritumomab-tiuxetan in tumor was significantly lower than the uptake of ^{227}Th -DOTA-p-benzyl-rituximab. The immunoreactivity of ^{125}I -ibritumomab-tiuxetan was 57%, which is acceptable. The tumor uptake in percentage of injected dose per gram 7 days after injection was 26% for ^{227}Th -DOTA-p-benzyl-rituximab, 3% for ^{125}I -ibritumomab-tiuxetan, and 19% for ^{125}I -rituximab (data not shown). Thus, labeling of rituximab with ^{125}I did not alter the tumor uptake significantly, indicating that ^{90}Y -tiuxetan-ibritumomab is not as

suitable for therapy of mice with lymphoma xenografts as radiolabeled rituximab. Consistently, Ma et al had to administer single injections of 278 to 370 MBq/kg ^{90}Y -tiuxetan-ibritumomab to achieve a significant increase in median survival time in a Ramos xenograft model.³⁰ The standard patient dosage of ^{90}Y -tiuxetan-ibritumomab is 15 MBq/kg. It is noteworthy that ^{227}Th -rituximab was significantly more effective than the clinically proven ^{90}Y -tiuxetan-ibritumomab.

The recently developed method yielding stable constructs of ^{227}Th -DOTA-p-benzyl-IgG in therapeutic quantities, and the demonstration of safe, efficacious use against a macroscopic tumor model, using modest dosages of isotope, suggest that clinical use of such targeted drugs is feasible. The 18.72-day half-life of ^{227}Th would allow the drugs to be manufactured at a central radiopharmacy and shipped throughout the world. Because of the extraordinary potency of the α -emitting ^{227}Th radionuclide, a limited amount of radioactivity would be required for therapeutic human use, permitting an economic and safe outpatient use. In addition, the half-life of ^{227}Th may allow time to maximize the uptake in macroscopic tumors.

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Authorship

Contribution: J.D. and R.H.L. designed the experiments. J.D. wrote the manuscript. J.D., J.B., and A.K.H. performed the research. J.D. did the data analysis. J.B. and R.H.L. performed research and contributed the ^{227}Th RICs. Ø.S.B., R.H.L., and T.J.J. contributed to the experimental design and writing of the manuscript. K.B.M., J.B., R.H.L., and J.D. performed research and did quality control of the ^{227}Th RICs. O.W.P. contributed in writing the manuscript.

Conflict-of-interest disclosure: J.B. is employed by Algeta ASA, who owns the patent for using ^{227}Th for radioimmunotherapy and has provided research support for this study. R.H.L. and Ø.S.B. are founders of Algeta ASA and have a significant stock interest. Ø.S.B. and O.W.P. are members of the scientific advisory board of Algeta ASA. J.D. and T.J.J. have a small amount of stocks in Algeta ASA. The remaining authors declare no competing financial interests.

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