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Initial evaluation of ^{227}Th-*p*-benzyl-DOTA-rituximab for low-dose rate α-particle radioimmunotherapy

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Abstract

Radioimmunotherapy has proven clinically effective in patients with non-Hodgkin's lymphoma. Radioimmunotherapy trials have so far been performed with β-emitting isotopes. In contrast to β-emitters, the shorter range and high linear energy transfer (LET) of α particles allow for more efficient and selective killing of individually targeted tumor cells. However, there are several obstacles to the use of α-particle immunotherapy, including problems with chelation chemistry and nontarget tissue toxicity. The α-emitting radioimmunoconjugate ^{227}Th-DOTA-*p*-benzyl-rituximab is a new potential anti-lymphoma agent that might overcome some of these difficulties. The present study explores the immunoreactivity, in vivo stability and biodistribution, as well as the effect on in vitro cell growth, of this novel radioimmunoconjugate. To evaluate in vivo stability, uptake in balb/c mice of the α-particle-emitting nuclide ^{227}Th alone, the chelated form, ^{227}Th-*p*-nitrobenzyl-DOTA and the radioimmunoconjugate ^{227}Th-DOTA-*p*-benzyl-rituximab was compared in a range of organs at increasing time points after injection. The immunoreactive fraction of ^{227}Th-DOTA-*p*-benzyl-rituximab was 56–65%. During the 28 days after injection of radioimmunoconjugate only, very modest amounts of the ^{227}Th had detached from DOTA-*p*-benzyl-rituximab, indicating a relevant stability in vivo. The half-life of ^{227}Th-DOTA-*p*-benzyl-rituximab in blood was 7.4 days. Incubation of lymphoma cells with ^{227}Th-DOTA-*p*-benzyl-rituximab resulted in a significant antigen-dependent inhibition of cell growth. The data presented here warrant further studies of ^{227}Th-DOTA-*p*-benzyl-rituximab.

Keywords: Alpha radiation; Radioimmunotherapy; Lymphoma; Thorium-227; Rituximab

1. Introduction

There is still no consensus as to the optimal initial therapy for follicular and low-grade non-Hodgkin's lymphoma (NHL) [1]. High-dose chemotherapy with stem cell support is effective only in patients with chemosensitive disease [2], and most patients with relapsed NHL are incurable by current conventional treatment. However, new treatment modalities, including immunotherapeutic approaches, have been developed. Therapeutic monoclonal antibodies (MoAbs) against the CD20 antigen present on mature B cells yield response rates of 30–50% in patients with relapsed B-cell NHL [3]. In addition, when MoAbs against CD20 are conjugated to the β-emitting isotope ^{131}I or ^{90}Y and used in patients, long-term responses in up to 86% of the patients have been observed [4–7].

Since NHL is often characterized with infiltrated bone marrow and/or lymph nodes with a significant presence of dispersed single-tumor cells, it would make sense to include radionuclides allowing a more cell-focused irradiation into the armamentarium. Alpha-emitting nuclides, characterized by short-range, high linear energy transfer (LET), and considerably higher radiobiological effectiveness than β-emitters, could be promising for arming tumor-seeking molecules useful for targeted therapy of NHL.

In a previous work, Aurlien et al. [8] have demonstrated cell-specific cytotoxicity of the chimeric MoAb rituximab labeled with the α emitter ^{211}At. Although this nuclide is

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promising for biomedical application, its short half-life (7.2 h) and the low production capability using current cyclotron technology make it challenging to apply ^{211}At in clinical settings. If MoAb is to be used as delivery agent in systemic therapy against NHL, a half-life exceeding that of ^{211}At could, in some respect, be beneficial. This could allow better targeting of tumor cells, since diffusion barriers exist, which tend to slow down the uptake. A few longer-lived α emitters are currently being evaluated for tumor therapy. Radium-223 ($t_{1/2}$=11.4 days) is currently being assessed in the treatment of skeletal metastases using the cationic form, but seems to be difficult to conjugate to MoAbs [9]. Actinium-225 ($t_{1/2}$=11.4 days) has shown promise when conjugated to MoAbs, but there are limitations on source material for the preparation of this nuclide [10,11]. Another issue would be that a radioimmunoconjugate would shed the daughter nuclides, which could then relocalize and may be associated with unwanted toxicity, e.g., kidney damage [12].

Rituximab has a mean serum half-life in patients of 3.2 days [13]. Conceivably, rituximab that binds to the CD20 antigen on B cells will stay in circulation in the body for a longer time than rituximab that is not bound. In other words, the proportion of rituximab that is bound to target cells will increase with time after injection as unbound rituximab is secreted from the body. Short-lived nuclides like ^{211}At will deposit most of the energy during the first 24 h after injection, which may be before the unbound antibody is secreted from the body. Thus, to reduce radiation damage to normal tissue, it is beneficial to use nuclides with a half-life that is longer than the half-life of the antibody. It is conceivable that nuclides with longer half-lives than the antibody will deposit a larger proportion of the energy in the target and not in untargeted cells.

A clinical safety study of the ^{227}Th-daughter ^{223}Ra showed acceptable myelotoxicity at a dosage level of 250 kBq/kg [14]. Based on the clinical tolerability of the daughter nuclide, we hereby propose to use ^{227}Th in radioimmunotherapy. In addition, ^{227}Th has a beneficial half-life of 18.7 days. However, B cells do not take up antibodies bound to the CD20 antigen, so the daughter nuclides may be redistributed in the body. Nevertheless, if a pure ^{227}Th-labeled radioimmunoconjugate is administered, it will take some time before a significant amount of ^{223}Ra has been generated. Because of the relatively long half-life of ^{223}Ra, this nuclide would, to a large extent, be excreted or become trapped in skeletal hydroxyapatite before decay occurs [14]. Thus, there may exist a therapeutic window where ^{227}Th could be used without causing nontolerable toxicity from the ^{223}Ra series generated.

Alpha particles from ^{227}Th have a mean energy of 5.9 MeV and their ranges in soft tissue are only 50–70 μm, i.e., a few cell diameters. The short range implies that the radiation dose is delivered to the cell or within the vicinity of the cell targeted by the MoAb. The short range also implies that the toxicity to normal tissue, which does not bind the MoAb, is lower for α-emitters than for β-emitters. The low γ components of ^{227}Th and the ^{223}Ra series indicate that the need for patient shielding is greatly reduced, allowing α-radioimmunotherapy to be given on an outpatient basis. Moreover, the high LET of α-particles of around 100 keV/μm is close to the optimum value for relative biological effectiveness. The effectiveness of high LET radiation is virtually independent of oxygen concentration, dose rate and position in the cell cycle [15]. The half-life of ^{227}Th is 18.7 days, which makes shipping of the radioimmunoconjugate from the production site to the hospital feasible.

The current study represents the first assessment of a ^{227}Th-labeled MoAb in vivo and was performed using ^{227}Th-DOTA-*p*-benzyl-rituximab as the model compound. The biodistribution in normal tissue of the radioimmunoconjugate was determined and compared with that of cationic ^{227}Th and ^{227}Th chelated with *p*-nitrobenzyl-DOTA in order to evaluate in vivo stability.

2. Materials and methods

2.1. Preparation of ^{227}Th

^{227}Ac was produced through thermal neutron irradiation of ^{226}Ra followed by β^- decay of ^{227}Ra ($t_{1/2}$=42.2 m) to ^{227}Ac [16]. ^{227}Th was selectively retained from a ^{227}Ac decay mixture in 7 M HNO_3 solution by anion exchange chromatography [17]. A column of 2 mm internal diameter, length 30 mm, containing 70 mg of AG-1×8 resin (200–400 mesh, nitrate form) was used. After ^{227}Ac, ^{223}Ra and daughters had eluted from the column, ^{227}Th was extracted from the column with 12 M HCl. The eluate containing ^{227}Th was evaporated to dryness and the residue resuspended in 0.01 M HCl.

2.2. Preparation of ^{227}Th-DOTA complexes

The ^{227}Th-DOTA complexes were prepared by adding 20 μl of 10 mg/ml solution of either *p*-NO_2-benzyl-DOTA or *p*-SCN-benzyl-DOTA (Macrocyclics, Dallas, TX) to a solution containing 20 μl (150 mg/ml) of L-ascorbic acid (Sigma-Aldrich, Gillingham, UK) and 20–150 μl of 100–300 MBq/ml ^{227}Th in HCl in a 2-ml glass vial. The pH was adjusted to about 5.5 by adding tetramethylammonium acetate (Aldrich Chem, Milwaukee, WI). Thereafter, the reaction mixture was incubated for 40 min at 55–60°C using a Thermomixer Comfort (Eppendorf, Hamburg, Germany). The *p*-SCN-benzyl-DOTA was used for antibody labeling, while the *p*-NO_2-benzyl-DOTA was purified on a column containing Sephadex CM C-25 (Aldrich) cation-exchange particulates (40–120 μm) using isotonic saline as eluting medium. It was verified from runs using reaction solutions without complexing agents that this system retained cationic ^{227}Th quantitatively. Typically, more than 95% of the activity was eluted in the saline fraction when the reaction solution was containing the complexing DOTA derivates.

2.3. Conjugation of ^{227}Th-*p*-isothiocyanato-benzyl-DOTA to rituximab

The reaction solution containing ^{227}Th and *p*-SCN-benzyl-DOTA was cooled down to 37°C, 1–2 mg rituximab (MabThera, Roche, Switzerland) in 100–200 μl was added and the pH was adjusted to 8–9 by adding 1 M $NaCO_3$/$NaHCO_3$ buffer. After 45 min of reaction, 20 μl of saturated diethylene triamine pentaacetic acid (DTPA, Fluka Chemie, Buchs, Switzerland) was added and the mixture further incubated for 5 min. Thereafter, the reaction mixture was purified by gel filtration (Econo-Pac10 DG, Bio-Rad, Hercules, CA) eluted with 1% bovine serum albumine (Sigma, St Louis, MI) in phosphate buffered saline, pH 7.4. Finally, the purified product was sterile filtered (Millex GV-13, Millipore, Bedford, IL) into a sterile 10-ml glass vial (Wheaton, Millville, NJ), which was subsequently capped with a sterile rubber cap. The specific activity of the radioimmunoconjugate was typically 500–1000 Bq/μg.

2.4. Measurement of immunoreactive fraction and cellular binding

The quality of the radioimmunoconjugate was measured using lymphoma cells and a modified Lindmo method [18]. Cell concentrations of up to 10^8 Raji cells per milliliter were used to compensate for the modest specific activity of the radioimmunoconjugate. The immunoreactive fractions of conjugates prepared in this manner were typically between 56% and 65%. Total binding of radioimmunoconjugate to cells was measured by incubating 2 million cells with increasing amounts of radioimmunoconjugate. Unspecific binding was determined by incubating some of the cells with 100 μg/ml cold antibody in addition to the radioimmunoconjugate. Specific binding was calculated by subtracting the unspecific binding from the total binding.

2.5. Biodistribution experiments

The biodistribution of ^{227}Th, ^{227}Th-*p*-nitrobenzyl-DOTA and ^{227}Th-DOTA-*p*-benzyl-rituximab was studied in Balb/c mice (Balb/c AnNHsd, Harlan UK, Oxfordshire, England). 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. Four- to 6-week-old Balb/c mice with an average body weight of 25 g at the start of the study were used. The preparations were administered by tail vein injection of 100 μl solution to each animal, resulting in a total activity injected of 1135, 913 or 974 kBq/kg, for ^{227}Th, ^{227}Th-*p*-nitobenzyl-DOTA or ^{227}Th-DOTA-*p*-benzyl-rituximab, respectively. This amount of radioactivity was necessary for the purpose of obtaining a suitable counting statistics from the tissue samples. However, for therapeutic purposes this could be too toxic in humans. Therefore, for radiation dose calculation purposes the distribution data were normalized to an injected dosage of 100 kBq/kg of bodyweight, which is also more in line with the levels of ^{223}Ra given in human studies. Preliminary data from ongoing studies in xenograft models indicate that therapeutic activity may be seen at similar dosage levels. The experiments were performed twice, each time with three animals per time point. The autopsy was performed after cervical dislocation at various time points after injection. The weight of each sample was determined, and the ^{227}Th and ^{223}Ra were measured by their most characteristic γ-rays employing 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) [19]. The count rates in the samples were also routinely measured using a γ counter (Crystal II Multidetector RIA System, Packard Instruments, Downers Grove, IL). Samples of the injectates were used as references in the measurement procedures.

2.6. Calculation of dose

The absorbed radiation doses were calculated assuming dose contributions coming only from α particle emissions with a mean energy of 5.9 MeV for ^{227}Th and 26.4 MeV for ^{223}Ra with daughters in equilibrium [19], and that there was a 100% absorption of the α dose in a tissue. The biodistribution data were normalized to an injection of 100 kBq/kg of bodyweight, and it was assumed that the radionuclides were uniformly distributed in the various tissues. For blood, the absorbed dose was calculated assuming 100% absorption of the α particle in the blood. This is a simplification since in the capillaries there will be escape of α particles beyond the blood. 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 activity concentration in various tissues vs. time [20]. Thus, the total dose to each organ could be calculated by Eq. (1):

$$\text{Dose} = \text{AUC}_0^\infty \cdot E_\alpha\left(^{227}\text{Th}\right) + \text{AUC}_0^\infty \cdot E_\alpha\left(^{223}\text{Ra} + \text{daughters}\right) \quad (1)$$

2.7. Cell growth experiments

Cell growth after treatment with ^{227}Th-DOTA-*p*-benzyl-rituximab was determined by seeding 10^6 Raji cells in 10 ml of medium and incubated for 8 days. Fifty percent of the medium was substituted with fresh medium after 1, 4 and 6 days to add nutrients and to simulate biological elimination of radioimmunoconjugate. The number of cells per milliliter of medium was determined at 1, 4, 6 and 8 days after the start of the incubation. Cells were incubated with medium alone, with medium containing 10 μg/ml cold antibody, with medium containing 10 μg/ml cold antibody and 200 Bq/ml ^{227}Th-DOTA-*p*-benzyl-rituximab

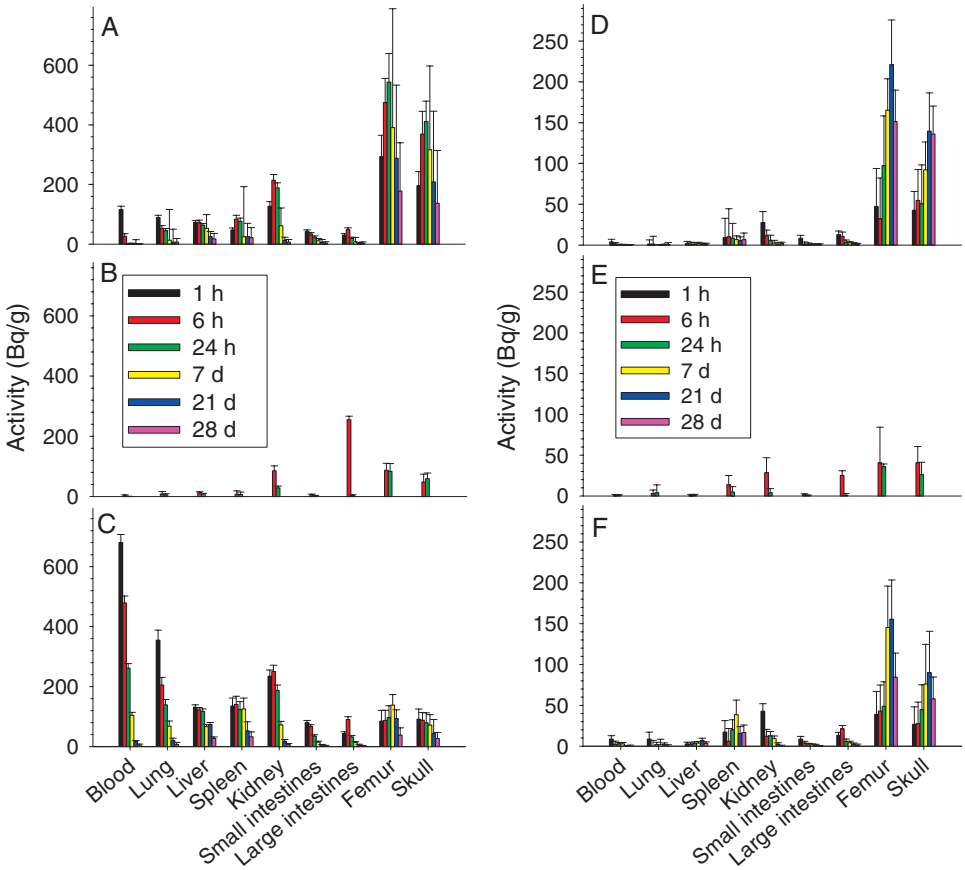

Fig. 1. Activity per gram measured using the 236-keV line of ^{227}Th (A–C) or the 154-keV line of ^{223}Ra (D–F) for various time points after injection of ^{227}Th (A, D), ^{227}Th-*p*-nitrobenzyl-DOTA (B, E) or ^{227}Th-*p*-benzyl-DOTA-rituximab (C, F). Error bars = standard error. Five to six mice per time point. The activity was normalized to 100 kBq/kg body weight for the different injectates.

or with medium containing 200 Bq/ml ^{227}Th-DOTA-*p*-benzyl-rituximab.

3. Results

3.1. Biodistribution of ^{227}Th, ^{227}Th-p-nitrobenzyl-DOTA and ^{227}Th-DOTA-p-benzyl-rituximab in Balb/c mice

The biodistribution of ^{227}Th, ^{227}Th-*p*-nitrobenzyl-DOTA and ^{227}Th-DOTA-*p*-benzyl-rituximab was studied in Balb/c mice at various time points after a single injection of the preparations. Distributions of the three different preparations in various organs and for increasing time points are shown in Fig. 1. Fig. 1A shows that 24 h after the injection of free thorium, most of it distributes to the bone. The uptake in the bone of thorium at 6 h was in accordance with previous publications [17]. There was also some uptake in the kidney at early time points. In the soft tissues, the highest long-term retention was found in the liver and spleen. Nevertheless, if ^{227}Th-DOTA-*p*-benzyl-rituximab is undergoing demetalation, most of the free thorium will redistribute to bony tissues. Thus, measurement of redistribution to the bone may be used to evaluate in vivo stability of the radioimmunoconjugate.

Fig. 1B shows the biodistribution of ^{227}Th-*p*-nitrobenzyl-DOTA. Compared with the free radionuclide, the uptake was lower and the clearance was faster for ^{227}Th-*p*-nitrobenzyl-DOTA than for free thorium, except for the large intestines, which had five times higher uptake of ^{227}Th-*p*-nitrobenzyl-DOTA than free thorium at 6 h. The high activity in the large intestines at 6 h and low value at 24 h could mean that a proportion of the preparation was cleared via the gastrointestinal system.

Fig. 1C shows the distribution of the radioimmunoconjugate ^{227}Th-DOTA-*p*-benzyl-rituximab. As expected, the retention in blood and blood-rich organs was higher for ^{227}Th-DOTA-*p*-benzyl-rituximab than for the other preparations. Fig. 2 shows the biodistribution calculated as percentage of injected dose per gram relative to the amount of injected activity at time point zero. Fig. 2A shows the distribution of all three preparations at 24 h. The biodistribution of the three preparations was fundamentally different at early time points. However, at 21 and 28 days after injection, the distribution of ^{227}Th and ^{227}Th-DOTA-*p*-benzyl-rituximab was more similar and dominated by retention in the bone, which may indicate some demetalation of ^{227}Th-DOTA-*p*-benzyl-rituximab (Fig. 1A–C and Fig. 2B–D). Nevertheless, the amount of activity in the

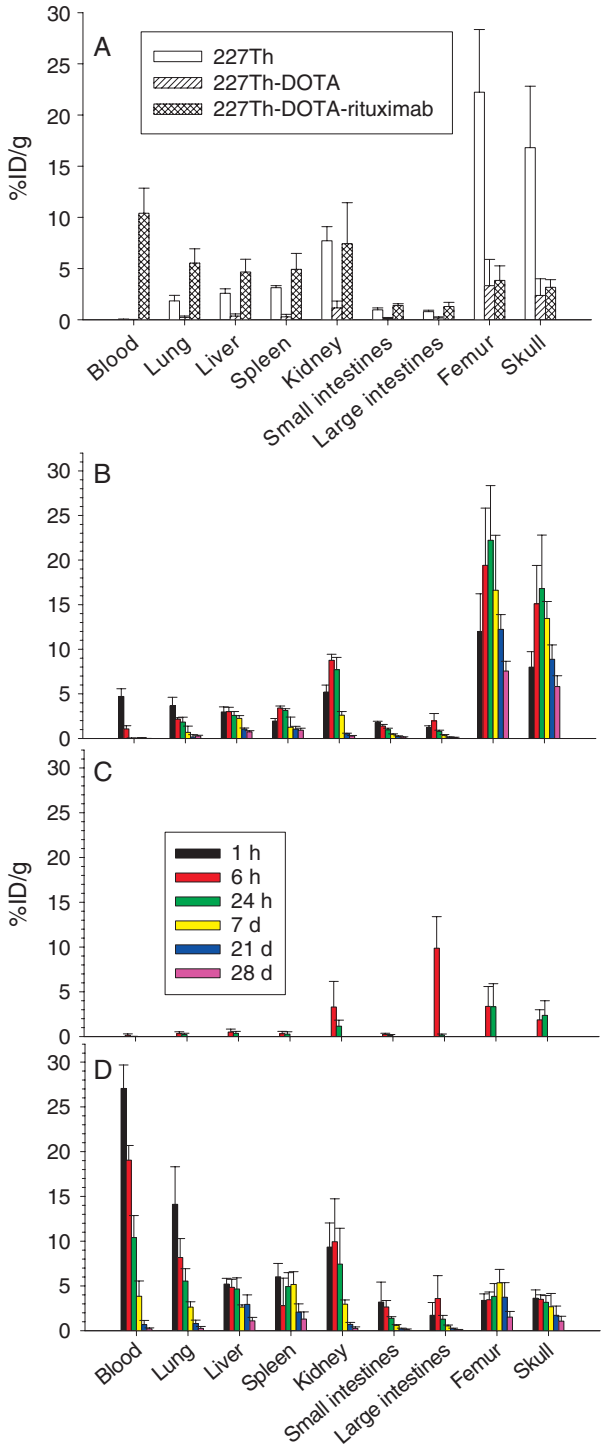

Fig. 2. Percentage of injected dose per gram measured using the 236-keV line of ^{227}Th. (A) Twenty-four hours after injection of ^{227}Th, ^{227}Th-*p*-nitrobenzyl-DOTA or ^{227}Th-*p*-benzyl-DOTA-rituximab. (B) Various time points after injection of ^{227}Th. (C) Various time points after injection of ^{227}Th-*p*-nitrobenzyl-DOTA. (D) Various time points after injection of ^{227}Th-*p*-benzyl-DOTA-rituximab. Error bars = standard deviation. Five to six mice per time point. Percentage of injected dose per gram was calculated relative to the amount of injected activity at the zero time point.

femur and skull 28 days after injection was four to six times higher for free thorium vs. ^{227}Th-DOTA-*p*-benzyl-rituximab. The activity of ^{227}Th-DOTA-*p*-benzyl-rituximab decreased with time in all organs except the femur. For the femur, the activity increased significantly with time up to 7 days and then it decreased. However, when the values were adjusted for physical decay of the radionuclide, there was a significant (*t*-test, $P<.05$) increase in activity in the femur from 24 h to 28 days after injection. There was also a trend of increased activity in the skull with time, but it was not statistically significant.

Fig. 1D–F shows the biodistribution of ^{223}Ra, measured using the characteristic 154-keV γ-line of ^{223}Ra after injection of ^{227}Th, ^{227}Th-*p*-nitrobenzyl-DOTA and ^{227}Th-DOTA-*p*-benzyl-rituximab. Panels D–F show that there was a small amount of radium present, corresponding to the ingrowth during a few hours of storage, when injecting the preparations, and that this radium was initially (1 h) mostly located in the kidneys, bone and intestines. The activity of ^{223}Ra increased with time in the femur and skull up to 21 days after injection and then decreased, while the amounts of ^{223}Ra were relatively low in the soft tissues, except some in the spleen. The biodistribution data for in vivo-generated ^{223}Ra were in good agreement with published data for intravenously injected ^{223}Ra [21].

3.2. Half-life of ^{227}Th-DOTA-p-benzyl-rituximab in blood

The half-life of ^{227}Th-DOTA-*p*-benzyl-rituximab in blood is an important parameter for future use of the radioimmunoconjugate in cancer therapy. The radioimmunoconjugate cleared from the blood with a typical biphasic clearance pattern as expected for an IgG (Fig. 3). From 1 to 24 h after injection, the biological half-life was 18.6 h. After 24 h, the biological half-life increased to 7.4 days. Thus, after Day 1 the effective half-life of ^{227}Th-DOTA-*p*-benzyl-rituximab in blood was 5.3 days. A time period of 28 days

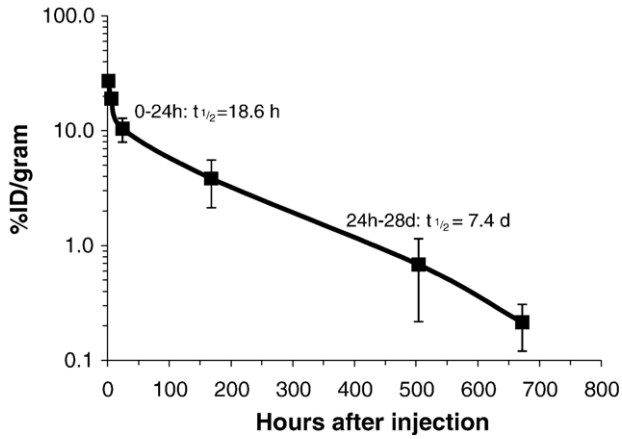

Fig. 3. Time course of the percentage of injected dose per gram in blood after injection of ^{227}Th-*p*-benzyl-DOTA-rituximab. Error bars = standard deviation. Five to six mice per time point.

thereby covers more than five effective half-lives of the radioimmunoconjugate.

3.3. Absorbed doses to organs

When using ^{227}Th in radioimmunotherapy, there is a potential problem of daughter nuclide toxicity. Therefore it is important to assess the dose contribution from the daughter nuclides as shown in Fig. 1D–F. Fig. 4A shows the cumulated activity of ^{227}Th and ^{223}Ra after injection of ^{227}Th-DOTA-*p*-benzyl-rituximab. The data were normalized to an injected amount of 100 kBq/kg, and the radium daughters were assumed to disintegrate in the same tissue as radium [22]. The cumulated activity of ^{227}Th was highest for femur, blood and spleen. The cumulated activity of ^{223}Ra was highest for femur, skull and spleen. Uptake of bone-seeking agents in the spleen may be specific for Balb/c mice and not characteristic of the compound [21,23–25]. Fig. 4B shows absorbed dose estimates for the various organs, calculated using the cumulated activities in Fig. 4A.

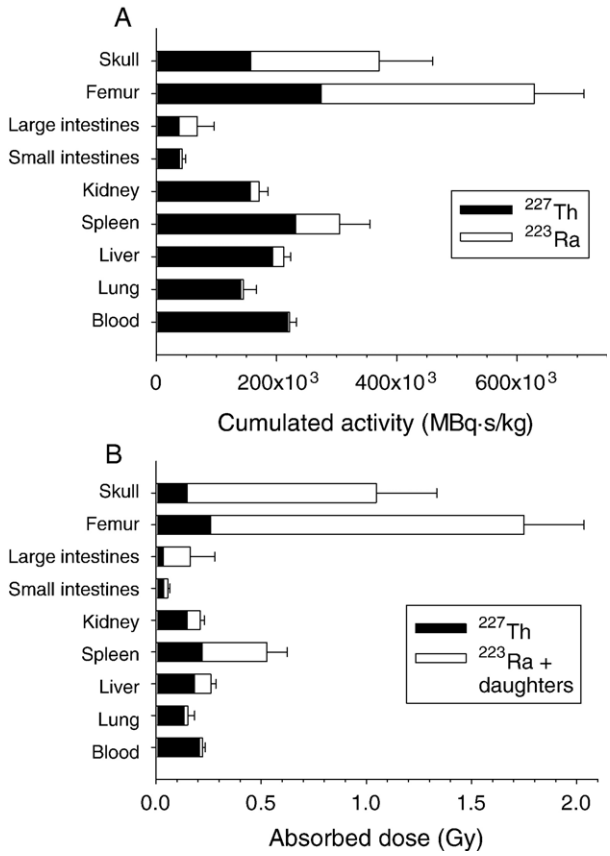

Fig. 4. (A) Cumulated activity of ^{227}Th and ^{223}Ra after injection of ^{227}Th-*p*-benzyl-DOTA-rituximab. The cumulated activity was determined by integration of the specific activity (Bq/g) for each organ over time from zero to infinity. (B) Absorbed dose in various organs after intravenous injection of ^{227}Th-*p*-benzyl-DOTA-rituximab. The dose was calculated assuming the dose contributions coming from alpha particle emissions with a mean energy of 5.9 MeV for ^{227}Th and a mean energy of 26.4 MeV for ^{223}Ra and daughters. It was assumed that the radium daughters deposited their energy in the same tissue as radium. The activity was normalized to 100 kBq/kg body weight. Error bars = standard error.

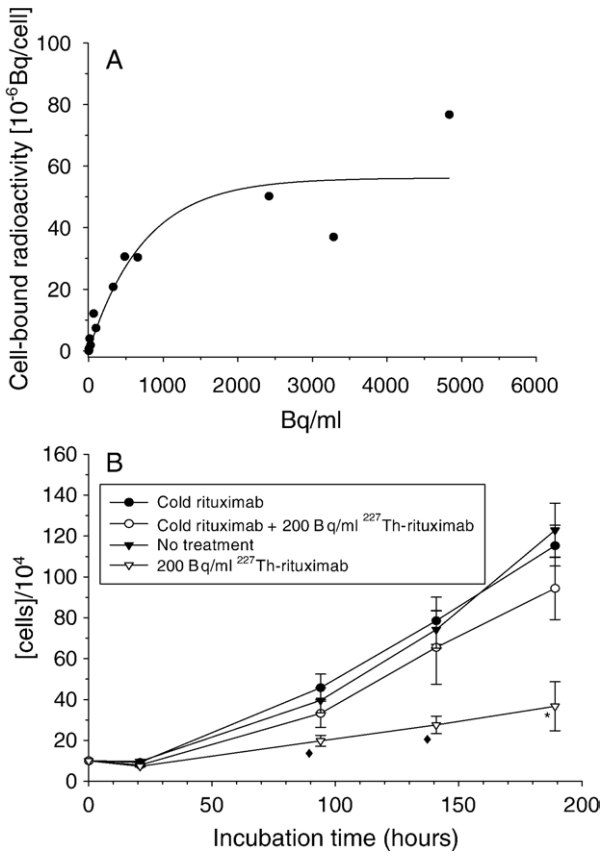

Fig. 5. (A) Cell bound ^{227}Th-*p*-benzyl-DOTA-rituximab related to the initial activity concentration. Two independent experiments were performed. The data points were fitted to an exponential expression using nonlinear regression ($R^2 = 0.87$). Resulting expression: cell bound activity $= (56 \pm 6) \times (1 - e^{-(0.0013 \pm 0.004) \times \text{initial activity concentration}})$. (B) Growth of Raji cells after incubation with ^{227}Th-*p*-benzyl-DOTA-rituximab. Circles: Binding of ^{227}Th-*p*-benzyl-DOTA-rituximab to the cells was blocked by incubation with cold rituximab (100 μg/ml). Triangles: No cold rituximab was used. Open circles and triangles: Cells were treated with 200 Bq/ml ^{227}Th-*p*-benzyl-DOTA-rituximab. Closed circles and triangles: Cells were not treated with radioactivity. *Significantly different from cells treated with cold antibody and 200 Bq/ml ^{227}Th-*p*-benzyl-DOTA-rituximab (*t*-test, $P < .05$). ♦Significantly different from untreated cells (*t*-test, $P < .05$). Error bars = standard deviation.

As expected, the femur and skull received the highest total absorbed radiation doses, calculated to 1.75 and 1.05 Gy, respectively. The total absorbed radiation dose to the spleen was also relatively high, 0.53 Gy. The blood received 97% (0.21 Gy) of the absorbed radiation dose from decay of ^{227}Th.

3.4. Cell binding and cell growth

Fig. 5A shows the amount of radioactivity bound to cells after 2 h of incubation at 4°C. The two curves show two independent experiments. For 200 Bq/ml, around 13 μBq was bound to each cell. Fig. 5B shows the effect of such an initial activity concentration on the growth of Raji cells. The cells were incubated with 200 Bq/ml for 8 days. The long incubation time is similar to the half-life of ^{227}Th-DOTA-*p*-benzyl-rituximab in blood (Fig. 3) and to

Table 1
Immunoreactive fraction

Days after production	Immunoreactive fraction±S.D.
1	59±15
5	59±8
12	65±18
19	56±26
26	59±15

the mean serum half-life of rituximab in patients (3.2 days, range 1.3–6.4 days) [13]. Changing 50% of the medium after 1, 4 and 6 days simulated the biological elimination of ^{227}Th-DOTA-*p*-benzyl-rituximab. Incubation with 200 Bq/ml of ^{227}Th-DOTA-*p*-benzyl-rituximab resulted in a significant inhibition of cell growth compared to cells not treated with radioactivity at 4, 6 and 8 days of cell growth. There was no effect of cold antibody alone. There appeared to be a small effect of nontargeted α-particles from treatment with ^{227}Th-DOTA-*p*-benzyl-rituximab diluted for binding blockage by cold rituximab (open circles) on cell growth, but it was not significant. Eight days after the addition of radioimmunoconjugate, the growth of cells treated was significantly inhibited as compared with the controls (i.e., treatment with ^{227}Th-DOTA-*p*-benzyl-rituximab after incubation with cold rituximab for blocking antigens).

4. Discussion

^{227}Th-DOTA-*p*-benzyl-rituximab is a new type of radioimmunoconjugate and has not yet been used in therapy. The labeling with ^{227}Th might potentially increase the therapeutic effect of rituximab by delivering highly toxic α particle radiation to the targeted tissue. The ^{227}Th-DOTA-*p*-benzyl-rituximab was shown to inhibit cell growth in vitro. The present study was designed to verify whether the integrity of the radioimmunoconjugate would be maintained in a timeframe compatible with the effective half-life of the radioimmunoconjugate. It was also a goal to obtain information regarding the fate of the main daughter radionuclide, ^{223}Ra, after prolonged circulation of ^{227}Th.

The in vitro data presented in Table 1 indicate that the immunoreactivity is maintained for a prolonged period of time, covering several biological half-lives for the radioimmunoconjugate in vivo. Biodistribution measurements from 1 to 24 h after injection of rituximab conjugated to the α emitter ^{211}At have been published [8]. In comparison with this, the present study showed slightly lower %ID/g in blood but similar uptake in lung, spleen, kidney and bone after 1 and 24 h. For the liver, the %ID/g was 50–60% lower for ^{227}Th-rituximab than for ^{211}At-rituximab after 1 h but similar after 24 h. The reason for the lower %ID/g in blood for ^{227}Th-rituximab might be the lower total blood volume, as in the study by Aurlien et al. [8], since slightly smaller mice were used. The biological half-life of ^{211}At-rituximab in blood was 24 h, while the biological half-lives of ^{227}Th-rituximab were 18.6 h the first day and 7.4 days between 1 and 28 days. In the patients, a mean serum half-life of 3.2 days (range 1.3–6.4 days) has been reported after intravenous infusion of rituximab [13].

Fig. 1A shows that free thorium was mainly distributed to the bone, suggesting that if ^{227}Th-DOTA-*p*-benzyl-rituximab is undergoing demetalation or if it is degraded in the liver, free thorium should, to a great degree, end up in the bone. This finding was used to evaluate the in vivo stability of the radioimmunoconjugate. The uptake in the bone of ^{227}Th-*p*-nitrobenzyl-DOTA was seven times lower than for free thorium. In general, if a product similar to ^{227}Th-*p*-nitrobenzyl-DOTA detaches from the radioimmunoconjugate it will likely be substantially cleared from the body within 24 h, and only a small fraction (around 3 %ID/g) would end up in the bone.

In the paper by Aurlien et al. [8], the uptake of rituximab labeled with ^{125}I or ^{211}At showed an uptake in the bone (femur) of about 3 %ID/g. These halogens have, by themselves, no affinity for bone. Therefore one may assume that the 3 %ID/g uptake reflects the radioimmunoconjugate distribution to the femur. If the amount of activity in the bone beyond 3 %ID/g is assumed to be mainly caused by free thorium that has detached from DOTA-*p*-benzyl-rituximab, it may be possible to obtain some information about the stability of the radioimmunoconjugate. The data show that the amount of ^{227}Th in the femur after administering the radioimmunoconjugate did not go much beyond the 3 %ID/g and therefore one may conclude that only very modest amounts of free ^{227}Th are generated from the radioimmunoconjugate in vivo (Fig. 2D). For the β-emitting ^{90}Y-Zevalin, a dose of 15 MBq/kg was administered [26], resulting in more than 10 times higher doses to human spleen, liver and lung than with injection of 100 kBq/kg ^{227}Th-*p*-benzyl-DOTA-rituximab in mice. However, the dose to kidneys was similar and the doses to bone were 50–70% lower for ^{90}Y-Zevalin than for ^{227}Th-*p*-benzyl-DOTA-rituximab. The doses to soft tissues and blood were similar to the doses found by Aurlien et al. [27] after injection of ^{211}At-rituximab, while the doses were 10 times higher for the bone in the present study with ^{227}Th-*p*-benzyl-DOTA-rituximab, mainly due to the generation of ^{223}Ra.

In the current study, the tissue distribution was the main focus, and the elimination pattern was not studied in detail. Some urine samples were collected, and they indicated that at least a fraction of the ^{227}Th from the radioimmunoconjugate had a renal clearance. Obtaining a detailed account of the clearance will probably require the use of metabolic cages, which was not used in this study. The details regarding elimination should therefore be evaluated in future studies.

There is, in general, limited knowledge about toxicity of internal α-emitting radioimmunoconjugates at potential therapeutic levels. However, Jaggi et al. [12] have

investigated the nephrotoxicity of internal irradiation of mice with α-particle-emitting actinium daughters. Injection of 720 kBq/kg ^{225}Ac bound to an antibody resulted in loss of renal function [12]. The calculated dose to kidney was 100 times higher in the study by Jaggi et al. than in the present study. A clinical safety study of the ^{227}Th daughter ^{223}Ra showed acceptable myelotoxicity at a dosage level of 250 kBq/kg [14]. Radium-223 is mainly causing irradiation of the bone surfaces, while a significant fraction of the bone marrow is beyond the reach from α particles emitted from bone surfaces [21]. Thus, the thorium daughters may probably be well tolerated in patients receiving ^{227}Th-*p*-benzyl-DOTA-rituximab. However, the toxicity of ^{227}Th-*p*-benzyl-DOTA-rituximab should be investigated in the future, since there would allegedly be additive toxicity from both ^{227}Th and ^{223}Ra when using the first in α-radioimmunotherapy.

The in vitro dosage-vs.-survival data show that it is possible to obtain specific single-cell kill with low dose rate α-immunotherapy using ^{227}Th. It should also be noted that the radioimmunoconjugate-specific activity was low, typically between 0.5 and 1.0 MBq/mg. The 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 [27]. That experiment fits well with experiments with microbeam irradiation of cells, which have shown that the probability of surviving an α-particle hit is 0.8^N, where N is the number of α-particle tracks through the nucleus [28]. Fig. 5B shows that 200 Bq/ml of ^{227}Th-DOTA-*p*-benzyl-rituximab will inhibit cell growth. At an initial activity concentration of 200 Bq/ml, 13 μBq of activity was bound to each cell (Fig. 5A), corresponding to 1.1 α-disintegrations per day, or about 9 after 8 days. However, the amount of radioimmunoconjugate will decrease with increasing incubation time [27]. Furthermore, only a third of the α-particles are expected to hit the nucleus of lymphoma cells, assuming a cellular radius of 5.5–8.5 μm and a nuclear radius of 4.5–7 μm [29]. Thus, with an initial activity of 200 Bq/ml, the nucleus of each cell was hit by around three targeted α-particles after 8 days. In addition, the cells were hit by α-particles from unbound radioimmunoconjugate, but this untargeted radiation had no significant effect (Fig. 5B, open circles) and from α-particles from daughter nuclides generated during the incubation time.

5. Conclusions

The study shows that rituximab labeled with ^{227}Th has acceptable immunoreactivity after labeling and retention time in blood, comparable to that of unlabeled rituximab in patients. The in vivo stability of ^{227}Th-*p*-benzyl-DOTA-rituximab seems promising. Further studies, including therapeutic studies in mice with CD20-positive lymphoma xenograft, are therefore warranted.

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