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ORIGINAL ARTICLE

In vitro characterization of ^{177}Lu-radiolabelled chimeric anti-CD20 monoclonal antibody and a preliminary dosimetry study

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Abstract
Purpose ^{131}I- and ^{90}Y-labelled anti-CD20 antibodies have been shown to be effective in the treatment of low-grade, B-cell non-Hodgkin's lymphoma (NHL). However, the most appropriate radionuclide in terms of high efficiency and low toxicity has not yet been established. In this study we evaluated an immunoconjugate formed by the anti-CD20 antibody rituximab and the chelator DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid). DOTA-rituximab was prepared as a kit formulation and can be labelled in a short time (<20 min) with either ^{177}Lu or ^{90}Y.
Materials and methods Immunoconjugates with different numbers of DOTA molecules per rituximab were prepared using p-SCN-Bz-DOTA. In vitro immunoreactivity and stability were tested and preliminary dosimetric results were acquired in two patients.
Results The immunological binding properties of DOTA-rituximab to the CD20 antigen were found to be retained after conjugation with up to four chelators. The labelled product was stable against a 10^5 times excess of diethylenetriaminepentaacetic acid (DTPA, 37°C, 7 days). Two patients with relapsed NHL were treated with 740 MBq/m^2 body surface ^{177}Lu-DOTA-rituximab. Scintigraphic images showed specific uptake at tumour sites and acceptable dosimetric results. The mean whole-body dose was found to be 314 mGy. The administration of ^{177}Lu-DOTA-rituximab was tolerated well.
Conclusion Our results show that DOTA-rituximab (4:1) can be labelled with ^{177}Lu with sufficient stability while the immunoconjugate retains its immunoreactivity. ^{177}Lu-DOTA-rituximab is an interesting, well-tolerated radiolabelled antibody with clinical activity in a low dose range, and provides an approach to the efficient treatment with few side effects for patients with relapsed NHL.

Keywords ^{177}Lu-DOTA-rituximab · Non-Hodgkin's lymphoma · Radioimmunotherapy · CD20$^+$ lymphoma

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Introduction

The monoclonal antibody rituximab has become a standard treatment for relapsed or refractory CD20-positive low-grade non-Hodgkin's lymphoma (NHL), along with chemotherapy [1]. Rituximab is a chimeric antibody, discovered in 1990 by IDEC Pharmaceuticals (San Diego, CA). It possesses a high binding affinity to the CD20 antigen. The CD20 antigen is expressed on the surface of normal and malignant B-lymphocytes but not on stem cells or other healthy tissues. Rituximab kills CD20-positive B-lymphocytes via a mechanism involving antibody-dependent cytotoxicity [2, 3].

Moreover, a mechanism such as apoptosis, which is independent of complement or antibody-dependent cytotoxicity, may also be triggered by cell surface coupling and then play a role in the tumoricidal effect [4].

Over recent years, radioimmunotherapy has been used in the treatment of $CD20^+$ lymphomas. Mostly low-grade NHL that has relapsed or that is refractory to the last standard therapy is treated. Radioimmunotherapy is used alone or in combination with other therapies with the goal of improving efficacy [5–7]. For this purpose beta-emitting radioisotopes are coupled to anti-CD20 antibodies. Lymphocytes and lymphoma cells are highly radiosensitive. Additionally, due to the millimetre-range of β-particles, the so-called "cross-fire" effect (in internal radiotherapy) allows cells devoid of radioactive-bound antibody to be irradiated and killed [8]. This is particularly relevant to lymphoma cells which lack antigens, or which cannot be reached due to poor vascularization and intratumoral pressure in a bulky tumour.

Radioimmunotherapy using anti-CD20 antibodies labelled with ^{131}I is a promising approach to the treatment of low-grade NHL [3]. A pilot study in 53 patients showed an overall response rate of 71%, including 38% of patients with complete remission and a median progression-free survival of 12 months (20.3 months for the complete responders) [9, 10]. Another, highly regarded study using radioimmunotherapy as first-line treatment for follicular lymphoma showed complete remission in 75% of all patients [11].

The pure, high-energy, β-emitting isotope, ^{90}Y is also used in radioimmunotherapy in relapsed NHL patients. For the preparation of the commercially available Zevalin (IDEC Pharmaceuticals, San Diego, CA), the murine form of rituximab (ibritumomab) is labelled with ^{90}Y. However, ^{90}Y-Zevalin is not suitable for dosimetry [12–14]. The use of ^{111}In as a surrogate for ^{90}Y is necessary for pretherapeutic dosimetry. Moreover, ^{90}Y delivers more energy to the tumour which might be advantageous in big tumours, but for micrometastases and consolidation therapy the energy is probably too high, resulting in the highest energy being absorbed outside the tumour. Thus, the combination of a high- and a low-energy β-emitting isotope, e.g. ^{131}I and ^{90}Y, as an "isotope cocktail" for the labelling of an anti-CD20 antibody might induce even higher rates of response and longer lasting responses in NHL patients who have failed to respond to treatment with chemotherapy, rituximab or a combination.

However, ^{131}I is not an ideal radioisotope for use in the clinic. It has the disadvantage of a high rate of emission of γ-radiation (364 keV, 82%; 637 keV, 7%). For instance, with respect to the European radiation protection law, this would result in prolonged hospitalization. Additionally, this may lead potentially to high radiation absorbed doses to staff and the family of the patient. Another inevitable drawback is the in vivo deiodination of antibodies [15]. A radiometal such as ^{177}Lu (E_{max} 0.497 MeV, $t_{½}$ 6.7 days) may be more suitable for the labelling of the antibody. Beside its β-emission, it features a low abundance of photons of almost ideal energy (113 keV, 6.5%; 208 keV, 11%) for imaging and posttherapeutic dosimetry.

In this study, we set out to attach an appropriate multiple number of DOTA molecules to rituximab and produce a reliable lyophilized kit formulation that would allow the rapid labelling of the antibody in a routine laboratory. We analysed the labelling efficiency of DOTA-rituximab with ^{177}Lu and ^{90}Y as well as the immunoreactivity before and after conjugation and labelling, respectively. The conjugation and labelling conditions were optimized to ensure that the final product could be obtained in high yield without loss of biological activity. Additionally, a dosimetric study in two patients with relapsed lymphoma treated with ^{177}Lu-DOTA-rituximab was performed.

Materials and methods

Materials

Chimeric anti-CD20 rituximab monoclonal antibodies were provided by Roche (Roche Pharma Schweiz, Basel, Switzerland). ^{57}Co was purchased from Amersham Bioscience (Piscataway, NJ). ^{177}Lu was purchased either from I. D.B. Holland (Baarle Nassau, The Netherlands) or from MURR (University of Missouri Research Reactor, Columbia, MO). ^{90}Y was purchased from PerkinElmer Life Sciences (Waltham, MA). Isothiocyanate-benzyl-DOTA (*p*-SCN-Bz-DOTA) was purchased from Macrocyclics (Dallas, TX). All chemicals were purchased from commercial sources and used without additional purification.

Cells

L-VB1 is a mouse fibroblast cell line transfected with the human CD20 cDNA, which was kindly provided by Dr. H. Kikutani (Osaka University, Japan). The cells were grown in RPMI-1640 medium supplemented with 2 m*M* L-glutamine and 10% heat-inactivated fetal calf serum (FCS). Prior to use, the cells were detached from the surface of the tissue culture flask by treatment with trypsin (0.05%) in PBS with EDTA (0.02%), and counted.

Antibody conjugation and kit formulation

A 5-ml aliquot of rituximab solution (10 mg/ml) was concentrated by centrifugation using an Amicon Ultra-15 filter (Millipore, MWCO 30,000). The solution was washed

three times with 0.2 *M* sodium carbonate buffer (coupling buffer), pH 9.5, to remove Tween-80 and sodium citrate and to exchange buffer. The final concentration was 100 mg/ml. The rituximab was then incubated with p-SCN-Bz-DOTA at 37°C for 1 h in molar ratios of 1:5 or 1:20. The coupling reaction was quenched by adjusting the pH to 7.0 using 0.25 *M* ammonium acetate buffer, pH 5.5, followed by centrifugation. The solution was then washed three to five times with 0.25 *M* ammonium acetate, pH 7.0; filtration and washing was continued until the volume of filtered solution was close to zero while the filtrate was monitored by UV spectrophotometry (Perkin Elmer Lambda2 UV/Vis spectrophotometer) at 280 nm. The concentration of rituximab was determined spectrophotometrically at 280 nm, and the immunoconjugate was diluted with 0.25 *M* ammonium acetate buffer, pH 7.0. at a concentration of 5 mg/ml.

The immunoconjugate solution (1 ml, 5 mg/ml) was prepared in kit form by lyophilization with 250 µl of mannitol (mannitol Ph Eur, 80 mg/ml in distilled water). Both the immunoconjugate solution and the mannitol solution were filtered through sterile 0.45-µm filters (Millipore) before lyophilization. The final product was a white pellet.

Determination of the number of chelators per antibody molecule

`A rituximab kit was reconstituted with 470 µl water for injection and the final volume was considered as approximately 500 µl, and the concentration of rituximab was re-determined by UV spectrophotometry. ^{57}Co was prepared in 3×10^{-4} *M* $CoCl_2$ (^{57}Co:$CoCl_2$ 1:10), and an aqueous solution of 3×10^{-4} *M* $^{nat}LuCl_3$ with a tracer amount of $^{177}LuCl_3$ was also prepared. Rituximab and $^{57}Co/CoCl_2$ solutions (molar ratio 1:5 or 1:10), as well as rituximab and $^{177}Lu/^{nat}LuCl_3$ solutions (molar ratio 1:10) were incubated at 37°C. After 1 h, 20 µl of labelled solution was taken and quenched by the addition of 3 µl of CaDTPA (1×10^{-4} *M*) in 25 µl of 0.25 *M* ammonium acetate, pH 7.0. The sample was then analysed by HPLC, using a TSK-Gel G3000SWXL size-exclusion column 7.8×300 mm, pre-equilibrated with 0.05 *M* phosphate buffer, pH 6.8, at a flow rate of 1 ml/min. The column eluent was passed through a UV detector (detection wavelength 280 nm) and then through a radioactivity detector. The number of chelators per antibody molecule was determined by calculating the area under the curve, which was measured radiometrically. The retention times were compared with those of the unlabelled and of the unconjugated antibody.

Matrix-assisted laser desorption ionization mass spectroscopy (MALDI-MS) measurements were performed on a Voyager sSTR equipped with a Nd:YAG laser (355 nm) (Applied BioSystems, Foster City, CA) for the determination of the number of chelators per antibody molecule, as an alternative to the tracer method. The unconjugated rituximab and the reconstituted kit of the immunoconjugate DOTA-rituximab were desalted by centrifugation at 4°C using Ultra-15 filters, and washed several times with milliQ water. The concentration was adjusted to 20–25 mg/ml. The samples were deposited directly on a metal MALDI target plate and sinapinic acid (10 mg/ml in 50:50 acetonitrile/water with 0.1% TFA) was used as the MALDI matrix at a sample to matrix volume ratio of 1:1.

^{177}Lu- and ^{90}Y-labelling of the DOTA conjugate

$^{177}LuCl_3$ and $^{90}YCl_3$ were used for the labelling of the DOTA-rituximab kit. The kit was reconstituted as described above and the designated amount of activity was added slowly to the vial through the septum using a sterile single-use syringe. The reaction mixture was incubated from 15 min to 4 h at 37°C. The amount of ^{177}Lu and ^{90}Y used was 1,480 MBq and 925 MBq per kit, respectively, and the volume ranged from 100 to 200 µl. The pH value of the reaction mixture was checked before incubation. At different time points, ranging from 15 min to 4 h, approximately 3 µl of the labelling mixture was taken and quenched by the addition of 5 µl of CaDTPA (1×10^{-4} *M*) in 500 µl of 0.25 *M* ammonium acetate buffer, pH 7.0. An aliquot of 10–20 µl was then used for the analysis by size-exclusion HPLC using the conditions described above.

Stability study

The stability of the radiolabelled immunoconjugate ^{177}Lu-DOTA-rituximab was evaluated in the presence of a competitor for the ^{177}Lu ions such as DTPA. For this purpose a 10^5 times excess of CaDTPA (1×10^{-4} *M*, pH 7.3) was added to the ^{177}Lu-DOTA-rituximab solution and kept at 37°C for 7 days. Samples of this mixture were removed at different time points (4 h, and 1, 3, and 7 days) and were analysed by size-exclusion HPLC as described above.

Immunoreactivity

The immunoreactivity of the DOTA-rituximab-immunoconjugate was analysed by flow cytometry before labelling and a binding assay was performed as described by Lindmo et al. [16] after labelling with ^{177}Lu.

Flow cytometry The binding of serial dilutions of DOTA-rituximab and unconjugated rituximab to CD20 transfectant L-VB1 cells was compared. Antibody dilutions ranging

from 50 μg/ml to 390 ng/ml were prepared in FACS buffer Dulbecco's PBS (Sigma-Aldrich) with 2% FCS and 0.1% sodium azide. Staining was performed in a 96-well microtitre plate (U-bottom). In each well 100 μl of the antibody was mixed with 50 μl cell suspension containing 5×10^5 L-VB1 cells and incubated for 1 h on ice. Cells were washed twice with FACS buffer by centrifuging the plate and siphoning off the supernatant. Subsequently, 100 μl per well goat antihuman IgG/Fc-FITC (Jackson ImmunoResearch, West Grove, PA) diluted 1:100 in FACS-buffer was added as second step reagent. The plate was incubated for 30 min on ice in the dark and cells were washed again twice as described above. Dead cells were discriminated by propidium iodide staining. Analysis was carried out on a FACScan cytometer (Becton Dickinson, Heidelberg, Germany) using CELLQUEST software.

Lindmo test L-VB1 cells were suspended in DMEM medium supplemented with 1% FCS at a concentration of 3.2×10^5 cells/ml. Four six-well microtitre plates were used for the study and 1.25 ml of the cell suspension (4×10^5 cells) was added to the first well of each plate. Serial 1:2 dilutions of the cells were added into the following five wells of each plate. The plates were incubated at 37°C overnight. The next day, the medium was removed and 1.25 ml of 4% paraformaldehyde in PBS was added to each well. The plates were incubated at room temperature for 20 min to fix the cells to the bottom of the wells. The paraformaldehyde solution was removed and the wells were washed twice with PBS (0.01 *M*, pH 7.3). The plates were additionally incubated at 37°C for 1 h with PBS supplemented with 2% FCS. To two of the four six-well microtitre plates was added 0.5 ml of commercial rituximab (2 μ*M*) in PBS/2% FCS in order to saturate the binding sites for subsequent measurement of the nonspecific binding of radiolabelled antibody. ^{177}Lu-DOTA-rituximab was diluted in PBS/2% FCS to a concentration of 2.5 ng/ml and 0.5 ml was added to each well of the four plates. The appropriate amount of PBS/2% FCS was added to each well to obtain a final volume of 1.25 ml per well. The final concentrations were 1 ng/ml for ^{177}Lu-DOTA-rituximab and 3.2, 1.6, 0.8, 0.4, 0.2 and 0.1×10^5 cells/ml. The plates were incubated at 37°C for 2 h. The solution was removed from each well, followed by two washing steps with PBS, as described above. The cells were digested from the wells by the addition of 1.25 ml NaOH (1 *N*), followed by incubation at 37°C for 30 min. The digested cell solutions were transferred to glass γ-counter tubes. The wells were washed with 1.25 ml NaOH (1 *N*) and the wash solutions were pooled to the corresponding tubes and measured in a γ-counter (CobraII, Auto-Gamma, Canberra, Meriden, CT), while 0.5 ml of ^{177}Lu-DOTA-rituximab was measured to determine the total applied radioactivity.

Dosimetry

Two patients were included in dosimetric studies. In accordance with the accepted protocols for Zevalin® and Bexxar®, patients were pretreated with rituximab at 250 mg/m^2 body surface on day −7 and 0. Later on day 0 they were injected with 740 MBq/m^2 ^{177}Lu-DOTA-rituximab. Whole-body scans were obtained 1, 2, 4, 24, 48, 72, 120, 168 and 357 h and 1, 2, 4, 24, 48, 96, 144 and 313 h after injection, respectively. Regions of interest were drawn manually around the whole body from the anterior and posterior projections. The Odyssey XP program was used. Background regions were placed close to the whole body for background correction. The geometric mean value of the counts from the anterior and posterior projections was taken and corrected for physical decay. Whole-body activity acquired 1 h after injection was defined as 100% of the injected activity (%IA). The patients did not void their bladder during this period. All data are expressed as %IA(t). The resulting time–activity data from the scans were fitted by a one-exponential curve. The activity in blood was fitted by a three-exponential curve. The residence times were determined by using these data and the half-life of ^{177}Lu. According to ICRP 23, the residence time for red marrow is 2% of the whole-body residence time, assuming that only the blood pool contributes to the total body activity [17, 18]. We assumed no specific uptake in the red marrow. Both patients had less than 20% infiltration of the bone marrow. The whole-body dose and the dose to the red marrow were calculated with the dose factors (S-values) published on www.doseinfo-radar.com.

Results

Multiple DOTA molecules on rituximab

For the preparation of the immunoconjugate, initially rituximab (10 mg/ml in 0.2 *M* sodium carbonate buffer, pH 9.0) was incubated for 2 h with a five- to tenfold excess of p-SCN-Bz-DOTA, resulting in a DOTA-rituximab conjugate with an approximate ratio of one DOTA molecule per antibody. However, this $(DOTA)_1$-rituximab kit was found to be unsuitable for labelling with the desired amount (1,480 MBq per 5-mg kit) of a radiometal, such as ^{177}Lu. The labelling yield was less than 95% after 4 h of incubation.

Modifying this coupling procedure by increasing the pH value of the coupling buffer to 9.5 and increasing the rituximab concentration (100 mg/ml) resulted in conjugation of multiple DOTA molecules per antibody. The number of DOTA molecules per antibody was determined by labelling of the $(DOTA)_X$-rituximab conjugates with $^{57}Co/CoCl_2$ (1:5

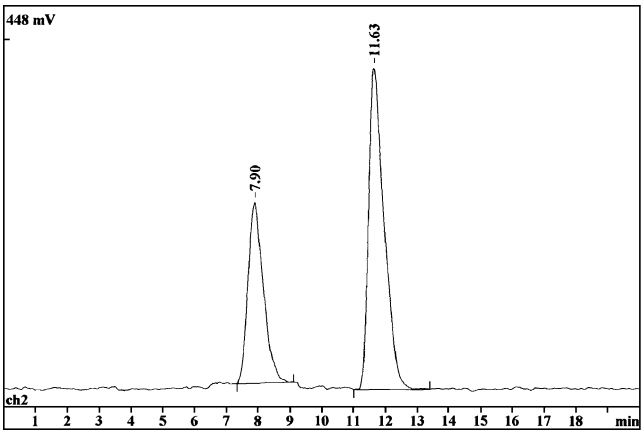


Fig. 1 Multiple DOTA molecules conjugated per rituximab molecule. An average of four DOTA molecules per rituximab was determined by calculation of the area under the curve (a 1:10 molar ratio of rituximab to $^{177/nat}$Lu resulted in 40% integration from the ^{1777nat}Lu-DOTA-rituximab; R_t=7.9 min for $^{177/nat}$Lu-DOTA-rituximab and R_t=11.63 min for $^{177/nat}$Lu-DTPA)

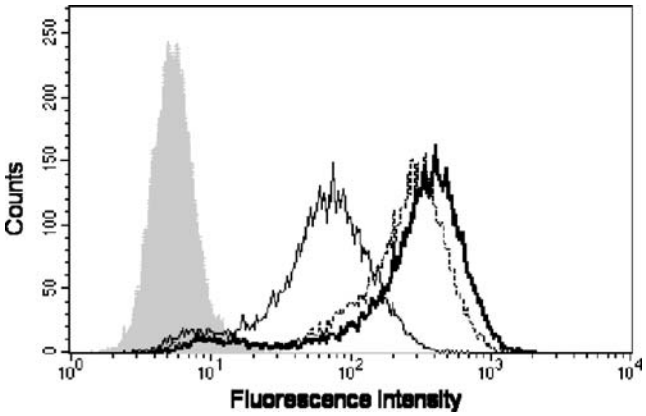


Fig. 3 Immunoreactivity of $(DOTA)_x$-rituximab conjugates. Cytofluorimetric comparison of unconjugated rituximab (*solid bold line*), $(DOTA)_4$-rituximab (*dotted line*) and $(DOTA)_8$-rituximab (*solid thin line*). The fluorescence intensity is presented as an overlay plot for identical antibody concentrations. The shaded histogram represents a negative control using irrelevant polyclonal human immunoglobulin

and 1:10 ratio), as well as with ^{177}Lu/^{nat}LuCl$_3$ (1:10 ratio). An average of four DOTA molecules per rituximab molecule [$(DOTA)_4$-rituximab] was found by HPLC analysis and calculation of the area under the curve (a 1:10 ratio of rituximab to $^{177/nat}$Lu resulted in 40% of $^{177/nat}$Lu-DOTA-rituximab; Fig. 1). Equivalent results were found for the ^{57}Co/CoCl$_2$ method.

As an alternative to the determination of the number of DOTA molecules per rituximab molecule, MALDI-MS was used. The results (Fig. 2) showed an average number of four DOTA molecules per rituximab. A peak at a mass-to-charge ratio (*m*/*z*) of 146,954 corresponded to unconjugated rituximab, while a peak at 149,146 corresponded to conjugated $(DOTA)_x$-rituximab. The mass difference between conjugated and unconjugated rituximab divided by the expected mass of single DOTA coupling (551) represents the average number of DOTA molecules conjugated to rituximab. The wider peak profile of the $(DOTA)_x$-rituximab is a result of the heterogeneous distribution of DOTA molecules per rituximab.

In order to evaluate the effect of the number of DOTA molecules per antibody and the immunoreactivity of the conjugate, we conjugated a higher number of DOTA molecules to rituximab using the modified method and a higher amount of p-SCN-BzDOTA (20-fold excess) during the coupling reaction. Under these conditions a DOTA-rituximab kit with an average number of eight DOTA molecules per antibody [$(DOTA)_8$-rituximab] was obtained.

Immunoreactivity of $(DOTA)_x$-rituximab immunoconjugates

DOTA-rituximab kits containing four or eight DOTA molecules per antibody molecule were compared side by side with unconjugated rituximab antibody. The binding of both preparations to cells of the CD20-transfected cell line L-VB1 was assessed using serial antibody dilutions (Fig. 3). At a concentration of 5 μg/ml unconjugated rituximab showed strong binding to the target cells (mean fluorescence intensity, MFI, 348). With $(DOTA)_4$-rituximab at the same concentration a slight shift of the histogram to

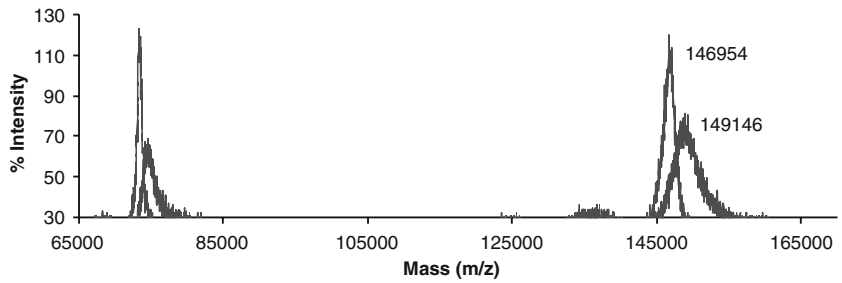


Fig. 2 Overlapping of MALDI-MS spectra of unconjugated rituximab and $(DOTA)_4$-rituximab immunoconjugates. The average number of DOTA molecules per rituximab molecule in the $(DOTA)_x$-rituximab conjugate can be estimated by the mass difference between unconjugated rituximab (146,954) and $DOTA_x$-rituximab immunoconjugate (149,146) divided by the mass of single DOTA coupling (551)

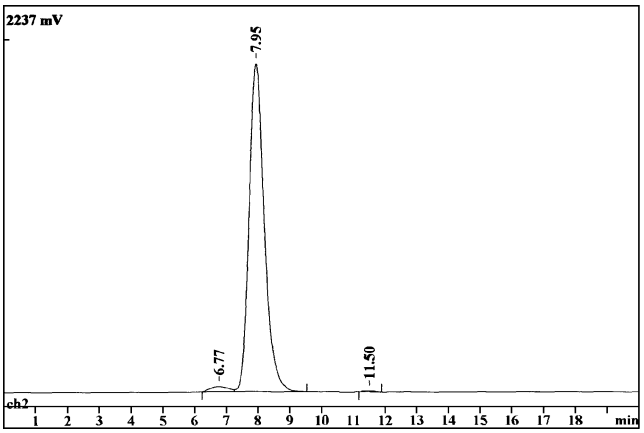


Fig. 4 HPLC analysis of ^{177}Lu-DOTA-rituximab. DOTA-rituximab (5 mg) was labelled with either ^{177}Lu or ^{90}Y at 37°C. High labelling yields (>99%) were achieved within 15 min for ^{177}Lu at pH 6.0 to 7.0, while a 1-h incubation period at pH 7.0 was necessary for ^{90}Y to achieve this high labelling yield. The HPLC profile for both radiolabelled compounds (^{177}Lu-DOTA-rituximab and ^{90}Y-DOTA-rituximab) was the same (R_t=6.8 min for dimer or polymer species, R_t=7.9 min for ^{177}Lu-DOTA-rituximab, and R_t=11.5 min for ^{177}Lu-DTPA)

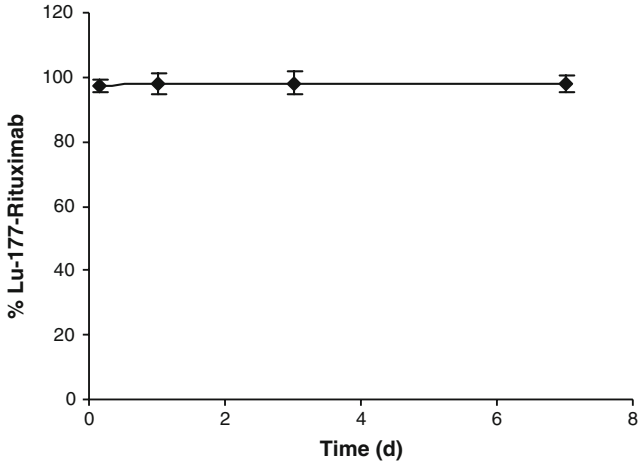


Fig. 5 Stability of ^{177}Lu-DOTA-rituximab against a high excess of DTPA. ^{177}Lu-DOTA-rituximab was incubated with a 10^5 times excess of CaDTPA (1×10^{-4} *M*, pH 7.3) at 37°C for up to 7 days. Samples of this solution were taken at 4 h, and 1, 3 and 7 days for analysis by gel filtration TSK-3000-HPLC. After 7 days ^{177}Lu was found to be still bound to DOTA-rituximab, while no free ^{177}Lu or ^{177}Lu-DOTA was detected

the left was observed (MFI 271), indicative of a moderate reduction of the binding activity. In contrast, the binding capacity of $(DOTA)_8$-rituximab was drastically impaired as demonstrated by a clear shift of the histogram towards the negative control (MFI 77).

Radiolabelling with ^{177}Lu or ^{90}Y

The $(DOTA)_4$-rituximab kit (5 mg per kit) was first labelled with ^{177}Lu. The pH of the reaction mixture was in the range 5.0 to 6.0. The reaction mixture was incubated at 37°C and at the preselected time points of 15, 30, 45, and 60 min, an aliquot (approximately 3 µl) was taken and analysed by HPLC as described above. The results revealed that a sufficient labelling yield (>99%) could be achieved within 15 min (Fig. 4). The radiolabelled antibody eluted at 7.9 min, and there was a small peak at 6.8 min (1–1.5%) which was probably due to dimer or polymer species formed during the conjugation reaction.

Labelling of the same $(DOTA)_4$-rituximab kit with ^{90}Y at pH<7.0 was insufficient. Adjustment of the pH to within the range 7.0–7.8 by reconstitution of the kit with 0.25 *M* ammonium acetate, pH 8.8, increased the labelling yield significantly. The labelling yield was found to be 96–97% at 30 min and increased to >99% at 1 h.

Stability of ^{177}Lu-DOTA-rituximab

The stability of ^{177}Lu-DOTA-rituximab with regard to transchelation was evaluated in the presence of a competitor in excess (10^5-fold excess of DTPA) at 37°C over a period of 7 days. The results are presented in Fig. 5. HPLC analysis at 4 h, and 1, 3 and 7 days showed that >97% of radioactivity was still bound to the antibody on day 7. No release of free ^{177}Lu or ^{177}Lu-DOTA was detected after 7 days, which indicates the high stability of the radioimmunoconjugate.

Immunoreactivity of ^{177}Lu-$(DOTA)_4$-rituximab

An immunoreactivity assay described by Lindmo et al. [16] was adopted to determine the immunoreactive fraction of the radiolabelled rituximab. The results are presented in Fig. 6a. The nonspecific binding was calculated by employing unlabelled rituximab to block the binding sites on the cell surface and this was subtracted from the total applied activity (TA) to calculate the specific binding (SB) of ^{177}Lu-DOTA-rituximab to cells of the lymphoma cell line L-VB1. Figure 6a is a plot of the ratio of specifically bound radiolabelled antibody to the total applied radioactivity (SB/TA) as a function of cell concentration. In this plot, the plateau value of SB/TA was >0.7 for the highest cell concentration, indicating that most of the radiolabelled antibody retained its immunoreactivity in high percentage (a plateau of 1.0 indicates 100% of immunoreactivity). However, the precise value of the plateau cannot be extracted from this plot.

A double inverse plot of [TA]/[SB] as a function of the inverse cell concentration (1/[cells]) was designed for the calculation of the immunoreactive fraction (Fig. 6b). Fitting a straight line through these data allows the determination of the intercept value at the ordinate, which equals 1/*r* where *r* represents the immunoreactive fraction of the total amount of antibody. Our data showed a linear relationship between TA/SB and 1/[cells], described by the equation

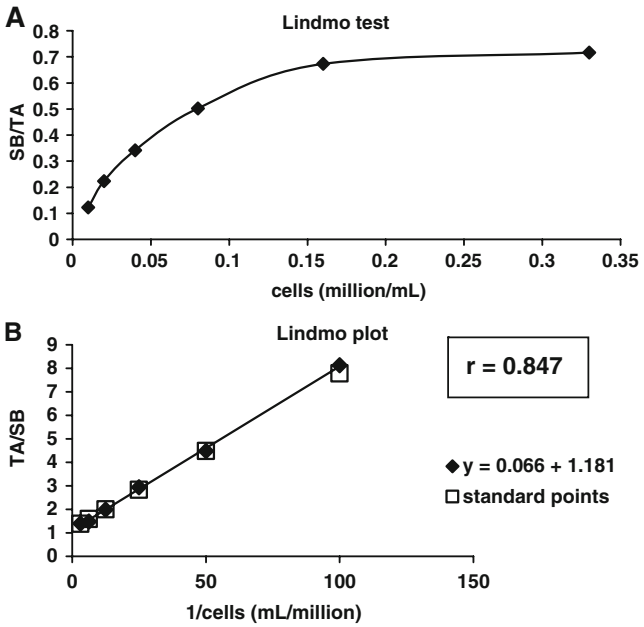


Fig. 6 Lindmo binding assay for the determination of the immunoreactive fraction of ^{177}Lu-(DOTA)$_4$-rituximab. The assay was set up using six concentrations of L-VB1 cells in a series of 1:2 dilutions from 0.33 to 0.01 million cells/ml. The final concentration of ^{177}Lu-(DOTA)$_4$-rituximab was 1 ng/ml. **a** Conventional plot of specific binding over total applied radioactivity as a function of cell concentration. **b** Double inverse plot. By extrapolating a fitted straight line to its intercept with the ordinate, the fraction of immunoreactive antibody is determined as the inverse of the intercept value (1/1.181=0.847)

$y = 0.066x + 1.181$. The immunoreactive fraction was found to be $r = 1/1.181 = 0.847$.

Dosimetry

A dosimetric study using (DOTA)$_4$-rituximab labelled with ^{177}Lu was performed in two patients with relapsed NHL. Both patients were injected with 740 MBq/m^2 ^{177}Lu-DOTA-rituximab. The total dose administered was 1,480 and 1,110 MBq, respectively. The injections were well tolerated by both patients. In both patients specific tumour uptake was seen over time. An example scintigraphic image acquired 4 days after injection together with the corresponding pretherapeutic ^{18}F-FDG-PET image are shown in Fig. 7. Whole-body activity determined by the scintigraphic images could be fitted monoexponentially. The effective half-lives were 136 h and 128 h, respectively. This resulted in whole-body doses of 351 mGy (0.24 mGy/MBq) and 277 mGy (0.25 mGy/MBq), respectively. The doses to the red marrow were calculated as 536 mGy (approximately 0.36 mGy/MBq) and 425 mGy (0.38 mGy/MBq), respectively.

Discussion

Rituximab labelled with radionuclides may provide a better therapeutic effect than rituximab alone in patients with relapsed or refractory B-cell NHL. Macrocyclic chelator molecules such as DOTA need to be coupled to rituximab to enable stable labelling with radiometals. Many methods

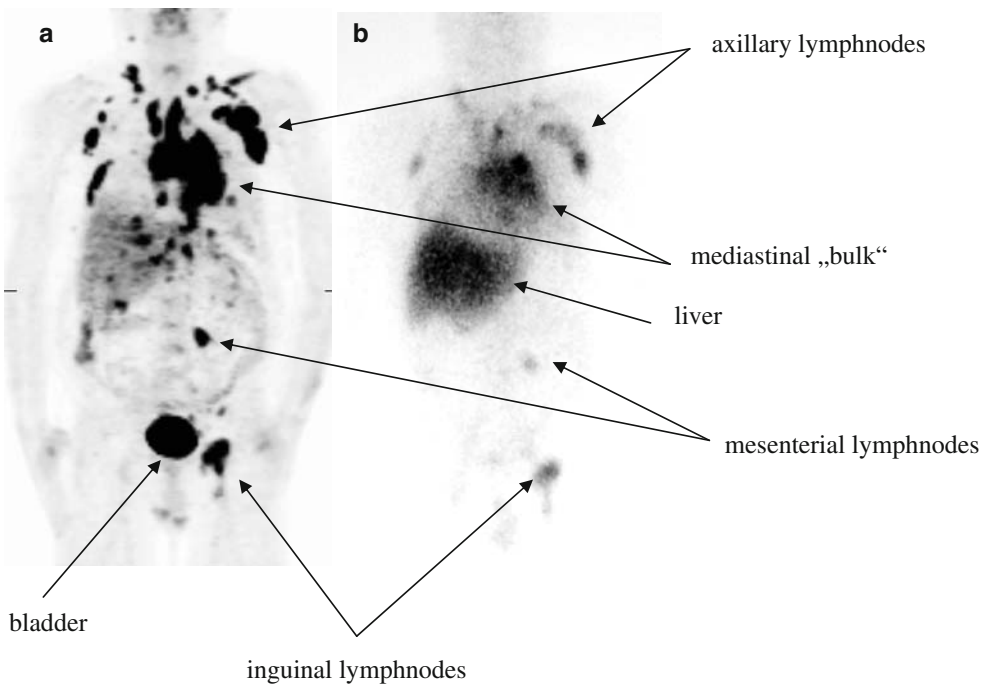
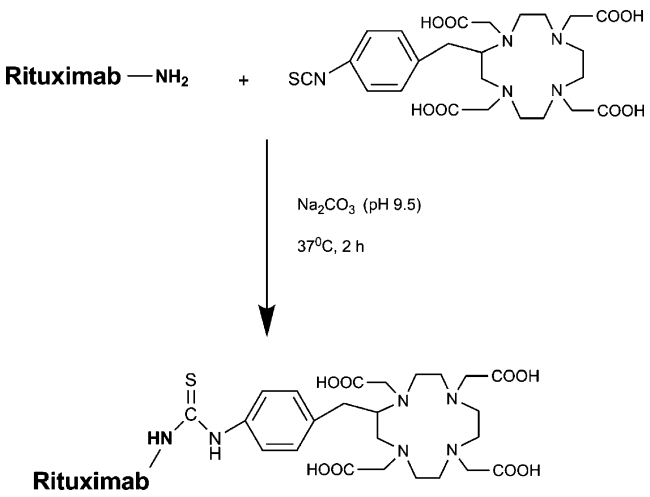


Fig. 7 **a** Pretherapeutic ^{18}F-FDG-PET image (MIP data) of a patient with grade IV follicular lymphoma (lymph node involvement above and below the diaphragm as well as liver infiltration). **b** Planar whole-body scintigraphic image of the same patient (anterior view) 4 days after injection of 40 mCi ^{177}Lu-DOTA-rituximab. Specific uptake at tumour sites as well as nonspecific accumulation of the radiopharmaceutical in the liver is seen

have been used to modify antibodies in order to get antibody-chelator immunoconjugates. In our study, rituximab was modified via thiourea bonds formed between ε-NH_2 groups of lysine residues on the antibody molecule and the isothiocyanate-benzyl-DOTA (Scheme 1). Using a pH of 9.0 for the coupling reaction and rituximab at a concentration of 10 mg/ml resulted in a conjugate with an approximate ratio of one DOTA molecule to one rituximab molecule, which was considered not to be sufficient for prompt labelling with the needed amount of radiometal (e.g. 1,480–3,700 MBq per 5 mg of antibody), for example ^{177}Lu. Increasing the pH from 9.0 to 9.5, and the concentration of antibody from 10 mg/ml to 100 mg/ml resulted in a conjugate with multiple DOTA molecules per antibody molecule [$(DOTA)_4$-rituximab]. Comparing the $(DOTA)_4$-rituximab immunoconjugate with commercially available rituximab, no significant influence of conjugation on the immunoreactivity with cells of the lymphoma cell line L-VB1 was found by FACS assay. This indicates strong binding of $(DOTA)_4$-rituximab to the CD20-expressing target cells and also indicates that rituximab is very robust even under reaction conditions of pH 9.5. However, when the ratio of DOTA molecules to antibody molecules was increased to 8:1 [$(DOTA)_8$-rituximab], the immunoreactivity was drastically reduced (50%), which indicates that conjugation of eight DOTA molecules per rituximab molecule affects the binding sites of the antibody. Therefore, the $(DOTA)_4$-rituximab conjugate in kit form appears to be suitable for use in radioimmunotherapy, whereas in the $(DOTA)_8$-rituximab conjugate most of the immunoreactivity of the antibody is lost and this conjugate cannot be used in patients. Rituximab is a very robust antibody with regard to chemical modification, since up to four chelator molecules per antibody molecule did not result in a significant loss of immunoreactivity.



Scheme 1 The production of DOTA-benzyl-rituximab

The most suitable radionuclide for radioimmunotherapy is not defined yet. ^{131}I is not an ideal radionuclide, mainly because of radiation protection issues. Recently, special attention has been paid to ^{177}Lu. ^{177}Lu has the advantage that besides its relatively low-energy β-emission a small amount of γ-radiation (113 keV 6.5%; 208 keV 11%), making it suitable for scintigraphic imaging, is emitted as well. This allows localization of the radiolabelled antibody conjugates in vivo and dosimetry to be performed. The labelling of $(DOTA)_4$-rituximab in a kit formulation with ^{177}Lu was straightforward (less than 20 min) and sufficient (1,480 MBq per 5 mg in a kit), making the product suitable for routine clinical use. ^{177}Lu appears to be ideal because of its low-energy β-emission. The low-energy β-emission makes it particularly suitable for imaging small or diffusely infiltrating tumours which are often found in NHL patients, since a lower energy β-emission results in a more favourable tumour to non-tumour ratio than higher energy β-emission [19, 20]. For example, the energy of the ^{90}Y β-radiation might be too high for these tumours. In calculations and measurements with human phantoms, Song et al. found that ^{131}I is superior to ^{90}Y, especially for tumours smaller than 2 cm [21].

Nevertheless, ^{90}Y was also evaluated for its radiolabelling efficiency in our study for possible use in patients with distinct bulky disease. In such patients, the use of a radionuclide "cocktail" might be optimal. ^{90}Y is a pure β-emitting high-energy radiometal. Compared to ^{131}I, ^{90}Y has a longer β-energy path and can thus deliver more energy to tumours (^{90}Y, 2.3 MeV; ^{131}I, 0.6 MeV), while it has a shorter half-life (2.7 days) and lack of γ-radiation, which anyway does not contribute to any therapeutic effect [13]. However, the labelling yield achieved with our $(DOTA)_4$-rituximab kit, reconstituted with water, was not sufficient with ^{90}Y, which may have been due to the low pH range (5.5–6.0) under these conditions. We therefore adjusted the pH of the kit to the range 7.0–7.8 by reconstitution with 0.25 *M* ammonium acetate buffer, pH 8.8. At this pH, 5 mg of $(DOTA)_4$-rituximab was successfully labelled with 925 MBq of ^{90}Y within 30 min. This indicates that the ^{90}Y chelation rate is more pH-dependent than the ^{177}Lu chelation rate.

The stability of the ^{177}Lu-DOTA-rituximab immunoconjugate was tested against a 10^5 excess of DTPA. HPLC showed that the radiolabelled immunoconjugate was stable for 7 days, which means that no free radiometal was detected. Thus DOTA-rituximab is capable of stably chelating ^{177}Lu, which is mandatory for using ^{177}Lu-radiolabelled rituximab for therapy in NHL patients.

Another prerequisite for the radiolabelled rituximab antibody for in vivo targeting is high binding affinity of the immunoconjugated and labelled rituximab to the CD20 antigen. An assay estimating immunoreactivity, designed

by Lindmo et al. [16], was performed to determine the immunoreactive fraction of the radiolabelled antibody. Effective systemic targeting of CD20-positive tumour cells was achieved with radiolabelled rituximab as illustrated in Fig. 6a, which shows that about 80% of the radiolabelled antibody was immunoreactive at the highest cell concentration. According to the law of mass action [16], a double inverse plot of TA/SB as a function of the inverse of cell concentration (1/[cells]) will yield a linear relationship based upon the assumption that the binding reaction reaches equilibrium. The immunoreactive fraction of the radiolabelled antibody can be determined from the intercept value obtained by linear extrapolation to the y-axis. Our result demonstrated such a linear relationship, described by the equation $y = 0.066x + 1.181$, where y represents the inverse SB/TA and x represents the inverse cell concentration (1/[cells]). Thus the immunoreactive fraction is determined as $r = 1/1.181 = 0.847$, which also indicates that most of the radiolabelled antibody is immunoreactive, possessing a high binding affinity for the CD20 antigen of lymphoma cells.

The above properties are strong indications for using this radiolabelled $DOTA_4$-rituximab immunoconjugate for in vivo studies and therapy in NHL patients. Dosimetry with ^{177}Lu-DOTA-rituximab in two patients in a therapeutic setting demonstrated effective delivery of radiation absorbed dose to the tumours by specific binding of the antibody to the tumours. The results of the dosimetry for the red marrow compare very well with the data reported by Scheidhauer et al. [18] who used iodinated rituximab. However, the whole-body dose appeared to be somewhat lower with our compound (0.25 mGy/MBq in our study vs. 0.37 mGy/MBq in the study with the iodinated rituximab). On the other hand, the doses for Zevalin (0.65 mGy/MBq for the red marrow and 0.54 mGy/MBq for the whole body) are clearly higher than ours [22]. This is related to the higher energy of ^{90}Y compared to ^{177}Lu. This becomes evident when comparing the effective half-life of the radiolabelled antibody. We found an effective half-life of 132 h, but the half-life of Zevalin has been found to be only 27 h [23]. Besides the shorter physical half-life of ^{90}Y compared to ^{177}Lu, the difference in effective half-life also appears to be related to the different antibodies. The use of a chimeric antibody results in a slower clearance.

In summary, this is the first report of stable binding of a sufficient activity of ^{177}Lu to rituximab as well as the first report of the dosimetry of this novel radioimmunoconjugate in patients. The evaluation of ^{177}Lu-DOTA-rituximab in a phase I/II clinical trial is in progress. However, the results of other studies using radiolabelled antibodies in NHL patients are very encouraging. Higher remission rates as well as longer lasting remissions could be achieved than with, for example, rituximab alone. Additionally, we showed that radioimmunotherapy can still be effective in patients who no longer respond to rituximab [22]. Further clinical trials should seek to determine at what time point in the course of the disease should radioimmunotherapy be given. The first results in a larger group of patients has indicated that it might be beneficial to use radiolabelled antibodies early [11]. However, these results need to be confirmed by other groups.

Conclusion

A $DOTA_4$-rituximab kit formulation was developed in this study, in which multiple DOTA molecules are conjugated to a rituximab antibody. The $(DOTA)_4$-rituximab kit is suitable for labelling with ^{177}Lu or ^{90}Y, and immunoreactivity is retained before and after labelling with the radiometal. Good uptake of the immunoconjugate was seen in two NHL patients. This $(DOTA)_4$-rituximab kit can produce high specific activity ^{177}Lu- and/or ^{90}Y-labelled antibody for clinical studies.

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