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Preparation, Quality Control and Biodistribution Studies of two [^{111}In]-Rituximab Immunoconjugates

Amir R. JALILIAN * [1], Darush SARDARI [2], Leila KIA [2], Pejman ROWSHANFARZAD [1], Javad GAROUSI [1], Mehdi AKHLAGHI [1], Saeed SHANEHSAZZADEH [1], Mohammad MIRZAII [1]

[1] Nuclear Medicine Research Group, Agricultural, Medical and Industrial Research School (AMIRS), P.O.Box: 31485-498, Karaj, Iran.

[2] Islamic Azad University, Science and Research Unit, P.O.Box: 14155-775, Tehran, Iran.

Abstract

In order to use Auger-electron therapeutic effects in CD20 antigen targeting in lymphomas, Mabthera™ (rituximab) was successively labeled with [^{111}In]-indium chloride (185 MBq) after conjugation with freshly prepared macrocyclic bifunctional chelating agent, *N*-succinimidyl-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetra-acetic acid (DOTA-NHS) and ccDTPA separately. Conjugated-Rituximab was obtained by the addition of 1 ml of a rituximab pharmaceutical solution (5 mg/ml, in phosphate buffer, pH=7.8) to a glass tube pre-coated with freshly prepared DOTA-NHS or ccDTPA (0.01–0.1 mg) at 25°C. Radiolabeling was performed at 37°C in 3h and room temperature for one hour for DOTA-conjugate and DTPA-conjugate respectively. HPLC showed an overall radiochemical purity of 97.5 and 95% for DOTA and DTPA-conjugates respectively (Specific activity =2800-5600 GBq/mM). The final isotonic ^{111}In-rituximab complexes were checked by gel electrophoresis for radiolysis. Preliminary biodistribution studies in normal rat model performed to determine radioimmunoconjugates distribution of up to 48h.

* Corresponding author: Tel. ++98-261-4411101; Fax:++98-261-4411106.
E-mail: ajalilian@nrcam.org (A. R. Jalilian).

Keywords

Rituximab • Indium-111 • Biodistribution • Radiolabeling • Targetted Therapy

Introduction

Several studies of radiolabeled anti-CD20 monoclonal antibodies at nonmyeloablative doses in treating B-cell NHL have been reported, and several are in progress. The agents for which most data are available are ^{131}I-tositumomab (Bexxar) and yttrium ^{90}Y-ibritumomab tiuxetan (Zevalin). These studies have reported response rates of 25% to 40% with median response duration of 6 to 18 months in most studies and some very durable responses of more than 5 years [1–4]. In some studies, rituximab has been labeled for metabolism and localization of CD20 antigens throughout the body and/or penetration of the antibody to specific organs [5].

In order to obtain an anti-CD20 conjugate for use in diagnostic/therapeutic studies, the ^{111}In-labeled antibody was prepared as a model of metal chelated immunoconjugate for preliminary dosimetric and biodistribution studies in rats.

As bi-functional ligands, *N*-succinimidyl-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (**3**, DOTA-NHS) [6] and freshly prepared ccDTPA were used.

DOTA ligand has already shown good biological performance when used in protein conjugation of various radioisotopes such as Ga-68 [7], Ga-66 [8], Ac-225 [9], Lu-177 [10] and lead radioisotopes [11].

Based on recent experiences on the preparation of radiometal-labeled rituximab for lymphoma imaging [12], we were interested in the preparation of an antiCD-20 immunoconjugate involving a cyclotron-produced radionuclide, i. e. In-111 (HL:67 h, major photopeak at 172 keV).

A precise labeling strategy was employed using freshly-prepared DOTA-NHS and ccDTPA and optimized radiolabeling methods for developing highly reactive conjugated anti-CD20 for radiometal studies has been has been introduced for diagnosis and therapy purposes.

Results and discussion

Preparation and structure confirmation of DOTA NHS

Various solvents can be used in the esterification reaction of DOTA and NHS, such as DMF, DMSO, etc. Due to the high boiling point of such solvents and the possibility of protein denaturing caused by these solvents, we tried to use dichloromethane based on the previous reports for DCC-mediated conjugation reactions [13]. This reaction is performed at room temperature and can be done overnight. Most of the reaction takes place in the first few hours. Considering the size exclusion chromatography performed in the next step of the process, all the starting materials can be separated. A more lipophillic species in the reaction mixture (monitored by TLC) is related to the formation of the ester. The exact stoichiometry of NHS:DOTA (1:1) is mandatory in order to avoid the formation of the di-succinimidyl ester.

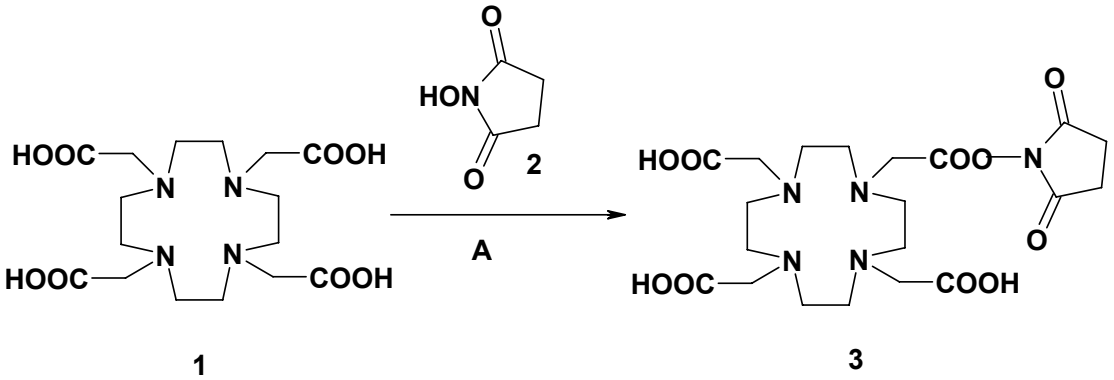

Fig. 1. Diagram of DOTA-NHS (**3**) synthesis. A: CH_2Cl_2, 25°C, 15h

The formation of mono-succinimidyl ester at R_f of 0.36 was observed. In case of longer reaction times another species at R_f=0.56-0.6 can be observed and is related to di-succinimidyl ester. All reactants remain at the R_f of 0.0-0.1.

Conjugation of rituximab with DOTA-NHS ester and radiolabeling of rituximab with ^{111}In

The conjugated DOTA-rituximab fractions containing the maximum protein content were labeled with ^{111}In-$InCl_3$ solution. The samples were checked by RTLC

to find the best time scale for labeling. After an hour, the free ^{111}In/conjugated ^{111}In ratio in the labeled sample remained unchanged at 2:98. The mixture could then be passed through another Sephadex G-50 size exclusion chromatography column in order to remove unbound ^{111}In cation and/or other low molecular weight impurities.

The eluted fractions were checked by Folin-Colciteau® reagent and for the presence of radioactivity in order to determine the ^{111}In-DOTA-rituximab containing fractions. Fraction with the highest radioactivity which contained the maximum color absorbance was chosen as the suitable final product with appropriate specific activity for animal tests. Instant thin layer chromatography using various mobile and stationary phases was performed in order to ensure the existence of only the desired radiolabeled antibody. Five different solvent systems and two stationary phases were tested. In all RTLC tests, radiolabeled antibody stayed at the origin while other species migrated to other R_fs depending on the mobile phase used. The R_fs of the possible occurring chemical species in chromatography of the reaction steps are summarized in Table 1 (n=5). As shown in the table 1, for In^{3+} detection the best eluent systems are system 5 and 6 resulting in Rfs of 0.8 and 0.9 respectively. For In-DOTA detection most of the systems used can demonstrate the amount of the component compared to the other fractions. However, systems 1-4 give very distinguishable Rfs in contrast to the others respectively 0.3, 0.5, 0.9 and 0.9. Due to the size and charge of the protein (≈150,000 D), ^{111}In-DOTA-rituximab remains at the origin in all systems used.

In HPLC studies using reverse phase column in 1mMDTPA as eluent. The fast eluting component (3.4 min) was shown to be a mixture of free ^{111}In and ^{111}InDTPA. Both compounds are ionic, so they are eluted at the same retention time. The radiolabeled protein was finally washed out at 15.86 minutes (Figure 2.).

Due to UV absorbance of DTPA moiety, the UV detector chromatogram can not be used for chemical purity determination, however using free DTPA and In-111 for control studies, it was shown that 98% of present DTPA at the reaction pH will form ^{111}In-DTPA species which can be analyzed by flow scintillation analyzer. Assuming the only impurity in whole process would be DTPA. In the production

Tab. 1. The R_f values of chemical impurities and DOTA-conjugates

	Chemical species	**Mobile phase**	**Stationary phase**	**R_f**
1	^{111}In-DOTA-rituximab	10% NH_4OAc:MeOH (1:1)	Silicagel	0.0
	$^{111}In^{3+}$	//	//	0.0
	^{111}In-DOTA	//	//	0.3
2	^{111}In-DOTA-rituximab	Normal saline	//	0.0
	$^{111}In^{3+}$	//	//	0.1
	^{111}In-DOTA	//	//	0.5
3	^{111}In-DOTA-rituximab	pyridine:ethanol:H_2O(1:2:4)	//	0.0
	$^{111}In^{3+}$	//	//	0.0
	^{111}In-DOTA	//	//	0.9
4	^{111}In-DOTA-rituximab	Methanol:ammonia (3:2)	//	0.0
	$^{111}In^{3+}$	//	//	0.1
	^{111}In-DOTA	//	//	0.9
5	^{111}In-DOTA-rituximab	Water:methanol (45:55)	Whatman 1.	0.0
	$^{111}In^{3+}$	//	//	0.8
	^{111}In-DOTA	//	//	0.9
6	^{111}In-DOTA-rituximab	10 mM DTPA (pH. 5)	//	0.0
	$^{111}In^{3+}$	//	//	0.9
	^{111}In-DOTA	//	//	0.9

step of DOTA(NHS)-MAb conjugate, the charge/size difference between DOTA(NHS)-MAb conjugate and unconjugated antibody is negligible with respect to 150000 Da molecular size, however the best specific activity can be obtained by the reaction of DOTA-NHS:antibody (3:1) molar ratio.

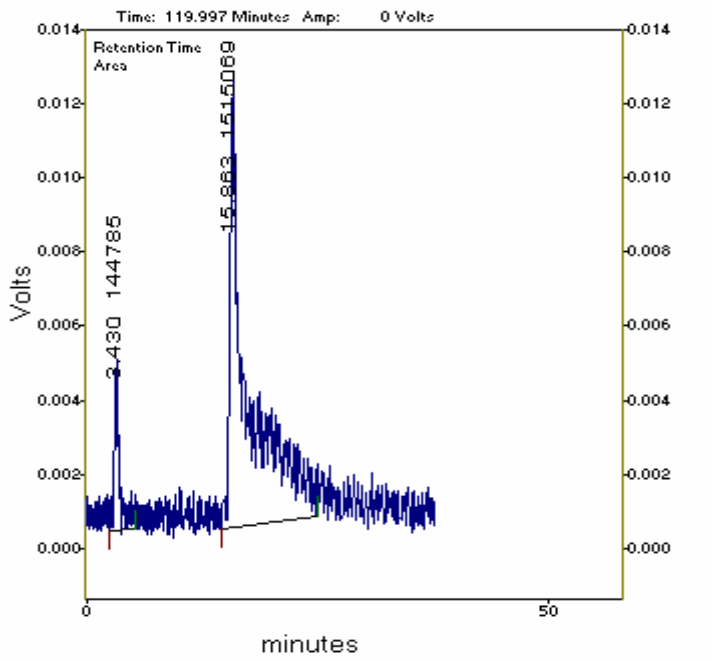

Fig. 2. HPLC chromatogram of final ^{111}In-DTPA-rituximab solution on a reversed phase column using a gradient of acetate/citrate buffer

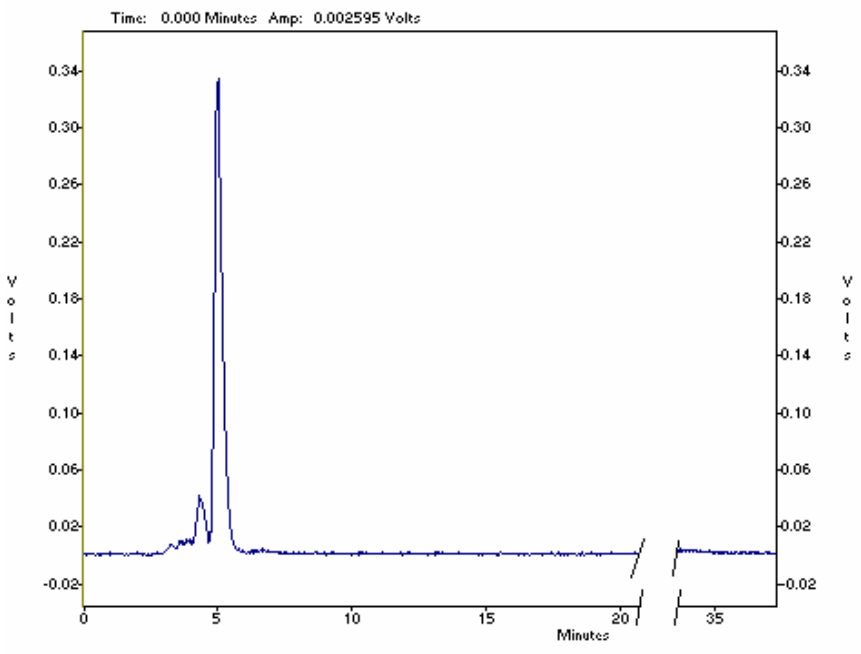

Fig. 3. HPLC chromatogram of final ^{111}In-DOTA solution on a reversed phase column using a gradient of acetate/citrate buffer

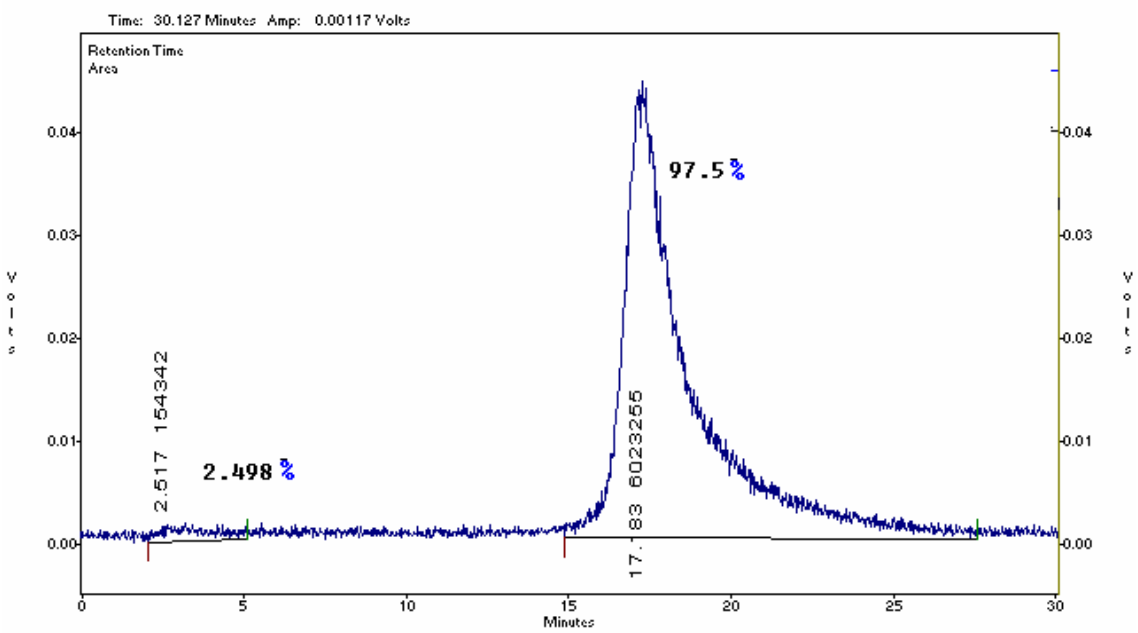

Fig. 4. HPLC chromatogram of final ^{111}In-DOTA-rituximab solution on a reversed phase column using a gradient of acetate/citrate buffer

Since radiolabeling of each antibody molecule with one radioisotope atom at optimal theoretical conditions can be satisfactory, we chose the 1:1 molar ratio for the molar ratio. The only possible way to check the yield was RTLC/HPLC analysis of final radiolabeled antibody at various molar ratios (Figures 3, 4).

Stability of Radiolabeled Protein in presence of human serum in vitro

After incubation of ^{111}In-DOTA-rituximab with freshly prepared human serum at described conditions in the methods, 96-98% of the radioactivity eluted at the same fraction as ^{111}In-DOTA-rituximab, using size exclusion chromatography. Thus, there was no evidence for either degradation or transchelation of ^{111}In to other serum proteins over a time period consistent with the normal blood clearance time of rituximab.

Protein integrity test using SDS-Polyacrylamide Gel Electrophoresis

In order to demonstrate the integrity of the protein after conjugation and radiolabeling gel electrophoresis was performed on the SDS PAGE gels using 16% bisacrylamide gel. The loaded samples were Rituximab commercial sample, DOTA-

rituximab and radiolabeled protein samples a week after the experiment while kept in the fridge. The 3 samples were showed to have similar pattern of migration in the gel electrophoresis.

Interestingly the SDS PAGE results were checked with a reported commercial Rituximab sample [16]. Figure 5. shows the SDS-PAGE patterns for both DOTA and DTPA conjugates in contrast to starting Rituximab sample and radiolabeled immunoconjugates.

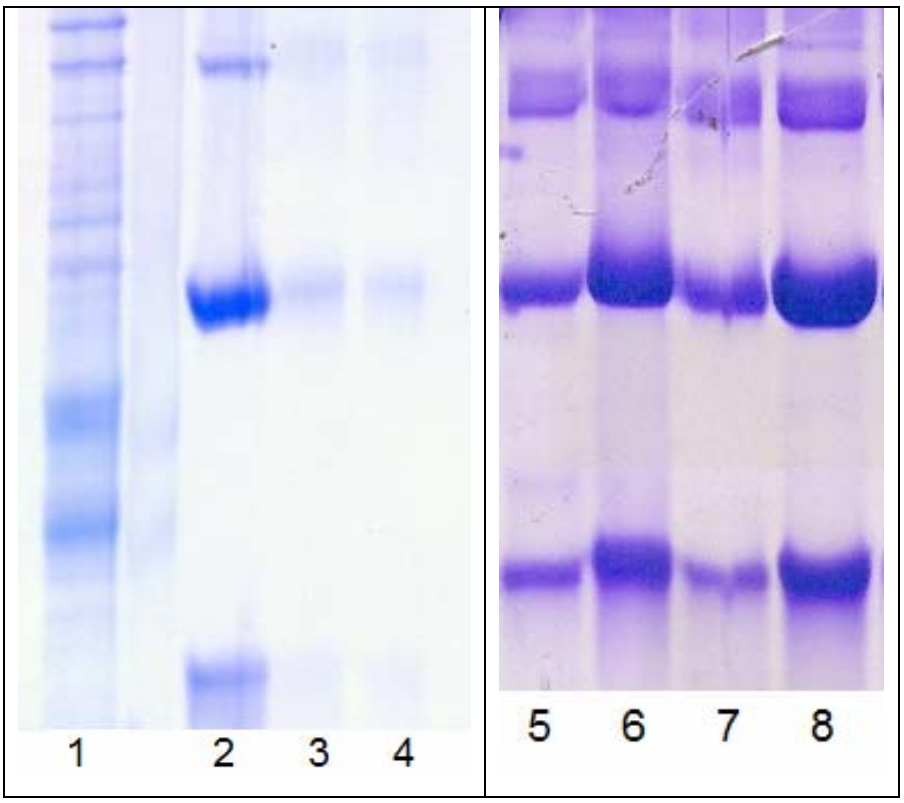

Fig. 5. SDS-PAGE lane patterns for Rituximab (2, 6, 8), DOTA-rituximab conjugate (3) and ^{111}In-DOTA-rituximab (4), DTPA-rituximab conjugate (5), ^{111}In-DOTA-rituximab (7) in contrast to in house-made standard ladder (1).

Biodistribution studies

The animals were sacrificed by ether asphyxiation at selected times after injection (24, 48 and 72h for DTPA conjugate and 2, 24 and 48h for DOTA conjugate). Dissection began by drawing blood from the aorta, followed by collecting blood, heart, spleen, kidneys, liver, intestine, stomach, lung and skin

samples. The tissue uptakes were calculated as the percent of area under the curve of the related photo peak per gram of tissue (% ID/g) (Figures 6, 7).

At two hours post injection, the activity is mainly in the blood which is in agreement with the other reported labeled antibodies [14], while the activity of the stomach, muscle, intestine and spleen is rather low.

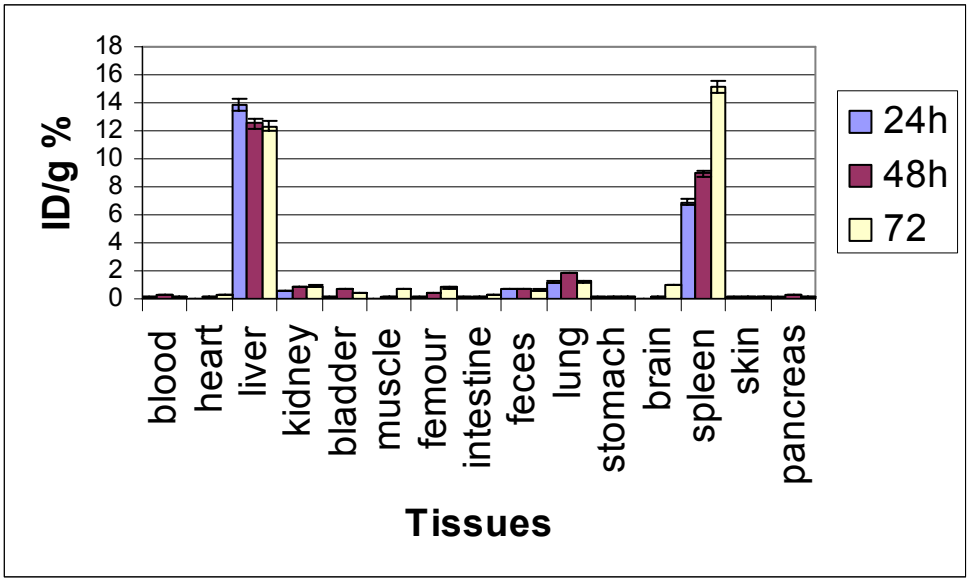

Fig. 6. Percentage of injected dose per gram (ID/g %) of ^{111}In-DTPA-rituximab in normal rat tissues at 24, 48 and 72 h post injection

The brain did not show any significant uptake over the period of time. This had already been shown by ^{123}I-antiCD20 conjugate biodistribution studies [5]. High uptake in spleen and reticuloendothelial organs was observed, which is due to the final accumulation of B lymphocytes carrying the radioimmunoconjugate on their surface. The increasing uptake of spleen as a course of time is a direct result of the depletion of circulating B cells occurring rapidly after administration to the mammals. This has been already shown in human, which is an important sign of therapy in lymphoma patients [15].

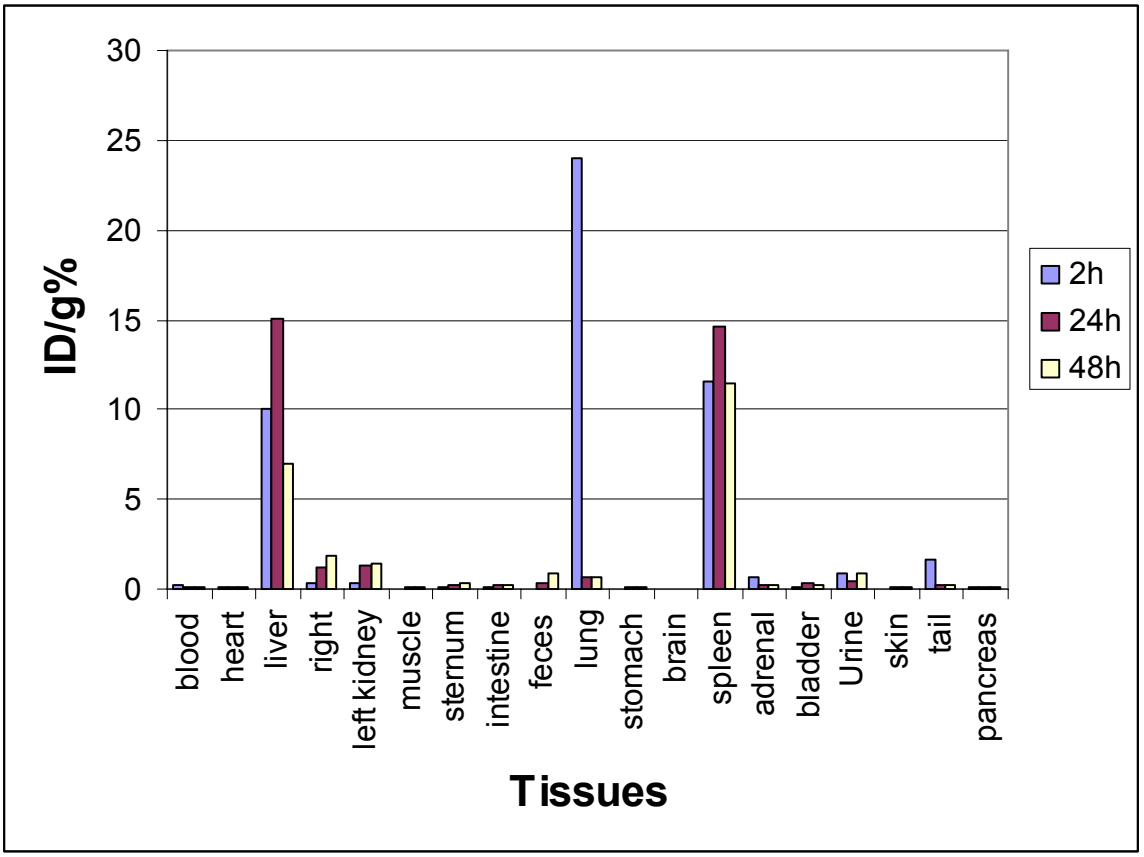

Fig. 7. Percentage of injected dose per gram (ID/g %) of ^{111}In-DOTA-rituximab in normal rat tissues at 2, 24 and 48 h post injection

As a natural reaction to the depletion of the lymphocytes the reticulloendothelial system including spleen will be the final possible reservoir of the depleted lymphocytes. However a direct resemblance of CD-20 antigen in human and rats has not been demonstrated. On the other hand, rituximab natural binding was found on lymphoid cells in the thymus, the white pulp of the spleen, and a majority of B-lymphocytes in peripheral blood and the lymph nodes in human being [16], this has been observed in our studies on the normal rats as well.

In some rats tested, accumulation in the lungs was observed. At the beginning it was concluded that this accumulation is caused by the non-specific immigration of the lymphocytes to the possibly infected bronchi of the objects which the infection. But the infection was not later confirmed by the lab tests post-mortem. Interestingly, we found reports of severe pulmonary reactions with pulmonary infiltrates or edema

in human casued by anti-CD20. Acute symptoms appear within 1-2 hours of the initiation of the 1st infusion [17].

Experimental

Production of ^{111}In was performed at the Agricultural, Medical and Industrial Research School (AMIRS), 30 MeV cyclotron (Cyclone-30, IBA) using ^{nat}Cd(p,x)^{111}In. Natural cadmium sulfate with purity of >95% was obtained from Merck Co. Germany. Sephadex G-50, sodium acetate, phosphate buffer components, methanol and cyclic DTPA dianhydride were purchased from Sigma-Aldrich Chemical Co. U.K. NHS DOTA was freshly prepared and kept under a blanket of dry N_2.

Rituximab was a pharmaceutical sample purchased from Roche Co. and was used without further purification. Radio-chromatography was performed by counting polymer-backed silica gel paper and/or C_{18} thin layer sheets using a thin layer chromatography scanner, Bioscan AR2000. Analytical HPLC to determine the specific activity was performed by a Shimadzu LC-10AT, armed with two detector systems, flow scintillation analyzer (Packard-150 TR) and UV-visible (Shimadzu) using Whatman Partisphere C-18 column 250 x 4.6 mm, Whatman Co. NJ, USA. Calculations were based on the 172 keV peak for ^{111}In. All values were expressed as mean ± standard deviation (Mean± SD) and the data were compared using student T-test.

Statistical significance was defined as $P<0.05$. Animal studies were performed in accordance with the United Kingdom Biological Council's Guidelines on the Use of Living Animals in Scientific Investigations, 2nd edn.

Production and quality control of ^{111}In-$InCl_3$ solution

Cadmium electroplating over a copper surface was performed according to previously reported method [18]. ^{111}In-indium chloride was prepared by 20 MeV proton bombardment of the natural Cd target [13]. The presence of copper and cadmium impurities in the final solution was checked using acidic dithizone solution

and alkaline dimethylglyoxime and NaK tartrate respectively according to the procedure [19, 20].

Conjugation of cyclic DTPA di-anhydride with rituximab

The chelator diethylenetriamine penta-acetic acid dianhydride was conjugated wth the Rituximab using a small modification of the well-known cyclic anhydride method [21]. Conjugation was performed at a 1:1 molar ratio. In brief, 20 µl of a 1 mg ml^{-1} suspension of DTPA anhydride in dry chloroform (Merck, Darmstadt, Germany) was pipetted under ultrasonication and transferred to a glass tube. The chloroform was evaporated under a gentle stream of nitrogen. Commercially available Rituximab (5 mg, 0.5 ml, pH 7.5) was subsequently added and gently mixed at room temperature for 60 min. Conjugation mixture was then passed through a Sephadex G-50 column (2×15 cm, 2 g in 50 ml of Milli-Q® water) separately and one-milliliter fractions were collected and checked for the presence of protein using UV absorbance at 280 nm or visible folin-phenol colorimetric assay. The fractions containing the highest concentration of the immunoconjugate were chosen and kept at 4°C and for radiolabeling.

Preparation of **N**-*succinimidyl-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetra-acetic acid (DOTA-NHS) (= 2,2',2''-(10-{2-[(2,5-dioxopyrrolidin-1-yl)oxy]-2-oxoethyl}-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid) for optimal protein conjugation*

This compound was prepared according to methods previously given in the literature with slight modifications [22]. For a single run, a mixture of DOTA in acidic form (4 mg, 0.01 mmol), *N*-hydroxysuccinimide (1.15 mg, 0.01 mmol) and dicyclohexyl carbodiimide (DCC) (2mg, 0.01 mmol) were dissolved in anhydrous CH_2Cl_2 (300-500 µl) under a blanket of N_2 in a glass vial. The mixture was vortexed for 30s and stirred at 25°C for 15 h. Thin layer chromatography using ethyl acetate:hexane (1:1) mixture as mobile phase was performed in order to monitor the reaction progress.

Conjugation of NHS-DOTA with the rituximab

The chelator *N*-succinimidyl-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA-NHS) prepared above, was conjugated to the antibody using a small modification of the DOTA-NHS method [23]. The residue mixture above was evaporated under a flow of N_2 gas in a glass tube producing a thin film coat of DOTA-NHS. The commercially available antibody Rituximab® (5mg/ml, pH.7.8, 0.5 ml) was added to the coated glass tube while gently mixed at room temperature for 30 s. The mixture was then incubated in a water bath at 25°C for 15–18 hours.

This conjugation mixture was then passed through a Sephadex G-50 column (2 × 15 cm, prepared by soaking 2g of the resin in 50 ml of Milli-Q® water overnight) separately and one-milliliter fractions were collected and checked for the presence of protein using UV absorbance at 280 nm or visible folin-phenol colorimetric assay. The fraction containing the highest concentration of the immunoconjugate was chosen and kept at 4°C and for radiolabeling.

Radiolabeling of the antibody conjugate with ^{111}In

The antibody conjugate was labeled using an optimization protocol according to the literature [24]. Typically, 40–400 MBq of ^{111}In-chloride (in 0.2M HCl) was added to a conical vial and dried under a flow of nitrogen. To the In-containing vial was added acetate buffer (200 µl, pH. 4.8) and the vial vortexed for 30 seconds. The protein containing fraction with the maximum protein content was added in 1 ml of phosphate buffer (0.1 M, pH= 8) to the vial and mixed gently for 30 seconds. The resulting solution was incubated at 37°C for 2–3 hours. Following incubation, the radiolabeled antibody conjugate was purified from free ^{111}In by size exclusion chromatography on a Sephadex G-50 column (15–20 ml bed volume) and eluted with PBS. Fractions (1 ml) were collected and the radioactivity of each fraction was measured by a recently calibrated radioisotope dose calibrator (CRC-7, Capintec Instruments, Ramsey, NJ). The protein presence in each fraction was determined using a fast protein assay method by mixing freshly prepared Folin-Colciteau®

reagent (5 μl prepared by mixing 25μl of fresh CuTartrate solution) and 10 μl of the eluted fractions. The fractions containing the proteins (visible blue color by naked eye) with the maximum radioactivity was tested for purity by ITLC using a radio TLC scanner. Control labeling experiments were also performed using $^{111}InCl_3$, and DOTA with $^{111}InCl_3$. Both reaction mixtures were passed through separate size exclusion chromatography columns and eluted with PBS. The fraction number 5-7 showed the presence of protein, which fraction 6 was used in the other experiments (n=3).

Preparation of ^{111}In-DOTA for control studies

For control studies, ^{111}In-DOTA was prepared for R_f and retention time studies in chromatographic methods [19]. For preparation, 37 MBq of ^{111}In-chloride (in 0.2M HCl) was added to a conical vial and dried under a flow of nitrogen. To the In-containing vial were added phosphate buffer (0.1 M, 200 μl, pH. 8) and the vial vortexed for 30 seconds (final pH. 7.5). A solution of DOTA (1 mg, 2.5 mM) in 300 μl of phosphate buffer (0.1 M, pH= 8) was added to the first vial and mixed gently for 30 seconds. The resulting solution was incubated at 37°C for 3 hours.

Quality control of ^{111}In-DOTA-rituximab

Thin layer chromatography

System I: A 5 μl sample of the final fraction was spotted on a silica gel paper and developed in a mixture of 10% ammonium acetate:methanol (1:1) as the mobile phase, in order to observe the Rfs of free $^{111}In^{3+}$ and ^{111}In-DOTA. Both In^{3+} and radiolabeled protein stay at R_f=0.0 [17].

System II: From the final product, 5 μl was applied to a silica-impregnated glass fiber sheet followed by developing in 0.9% NaCl for 5 min. Radioactivity was determined by a chromatography scanner equipped with an HPGe crystal. ^{111}In-DOTA will move to the front, the ^{111}In-labelled monoclonal antibody remains at the starting position [9].

System III: Another system was performed on silica-impregnated glass fibre sheets. From the final product, 5 μl was applied to the ITLC strip that was

developed with a mixture of pyridine:ethanol:water (1:2:4) for 5 min. Radioactivity was determined by a chromatography scanner equipped with an HPGe crystal. ^{111}In-DOTA will move to the front, the ^{111}In-labelled monoclonal antibody remains at the starting position [18].

Paper chromatography

Paper chromatography [Whatman No. 1 (Whatman, Maidstone, UK), methanol/water (55:45)] of the eluate showed that >94% of the activity remained at the origin corresponding to the ^{111}In-DOTA-conjugate. The labeling yield was 45±5% (n =3), and a specific activity of 300-500 Bq per 1 mg DOTA-conjugate was obtained [15].

High performance liquid chromatography

HPLC was performed on the final preparation using acetate buffer solution (50 mM pH. 5.5) as eluent (flow rate: 1ml/min pressure: 130 KgF/cm^2) for 20 min in order to elute low molecular weight components.

Radiolabeled antibody was eluted using a gradient of the latter solution (100 to 0%) and citrate buffer solution (50mM, pH.4, 0 to 100%) using reverse stationary phase. Any remaining free In^{3+} cation can be complexed with citrate anion; however pre-complexed In-DTPA-rituximab was not challenged with the addition of citrate chelate [25].

SDS-Polyacrylamide Gel Electrophoresis

The radioimmunoconjugates were analyzed for integrity by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The radiolabeled mAb was evaluated with and without reduction by 2-mercaptoethanol. Approximately 200,000 cpm of each preparation was applied per lane and the 4–20% polyacrylamide were run according to the method of Laemmli [26].

Stability testing of the radiolabeled compound in final formulation

Stability of ^{111}In-DOTA-rituximab in PBS was determined by storing the final solution at 4°C for 14 days and performing frequent ITLC analysis to determine

radiochemical purity. The stability of the conjugated DOTA-rituximab stored at −20 °C for more than 3 months was also investigated. ITLC analysis of the conjugated product was performed to monitor for degradation products or other impurities. After subsequent ^{111}In-labeling of the stored conjugated product, both labeling efficiency and radiochemical purity were determined.

Stability testing of the radiolabeled compound in presence of human serum

Radiolabel stability was assessed by size exclusion chromatography on a Sepharose column (1 × 30 cm). The column was equilibrated with PBS and eluted at a flow rate of 0.5 mL/min at room temperature; 1 mL fractions were collected.

Biodistribution of ^{111}In-radioimmunoconjugates to normal rats

To determine its biodistribution, ^{111}In-DOTA/DTPA-rituximab was administered to normal rats. A volume (50-100 μl) of final ^{111}In-DOTA/DTPA-rituximab solution containing 20±5 μCi radioactivity was injected intravenously to rats through their tail vein. The animals were sacrificed at the exact time intervals (2–48 h), and the specific activity of different organs was calculated as percentage of injected dose per gram using a radiometer.

Conclusion

Two separate rituximab conjugates were prepared and their behaviour in radiolabeling, quality control radiochemical purity were compared. For control studies ^{111}In-DOTA was prepared and was checked using RTLC and HPLC. Total labeling, formulation and quality control of [^{111}In]-DOTA-rituximab took about 3 hours, with a yield of 97.5 % while using the optimized conditions. For [^{111}In]-DTPA-rituximab the chepurity was 95% and the labeling and quality control took one hour. The radio-labeled complexes were stable in human serum for at least 24 hours and no significant amount of free ^{111}In as well as ^{111}In-DOTA was observed. The final preparations were administered to normal rats and biodistribution of the radiopharmaceutical was checked 2–48 hours later. [^{111}In]-rituximab is potentially a good probe for diagnosis and therapy of lymphomas. Using the DOTA conjugate

the incorporation of other therapeutic nuclides such as lanthanides and yttrium can be performed.

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